Increasing the Water Use Efficiency (WUE) of Tomato (*S. lycopersicum*) via Manipulation of the Abscisic Acid (ABA) Biosynthesis Pathway

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ABSTRACT

Breeding plants that produce equivalent growth with reduced water input (improved water use efficiency (WUE)) is necessary for sustained future crop production. Water deficit induces redistribution and synthesis of the phytohormone abscisic acid (ABA) thereby restricting transpiration. ABA is synthesised via cleavage of oxygenated carotenoids (xanthophylls). This work involved two approaches and three key rate-limiting enzymes in the ABA biosynthesis pathway: 9-*cis*-epoxycarotenoid dioxygenase (NCED); β-carotene hydroxylase (BCH); and phytoene synthase (PSY). Increasing ABA production under optimal conditions by overexpressing *SlNCED1* has been shown to reduce stomatal conductance and improve WUE. The first approach, part of a larger programme exploring the allelic variation of *NCED1* in wild tomato species, describes the introgression of *S. galapagense* and *S. neorickii NCED1* alleles into the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig. Plants homozygous for *SgNCED1*, *SnNCED1* and *SlNCED1* alleles were compared for water use gravimetrically. It was concluded that *SgNCED1* and *SnNCED1* did not improve WUE.

The second approach involved creating a transgenic rootstock that might provide sufficient extra root-sourced ABA under non-stressed conditions to improve WUE of a non-transgenic scion. Root tissues contain less carotenoids than photosynthetic tissues, which may limit increases in root ABA biosynthesis. To increase precursor flux through the pathway, transgenic lines simultaneously over-expressing *SlPSY1*, *SlBCH2*, and *SlNCED1* were created by crossing two double transgenic lines. Isolated roots of a selected triple line (H-22-8-8) accumulated increased concentrations of carotenoids, ABA and ABA catabolites. Gravimetric water use trials revealed that WT/H-22-8-8 (scion/rootstock) grafts frequently showed improved; TE_p, $\delta^{13}C$, exudate flow rate and ABA concentration. H-22-8-8 rootstock conferred a consistent, modest, daily water saving; significant in the absence of water stress. This indicates that the triple rootstock is capable of restricting stomatal opening and water use, without reducing biomass production.

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In memory of
Brian White

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35S	Cauliflower mosaic virus promoter
8´-OH-ABA	8´-hydroxy-ABA
A	Assimilation rate
AAIP1	AAPK interacting protein1
AAO	Abscisic acid aldehyde oxidase
AAPK	Activated serine-threonine protein kinase
ABA	Abscisic acid
ABAH	undissociated form of ABA
ABAR	Abscisic acid receptor
ABC	ATP-binding cassette
ABC	ABC transporter protein
ABF	ABA binding factor
ABI	ABA insensitive
ABRE	ABA-responsive element
ACC	1-aminocyclopropane-1-carboxylic acid
ACCd	ACC deaminase
ACMC	Amber coloured microfuge tubes
AHA1	*Arabidopsis* H^+ ATPase1
AM	Arbuscular mycorrhiza
ANOVA	Analysis of variance
AO	Aldehyde oxidase
AOG	ABA glucosyltransferase
AREBs	ABA Responsive Element Binding proteins
ARV8	*Achromobacter piechaudii*
AS	Artificial sap
AS-AO	ABA specific aldehyde oxidase
AS-SDR	ABA-specific short-chain dehydrogenase/reductase
ATP	Adenosine triphosphate
ats	*Aberrant testa shape*
Au	Absorbance units
AVRDC	Asian Vegetable Research and Development Centre
B	*Beta* tomato mutant
BC	Backcross
BCH	Beta-carotene hydroxylase (also known as CrtR-B)
BG1	β-glucosidase1
BHQ	Black Hole Quencher
bp	Base pair
BSA	Bovine serum albumin
b-ZIP	basic-leucine zipper
C_a	CO_2 Concentration in ambient air
CaMV	Cauliflower mosaic virus
CAPS	Cleaved amplified polymorphic sequence
CCD	Carotenoid cleavage dioxygenase
cDNA	Complementary DNA
CDPKs	Ca^{2+}-dependent protein kinases
CE	Coupling element
CHLH	Mg-chelatase H subunit
C_i	Concentration of CO_2 inside of the leaf
cpm	counts per minute
CRTISO	Carotenoid isomerase
CrtL-B	Lycopene β-cyclase (LCY-B/CrtL-B)
CrtR-B	See BCH
ct	Threshold cycle
CTAB	Hexadecyltrimethyl ammonium bromide
cv.	Cultivar
CYP	Cytochrome P450
$\delta\ ^{13}C$	Carbon isotope composition
$\Delta\ ^{13}C$	Oxygen isotope discrimination
$\delta\ ^{18}O$	Oxygen isotope composition
$\Delta\ ^{18}O$	Carbon isotope discrimination
DDB1	DNA binding protein1
DEFRA	Department for Environment, Food and Rural Affairs
DET	de-etiolated1
DI	Deficit Irrigation
DMAPP	Dimethylallyl pyroposhophate
DNA	Deoxyribonucelic acid
DNase	Deoxyribonuclease
DOG	Delay of Germination
DPA	Dihydrophaseic acid
dpm	disintegrations per minute
DWt	Dry weight
DXP	1-deoxy-D-xylulose-5-

	phosphate
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
E	Transpiration rate
ECH	ε-ring hydroxylase (also as CrtR-e)
EDTA	Ethylenediamine tetraacetic acid
FAM	Fluorescein
FCA	Flowering time control protein A
flc	*flacca* mutant of tomato
FPP	farnesyl diphosphate
FWt	Fresh weight
GA	Gibberellic acid
GA-3-P	D-glyceraldehyde 3-phosphate
galaneo	An interspecific hybrid cross between *S. galapagense x S. neorickii*
GAs	Gibberellins
GBSSI	Granule-bound starch synthase gene
GCAC1	Guard cell anion channel1
GCR2	G protein-coupled receptor2
GDP	Guanosine diphosphate
GE	Glucose ester
GFP	Green fluorescent protein
GGPP	Geranylgeranyl pyrophosphate
gia3	growth insensitive to ABA Arabidopsis mutant
GM	genetically modified
GORK	guard cell outward rectifying K^+ channel
GPA1	G protein α subunit
GPCR	G protein-coupled receptor
GPP	Geranyl diphosphate
g_s	stomatal conductance
Gtase	Glycosyltransferase
GTG	GPCR-type G proteins
GTP	Guanosine triphosphate
GUN5	GENOME UNCOUPLED5 gene locus
GUS	b-glucuronidase
HAB	Homology to ABI
HDR	Hydroxymethylbutenyl diphosphate reductase
hetero	Heterozygous
HEX	Hexachlorofluorescein
homo	Homozygous
hp	*high-pigment* tomato mutant
HPLC (PDA)	High performance liquid chromatography (photo diode array)
IL	Introgression Line
IPP	Isopentenyl pyrophosphate
KAT	K^+_{in} channel α-subunit
KEG	keep on going
LCY-B/E	Lycopene beta/epsilon-cyclase
LEA	Late embyrogenesis abundant protein
Lp_r	Root hydraulic conductivity
LSD	Least significant difference
MAC252	Antibody
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAS	Marker assisted selection
MEE26	*Maternal Embryo Effect 26*
MEP	2-C-methyl-D-erythritol 4-phosphate
MoCo-O/S	Di/mono-oxygenated molybdenum cofactors
MRM	Multiple Reaction Monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS	Murashige and Skoog mineral salts
MTBE	Methyl-tert-butyl ether
NCED	9-*cis*-epoxycarotenoid dioxygenase
NCEI	9-*cis*-epoxycarotenoid-forming isomerase
NEC	No enzyme control
neo	Pure *S. neorickii* parental line
neoPA	*neo*phaseic ABA
NMR	Nuclear magnetic resonance
not	*Notabilis* tomato mutant
NPQ	Non-photochemical quenching
npq2	*Non-photochemical quenching2 Arabidopsis* mutant
nptII	*Neomycin phosphotransferase II*

NR	Nitrate reductase
NSY	Neoxanthin synthase
NTC	No template control
NXS	Neoxanthin synthase
og	*Old-gold* tomato mutant
og^c	*old-gold-crimson* tomato mutant
ORF	Open reading frame
OST	Open Stomata *Arabidopsis* mutant
P	Probability
P5CS	Δ^1-Pyrroline-5-Carboxylate Synthase
PA	Phaseic acid
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PDR3	protein pleiotropic drug resistance 3
PDS	Phytoene desaturase
PGPB	Plant growth-promoting bacteria
pH_{cyt}	Cytosolic pH
PIPs	Plasma membrane Intrinsic Proteins
pKa	Acid dissociation constant
PP2Cs	protein phosphatase type 2Cs
PRD	Partial root-zone drying
PSI	Photosystem I
PSY	Phytoene synthase
PVP	Poly vinyl pyrrolidone
PYL	PYR1-Like
PYR	Pyrabactin resistance
qPCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative trait loci
r	*yellow-flesh* mutant of tomato
RCAR	Regulatory Component of ABA Receptors
RIA	Radio-immuno assay
RNA	Ribonucleic acid
RNaseA	Ribonuclease A
ROS	Reactive oxygen species
R-type	Rapid-type
SAPK	Stress-Activated Protein Kinases
SDR	Short-chain alcohol dehydrogenase/reductase
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
SLAC1	Slow anion channel-associated 1
SnRK2s	Sucrose non-fermented-related protein kinases 2
SO	Sulfite oxidase
sp	Super-promoter
SSC	Saline-sodium citrate
START	Star-related lipid-transfer domain proteins
S-type	slow-type
SUMO	Small ubiquitin-related modifier
SV	Slow vacuolar
TAO	Tomato aldehyde oxidases
TBE	Tris/Borate/EDTA
T-DNA	Transfer DNA
TE	Transpiration efficiency
TE_i	Intrinsic transpiration efficiency
TE_p	Transpiration efficiency (whole plant)
TMV	Tomato mosaic virus
TSS	Total soluble solid
ttg	*Transparent testa glabra Arabidopsis* mutant
UPP	Undigested PCR product
v/v	Volume for volume percentage relationship
VDE	Violaxanthin de-epoxidase
VI	Vacuolar invertase
VK	Vacuolar K^+ selective channel
vp	*viviparous* maize mutant
VPD	Vapour pressure deficit
w/v	Weight for volume percentage relationship
wf	*White-flower* tomato mutant
WT	Wild type
WUE	Water use efficiency
XDH	Xanthine dehydrogenase
y9	*pale yellow9* maize mutant
ZDS	z-carotene desaturase
ZEP	Zeaxanthin epoxidase
Z-ISO	Carotenoid isomerase 15-*cis*-ζ-CRTISO

CHAPTER 1:

GENERAL INTRODUCTION

Agriculture consumes 70% of available fresh water resources, and this requirement is predicted to increase 17% by 2025, according to the International Water Management Institute (IWMI, http://www.iwmi.cgiar.org/). The Food and Agriculture organisation (FAO, http://www.fao.org/) reports that by the year 2025, 1,800 million people will be living in countries or regions with absolute water scarcity, and two-thirds of the worlds population could be under stress conditions. Additionally, global climate change is projected to decrease agricultural yields as a consequence of increasing temperatures and lower, more erratic, rainfall (Parry *et al.*, 2005). There is therefore an urgent need to develop integrated and sustainable approaches to increase agricultural production per unit area with lower input of water resources, if global food security is to be achieved. A second agricultural 'green' revolution has been called for; this has been termed a 'blue revolution' in which water use productivity is much more closely linked to land-use productivity (Bourlaug, 2007). One approach towards achieving 'more crop per drop' is breeding more water use efficient crops.

The long distance transport of water and minerals in plants takes place in the xylem, which extends from root to leaf. Water is taken up from the soil by the roots where it enters the xylem, and then travels through it via the mechanisms postulated in the 'cohesion-tension theory'. When water reaches the leaves it evaporates from the surfaces of the mesophyll cells into the intracellular spaces. When stomatal pores are open, water vapour rapidly diffuses from the intracellular space into the atmosphere, a process known as transpiration (Raven *et al.*, 1999). The availability of light and carbon dioxide for photosynthesis, and the need to maintain plant water status, are major factors affecting the regulation of stomatal aperture and the optimisation of plant growth. There

is a clear positive relationship between the total amount of water transpired by a crop and its yield (Bacon, 2004).

1.1 WATER USE EFFICIENCY

The positive relationship between transpiration and yield was first revealed in the 'Akron series' created by Briggs and Shantz in 1914, in which the transpiration ratio, the ratio of water transpired to dry weight produced, was determined for 62 plant species (Briggs and Shantz, 1914; Bacon, 2004). Water use efficiency (WUE) is either discussed in terms of instantaneous measurement of the efficiency of carbon gained to water loss; or as an integral of such efficiency over time, frequently expressed as a ratio of water use to biomass accumulation or harvestable yield (Bacon, 2004). The transpiration efficiency (TE; the leaf contribution to water use efficiency at the plant, crop, or ecosystem level; Farquhar *et al.*, 2007) can be defined; either at the whole plant level (TE_p), as biomass accumulation or crop yield per unit of water transpired; or at the leaf level as, the ratio A/g_s between CO_2 assimilation rate (A) and stomatal conductance (g_s) gives TE_i, the intrinsic transpiration efficiency (Condon *et al.*, 2002). N.B. The terms WUE and TE (and other associated terms) are used interchangeably in the literature and their definitions are contentious since the same terms have been differently defined by different authors.

It is now accepted that the concentration gradient between CO_2 outside and inside of the leaf (C_i/C_a), may be evaluated indirectly by measuring the carbon isotope composition of plant (C_3 species) dry matter (Farquhar and Richards, 1984; Farquhar *et al.*, 1989) and that C_i/C_a is related to TE_i because it is influenced by both stomatal aperture and rate of photosynthesis. The ^{13}C isotope of carbon comprises about 1% of the carbon in atmospheric CO_2. The proportion of ^{13}C to ^{12}C in dry matter of plants is less than in atmospheric CO_2 because the plant discriminates against ^{13}C during photosynthesis. Carbon isotope discrimination ($\Delta^{13}C$) is a measure of the $^{13}C/^{12}C$ ratio in

plant material relative to the same ratio in the atmosphere. Tissues with lower $\Delta^{13}C$ exhibit enhanced intrinsic WUE (TE_i) (Bacon, 2004). A low $\Delta^{13}C$ could be the result of reduced stomatal conductance, increased photosynthetic capacity, or both (Farquhar *et al.*, 1982). Therefore, using $\Delta^{13}C$ alone does not give any information about the relative contribution of photosynthetic or stomatal control to the internal CO_2 concentration in determining WUE. The use of isotopes of oxygen can assist in this matter. When water evaporates from the sub-stomatal cavity, the leaf becomes enriched in O^{18} due to the enhanced evaporation of H_2O^{16} molecules relative to the heavier H_2O^{18} molecules. The overall effect is that increased stomatal conductance results in a decrease in H_2O^{18} enrichment. The clear relationship between transpiration and productivity makes screening for genotypes with low $\Delta^{18}O$ (high transpiration) and low $\Delta^{13}C$ (high WUE) a powerful tool for identifying high yielding water efficient crops (Bacon, 2004).

In the context of the research reported herein it is important to note that since crop yield and/or biomass accumulation are often positively related to the total amount of water transpired, it is possible that increasing abscisic acid (ABA) production to reduce g_s and limit transpiration could simply suppress biomass production and affect crop yield (Boyer, 1982; Condon *et al.*, 2004; Blum, 2005). However, there is also evidence for excessive, non-productive transpiration under well-watered conditions; for instance a computer simulation, in which a limit was placed on maximal *E* (transpiration rate) in sorghum (*S. bicolor*) resulted in a slight increase in yield over the 115 seasons included in the trial. This increase was mainly due to improved yield in dry, low-yielding years (Sinclair *et al.*, 2005). This was associated with an improved water use efficiency since transpiration had been reduced during periods when atmospheric demand (VPD; vapour pressure deficit) was greatest, i.e. when water loss would be greatest.

Additionally, a decline in g_s and transpiration is generally proportionally greater than the associated reduction in *A* (Jones, 1976; Hetherington and Woodward, 2003); an increase in TE_i is therefore often observed as g_s decreases. As a result of this

nonlinear relationship, a given reduction in g_s has a small effect on *A* at high g_s but has a much larger effect on *A* at lower values of g_s, which was demonstrated experimentally by the shape of the *A*/g_s curve obtained from WT and a range of transgenic tomato lines overexpressing *SlNCED1* (Thompson *et al.*, 2007a). Interestingly, in these transgenic tomato lines, and conforming to theoretical expectations (Farquhar *et al.*, 2007), the increased ABA content was associated with a decrease in g_s, reflected in an increase in organic $\delta^{18}O$ (oxygen isotope composition), and an increase in WUE with the associated increase in $\delta^{13}C$ (Farquhar *et al.*, 2007; Thompson *et al.*, 2007a). It is possible that ABA may reduce assimilation by limiting g_s in some situations, but it may also benefit growth and development via improved plant water status, and/or through its effect on signalling pathways and interactions with other hormones (Thompson *et al.*, 2007a).

1.2 ABSCISIC ACID

ABA is often called a stress hormone as it is necessary for the induction of a variety of adaptive responses to environmental stress, particularly drought (section 1.8.1) and salinity (Nambara and Marion-Poll, 2005). ABA also appears to be involved in: promoting and or inhibiting growth during vegetative development (section 1.8.2) (Barrero *et al.*, 2005); abscission and vegetative dormancy (Roberts *et al.*, 2002; Schwartz *et al.*, 2003); and it undoubtedly plays a major role in the development, germination and dormancy of seeds (section 1.5) (Hilhorst and Karssen, 1992; Bewley, 1997b). The level of ABA in different tissues is controlled by the rates of ABA biosynthesis (section 1.3), catabolism (section 1.4) and redistribution (section 1.8) (Nambara and Marion-Poll, 2005).

ABA is a weak acid (pKa = 4.8) and therefore distributes according to the Henderson-Hasselbach equation, which results in the accumulation of ABA in alkaline anion traps (section 1.8.1) (Hartung and Davies, 1991). The chemical formula of ABA is $C_{15}H_{20}O_4$ and the naturally occurring form of ABA is the 2-*cis*, 4-*trans* geometrical

isomer (Figure 1.2.1). On exposure to UV light the 2-*cis*-double bond is reversibly isomerised to the inactive *trans* form 2-*trans*,4-*trans* ABA (Figure 1.2.1) (Buchanan *et al.*, 2000; Cutler *et al.*, 2010).

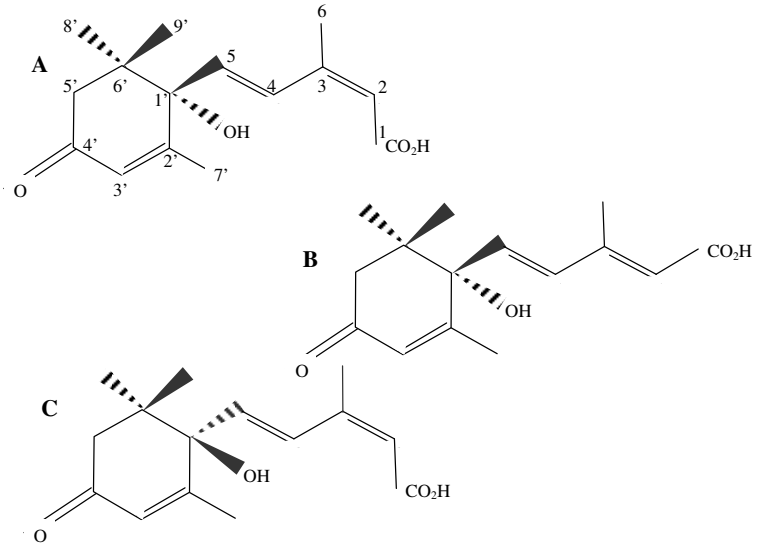

Figure 1.2.1 The structure of ABA isomers. **A**: The naturally occurring *S*-(+)-abscisic acid enantiomer, 2-*cis*, 4-trans-ABA. **B**: The biologically inactive reversibly photoisomerised (*S*)-(+)-2-*trans*, 4-trans ABA. **C**: The synthetic *R*-(-)-2-*cis*, 4-trans abscisic acid enantiomer. Adapted from Abrams et al., (1989); Lin et al., (2005); and Zaharia et al., (2005)

ABA exists as two enantiomers (stereoisomers that are mirror images of each other) the naturally occurring *S*-(+)-ABA and the synthetic optical isomer *R*-(-)-ABA form (Figure 1.2.1) which has not been reported in nature (Lin *et al.*, 2005; Zaharia *et al.*, 2005). *R*-(-)-ABA and racemic ABA (a synthetic commercially available 1:1 mixture of the two enantiomers) do not necessarily have the same effects as the natural *S*-(+)-ABA, they can differ in eliciting physiological responses and inducing gene expression (Walker-Simmons *et al.*, 1992); other differences are reviewed by Cutler *et al.,* (2010), Lin *et al.,* (2005) and Zaharia *et al.,* (2005). It should be noted that: *R*-(-)-ABA is metabolised at a slower rate than *S*-(+)-ABA (Abrams *et al.*, 1989), that *R*-(-)-ABA has little effect on stomatal aperture (Walton, 1983 cited in Zaharia *et al.*, 2005), but that in seed germination studies in wheat (Walker-Simmons *et al.*, 1992) and *Arabidopsis* (Nambara *et al.*, 2002) the two enantiomers had similar activity.

R-(-)-ABA and *S*-(+)-ABA are metabolised to different products: *S*-(+)-ABA is principally converted to phaseic (PA) and dihydrophaseic (DPA) acid, and the unnatural *R*-(-)-ABA is metabolised at a slower rate, primarily to 7′-hydroxy ABA (7′-OH ABA) (Hampson *et al.*, 1992; Lin *et al.*, 2005; see section 1.4 for further information on ABA catabolism). Lin *et al*., (2005) supplied deuterium labelled ABA enantiomers to *Marsilea quadrifolia* (four leaf clover) plants to trace the fate of applied ABA and to distinguish the applied ABA from endogenous ABA. They reported that *R*-(-)-ABA can induce the biosynthesis of *S*-(+)-ABA in plants tissues, as the levels of *S*-(+)-ABA and its metabolites increased.

1.3 ABA BIOSYNTHESIS

ABA is a sesquiterpenoid (C_{15}) molecule which is synthesized in plants via an indirect pathway using carotenoid (C_{40}) precursors (Zeevaart and Creelman, 1988). Carotenoids are plant pigments that, as well as being phytohormone precursors, also function as colourants, antioxidants and essential components of the photosynthetic apparatus (Howitt and Pogson, 2006). Carotenoids are synthesized in all types of plastids, including more or less 'colourless' plastids such as leucoplasts in roots (Hirschberg, 2001), but excluding the undifferentiated progenitor of all plastids, the proplastid (Howitt and Pogson, 2006). Plastids are present in most plant tissues and therefore carotenoids are found in most plant tissues. Genes encoding the enzymes for most of the steps in the ABA biosynthetic pathway have been cloned, and their functions confirmed using ABA-deficient mutants (North *et al.*, 2007).

1.3.1 The Plastidial/MEP Pathway

The C_{40} precursors of ABA are synthesized from the colourless carotenoid phytoene, which is formed in plastids by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. A schematic outline of part of the plastidial/MEP pathway can be seen in Figure 1.3.1.

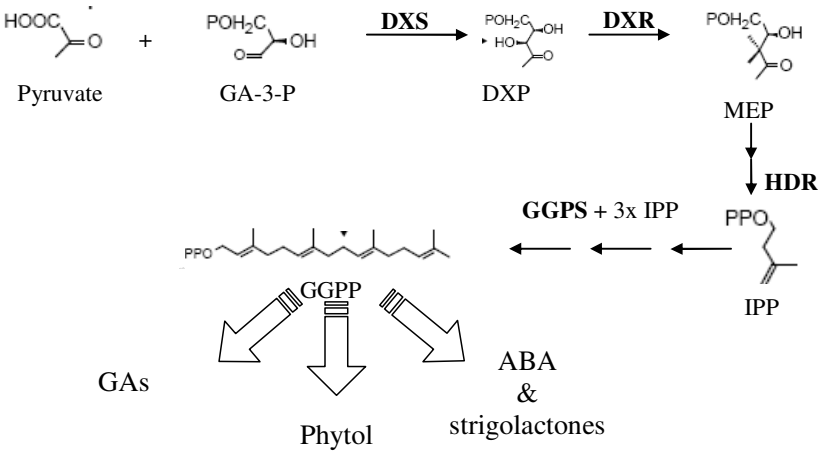

Figure 1.3.1 Outline of part of the plastidial/MEP pathway in higher plants leading to the C_{20} compound geranylgeranyl pyrophosphate (GGPP) and the subsequent involvement of GGPP in pathways leading to the production of ABA, phytol and gibberellins. Abbreviations of enzyme names are given in bold: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HDR, hydroxymethylbutenyl diphosphate reductase; GGPS, geranylgeranyl diphosphate synthase. GA-3-P, D-glyceraldehyde 3-phosphate; GAs, gibberellic acids. Note that GGPS performs three successive condensation reactions: isopentenyl diphosphate (IPP) is first condensed with its isomer DMAPP (not shown) forming GPP, before successively condensing each product with IPP to form FPP, and finally GGPP. Adapted from Hirschberg (2001), Taylor *et al.,* (2005) and Fray *et al.,* (1995).

Condensation of pyruvate and D-glyceraldehyde 3-phosphate (GA-3-P) to form 1-deoxy-D-glucose 5-phosphate (DXP) is catalysed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS). The conversion of DXP to 2-C-methyl-D-erythritol 4-phosphate (MEP) is catalysed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) (Taylor *et al.*, 2005). The enzyme hydroxymethylbutenyl diphosphate reductase (HDR) controls a branch point which results in the independent formation of the C_5 compound isopentenyl diphosphate (IPP) and its isomer DMAPP. IPP is condensed in three successive reactions with its isomer DMAPP to form the following compounds; C_{10} geranyl diphosphate (GPP), C_{15} farnesyl diphosphate (FPP), and C_{20} geranylgeranyl diphosphate (GGPP).

The C_{20} compound GGPP is precursor for the biosynthesis of: gibberellins, the phytol tail of chlorophylls, the phylloquinones involved in photosystem I (PSI), and

tocopherols, otherwise known as vitamin E (Taylor *et al.*, 2005). These pathways are all potentially in competition with the carotenoid biosynthesis pathway for the supply of GGPP (Taylor *et al.*, 2005).

The first committed step in carotenoid biosynthesis is the condensation of two molecules of (GGPP) to form the colourless C_{40} compound phytoene (Figure 1.3.2) which does not normally accumulate in tissues (Hirschberg, 2001; Howitt and Pogson, 2006). This reaction is catalysed by phytoene synthase (PSY), which is encoded by the *CrtB* gene in bacteria (Dogbo *et al.*, 1988; Bartley *et al.*, 1992; Armstrong, 1994). A fruit-ripening related PSY was isolated from pepper (*Capsicum annum*) chromoplast stroma by Dogbo *et al,* (1988).

All phytoene synthases are membrane associated, but are not integral membrane proteins (Fraser and Bramley, 2004). PSY has been identified as a key regulatory enzyme of carotenoid biosynthesis in ripening tomato fruits (Ray *et al.*, 1992; Fraser *et al.*, 1994) and in the seeds of members of the grass (*Poaceae*) family (Gallagher *et al.*, 2004). There is evidence that PSY gene expression can be regulated by phytochrome (red-light), in deetiolating white mustard (*Sinapis alba*) and *Arabidopsis* seedlings. In these two species PSY encoding transcripts were upregulated in response to light whereas other genes encoding other enzymes in the early carotenoid biosynthesis pathway, such as PDS were not (von Lintig *et al.*, 1997; Welsch *et al.*, 2000). Additionally, the *Arabidopsis PSY* promoter region showed G-box-like elements mediating light regulation (Welsch *et al.*, 2003).

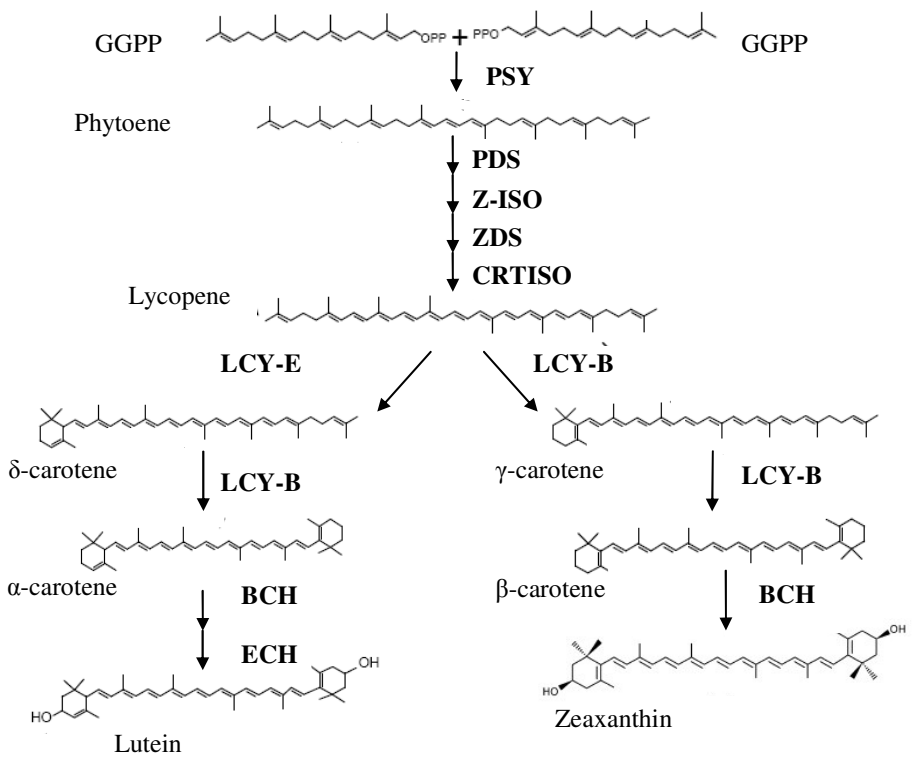

Figure 1.3.2 Schematic of carotenoid biosynthesis pathway in higher plants, including the branch that leads to lutein and the branch that leads to ABA via zeaxanthin. Abbreviated enzyme names are written in bold: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; Z-ISO, carotenoid isomerase 15-*cis*-ζ-CRTISO; LCY-B, lycopene-β-cyclase; LCY-E, lycopene-ε-cyclase; ECH, ε-ring hydroxylase; BCH, β-carotene hydroxylase. N.B. Schematic only includes structure of all-*trans*-isomers, for simplicity of layout. Adapted from Hirschberg (2001) and Taylor *et al.,* (2005)

There is also relatively recent evidence for a feedback mechanism in which PSY can affect metabolic flux by feedback regulation to DXS; transgenic overexpression of PSY in etiolated *Arabidopsis* seedlings resulted in increased carotenoid levels via a post-transcriptional accumulation of DXS mRNA (and presumably also directly due to the increased *PSY* expression), while the overexpression of just DXS did not result in increased carotenoid accumulation, which suggests a feedback mechanism by PSY to supply MEP substrates (Rodriguez-Villalon *et al.*, 2009a,b; Cazzonelli and Pogson, 2010).

Arabidopsis has only one *PSY* gene, while in other plant species there can be two or more PSY homologues. In tomato there are two genes for PSY; encoding PSY1,

found in chromoplasts; and PSY2 found in chloroplasts (Bartley and Scolnick, 1993; Fraser *et al.*, 1999; Fraser *et al.*, 2000). In tomato, *PSY1* is encoded by the *R* gene and the *yellow flesh* (*r*) tomato mutant has an almost carotenoid-free pericarp in the ripe fruit together with pale yellow mature petals (Fray and Grierson, 1993). Two PSY genes have also been identified in carrot (*Daucus carota*; Just *et al.*, 2007) and cassava (*Manihot esculenta*, unpublished data reported in Welsch *et al.*, 2008 and Mass *et al.*, 2009). A third member of the *PSY* gene family, *PSY3*, has been relatively recently identified in members of the grass family (Poaceae) including; rice, maize and sorghum (Li *et al.*, 2008; Welsch *et al.*, 2008). In maize, *PSY3* expression has been found to influence root carotenogenesis and is up-regulated in response to salt and drought stress (Li *et al.*, 2008). There were similar findings in rice (*Oryza sativa*) where *OsPSY3* transcripts were found to be up-regulated in response to salt treatment and drought, especially in roots (Welsch *et al.*, 2008). Additionally, it appeared that there may be a level of positive feedback regulation by ABA on *OsPSY3* gene expression (Welsch *et al.*, 2008) (PSY overexpression is discussed in section 1.9.3).

Four desaturation reactions catalyse the conversion of the colourless pigment phytoene into the red carotenoid lycopene by successively introducing double bonds to create the characteristic polyene chain/chromophore. In bacteria, these reactions are mediated by a single enzyme, CrtI (Linden *et al.*, 1991). In plants, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) each catalyse two of the desaturation reactions. PDS introduces two *trans* double bonds transforming 15-*cis*-phytoene to 9,9′-di-*cis*-ζ-carotene. ZDS then introduces two *cis* double bonds to form a tetra-*cis*-lycopene known as prolycopene (poly-*cis*-lycopene) (Hirschberg, 2001; Taylor *et al.*, 2005; Howitt and Pogson, 2006). Additional isomerization reactions are also required to generate the correct geometric isomer substrates for the desaturases and the following cyclization steps (Beyer *et al.*, 1989). In the chloroplasts of green tissues photoisomerisation of ζ-carotene, neurosporene, and prolycopene is associated with the photosynthetic apparatus

(Isaacson *et al.*, 2002). The product of the first desaturation is 9,15,9′-tri-*cis*-ζ-carotene which is isomerised by light and a relatively newly discovered isomerase 15-*cis*-ζ-CRTISO (Z-ISO; Li *et al.*, 2007) to yield 9,9′-di-*cis*-ζ-carotene, the substrate for ZDS (Breitenbach and Sandmann, 2005). Characterisation of the maize (*Zea mays*) *pale yellow9* (*y9*) locus (Li *et al.*, 2007) provided evidence supporting Z-ISO activity; in the absence of light, the 9,15,9′-tri-*cis*-ζ-carotene isomer accumulates in etiolated leaves and roots of *y9* maize plants, in contrast to accumulation of 9,9′-di-*cis*-ζ-carotene in a maize mutant defective in ZDS activity, *viviparous9* (*vp9*). However, it remained unclear from this study as to whether *y9* encoded Z-ISO or regulated it's expression (Li *et al.*, 2007). Analysis of additional *Z-ISO* maize and *Arabidopsis* mutants allowed the gene to be identified, and functional testing of the gene product in *E. coli* showed isomerization of the 15-*cis* double bond in 9,15,9′-tri-*cis*-ζ-carotene (Chen *et al.*, 2010).

It was previously suggested that photosynthetic tissues may not require Z-ISO because light could trigger photoisomerization, but there is evidence that Z-ISO may also be active in photosynthetic tissues to ensure optimal levels of isomerisation. Light-exposed homozygous *y9* plants had reduced carotenoids in pale green striped regions, a phenotype which was enhanced in response to temperature fluctuations (Robertson, 1975; Janick-Buckner *et al.*, 2001). *Arabidopsis* mutant plants impaired in Z-ISO activity exhibit a delayed greening phenotype and contain lower levels of carotenoids and chlorophylls. Additionally, the gene encoding Z-ISO was found to be highly expressed in green leaf tissue (Chen *et al.*, 2010). These results suggest that photoisomerisation can not entirely account for isomerization in photosynthetic tissues, and indicates that Z-ISO is important in these tissues, particularly during abiotic stress (Li *et al.*, 2007; Chen *et al.*, 2010).

The first plant isomerase to be identified, carotenoid isomerase (CRTISO), was characterised through map based cloning of the tomato fruit mutant *tangerine*, which has mature orange fruit that accumulate prolycopene instead of all-*trans*-lycopene

(Isaacson *et al.*, 2002). An *in vitro* enzymatic assay of purified tomato CRTISO polypeptide overexpressed in *E. coli* demonstrated that CRTISO isomerase activity followed ζ-carotene desaturation, being specific for the conversion of prolycopene to all-*trans*-lycopene (Isaacson *et al.*, 2004). The *Arabidopsis* orthologue of *tangerine* is *Ccr-2* (Park *et al.*, 2002).

1.3.2 The α- and β- Branch Points of the Pathway

Different routes for the cyclization of lycopene represent an important branch point in the carotenoid biosynthetic pathway (Figure 1.3.2): the β,β branch leads to β-carotene and its derivatives, while the β,ε branch leads to α-carotene and its derivatives, notably lutein (Hirschberg, 2001; Taylor *et al.*, 2005). These alternative routes can be more simply referred to as the α- and β- branches of the pathway. Lycopene β-cyclase (LCY-B/CrtL-B) catalyses the formation of β-carotene by introducing a β-ring onto either end of lycopene via the intermediate γ-carotene (Howitt and Pogson, 2006). The tomato *Beta* (*B*) mutant causes the continued expression of lycopene β-cyclase (LYC-B) in fruit chromoplasts, which results in orange coloured ripe fruit due to the accumulation of β-carotene and depletion of lycopene (Ronen *et al.*, 2000; Galpaz *et al.*, 2006). The *old-gold* (*og*) and *old-gold-crimson* (og^c) mutants of tomato are null mutants in the *B* locus which normally encodes (LYC-B). The inability of *og* and og^c mutant homozygotes to synthesise β-carotene in chromoplasts of mature flowers causes a build up of lycopene, which results in mature petals having a reddish gold colouration (Ronen *et al.*, 2000; Galpaz *et al.*, 2006). In the α- branch, lycopene ε-cyclase (LCY-E/CrtL-E) introduces an ε-ring to one end of the lycopene molecule to form δ-carotene and a β–ring is introduced by LCY-B to the other end to form α-carotene (Cuttriss and Pogson, 2004). In tomato mutant *Delta* (*Del*), the mature fruit is orange as a result of the accumulation of δ-carotene, rather than lycopene, due to the upregulation of the gene encoding LCY-E during fruit ripening. (Ronen *et al.*, 1999). Carotenoid synthesis via the β- and α-

branches appears to be tightly regulated, balanced and compensatory (Taylor *et al.*, 2005). β-carotene represents a major fraction of the total carotenoid content of photosynthetic tissues, and the importance of conserving pool size for this compound has been noted (Taylor *et al.*, 2005). It should be noted that only the β-branch of the pathway leads to the formation of ABA.

1.3.3 Xanthophyll Synthesis and Cleavage

α-carotene and β-carotene can be further modified to produce xanthophylls, oxygenated carotenoids, some of which are accessory pigments in the light harvesting antennae of chloroplasts and are capable of transferring energy to the chlorophylls (Horton *et al.*, 1996; Niyogi, 1999). They also quench triplet excited states in chlorophyll molecules by dissipating excess excitation energy in a non-radiative manner, a process known as non-photochemical quenching (NPQ) (Hirschberg, 2001). Hydroxylation of the β- and ε-rings are carried out by β-carotene hydroxylase (BCH/CrtR-b) and ε-carotene hydroxylase (ECH/CrtR-e) enzymes respectively (Howitt and Pogson, 2006). The action of these two enzymes leads to the formation of lutein, the major end product of the β,ε branch (Figure 1.3.2). In the β- branch, BCH (CrtR-B) enzymes catalyse two steps, forming zeaxanthin via β-cryptoxanthin (Howitt and Pogson, 2006). The C_{40} precursors of ABA are the major β-xanthophylls zeaxanthin, violaxanthin, and neoxanthin (North *et al.*, 2007).

Two genes encoding β-carotene hydroxylase (BCH/CrtR-B) have been reported in: *Arabidopsis* (Sun *et al.,* 1996; Tian & DellaPenna, 2001) pepper (*Capsicum annuum* L.) (Bouvier *et al.*, 1998), and tomato (Galpaz *et al.*, 2006). The *white-flower* (*wf*) tomato mutant which has white/beige petals and pale anthers in mature flowers is caused by a mutation of the *SlBCH2* (*CrtR-b2*) gene locus which somehow prevents xanthophyll accumulation in mature petals, but does not affect xanthophyll accumulation in leaves (Galpaz *et al.*, 2006). In contrast, the *SlBCH1* (*CrtR-b1*) gene expression was

reported to be high in leaves and low in mature petals (Galpaz *et al.*, 2006). There is evidence that the expression of *BCH1* has a circadian rhythm in photosynthetic tissues (Sonneveld *et al.*, unpublished data); its mRNA accumulation anticipated the light period similar to that of *ZEP* and *LHCII* (Thompson *et al.*, 2000a) but differed from *ZEP* and *LHCII* in that peak mRNA levels were detected 11 h into the dark period (an hour before the light period; Sonneveld *et al.*, unpublished data). This diurnal pattern of expression suggests that the *BCH1* gene may primarily be involved in photosynthesis related processes, providing zeaxanthin and other xanthophylls for photoprotection and as accessory pigments, rather than ABA biosynthesis (Sonneveld *et al.*, unpublished data). There is additional evidence that, in vegetative tissues, BCH2 may function to supply xanthophyll precursors required to sustain water stress-induced ABA biosynthesis, since dehydration induces the expression of *BCH2* mRNA, even in leaves (Sonneveld *et al.*, unpublished data) (The overexpression of BCH2 is discussed in section 1.9.2).

Zeaxanthin can in some ways be regarded as the starting point of the ABA biosynthetic pathway, since mutants blocked downstream of zeaxanthin have symptoms typical of ABA rather than carotenoid deficiency (Taylor, 1991; see Figure 1.3.3). The two epoxidation reactions involved in the conversion of zeaxanthin to violaxanthin via the intermediate antheraxanthin are catalysed by zeaxanthin epoxidase (ZEP) (Marin *et al.*, 1996; North *et al.*, 2007). Zeaxanthin and violaxanthin are interconvertable; the interconversion of these xanthophylls is known as the xanthophyll cycle (Hirschberg, 2001). Studies involving *Nicotiana plumbaginifolia* (Audran *et al.,* 1998) and tomato (Thompson *et al.*, 2000a) have revealed that steady state *ZEP* mRNA levels in leaves are under the control of a circadian oscillator, as mentioned earlier in the context of *BCH2* transcript levels. *ZEP* mRNA peaks during the day and falls before the dark period, increasing again about an hour before dawn (Taylor *et al.*, 2005). In photosynthetic tissues no increase in *ZEP* mRNA was detected in response to water deficit, the circadian

control appears to result in an adequate supply of violaxanthin for stress responses in these tissues.

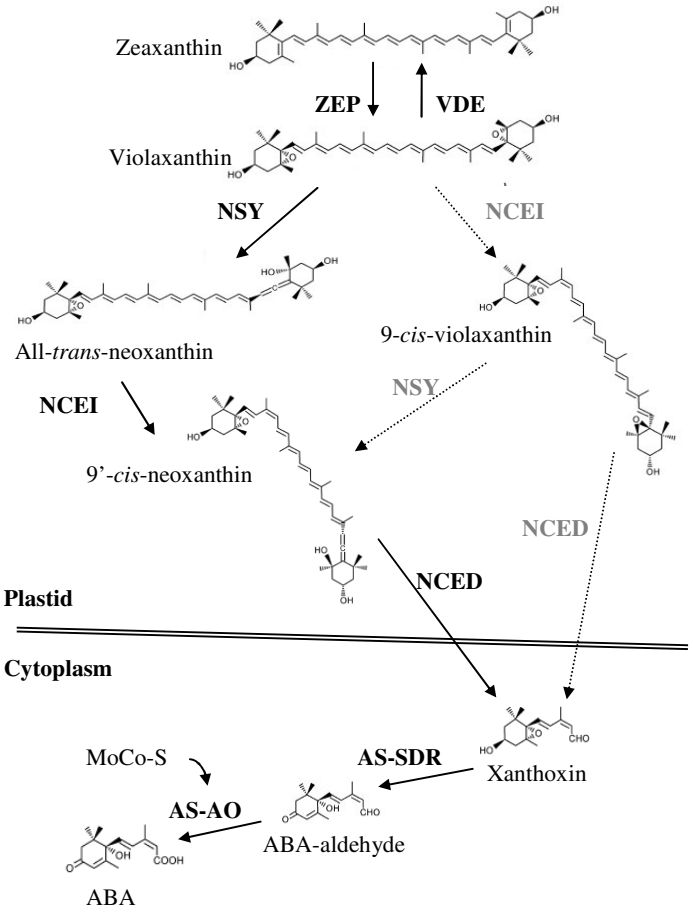

Figure 1.3.3 Schematic of ABA biosynthesis pathway in higher plants downstream from zeaxanthin, excluding the branch that leads to lutein and only featuring the branch that leads to ABA. Abbreviated enzyme names are written in bold: ZEP, zeaxanthin epoxidase; VDE, violaxanthin deepoxidase NSY, neoxanthin synthase; NCEI, 9-*cis*-epoxycarotenoid-forming isomerase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; AS-SDR, ABA-specific short-chain dehydrogenase/reductase; AS-AO, ABA specific aldehyde oxidase; MoCo-S, mono-oxo Molybdenum Cofactor. Dashed arrows, as opposed to solid arrows, represent the less favoured route. Adapted from Hirschberg (2001), Nambara & Marion-Poll (2005) and Taylor *et al*., (2005)

The situation is different in root tissues because they have much lower levels of carotenoids in comparison to leaves (Parry and Horgan, 1992). It has been found that steady state *NpZEP* mRNA (Audran *et al*, 1998) and *SlZEP* mRNA (Thompson *et al.*, 2000a) levels in roots increased in response to water stress. It therefore appears that the control of *ZEP* gene expression is one of the rate-determining steps in ABA biosynthesis

in non-photosynthetic tissues, where violaxanthin is not already in abundance (Thompson *et al.*, 2004; Taylor *et al.*, 2005).

Under high light stress, violaxanthin de-epoxidase (VDE) catalyses the de-epoxidation of violaxanthin back to zeaxanthin (Pfundel and Bilger, 1994). The pH-catalysed post-translational activation of VDE (Gilmore, 2001) enables plants to cope with sudden increases in light levels, by rapid elevation of zeaxanthin content to prevent photobleaching (Taylor *et al.*, 2005). Long-term high light intensities decrease *VDE* mRNA levels and increase *BCH* mRNA levels (Rossel *et al.*, 2002), which indicates that increasing zeaxanthin synthesis via β-carotene, rather than from violaxanthin, represents a more appropriated longer term response to high light intensities (Taylor *et al.*, 2005).

After the synthesis of violaxanthin there is some ambiguity as to the exact route of ABA synthesis prior to the oxidative cleavage that forms xanthoxin, (Taylor *et al.*, 2005) as indicated in Figure 1.3.3. Two aspects of the pathway remain to be clarified: firstly, the identity of the enzymes involved in neoxanthin synthesis and isomerization; secondly, the *in vivo* substrates for oxidative cleavage to form xanthoxin. Violaxanthin is converted to neoxanthin by neoxanthin synthase (NXS). In light-grown tomato leaves all-*trans*-violaxanthin was reported to represent approximately half (49.9 %) of the total epoxyxanthophyll content, with 9′-*cis*-neoxanthin making up most of the other half (46.6%). The all-*trans*-isomer of neoxanthin and 9-*cis*-violaxanthin represented only 0.85% and 0.34% respectively (Parry *et al.*, 1990). Two enzymes are probably responsible for synthesizing 9′-*cis*-neoxanthin from this large pool of all-*trans*-violaxanthin: neoxanthin synthase (NSY) and a putative nine-*cis* epoxycarotenoid-forming isomerase (NCEI) (Taylor *et al.*, 2005). Two homologous genes cloned from either tomato or potato were initially proposed to be putative neoxanthin synthases (NSY) (Al-Babili *et al.,* 2000; Bouvier *et al.*, 2000). ABA biosynthesis is significantly reduced in *aba4* mutants of *Arabidopsis* in response to dehydration compared with the WT and leaves of this mutant have high levels of all-trans-violaxanthin and abnormally low

amounts of 9′-cis-neoxanthin. North *et al*. (2007) concluded that *ABA4* encodes a membrane protein involved in neoxanthin synthesis, although they were unable to express a functional version of the enzyme in *E. coli*.

The first committed step in ABA biosynthesis as a specific reaction is the oxidative cleavage of 9-*cis*-epoxycarotenoids to produce the first C_{15} intermediate, xanthoxin, which is transited from the plastid to the cytoplasm (North *et al.*, 2007). The maize (*Zea mays*) *VP14* mutant is mutated in a 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) gene (Schwartz *et al.,* 1997; Tan *et al.*, 1997). *NCED* genes have subsequently been identified in several other plant species, such as tomato (Burbidge *et al.*, 1997; Burbidge *et al.*, 1999), bean (*Phaseolous vulgaris*) (Qin and Zeevaart, 1999), cowpea (*Vigna unguiculata*) (Iuchi *et al*., 2000), avocado (*Persea americana*) (Chernys & Zeevaart, 2000) and *Arabidopsis* (Iuchi *et al.,* 2001).

Plant *NCED* genes typically form multigene families which show different tissue specific patterns of expression and environmental responsiveness in different family members. Of the nine genes expressing sequence similarity to *VP14* in *Arabidopsis, AtNCED 2, 3, 5, 6* and *9* appear to encode enzymes with NCED activity and are likely to be involved in ABA synthesis (Iuchi *et al.*, 2001; Tan *et al.*, 2003). It has been demonstrated that the ABA-deficient wilty tomato mutant *notabilis* is a null allele of *SlNCED1,* containing a single A/T base pair deletion (Burbidge *et al.*, 1999). *SlNCED1* has a diurnal cycle of expression in non-stressed plants (Thompson *et al.*, 2000a), with expression peaking during the light period and with no circadian component. This is consistent with either a direct response to light or perhaps the drop in water status resulting from the imbalance of water uptake and transpiration during the light period (Taylor *et al.*, 2005). During drought stress, *SlNCED1* mRNA rapidly increases in both leaves and roots of tomato (Thompson *et al.*, 2000a) and *AtNCED3* is the orthologous member of the *Arabidopsis NCED* family which is most strongly responsive to water stress (Iuchi *et al.*, 2001) (Section 1.9.1).

In most plant tissues, 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin are the only 9-*cis* epoxycarotenoid isomers found, so they are the most likely NCED substrates (Schwartz *et al.*, 2003). The oxidative cleavage of both 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin can occur *in vitro* but the former is probably the preferred substrate *in planta*, as when it is absent, e.g. in *aba4* mutants, insufficient ABA is synthesized in response to water deficit (North *et al.*, 2007).

1.3.4 The C15 Level of the Pathway

In Figure 1.3.3 it can be seen that xanthoxin is converted to abscisic aldehyde by an ABA specific short chain dehydrogenase/reductase (AS-SDR). In *Arabidopsis* this is catalysed by SDR1 (which is encoded by the *ABA2* gene) (Rook *et al.*, 2001; Cheng *et al.*, 2002; González-Guzmán *et al.*, 2002). The oxidation of abscisic aldehyde by an abscisic aldehyde oxidase (AS-AO) to ABA is the final step in ABA biosynthesis (Sindhu and Walton, 1988; Taylor *et al.*, 1988). The aldehyde oxidase requires a molybdenum cofactor (MoCo) for its catalytic activity (Leydecker *et al.*, 1995). Two wilty mutants of tomato, *sitiens* and *flacca,* were the first genetic lesions shown to be impaired in this final stage of ABA biosynthesis (Taylor *et al.*, 1988; Leydecker *et al.*, 1995). Four aldehyde oxidase (AO) genes were investigated in *Arabidopsis* and only one of these, *AAO3*, encoded an ABA-specific aldehyde oxidase (AS-AO). It appears that AAO3 is the major AO catalysing ABA synthesis in leaves in response to water stress (Seo *et al.*, 2000). The *sitiens* tomato mutant may be impaired in an AS-AO (Marin and Marion-Poll, 1997); in support of this, constructs based on the *AtAAO3* gene were found to complement the mutation (Okamoto *et al.*, 2002) but although putative tomato aldehyde oxidases (TAO's) have been identified (Ori *et al.*, 1997; Min *et al.*, 2000) the gene for *sitiens* has remained elusive (Seo *et al.*, 2000).

The *flacca* mutant allele, on the other hand, has been shown to be a 6 bp out-of-frame deletion in the MoCo sulfurase (Sagi *et al.*, 2002) and is orthologous to the

Arabidopsis aba3 mutation which is also impaired in MoCo sulfurase (Bittner *et al.*, 2001). Rapid induction of the *ABA3* gene was reported upon drought and salt stress in *Arabidopsis* as well as upon ABA treatment (Xiong *et al.*, 2001). MoCo sulfurase converts the di-oxo form of MoCo (MoCo-O) to the mono-oxo form of MoCo by adding a terminal inorganic sulphur (MoCo-S) (Bittner et al., 2001). A mutational block in any of the early steps in MoCo biosynthesis can lead to the combined loss of function of all four of the known plant molybdenum dependent enzymes: aldehyde oxidase (AO), nitrate reductase (NR), peroxisomal sulfite oxidase (SO) and xanthine dehydrogenase (XDH). Mutants resulting in the loss of MoCo-dependent AO activity, will therefore also be impaired in ABA biosynthesis. There are numerous reviews covering MoCo biosynthesis (for example: Schwarz, 2005; Mendel and Bittner, 2006; Mendel, 2007; Schwarz *et al.*, 2009) and the reader is directed to these for further information on MoCo biosynthesis genes and any mutations that may then affect the production of ABA.

1.4 ABA CATABOLISM

ABA catabolism involves hydroxylation and/or conjugation, both of which play a role in determining endogenous ABA levels. The main ABA catabolites are shown in Figure 1.4.1. The major ABA catabolism pathway begins with the hydroxylation of ABA by ABA 8′-hydroxylase, a cytochrome P450, to produce 8′-hydroxyl ABA. This unstable compound readily isomerizes to phaseic acid (PA), which can be further catabolised to dihydrophaseic acid (DPA) by an unknown reductase (Gillard and Walton, 1976; Krochko *et al.*, 1998; Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005; Okamoto *et al.*, 2009). It has been shown that exogenously applied ABA can be rapidly degraded by this route (Huang *et al.*, 2007) and inhibitor and labelling studies have suggested a rapid turnover of ABA in both unstressed and dehydrated plant tissue (Cornish and Zeevaart, 1984; Priest *et al.*, 2006; Ren *et al.*, 2007). Overexpression of *AtNCED3* in transgenic plants resulted in increases in ABA content under stress

conditions, and also produced increased levels of PA (Iuchi *et al.*, 2001; Qin and Zeevaart, 2002; Priest *et al.*, 2006). While, the overproduction of a bean NCED1 enzyme (PvNCED1) in tobacco (*N. plumbaginifolia*) also resulted in increased levels of PA.

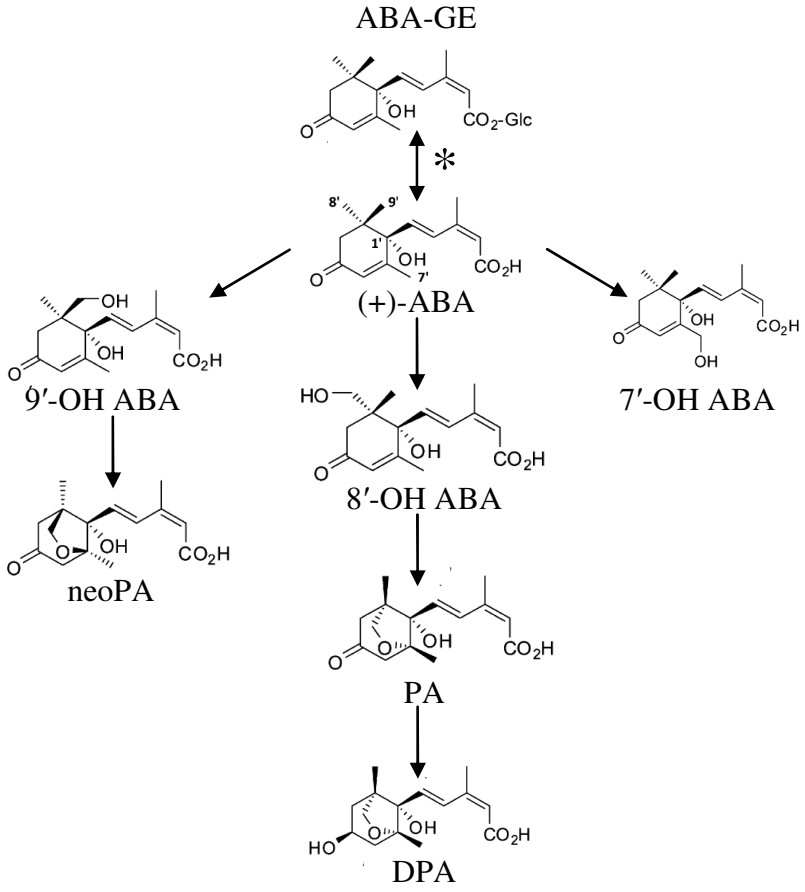

Figure 1.4.1 Schematic of the main catabolic pathways of ABA in plants. The major hydroxylation pathway is catalysed by 8′-hydroxylase to produce 8′-hydroxy-ABA (8′-OH ABA), which isomerizes to phaseic acid (PA), which is then converted to dihydrophaseic acid (DPA). The minor hydroxylation pathways result in: 9′-hydroxy-ABA (9′-OH ABA), which is then converted to neophaseic ABA (neoPA); and 7′-hydroxy-ABA (7′-OH ABA). The major ABA conjugate is the ABA glucose ester (ABA-GE). * Related glucose esters of other ABA metabolites e.g. PA-GE, can also be formed along with various glucosides. Adapted from Shimomura *et al*., (2007).

In *Arabidopsis* the *CYP707A* four member gene family encodes ABA 8′-hydroxylases (Kushiro et al., 2004; Saito et al., 2004). During drought stress, all *CYP707A* genes were upregulated and upon rehydration a continued elevation in mRNA level was observed (Kushiro et al., 2004). The transcripts of *CYP707A* genes were also observed to increase in response to salt, osmotic and dehydration stresses, as well as

exogenous ABA (Saito *et al.*, 2004). The expression of the *CYP707A3* gene was particularly responsive to dehydration, the *cyp707a3* mutant contains higher ABA levels than wild type during dehydration, suggesting that CYP707A3 functions to primarily catabolise ABA during water stress (Umezawa *et al.*, 2006). Each *CYP707A* gene appears to play a distinct role in seed development and post-germination growth (section 1.5) (Okamoto *et al.*, 2006). Relatively recent evidence has suggested that CYP707A1 is essential for ABA catabolism inside guard cells (section 1.7), since stomatal closure in the *Arabidopsis cyp707a1* mutant, but not *Arabidopsis cyp707a3* mutant was reported to be ABA hypersensitive when epidermal peel was treated with exogenous ABA (Okamoto *et al.*, 2009).

Analysis of spatial expression patterns using transgenic plants with the *CYP707A1* and *CYP707A3* promoters driving the β-glucuronidase reporter gene indicated that high-humidity induced expression of *CYP707A1* occurred primarily in the guard cells, while the *CYP707A3* construct was primarily expressed in the vascular tissues (Okamoto *et al.*, 2009). Taken together these results imply that CYP707A3 reduces the amount of ABA in vascular tissues, whereas CYP707A1 inactivates ABA inside the guard cells (Okamoto *et al.*, 2009).

Two other minor oxidation pathways also exist: one of these involves the hydroxylation of the 7′-methyl group of ABA producing 7′-hydroxyl ABA (Zaharia *et al.*, 2005 and references therein), which has hormonal activity in ABA assays (Hill *et al.*, 1995) although catabolites of 7′-hydroxyl ABA have not yet been identified (Zaharia *et al.*, 2005); the other minor oxidative pathway involves the hydroxylation of ABA at the 9′-methyl group of ABA and was discovered during a mass spectrometric (MS) analysis of ABA metabolism in *Brassica napus* siliques (Zhou *et al.*, 2004). These results indicated that hydroxylation of ABA had occurred at the 9′-methyl group, as well as at the 7′- and 8′-methyl groups to produce the cyclized form of 9′-hydroxyl ABA, which was isolated from the plant extract and called *neo*phaseic ABA (neoPA) (Zhou *et al.*,

2004). NeoPA was also detected in fruits of orange (*Citrus sinensis*) and tomato, in *Arabidopsis*, and in chickpea (*Cicer arietinum*), as well as in drought-stressed barley (*Hordeum vulgare*) and *B. napus* seedlings (Zhou *et al.*, 2004). The open 9′-hydroxyl ABA, i.e. not the cyclised form, neoPA, showed ABA-like hormonal activity; for example it was able to inhibit *Arabidopsis* seed germination (Zhou *et al.*, 2004).

ABA and the metabolites of the major oxidative pathway can be conjugated to glucose (as well as other sugars) to form the biologically inactive compound ABA-glucose ester (ABA-GE) and other related compounds (Figure 1.4.1) (Cutler and Krochko, 1999). The glucose conjugation reaction can be catalyzed by glycosyltransferases (GTases). An ABA-inducible GTase from adzuki bean (*Vigna angularis*), encoded by the *AOG* gene, was identified and the level of the mRNA transcribed from this gene was observed to increase in response to both the application of ABA and water stress (Xu *et al.*, 2002). ABA-GE is found mostly in the vacuole and intercellular spaces. Contrary to earlier assumptions, it has been shown that ABA can be released by hydrolysis of ABA-GE by specific β-glucosidases (Lehmann and Vlasov, 1988; Dietz *et al.*, 2000; Sauter *et al.*, 2002; Lee *et al.*, 2006). The *Arabidopsis* β-glucosidase1 (AtBG1) enzyme is localized in the endoplasmic reticulum (Lee *et al.*, 2006) which is where many cellular stresses are monitored (Hirayama and Shinozaki, 2007). Dehydration quickly induces AtBG1 polymerization, which triggers the release of ABA from ABA-GE in a stress-dependent manner (Lee *et al.*, 2006). These results indicate that ABA catabolism plays a complex role in altering ABA concentration in response to external stimuli and developmental cues (Verslues and Zhu, 2007; Wasilewska *et al.*, 2008).

It has been suggested that the conjugated forms of ABA could act in the long distance transport of ABA (Hartung *et al.*, 2002; Wilkinson and Davies, 2002) and it has been shown that drought can increase ABA-GE concentrations in the xylem sap (Munns and King, 1988; Sauter *et al.*, 2002; Jiang and Hartung, 2008; Schachtman and Goodger,

2008). ABA biosynthesis and catabolism can both be affected by stress as well as being important in developmentally determined ABA accumulation (Verslues and Zhu, 2007).

1.5 ABA & SEEDS

Seed germination starts with imbibition of water by the quiescent dry seed and ends with the rupture of the covering layers, elongation of the embryonic axis and radicle emergence at which point germination is considered completed (Bewley, 1997b; Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008). Seed dormancy is the incapacity of a viable seed to germinate under favourable conditions and can involve coat imposed dormancy and/or embryo dormancy (Finch-Savage and Leubner-Metzger, 2006). The seeds of many angiosperms are dormant at maturity and this dormancy must be broken before germination can occur (Bewley, 1997b). Environmental factors such as light intensity and temperature are known to affect seed dormancy (Holdsworth *et al.*, 2008) and antagonistic regulatory roles for ABA and gibberellins (GAs) in dormancy and germination have been widely reported (Bewley, 1997b; Holdsworth *et al.*, 2008). ABA is involved in embryogenesis, the induction of dormancy during seed maturation, dehydration and the maintenance of the dormant state in fully imbibed seeds (Hilhorst and Karssen, 1992; Hilhorst and Downie, 1996; Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003).

Germination can be regarded as triphasic: initially, in phase I, there is a rapid and passive imbibition of water; phase II involves a plateau of water uptake and the initiation of metabolism during which new mRNAs and proteins are synthesised; in phase III an increase in water uptake occurs as the embryo axis elongates and breaks through the endosperm covering layers and testa to complete germination (Schopfer and Plachy, 1984; Manz *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006).

Baskin and Baskin (2004) defined a dormant seed as one that, although viable, does not have the capacity to germinate in a specified period of time under any

combination of physical/environmental factors that are normally favourable for germination. Finch-Savage and Leubner-Metzger (2006) pointed out that dormancy should not just be associated with the absence of germination, rather that it is a characteristic of the seed that defines the conditions required for germination (Finch-Savage and Leubner-Metzger, 2006 and references therein). Primary dormancy is the major form of dormancy in most seed model species (including plant family *Solanaceae*) and therefore will be the main focus of this section (for information on other forms of dormancy the reader is directed to Baskin and Baskin (2004) or Finch-Savage and Leubner-Metzger (2006)). Generally, primary dormancy can be broken either by after-ripening, which consists of a prolonged warm temperature treatment of dry seeds and/or by stratification, which consists of a low temperature treatment to imbibed seeds which may, or may not, have previously undergone an after ripening process. Secondary dormancy occurs when essentially non-dormant seeds are subjected to a period of dormancy maintained by environmental conditions unfavourable for germination. (Bewley, 1997b; Grappin *et al.*, 2000; Baskin and Baskin, 2004).

Seed germination has been proposed to be under the control of two distinct and independent programmes; one driving radicle emergence and the other driving reserve mobilization (Pritchard *et al.*, 2002). ABA is involved in the radicle emergence aspect of germination, acting to inhibit phase III. In order for the radicle to emerge from the seed and complete germination, it has to break through the surrounding endosperm and testa (Figure 1.5.1).

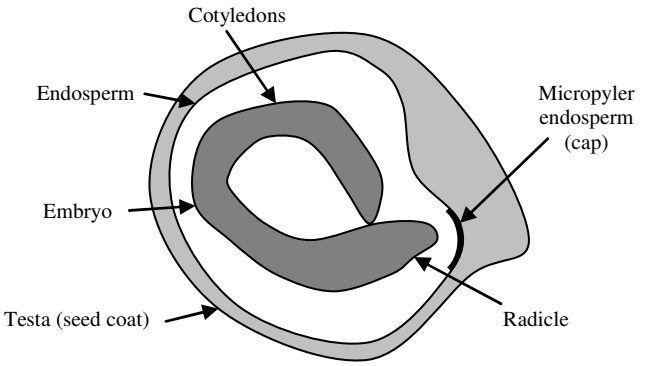

Figure 1.5.1 Simplified diagram of a section through a mature seed from the Solanoideae subgroup of family Solanaceae, to which tomato (*Solanum lycopersicum*) belongs. The embryos are curved and flattened, and the seeds have a discoid shape. The embryo has well-developed cotyledons and is surrounded by a thick walled endosperm and a thinner walled micropylar endosperm (cap). The testa (seed coat) surrounds the endosperm. The micropylar endosperm covers the radicle tip, and is the site of radicle emergence during germination. Adapted from 'the seed biology place' (http://www.seedbiology.de) and Black *et al*., 2006.

In most species, these structures are potential barriers to radicle emergence and can contribute to seed dormancy (Finch-Savage and Leubner-Metzger, 2006). Nuclear magnetic resonance (NMR) micro-imaging revealed the pattern of hydration of imbibed tobacco (Manz *et al.*, 2005) and wheat (Rathjen *et al.*, 2009) seeds. Using NMR it has been observed that the micropylar endosperm and the radicle tip are the initial sites of hydration (Nonogaki *et al.*, 2010). It should be pointed out that this pattern of water uptake appears not to occur in all plant species; for example, the cotyledons were reported to be the site of initial hydration in western white pine (Terskikh *et al.*, 2005).

In some plant families, including the *Solanaceae* and *Brassicaceae*, a distinct 'two step' germination process exists, where testa rupture and endosperm rupture are two successive visible events (Manz *et al.*, 2005; Muller *et al.*, 2006; Muller *et al.*, 2009). There is evidence that testa imposed dormancy is maternally controlled; some *Arabidopsis* testa mutants, such as *aberrant testa shape* (*ats*) and *transparent testa glabra* (*ttg*), have a maternally inherited reduction in dormancy due to alterations in testa

morphology (Leon-Kloosterziel *et al.*, 1994; Koornneef *et al.*, 2002; Finch-Savage and Leubner-Metzger, 2006 and referenced therein).

There is also evidence that ABA may influence testa development during embryogenesis; the ABA deficient mutant *sitiens* (*sit*) has a thinner (one cell thick) testa, compared to the WT seed testa (five cells thick) (Hilhorst and Downie, 1996). In transgenic tomatoes strongly overexpressing an ABA biosynthesis gene, it was observed that post germination cotyledon emergence from the surrounding testa was severely hampered (Tung *et al.*, 2008). This may be further evidence that ABA can influence testa morphology or it may be due to cotyledon expansion. It was shown, through reciprocal crosses between ABA overexpressing and WT tomatoes (Tung *et al.*, 2008) that maternal ABA phenotype was not solely responsible for altered testa morphology. Furthermore, imbibition with an aqueous norflurazon solution could not reverse the phenotype. Together these results indicate that increased embryonic ABA biosynthesis during seed development influenced testa development and the subsequent ability of the testa to rupture (Tung *et al.*, 2008).

Embryo elongation, leading to radicle protrusion, is due to cell expansion rather than cell division. In *Arabidopsis* a discrete cell elongation region immediately proximal to the radicle i.e., the hypocotyl-radicle transition zone, was identified (Sliwinska *et al.*, 2009; Nonogaki *et al.*, 2010). Embryo axis elongation and radicle emergence are turgor driven processes that also require cell wall loosening. In germinating tomato seeds, the expansin gene *SlEXP8* is expressed in a region similar to the *Arabidopsis* elongation zone, indicating that localized cell wall modification facilitates cell expansion (Chen and Bradford, 2000; Chen *et al.*, 2001; Nonogaki *et al.*, 2010). Analysis of the impact of ABA on oilseed rape (*Brassica napus*) seeds demonstrated that cell-wall loosening was prevented, resulting in the inhibition of germination (Schopfer and Placy, 1985; Bewley, 1997b). In sunflower (*Helianthus*

annus), application of ABA to embryos prevented radicle extension, and the removal of ABA led to the completion of germination (LePage-Degivry and Garello, 1992).

After testa rupture, the micropylar endosperm (Figure 1.5.1) must weaken to allow radicle emergence. Surgical removal of the micropylar endosperm in intact tomato seeds permits germination even in the presence of ABA (Liptay and Schopfer, 1983). It has been reported that endosperm weakening occurs prior to endosperm rupture in Garden Cress (*Lepidium sativum*) and is controlled by the GA:ABA ratio, where relatively high ABA levels prevent endosperm weakening and inhibit germination (Muller *et al.*, 2006). It has recently been shown that endosperm rupture in *Arabidopsis*, which is inhibited by ABA, involves the expression of the ABI3 and ABI5 transcription factors (Piskurewicz *et al.*, 2008; Piskurewicz *et al.*, 2009) and studies using an ABI5:GUS reporter gene have indicated that it is specifically activated in the endosperm region surrounding the radicle (Penfield *et al.*, 2006; Piskurewicz *et al.*, 2008). Graeber *et al.,* (2010) have found that orthologues of the *Arabidopsis* ABA induced dormancy gene, *Delay Of Germination 1* (*DOG1*) (Bentsink *et al.*, 2006), exist in *L. sativum* and *Brassica rapa* (*LsDOG1* and *BrDOG1* respectively). Tissue specific analysis of *LsDOG1* transcript levels in the radicle and micropylar endosperm revealed that they are ABA inducible and regulated by imbibition. *LsDOG1* transcript abundance was associated with radicle extension and endosperm weakening (Graeber *et al.*, 2010).

In germinating *L. sativum* seeds, it was shown *in vivo* that hydroxyl radical (·OH)-mediated cell wall loosening occurs during seed germination and seedling growth. The production and action of ·OH increased during endosperm weakening and radicle elongation and was inhibited by ABA and both effects were reversed by gibberellins (Muller *et al.*, 2009). In tomato and *Datura ferox* seeds, endosperm weakening can be linked to an increase in activity of the hydroxylase endo-β-mannanase (Bewley, 1997a; Nonogaki *et al.*, 2000). Correlation between β-1,3-glucanase (βGlu) induction and

endosperm rupture in tobacco suggests that βGlu may also promote radicle protrusion (Leubner-Metzger, 2003). The expression of β-1,3-glucanase in the micropylar endosperm, its specific inhibition by ABA and the more general inhibition of endosperm rupture by ABA, have been widely reported among the *Solanaceae* (Leubner-Metzger, 2003; Petruzzelli *et al.*, 2003; Finch-Savage and Leubner-Metzger, 2006). Finch-Savage and Leubner-Metzger (2006) speculate that β-1,3-glucanases facilitate endosperm rupture by breaking intercellular adhesion and causing cell separation.

During phase III of germination (i.e. endosperm rupture and embryonic axis elongation) and the early post-germination growth processes of cotyledon expansion, greening, and the onset of vegetative growth, sudden osmotic stress or exogenous ABA application can severely delay or arrest seedling growth (Lopez-Molina *et al.*, 2001). ABA can arrest growth only during a 48h window during which, in response to ABA, the ABI5 transcription factor stimulates transcription of the late embryogenesis abundant (LEA) genes *AtEm1* and *AtEm6*. It should be noted that *abi5* mutants are insensitive to the ABA-dependent inhibition of endosperm rupture (Finkelstein and Lynch, 2000; Lopez-Molina and Chua 2000; Piskurewicz *et al.*, 2008). A recessive mutation in *Arabidopsis*, termed *growth insensitive to ABA3* (*gia3*) that mediates the ABA-dependent repression of cotyledon expansion and greening, was relatively recently identified (Kinoshita *et al.*, 2010). Microarray analysis revealed that expression of the mid-embryogenesis gene *Maternal Embryo Effect 26* (*MEE26*) is induced by ABA during early seedling growth in WT and *abi5* plants but not in *gia3* mutants (Kinoshita *et al.*, 2010). These authors also show that the *GIA3* locus controls ABA-dependent gene expression responses that partially overlap with those controlled by *ABI5* (Kinoshita *et al.*, 2010).

Seeds integrate different external cues to determine if the surrounding environment is favourable for germination. Light intensity and temperature are two

critical external factors influencing germination. High temperatures can inhibit germination, a process known as thermoinhibition; and contrariwise, exposure of imbibed seeds to cold temperature, a process known as stratification, can break dormancy and thereby stimulate germination in many plant species. A discussion of seed perception and response to varying environmental cues influencing germination is beyond the scope of this work, and the reader is directed to Baskin and Baskin (2004) or Finch-Savage and Leubner-Metzger (2006).

Within the seed of a typical dicotyledonous species e.g. tomato (Figure 1.5.1), the triploid endosperm and diploid embryo tissues are differentially derived from both parents, as opposed to the diploid testa which is exclusively maternally derived tissue. ABA in the seed may have been transferred from the maternal parent or endogenously produced. During early seed development, ABA levels are initially low and usually peak around the mid point of maturation (Bewley, 1997b; Kermode, 2005 and references therein). It has been revealed using ABA deficient mutants of the diploid tobacco relative *N. plumbaginifolia* (commonly known as 'Tex-Mex' tobacco or 'curl-leaved' tobacco: Knapp and Clarkson, 2004 and references therein) that during early embryogenesis, maternal ABA can function to promote initial seed development and growth (Cheng *et al.*, 2002; Frey *et al.*, 2004). Endogenous ABA within the seed is important, despite being present in relatively low amounts during early embryogenesis. Seed ABA content increases to a peak during mid-maturation and promotes the accumulation of storage reserves at that time (Bewley, 1997b). During the final stage of embryogenesis the embryo undergoes programmed desiccation, which is positively regulated by ABA and then ABA levels typically fall during the later stages of dehydration and seed maturation. Desiccation tolerance is conferred by the accumulation of osmoprotectants and various proteins that protect cell components from dehydration, such as LEA proteins.

Experiments with sorghum (*Sorhum bicolor* (Steinbach *et al.*, 1997)) and maize (*Zea mays*) ABA deficient and insensitive mutants (White *et al.*, 2000; White and

Rivin, 2000) support the idea that ABA and GA act simultaneously and antagonistically on both dormancy and germination. ABA downregulates GA biosynthesis and upregulates GA deactivation (Seo *et al.*, 2006). The physiological analysis of ABA deficient mutants has highlighted that during late embryogenesis ABA limits further embryo expansion by counteracting the growth and germination promoting action of GA (White *et al.*, 2000; Raz *et al.*, 2001). Within the seed, it appears that the relative ratio of ABA to GA, rather than the absolute hormone concentrations, determines germination capacity (Finch-Savage and Leubner-Metzger, 2006; Seo *et al.*, 2009).

In non-dormant fully imbibed seeds, germination is preceded by a decrease in ABA resulting from both a reduction in ABA biosynthesis (section 1.3) and an increase in ABA catabolism (section 1.4) (Grappin *et al.*, 2000; Kushiro *et al.*, 2004; Kermode, 2005; Finch-Savage and Leubner-Metzger, 2006). As the ABA concentration decreases in these seeds, increases in the ABA hydroxylation products, phaseic acid (PA) and dihydrophasic acid (DPA) have been observed in a number of species including barley, lettuce, and *Arabidopsis* (Nambara and Marion-Poll, 2005 and references therein). In contrast, fully imbibed dormant seeds maintain relatively constant ABA concentration and *de novo* ABA biosynthesis during imbibition. This has been demonstrated in many plant species including sunflower (*Helianthus annus*; LePage-Degivry and Garello, 1992) and barley (*Hordeum vulgare*; Wang *et al.*, 1995).

In *Arabidopsis,* functional and expression analyses indicated that two members of the key regulatory *NCED* family of ABA biosynthesis genes, *AtNCED6* and *AtNCED9* (section 1.3.3) are both strongly expressed in seeds. Expression of *AtNCED6* was observed to occur almost exclusively in the endosperm during seed development, and *AtNCED9* was expressed in both the embryo and the endosperm during the mid-development phase (Lefebvre *et al.*, 2006) and this was also found to be responsive to water stress in leaves. Consistently, high levels of *HvNCED1* transcripts were detected in dormant barley embryos (Millar *et al.*, 2006). Further evidence indicating that the

maintenance of dormancy in imbibed seeds is an active process involving *de novo* ABA synthesis has been provided by experiments involving inhibitors of carotenoid biosynthesis, such as fluridone and norflurazon, which can break seed dormancy and promote germination (Debeaujon and Koornneef, 2000; Grappin *et al.*, 2000).

ABA deficient or insensitive mutants exhibit reduced seed dormancy and can also be associated with precocious germination in some species e.g. seeds of the maize *viviparous* (*vp*) mutants, which germinate precociously in some cases due to reduced ABA levels or decreased ABA sensitivity in developing embryos (McCarty *et al.*, 1989). Additionally, *Arabidopsis* ABA deficient mutants, such as *aba1*, *aba2* and *aao3* and the ABA insensitive mutants *abi1*, *abi2* and *abi3*, all have reduced primary dormancy (Leon-Kloosterziel *et al.*, 1996; Finkelstein *et al.*, 2002; Himmelbach *et al.*, 2003; Kushiro *et al.*, 2004; Nambara and Marion-Poll, 2005).

Freshly harvested seeds of *N. plumbaginifolia* exhibit primary dormancy which results in delayed germination and the seeds require longer after ripening and/or stratification before they will germinate. Seeds of the ABA deficient mutant *aba2-s1* (mutated in the *ABA2* gene encoding zeaxanthin epoxidase (ZEP: section 1.3.3)) do not exhibit primary dormancy and germinate rapidly compared to wild type seeds (Marin *et al.*, 1996; Frey *et al.*, 1999). Seeds of tomato plants, on the other hand, do not exhibit primary dormancy and freshly harvested seeds will readily germinate. Therefore decreasing seed ABA levels, via mutants or knockout lines, in *N. plumbaginifolia* will have a more dramatic effect than in tomato. Conversely increasing seed ABA levels, via the transgenic overexpression of ABA biosynthesis genes, will have a minor effect in *N. plumbaginifolia* and a more dramatic effect in tomato plants, inducing dormancy in otherwise essentially non-dormant seed.

For example, transgenic *N. plumbaginifolia* plants overexpressing *ABA2* exhibited a relatively minor delay in germination (three days) in already dormant seed, whereas the antisense induced down-regulation of *ABA2* expression resulted in reduced

levels of ABA in the seeds and complete loss in dormancy, resulting in rapid germination (Frey *et al.*, 1999). On the other hand, the overexpression of *SlNCED1* in tomato increased the accumulation of ABA in the seed and increased dormancy. In the less severe *SlNCED1* overexpressing line (sp12) germination was delayed by between five to ten days, and the final germination rate was just 15% (Thompson *et al.*, 2000b). Typically less than 5% of seed of the more severe *SlNCED1* overexpressing line (sp5) will germinate, unless the seeds are imbibed with a norflurazon solution (Thompson *et al.*, 2000b). Other transgenic lines, producing higher amounts of ABA than the aforementioned *SlNCED1* overexpressors, have seed which has an absolute requirement for norflurazon to be able to germinate and is therefore effectively permanently dormant (Sonneveld *et al.*, unpublished data).

ABA catabolism (section 1.4) also plays a role in germination e.g. the concentration of ABA in the seed can be reduced through the formation of ABA glucose ester (ABA-GE), which results in enhanced germination, conversely the hydrolysis of ABA-GE can reactivate ABA and negatively affect germination (Chiwocha *et al.*, 2005). Recently an ABA catabolism pathway in seeds involving regulation by NO, H_2O_2, and the ABA 8′-hydroxylase CYP707A2 (section 1.4) has emerged. A role for NO in germination has been previously demonstrated in *Arabidopsis* (Bethke *et al.*, 2004; Bethke *et al.*, 2006), barley (Bethke *et al.*, 2004) and lettuce (Beligni and Lamattina, 2000). Fluorescent imaging of NO in seeds demonstrated that, in *Arabidopsis*, rapidly formed NO is released in the endosperm during imbibition and this preceded increases in ABA catabolism (Liu *et al.*, 2009). The NO-induced ABA decrease correlated with the regulation of *CYP707A* transcription and protein expression. *CYP707A2* transcript levels increased within six hours of imbibition, and *cyp707a2* mutant seeds accumulated six-fold more ABA than WT seeds and had increased dormancy (Kushiro *et al.*, 2004; Saito *et al.*, 2004; Okamoto *et al.*, 2006; Liu *et al.*, 2009). Liu *et al.*, (2009) analysed the *cyp707a* mutant and found that it does not show the NO response, implicating CYP707A

in ABA catabolism during the first stage of imbibition, although the mechanism of NO accumulation is still unclear (Liu *et al.*, 2009).

Exogenous H_2O_2 decreases seed dormancy both by increasing ABA catabolism through increased expression of *CYP707A* genes, and through the promotion of GA biosynthesis by enhanced expression of GA biosynthesis genes e.g. *GA3ox* and *GA20ox* (Liu *et al.*, 2010). The up-regulation of *CYP707A* genes by H_2O_2 apparently required NO, as indicated by the effect of applying an NO scavenger (c-PTIO). Conversely, the inhibition of H_2O_2 decreased the expression of *CYP707A*, *GA3ox* and *GA20ox* genes and enhanced seed dormancy and these effects could be reversed by application of a NO donor (Liu *et al.*, 2010).

Generally, there is very strong experimental support, from a range of diverse studies that collectively indicate that a substantially elevated amount of ABA in the seed can act to prevent seed germination.

1.6 ABA TRANSPORT, PERCEPTION & CELL SIGNALLING

1.6.1 ABA Transport

The initial steps of ABA perception and transport within plant cells are only just beginning to be understood, and recently ABC (ATP-binding cassette) proteins have been implicated in stomatal responses and have been suggested as ABA transporters (Sirichandra *et al.*, 2009b). Most ABC proteins are integral membrane proteins, and they are well known to act as ATP driven transporters for a very wide range of substrates (Rea, 2007). A number of *Arabidopsis* mutations in some of the genes encoding ABC transporter proteins have lead to perturbed stomatal functioning and four of these will be briefly discussed.

The first is the ABC transporter known as AtABCB14, which has been proposed to be a malate importer that modulates stomatal response to CO_2 (Lee *et al.*,

2008). The usual high [CO_2] induced stomatal closure was found to be accelerated in plants lacking AtABCB14 (Lee *et al.*, 2008). In isolated epidermal strips containing only guard cells, malate-dependent stomatal closure was faster in plants lacking AtABCB14 and slower in AtABCB14 overexpressing transgenic plants. This is consistent with the suggestion that AtABCB14 has an important role in the transport of malate from the apoplast into guard cells (Lee *et al.*, 2008). Therefore these authors have proposed that AtABCB14 modulates stomatal movement by transporting malate from the apoplast into guard cells.

The second ABC transporter is, known as *AtPDR3* (pleiotropic drug resistance 3) and was discovered in a screen designed to identify genes preferentially expressed in guard cells (Galbiati *et al.*, 2008). Preliminary evidence appears to indicate that disruption of this gene can lead to reduced sensitivity of guard cells to ABA, and may therefore have a role as a positive regulator of ABA signalling in guard cells (Galbiati *et al.*, 2008);

The third ABC transporter gene is known as *AtABCG25* and was identified during a genetic screen for ABA sensitivity (Kuromori *et al.*, 2010). *AtABCG25*, is expressed mainly in vascular tissues, but a fluorescent protein fused to AtABCG25 revealed that it was also localized to the plasma membrane. In membrane vesicles from AtABCG25-expressing insect cells, AtABCG25 exhibited ATP-dependent ABA transport (Kuromori *et al.*, 2010). *AtABCG25* overexpressing transgenic plants had higher leaf temperatures, which might imply stomatal closure. The authors conclude that their results suggest that the *AtABCG25* gene encodes a protein responsible for ABA transport; an exporter of ABA involved in the ABA intercellular signalling pathway (Kuromori *et al.*, 2010).

The fourth, ABC transporter gene is known as *AtPRD12*, and has been suggested to be an ABA uptake transporter (Kang *et al.*, 2010). Yeast and cultured tobacco BY2 cells expressing/overexpressing the *AtPDR12* gene took up ABA

consistently faster than controls, and ABA uptake into protoplasts of *Atprd12* mutant plants was decreased (Kang *et al.*, 2010). After exposure to exogenous ABA, the up-regulation of ABA responsive genes was strongly delayed in *Atprd12* mutant plants compared to non-mutant control plants, indicating that AtPRD12 is necessary for rapid closure of stomata in response to ABA. The stomata of loss of function *Atprd12* mutant plants closed more slowly, lateral root formation was delayed in these plants and they also had impaired ABA regulation of seed germination (Kang *et al.*, 2010). These authors postulate that this and other ABA transporters are required, in addition to ABA passive diffusion across the plasma membrane, for rapid and efficient ABA signalling under stress conditions (Kang *et al.*, 2010).

1.6.2 ABA Perception

The identity of specific ABA receptors has remained a mystery for many years, despite the publication of a number of studies providing apparent evidence for the existence of several different extra- and intra-cellular ABA receptors (Hornberg and Weiler, 1984; Allan *et al.*, 1994; Anderson *et al.*, 1994; MacRobbie, 1995; Leung and Giraudat, 1998; Finkelstein *et al.*, 2002; Yamazaki *et al.*, 2003; Christmann *et al.*, 2006; Huang *et al.*, 2007; Verslues and Zhu, 2007; Wang and Zhang, 2008). To date, a total of five putative ABA receptors have been reported and their relative importance has been intensely debated: The first (Razem *et al.*, 2006) is a nuclear receptor named flowering time control protein A (FCA), but as the suggestions in the original papers have now been effectively withdrawn by the authors (Razem *et al.*, 2008; Razem *et al.*, 2010) this will not be considered in further detail; the second (Shen *et al.*, 2006) is a plastid receptor, the H subunit of the magnesium-proto-phorphyrin IX chelatase (ABAR/CHLH); the third (Liu *et al.*, 2007b) and fourth (Pandey *et al.*, 2009) are both G protein plasma membrane receptors in the form of a G protein (GTP binding protein) coupled receptor (GPCR) and GPCR-type G proteins (GTGs); and the fifth (Ma *et al.*, 2009; Park *et al.*, 2009) putative

ABA receptor is a family of intracellular receptors, in the form of the PYR/PYL/RCAR subfamily of the Bet v I-fold/START protein superfamily.

The second putative ABA receptor (Shen *et al.*, 2006), the H subunit (CHLH) of the magnesium-proto-phorphyrin IX chelatase (Mg-chelatase), appears to be a key component in chlorophyll synthesis and plastid to nucleus signalling (Zhang *et al.*, 2002; Shen *et al.*, 2006). This plastid localized putative ABA receptor was originally identified in broad bean (*Vicia faba* L.) as an ABA-specific-binding protein potentially involved in stomatal signalling and was designated ABAR (abscisic acid receptor) (Zhang *et al.*, 2002). In *Arabidopsis*, the CHLH subunit is encoded by *GENOME UNCOUPLED5 (GUN5)* gene locus, which had previously been implicated in plastid to nucleus signalling under stressful conditions (Mochizuki *et al.*, 2001). Purified yeast-expressed CHLH was demonstrated to specifically bind to ABA and transgenic *Arabidopsis* which had been down regulated for CHLH expression were reported to be insensitive to ABA; in the reverse experiment, CHLH overexpressing transgenic *Arabidopsis* plants apparently displayed hypersensitive ABA phenotypes (Shen *et al.*, 2006).

Two CHLH *Arabidopsis* mutants (*abar*-2 and *abar*-3) show altered ABA phenotypes with regard to germination and seedling growth but not stomatal movement (Wu *et al.*, 2009). This additional evidence appears to support the original suggestion that CHLH is an ABA receptor. However, the barley (*Hordeum vulgare*) magnesium chelatase large subunit, XANF, apparently does not bind to ABA and *xanF* loss-of-function mutants have been reported to display normal ABA phenotypes, questioning the proposed ABA receptor function of this subunit (Muller and Hansson, 2009). It remains a possibility that the CHLH protein may function differently in monocotyledonous and dicotyledonous plants (Cutler *et al.*, 2010), but the role of CHLH as an ABA receptor remains uncertain and clearly requires further investigation involving a wider range of plant species.

The third and fourth putative ABA receptors are heterotrimeric G proteins, a class of proteins which are well known to be associated with signal transduction in many systems in animals as well as plants (section 1.6.3). In the inactive state, a G protein exists as a trimer consisting of an α subunit (Gα) bound to GDP, a β subunit (Gβ), and a γ subunit (Gγ). When a ligand binds to a G protein coupled receptor (GPCR) a conformational change occurs resulting in the exchange of GDP for GTP and the dissociation of Gα-GTP from the Gβγ dimer. These dimers can then target downstream effectors, such as protein kinases or phospholipases. The dimers become inactive when the GTPase activity of the Gα subunit results in the hydrolysis of GTP to GDP, allowing the trimer reassociate (Chen *et al.*, 2006; Grill and Christmann, 2007; Cutler *et al.*, 2010; Gao *et al.*, 2010; Nilson and Assmann, 2010) Much of the recent work on G-proteins has been centred around the only G protein α-subunit identified in *Arabidopsis,* GPA1, and its potential role in ABA signal transduction (section 1.6.3).

A putative G protein coupled receptor (GPCR), termed GCR2, in *Arabidopsis*, became the third candidate ABA receptor and has been reported to be located in the plasma membrane (Liu *et al.*, 2007b). It was found that in *Arabidopsis*, GCR2 could apparently interact with the G protein α subunit (GPA1) to mediate ABA responses, and that the binding of ABA to GCR2 disrupted the GCR2/GPA1 interaction. The overexpression of GCR2 in transgenic *Arabidopsis* plants was reported to result in an ABA hypersensitive phenotype; while loss of function *gcr2* mutants apparently display defective responses to ABA (Liu *et al.*, 2007b). The authors also provided evidence indicating that GCR2 binds to ABA, and that the binding of ABA can disrupt the GCR2 GPA1 complex in yeast (Liu *et al.*, 2007b). In a subsequent paper these authors suggest that although some of the properties of GCR2 may not be consistent with classic GPCRs, GCR2 may define a new type of non-classical GPCR (Liu *et al.*, 2007a). However, other research groups have published evidence, that *GCR2* is not a transmembrane protein or a

G-protein coupled receptor and that it is in fact a plant homologue of bacterial lanthionine synthases (Johnston *et al.*, 2007; Illingworth *et al.*, 2008). Risk *et al*., (2009) reported biochemical binding assays showing that GCR2 does not bind ABA; from which they concluded that GCR2 is not a plasma membrane ABA receptor. At the time of writing, the status of GCR2 remains controversial with different research groups drawing their own conclusions (Pennisi, 2009; Santner and Estelle, 2009).

The closely related fourth set of putative ABA receptors were identified by Pandey *et al*., (2009) as two membrane associated GPCR-type G (GTG) receptor proteins, termed GTG1 and GTG2, based on their similarity to known GPCRs. GTG1 and GTG2 are targeted to the plasma membrane and like GCR2, have been reported to interact with the G protein α subunit GPA1 (Pandey *et al.*, 2009; Kim *et al.*, 2010). Both GTG1 and GTG2 appeared to bind to ABA specifically and binding was stimulated by GDP, suggesting that the GDP-bound form of the receptor is the high-affinity binding state (Cutler *et al.*, 2010). Additionally, it was found that *Arabidopsis gtg1/gtg2* double mutants have reduced sensitivity to ABA in seed germination, root growth and stomatal responses. Pandey *et al*., (2009) concluded that the two GTGs are plasma membrane localized GPCRs that control ABA signalling. Risk *et al*., (2009) have questioned the results and methods used to demonstrate ABA binding to GTG1 and GTG2. They point out that one of the GPCRS which was used as a basis for the identification of the two GTGs has more recently been identified as an anion channel (Maeda *et al.*, 2008). Obviously further research is needed to precisely resolve the functions, if any, of GCR2, the two GTGs and any other GPCRs in ABA perception and signal transduction (McCourt and Creelman, 2008; Risk *et al.*, 2009; Cutler *et al.*, 2010).

The fifth and most recent set of proteins proposed to be ABA receptors have become known as the PYR/PYL/RCAR family, which were independently identified in *Arabidopsis* by two research groups using different approaches; the Cutler group (Nishimura *et al.*, 2009; Park *et al.*, 2009; Santiago *et al.*, 2009a) and the Grill group (Ma

et al., 2009). Park *et al.,* (2009) based their approach on the fact that pyrabactin, a synthetic growth inhibitor, can act as a selective agonist of ABA response in terms of seed germination. A germination based screen for pyrabactin targets led to the identification of Pyrabactin resistance 1 (PYR1), a member of a subfamily of star-related lipid-transfer (START) domain proteins (Iyer *et al.*, 2001), recently called the Bet v I-fold superfamily (Radauer *et al.*, 2008). In addition to PYR1, Park *et al.,* (2009) identified 13 similar proteins encoded by a large gene family in *Arabidopsis* and designated them PYL (PYR1-Like) proteins PYL1 to PYL13. Simultaneously, the Grill group identified this same 14 member gene family while screening for ABI1 and ABI2 interacting proteins using a yeast two-hybrid approach (Ma *et al.*, 2009). They called these proteins Regulatory Component of ABA Receptor, (RCAR1 to RCAR14) and RCAR1 turned out to be identical to the protein designated PYL9 by the Cutler group. To prevent confusion, only the PYR/PYL numbering system will be used from this point on.

Park *et al.,* (2009) demonstrated that PYR1 can bind to ABA, and the resulting complex can bind to and inhibit the protein phosphatase type 2Cs (PP2Cs); encoded by ABI1 (ABA insensitive1), ABI2, and HAB1 (Homology to ABI1). The two PP2Cs, *ABI1* and its homolog *ABI2*, act in a negative feedback regulatory loop in the ABA signalling pathway (Merlot *et al.*, 2001), and have emerged as a hub in the network of ABA signal transduction (Yang *et al.*, 2006; Moes *et al.*, 2008 section 1.6.3). Park *et al.,* (2009) proposed that the PYR1/PYLs are a family of ABA receptors that function as a complex with PP2Cs, in a negative regulatory pathway in ABA signalling. Ma *et al.,* (2009) demonstrated that in the presence of PYL9, ABA instantaneously and almost fully blocked ABI2 phosphatase activity and that there was a physical interaction between PYL9 and HAB1, ABI1 and ABI2. PYL9 was shown to be expressed in *Arabidopsis* roots, stomata and parenchyma cells of the vasculature (Ma *et al.*, 2009). The authors concluded that the PP2C - PYL9 complex had the "hallmark of a receptor": selective recognition and transmission of the signal, in this case via ABA control of protein

phosphatase activity. This soluble ABA receptor complex was detected in both the cytosol and the nucleus and further analysis indicated the binding of one ABA molecule per PYL9 receptor (Ma *et al.*, 2009).

Single mutants (apart from *pyr1* which differs from WT in enabling seed to germinate in the presence of the pyrobactin) have no obvious ABA phenotype due to functional redundancy. However, triple (*pyr1;pyl1;pyl4*) and quadruple (*pyr1;pyl1;pyl2;pyl4*) mutant plants were reported to have phenotypes consistent with reduced ABA sensitivity with regard to seed germination, root growth, and reduced ABA activation of sucrose non-fermented-related protein kinases 2 (SnRK2s) (Park *et al.*, 2009; Nishimura *et al.*, 2010). The quadruple mutants were also impaired in ABA-induced stomatal closure and ABA inhibition of stomatal opening, but not in Ca^{2+}-induced stomatal closing. It has been reported that the overexpression of one gene family member (*PYL5*) in *Arabidopsis* conferred drought resistance on the resulting transgenic plants (Santiago *et al.*, 2009b).

Several studies appear to have elucidated not only the structure of the various PYR1/PYL proteins, but also the mechanism of their involvement in ABA perception and initiation of signal transduction (Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Nishimura *et al.*, 2009; Santiago *et al.*, 2009a; Yin *et al.*, 2009). The various PYR1/PYL proteins can all form homodimers, confirmed *in planta* by coimmunoprecipitation (Nishimura *et al.*, 2009), one of the subunits of which binds specifically to ABA. In the unbound receptor an open ligand-binding pocket is flanked by two highly conserved loops, which form what have been called the ‘gate and latch’ (Melcher *et al.*, 2009). When one ABA molecule binds in the pocket, conformational changes cause the ‘gate’ (which others call the ‘lid’) to close over the pocket and form the ‘gate/latch interface’ which becomes a binding site for the PP2Cs. A conserved tryptophan in the PP2Cs specifically interacts with the trapped ABA molecule, between the ‘gate’ and ‘latch’ loops, and the ‘gate’ loop comes into contact with the active site of the PP2Cs, thereby inhibiting their phosphatase

activity (Miyazono *et al.*, 2009). Differences in the specific binding pockets of the individual PYR/PYL family members would allow for some specialisation in receptor activation and could explain the different receptor selectivity observed for one or both of the optical isomers, (-)-ABA and (+)-ABA, and the ABA agonist pyrabactin (Cutler *et al.*, 2010).

1.6.3 Later Events in ABA Signal Transduction

Once the ABA signal has been perceived within a cell, a complex network of signalling pathways is activated, resulting not only in stomatal closure (section 1.7), but also in altered tissue specific expression of numerous ABA responsive genes. The specific details of the various signalling cascades that have been reported to be associated with ABA are far beyond the scope of this thesis, but some of the most pertinent findings related to ABA signalling during water deficit, and guard cell responses to ABA will be highlighted. For further information the reader is directed to the many recently published reviews on this subject (for example; Christmann *et al.*, 2006; Wang and Song, 2008; Santner and Estelle, 2009; Sirichandra *et al.*, 2009b; Cutler *et al.*, 2010; Hey *et al.*, 2010; Kim *et al.*, 2010).

As mentioned in the previous section, reversible protein phosphorylation, catalysed by protein kinases and phosphatases, is a vital early event in ABA signal transduction (Leung *et al.*, 1997; Himmelbach *et al.*, 2002; Sokolovski *et al.*, 2005; Mishra *et al.*, 2006; Zhu *et al.*, 2007; Fujii *et al.*, 2009). There is evidence that several PP2Cs are negative regulators of these pathways in *Arabidopsis* (Umezawa *et al.*, 2009; Vald *et al.*, 2009), and that ABA activated SnRK2s and Ca^{2+}-dependent protein kinases (CDPKs) are positive regulators in ABA signalling (Li *et al.*, 2000; Mustilli *et al.*, 2002; Yoshida *et al.*, 2002; Umezawa *et al.*, 2009). Fuji *et al.,* (2009) revealed by co-expression in *Arabidopsis* protoplasts, that a minimal core set of four components was required for ABA induced gene expression; a PYR/PYL ABA receptor protein (e.g. PYR1) which

forms a complex with a PP2C (e.g. ABI1), a serine/threonine protein kinase (e.g. OST1/SnRK2.6) and a transcription factor (e.g. AREB1/ABF2). The discovery of the PYR1/PYL protein family (section 1.6.2) has brought focus on the function of the PP2Cs as part of the ABA receptor complex. The PP2Cs are usually described as Mg^{2+}- and Mn^{2+}-dependent serine/threonine phosphatases type 2C. There are 76 PP2Cs in *Arabidopsis*, separated into ten groups (A – J) (Schweighofer *et al.*, 2004) and six of the nine PP2Cs in group A have been identified as negative regulators of ABA responses (Merlot *et al.*, 2001; Saez *et al.*, 2006; Yoshida *et al.*, 2006b; Nishimura *et al.*, 2007).

Two of these six PP2Cs, ABI1 (ABA insensitive1) and its close homologue ABI2, have long been known to be crucial for ABA-mediated stomatal regulation (Leung *et al.*, 1997; Gosti *et al.*, 1999; Merlot *et al.*, 2001). They were discovered through genetic screens for *Arabidopsis* mutants insensitive to ABA inhibition of seed germination (Koornneef *et al.*, 1984). Plants carrying the dominant mutant alleles, *abi1-1* and *abi2-1*, are insensitive to ABA in seed germination, root growth, stomatal closure and in ABA responsive gene regulation (Koornneef *et al.*, 1984; Leung *et al.*, 1994). The ABI1 protein has been found to be located in both the nucleus and the cytosol, as are the other parts of the ABA receptor complex i.e. the PYR1/PYL proteins (Moes *et al.*, 2008). ABI1 has been shown to physically interact with a number of ABA signalling components, such as the protein kinase OST1 (open stomata1), (Yoshida *et al.*, 2006a), and the HD-Zip transcription factor known as AtHB6 (Himmelbach *et al.*, 2002). The role of PP2Cs similar to ABI2 in plant species other than *Arabidopsis* are not as well defined (Komatsu *et al.*, 2009), but there is evidence that the negative regulation of ABA signalling by ABI1-related PP2Cs has been conserved throughout the evolution of land plants (Komatsu *et al.*, 2009).

Some protein kinases can act as positive regulators in ABA signalling and are the other half of the reversible protein phosphorylation that forms the basis of early ABA signal transduction. The first putative ABA-activated serine-threonine protein kinase

(AAPK) was purified from *Vicia faba*. This guard cell-specific kinase is a member of the SnRK2 family (Li and Assmann, 1996; Li *et al.*, 2000) which are known to be involved in the regulation of ABA-induced stomatal closure (Li *et al.*, 2002). In *Arabidopsis* the SnRK2 family consists of 10 members (SRK2A-J/SnRK2.1–10) (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002), classified into three subgroups (Kobayashi *et al.*, 2004). OST1 along with SnRK2.2/SRK2D and SnRK2.3/SRK2I, comprise three members of subclass III which are strongly ABA-induced positive regulators of ABA signalling (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002; Boudsocq *et al.*, 2004; Yoshida *et al.*, 2006a; Fujii *et al.*, 2007; Fujii and Zhu, 2009; Nakashima *et al.*, 2009). OST1 (*Arabidopsis* orthologue of *V. faba* AAPK also known as SnRK2.6/Srk2E Merlot *et al.*, 2002; Mustilli *et al.*, 2002; Yoshida *et al.*, 2002; Assmann, 2003) functions in ABA induced stomatal closure (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002) while SnRK2.2 and SnRK2.3 have been reported to regulate ABA responses in seed germination, root growth and gene expression (Fujii *et al.*, 2007). These three SnRK2s function as the main positive regulators of the recently defined signalling pathway associated with the PYR/PYL ABA protein/PP2C receptor complex (section 1.6.2) (Fujii *et al.*, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; Vald *et al.*, 2009). The SnRK2 family is similarly constructed in rice (*Oryza sativa*) consisting of ten Stress-Activated Protein Kinases (SAPK) and three of these have been demonstrated to be ABA inducible (Kobayashi *et al.*, 2004; Hirayama and Shinozaki, 2007).

Many other protein kinases have been implicated in ABA signalling, for example: the SnRK subgroup 3 (SnRK3s/CIPKs/PKSs) act in a calcium-dependent manner to negatively regulate ABA signalling (Cutler *et al.*, 2010); some members of the mitogen-activated protein (MAP) kinase (MAPK) family have also been reported to be ABA activated and involved in ABA signalling (Lu *et al.*, 2002; Jammes *et al.*, 2009; Wang *et al.*, 2010); another family of Ca^{2+}-dependent protein kinases (CDPKs/CPKs) act to positively regulate ABA signalling; these kinases have both kinase and calcium sensor

domains, so they are directly activated by calcium and can then phosphorylate the ABA-Responsive element binding factors (ABFs) (Zhu *et al.*, 2007; Cutler *et al.*, 2010; Kim *et al.*, 2010). Many CDPK genes contain an ABA-responsive element (ABRE) in their promoter and therefore can be induced at the transcriptional level by ABA (Hey *et al.*, 2010).

Several protein kinases, like the CDPKs, function to directly phosphorylate basic-leucine zipper (b-ZIP) transcription factors, including ABA Responsive Element Binding proteins (AREBs), ABA binding factors (ABFs), and ABA insensitive 5 (ABI5) (Kobayashi *et al.*, 2005; Furihata *et al.*, 2006; Fujii *et al.*, 2007; Johnston *et al.*, 2007). Members of the b-ZIP transcription factor family interact with ABREs in the promoter regions of many ABA inducible genes to upregulate gene expression (Giraudat, 1995; Busk and Pages, 1998; Grill and Himmelbach, 1998). For optimal ABA responsiveness usually more than one ABRE is required; either additional ABREs or a coupling element (CE) may also be present in the promoter region of ABA responsive genes (Shen and Ho, 1995; Shen *et al.*, 1996).

Furihata *et al.* (2006) demonstrated that the ABA-dependent SnRK2 kinases phosphorylate the b-ZIP transcription factors AREB1/ABF2, which can then activate ABRE dependent gene expression. The *Arabidopsis* transcription factors AREB1, AREB2 and ABF3 interact with ABREs to enhance drought stress tolerance (Uno *et al.*, 2000; Fujita *et al.*, 2005), and the constitutive overexpression of *ABF3* or *ABF4*/*AREB2* in *Arabidopsis* resulted in ABA hypersensitivity as well as reduced transpiration rates and enhanced drought tolerance (Kang *et al.*, 2002). Three of the ABA insensitive genes (*ABI3*, *ABI4*, and *ABI5*) have been found to encode transcription factors belonging to the B3, AP2 and bZIP families respectively (Giraudat *et al.*, 1992; Finkelstein *et al.*, 1998; Finkelstein and Lynch, 2000). Phenotypic analysis of plants carrying mutations in these genes defined their roles as mostly influencing seed related ABA responses (Parcy *et al.*, 1994; Nambara *et al.*, 2002). The *ABI3* gene is homologous to the maize seed specific

transcription factor VP1 (Nambara *et al.*, 2002 and references therein) and the b-ZIP transcription factor ABI5 and its rice homologue, TRAB1, have also been shown to mediate ABA responses in seed germination (Finkelstein and Lynch, 2000; Uno *et al.*, 2000; Brocard *et al.*, 2002).

The abundance of the ABI3 and ABI5 transcription factors can also be regulated by the ubiquitin-proteasomal pathway (Santner and Estelle, 2009; Cutler *et al.*, 2010; Kim *et al.*, 2010). Two RING E3 ligases, ABI3 interacting protein (AIP2; Zhang *et al.*, 2005) and Keep On Going (KEG; Stone *et al.*, 2006) have been shown to ubiquitinylate ABI3 and ABI5 respectively, targeting them for degradation by the 26S proteasome (Santner and Estelle, 2009; Cutler *et al.*, 2010; Kim *et al.*, 2010). ABA increases AIP2 levels which promotes ABI3 degradation and downregulates ABA signal transduction (Zhang *et al.*, 2005). On the other hand, ABA treatment or stress results in the stabilization of ABI5, and consistently *keg* T-DNA insertion mutants had increased ABI5 protein levels (Stone *et al.*, 2006). It has been proposed that ABA might prevent the recognition or the unbiquitination of ABI5 by KEG (Stone *et al.*, 2006). In addition to KEG, the SUMO (small ubiquitin-related modifier) E3 ligase SIZ1 (which is the sole locus encoding a SUMO E3 ligase in *Arabidopsis*) stabilizes but inactivates ABI5 by sumoylation and attenuates ABA responses during seed germination and negatively regulates ABA signalling (Miura *et al.*, 2009). In addition to ABI5, other sumoylated proteins accumulate in response to drought stress (Catala *et al.*, 2007; Cutler *et al.*, 2010; Kim *et al.*, 2010). Further research is needed to determine other transcription factors that are degraded and/or stabilized during ABA signalling and how these mechanisms mediate the ABA control of stomatal movements (Santner and Estelle, 2009; Kim *et al.*, 2010).

1.7 ABA & STOMATA

Stomata in the epidermis of plant aerial tissues are formed by pairs of guard cells which are typically dumbbell shaped in grasses and sedges, and kidney shaped in dicotyledonous plants (Salisbury and Ross, 1992; Hetherington and Woodward, 2003). Guard cells dynamically regulate the size of the stomatal aperture to allow sufficient CO_2 influx for optimal photosynthesis while minimising transpirational water loss. Stomata also influence leaf temperature through transpirational evaporation (Acharya and Assmann, 2009; Kim *et al.*, 2010). The size of the stomatal aperture is determined by reversible changes in guard cell turgor; guard cells actively take up solutes, such as K^+ and Cl^-, and synthesize osmolytes, such as malate and sucrose. As a result of this and other factors water diffuses into the cell (Grill and Ziegler, 1998; Raven *et al.*, 1999; Fan *et al.*, 2004; Vavasseur and Raghavendra, 2005; Nilson and Assmann, 2007) causing the guard cells to become turgid and swell. Asymmetric distribution of cellulose microfibrils in the guard cell wall cause the cells to separate as they swell, widening the stomatal aperture (Salisbury and Ross, 1992; Raven *et al.*, 1999; Fan *et al.*, 2004). Conversely, when guard cells loose solutes and water they become flaccid and the aperture closes (Grill and Ziegler, 1998; Raven *et al.*, 1999; Pei and Kuchitsu, 2005).

Complex networks of signal transduction pathways combine to modulate the size of the stomatal aperture in response to various and multiple internal and environmental signals. Generally, stomata open in response to blue and red light, low CO_2 concentrations and high atmospheric humidity, and close in response to darkness, high CO_2 concentrations, stress-induced ABA, and elevated ozone (Salisbury and Ross, 1992; Raven *et al.*, 1999; Hetherington and Woodward, 2003; Kim *et al.*, 2010). During drought stress ABA concentrations can increase up to 30-fold, depending on the pre-stress/basal levels (Outlaw, 2003). ABA functions to both promote stomatal closure and inhibit stomatal opening (Schroeder *et al.*, 2001); it should be noted that these two effects

are not simply the reverse of each other (Li *et al.*, 2000; Wang *et al.*, 2001), but ultimately the result is to restrict transpiration and thereby limit water loss.

The perception of ABA (section 1.6.2) both inside and outside (although perception outside is still not well established) the guard cells, triggers multiple simultaneous signal cascades (section 1.6.3) to: regulate ABA responsive genes; to promote stomatal closure by the efflux of K^+, anions, and the production of osmotically inactive substances (e.g. starch) to reduce guard cell turgor and trigger water loss; and to inhibit stomatal opening mainly by inhibiting K^+ uptake. Figure 1.7.1 summarises the guard cell signalling and ion channel regulation triggered by ABA. Guard cell signal transduction and responses to ABA and other environmental factors have been described in detail in Kim *et al.,* (2010), Sirichandra *et al.,* (2009), Hetherington and Woodward (2003) and Schroeder *et al.,* (2001). Only the major guard cell responses to ABA resulting in stomatal closure and the inhibition of stomatal opening will be highlighted below.

ABA increases the levels of reactive oxygen species (ROS) (Pei *et al.*, 2000; Kwak *et al.*, 2003) such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) in guard cells. ROS activate Ca^{2+} permeable non-selective cation channels (I_{Ca}) in guard cell plasma membranes (Pei *et al.*, 2000; Kwak *et al.*, 2003). In *Arabidopsis*, two guard cell expressed NADPH oxidases, known as AtRBOHF and AtRBOHD, are involved in ABA-induced ROS production (Pei *et al.*, 2000; Kwak *et al.*, 2003). In the *Arabidopsis ost1* mutant (section 1.6.3) ABA induced ROS production is disrupted but applied H_2O_2 induces stomatal closure, indicating that OST1 functions upstream of ROS production in ABA signalling (Schroeder *et al.*, 2001). Notably, OST1 has now been shown to phosphorylate AtRBOHF (Sirichandra *et al.*, 2009a).

The generation of nitric oxide (NO) in guard cells in response to ABA has also been shown to be critical for stomatal closure (Neill *et al.*, 2002). In plants NO can be produced from arginine by NO synthase and from nitrate via nitrite by nitrate reductase

(NR). An *Arabidopsis* double mutant in the NR genes, known as NIA1 and NIA2, did not produce ABA induced NO or stomatal closure (Desikan *et al.*, 2002; Neill *et al.*, 2008). The *Arabidopsis abi1-1* and *abi1-2* (section 1.6.3) mutants still produced NO in response to ABA, placing ABI1 either downstream of NIA1 and NIA2 or in a parallel branch of ABA signalling (Desikan *et al.*, 2002). In response to ABA, dominant *abi1-1* mutant plants do not generate ROS, but the dominant ABA-insensitive *abi2-1* mutant plants do generate ROS, which suggests that ABI1 acts upstream and ABI2 downstream of ROS production in guard cell ABA signalling (Murata *et al.*, 2001b). In *Arabidopsis* and *Vicia faba,* the production of NO in response to ABA occurs downstream of ROS production by AtRBOHD/F (Bright *et al.*, 2006). These two stress signalling intermediates, H_2O_2 and NO, can close stomata in their own right, since H_2O_2 and NO donors can induce stomatal closure independently of ABA (Neill *et al.*, 2008; Wang and Song, 2008). Alternatively, they can reinforce ABA-induced stomatal closure (Wilkinson and Davies, 2010) where, ABA induces H_2O_2 production, which in turn induces NO production.

ROS activate both non-selective cation and Ca^{2+} channels (Hamilton *et al.*, 2000; Pei *et al.*, 2000; Murata *et al.*, 2001a; Kwak *et al.*, 2003) leading to increased Ca^{2+} influx (Blatt, 2000; Hetherington and Brownlee, 2004; McAinsh and Pittman, 2009) which increases the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). The ubiquitous nature of Ca^{2+} signalling in cells necessitates a means of encoding the specific trigger that initiated the signal. Two pathways have been proposed: one, that different triggers have specific Ca^{2+} signatures that are recognised by downstream components; and two, that Ca^{2+} signals are accompanied by Ca^{2+}-independent signals that determine the specificity. The existence of these pathways can partially explain the response of guard cells to ABA in both the presence or absence of Ca^{2+} signals (Roelfsema and Hedrich, 2010). Increases in $[Ca^{2+}]_{cyt}$ usually occur as repetitive oscillations, where the frequency, amplitude and shape are specific to the stimulus and create a signal specific calcium signature (McAinsh and Hetherington, 1998; Allen *et al.*, 2001; McAinsh and Pittman, 2009; Dodd *et al.*,

2010). The calcium signature is recognised by Ca^{2+} dependent proteins, leading to specific downstream responses.

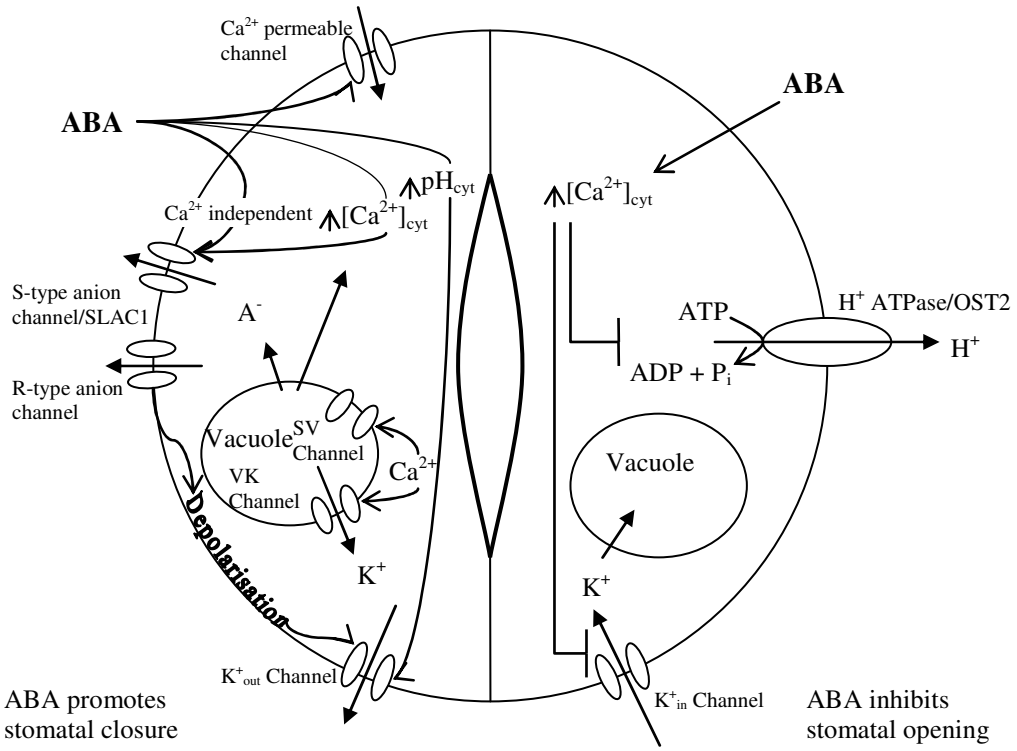

Figure 1.7.1 Summary of guard cell signalling via ion channel regulation by ABA. The left side shows the major ion channels and effectors involved in ABA promotion of stomatal closure. The right side shows the parallel effects of ABA inhibition of stomatal opening. Abbreviations: A-, anions; ABA, abscisic acid; $[Ca^{2+}]_{cyt}$, free cytosolic Ca^{2+} concentration; pH_{cyt}, cytosolic pH; S-type, slow-type; SLAC1, slow anion channel associated1; R-type, rapid-type; SV, slow vacuolar; VK, vacuolar K^+ selective channel, AHA1, Arabidopsis H^+ ATPase1; OST2, open stomata 2. Adapted from Kim *et al*., (2010) and Schroeder *et al*., (2001).

There is also a hypothesis that pre-exposure of guard cells to ABA can 'prime' Ca^{2+} sensors, increasing their sensitivity to subsequent Ca^{2+} increases (Israelsson *et al.*, 2006; Siegel *et al.*, 2009). The mechanistic basis for this priming is unclear, but could involve an ABA- responsive protein or protein kinase (Dodd *et al.*, 2010). If Ca^{2+} sensors are primed, the resting $[Ca^{2+}]_{cyt}$ might be sufficient to activate guard cell ion efflux (Levchenko *et al.*, 2005). Experimental application of repeated hyperpolarizations negative of -120 mV induced repetitive $[Ca^{2+}]_{cyt}$ transients in guard cells (Grabov and Blatt, 1998). The application of ABA shifted the threshold of hyperpolarization-activated Ca^{2+} elevations to -80 mV, indicating that an early event in ABA signalling is the

sensitization of Ca^{2+} influx to membrane potential. Lowering the $[Ca^{2+}]_{cyt}$ in guard cells inhibits ABA-induced activation of anion channels (Levchenko *et al.*, 2005) and therefore inhibits stomatal closure (Siegel *et al.*, 2009). It appears that a certain threshold $[Ca^{2+}]_{cyt}$ is required to enable ABA responses in guard cells (Roelfsema and Hedrich, 2010).

Increased Ca^{2+} concentrations can activate two types of Ca^{2+}-dependent plasma membrane anion channels, the slow activating sustained (S-type) and the rapid transient (R-type) (Schroeder and Keller, 1992). The S-type anion channels generate slow and sustained anion efflux and are activated by increases in $[Ca^{2+}]$ (Schroeder and Hagiwara, 1989; Linder and Raschke, 1992; Schroeder and Keller, 1992; Allen *et al.*, 1999). R-type anion channels such as the *Vicia faba* guard cell anion channel1 (GCAC1) are transiently activated, within 50 ms, in response to increases in external $[Ca^{2+}]$ (Hedrich *et al.*, 1990; Schroeder and Keller, 1992). The R-type anion channels, are not regulated by phosphorylation events like the S-type, but by nucleotide binding activities (Hedrich *et al.*, 1990; Schulz-Lessdorf *et al.*, 1996). A gene encoding the anion-conducting subunit of S-type anion channels has recently been identified *SLAC1* (slow anion channel-associated 1) (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). The *slac1* mutant is impaired in the ABA-activation of S-type anion channels and in ABA induced stomatal closure (Vahisalu et al., 2008). R-type anion channel activities are maintained in *slac1* mutants, which is consistent with the proposed presence of two types of guard cell anion channels (Schroeder and Keller, 1992).

A serine/threonine protein kinase inhibitor abolished anion channel activity and ABA-induced stomatal closing (Schmidt *et al.*, 1995), and the presence of a dominant allele at the *AAPK* gene locus in *Vicia faba* (section 1.6.3) (Li and Assmann, 1996; Li *et al.*, 2000) prevents ABA activation of S-type anion channels, and demonstrates that the final phosphorylation event in anion channel activation could be Ca^{2+}-independent (Schroeder *et al.*, 2001). More than 90% of the solutes released from

guard cells, originate from the vacuoles (MacRobbie, 1998) and the $[Ca^{2+}]_{cyt}$ can also activate vacuolar K^+ (VK) and slow vacuolar (SV) channels triggering the release of K^+, Ca^{2+} and anions from the vacuole into the cytoplasm (Ward and Schroeder, 1994; Gobert *et al.*, 2007). The plasma membrane anion channels transport chloride and malate anions, which have been released into the cytoplasm from the vacuole, across the plasma membrane which results in guard cell depolarisation.

Depolarisation of the guard cells triggers the activation of outward-rectifying K^+ channels (K^+_{out}) and the release of K^+ from guard cells (Schroeder *et al.*, 1987). K^+_{out} channels are largely insensitive to the increases in $[Ca^{2+}]_{cyt}$ that occur during ABA signalling (Schroeder and Hagiwara, 1989). A guard cell outward rectifying K^+ channel, GORK, was isolated from *Arabidopsis* and when expressed in *Xenopus* oocytes mediated depolarization-activated K^+ currents (Ache *et al.*, 2000). A T-DNA knock-out in the *GORK* gene fully suppressed K^+_{out} channel activity, indicating that the *GORK* gene encodes the major voltage-gated K^+_{out} channel in *Arabidopsis* guard cell membranes (Hosy *et al.*, 2003).

ABA can also induce increases in the cytosolic pH (pH_{cyt}) of guard cells (Blatt and Armstrong, 1993; Grabov and Blatt, 1997). Experiments in *Vicia* guard cells demonstrated that K^+_{out} currents can be enhanced by increased pH_{cyt} (Blatt, 1992; Blatt and Armstrong, 1993); ABA-induced increases in K^+_{out} currents can be inhibited by acidification or buffering of the guard cell pH_{cyt}. The ABA induced alkalization of the cytosol, activates K^+_{out} channels promoting K^+ efflux. It has been suggested, based on time course analysis, that cytosolic alkalization precedes NO production (Gonugunta *et al.*, 2008). Protein phosphorylation also plays a role in K^+_{out} activity; guard cells from tobacco plants transformed with constructs involving the dominant phosphatase mutant allele *abi1-1* (section 1.6.2) have K^+_{out} currents two to four fold lower than corresponding untransformed wild type, and are thereby rendered insensitive to ABA (Armstrong *et al.*, 1995). The efflux of cations and anions from the guard cells reduces the solute

concentration, which leads to water efflux from the guard cells which become less turgid resulting in stomatal closure (MacRobbie, 1991; McAnish *et al.*, 1991; Grill and Ziegler, 1998).

Actin microfilaments also participate in guard cell regulation of the stomatal aperture (Kim *et al.*, 1995). In closed stomata, actin microfilaments have a random array throughout the cytoplasm (Gao *et al.*, 2008) and during stomatal opening actin microfilaments are radially orientated and transiently bundled. These changes have been shown to be crucial for stomatal opening (Kim *et al.*, 1995; Gao *et al.*, 2008; Higaki *et al.*, 2010). Treatments with ABA reorganize the actin structure from the radial pattern observed in open stomata to the randomly oriented and short-fragmented pattern seen in closed stomata (Eun and Lee, 1997). Lemichez *et al*,. (2001) found that ABA inactivates a small GTPase encoded by the *AtRAC1* gene which results in the breakdown of the actin cytoskeleton in stomatal guard cells. Transgenic *Arabidopsis* plants expressing a dominant-positive *AtRac1* mutation blocked the ABA-mediated effects on actin cytoskeleton and stomatal closure. Conversely, the expression of a construct based on a dominant *Atrac1* mutant allele showed constitutive ABA-induced actin reorganization and increased ABA sensitivity of stomatal closure. Additionally, in the ABA-insensitive *abi1-1* mutant, which is impaired in stomatal closure, ABA treatment failed to inactivate the AtRAC GTPase, disrupt the actin cytoskeleton or to close stomata (Lemichez *et al.*, 2001). AtRac1 inactivation by ABA is dependent on the ABI1 phosphatase and is another important step in the ABA-signalling cascades which leads to stomatal closure.

ABA not only promotes stomatal closure but also functions to inhibit stomatal opening, see Figure 1.7.1. Stomatal opening is initiated by the hyperpolarisation of the guard cell membrane by H^+-ATPases (proton pumps coupled to ATP hydrolysis). The *Arabidopsis OPEN STOMATA2* (*OST2*) gene encodes the major plasma membrane H^+-ATPase previously called AHA1 (Harper *et al.*, 1989). The dominant *ost2–1* and *ost2–2* mutants produce constitutively activated H^+-ATPases, and have persistently open stomata

and are thus insensitive to ABA (Merlot *et al.*, 2007). The hyperpolarisation of the plasma membrane activates voltage dependent inward rectifying K^+ (K^+_{in}) channels, resulting in K^+ influx. The functional form of these channels is composed of four α-subunits, in *Arabidopsis* guard cells. The genes known as *KAT1* and *KAT2* encode the majority of these α subunits (Sirichandra *et al.*, 2009b and references therein). The influx of K^+ along with other anions such as Cl^-, and the production of malate from starch, causes an increase in guard cell turgor and stomatal opening.

The ABA induced Ca^{2+} signal restricts activity of the plasma membrane proton (H^+) pumps and inhibits the activity of K^+ inward-rectifying (K^+_{in}) channels (Schroeder and Hagiwara, 1989; Kelly *et al.*, 1995) and the ABA induced guard cell depolarisation further deactivates the K^+_{in} channels (Schroeder *et al.*, 1987). Additionally, ABA triggers the endocytotic internalization of the K^+_{in} channel α-subunit KAT1 (Sutter *et al.*, 2007). KAT1 movement from the plasma membrane to the endosome contributes to a reduction in K^+_{in} channel activity (Sutter *et al.*, 2007). It has also been discovered that actin filament depolymerising agents can activate K^+_{in} channels and enhance light-induced stomatal opening; conversely an actin stabilizer can inhibit K^+_{in} channel activity and therefore stomatal opening (Hwang *et al.*, 1997). Higaki *et al*., (2010) have proposed that the dissolution of microfilament bundles enhances stomatal opening via the activation of potassium channels (Higaki *et al.*, 2010). The ABA-induced inhibition of K^+_{in} channels may secondarily inhibit the dissolution of microfilament bundles and reinforce ABA inhibition of stomatal opening. Therefore, the maintenance of guard cell depolarization and the inhibition of K^+ influx by ABA functions to inhibit stomatal opening.

1.8 ABA & THE WHOLE PLANT

1.8.1 Long Distance Signalling of Water Deficit

The concentration of ABA in the apoplast surrounding the guard cells is pivotal for the control of the stomatal aperture and transpiration rate. It is a better indicator of the stomatal aperture than bulk apoplastic, bulk leaf ABA concentration ($[ABA_{leaf}]$) or xylem sap ABA concentration ($[ABA_{xyl}]$) (Zhang and Outlaw, 2001a; Zhang and Outlaw, 2001b). An increase in xylem sap or leaf ABA concentration can be associated with drying soil in contact with the root system (Zhang and Davies, 1989; Zhang and Davies, 1990), or alternatively with exposure to drying air around the shoot (Wilkinson and Davies, 2008), which can be measured by an increase in VPD (Vapour Pressure Deficit) (Trejo *et al.*, 1995; Nejad and Van Meerteren, 2007). Rapid drought-induced stomatal closure often precedes an increase in overall ABA accumulation in the shoot (Beardsell and Cohen, 1975) and can be closely related to changes in soil water availability, even when leaf water status is not affected (Munns and King, 1988; Davies *et al.*, 2005). Stress dependent ABA biosynthesis (section 1.3) has been reported to start with a lag phase of 30-60 min (Dorffing *et al* 1980). ABA biosynthesis in the leaf only starts to be induced when leaf water potential (Ψ_{leaf}) falls to the point where turgor reaches zero (Pierce and Raschke, 1980; Creelman and Zeevaart, 1985; Sauter *et al.*, 2001).

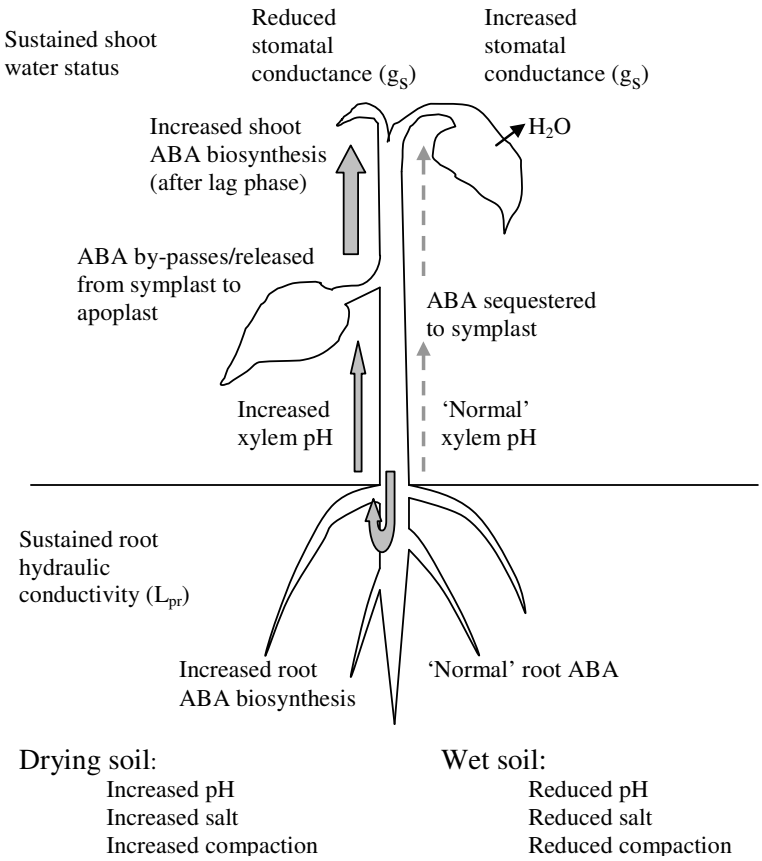

Figure 1.8.1 Diagrammatic representation of the long distance signalling of soil water status by apoplastic pH and ABA concentration. Arrows indicate ABA movement. The left side shows the signals in response to drying soil; causing sap alkalinization (in some species), concentrating ABA in the apoplast, and increasing ABA biosynthesis in the roots and after a lag phase the shoot. The right side shows the effects of wet soil; xylem sap maintains normal pH and ABA is sequestered to the symplast. The ABA concentration finally perceived by stomata is based on the integration of signals from the whole plant. Adapted from Jia & Davies (2007) and Wilkinson & Davies (2008).

The long distance signalling mechanisms plants use to relay information about soil water deficit to the shoot fall into two broad categories; hydraulic/physical signals and chemical signals (Wilkinson and Davies, 2010). Hydraulic/physical signals are internal changes in water relations (water pressure 'waves' and the mass flow of water (Malone, 1993)) that can, in theory, spread rapidly from one cell/organ to another, such as from the roots in drying soil to the leaves (Christmann *et al.*, 2007; Wilkinson and Davies, 2010). Chemical signals may involve changes in the transport of hormones and other chemicals from one cell/organ to another. One possibility is that the increased biosynthesis and/or availability of ABA in roots sensing drying soil, results in ABA

being transported in the xylem to the shoot (Davies and Zhang, 1991; Wilkinson and Davies, 2002; Dodd *et al.*, 2008a). Figure 1.8.1 summarises some of the major long distance signalling mechanisms, discussed below, that are likely to function in response to water deficit throughout the whole plant to link perception of the soil environment and that of the aerial environment.

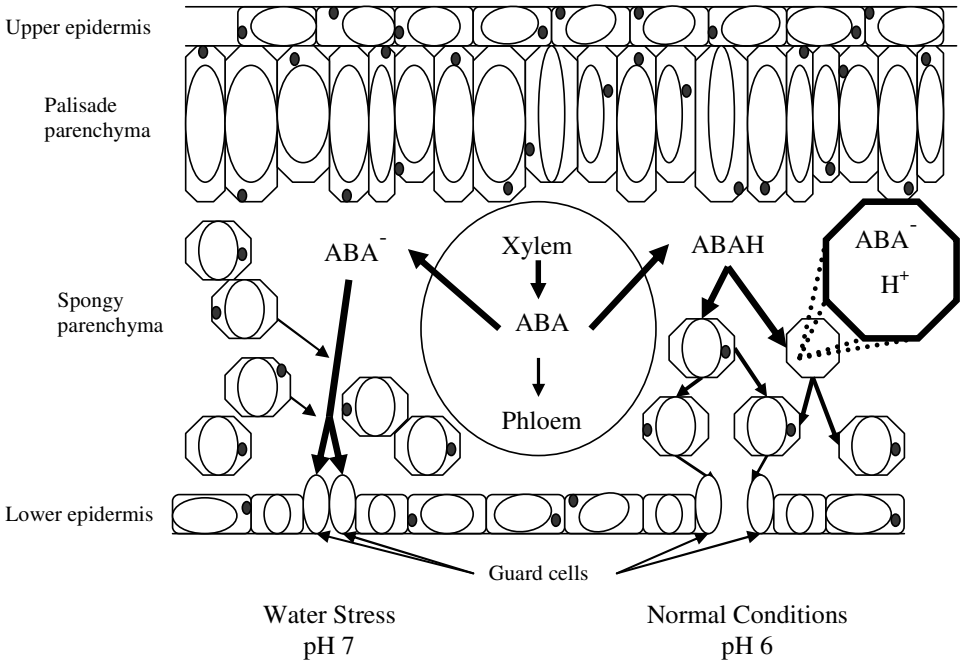

Figure 1.8.2 Schematic representation of ABA redistribution within a leaf. Under normal conditions (right hand side) the apoplast and therefore xylem sap is slightly acidic, usually around pH 6. Protonated ABA arriving in the xylem sap is sequestered into alkaline symplast compartments where ABAH dissociates to ABA^- and a proton (see magnified cell). This alkaline trapping restricts the amount of ABA that can reach the guard cell apoplast. Alkalization, usually around pH 7, of the apoplast is a common response to soil drying (left hand side). ABA is dissociated in the apoplast and ABA moves from the symplast to the apoplast where it is transported directly to the guard cells in the transpiration stream, causing stomatal closure. ABA in the leaves can also be loaded into the phloem and transported to the roots. Adapted from Wilkinson (1999) & Hartung *et al.*, (2002).

ABA is a weak acid (pKa = 4.8) and therefore distributes according to the Henderson-Hasselbach equation, which results in the accumulation of ABA in alkaline anion traps (Hartung *et al.*, 1981; Hartung and Davies, 1991; Slovick *et al.*, 1992; Slovick and Hartung, 1992b,a). The xylem sap, and therefore the apoplast, of well watered plants

is slightly acid (usually between pH 5 and 6), which causes the undissociated form of ABA, in this context termed ABAH, arriving in the transpiration stream to partition into alkaline compartments in the leaf, i.e. the symplast of the leaf cells and the phloem, see Figure 1.8.2. Once inside the more alkaline cell cytoplasm and inside chloroplasts in the light, ABAH dissociates to form the anion ABA^- and a proton (Sauter *et al.*, 2001). Lipid membranes are impermeable to this negatively charged ABA anion and so ABA in this form (ABA^-) becomes trapped inside the cell or compartment, where it can either be metabolised or stored (Kaiser and Hartung, 1981; Davies *et al.*, 2002). This alkaline trapping restricts the amount of ABA that reaches the guard cell apoplast and therefore the guard cell ABA receptors (Jia and Davies, 2007). It should be noted that the recently discovered ABA transporters (section 1.6.1) and receptors (section 1.6.2) may alter the interpretation of the effect of apoplast/symplast partitioning.

Hartung *et al.,* (1988) found that pressure induced dehydration increases the pH and ABA content of exuded sap from leaves and that the pH gradients between the leaf cytosol and the apoplast were reduced (Hartung *et al.*, 1988). Alkalization (usually to between pH 6.4 and 7.2) of xylem sap and therefore the apoplast, has been observed as a common response to soil drying (Sauter *et al.*, 2001; Davies *et al.*, 2005; Jia and Davies, 2007; Wilkinson and Davies, 2008). Increased xylem sap pH causes a redistribution of ABA from storage in the symplast, to the apoplast where ABA can be transported in the transpiration stream (Sobeih *et al.*, 2004) and can potentially access the guard cells, see Figure 1.8.2 (Jia and Davies, 2007; Wilkinson and Davies, 2008).

Xylem sap leaving the root system is generally more acidic than leaf apoplastic sap (Jia and Davies, 2007) because protons are removed as the sap moves up the transpiration stream, resulting in alkalization. The more rapid the rate of transpiration, and the less time the sap spends in the apoplastic pathway, the smaller the impact of proton removal. This redistribution of ABA may cause stomatal closure without any *de novo* ABA synthesis (Hartung and Davies, 1991), which suggests that there is normally

enough ABA present in the leaf to regulate stomatal behaviour in wild type (WT) plants (Sobeih *et al.*, 2004).

There is a lot of evidence supporting the suggestion that changes in xylem sap pH may be an early indication of declining water availability to the roots (Wilkinson *et al.*, 1998; Jia and Davies, 2007). It has been demonstrated that stomatal closure in response to xylem sap alkalization is indirect, in that it is ABA dependent (Sobeih *et al.*, 2004). Unlike WT controls, leaves from the ABA-deficient tomato mutant *flacca* did not exhibit stomatal closure when fed with pH 7 buffers (Wilkinson *et al.*, 1998). Additionally, in a barley (*Hordeum vulgare*) ABA deficient mutant, an exogenous supply of ABA was necessary for a change in pH to cause stomatal closure but not in the WT control (Bacon *et al.*, 1998). Wilkinson and Davies (2008) reported that the spraying of alkaline buffers of pH 6.4 or above onto *Forsythia* and tomato plants elicited the same responses as plants experiencing water deficit. Therefore, changes in xylem sap and apoplastic pH elicit ABA dependent stomatal closure without increased ABA concentration (Sobeih *et al.*, 2004; Davies *et al.*, 2005).

The extent of this alkalization and its effect on ABA distribution is species dependent. For example, the pH of sunflower xylem sap did not change significantly in response to soil drying, while tomato sap pH increased as water availability in the soil declined (Jia and Davies, 2007). Another study compared the Asiatic dayflower (*Commelina communis* L.) with *Arabidopsis* and found that in *C. communis* the stomatal aperture was ~27% greater at pH 7, compared with pH 5, while *Arabidopsis* stomata exhibited little difference (1%) in stomatal opening between these two pHs (Prokic *et al.*, 2006). Both species responded similarly to ABA, and in both species stomata were more sensitive to ABA at the more alkaline pH 7 (Prokic *et al.*, 2006). In soybean (*Glycine max*) xylem sap pH did not increase in response to initial soil drying, and therefore was not correlated with stomatal conductance (Liu *et al.*, 2003). Jia and Davies (2007) found

an apoplastic pH gradient, increasing from stem base to leaves, in *C. communis*, sunflower and tomato (Jia and Davies, 2007).

A relatively recent study by Sharp and Davies (2009) considered the universality/non-universality of the pH response to drying soil in a range of perennial species from a diverse range of plant families; this is contrasted with previous studies which had mostly been performed on herbaceous annuals. These authors found that xylem sap alkalization in response to soil drying was far from universal; species either alkalize, do not alter their pH, or acidify xylem sap pH (Sharp and Davies, 2009). There was no clear evolutionary relationship between apoplastic pH responses, and the findings corresponded with different species being categorized as either isohydric (e.g. maize) which tightly regulate leaf water status, or anisohydric (e.g. sunflower) which do not (Tardieu and Davies, 1992; Tardieu and Simonneau, 1998). Species that exhibited the sap alkalization response tended to be isohydric, i.e. they showed the greatest control of water status under water deficit (Sharp and Davies, 2009). Sharp and Davies (2009) hypothesised that the xylem sap pH response to water deficit is an intensified version of the natural pH gradients that occur in the transpiration stream, indicating that there is a necessity for a plant species to have an alkalization process under well-watered conditions for it to be up-regulated during water deficit. Although species that do not alkalize their sap in response to water stress, can still respond to an alkaline foliar spray (Sharp and Davies, 2009) and it has been found that the application of alkaline sprays to plants can result in water savings of up to 20% (unpublished results of Sharp and Davies, reported in Wilkinson and Hartung, 2009).

The non-hydraulic (i.e. independent of shoot water relations) chemical root to shoot signalling of drying soil was demonstrated with maize (*Zea mays*) by Blackman and Davies (1985). These authors used a technique which has subsequently become known as partial root zone drying (PRD). Blackman and Davies (1985) split the plant root system into two halves, one of which was well-watered and the other allowed to dry.

The irrigated roots were able to supply the shoot with sufficient water to prevent deficit, while the drying roots produced chemical or other signals that were transmitted to the shoot to limit water use (Blackman and Davies, 1985). It was observed that in apple (*Malus* x *domestica* Borkh) plants grown on a similar split root system, the inhibition of leaf growth by soil drying could be prevented by either re-watering or excising the dried root portion (Gowing *et al.*, 1990). It was concluded that the roots in contact with drying soil could be a source of chemical signals from the root to the shoot which were independent of shoot water relations, and that the removal of these roots removes the source of the chemical signal (Gowing *et al.*, 1990). Experiments with field grown and potted grapevine (*Vitis vinifera*) showed that both shoot growth and transpiration could be significantly reduced by PRD (Dry and Loveys, 1999; Dry *et al.*, 2000a,b and references therein). Various versions of the PRD technique appear to be capable of inducing partial stomatal closure in the absence of changes in leaf water potential (Sobeih *et al.*, 2004; Davies *et al.*, 2005).

To sustain the effect of PRD during long term growth of a crop it is necessary to alternate the wet and dry parts of the root system. This is essential to maintain both the transport and 'strength' of the water stress signal (ABA) and the growth of the root system as a whole (Dodd *et al.*, 2006; Dodd *et al.*, 2008b; Dodd *et al.*, 2008a). Modelling has been used to predict that the specific dry soil water potential (Ψ_{dry}) required to maximise the water stress signal from the whole root system varied according to the wet soil water potential (Ψ_{wet}) (Dodd *et al.*, 2008b), which would inevitably affect the timing of alternate watering schedules (Dodd, 2009). Agricultural applications of PRD and the technically simpler system of DI (deficit irrigation) have been most successful with tree crops and in viticulture (dos Santos *et al.*, 2003; Fereres and Soriano, 2007). Dodd (2009) reviewed 15 separate studies comprising ten different crop species, and reported that in no case did PRD significantly reduce yield and in six of these studies yield had increased

by more than 15%. The economic value of a crop can also depend on factors other than yield and in this case PRD has been reported to have additional agronomic benefits.

The main effects of PRD in grapevine are that water use efficiency is increased and vegetative vigour is reduced, but crop yield and berry size are not significantly reduced (Stoll *et al.*, 2000). The reduction in canopy density of the vines can result in better light penetration to the bunch zone and as a consequence can increase grape anthocyanin content, leading to an increase in grape quality and wine of greater value (Dry and Loveys, 1999; Davies *et al.*, 2000; Davies *et al.*, 2002). PRD can improve water use efficiency of wine grape production by up to 50% without significant crop reduction (Stoll *et al.*, 2000).

PRD has also been shown to improve tomato crop quality; one study found that the PRD regime increased WUE by 50% and could account for around a 6% increase in total soluble solid (TSS) accumulation (Refractometer brix (Brix) values) independent of the significant reduction in fruit size (Davies *et al.*, 2000). Another study (Campos *et al.*, 2009) found that tomatoes grown under PRD accumulated less vegetative dry matter, had increased WUE of up to 33% and increased fruit firmness of up to 31%, with no detrimental affect on yield. PRD has also been reported to promote earlier crop maturity in tomato (Zegbe-Dominguez *et al.*, 2003). Davies *et al.,* (2000) suggested that the characteristic PRD effect of reducing vegetative growth of tomato and grape crops, without a corresponding loss of economic yield, could be explained by the fruit hydraulic architecture. In both grape and tomato, there is a change from water supply to the fruit coming from the xylem in early development to water supply from the phloem occurring later in fruit development (Davies *et al.*, 2000). This restricts the influence of potentially growth retarding xylem-borne chemical signals reaching the fruit, potentially explaining the reduced effect on fruit size and yield relative to the vegetative parts of the plant (Davies *et al.*, 2000).

Root sourced signals are able to act independently of the straightforward hydraulic effects of drought on shoot water relations (Gollan *et al.*, 1986). Shoot and/or root sourced hydraulic physical signals (Malone, 1993) could in theory vary concurrently with chemical signals, and could either reinforce or moderate ABA-based signals (Wilkinson and Davies, 2010). Under prolonged water deficit, hydraulic effects will increasingly contribute to regulating stomatal conductance (g_s) and growth (Sharp and Davies, 2009 and references therein). There is evidence for an ABA requirement in stomatal closure in response to soil drying; both ABA deficient and insensitive mutants are known to keep stomata relatively wide open compared to WT plants under conditions of dehydration. For example, in response to soil water stress, the *aba2* and *abi1* *Arabidopsis* mutants revealed a rapid hydraulic response (in terms of mesophyll cell turgor) comparable to that of WT while their stomata did not close to the same extent (Christmann *et al.*, 2007). The authors surmised that this provided evidence in support of a long distance root to shoot hydraulic signal.

Roots can synthesize ABA in response to dehydration by the same biosynthesis pathway as that in photosynthetic tissues (Robertson, 1990; Hartung and Davies, 1991; Parry *et al.*, 1992). It has been reported that in roots of the Asiatic dayflower (*Commelina communis*) and pea *(Pisum sativum*) ABA is synthesized predominantly near the tips (Zhang and Tardieu, 1996) and that the main site of ABA synthesis occurs somewhere between the root tip itself and 3 cm region distal to the tip (Zhang and Davies, 1987). The ABA biosynthetic genes *AtNCED2* and *AtNCED3* (involved in the conversion of 9-*cis*-epoxycarotenoid to xanthoxin (section 1.3.3) were reported to be expressed in the pericycle, around the site of lateral root initiation (Tan *et al.*, 2003), *ABA2* (involved in the conversion of xanthoxin to ABA-aldehyde) appears to be expressed at the branching points of lateral and mature roots and the Abscisic Aldehyde Oxidase 3 (*AtAAO3)* (involved in the conversion of ABA-aldehyde to ABA) gene has been reported to be expressed in root tips and root vascular bundles of

Arabidopsis plants (Cheng *et al.*, 2002; Tan *et al.*, 2003; Koiwai *et al.*, 2004). This evidence supports the earlier suggestion that roots can synthesise ABA by the same biosynthesis pathway as that in photosynthetic tissues.

The ABA content in roots has been correlated with soil moisture and root water content (Zhang and Davies, 1989; Liang *et al.*, 1997), but the precise role of root-sourced ABA is still controversial (e.g. see Christmann *et al.*, 2007). ABA synthesized in the leaves and shoots can be loaded into the phloem (Jiang *et al.*, 2004) and transported to the roots, where it can be stored, metabolised, or loaded into the xylem for redistribution (Figure 1.8.2 (Loveys, 1984; Davies *et al.*, 2005)). ABA recirculation has been detected in white lupins (*Lupinus albus*), castor beans (*Ricinus communis*) and maize (*Zea mays*) (Sauter *et al.*, 2001). The redistribution of ABA originally synthesized in the shoot and 'recycled' could represent a significant proportion of what is often called the 'root-sourced signal' (Wolf *et al.*, 1990; Neales and McLeod, 1991) as could shoot-biosynthesized ABA stored in the root (Slovick *et al.*, 1995).

Christmann *et al*., (2005) attempted to track ABA distribution non-invasively in whole *Arabidopsis* plants using transgenes constructed with ABA-responsive promoters fused to reporter genes (e.g. *pAtHB6::LUC*). In the absence of water stress, low levels of ABA-dependent reporter activation were observed in the columella cells and quiescent centre of the root as well as in the vascular tissues and stomata of cotyledons, perhaps indicating a non stress-related developmental role for ABA in these cell types (Christmann *et al.*, 2005). Water stress induced reporter expression was detected in the leaf vasculature and stomata, before expression was induced in the root system (Christmann *et al.*, 2005).

Grafting experiments have been used to attempt to determine the source of ABA which is specifically involved in drought-induced stomatal closure. Some experiments have been interpreted as suggesting that shoot-sourced ABA is more important than root-sourced ABA for stomatal closure (Holbrook *et al.*, 2002;

Christmann *et al.*, 2007; Thompson *et al.*, 2007b); while other evidence indicates that root sourced ABA, transported to the shoot in response to soil drying, is capable of restricting stomatal opening (Davies and Zhang, 1991; Borel *et al.*, 2001; Davies *et al.*, 2002; Soar *et al.*, 2006).

Using ABA deficient *aba2-1* mutant reporter (e.g. *pAtHB6::LUC*) *Arabidopsis* lines (first described in Christmann *et al.*, 2005) it was observed, from the comparison of WT/WT (scion/rootstock) and WT/*aba2-1* grafts, that roots which were water stressed by contact with an osmotic stress medium containing mannitol could elicit a response in the shoot in the absence of root synthesised ABA. It was also reported that in response to root water stress caused by the same osmotic treatment the stomata of both *aba2/aba2* and *aba2/*WT remained open and no ABA-dependent reporter induction was detected, irrespective of the rootstock capacity to synthesise ABA (Christmann *et al.*, 2007). The authors concluded from this that ABA biosynthesis in the shoot alone was sufficient and a necessary requirement for stomatal closure of whole plants in response to water stress at the roots (Christmann *et al.*, 2007); although shoot synthesized ABA recirculation was not measured (Wilkinson and Davies, 2008).

These results highlight that the proportion of whole plant ABA synthesised by WT roots alone is insufficient to impact upon stomatal aperture and to supply the whole plant with sufficient ABA during water stress, in the absence of normal shoot ABA biosynthetic capability. The use of a genotype that produces increased amounts of ABA as a rootstock, as opposed to a WT rootstock, may have had an impact on the stomatal aperture of the ABA deficient scion. However, these results do not necessarily exclude root sourced ABA (synthesized, recirculated and/or stored) or other compounds from acting as a water stress signal under sufficient whole plant ABA concentrations i.e. with a WT scion capable of ABA synthesis.

To further explore the nature of the long distance signal, Christmann *et al.*, (2007) attenuated water stress signalling by water feeding leaves i.e. water droplets (3-5

µl) and microdroplets (0.4 µl) were applied to the adaxial side of leaves or cotyledons. The water feeding conditions were predicted to allow net water influx into the leaf tissue via the hydathodes. This water feeding was observed to reduced/abolish stomatal closure in response to water stress. The exogenous application of ABA (30 µM) via the roots fully restored the stomatal response in water fed leaves, and six hours after the onset of root water stress the stomata were closed to the same extent as stomata from water stressed seedlings that were not water fed (Christmann *et al.*, 2007). This shows that the leaf responded to transported ABA from the root system, while the response to water feeding provided evidence for a hydraulic signal. This was similar to the split root experiments previously described by Gowing *et al*., (1990), where half the root system was well watered and half was allowed to dry, but the conclusion was in support of a chemical signal, i.e. the conclusions were opposite.

Christmann *et al.,* (2007) also measured mesophyll cell turgor with a pressure probe, while water deficit was rapidly imposed on the root systems of whole plants by lowering the soil water potential using a sorbitol solution (-0.8 MPa). This was found to reduce the turgor pressure of mesophyll cells to zero within 200 sec of application, the subsequent drainage of the sorbitol from the soil by excessive watering reversed the effect and almost recovered the initial turgor values (~0.31 MPa). Stomatal responses were more delayed and started to close within ten minutes of applying the stress and were fully closed after one hour.

When the leaves were fed with water, as previously described, a decrease in soil water potential generated only minor changes; the mesophyll cells remained turgid (0.3 MPa) and the stomata did not close. When the osmotic potential of the sorbitol solution was further lowered (-2 MPa) and leaves fed with water there was a complete loss of turgor, and a subsequent closure of stomata occurred (Christmann *et al.*, 2007). The identification of changes in mesophyll turgor pressure within minutes of root water

stress provided evidence in favour of a hydraulic signal. Since stomatal closure in response to water stress required both ABA and a hydraulic signal in the shoot, the authors concluded that this data supports a root-to-shoot signalling pathway where ABA acts downstream of a rapid long distance hydraulic signal from the root to the shoot.

An alternative explanation could be that the sudden addition of sorbitol blocks water uptake by the roots, the continuation of transpiration leads to reduced shoot water potential, the ABA signal would then close stomata. However, under natural conditions imposition of water deficit is slower and will develop over only part of the root system as soil dries, preventing the sudden block in water uptake, so ABA signals will be generated before the drop in shoot water potential.

Other putative long-distance chemical signals of soil drying may include: mobile C_{15} precursors of ABA (e.g. xanthoxin and ABA aldehyde); ABA-conjugates, such as ABA-glucose ester (ABA-GE) (Jiang and Hartung, 2008; Wilkinson and Hartung, 2009) and bio-active products of ABA catabolism (section 1.4) such as phaseic acid (Davies *et al.*, 2005; Wilkinson and Hartung, 2009). Changes in the concentration of nitrate and the products of nitrate reductase activity, which may impact on pH and/or cellular sensitivity to ABA, may also be important in the root stress signal (Wilkinson, 2004; Wilkinson *et al.*, 2007; Neill *et al.*, 2008; Wilkinson and Davies, 2010).

The whole plant response to water deficit is perhaps based on the integration of both hydraulic and chemical signals from the roots; the latter involving contributions from both root and shoot biosynthesised ABA (Wilkinson and Hartung, 2009; see Figure 1.8.1). The degree of response would depend on the extent and speed of water reduction, the nutritional, hormonal (probably both ABA and ethylene) and water history of the plant, the aerial microclimate, and the developmental stage, structure and form of the plant (Wilkinson and Davies, 2002,2008; Wilkinson and Hartung, 2009).

1.8.2 ABA & Plant Growth

The effects of ABA on plant growth during water deficit are complex and often appear to be contradictory. There is evidence for a role for ABA as a growth inhibitor, for example causing a reduction in leaf growth, from endogenous ABA (Creelman *et al.*, 1990; Gowing *et al.*, 1990; Zhang and Davies, 1990), via exogenous ABA application (Dodd and Davies, 1996; Bacon *et al.*, 1998; Wilkinson *et al.*, 2007; Wilkinson and Davies, 2008), or in transgenic plants that produce more ABA in unstressed plants than in corresponding controls (Tung *et al.*, 2008). There is also evidence that ABA may in some circumstances function to promote or sustain shoot growth, through the analysis of ABA deficient mutants, exogenous ABA application and the chemical inhibition of endogenous ABA production (Mulholland *et al.*, 1996b; Mulholland *et al.*, 1999; Sharp, 2002; Sharp and LeNoble, 2002) or in transgenic plants that overproduce ABA (Thompson *et al.*, 2007a; Parent *et al.*, 2009).

Despite this, it is generally accepted that water deficit can reduce leaf expansion, but there is no consensus on the direct involvement of ABA in this reduction (Wilkinson and Davies, 2010). It should be noted that shoot and root growth are differentially sensitive to water stress (Sharp, 2002) and that plants experiencing water deficit respond differently to ABA than well watered plants. Additionally, the interpretation of results from the endogenous application of ABA are complicated by the uncertainty that the effects of applied ABA are indicative of the role of endogenous ABA (Sharp *et al.*, 1994).

The inhibition of shoot growth in response to soil drying can occur before a decrease in water potential in the aerial parts of the plant occurs (Saab and Sharp, 1989; Gowing *et al.*, 1990). The split root techniques implemented by Gowing *et al.,* (1990) (section 1.8.1) demonstrated that plants with half of the root system growing in dry soil had reduced growth rate compared to well watered controls. The subsequent excision of these dried roots resulted in shoot growth rates returning to those of control plants and

such observations have led to much interest in the involvement of non-hydraulic regulatory signals, e.g. ABA, from the roots (Davies and Zhang, 1991; Davies *et al.*, 2000 discussed in more detail in section 1.8.1). It should be mentioned that Tardieu *et al*., (2010) have proposed an alternative hydraulic explanation for the reduction in leaf growth in split root systems: that roots in the dry soil compartment act as a second 'water sink' in addition to transpiration, resulting in water transfer from the roots in wet soil to the roots in dry soil. The authors suggest that the xylem water potential may, as a result, be lower in PRD plants compared to controls, while leaf water potentials remain similar. This may then affect leaf growth rate via a hydraulic mechanism linked to changes in xylem water potential (Tardieu *et al.*, 2010 and references therein).

Different experimental designs have been used to try to separate the effects of ABA and plant water status on plant growth, such as ABA feeding, transgenic over- and under-expressing ABA plants, pressurisation of the root system or PRD. Some of the early work involved in elucidating the role of ABA on plant growth was based on the phenotypes of ABA deficient mutants. Generally, ABA deficient mutants have reduced root hydraulic conductance (Tal and Nevo, 1973; Bradford, 1983; Nagel *et al.*, 1994), decreased leaf area (Jones *et al.*, 1987; Sharp *et al.*, 2000; Dodd, 2003) and are often shorter than corresponding wild type plants. These phenotypes can be more or less completely restored to normal with exogenous ABA treatment, for example, it has been reported that leaf and stem growth of the ABA deficient tomato mutants, *flacca* (*flc*), *notabilis* (*not*) and *sitiens* (*sit*), can be restored by applying ABA (Imber and Tal, 1970; Tal and Nevo, 1973; Bradford, 1983; Neill *et al.*, 1986; Nagel *et al.*, 1994).

When the *flc* and *not* ABA-deficient mutants of tomato were grown under controlled humidity conditions to maintain their leaf water potentials equivalent to well watered WT plants (Sharp *et al.*, 2000) most parameters of shoot growth remained impaired and root growth was reduced. Additional experiments with *flc* showed that shoot growth recovered when WT levels of ABA were restored by treatment with

exogenous ABA, even though changes in leaf water potential were prevented (Sharp *et al.*, 2000). The ability of applied ABA to increase growth was greatest for leaf expansion (Sharp *et al.*, 2000). These results appear to demonstrate that normal levels of endogenous ABA are required to maintain shoot development, particularly leaf expansion, in well-watered tomato plants, independently of the effects of plant water status (Sharp *et al.*, 2000). These results were reported to be consistent with findings that the inhibition of leaf growth of barley plants, grown in compacted soil, were greater in an ABA deficient mutant than in WT, where evidence indicated that plant water relations were not the cause (Mulholland *et al.*, 1996a; Mulholland *et al.*, 1996b). Contrastingly it has been shown that ABA deficiency in maize seedlings at low water potentials is associated with increased shoot growth, indicating the ABA accumulation was related to the inhibition of shoot growth (Saab *et al.*, 1990).

Plants fed with artificial ABA or genetically enhanced to overproduce ABA typically have a higher degree of leaf hydration compared to control plants (Thompson *et al.*, 2007a; Parent *et al.*, 2009), as mentioned in the previous paragraph the opposite occurs with mutants or transgenics that underproduce ABA (Dodd *et al.*, 2009b; Parent *et al.*, 2009). A mild ABA overexpressing transgenic tomato line had larger leaf areas and greater root hydraulic conductivity than WT plants despite lower assimilation rates per unit leaf area (Thompson *et al.*, 2007a). While, on the other hand, stronger ABA overexpressing transgenic tomato lines exhibited strongly reduced shoot growth (Tung *et al.*, 2008) perhaps indicating that there may be an optimal range of endogenous ABA concentration that promotes leaf growth so that concentrations either above or below this range inhibit growth.

ABA also has an effect on root architecture, which determines the spatial distribution of roots. The application of exogenous ABA tends to maintain root growth under water deficit (Saab *et al.*, 1990) but decreases the number of lateral roots (Guo *et al.*, 2009). The resulting phenotype is a less dense but deeper root system, favourable for

water uptake from deep soil water (de Dorlodot *et al.*, 2007). Additionally, it has been reported that ABA deficient and ABA insensitive mutants of *Arabidopsis* in drying soil have reduced root biomass compared to WT plants (Vartanian *et al.*, 1994). ABA is also known to increase root hydraulic conductivity (L_{Pr}) (Glinka, 1980; Hose *et al.*, 2000; Tardieu *et al.*, 2010) and this may be partly explained by the upregulation of aquaporin genes (Zhu *et al.*, 2005; Tardieu *et al.*, 2010). Consistent with this, ABA-deficient tomato mutants are known to have decreased L_{pr} (Tal and Nevo, 1973) and ABA overproducing transgenic tomato plants have increased L_{pr} (Thompson *et al.*, 2007a; Thompson *et al.*, 2007b).

ABA overproducing transgenic plants have also been observed to overguttate through the hydathodes (Thompson *et al.*, 2000b) which was often accompanied by flooding of intercellular air spaces (Thompson *et al.*, 2007a). It was proposed that because these phenotypes were observed when vapour pressure deficit (VPD) was low, that the combination of this low water output and the increased water input from an increase in L_{pr} caused an imbalance in water supply which can overload the hydathode guttation system and result in leaf interveinal flooding (Thompson *et al.*, 2007a). It has relatively recently been demonstrated in maize (Parent *et al.*, 2009) and tobacco (*Nicotiana tabacum*) (Mahdieh and Mostajeran, 2009) that ABA can increase aquaporin activity and the hydraulic conductivity of both roots and shoots, which results in increased tissue water status and, in maize, greater leaf elongation rates (Parent *et al.*, 2009).

Parent *et al.,* (2009) investigated transgenic maize lines with contrasting rates of ABA biosynthesis generated by overexpressing (sense) or repressing (antisense) *NCED* (a key enzyme in ABA biosynthesis (section 1.3.3)). These plants were kept at constant leaf water potential and it was reported that ABA levels affected aquaporin activity and hydraulic conductivity in both root and shoot tissues. The ABA overproducing plants (high hydraulic conductivity) exposed to water deficit recovered

more rapidly after rewatering than both WT and ABA deficient antisense plants (lower hydraulic conductivity) (Parent *et al.*, 2009). These differences in hydraulic conductance appeared to confer a faster recovery of leaf growth (Parent *et al.*, 2009).

ABA deficient mutants of tomato have been reported to exhibit morphological symptoms characteristic of excess ethylene, such as epinasty and adventitious rooting (Tal, 1966; Nagel *et al.*, 1994) and enhanced ethylene production has been reported in the ABA deficient tomato mutants *not* and *flc* (Tal *et al.*, 1979; Hussain *et al.*, 2000) even when grown at an equivalent or higher shoot water status than WT plants (Sharp *et al.*, 2000). ABA deficient *Arabidopsis* mutants (*aba2-1*) have also been observed to produce excess ethylene (Rakitina *et al.*, 1994; Sharp *et al.*, 2000; LeNoble *et al.*, 2004). Using ethylene deficient transgenic antisense tomato plants with impaired 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase activity ($ACO1_{AS}$), it was found that excess ethylene production was a major cause of shoot growth inhibition in WT tomato plants (Hussain *et al.*, 1999), and further treatment with supplemental ABA partly reduced shoot ethylene production and partly restored shoot growth in WT plants (Hussain *et al.*, 2000). However, this topic remains controversial due to contrasting research findings e.g. the regulation of maize leaf expansion by water deficit during the night did not appear to be regulated by either ABA or ethylene (Voisin *et al.*, 2006).

Foliar ABA sprays decreased ethylene evolution and increased leaf area of *flc* (Sharp *et al.*, 2000) and *aba2-1* (LeNoble *et al.*, 2004) plants. Blocking ethylene perception in ABA-deficient mutants chemically (the foliar sprays of the ethylene inhibitor silver thiosulphate (Sharp *et al.*, 2000)); or genetically (using an *aba2-1 etr1-1* double mutant line which is both ABA deficient and ethylene insensitive (LeNoble *et al.*, 2004)) increased leaf area, once again suggesting that an important role of endogenous ABA is to limit the growth inhibitory action of ethylene (Dodd, 2009). ABA appears to restrain the production of ethylene (Sharp and LeNoble, 2002; Benschop *et al.*, 2007).

Ethylene has been noted to usually be inhibitory to plant shoot and root growth (Sharp *et al.*, 2000) and soil drying can increase the generation of ethylene in shoots by upregulating the synthesis and root to shoot xylem transport of the ethylene precursor ACC (Sobeih *et al.*, 2004). Ethylene has also been reported to induce ABA biosynthesis (Grossmann and Hansen, 2001; Chiwocha *et al.*, 2005) which suggests a self-maintained negative regulatory loop involving these two hormones (Wasilewska *et al.*, 2008). However, ethylene was not found to induce *NCED* expression (A.J. Thompson personal communication). The leaf area of the *aba2-1 etr1-1* double mutant was found to be intermediate between *aba2-1* and WT plants which may indicate the existence of a direct, ethylene-independent, mechanism for the promotion of leaf area by ABA (LeNoble *et al.*, 2004).

Interesting results were obtained from studies using the ABA biosynthesis inhibitor fluridone (similar mode of action to norflurazon see section 1.5) and the *vp5* maize mutant (blocked at the same step as that is blocked by fluridone i.e. phytoene desaturase is inhibited (Saab *et al.*, 1990; Saab *et al.*, 1992; Sharp *et al.*, 1994; Spollen *et al.*, 2000)) and similar studies with the *vp14* maize mutant (defective in the synthesis of xanthoxin (Tan *et al.*, 1997)). These experiments gave consistent results: at high water potentials (well-watered) root elongation rates and ABA contents were minimally affected; in contrast, at low water potentials (during water deficit), ABA deficiency was associated with inhibition of root elongation compared to WT or untreated seedlings. Root elongation rates fully recovered when the ABA content of the elongation zone was restored to normal levels with exogenous ABA. Taken together these results strongly indicate that one function of endogenous ABA is in the maintenance of primary root growth under water deficit by prevention of excess ethylene production. This confirmed ideas suggested by earlier research (Wright, 1980 cited in Sharp *et al.*, 2000) which were based on the finding that pre-treatment with exogenous ABA prevented the normal increase in ethylene production caused by wilting of excised wheat leaves. These findings

from *Arabidopsis*, maize and tomato collectively suggest that the restriction of ethylene production may be a widespread function of ABA and that endogenous ABA may often function to maintain rather than inhibit plant growth during stress (Sharp, 2002).

To evaluate the role of root sourced ABA in regulating growth, as well as stomatal behaviour, Dodd *et al.,* (2009b) studied reciprocal grafts of the ABA-deficient tomato mutant *flc* and WT plants; a subset of which were grown under misting to minimise differences in shoot water status (Dodd *et al.*, 2009b). WT root stocks increased xylem ABA concentration in *flc* scions, relative to *flc* self grafts, leading to partial closure of the *flc* stomata. This resulted in an increase in leaf water potential to a level still lower than in WT scions (Dodd *et al.*, 2009b). These differences in leaf water potential were reported to be consistent with those reported for reciprocally grafted *wilty* and WT sunflower (*Helianthus annuus*) plants (Fambrini *et al.*, 1995). Dodd *et al.,* (2009b) suggest that it is the leaf transpiration rate that controls leaf water potential and not vice versa, and that since xylem ABA concentration controls leaf transpiration rate, scions with the lowest xylem ABA concentration also have the lowest leaf water potential (Dodd *et al.*, 2009b). The 1.6 fold increase in leaf area of *flc*/WT (scion/rootstock) plants (relative to *flc* self grafts) was independent of shoot water status, because the relative effects of the different graft combinations were similar in both the presence and absence of shoot misting. The WT rootstock resulted in decreasing *flc* scion xylem ACC concentrations and detached leaf ethylene evolution in the mutant tissue returned to levels similar to those of WT scions (Dodd *et al.*, 2009b). Since the WT rootstock completely normalized shoot ethylene levels but only partially restored the leaf area of *flc* scions, relative to WT scions, Dodd *et al*., (2009b) concluded that shoot ABA biosynthesis can directly promote leaf area (this was also observed in tomato plants with high [ABA] which had a greater leaf area; Thompson *et al.*, 2007a); perhaps via an unknown, ethylene-independent, mechanism (Dodd *et al.*, 2009b). Whether root-synthesised ABA can also directly promote leaf area, independently of ethylene, awaits further experiments

where ethylene perception of *flacca*/WT plants is blocked e.g., with silver thiosulphate (Dodd *et al.*, 2009b).

Tardieu *et al*., (2010) recently reviewed these (and other) often contradictory findings of the effects of ABA on leaf growth under water deficit and proposed that ABA has mainly three effects on growth. These proposed effects of ABA will be summarised as follows: The first effect is that through controlling transpiration rate and the maintenance of leaf water status, increased ABA can have a positive, but possibly only short term, effect on leaf expansive growth. It has been suggested that the theoretically negative effects of ABA on biomass production via partial restriction of stomatal opening implies that the positive effect might not be sustainable beyond a few weeks (Tardieu and Davies, 1992). However, it should be considered that a larger leaf area and a greater specific leaf area (cm^2/g) will give better radiation use. The second effect, that ABA can promote growth hydraulically i.e. stimulates leaf expansion as a result of increased tissue hydraulic conductivity in roots and leaves, via alterations in root architecture and aquaporin activities (Tardieu and Davies, 1992; Tardieu *et al.*, 2010). The third effect is proposed to be non-hydraulic, where specifically in maize, endogenous ABA tends to reduce leaf growth. This non-hydraulic negative effect can also act on shoot growth in plants subject to water deficit, fed with artificial ABA, or manipulated for increased ABA biosynthesis; but this effect can be positive in well-watered plants deficient in ABA. These authors also acknowledge the possibility of a positive non-hydraulic effect of ABA on leaf growth in some species via the ethylene-ABA opposition as discussed earlier in this section (Tardieu and Davies, 1992; Tardieu *et al.*, 2010).

The overall effect of ABA on leaf growth probably depends on a combination of these and possibly other mechanisms; depending on the extent and duration of the water deficit, the plant developmental stage and prior stress history (Tardieu *et al.*, 2010; Wilkinson and Davies, 2010). Additionally, it should be noted that various plant species may react differently to water deficit, depending on their evolved strategy of drought

resistance, i.e. dehydration tolerance/avoidance to fit their habitat (Wilkinson and Davies, 2010).

1.8.3 Soil Micro-Organisms can Affect Plant ABA

ABA in the xylem can originate not only from internal sources (biosynthesis; section 1.3) but also from external sources (Jiang and Hartung, 2008; Wilkinson and Hartung, 2009). The external rhizosphere can indirectly affect root ABA accumulation; salt stress, the nutritional status of the soil, soil compaction and soil water status can all influence the rate of root ABA biosynthesis. However, soil micro-organisms can directly affect root ABA levels by synthesizing ABA themselves which is subsequently taken up by plant roots.

Plant growth-promoting bacteria (PGPB) are free living soil bacteria that form associations with many plant species and are commonly present in many soil environments (Patriquin *et al.*, 1983; Cohen *et al.*, 2008). The inoculation of some crops with these rhizobacteria has been shown to increase yield by apparently enhancing salt and water stress tolerance (Creus *et al.*, 1997; Mayak *et al.*, 2004; Cohen *et al.*, 2008). The inoculation of *Arabidopsis* with *Azospirillum brasilense* Sp 245 was found to increase ABA concentration by two-fold (Cohen *et al.*, 2008), which may partially explain previously beneficial results of inoculation with *Azospirillum* sp. on plants under osmotic and salt stress. Inoculation with *A. brasilense* Sp 245 improved the water status of wheat seedlings under salt and osmotic stresses (Creus *et al.*, 1997), and the inoculation of maize seedlings with *A. lipoferum* partially reversed the effect of fluridone (an ABA inhibitor) in blocking ABA synthesis, suggesting the bacterial capacity to synthesize the hormone (Cohen *et al.*, 2001). Additionally, increases in shoot ABA were observed in lettuce 15 days after inoculation with *Bacillus subtilis*, a cytokinin-producing bacteria, the increased accumulation of cytokinins in the plant was thought to have modified ABA biosynthesis (Arkhipova *et al.*, 2005).

Bacteria containing the enzyme ACC deaminase (ACCd) that degrade the ethylene precursor ACC have been investigated (Glick *et al.*, 1998). Bacterial uptake of rhizospheric ACC, which can be used as a source of carbon and nitrogen, decreases root ACC concentration and root ethylene production and can increase root growth (Glick *et al.*, 1998). Inoculation with *Achromobacter piechaudii* ARV8, containing ACCd, significantly increased the fresh and dry weights of tomato and pepper seedlings exposed to transient water stress. Ethylene production was reduced in inoculated tomato seedlings following water stress. Inoculation of tomato plants with the bacterium did not influence relative water content, but resulted in continued plant growth during the water stress and after watering was resumed (Mayak *et al.*, 2004). Another ACCd containing rhizobacterium receiving attention is *Variovorax paradoxus*. The inoculation of pea (*Pisum sativum*) with *V. paradoxus* 5C-2 increased growth, yield and water use efficiency of droughted peas, possibly by attenuating a drought induced increase in xylem ACC concentration (Belimov *et al.*, 2009; Dodd, 2009). An amplified soil drying-induced increase of xylem abscisic acid (ABA) concentration was also observed in the inoculated pea plants (Belimov *et al.*, 2009). The possible mediation of ABA levels by *V. paradoxus* 5C-2 was further investigated in maize plants (Dodd *et al.*, 2009a), in which the xylem ABA concentration of both inoculated and uninoculated maize plants increased similarly as Ψ_{leaf} decreased. Dodd *et al.,* (2009) concluded from these experiments that *V. paradoxus* 5C-2 does not intensify ABA signalling. It is hypothesized that species differences may account for the different impacts of *V. paradoxus* 5C-2 on ABA signalling in pea (Belimov *et al.*, 2009) and maize (Dodd *et al.*, 2009a). Field studies with this bacteria (unpublished results by Teijeiro, Dodd and Davies reported in Dodd, 2009) resulted in a 20% increase in pea pod biomass at harvest despite the bacteria population decreasing by 60% over the growing season.

There are potential problems with PGPB or *Arbuscular mycorrhiza* (AM) inoculation on a field scale, including unfavourable competition with indigenous soil

populations and poor persistence in the rhizosphere (Dodd, 2009). Despite the need for further research into the area, the manipulation of soil micro-organisms to affect plant hormone signalling might provide a simpler and less controversial alternative to the use of genetically modified plants. They could also be used to complement watering regimes such as PRD and DI, to increase water use efficiency, growth and crop yield (Dodd, 2009; Wilkinson and Hartung, 2009).

Arbuscular mycorrhiza (AM fungi) exhibit mutualistic interactions, usually between fungal species belonging to the taxonomic group known as the *Zygomycota* and the roots of most terrestrial plants (Smith and Read, 1997). AM fungi, e.g. *Glomus intradices*, grow into the root cortex forming intracellular hyphae and arbuscules (Smith and Read, 1997; Strack *et al.*, 2003). Extensive networks of root plastids cover the arbuscules during their development (Fester *et al.*, 2001; Strack *et al.*, 2003). Apocarotenoids (the oxidative cleavage products of carotenoids) accumulate in AM colonised roots, indicating an increase in carotenoid biosynthesis (Fester *et al.*, 2001; Strack *et al.*, 2003; Akiyama, 2007). Products of fungal metabolism such as mycorradicin and cyclohexanone derivatives accumulate during root colonisation. Mycorradicin has the chromophore mainly responsible for the yellow coloration of many AM associated roots and this yellow pigment is usually esterified in a complex way to other compounds (Fester *et al.*, 2002; Fester *et al.*, 2005; Akiyama, 2007; Walter *et al.*, 2007). The relevance and function of the accumulation of this and other pigments is still not understood (Walter *et al.*, 2007). The arbuscules are a major site of nutrient exchange; carbohydrate from the plant to the fungus and mineral nutrients (especially phosphate) and water from the fungus to the plant (Smith and Read, 1997; Strack *et al.*, 2003). In this way AM symbiosis enhances plant growth, fitness and tolerance to stresses such as water deficit (Auge, 2001; Ruiz-Lozano, 2003; Aroca *et al.*, 2008).

It has been demonstrated using the ABA deficient tomato mutants, *notabilis* and *sitiens*, that ABA plays a contributing role in the establishment of functional AM

symbiosis (Herrera-Medina *et al.*, 2007; Martın Rodriguez *et al.*, 2010). The *sitiens* mutants, which have ABA level reduced to around 8% that of unstressed WT tissues (Herde *et al.*, 1999), were less susceptible to root colonization by the AM fungus *Glomus intradices* than corresponding WT plants (Herrera-Medina *et al.*, 2007). Both ABA and silver thiosulphate (STS; which blocks ethylene perception) application to roots increased the susceptibility of both WT and *sitiens* to AM infection (Herrera-Medina *et al.*, 2007). Herrera-Medina *et al.,* (2007) pointed out that ethylene perception is crucial for AM colonisation, and suggested that impairment of mycorrhizal development in the ABA-deficient mutant *sitiens* is partly due to the excess ethylene levels in this mutant. Martin-Rodriguez *et al.,* (2010) found similar results in the *sitiens* mutant i.e. that there was limited colonisation (mycorrhizal intensity and arbuscule formation) and that the application of exogenous ABA could recover arbuscular frequency. The *notabilis* plants, which appeared to maintain normal root ABA levels under the specific experimental conditions, were less affected and only showed a decrease in mycorrhizal intensity (Martın Rodriguez *et al.*, 2010). Mycorrhization in WT plants was reduced by blocking ABA formation with tungstate application, and this could be reversed by the subsequent application of ABA (Martın Rodriguez *et al.*, 2010). Another recent study (Fiorilli *et al.*, 2009) has suggested that the balance between ABA biosynthesis and catabolism is crucial for the formation of arbuscules. Collectively these results suggest that ABA has a direct and critical effect on AM formation and functioning, while further work is required to elucidate the specific roles of ABA and ethylene in AM colonisation (Fiorilli *et al.*, 2009; Martın Rodriguez *et al.*, 2010).

Drought stressed AM-plants express stress-related genes differently than non-AM plants (Ruiz-Lozano *et al.*, 2006). The altered genes include: LEA (Late Embryogenesis-Abundant proteins), the function of these genes during drought stress is not fully understood but may include protein stabilization, ion binding and antioxidant function; P5CS (Δ^1-Pyrroline-5-Carboxylate Synthase), which catalyses the rate limiting

step in proline biosynthesis, proline is an osmotic agent; plasma membrane aquaporin proteins such as, PIPs (Plasma membrane Intrinsic Proteins), aquaporins facilitate and regulate passive water flow down an osmotic gradient; and *NCED* genes (Saez *et al.*, 2006; Aroca *et al.*, 2008; Jahromi *et al.*, 2008). AM-plants appear to be able to more rapidly regulate ABA levels than non-AM plants during drought stress and recovery (Aroca *et al.*, 2008).

1.9 GENETIC MANIPUATION OF THREE KEY GENES IN THE CAROTENOID/ABA BIOSYNTHESIS PATHWAY

Three of the rate-limiting enzymes in the C_{40} pathway leading to the biosynthesis of ABA: 9-*cis*-epoxycarotenoid dioxygenase, β-carotene hydroxylase and phytoene synthase, provide a major focus of the present research programme. These enzymes and the genes encoding them will be considered in the reverse order in which they appear in the biosynthetic pathway.

1.9.1 Nine-Cis-Epoxycarotenoid Dioxygenase

The first committed enzyme in ABA biosynthesis is nine-*cis*-epoxycarotenoid dioxygenase (NCED), which cleaves 9-*cis*-epoxycarotenoids within plastids (section 1.3.3) to release xanthoxin, which is exported to the cytoplasm where it is oxidized in two steps to produce ABA (section 1.3.4). *NCED* genes form a multi gene family and most plant species seem to have at least one *NCED* gene that responds strongly and rapidly to leaf dehydration, for example: *Vp14* in maize (Tan *et al.*, 1997); *PvNCED1* in bean (Qin and Zeevaart, 1997), *SlNCED1* in tomato (Burbidge *et al.*, 1997; Thompson *et al.*, 2000a), *VuNCED1* in cowpea (Iuchi *et al.*, 2000), *PaNCED1* in avocado (Chernys and Zeevaart, 2000); and *AtNCED3* in *Arabidopsis* (Iuchi *et al.*, 2001; Tan *et al.*, 2003). Increases in NCED mRNA in response to water deficit have been detected in leaves and roots of: tomato (Burbidge *et al.,* 1997; Thompson *et al.*, 2000a), bean (Qin & Zeevaart,

1999), and common stylo (*Stylosanthes guianensis*) (Yang and Guo, 2007); and similar increases have been reported in the leaves of maize (Tan *et al,* 1997) and *Arabidopsis* (Iuchi *et al.*, 2001). *SlNCED1* was shown to be the wild type allele of the wilty tomato *notabilis* mutant (Burbidge *et al.*, 1999); this mutant was first described by Stubbe, (1958). Two other *Arabidopsis NCED* genes, *AtNCED5* and *AtNCED9,* have been reported to respond to leaf dehydration after a 6 hour treatment, but these genes are 'slow' in comparison with *AtNCED3* which responded to a water stress stimulus after only 35 minutes (Tan *et al.*, 2003). The rapidly responding genes (*AtNCED3, SlNCED1, PvNCED1,* and *Vp14*) have a conserved lack of introns (Tan *et al.*, 2003; Thompson *et al.*, 2004), which may facilitate rapid expression in response to stress induction (Taylor *et al.*, 2005).

Relatively recently, it has been reported that drought-induced *AtNCED3* action appears to be mainly restricted to the vascular parenchyma cells of dehydrated leaves (Endo *et al.*, 2008). In this context it is interesting to note that two other ABA biosynthesis proteins, AtABA2 and AAO3, have also been reported to be localized in the vascular parenchyma cells (Koiwai *et al.*, 2004; Endo *et al.*, 2008), highlighting the importance of the vascular cells in drought induced ABA biosynthesis (Endo *et al.*, 2008).

Thompson *et al*., (2000b) provided the first directly conclusive evidence that NCED is a key regulatory enzyme in ABA synthesis. Overexpression of *SlNCED1* in transgenic tobacco using an inducible promoter, and in tomato transformants using a strong constitutive promoter, lead to increased ABA content (Thompson *et al.*, 2000b) and phenotypes associated with the effects of increased ABA (Thompson *et al.*, 2000b). Dex-inducible overexpression of *PvNCED1* in tobacco also caused an increase in leaf ABA content (Qin and Zeevaart, 2002). Additionally, constitutive overexpression of *AtNCED3* caused doubling of ABA content in transgenic *Arabidopsis* plants (Iuchi *et al.*, 2001). Tomato plants constitutively overexpressing *SlNCED1* show greatly reduced

stomatal conductance and grafting experiments confirmed that this was predominantly due to increased leaf ABA biosynthesis (Thompson *et al.*, 2007b). These *SlNCED1* overexpressing lines have a higher transpiration efficiency, due to a greater reduction in stomatal conductance than in assimilation (Thompson *et al.*, 2007a).

Transgenic tomato plants strongly overexpressing *SlNCED1* in a light driven/circadian manner, revealed possible adverse effects of long term high level ABA accumulation (Tung *et al.*, 2008). These plants displayed: reduced CO_2 assimilation due to excessive stomatal closure; a reduction in chlorophyll and carotenoid content; interveinal chlorosis due to leaf flooding; photobleaching; severely reduced growth; and perturbed cotyledon release from the testa (Tung *et al.*, 2008). The levels of ABA in unstressed leaves of these transgenic plants were approximately half the ABA levels typically accumulated in dehydrated WT leaves; but nevertheless appeared to be too high to maintain normal growth rates under well-watered conditions in the long term (Tung *et al.*, 2008). Coupling this data with that for the constitutively overexpressing *SlNCED1* lines (Thompson *et al.*, 2007a) allowed boundaries to be set for the optimal levels of *NCED* up-regulation and ABA accumulation in whole plants that appeared to be beneficial to WUE, without incurring severely adverse negative effects on growth, possibly resulting from the depletion of β-xanthophyll pools in photosynthetic tissues (Tung *et al.*, 2008).

In non-photosynthetic tissues, such as roots, it is much more likely that the supply of xanthophyll precursors required by NCED will be rate limiting when the rate of ABA synthesis increases due to *NCED* overexpression in the absence of a water stress stimulus. It is likely to be necessary to combine the overexpression of gene constructs encoding NCED with the overexpression of constructs encoding other rate limiting enzymes upstream in the ABA synthesis pathway in order to commit β-xanthophylls in root plastids to ABA production, thereby achieving higher amounts of root biosynthesized ABA (Taylor *et al.*, 2005).

1.9.2 β-Carotene Hydroxylase

A second key regulatory enzyme, BCH, catalyses the formation of the first xanthophyll in the β-branch of the pathway, performing the two reactions to convert β-carotene to zeaxanthin (section 1.3.2). A pair of closely homologous genes (the *BCH*/*CrtR-b* genes), both encoding β-carotene hydroxylases have been reported in: *Arabidopsis* (Sun *et al.,* 1996; Tian & DellaPenna, 2001), pepper (*Capsicum annulum* L.) (Bouvier *et al.*, 1998) and tomato (Galpaz *et al.*, 2006).

In tomato, *SlBCH1* (*CrtR-b1*) has been reported to be chloroplast specific and therefore predominantly expressed in photosynthetic tissues (Galpaz *et al.*, 2006). There is evidence that the expression of the *BCH1* gene in tomato has a circadian rhythm (Sonneveld *et al.*, unpublished data) as described in section 1.3.3. In contrast, *SlBCH2* (*CrtR-b2*) has been described as chromoplast specific as it is strongly expressed in yellow coloured mature flower organs (Ronen *et al.*, 2000,Galpaz *et al.*, 2006;Taylor *et al.*, 2005). However, it has more recently been observed that in response to water stress *SlBCH2* (*CrtR-b2*) is up-regulated in leaves (Sonneveld *et al.*, unpublished data) as well as in roots (Thompson *et al.*, 2007b), demonstrating that its expression is not exclusively chromoplast specific. When Davison *et al.,* (2002) overexpressed *chyB,* encoding a β-carotene hydroxylase from *Arabidopsis* they reported substantial increases in β-epoxycarotenoid pool sizes, notably in all-*trans*-violaxanthin. These *chyB* overexpressing transgenic *Arabidopsis* plants had increased tolerance to high light stress, presumably due to the additional photoprotection provided by the increased xanthophylls (Davison *et al.*, 2002). The constitutive overexpression of *SlBCH2* (*CrtR-b2*) in transgenic tomatoes also resulted in increases in leaf all-*trans*-violaxanthin (Sonneveld *et al.*, unpublished data). There was no consequential reduction in the β-carotene pool in either of these transgenic lines, despite the additional demand for substrate resulting from the overexpression of *chyB* (Davison *et al.*, 2002) and the *SlBCH2* gene (Taylor *et al.*, 2005). The gain in β-ring

xanthophylls, notably all-*trans*-violaxanthin, is not simply occurring at the expense of the β-carotene pool, which appears to indicate that earlier steps in the pathway are up-regulated to prevent excessive depletion of this important carotenoid in photosynthetic tissues.

The overexpression of the *SlBCH2* gene in tomato also resulted in an orange fruit phenotype, due to reduced lycopene and total carotenoid levels compared to ripe WT fruits. Interestingly, the constitutive overexpression of *SlBCH2* in tomatoes also appeared to confer a small, but significant, increase in leaf ABA content and transpiration efficiency (TE_p) (Sonneveld *et al.*, unpublished data).

1.9.3 Phytoene Synthase

A third key regulatory enzyme in the ABA biosynthesis pathway is involved in the first dedicated step of carotenoid biosynthesis. Phytoene synthase converts the C_{20} substrate GGPP to the C_{40} colourless compound known as phytoene (section 1.3.1). *Arabidopsis* has only one *PSY* gene, while in other plant species there can be two or more *PSY* homologues. In tomato there are two genes encoding PSY enzymes: *SlPSY1* is predominantly a mature fruit and flower (i.e. chromoplast) expressed gene (Bartley *et al.*, 1992) in contrast to *PSY2* which is usually expressed in chloroplasts and therefore functions in all green tissues, including immature petals and unripe tomato fruit (Bartley and Scolnick, 1993; Fraser *et al.*, 1999). More recently a third PSY encoding gene, *PSY3*, has been identified in members of the grass family (Poaceae) (Li *et al.*, 2008; Welsch *et al.*, 2008) and was reported to influence root carotenogenesis and to be up-regulated in response to salt and drought stress (Li *et al.*, 2008). Li *et al.,* (2008) found that in response to drought, elevated ABA in leaves and roots of maize plants was accompanied by elevated *PSY2* and *PSY3* mRNA in leaves, of which *PSY2* was the most abundant, and only *PSY3* mRNA increased in roots (Li *et al.*, 2008).

The constitutive overexpression of *PSY1* in transgenic tomato plants was reported to be associated with abnormal carotenoid production in a variety of organs including the cotyledons, leaves and fruit (Fray *et al.*, 1995). There was also a complex inverse relationship between *PSY1* mRNA content and plant height. The most severely stunted plants had the most substantially increased levels of phytoene and lycopene, which was correlated with a reduction in several gibberellic acids (GAs) which may have contributed to the reduced height of these transgenic plants. Fray *et al.,* (1995) suggested that the overexpression of *PSY1* could have altered the flux through different branches of the plastidial (MEP) terpenoid biosynthesis pathway, with high levels of phytoene and other C_{40} compounds being produced at the expense of the C_{20} precursors of GAs. This dwarfism could also in part be related to a reduction in phytol, which is required for chlorophyll formation and is also formed from the same GGPP substrate pool (Fray *et al.*, 1995).

The fruit of these plants produced lycopene much earlier in development than is normal, but the final concentrations of lycopene in ripe fruit were lower in these plants than in the wild type (Fray *et al.*, 1995). The changes in carotenoid composition in the fruit are associated with alterations in plastid type; chromoplast-like structures could be detected prematurely, i.e. during early development of the unripe fruit, and were not connected with the normal ripening process (Fraser *et al.*, 2007).

Tissue specific overexpression of constructs encoding *PSY1* in tomato fruits resulted in altered carotenoid content, while avoiding the negative side effects of constitutive *PSY* overexpression (Fraser *et al.*, 2002). The manipulation of PSY has also been achieved in oilseed rape (*Brassica napus*) in which *CrtB*, a gene encoding PSY from *Erwinia*, was expressed in a seed-specific manner and resulted in a 50-fold increase in carotenoid content in mature seeds, mainly in the form of α- and β-carotene (Shewmaker *et al.*, 1999). Additionally, the tissue specific overexpression of PSY in *Arabidopsis* seeds (Lindgren *et al.*, 2003) resulted in increased carotenoid content

(Lindgren *et al.*, 2003). In both *Arabidopsis* and oilseed rape, *PSY* overexpression resulted in decreased seed germination rate, which was suggested to be due to the elevation of ABA levels (Shewmaker *et al.*, 1999; Lindgren *et al.*, 2003). PSY encoding constructs were also used in the production of golden rice (Burkhardt *et al.*, 1997) and have been utilised in similar work in potato tubers (Ducreux *et al.*, 2005; Diretto *et al.*, 2007) to increase the carotenoid content of these non-photosynthetic underground organs.

Another phenotype of constitutive *PSY1* overexpression is increased root pigmentation. The root systems of *SlPSY1* overexpressing transgenic tomato lines (Z171; Fray *et al.*, 1995) appeared orange, which was considered to be due to the accumulation of β-carotene and lycopene (Jones, 2008). This finding was consistent with the accumulation of lycopene and the increased total carotenoid concentration in vegetative tissues of these transgenic plants (Fray *et al.*, 1995). Additionally, an increase in the total root carotenoid content, mostly in the form of lycopene and β-carotene, was observed in double transgenic tomato lines constitutively overexpressing both *SlPSY1* and *SlNCED1* (Jones, 2007). Interestingly, an increase in root ABA concentration was also observed in both the single *SlPSY1* overexpressor and the double transgenic line overexpressing both *SlPSY1* and *SlNCED1* (Jones, 2007). It appears that the overexpression of *SlPSY1* can increase the supply of carotenoid precursors in roots, which can have a positive impact on root ABA biosynthesis. This indicates that PSY catalyses an important rate-determining step in root carotogenesis (Taylor *et al.*, 2005).

1.10 AIMS & OBJECTIVES

The overall aim of the project was to improve the water use efficiency of tomatoes via increased ABA biosynthesis in the absence of water stress. Two approaches were taken in order to realise this aim. The first involved the use of wild species of tomato in a classical backcrossing programme, as part of a larger joint Nottingham/Warwick-HRI DEFRA funded research project, to explore the natural

variation of the *NCED1* gene. It was hypothesised that wild relatives of tomato might have evolved differently expressed *NCED1* alleles due to the unique habitats of their areas of speciation. Two wild species closely related to tomato; *S. galapagense* and *S. neorickii* were selected for investigation within this thesis, with the intention of introgressing the divergent *SgNCED1* and *SnNCED1* alleles into the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig. The aim of this intogression programme was to assess the effect of the wild species *NCED1* alleles on whole plant water use efficiency.

The second approach involved the development of a triple transgenic line for use as a rootstock. Non photosynthetic tissues, such as roots, have much lower carotenoid levels than photosynthetic tissues, such as leaves, and therefore the supply of carotenoid precursors may become limiting to overall increases in root ABA biosynthesis. It was postulated that the constitutive overexpression of three key rate-limiting enzymes in the carotenoid/ABA biosynthesis pathway; *SlPSY1*, *SlBCH2*, and *SlNCED1*, would increase carotenoid precursor flux through the biosynthetic pathway, potentially resulting in increased ABA production throughout the plant.

The hypothesis was that this selected genotype could be used as a triple transgenic rootstock, which could be grafted to a non-transgenic commercial cultivar as a scion. It was anticipated that this could conceivably provide sufficient extra root-sourced ABA, under non-stressed conditions, to reduce stomatal conductance and increase whole plant water use efficiency. This unique 'semi-GM' approach may circumvent some of the objections made against the use of genetically modified crops; the edible part of the crop (tomato fruit) is unmodified and, the risk of transgene transfer to the environment would be greatly reduced since the transgenic material is limited to vegetative growth as a rootstock.

These two independent approaches were each pursued with specific objectives:

I. The Non-Transgenic Approach

i. To introgress the *SgNCED1* and *SnNCED1* alleles into the cultivated tomato *S. lycopersicum* cv. Alisa Craig genetic background with four backcrosses, followed by selfing to produce lines homozygous for the respective *NCED1* alleles

ii. To design CAPS (cleaved amplified polymorphisms) markers to identify the wild species *NCED1* alleles.

iii. To assess the effect of the *SgNCED1* and *SnNCED1* alleles on WUE in comparison to the *SlNCED1* allele.

II. The Transgenic Rootstock Approach

i. To generate a transgenic tomato line which contains three constructs *sp::SlNCED1*, *35S::SlCrtRb2/BCH2*, *35S::SlPSY1*, preferably homozygous at all three T-DNA insertion loci.

ii. To investigate the effect of this novel combination of transgenes on the carotenoid biosynthesis pathway in root tissues via HPLC-PDA analysis.

iii. To determine the extent to which root-synthesized ABA can be elevated by the combination of these three transgenes.

iv. To assess the effect of the selected triple transgenic line, when used as a rootstock, on daily water loss and WUE of non-GM scions.

CHAPTER 2:

GENERAL MATERIALS & METHODS

2.1 PLANT MATERIAL & GROWTH CONDITIONS

2.1.1 Plant Material

The non-transgenic line Tm2a is homozygous for the tomato mosaic virus (TMV) resistance gene (*Tm-2*^{a} synonymous with *Tm-2*^{2} locus AF536201; Lanfermeijer *et al.*, 2003), in the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig (GCR267). This line or the standard TMV susceptible Alisa Craig variety were used as wild type cultivars. The transgenic tomato lines overexpressing ABA biosynthetic genes (some resulting from transformation of the Tm2a line and others from the standard Ailsa Craig tomato variety) are described in section 4.2.1 and are listed in Appendix I Table 7.1.1. The wild species closely related to tomato which were used in the non-transgenic approach are listed in section 3.2.1, along with the ABA deficient mutant used.

2.1.2 Seed Harvest

Seeds were removed from the locules of ripe tomato fruit, rinsed in a sieve to remove any pulp and incubated overnight in 12 mM hydrochloric acid (Fisher Scientific, Leicestershire, UK) plus 1 g l^{-1} pectinase (Youngs, UBrew, West Midlands, UK). They were then rinsed and left to dry on Whatman® filter paper (No.1 qualitative) at ambient humidity, then stored in seed envelopes at room temperature.

2.1.3 Glasshouse Conditions

Glasshouse conditions were set at: 24/20°C (± 0.3°C) day/night temperature, ventilation at 26°C (± 0.5°C) and, supplementary lighting for a 16/8 h day/night cycle. Pre-imbibed seeds were sown directly ~1 cm below the surface of the compost in 7 cm diameter pots of Levington's F2 compost (Levington Horticulture, Suffolk, UK) with 9

cm Petri dish lids placed over the top of the pots to create a humid environment until the hypocotyl emerged and the hook started to straighten, at which point the lids were removed. Seeds requiring norflurazon treatment to break ABA-induced dormancy were imbibed in 0.1 mgL^{-1} norflurazon for specific periods of time, depending on seed genotype (Appendix I Table 7.1.2) and then rinsed carefully and thoroughly (approximately one minute) in water before sowing. In all cases care was taken to ensure that the seed was covered with compacted moist compost to prevent drying in subterranean air spaces. Plants were transferred to 13 cm pots (and then potted on into larger pots as required) of Levington's M3 compost during their growth period. Plants were irrigated regularly with Vitafeed 214 (Vitax Ltd., Leicestershire, UK) unless otherwise stated.

2.2 GRAVIMETRIC WATER USE EXPERIMENTS

The seeds of each genotype were sown (with/without norflurazon pre-treatment) at varying times to synchronise plant development at the end of the seedling establishment phase (Appendix I Table 7.1.3); some of the different transgenic ABA overexpressing lines typically exhibited a delay in seedling establishment. The plants were re-potted into 5 L pots in Levington's M3 compost, and after a few days establishment those plants most similar in size were selected for inclusion in the experiment. The soil was watered until saturated i.e. water was running out of the bottom of the pots and Uvi ground cover disks (Growing Technologies, Derby, UK) were placed on top of the compost to minimise soil moisture evaporation. The plants were arranged in a randomised block design and a 'blank' pot which did not contain a plant was included within each block. These pots were also watered to field capacity and weighed every day. The blank pots allowed an estimate to be made of the daily water loss per pot due to water evaporation from the soil surface and the average daily weight loss from these pots was deducted from the daily measurements of transpiration.

Once the excess water had drained from the pots, each was weighed to ascertain the weight at field capacity. At the beginning of the experimental period a number of plants of each genotype (varied according to each specific experiment) were destructively harvested. The height, fresh weight and dry weight were measured. Individual pots were weighed daily throughout the experimental period (30 days) to determine the quantity of water used by each plant over the course of the day. Each pot was then watered back to its individual weight at field capacity (as measured the day before). At the end of the experimental period, all plants were destructively harvested and above-ground fresh and dry weights were recorded. A number of other morphological/growth parameters were also measured, (not all were measured in each experiment) typically including; plant height, leaf length, leaf area, and stem diameter.

2.3 DNA EXTRACTION: CTAB DNA MINI-PREP

Young expanding leaflets (~1 cm long) were harvested in 1.5 ml Eppendorf tubes and immediately frozen in liquid nitrogen. Samples were ground to a fine powder and 500 µl pre-warmed (65 °C) 2% CTAB buffer was added. The samples were briefly mixed and incubated in a water bath at 65°C for 30 min. The homogenate was inverted twice during incubation to mix the sample. To the homogenate, 500µl phenol:chloroform:isoamyl alcohol (25:24:1) was added and inverted to mix the phases. The samples were centrifuged at 13,000 rpm for 10 min; the aqueous supernatant layer was then carefully transferred to a fresh 1.5 ml Eppendorf tube and 300 µl of ice-cold isopropanol was added. The tube contents were mixed by inverting the tube several times and left to stand at -20 °C for 15 min to precipitate the DNA. The tubes were centrifuged at 13,000 rpm for 10 min, and the supernatant was decanted off, and any remaining supernatant removed by pipetting. The pellet was washed by adding 500 µl of cold wash buffer (Appendix II section 7.2.6); the tube was inverted to mix and left to stand for two minutes. The sample was centrifuged at 13,000 rpm for 3 min and the wash step repeated.

The pellet was briefly centrifuged again (13,000 rpm for 1 min), and any remaining buffer was removed by pipette. The pellet was re-dissolved in 75 µl of TE buffer (Appendix II in section 7.2.7) containing 0.3 µg μl^{-1} RNase A. Samples were stored at -20 °C.

2.4 POLYMERASE CHAIN REACTION (PCR)

PCR was used to identify plant genotypes, confirming the presence of a gene/transgene either using pairs of primers (Listed in Table 7.3.1) to obtain a direct amplicon or subsequently treating the PCR product with a restriction endonuclease to obtain cleaved amplified polymorphic sequence (CAPS) markers (Listed in Table 7.3.3) specific for the appropriate gene. For a typical 20 µl PCR reaction (set up in 0.2 ml PCR tubes): 1 µl of ~50 ng of template DNA ; 1 µl each of 40 pmol forward and reverse primers; 10 µl MangoMix (a 2x pre-optimized reaction mix containing Mango*Taq*™ DNA Polymerase from Bioline, London, UK), and 7 µl of SDW. PCR cycling conditions varied depending on the primer pairs in use and the amplicon size (specific PCR cycling details are listed in Appendix III Table 7.3.4). Typical cycling conditions were: 95°C for 5 min to completely denature the template before 35 cycles of PCR amplification (denaturation 95°C for 60 seconds; annealing 50 – 65 °C for 30 seconds; extension 72°C for 60 sec). A final incubation for 10 min at 72°C completed the reaction.

The PCR product was then visualized by gel electrophoresis; the gel composition used depended on the amplicon size, but typically a 1% (w/v) agarose gel in 1x TBE buffer (Appendix II in section 7.2.9) was used and 4 µl ethidium bromide was mixed into the gel solution before it was set, to allow subsequent visualization of the PCR product. Between 5 – 10 µl of the PCR product was loaded onto the gel, with an appropriate DNA ladder (Bioline, London, UK), and run in 1x TBE buffer at 100 V/ 150 A for 45 min. The gel was then visualized under a UV-transilluminator.

2.5 RESTRICTION DIGESTS

PCR products were amplified with the appropriate primer pairs. A master mix was prepared on ice so that each tube would contain; 1 µl of enzyme appropriate buffer (supplied with the enzyme (New England Biolabs, Hertfordshire, UK)) (vortexed buffer before use); 0.1U of appropriate restriction enzyme (between 0.1 - 0.5 µl); 3.5 – 3.9 µl sterile water (total volume 5 µl). The master mix was aliquoted (5 µl) into 0.5 ml Eppendorf tubes on ice. A 5 µl aliquot of the appropriate PCR product was added to each tube. Both a positive control (a PCR product previously shown to successfully digest), and a negative control (no enzyme control) were used to check for enzyme activity and contamination of the reagents respectively. The tubes were briefly vortexed to mix, and briefly centrifuged to pool the contents. The tubes were incubated for at least 2 h (no longer than overnight). The digest products were visualized on a 2 – 4 % (w/v) agarose gel in 1x TBE buffer (Appendix 7.2.9), depending on the expected product size. A 5 µl aliquot of the digest product was mixed with 5 µl Orange G 1x loading dye, and then loaded onto the gel, with a 100 bp DNA ladder (HyperLadder™ IV, Bioline. London, UK) and an uncut control (undigested PCR product for comparison of the effect of the enzyme). The gel was run in 1x TBE buffer at 120V for 1 hr. The gel was visualized under a UV-transilluminator.

2.6 ABA RADIOIMMUNOASSAY

Samples were extracted and assayed as previously described (Thompson *et al.*, 2000a). Leaf samples were collected in 2 ml amber coloured microfuge tubes (ACMC tubes, VWR-International, UK) and immediately frozen in liquid nitrogen. Root material was collected in 7 ml Bijous (Fisher Scientific, Leicestershire, UK), and freeze-dried for 48 h in a freeze-dryer (CHRIST, Freeze Drying Solutions, Shropshire, UK) at -90°C. The samples were ground to a fine powder under liquid nitrogen before adding either 500 µl

or 1500 µl of distilled water per 100 mg of leaf or root material respectively. The samples were then extracted overnight on a shaker at 4°C in the dark.

2.6.1 Preparation of Antibody & ABA Standards

A stock of lyophilised monoclonal antibody AFRC MAC252 (Quarrie *et al.*, 1988) was rehydrated with water according to the manufacturer's instructions (Babraham Bioscience Technologies Ltd, Cambridgeshire, UK). The stock antibody solution was diluted 1:10 in MAC252 buffer (Appendix II in section 7.2.19). The diluted MAC252 antibody was then transferred to 1.5 ml Eppendorf tubes in 1 ml aliquots and stored at -20 ºC. This was used as the working solution. For use in the assay, 100 µl of the working antibody solution was mixed with 20 ml of MAC252 buffer. A set of unlabelled (+)-*cis*-*trans*-ABA (Sigma-Aldrich, Dorset, UK) standards diluted to 2,000, 1,000, 500, 250, 125 pg per 50 µl, were prepared for use in construction of a standard curve, from which tissue ABA content was calculated.

2.6.2 ABA-RIA Assay

The ABA standards, a blank of SDW, and the samples (50 µl aliquots) were added to 2 ml screw top vials (Fischer Scientific, UK) each tested in triplicate. To these were added 100 µl ^{3}H-ABA (Appendix II in section 7.2.18), 100 µl MAC 252 buffer (Appendix II in section 7.2.19) and 200 µl 50% PBS (Appendix II in section 7.2.17). The samples were then covered with aluminium foil and incubated at -4 ºC for 45 min, after which 500 µl of saturated ammonium sulphate (Appendix II in section 7.2.20) was added. The rack was then inverted to mix the solution, and the samples were covered with foil and incubated at room temperature for 30 min. The samples were centrifuged at 13,000 rpm for 4 min, and the supernatant decanted off and the vial blotted dry on paper towels. The pellet was mixed with 1 ml of 50% saturated ammonium sulphate and centrifuged at 13,000 rpm for 4 min. The supernatant was decanted off and the tubes blotted dry as before. The remaining pellet was dissolved in 100 µl of distilled water, and allowed to

stand for 5 min before being vortexed briefly. Then the samples were mixed with 1.2 ml of scintillation cocktail (National Diagnostics, East Yorkshire, UK). The 2 ml sample vials were placed into 20 ml high density polyethylene vials (Fischer Scientific, Leicestershire, UK) before being loaded into a liquid scintillation analyser (Tri-Carb 2800TR; PerkinElmer, Cambridgeshire, UK). The radioactivity estimates were given as either counts per minute (cpm) or disintegrations per minute (dpm). Tissue ABA content was calculated by substituting the values for cpm or dpm into the equation that was derived from the standard curve.

2.7 CAROTENOID/XANTHOPHYLL ANALYSES

Carotenoids were extracted using a two stage solvent extraction method. Samples were collected in 2ml amber coloured microfuge tubes (ACMC tubes, VWR-International, West Sussex, UK) and immediately frozen in liquid nitrogen. Frozen material (~60 mg leaf material; ~100 mg (freeze dried) root material; ~200 mg isolated tissue culture (freeze dried) root material) was ground to a fine powder, either with a glass rod in the tube for leaf material, or with a pestle and mortar for root material which was then transferred to a 2 ml amber microfuge tube. To the ground sample 1 ml of 100% acetone was added. Samples were vortexed for 5 min and then centrifuged at 15,000 rpm for either 5 min or 10 min for leaf or root material respectively. The supernatant was transferred to a fresh tube, being careful not to disturb the pellet. The second extraction involved adding 1 ml of 100% acetone to the remaining pellet, vortexing for 5 min and then re-centrifuging for either 5 min or 10 min at 15,000 rpm for leaf or root material respectively. The resulting second supernatant was carefully added to the tube containing the first supernatant. Samples from root material were further centrifuged at 15,000 rpm for 10 min, and the supernatant carefully transferred to a fresh tube, ensuring that no debris was also transferred. The acetone was evaporated to dryness using a gyro-vap (CHRIST, Freeze Drying Solutions, Shropshire, UK) for 4 h at 30°C.

2.7.1 Reverse (C_{30}) Phase HPLC Analysis

The powdered samples were re-suspended in 300 µl (or 240 µl for cultured root material) ethyl acetate by mixing. Samples were then centrifuged for 5 min at 15,000 rpm to pellet any debris. Following this a 150 µl (120 µl for cultured root material) aliquot of the supernatant was transferred to HPLC vials (Chromacol Ltd, Thermo Scientific, Hertfordshire, UK). Samples were analysed using a Waters Alliance 2695 machine (Waters, Hertfordshire, UK) in conjunction with Empower software. A C_{30} HPLC column (Ultracarb 5µm particle size ODS (30) 250 x 4.6 mm i.d. 152720-8, Phenomenex, Cheshire, UK cat no.00g-0351-E0,) was wet primed with 95% solvent A (100% methanol) to 5% solvent B (2% ammonium acetate w/v in methanol: water 80:20 v/v), at a flow rate of 1 ml min^{-1} for 10 min at 300-500 psi. A 50 µl aliquot of each sample was automatically injected on to the column and eluted according to the solvent gradient regime outlined in Appendix V Table 7.5.1. De-gasser pressure was maintained at 1800 psi and column temperature at 21°C. All pigments were analysed by photo diode array detector and quantified at wavelength 440 nm.

2.7.2 Forward (Adsorption) Phase HPLC Analysis

The remaining sample extract in ethyl acetate was evaporated to dryness using the gyro-vap (CHRIST, Freeze Drying Solutions, Shropshire, UK) for 4 h at 30°C. The sample was then re-suspended in 150 µl (120 µl for cultured root material) hexane by vortexing for 5 min. The samples were then centrifuged at 15,000 rpm for 5 min to pellet any debris, 150 µl (120 µl for cultured root material) of the supernatant was transferred to HPLC vials. The samples were then run on a silica 5 µm column (5 µm particle size, 250 x 4.6 mm i.d. Luna range Phenomenex, Cheshire, UK cat no. 00G-4274-EO), with a wet prime of 95% hexane, 5% hexane/isopropanol (60:40) at a flow rate of 1 ml min^{-1} at a pressure of 300-500 psi for 10 min. A 50 µl aliquot of each sample was automatically injected on to the column and eluted according to the solvent gradient regime outlined in

Appendix V Table 7.5.1. De-gasser pressure was maintained at 600 psi and column temperature at 21°C.

2.7.3 Carotenoid Identification and Quantification

Identification of the carotenoids under investigation involved the identification of specific peaks from light absorbance spectra including λ-maxima values. The order in which specific pigments were separated on the reverse and forward (adsorption phase) system used was highly conserved, and the retention time at which they were separated also remained relatively static. These known peak patterns, values for retention time, and λ-maxima values (previously validated with standards) were used for the identification of the carotenoids present in each sample. Typical chromatographs, obtained using the forward (adsorption) phase and the reverse phase systems, are shown in Appendix VI in section 7.6.1. Typical absorption spectra obtained using the photo diode array detector for the investigated carotenoids are included in Appendix VI in section 7.6.2. The Absorbance value at 440 nm was used to quantify the carotenoid concentration in each sample, and this value was input to either: Equation 1, for β-Carotene; Equation 2 for lycopene; Equation 3 for violaxanthin; or Equation 4 for Lutein, zeaxanthin, and isomers of neoxanthin; where: α is the extraction factor, x is the sample weight (mg) and z is the peak area.

Equation 1

$$\text{Carotenoid Concentration}\,(\mu\text{g/gFWt}) = ((z / (x / \alpha)) / 19463) / 1000$$

Equation 2

$$\text{Carotenoid Concentration}\,(\mu\text{g/gFWt}) = ((z / (x / \alpha)) / 10665) / 1000$$

Equation 3

$$\text{Carotenoid Concentration}\,(\mu\text{g/gFWt}) = ((z / (x / \alpha)) / 24133.68) / 1000$$

Equation 4

$$\text{Carotenoid Concentration}\,(\mu\text{g/gFWt}) = ((z / (x / \alpha)) / 21200) / 1000$$

2.8 STATISTICAL ANALYSES

To assess the significance of the experimental results statistical analyses were performed to a significance of $P<0.05$ using SPSS 15.0 for Windows (SPSS Inc. Chicago, USA 2006). The specific analyses performed are stated in the text and include: Pearsons χ^2 test; analysis of variance (ANOVA), with various *post hoc* tests: Least Significant Difference (LSD) when the ANOVA was significant and population variances were equal; Games-Howell when sample sizes were not equal or the overall ANOVA was not significant; Dunnett's test to compare means against a control mean and linear regression analysis. Error bars presented on graphical plots represent the standard error of the mean.

CHAPTER 3:

A NON-TRANSGENIC APPROACH TO MANIPULATING ABA BIOSYNTHESIS

3.1 INTRODUCTION

The non-transgenic approach adopted in the present research programme involved the use of wild species of tomato in a classical backcrossing procedure. It was hypothesised that different wild species related to the commercial tomato might have evolved different strategies to cope with limited water availability associated with the unique habitats of their areas of speciation and in some cases their geographical isolation from other wild tomato populations. The wild species may have adapted different methods of modulating ABA biosynthesis and therefore may have evolved changes in their promoters potentially resulting in differently expressed alleles related to *SlNCED1* of the commercial tomato. Natural allelic variation in *NCED1* activity in sexually compatible *Solanum* species could be exploited as a potential means of improving WUE of cultivated tomato varieties, potentially providing natural equivalents of the *SlNCED1* overexperssing transgenic lines (sp12 and sp5 ; Thompson *et al.*, 2000b; Thompson *et al.*, 2007a; Thompson *et al.*, 2007b).

Wild relatives of the tomato are native to western South America and grow in a variety of habitats from near sea level to over 3,300 m in elevation (Rick, 1973; Taylor, 1986). This group of species is distributed from central Ecuador, through Peru to northern Chile, and in the relative isolation of the Galapagos Islands (Darwin *et al.*, 2003; Spooner *et al.*, 2005). The wild cherry tomato *S. lycopersicum* (*S. lycopersicum* var. *cerasiforme*) is considered to be the immediate ancestor of cultivated tomatoes and this group within the species occurs/re-evolves as a weedy escape from cultivation worldwide (Spooner *et*

al., 2005). There are presently two competing hypotheses for the original place of domestication; the Mexican hypothesis and the Peruvian hypothesis. Neither of these hypotheses, nor the idea of parallel domestication, can be discounted at present (Peralta and Spooner, 2006). There has been much controversy surrounding the taxonomy of tomato and its close relatives, including the nomenclature of the genus, the number of species in the group and their inter-relationships. This will be briefly outlined herein.

When the tomato was introduced to Europe in the sixteenth century early taxonomists placed tomatoes in the genus *Solanum*, commonly referring to the crop species as *S. pomiferum* (Luckwill, 1943 as cited in Peralta *et al.*, 2006). Apparently Tournefort was the first to name cultivated tomatoes as *Lycopersicon*, meaning 'wolf peach' in Greek (Tournefort, 1694 as cited in Peralta *et al.*, 2006). Müller (1940) and Luckwell (1943) produced two taxonomic monographs of wild tomatoes based on morphological data, and placed them in the genus *Lycopersicon*. Linnaeus (1753), on the other hand, classified tomatoes in the genus *Solanum*, although only a short time afterwards Miller (1754 as cited in Peralta *et al.*, 2006) formally described the genus *Lycopersicon* based on Tournefort's previous classification. This classification system, which was supported by inter-breeding criteria, notably the severity of barriers to gene exchange between species, within and outside the genus, (Rick, 1963,1979) prevailed until relatively recently (Taylor, 1986; Rick *et al.*, 1990).

Breeding systems have played an important role in wild tomato species evolution, and they vary from allogamous self-incompatible, to facultative allogamous and self-compatible, to autogamous and self-compatible (Rick, 1963,1979,1986). All wild species closely related to tomato are diploid (genome formula $2n=2x=24$) and have been crossed, albeit sometimes with difficulty, to the cultivated tomato (Rick, 1979). Rick (1963, 1979, 1986), using the *Lycopersicon* nomenclature, described two subgenera based on crossing relationships: the Esculentum complex which included seven intercrossable species, and the Peruvianum complex, which included two self-

incompatible species that intercrossed but had severe breeding barriers with the Esculentum complex (Rick, 1963,1979,1986). Later work by Child (1990), using the *Solanum* classification, divided tomatoes into three groups: *Lycopersicon, Eriopersicon*, and *Neolycopersicon*, the first two corresponded to the subgenera *Eulycopersicon* and *Eriopersicon* used by Müller (1940) and Luckwill (1943), while the latter only included *S. pennellii* (reviewed in Esquinas-Alcazar and Nuez (2001); described in Zuriaga *et al.*, 2009).

Relatively recently a new taxonomic monograph has been proposed (Peralta *et al.*, 2008) which once again places tomatoes within in the large genus *Solanum*, rather than as the small genus *Lycopersicon* and the taxonomic approach here is similar to that proposed by Child (Child, 1990). This new nomenclature has been based on plant morphology, distribution data (Peralta and Spooner, 2006), and evidence from studies of DNA sequences including; chloroplast DNA restriction site and sequence data (Spooner *et al.*, 1993; Bohs and Olmstead, 1997; Olmstead and Palmer, 1997) and nuclear granule-bound starch synthase gene (GBSSI or waxy) sequence data (Peralta and Spooner, 2001). Thirteen species of wild tomato, including the cultivated tomato (*S. lycopersicum*), plus four closely related species, have been described in this new monograph (Peralta *et al.*, 2008). This is an increase from the nine species traditionally recognised (Rick *et al.*, 1990). The majority of taxonomists, breeders and the genomics community have now re-adopted *Solanum* as the generic name for tomatoes (Peralta *et al.*, 2005,2006).

This latest monograph divides the section into four groups: Lycopersicon (comprising *S. lycopersicum, S. pimpinellifolium, S. cheesmaniae* and *S. galapagense*), Neolycopersicon (containing only *S. pennellii*), Eriopersicon (comprising *S. peruvianum, S. corneliomulleri, S. huaylasense, S. habrochaites* and *S. chilense*) and Arcanum (*S. arcanum, S. chmielewskii* and *S. neorickii*). It should be mentioned that these groupings are not definitive and debate about the classification of some species is still ongoing (Zuriaga *et al.*, 2009).

Introgression lines for some wild species of tomato are publicly available including; *S. pennellii* (formerly *L. pennellii* (Eshed *et al.*, 1992)), *S. lycopersicoides* (formerly *L. lycopersicoides* (Canady *et al.*, 2005)) and *S. habrochaites* (formerly *L. hirsutum* (Monforte and Tanksley, 2000)). These introgression lines, along with the fast growing range of bioinformatics data available from sources such as the Solanaceae Genomics Network (SGN, http://solgenomics.net), and sequencing of the tomato genome (Mueller *et al.*, 2005a; Mueller *et al.*, 2005b; Mueller *et al.*, 2009) provide powerful tools for genome analysis and breeding programmes (Fridman *et al.*, 2004; Chetelat and Ji, 2006). The use of the wild relatives of crops in breeding programmes as sources of disease resistance and desirable agronomic traits is not new, but the advance of molecular techniques has made the process more directed and efficient.

The work described in this chapter is part of a larger DEFRA-funded programme based on the analysis of alleles of *SlNCED1* in ten wild species (13 different accessions) of tomato. The full coding sequences of alleles of *SlNCED1* in these wild species accessions and two cultivated varieties have been obtained and a phylogenic tree constructed (Harrison *et al.,* unpublished data). A sub-set of these wild species accessions have been chosen for introgression of the wild species alleles of *SlNCED1* into the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig. The work described herein specifically relates to the backcrossing and phenotypic analysis of alleles of *SlNCED1* found in the species *S. galapagense* (Darwin *et al.*, 2003 (formerly *L. cheesmaniae* forma or var. *minor*: Hooker, 1847; Müller and Hooker, 1940) and *S. neorickii* (Spooner *et al.*, 1993 (formerly *L. parviflorum*: Rick *et al.*, 1976)).

S. galapagense is closely related to *S. cheesmaniae* (there is still some suggestion that these may be better regarded as simply two forms of a single species Zuriaga *et al.*, 2009) and endemic/restricted to the Galápagos Islands. *S. galapagense* mostly occurs on coastal lava within the range of sea spray and therefore is salt tolerant. Its distribution ranges from close to sea level to 50m. It has been occasionally found

inland on volcanic slopes, where it extends its range up to 1500m. *S. galapagense* flowers and fruits throughout the year in response to moisture, is self-compatible and exclusively autogamous (Peralta *et al.*, 2005; Peralta and Spooner, 2006; Peralta *et al.*, 2008). *S. galapagense* and *S. cheesmaniae* have orange coloured fruits which is a distinguishing characteristic of these two species (Rick, 1971; Peralta and Spooner, 2001). *S. neorickii* is distributed from South Ecuador to South Peru in dry inter-Andean valleys, and found in moist and well-drained rocky environments ranging from 1950 – 2600m. Rick *et al*., (1976) postulated that *S. neorickii* evolved from *S. chmielewskii* (Peralta *et al.*, 2008). *S. neorickii* flowers and fruits sporadically throughout the year, is self-compatible and exclusively autogamous and therefore has low intra-populational genetic variation (Peralta *et al.*, 2005; Peralta and Spooner, 2006; Peralta *et al.*, 2008).

The aim of this introgression programme is to assess the expression of the wild species *NCED1* alleles in a cultivated tomato genetic background for any conferred increase in ABA production and/or reduced water use.

3.2 MATERIALS & METHODS

3.2.1 Plant Material

The ABA deficient *notabilis* (*not*) mutant, containing a null mutation in the *SlNCED1* gene (Burbidge *et al.*, 1999) in the cultivated tomato genetic background *S. lycopersicum* cv. Ailsa Craig, was initially used in a backcross breeding programme to allow easy visual selection for allelic introgression. Progeny containing the functional wild species *NCED1* allele were easily identified as non-wilty, due to replacement of at least one copy of the recessive null mutant allele. This allowed recurrent selection for heterozygotes in each successive backcross to *notabilis* mutant homozygotes (*not*). The two wild species of tomato used in this part of the programme were: *S. neorickii* (Spooner *et al.*, 1993 (formerly *L. parviflorum*: Rick *et al.*, 1976)) accession LA2113 and, *S. galapagense* (Darwin *et al.*, 2003 (formerly *L. cheesmaniae* forma or var. *minor*: Hooker,

1847; Müller and Hooker, 1940) accession WJW. The F_1 progeny of a *S. galapagense x S. neorickii* interspecific hybridisation were used for the initial cross, as opposed to pure *S. galapagense.* This was primarily because the F_1 progeny germinate more easily and flower more reliably than the pure *S. galapagense* parent. The *S. lycopersicum* cv. Alisa Craig commercial line was used as one control line, but the Tm2a line, homozygous for the tomato mosaic virus (TMV) resistance gene (*Tm-2^a/Tm-2^2* locus AF536201; Lanfermeijer *et al.*, 2003), in the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig (GCR267) was used as the genetic background into which the wild species *NCED* alleles were finally introgressed.

3.2.2 CAPS Markers

Two sets of CAPS markers were designed to differentiate between the different commercial tomato and wild species *NCED1* alleles being investigated. The primers were designed around a polymorphic restriction endonuclease site, and following PCR the amplicons were subsequently digested with the appropriate restriction enzyme required to reveal the predicted allelic variation. CAPS markers were designed using BlastDigester software (Ilic *et al.*, 2004): The two sequences to be distinguished between were aligned using nucleotide BLAST (BLASTn; Zhang *et al.*, 2000; NCBI, http://www.ncbi.nlm.nih.gov/). The BLASTn alignment output was then used with the BlastDigester software (Ilic *et al.*, 2004) to identify potential restriction cutting sites. Once a specific polymorphic restriction endonuclease was chosen, the link in the software was followed to the Primer3 programme (Rozen and Skaletsky, 2000) to design potential CAPS primers around the selected cutting site.

The first CAPS primer pair for the CAPS marker Cap_NCED1_Neo (Appendix III Table 7.3.3) was designed to differentiate between the different *NCED1* DNA sequences obtained from *S. neorickii* and *S. galapagense*. They were designed so that the amplified PCR product (118 bp) from the *SnNCED1* DNA sequence was cut (81

bp and 37 bp) and the equivalent amplified PCR product (118 bp) from *S. galapagense* (or *S. lycopersicon*) was not cut (schematic diagram of the primer design in Figure 3.3.6). The second primer pair for the CAPS marker Cap_NCED1_Gala (Appendix III Table 7.3.3) was designed to differentiate between the different *NCED1* DNA sequences obtained from *S. galapagense* and *S. lycopersicum*. They were designed so that the amplified PCR product (127 bp) from the *SlNCED1* DNA sequence was cut (73 bp and 54 bp), and the equivalent amplified PCR product (127 bp) from *S. galapagense* DNA (or *S. neorickii* DNA), as not cut (schematic diagram of the primer design in Figure 3.3.9).

3.2.3 Detached Leaf Dehydration

Plants were grown in the glasshouse to varying growth stages. The first and/or second fully expanded leaves were removed from the plants, and pre-treated overnight in the glasshouse with artificial sap (AS) pH 6 (Wilkinson and Davies, 1997). Control samples were weighed and placed in polythene bags containing moist tissue paper. Leaves to undergo dehydration were weighed and then placed abaxial side uppermost on the glasshouse bench and allowed to dry to between -9 and -11% of their initial fresh weight (FWt). At this point the leaves were placed in polythene bags containing moist tissue paper to prevent further moisture loss. The samples were incubated on the glasshouse bench until leaflets were removed and frozen in liquid nitrogen at indicated time points.

3.2.4 Northern Blotting, Hybridization and mRNA Quantification

Leaf samples (~1 cm long, newly expanded leaflet) were harvested in 1.5 ml Eppendorfs and flash frozen in liquid nitrogen. Samples were ground to a fine powder under liquid nitrogen in the 1.5 ml Eppendorfs with glass rods pre-cooled in liquid nitrogen. Total RNA was extracted from sampled leaf material using RNeasy mini kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. Briefly, weighed tissue samples (100 mg) were placed into a 2 ml Eppendorf tube to which 450 µl of

Buffer RLT (containing 10 µl of β-mercaptoethanol per 1 ml of Buffer RLT) was added, and vortexed. The lysate was then transferred to a QIAshredder spin column, placed in a 2 ml collection tube and centrifuged at 13,000 rpm for 2 min. The supernatant from the flow-through was transferred to a clean 2.0 ml Eppendorf tube, 225 µl of ethanol was added and mixed gently by pipetting. The mixture was transferred to an RNeasy spin column, placed in a 2 ml collection tube and centrifuged at 13,000 rpm for 15s. The flow-through was discarded and 700 µl of Buffer RW1 was added to the RNeasy spin column. The column was then centrifuged at 13,000 rpm for 15s and the flow-through discarded. Subsequently 500 µl Buffer RPE was added to the column, which was centrifuged at 13,000 rpm for 15s, the flow-through discarded and washed a second time with 500 µl of Buffer RPE, this time centrifuging for 2 min. The column was transferred to a clean 1.5 ml Eppendorf tube and 30 µl of RNase-free water (containing 0.1 mg ml^{-1} RNase inhibitor) was added to the column. The tube was centrifuged at 13,000 rpm for 1 min. The flow-through was pipetted back onto the column and centrifuged for a further 1 min. The flow-through, which contained total RNA, was either used immediately or stored at -20 ºC.

RNA content was quantified using a NANODROP UV-Vis spectrophotometer, and the samples stored at -20°C. Procedures for Northern blotting, hybridization to *in vitro* transcribed radiolabelled RNA probes and quantification by phosphorimaging were as previously described (Thompson *et al.*, 2000a) except for a few adaptations. Total RNA (10 µg) was taken up in to 10 µl of sterile distilled water (SDW) and mixed with an equal volume of filter-sterilised ethidium bromide loading buffer (Appendix II in section 7.2.10) in a 1.5 ml Eppendorf tube. The mixture was then incubated at 65 °C for 15 min, cooled on ice for 5 min then centrifuged briefly to collect the contents at the bottom of the tube. The RNA was subsequently mixed with 4 µl of 2x RNA gel loading buffer and loaded onto a 1% (w/v) denaturing agarose gel (Appendix II

in section 7.2.12). The gel was electrophoresed at 100 V for 45 min in gel running buffer (Appendix II in section 7.2.13), the buffer was mixed every 15 min to aid circulation.

The gel was then photographed under UV light with a UV-transilluminator, to confirm that the gel was equally loaded, before being capillary blotted overnight onto a Zetaprobe GT nylon membrane (Bio-Rad, Hertfordshire, UK) in sodium phosphate buffer (0.025 M, pH 6.5). The blotted membrane was photographed under UV light to check the transfer of RNA from the gel to the membrane. The RNA was subsequently UV-fixed onto the nylon membrane by crosslinking with 120,000 μJ UV light for 15s using a UV-crosslinker (UV Stratalinker 2400; Stratagene, Cheshire, UK). The membrane was then prehybridised in a roller tube (Techne, Staffordshire, UK) containing 20 ml of prehybridisation buffer (Appendix II in section 7.2.14) for at least 4 h at 65 °C. The *SlNCED1* probe used was the full open reading frame (ORF) as described by Thompson *et al.*, 2000a. The RNA probe was synthesised from plasmid DNA (20 ng) (Appendix II in section 7.2.15) by transcription with T7 RNA polymerase, using a Rediprime kit (Amersham Biosciences, Buckinghamshire, UK) in the presence of 2.5 μl of [α-^{32}P]-UTP to produce antisense RNA probes (Appendix II in section 7.2.15). The riboprobe was boiled for 1 min, cooled on ice and then added to the prehybridisation solution. Hybridisation was performed overnight at 65 ºC. The membrane was washed twice in a 1 x saline-sodium citrate (SSC), 0.5% sodium dodecyl sulphate (SDS) wash, once at room temperature and once at 65°C; and then washed a third time at higher stringency in 0.1x SSC, 0.5% SDS at 65°C. The membrane was subsequently sealed in plastic sheeting and placed in an autoradiography cassette with two X-OMAT films (KODAK) placed one on top of the other, and stored for 48 h at -70 ^{o}C. The exposed films were developed for 4 min in developer solution (KODAK) and for a further 4 min in a fixer solution (KODAK). The mRNA was quantified by exposing the membrane to phosphorscreens for 4h, the images were read on a cyclone phosphorimager and the relative signal in each

band was determined (cyclone storage phosphor screen and accompanying software, Packard).

3.3 RESULTS

3.3.1 The Wild Species Preliminary Data

An interspecific hybrid cross between *S. galapagense x S. neorickii* (abbreviated to *galaneo*) was performed and the F_1 progeny used, as opposed to pure *S. galapagense*, as the F_1 seed germinate more easily and the F_1 plants flower more reliably. The pure *S. neorickii* parental line (abbreviated to *neo*), which unlike *S. galapagense* has no germination or flowering problems, was also used in this research, together with the WT control line *S. lycopersicum* cv. Alisa Craig.

Table 3.3.1 The leaf carotenoid content (μg g^{-1} FWt) of *S. lycopersicum* cv. Alisa Craig (WT); *S. neorickii* (*neo*); and *S. galapagense x S. neorickii* (*galaneo*) F_1 interspecific hybrid plants 24 d after germination. ANOVA probability (*P*) values are given, values with a different letter are significantly (*=*P*<0.05 level or the **=*P*<0.01) different according to LSD *post hoc* test (n = 4 ± S.E.).

GENOTYPE	THE MEAN LEAF CAROTENOID CONTENT (μg g^{-1}FWt)						
	Lutein	Zeaxanthin	Violaxanthin	9'-cis-neoxanthin	All-*trans*-neoxanthin	β-Carotene	Total
WT	101.37 ± 1.6	0.74 ± 0.2	7.84 ^{a*} ± 1.38	22.32 ± 0.37	5.3 ^{a} ± 0.74	69.07 ± 0.98	199.78 ± 5.76
neo	86.19 ± 12.2	0.79 ± 0.11	5.74 ^{ab} ± 1.44	17.5 ± 0.28	2.91 ^{b*} ± 0.28	61.41 ± 8.11	174.53 ± 22.29
galaneo	102.20 ± 3.3	1.2 ± 0.32	2.55 ^{b*} ± 0.44	20.35 ± 1.15	1.73 ^{b**} ± 0.3	73.15 ± 4.06	201.17 ± 8.8
p	0.274	0.325	0.033	0.166	0.002	0.324	0.371

The carotenoid content of fully expanded leaflets of the two wild species genotypes and the WT control 24 d after germination is displayed in Table 3.3.1. The three genotypes had similar amounts of total leaf carotenoids and similar amounts of lutein, β-carotene, zeaxanthin and 9'-*cis*-neoxanthin. The leaf violaxanthin content of WT was significantly greater (*P*<0.05) than that of *galaneo* (7.8 and 2.6 μg g^{-1}FWt respectively) while the concentration of violaxanthin in *neo* was not significantly

different from the other two genotypes. The WT also contained significantly more all-*trans*-neoxanthin (5.3 μg g^{-1}FWt) in its leaves than *galaneo* (1.7 μg g^{-1}FWt, $P<0.01$) and *neo* (2.9 μg g^{-1}FWt, $P<0.05$).

The relative abundance of *NCED1* mRNA is presented in Table 3.3.2; there was no significant difference between the relative abundance of *NCED1* mRNA in the expanded leaves of WT, *neo* and *galaneo* plants 25 d after germination (Table 3.3.2), although WT had the highest mRNA levels at 77% maximum. The concentration of leaf ABA ($[ABA]_{leaf}$) in the leaves of the three genotypes is presented in Table 3.3.2. There were no statistically significant differences in ABA content between the genotypes, although WT appeared to have the lowest $[ABA]_{leaf}$ with 192 ng g^{-1} FWt, and *galaneo* the highest with 396 ng g^{-1} FWt. The environmental stress history for each plant was not tightly controlled and this may account for the high level of variation found in these preliminary results.

Table 3.3.2 The mean percentage maximum of steady state *NCED1* mRNA abundance and the mean ABA content (ng g^{-1} FWt) in the fully expanded unstressed leaves of *S. lycopersicum* (WT); *S. neorickii* (*neo*); and *S. galapagense x S. neorickii* F_1 interspecific hybrid (*galaneo*) plants, 25 d after germination. ANOVA probability (*P*) values are given (n = 3 and 2 respectively ± S.E.).

GENOTYPE	MEAN *NCED1* mRNA (% MAXIMUM)	MEAN LEAF ABA (ng g^{-1} FWt)
WT	76.67 ± 11.68	191.95 ± 33.25
neo	66 ± 0.58	260.9 ± 85.4
galaneo	55.33 ± 6.64	395.9 ± 90.7
P	0.231	0.288

A detached leaf wilt experiment was used to investigate the short term (4 h) effect of dehydration on leaf *NCED1* mRNA steady state levels and also on ABA content. The amount of *NCED1* mRNA at time point zero appeared to be higher in the WT than in either of the two wild species (Figure 3.3.1), which is consistent with the previous mRNA levels observed (Table 3.3.2).

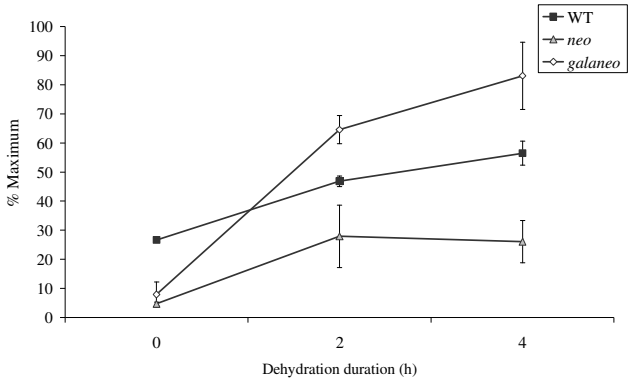

Figure 3.3.1 The mean percentage maximum of *NCED1* mRNA in detached leaves of *S. lycopersicum* (WT), *S. neorickii* (*neo*); and *S. galapagense x S. neorickii* F_1 interspecific hybrid (*galaneo*) plants 58 d after germination. Leaves were dehydrated to ~90% FWt and then leaflets were harvested at zero, two and four hours after dehydration (n= 3 ± S.E.).

A regression analysis revealed that the dehydration had a statistically significant (P<0.05) effect on the *NCED1* mRNA levels in WT and *galaneo*, $R^2 = 0.88$ and 0.76 respectively. The gradient of the regression line for *galaneo* was more than twice as steep as that of WT (*galaneo NCED1* [mRNA] = 18x +16.7 and WT *NCED1* [mRNA] = 7.6x + 28.1) demonstrating a more rapid increase in *NCED1* mRNA, albeit from a lower initial level. On the other hand, the dehydration did not appear to have a significant effect on *NCED1* mRNA abundance in *neo*, $R^2 = 0.23$ (*neo NCED1* [mRNA] = 5.3x + 9.2), although there appeared to be a slight increase between the 0 to 2 h time points. The *NCED1* mRNA abundance in *neo* remained below the amount initially found in WT throughout the duration of the dehydration treatment.

The mean $[ABA]_{leaf}$ increased rapidly within the first two hours of dehydration treatment only in the *galaneo* samples (Figure 3.3.2). The amount of ABA in *galaneo* leaves increased even more after four hours of responding to dehydration, rising from 252 ng g^{-1} FWt initially to eventually reach 1465 ng g^{-1} FWt. A regression analysis confirmed that the dehydration treatment had a very significant (P<0.01) effect in quickly increasing the amount of ABA in the detached *galaneo* leaves, $R^2 = 0.85$ (*galaneo* [ABA] = 181.2x + 307).

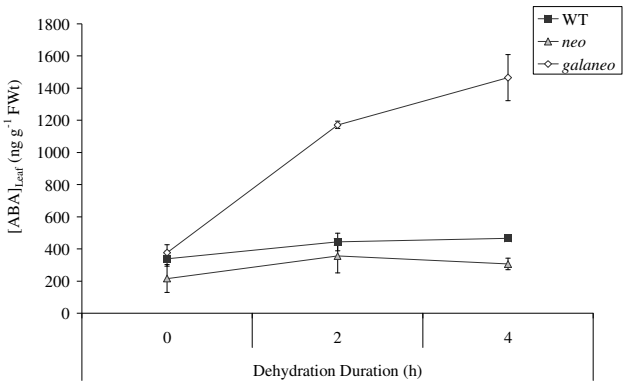

Figure 3.3.2 The ABA content (ng g^{-1} FWt) in detached leaves of *S. lycopersicum* (WT), *S. neorickii* (*neo*) and *S. galapagense x S. neorickii* F_1 hybrid (*galaneo*) plants 58 d after germination. Leaves were dehydrated to ~90% FWt after which leaflets were harvested at zero, two and four hours after dehydration. (n= 3 ± S.E.)

The $[ABA]_{leaf}$ in WT and *neo*, on the other hand did not respond in a statistically significant way to the dehydration treatment, with WT appearing to increase slightly from 229 to 311 ng g^{-1} FWt over the four hour treatment. Although the $[ABA]_{leaf}$ in *neo* plants apparently increased slightly over the first two hours (144 to 237 ng g^{-1} FWt) the content then appeared to drop to 204 ng g^{-1} FWt over the second two hour period.

The results for *NCED1* mRNA abundance (Figure 3.3.1) and for $[ABA]_{leaf}$ (Figure 3.3.2) both appear to indicate that *galaneo* shows a stronger response and reacts in a different manner to leaf dehydration, than either *neo* or the *S. lycopersicum* WT.

3.3.2 The Backcrossing Programme: Using *notabilis* as the Recurrent Parent - The F_1 Generation

The pure *S. neorickii* (*neo*), the interspecific F_1 hybrid (*galaneo*) and the WT (*S. lycopersicum*) control were initially crossed with a tomato (*S. lycopersicum*) line homozygous for the ABA deficient mutant allele, *notabilis* (*not;* containing a null mutation due to an A/T base pair deletion in the *NCED1* gene; Burbidge *et al*., 1999). This was in order to allow easy selection for the replacement of the non-functional *NCED1 not* allele with a substitute functional allele obtained from a wild species genome. Three crosses were performed: *notabilis x S. lycopersicum* (*not*-WT), *notabilis x*

S. neorickii (*not-neo*) and *notabilis x* the interspecific F_1 hybrid between *S. galapagense x S. neorickii* (*not-galaneo*).

The carotenoid content of fully expanded leaf tissue from the progeny of the three *not* crosses 29 d after germination is shown in Table 3.3.3. The three F_1 genotypes contained similar amounts of total leaf carotenoids and similar amounts of lutein, zeaxanthin, and 9'-*cis*-neoxanthin. The total amount of leaf carotenoids found in these *not* crosses (Table 3.3.3) was ~28% lower than in the original parental lines (Table 3.3.1), which appears to be mostly accounted for by 41% less lutein and 61% lower levels of the ABA precursor and NCED substrate, 9'-*cis*-neoxanthin. Some variation might be expected as environmental conditions were different for the two sets of plants.

Table 3.3.3 The leaf carotenoid content (μg g^{-1} FWt) in *notabilis x S. lycopersicum* (*not-*WT_{F1}); *notabilis x S. neorickii* (*not-neo*$_{F1}$) and *notabilis x* F_1 interspecific hybrid (*S. galapagense x S. neorickii*) (*not-galaneo*$_{F1}$) plants 29 d after germination. ANOVA probability (*P*) values are given, values with a different letter are significantly different (P<0.05) from each other as determined by LSD *post hoc* test (n = 7 ± S.E.).

GENOTYPE	THE MEAN LEAF CAROTENOID CONTENT (μg g^{-1}FWt)						
	Lutein	Zeaxanthin	Violaxanthin	9'-cis-neoxanthin	All-*trans*-neoxanthin	β-Carotene	Total
not-WT_{F1}	59.09 ± 3.1	1.28 ± 0.13	7.75 [a] ± 1.26	7.29 ± 1.03	5.06 [a] ± 0.82	58.46 [a] ± 2.55	138.95 ± 6.02
not-neo$_{F1}$	56.95 ± 2.45	1.19 ± 0.13	4.84 [b] ± 0.46	8.27 ± 1.03	3.13 [b] ± 0.25	68.87 [b] ± 2.74	139.19 ± 3.96
not-galaneo$_{F1}$	55.26 ± 1.68	1.11 ± 0.08	5.37 [b] ± 0.26	7.62 ± 0.43	3.67 [ab] ± 0.15	66.17 [b] ± 2.12	143.25 ± 5.2
P	0.559	0.600	0.039	0.732	0.038	0.022	0.798

Comparisons between lines within the F_1 generation indicate that *not*-WT_{F1} plants had significantly (*P*<0.05) lower amounts of β-carotene (58.5 μg g^{-1} FWt) than both *not-neo*$_{F1}$ and *not-galaneo*$_{F1}$ (68.9 and 66.2 μg g^{-1} FWt respectively). Conversely, *not*-WT_{F1} plants had significantly (*P*<0.05) higher concentrations of violaxanthin (mean of 7.8 μg g^{-1} FWt) than the other two genotypes (means of 4.8 and 3.6 μg g^{-1} FWt respectively). The *not*-WT_{F1} plants also had significantly more leaf all-*trans*-neoxanthin than *not-neo*$_{F1}$ plants (means of 5.1 and 3.1 μg g^{-1} FWt respectively).

The mean $[ABA]_{leaf}$ of the F_1 plants is displayed in Figure 3.3.3; *not*-WT_{F1} (pure *S. lycopersicum*) control plants had the highest mean $[ABA]_{leaf}$ (602 ng g^{-1} FWt) which was ~2 times greater (P<0.05) than that of *not-neo*$_{F1}$ (mean of 292 ng g^{-1} FWt) and ~2.5 times greater (P<0.05) than *not-galaneo*$_{F1}$ (mean of 240 ng g^{-1} FWt).

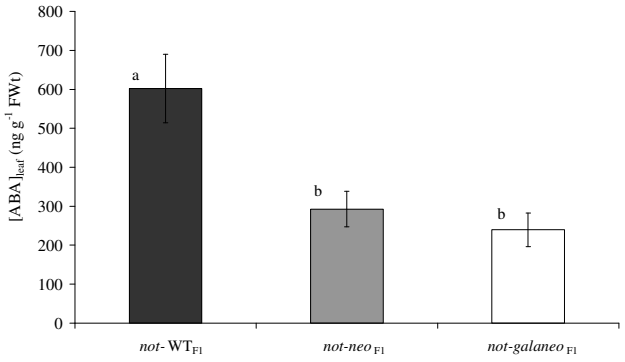

Figure 3.3.3 The mean ABA concentration ($[ABA]_{leaf}$ ng g^{-1} FWt) in fully expanded leaves of: *notabilis x S. lycopersicum* (*not*-WT_{F1}); *notabilis x S. neorickii* (*not-neo*$_{F1}$) and *notabilis x* (*S. galapagense* x *S. neorickii)* (*not-galaneo*$_{F1}$) plants 35 d after germination. Bars marked with a different letter are significantly different (P<0.05) from each other as determined by LSD (n = 6 ± S.E).

The F_1 generation $[ABA]_{leaf}$ appear quite high relative to the WT $[ABA]_{leaf}$ of 192 ng g^{-1}FWt previously reported in Table 3.3.2 and the unstressed ABA level of 229 ng g^{-1}FWt quantified at the beginning of the detached leaf wilt experiment (Figure 3.3.2). As previously stated, the water stress history of these plants was not known or very precisely controlled and this, together with other environmental variation may account for this and any other differences in ABA concentration.

3.3.3 The Backcrossing Programme: Using *notabilis* as the Recurrent Parent – The First Backcross

During the first backcross (BC1) six individual plants of *not-galaneo* (designated *not-galaneo1 – not-galaneo6*) were backcrossed to the *S. lycopersicum* cv. Ailsa Craig line homozygous for the *not* mutant allele. The use of six plants was thought likely to give a realistic probability of retaining both the *S. galapagense* and the *S. neorickii NCED1* alleles (*SgNCED1* and *SnNCED1* respectively) and to explore any

novel water stress related phenotypes that may have arisen from the crossing of these two geographically isolated wild tomato relatives. The probability that one of the alleles (*SnNCED1* or *SgNCED1*) would be missing from all six lines was one in sixty four, but this was thought to be worth the risk considering the easier propagation of the F_1 interspecific hybrid (*galaneo*) progeny as opposed to pure *S. galapagense*. In addition, the *not-neo* line was directly backcrossed to the *S. lycopersicum* cv. Ailsa Craig *notabilis* mutant line for comparison with any of the *not-galaneo* derived backcross lines which could be subsequently demonstrated to carry the *SnNCED1* allele. The *not*-WT (heterozygous) control line was also backcrossed to the homozygous *not* mutant in a pure *S. lycopersicum* cv. Ailsa Craig genetic background.

The mean leaf carotenoid content from the first backcross (BC1) generation of plants, harvested 80 d after germination, is displayed in Table 3.3.4. This BC1 generation of plants appeared to have lower total carotenoids than the original F_1 progeny of the first *not* cross (Table 3.3.3); *not*-WT_{BC1} had 25% less and *not-neo*$_{BC1}$ had 39% less total carotenoids than their equivalents in the previous generation; although it should be noted that different environmental conditions were prevailing at the times of year that plants of these two genotypes were grown. The *not-galaneo*$_{BC1}$ plants as a group (there was no significant difference between the individual genotypes regarding total leaf carotenoid contents) had 52% less total leaf carotenoids than was determined in the previous generation (i.e. *not-galaneo*$_{F1}$). This reduction appears to be mostly accounted for by lower amounts of violaxanthin, all-*trans*-neoxanthin and β-carotene: The *not*-WT_{BC1} plants had 83%, 79% and 24% less of each of these carotenoids respectively, and the *not-neo*$_{BC1}$ plants had 77%, 68% and 50% less respectively. While the *not-galaneo*$_{BC1}$ plants, as a group (which also showed no significant differences between the individual lines with regard to violaxanthin, all-*trans*-neoxanthin and β-carotene) had 75%, 74% and 56% less of these carotenoids respectively.

Table 3.3.4 The mean leaf carotenoid content (μg g^{-1} FWt) in the first backcross (BC1) of: *notabilis x S. lycopersicum* (*not*-WT_{BC1}); *notabilis x S. neorickii* (*not-neo*$_{BC1}$) and *notabilis x* (*S. galapagense* x *S. neorickii1 - 6*) (*not-galaneo1*$_{BC1}$ - *6*$_{BC1}$) plants 80 d after germination. ANOVA probability (*P*) values are given, values marked with a different letter are significantly different (*=*P*<0.05, **=*P*<0.01) from each other as determined by either [1]Games-Howell or [2]LSD *post hoc* test (n = 3 ± S.E).

GENOTYPE	THE MEAN LEAF CAROTENOID CONTENT (μg g^{-1}FWt)						
	Lutein[1]	Zeaxanthin[2]	Violaxanthin	9'-cis-neoxanthin[1]	All-*trans*-neoxanthin	β-Carotene	Total[1]
not-WT_{BC1}	46.77 ^{a*} ± 1.1	1.02 ^{a**cde} ± 0.03	1.34 ± 0.48	8.48 ^{a**} ± 0.24	1.08 ± 0.14	44.84 ± 4.14	103.54 ^{a*} ±4.98
not-neo $_{BC1}$	41.34 ^{ab} ± 5.41	0.73 ^{ac*e} ± 0.08	1.1 ± 0.27	7.33 ^{ab} ± 0.71	1.01 ± 0.13	34.13 ± 4.97	85.64 ^{ab} 10
not-galaneo1 $_{BC1}$	24.29 ^{b*} ± 2.44	0.54 ^{b**cdf**} ± 0.06	0.65 ± 0.22	3.04 ^{b**} ± 0.42	0.58 ± 0.11	20.54 ± 2.23	49.63 ^{b*} ±5.29
not-galaneo2 $_{BC1}$	31.47 ^{ab} ± 4.36	0.59 ^{b**cdf} ± 0.01	1.6 ± 0.75	5.84 ^{ab} ± 0.80	1.02 ± 0.05	31.90 ± 6.19	72.43 ^{ab} ±11.4
not-galaneo3 $_{BC1}$	26.58 ^{ab} ± 5.84	0.42 ^{b**d*f*} ± 0.08	0.98 ± 0.1	4.11 ^{ab} ± 1.09	0.86 ± 0.12	21.82 ± 5.12	54.77 ^{ab} ±12.31
not-galaneo4 $_{BC1}$	26.09 ^{b**} ± 0.27	0.56 ^{b**cde} ± 0.08	0.7 ± 0.07	3.8 ^{ab} ± 0.54	0.81 ± 0.06	20.27 ± 2.03	52.23 ^{b*} ±3.04
not-galaneo5 $_{BC1}$	39.86 ^{ab} ± 8.89	0.89 ^{a**cde*} ± 0.22	2.24 ± 0.3	6.21 ^{ab} ± 2.52	1.31 ± 0.27	38.92 ± 7.61	89.43 ^{ab} ±19.65
Not -galaneo6 $_{BC1}$	39.03 ^{ab} ± 6.99	0.58 ^{b**cdf*} ± 0.08	1.95 ± 0.68	6.78 ^{ab} ± 2.01	1.22 ± 0.35	40.06 ± 8.94	89.62 ^{ab} 18.99
P	0.082	0.012	0.068	0.131	0.238	0.057	0.065

Regarding comparisons between lines in this generation, the *not*-WT_{BC1} plants were found to have the highest amount of total leaf carotenoids (103.5 μg g^{-1} FWt) which was found to be significantly (*P*<0.05) more than the amount found in *not-galaneo1*$_{BC1}$ and *not-galaneo4*$_{BC1}$ plants (49.7 and 52.2 μg g^{-1} FWt respectively). The *not*-WT_{BC1} plants with the pure *S lycopersicum* cv Alisa Craig genetic background had the greatest mean concentration of lutein (46.8 μg g^{-1} FWt) in the leaves and this was significantly more than *not-galaneo1*$_{BC1}$ (24.3 μg g^{-1} FWt *P*<0.05) and *not-galaneo4*$_{BC1}$ (26.1 μg g^{-1} FWt *P*<0.01). The *not*-WT_{BC1} also had the highest zeaxanthin levels (1 μg g^{-1} FWt) which were significantly greater statistically (*P*<0.01) than those found in *not-galaneo1*$_{BC1}$, *2, 3, 5* and *6* plants, which had mean leaf zeaxanthin levels of 0.5, 0.6, 0.4, 0.6, and 0.6 μg

g^{-1} FWt respectively. The *not-neo*$_{BC1}$ plants had significantly (P<0.05) more zeaxanthin than *not-galaneo3*$_{BC1}$ (means of 0.7 and 0.4 µg g^{-1} FWt respectively).

Additionally, *not-galaneo5*$_{BC1}$ was found to have significantly (P<0.01) greater zeaxanthin levels than *not-galaneo1*$_{BC1}$ (the means were 0.9 and 0.5 µg g^{-1} FWt respectively) and greater (P<0.05) levels than *not-galaneo2*$_{BC1}$, *3*$_{BC1}$ and *6*$_{BC1}$ (0.6, 0.4 and 0.6 µg g^{-1} FWt respectively). The *not*-WT$_{BC1}$ also had the greatest leaf 9'-*cis*-neoxanthin concentration (8.5 µg g^{-1} FWt) which was significantly (P<0.01) greater than *not-galaneo1*$_{BC1}$ which had the lowest concentration of 9'-*cis*-neoxanthin (3 µg g^{-1} FWt).

The mean $[ABA]_{leaf}$ of the BC1 plants is presented in Figure 3.3.4; the mean $[ABA]_{leaf}$ of pure *S lycopersicum* cv. Ailsa Craig *not*-WT$_{BC1}$ plants was 45% less than that previously reported for the *not*-WT$_{F1}$ plants in the same genetic background (Figure 3.3.3); but similar to those reported for the WT control at the start of the detached leaf wilt experiment (Figure 3.3.2). The *not-galaneo6*$_{BC1}$ plants had the highest mean $[ABA]_{leaf}$ at 509 ng g^{-1} FWt and *not-galaneo3*$_{BC1}$ the lowest at 221 ng g^{-1} FWt, although none of the lines were found to have statistically significant differences in $[ABA]_{leaf}$.

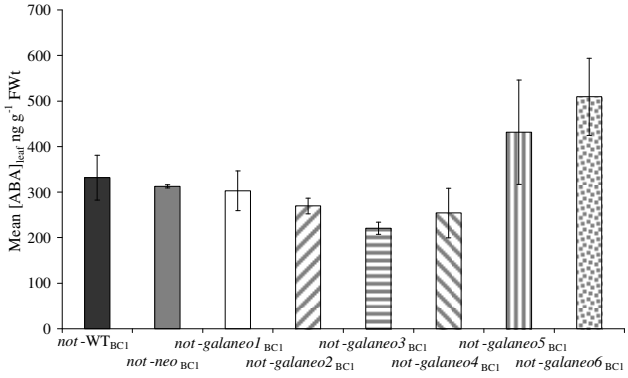

Figure 3.3.4 The mean leaf ABA concentration ([ABA] ng g^{-1} FWt) in the first backcross (BC1) of: *notabilis x S. lycopersicum* (*not*-WT$_{BC1}$); *notabilis x S. neorickii* (*not-neo*$_{BC1}$) and *notabilis x* the F_1 interspecific hybrids (*S. galapagense* x *S. neorickii1*$_{BC1}$ – *6* $_{BC1}$) (*not-galaneo1*$_{BC1}$ – *6*$_{BC1}$) plants 46 d after germination (n=3 ± S.E.).

3.3.4 The Backcrossing Programme using *notabilis* as the Recurrent Parent – The Second Backcross

During the second backcross (BC2), *not-neo*$_{BC1}$ and five of the six *not-galaneo*$_{BC1}$ lines were backcrossed to the *S. lycopersicum* cv. Alisa Craig line homozygous for the *not* mutant allele, as the recurrent parent. The line *not-galaneo4*$_{BC1}$ was dropped from the programme because it had an extreme necrosis/hypersensitive reaction to tomato mosaic virus (TMV). This reaction can be beneficial in the wild by preventing infection of an entire population through the sacrifice of one individual, but in cultivated conditions this response is not desirable. The *not-neo*$_{BC1}$ plants were also severely infected with TMV, which adversely affected their ability to shed pollen and as a result had to be re-sown for crossing. Therefore the *not-neo*$_{BC2}$ leaf carotenoid and ABA data is not presented here. The *not*-WT$_{BC1}$ was used as the control instead of forming an essentially identical *not*-WT$_{BC2}$ generation since, in this case, the pure *S. lycopersicum* cv. Ailsa Craig genetic background would remain unchanged.

The mean leaf carotenoid content from the second backcross (BC2) generation of plants, harvested 55 d after germination, is displayed in Table 3.3.5. The concentrations of the two neoxanthin isomers are presented together for this generation due to technical difficulties in separating these two compounds during the HPLC run (N.B. a new column was subsequently fitted). The mean total leaf carotenoid content found in *not*-WT$_{BC1}$ grown with this generation was 14% less than the total leaf carotenoid content previously found in *not*-WT$_{BC1}$ (Table 3.3.4). In contrast, the total leaf carotenoid levels in this generation of wild species *not* crosses appear to have increased compared to the BC1 generation; *notgalaneo1*$_{BC2}$ showed the largest increase of 167%, *notgalaneo2*$_{BC2}$ 29%, *notgalaneo3*$_{BC2}$ 57%, and *notgalaneo6*$_{BC2}$ showed the smallest increase of 5%. While *notgalaneo5*$_{BC2}$ showed a 17% decrease in the total carotenoids found in the BC1 generation. The changes in total leaf carotenoids are accounted for mainly by increases in lutein, changes in β-carotene levels, and surprisingly large

decreases in violaxanthin, i.e. apart from in the *not-galaneo1*$_{BC2}$ samples, the violaxanthin content was between 62% and 91% less than that found in the previous generation (Table 3.3.4).

Table 3.3.5 The mean leaf carotenoid content (µg g^{-1} FWt) of: *notabilis x S. lycopersicum* (*not*-WT$_{BC1}$) control plants and the second backcross (BC2) plants of: *notabilis x S. neorickii* (*not-neo* $_{BC2}$) and *notabilis x* (*S. galapagense x S. neorickii1 – 3, 5, 6*) (*not-galaneo1* $_{BC2}$ *- 3* $_{BC2}$*, 5* $_{BC2}$*, 6* $_{BC2}$) 55 d after germination. ANOVA Probability (*P*) values are given, values marked with a different letter are significantly different (*=*P*<0.05, **=<*P*<0.01) from each other as determined by LSD *post hoc* test (n = 4 ± S.E).

GENO TYPE	THE MEAN LEAF CAROTENOID CONTENT (µg g^{-1}FWt)					
	Lutein	Zeaxanthin	Violaxanthin	Neoxanthin Isomers	β-Carotene	Total
not-WT $_{BC2}$	54.84 ^{a**} ± 3.59	0.67 ± 0.04	0.42 ^{a} ± 0.08	9.06 ^{a*c**} ± 0.68	24.19 ^{a**cd} ± 0.75	89.22 ^{a**} ± 4.9
not-galaneo1 $_{BC2}$	80.70 ^{b*} ± 3.52	0.68 ± 0.6	0.63 ^{b*} ± 0.09	14.7 ^{b**d**} ± 0.79	35.94 ^{b**cd} ± 1.02	132.65 ^{b**} ± 4.38
not-galaneo2 $_{BC2}$	58.48 ^{a**} ± 7.3	0.55 ± 0.07	0.33 ^{a**} ± 0.02	7.84 ^{ac**} ± 1.08	26 ^{a**c**} ± 2.19	93.19 ^{a**} ± 8.02
not-galaneo3 $_{BC2}$	54.25 ^{a**} ± 3.31	0.49 ± 0.02	0.36 ^{a*} ± 0.11	6.2 ^{ac**} ± 1	24.88 ^{a**cd} ± 1.5	86.01 ^{a**} ±4.54
not-galaneo5 $_{BC2}$	47.60 ^{a**} ± 3.87	0.87 ± 0.27	0.22 ^{a*} ± 0.04	4.51 ^{b*c**} ± 0.70	21.07 ^{a**d**} ± 1.45	74.27 ^{a**} ±5.77
not-galaneo6 $_{BC2}$	62.23 ^{a*} ± 8.19	0.7 ± 0.09	0.36 ^{a*} ± 0.07	7.7 ^{ac**} ± 2.2	23.98 ^{a**cd} ± 1.88	93.95 ^{a**} ± 12.04
P	0.008	0.462	0.022	<0.001	<0.001	0.001

In this generation the *not-galaneo1* line was found to have significantly (*P*<0.01) more leaf carotenoids statistically than all the other lines; the mean for the line was 89.2 µg g^{-1} FWt (Table 3.3.5). The leaves of *not-galaneo1*$_{BC2}$ had a significantly (*P*<0.05) greater concentration of lutein (mean of 80.7 ng g^{-1} FWt, which was 232% more than that found in the previous generation) in its leaves than *not*-WT$_{BC1}$, *not-galaneo2*$_{BC2}$, *not-galaneo5*$_{BC2}$, (means of 54.8, 58.5 and 47.6 ng g^{-1} FWt respectively, *P*<0.01) and *not-galaneo6*$_{BC2}$ (mean of 62.2 ng g^{-1} FWt, *P*<0.05). The *not-galaneo1*$_{BC2}$ line had significantly (*P*<0.05) more leaf violaxanthin (mean of 0.63 ng g^{-1} FWt) than the other lines, of which *not-galaneo5*$_{BC2}$ had the least with a mean of 0.22 ng g^{-1} FWt, which was

91% lower than the mean violaxanthin concentration found in the *not-galaneo5*$_{BC1}$ plants (Table 3.3.4).

The *not-galaneo1*$_{BC2}$ line also had significantly (P<0.01) the greatest concentration of leaf neoxanthin isomers with a mean of 14.7 ng g^{-1} FWt (a 306% increase compared to the BC1 generation, Table 3.3.4). The *not-galaneo5*$_{BC2}$ line had the lowest concentration of neoxanthin isomers, i.e. 4.5 ng g^{-1} FWt (a 40% reduction from the previous generation, Table 3.3.4) which was significantly (P<0.05) lower than the *not*-WT$_{BC1}$ control line. Furthermore, the *not-galaneo1*$_{BC2}$ line also had significantly (P<0.01) greater concentrations of β-carotene in its leaves than the other lines, with a mean of 35.9 ng g^{-1} FWt (a 75% increase compared to the BC1 generation, Table 3.3.4). The *not-galaneo5*$_{BC2}$ line contained the smallest amount of β-carotene in its leaves, with a mean of 21.1 ng g^{-1} FWt, (which was 46% less than in the BC1 generation) which was significantly (P<0.01) lower than the β-carotene concentration found in *not-galaneo2*$_{BC2}$.

The mean [ABA]$_{leaf}$, presented in Figure 3.3.5, in *not*-WT$_{BC1}$ was 680 ng g^{-1} FWt, which was twice that observed previously (Figure 3.3.4) in *not*-WT$_{BC1}$ plants and WT leaves at the start of the detached leaf wilt (Figure 3.3.2), but similar to [ABA]$_{leaf}$ in *not*-WT$_{F1}$ (Figure 3.3.3). The *not-galaneo3*$_{BC2}$, *not-galagneo5*$_{BC2}$, and *not-galaneo6*$_{BC2}$ [ABA]$_{leaf}$ were all significantly lower than the *not*-WT$_{BC1}$ control plants (429 ng g^{-1} FW, P<0.05, 447 ng g^{-1} FW P<0.05, and 392 ng g^{-1} FW P<0.01 respectively). Additionally, *not-galaneo1*$_{BC2}$ had a [ABA]$_{leaf}$ of 600 ng g^{-1} FWt which was significantly (P<0.05) greater than *not-galaneo6*$_{BC2}$. These results may indicate that *not-galaneo3*$_{BC2}$, *not-galaneo5*$_{BC2}$ and *not-galaneo6*$_{BC2}$ were either unaffected by, responded differently to or avoided the possible water stress which may have caused the elevated [ABA]$_{leaf}$ in this generation of plants.

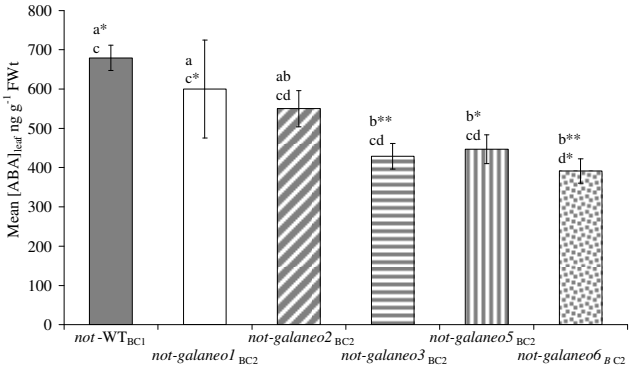

Figure 3.3.5 The mean $[ABA]_{leaf}$ (ng g^{-1} FWt) in the fully expanded leaflets of: *notabilis x S. lycopersicum* (*not*-WT_{BC1}) control plants and second backcross (BC2) plants of: *notabilis x S. neorickii* (*not-neo*$_{BC2}$); *notabilis x* (*S. galapagense x S. neorickii1 – 3, 5, 6*) (*not-galaneo1*$_{BC2}$ *–* 3_{BC2}, 5_{BC2}, 6_{BC2}) 55 d after germination. Bars marked with a different letter are significantly different (*=P<0.05, **=<P<0.01) from each other as determined by LSD *post hoc* test (n = 4 ± S.E).

3.3.5 Identification of *NCED1* Alleles

As a result of work carried out on a joint Nottingham/Warwick-HRI DEFRA funded research programme the DNA sequences of a number of *NCED1* alleles from wild tomato species became available at this stage of the research (Harrison, E., personal communication). As a result, the BC2 lines were subjected to a cleaved amplified polymorphic sequence (CAPS) marker selection process, designed to identify which of the *not-galaneo* lines contained the *SnNCED1* allele and, initially by the process of elimination, allowed the lines containing the *SgNCED1* allele to be identified. The CAPS Marker (Cap_NCED1_Neo, Appendix III Table 7.3.3) was designed using BLASTn (Zhang *et al.*, 2000), BlastDigester software (Llic *et al.*, 2004) and the Primer3 programme (Rozen and Skaletsky, 2000) as described in section 3.2.2. A schematic diagram of the CAPS Marker Cap_NCED1_Neo is presented in Figure 3.3.6.

```
                                                                                >
S.lycopersicum(1081) gtcattcctgatcaacaagtcgttttcaagatgtctgaaatgatccgtggaggttcaccg
Alignment            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S.neorickii   (1081) gtcattcctgatcaacaagtcgttttcaagatgtctgaaatgatccgtggaggttcaccg

                     >>>>>>>>>>>>>>>>>>>>
S.lycopersicum(1141) gtggtttacgacaagaacaaagtttcccgatttggtattctggataagtacgcgaaagat
Alignment            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
S.neorickii   (1141) gtggtttacgacaagaacaaagtttcccgatttgg GATTCtggataagtacgcgaaagat
                                                       HinfI

S.lycopersicum(1201) gggtctgatttgaaatgggttgaagtacctgattgtttctgtttccacctctggaatgct
Alignment            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
S.neorickii   (1201) gggtctgatttgaaatgggttgaagtacctgattgtttctgtttccacctctggaatgct
                                                     <<<<<<<<<<<<<<<<<<<<
```

Figure 3.3.6 A schematic diagram of the CAPS Marker Cap_NCED1_Neo, used to identify the *SnNCED1* allele. The 1081 – 1201 bp section of the two aligned 1818 bp *NCED1* allele sequences from *S. lycopersicum* and *S. neorickii* are shown: | indicates sequence similarity, whereas gaps indicate sequence differences. The 22 bp CAPS forward (>>>>) and reverse (<<<<) primers were designed to amplify a 118 bp PCR product containing the *Hinf*I restriction endonuclease recognition site (bold higher case, with the cutting site indicated). Digestion with *Hinf*I is predicted to cut the amplified PCR product from the *SnNCED1* allele into two fragments of 81 bp and 37 bp in length.

The CAPS Marker Cap_NCED1_Neo amplifies a 118 bp section of the *NCED1* allele which, when digested with the *Hinf*I restriction endonuclease (R.E.) cuts the PCR amplicon from the *SnNCED1* allele to form two fragments of 81 bp and 37 bp in length. *Hinf*I does not cut the 118 bp PCR amplicon from the *S. lycopersicum NCED1* allele (*SlNCED1*) (including the defective *not* allele, designated *SlNCED1*^{not}) or the *SgNCED1* allele (N.B. the *SgNCED1* sequence when aligned with either *SnNCED1* or *SlNCED1* showed no potential CAPS restriction endonuclease cutting sites, i.e. any potential CAPS cutting sites were present in the other sequence).

It can be seen in Figure 3.3.7 that, as expected, the full length 118 bp *NCED1* amplicon obtained from *not*-WT DNA (negative control) was not cut. The heterozygous *not-neo*$_{BC2}$ DNA (positive control) was cleaved by the *Hinf*I R.E. resulting in three bands. The two smaller bands (37 bp band was too faint to be visible on the 3.5 % agarose gel) are from the cleaved *SnNCED1* allele, the larger uncut band (full amplicon of 118 bp) is from the *SlNCED1*^{not} allele. The presence of one 118 bp uncut band in the *not-galaneo*$_{BC2}$ lines (lanes 1 – 5) is either from a DNA template of a *SlNCED1*^{not} homozygote or from a heterozygote containing a mixture of the *SlNCED1*^{not} allele and the *SgNCED1* allele. The

amplicons obtained from *not-galagneo1*$_{BC2}$ and *3*$_{BC2}$ DNA templates were cleaved (lanes 1 and 3), indicating that the wild species *NCED1* allele inherited by these lines was *SnNCED1*. The lack of cleavage in the equivalent amplicons obtained from *not-galaneo2*$_{BC2}$, *5*$_{BC2}$ and *6*$_{BC2}$ DNA templates (lanes 2, 4 and 5) indicates that they must have inherited the *SgNCED1* allele from the original *galaneo* F_1 interspecific hybrid parent.

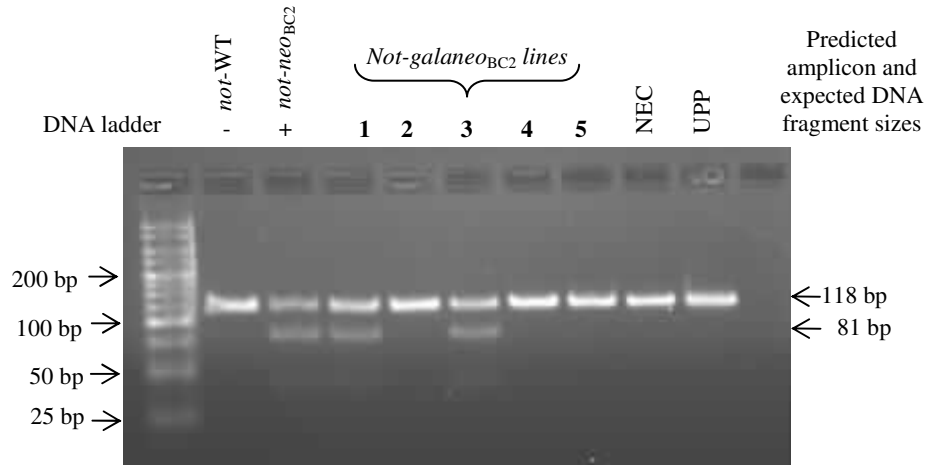

Figure 3.3.7 A gel electrophoresis image showing the discrimination between the *SnNCED1* and the *SgNCED1* alleles in plants from the second *notabilis* backcross. The restriction digest of the 118 bp *NCED1* amplicon with *Hinf*I, cleaves the *SnNCED1* allele to form two DNA fragments of 81 bp and 37bp (too faint to be visible on this 3.5% agarose gel): -, negative control, *notabilis x S. lycopersicum* (*not*-WT); +, positive control, *notabilis x S. neorickii*$_{BC2}$; (*not-neo*$_{BC2}$); NEC, no enzyme control; UPP, undigested PCR product of *NCED1* amplicon using *not-neo* DNA as a template; Lane **1**, *notabilis x* (*S. galapagense x S. neorickii*)*1*$_{BC2}$ (*not-galagneo1*$_{BC2}$); **2**, *not-galagneo2*$_{BC2}$; **3**, *not-galagneo3*$_{BC2}$; **4**, *not-galagneo5* $_{BC2}$; **5**, *not-galaneo6* $_{BC2}$.

At this point in the backcrossing programme four lines were chosen to be taken forward: two containing the *SnNCED1* allele; one from *not-neo* and the other derived from the F_1 interspecific hybrid cross via *not-galaneo1*; and the other two lines containing the *SgNCED1* allele, one from *not-galaneo2* and the other from *not-galaneo6*. The line *not-galaneo1* was chosen over *not-galaneo3* because it was found to have different leaf carotenoid composition from the control *not*-WT$_{BC1}$ in both the BC1 and BC2 generations, whereas *not-galaneo3* did not: i.e. in BC1 (Table 3.3.4), *not-galaneo1*$_{BC1}$ had consistently lower amounts of the carotenoids analysed than *not*-WT$_{BC1}$,

which resulted in *not-galaneo1*$_{BC1}$ having significantly (P<0.01) less total leaf carotenoids (mean of 50 μg g^{-1} FWt compared to *not*-WT$_{BC1}$ with a mean of 104 μg g^{-1} FWt) (Table 3.3.4); In BC2 (Table 3.3.5) *not-galaneo1*$_{BC2}$ had consistently greater amounts of all the carotenoids analysed than *not*-WT$_{BC1}$, which resulted in *not-galaneo1*$_{BC2}$ having significantly (P<0.01) higher total leaf carotenoids (mean of 133 μg g^{-1} FWt compared to *not*-WT$_{BC1}$ with a mean of 89 μg g^{-1} FWt). Additionally *not-galaneo1* had greater mean $[ABA]_{leaf}$ in BC1 (Figure 3.3.4) and BC2 (Figure 3.3.5) than *not-galaneo3*$_{BC2}$.

The line *not-galaneo2* was chosen from the three lines found to carry the *SgNCED1* allele because it consistently had a different $[ABA]_{leaf}$ from the other two lines found to carry the *SgNCED1* allele, *not-galaneo5* and *not-galaneo6*, which consistently appeared to have similar amounts of $[ABA]_{leaf}$: In the BC1 generation (Figure 3.3.4) *not-galaneo2*$_{BC1}$ had less $[ABA]_{leaf}$ (mean of 303 ng g^{-1} FWt) than *not-galaneo5*$_{BC1}$ and *not-galaneo6*$_{BC2}$ (means of 432 ng g^{-1} FWt and 509 ng g^{-1} FWt respectively). In the BC2 generation (Figure 3.3.5) *not-galaneo2*$_{BC2}$ had greater $[ABA]_{leaf}$ (mean of 550 ng g^{-1} FWt) than *not-galaneo5*$_{BC1}$ and *not-galaneo6*$_{BC2}$ (means of 447 ng g^{-1} FWt and 392 ng g^{-1} FWt respectively). The *not-galaneo6* line was chosen as opposed to *not-galaneo5* because it had the lowest $[ABA]_{leaf}$ of the two lines, and therefore was the most different of the two lines from *not-galaneo2*$_{BC2}$ with regard to its $[ABA]_{leaf}$ (Figure 3.3.5). The *not-galaneo6*$_{BC2}$ line was also found to have significantly (P<0.05) lower $[ABA]_{leaf}$ than the *not-galaneo1*$_{BC2}$ line, identified as carrying the *SnNCED1* allele, and chosen (as described above) to continue in the programme. Therefore the line *not-galaneo6* was selected as opposed to the *not-galaneo5* line in an attempt to keep the broadest range of 'ABA biosynthesis' phenotypes identified earlier in the programme.

3.3.6 The Backcrossing Programme: using *notabilis* as the Recurrent Parent – The Third Backcross

During the third backcross (BC3), *not*-WT, *not-neo*$_{BC2}$, and the three chosen *not-galaneo*$_{BC2}$ lines were once again backcrossed to the *S. lycopersicum* cv. Ailsa Craig line homozygous for the mutant *not* allele.

3.3.7 Allelic Identification

The CAPS marker (Cap_NCED1_Neo, Appendix III Table 7.3.3) was used to distinguish the lines containing the *SnNCED1* allele from those carrying either the *SgNCED1* allele or the defective *SlNCED1*^{not} allele. It can be seen in Figure 3.3.8 that, as expected (from the schematic in Figure 3.3.6), the *NCED1* amplicon obtained from *not-neo*$_{BC3}$ DNA was cleaved by the *Hinf*I R.E., resulting in three bands, (two visible on the 3.5% gel). Additionally, equivalent amplicons obtained from *not-galagneo1* and *2* DNA templates were also cleaved (lanes 1 and 2), indicating that these lines had inherited the *SnNCED1* allele. This result for *not-galaneo2*$_{BC3}$ was surprising as *not-galaneo2*$_{BC2}$ had been previously identified as carrying the *SgNCED1* allele since the amplicon was not cleaved (Figure 3.3.7). The lack of cleavage in the equivalent amplicon obtained from the *not-galaneo6* DNA template indicates that it must have inherited the *SgNCED1* allele from the original *S. galapagense x S. neorickii* interspecific hybrid parent.

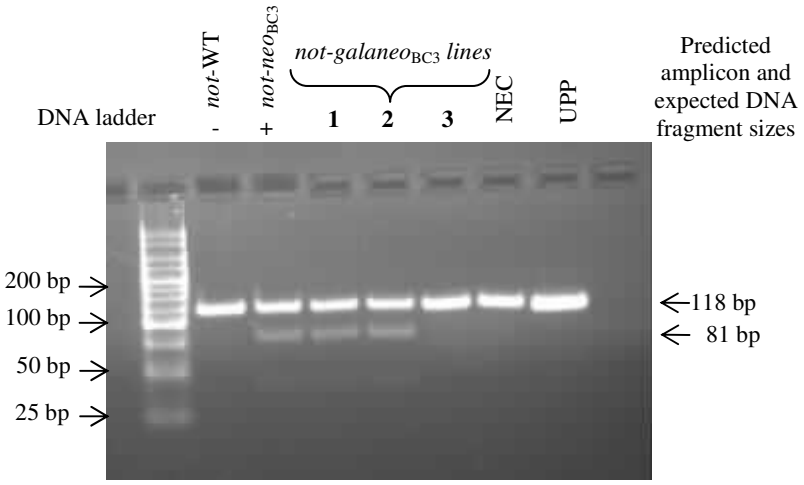

Figure 3.3.8 A gel electrophoresis image showing the identification of the *SnNCED1* allele, in comparison to *SlNCED1/SlNCED1*^{not} and *SgNCED1* alleles in plants from the third *notabilis* backcross. The restriction digest of the 118 bp *NCED1* amplicon with *Hinf*I, cleaves amplicons derived from the *SnNCED1* allele to form two DNA fragments of 81 bp and 37 bp (too faint to be visible on this 3.5% agarose gel): -, negative control, *notabilis x S. lycopersicum* (*not*-WT); +, positive control, *notabilis x S. neorickii*$_{BC3}$; (*not-neo*$_{BC3}$); NEC , no enzyme control; UPP, undigested PCR product of *NCED1* amplicon using the *not-neo* DNA as a template; Lane **1**, *notabilis x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC3}$); **2***, not-galaneo2* $_{BC3}$; **3**, *not-galaneo6* $_{BC3}$.

The CAPS Marker (Cap_NCED1_Gala, details given in Appendix III Table 7.3.3) was designed using BLASTn (Zhang *et al.*, 2000), BlastDigester software (Llic *et al.*, 2004) and the Primer3 programme (Rozen and Skaletsky, 2000) as described in section 3.2.2. A schematic diagram of the CAPS Marker Cap_NCED1_Gala is presented in Figure 3.3.9. The CAPS Marker Cap_NCED1_Gala amplifies a 127 bp section of the NCED1 allele, which when digested with the *Hind*III R.E. cuts the PCR amplicon from the *SlNCED1* allele to form two fragments of 73 and 54 bp in length. *Hind*III does not cut the 127 bp PCR amplicon from the *SgNCED1* allele or the *SnNCED1* allele.

Figure 3.3.9 A schematic diagram of the CAPS Marker Cap_NCED1_Gala, used to identify the *SlNCED1* allele. The 1621 – 1741 bp section of the two aligned 1818 bp NCED1 allele sequences from *S. lycopersicum* and *S. galapagense* are shown, | indicates sequence similarity, whereas gaps indicate sequence differences. The 23 bp CAPS forward (>>>>) and 22 bp CAPS reverse (<<<<) primers were designed to amplify a 127 bp PCR product containing the *Hind*III restriction endonuclease recognition site (bold higher case, with the cutting site indicated). Digestion with *Hind*III is predicted to cut the amplified PCR product from the *SlNCED1* allele into two fragments of 73 bp and 54 bp in length.

Ideally this CAPS marker would have been designed to produce a unique cut characteristic of the *S. galapagense* allele, but unfortunately, as previously mentioned, the *SgNCED1* sequence did not contain any predicted restriction enzyme cutting sites when compared to either *SnNCED1* or *SlNCED1* i.e. any predicted CAPS cutting sites identified were present in the other sequence.

It can be seen in Figure 3.3.10 that, as expected, the *NCED1* amplicon obtained from the homozygous *not*-WT DNA was cleaved by the *Hind*III R.E. resulting in two bands. The equivalent amplicons obtained from *not-neo*$_{BC3}$, *not-galagneo1*$_{BC3}$, *2*$_{BC3}$ and *6*$_{BC3}$ DNA templates were also cleaved, but gave 3 bands (lanes 1 - 4), indicating that these were heterozygous lines which had inherited the *SlNCED1*^{not} allele (the two smaller size bands) and also had an additional *NCED1* allele derived from one of the two wild species included at the start of the backcrossing programme.

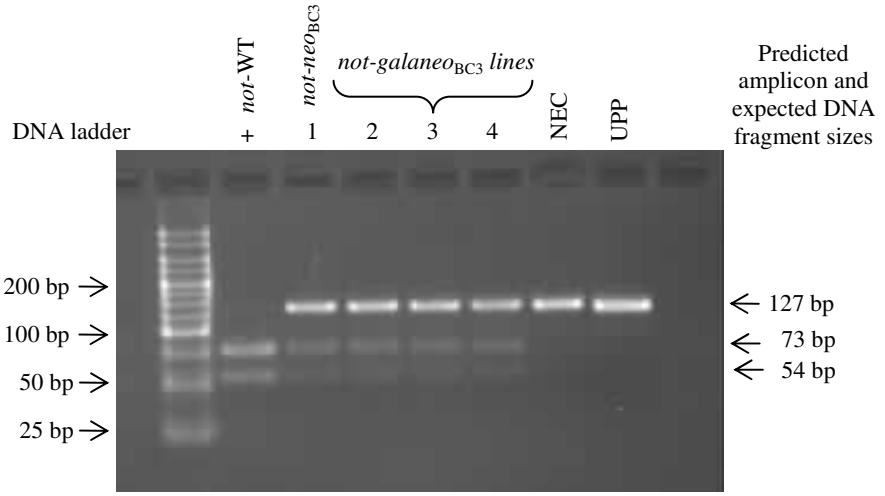

Figure 3.3.10 A gel electrophoresis image showing the identification of the *S. lycopersicum NCED1* allele in comparison to wild species *NCED1* alleles in plants from the third *notabilis* backcross. The restriction digest of the 127 bp *NCED1* amplicon with *Hind*III, cleaves the *SlNCED1/SlNCED1*^{not} and allele to form two DNA fragments of 74bp and 54 bp (3.5% agarose gel): +, positive control, *notabilis x S. lycopersicum* (*not*-WT); NEC, no enzyme control; UPP, undigested PCR product of *NCED1* amplicon using *not*-WT DNA as a template; Lane **1**, *notabilis x S. neorickii*$_{BC3}$ (*not-neo*$_{BC3}$); **2**, *notabilis x* (*S. galapagense x S. neorickii*)*1*$_{BC3}$ (*not-galaneo1*$_{BC3}$); **3**, *not-galaneo2*$_{BC3}$; **4**, *not-galaneo6*$_{BC3}$.

3.3.8 The Backcrossing Programme: Using Tm2a as the Recurrent Parent – The Fourth Backcross

At this point in the backcrossing programme the tomato genotype known as Tm2a, a non-transgenic line homozygous for the tomato mosaic virus (TMV) resistance gene (*Tm-2a/Tm-2*^{2} locus AF536201; Lanfermeijer *et al.*, 2003), in the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig (GCR267) was substituted for the *not* mutant line as the recurrent parent. This has the same genetic background as the mutant homozygous for the *not* allele and was used in order to eliminate the mutated *SlNCED1*^{not} allele from the programme now that CAPS markers were available for identifying the functional wild species alleles. The Tm2a line was also included at this point to confer TMV resistance on the lines, as this disease had been a recurring problem in earlier generations. Since Tm2a and the *not* mutant share the same genetic background, this is

essentially the fourth backcross, albeit strictly speaking an outcross, and this generation was therefore simply designated BC4.

The *not-neo*$_{BC4}$, *not-galaneo1*$_{BC4}$, *not-galaneo2*$_{BC4}$, and *not-galaneo6*$_{BC4}$ plants were subject to CAPS marker selection (Appendix III Table 7.3.3) for both the *SnNCED1* and *SlNCED1* alleles (A summary of the individual plants and their specific *NCED1* allelic genotype is presented in Appendix VIII Table 7.8.1; the accompanying gel electrophoresis images can be found in Appendix VIII section 7.8.1). The BC4 plants segregated in an expected 1:1 Mendelian genetic ratio for homozygous and heterozygous F_1 plants, χ^2(d.f. = 3, n = 78) = 0.42, P>0.05.

At this stage the *not-galaneo2* line was dropped from the programme in order to continue with two selected lines originally derived from the *S. galapagense x S. neorickii* interspecific F_1 hybrid cross; one known to carry the *SgNCED1* allele and one known to carry the *SnNCED1* allele. The three BC4 lines were selfed to allow homozygous plants to be selected for use in a gravimetric water use trial. (A summary of the individual plants and their specific *NCED1* genotype is present in Appendix VIII Table 7.8.2 the accompanying gel electrophoresis images can be found in Appendix VIII section 7.8.2). This generation of selfed BC4 plants segregated as expected in a 1:2:1 Mendelian F_2 ratio χ^2(d.f. = 4, n = 144) = 2.02, P>0.05.

3.3.9 Gravimetric Water Use Efficiency

Selected paired homozygous plants, i.e. plants homozygous for one or other of the two wild species *NCED1* alleles (*SnNCED1* or *SgNCED1*) paired with plants homozygous for the *SlNCED1* allele derived from the same segregating F_2 seed. This approach was used because the BC4 generation of plants was expected to still have a lot of genetic variation; the use of individual homozygous plants selected from the same segregating F_2 population would ensure that each of the two sets of plants should vary at random with regard to the genetic background, while being homozygous for the

specifically selected *NCED1* allele. These paired groups of selected homozygous plants allowed the wild species *SnNCED1* and *SgNCED1* alleles to be analysed in an unbiased way for their effect, if any, on plant water use and leaf carotenoid content; against an equivalent set of selected control plants from the same segregating F_2 population, which were homozygous for the *SlNCED1* allele.

The paired set of selected homozygous plants were then subjected to a 30 d gravimetric water use trial. As can be seen in Table 3.3.6, there were no significant differences (P>0.05) either between the three sets of paired homozygous genotypes, or overall between any of the genotypes. The TE_p ranged from a mean of 4.8 g DWt kg H_2O^{-1} in *not-neo* plants homozygous for *SlNCED1* to 4.4 DWt Kg H_2O^{-1} in *not-galaneo1* plants homozygous for *SlNCED1*. The component measurements (Table 3.3.6) from which TE_p was calculated also revealed no significant differences between the genotypes: TE_p is based on two components, the amount of biomass accumulated and the amount of water transpired over the experimental period. It should be noted that the above ground biomass produced during the 30 d trial was determined as the difference between the initial biomass g DWt at the start of the trial and the final biomass at the end of the trial. The different genotypes all had a similar biomass (Table 3.3.6) at the start of the experimental period.

The genotypes produced similar amounts of above ground biomass over the 30 day period (Table 3.3.6), ranging from a minimum mean of 57.8 g DWt in *not-neo* homozygous for *SlNCED1* to a maximum mean of 63.7 g DWt in *not-galaneo1* homozygous for *SlNCED1*. The different genotypes also transpired similar amounts of water (kg H_2O; Table 3.3.6) over the 30 d trial period; which ranged from a minimum mean of 12.1 kg H_2O in *not-neo* homozygous for *SlNCED1* and to a maximum mean of 14.6 kg H_2O in *not-galaneo1* homozygous for *SlNCED1* (Table 3.3.6).

Table 3.3.6 The effect of the wild species *NCED1* alleles on whole plant transpiration efficiency (TE_P, g DWt kg H_2O^{-1}) determined gravimetrically over a period of 30 days. The above ground biomass production (g DWt) during the trial period was determined as the difference between the initial and final above ground biomass (g DWt). Three sets of paired homozygous F_2 plants derived from backcross four lines: *S. lycopersicum x S. neorickii* (*not-neo*$_{BC4}$ *x* self), *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$ *x* self) and *S. lycopersicum x* (*S. galapagense x S. neorickii*)*6* (*not-galaneo6*$_{BC4}$ *x* self): F_2 individuals selected to be homozygous for either the wild species *SnNCED1* or *SgNCED1* allele were compared to control plants from the same F_2, selected to be homozygous for the *SlNCED1* allele. Both 'between pairs of homozygotes' independent *t*-test (1-tailed) and overall ANOVA probability (*P*) values are given (n = 4 ± S.E.).

Segregating F_2 Population	Selected Genotypes of F_2 Plants	initial Biomass (g DWt)	Biomass Produced (g DWt)	Total Transpiration (Kg H_2O)	TE_P, (g DWt kg H_2O^{-1})
not-neo$_{BC4}$ *x* self	*SlNCED1 SlNCED1*	0.83 ± 0.03	57.78 ± 1.7	12.06 ± 0.97	4.86 ± 0.32
	SnNCED1 SnNCED1	0.9 ± 0.04	60.25 ± 3.27	13.35 ± 0.22	4.51 ± 0.19
t-test *P*		0.168	0.264	0.140	0.186
not-galaneo1$_{BC4}$ *x* self	*SlNCED1 SlNCED1*	0.63 ± 0.03	63.67 ± 5.34	14.57 ± 1.14	4.36 ± 0.04
	SnNCED1 SnNCED1	0.8 ± 0.12	62.2 ± 5.01	13.58 ± 0.8	4.57 ± 0.14
t-test *P*		0.119	0.424	0.252	0.103
not-galaneo6$_{BC4}$ *x* self	*SlNCED1 SlNCED1*	0.78 ± 0.13	58.93 ± 3.59	13.11 ± 1.11	4.54 ± 0.21
	SgNCED1 SgNCED1	0.63 ± 0.09	58.21 ± 4.08	12.87 ± 1.02	4.54 ± 0.07
t-test *P*		0.188	0.449	0.438	0.500
ANOVA *P*		0.187	0.881	0.570	0.569

As shown in Table 3.3.7, there was a significant difference ($P<0.05$) in initial height (cm) between the paired sets of homozygous *not-neo* plants, with the paired controls homozygous for *SlNCED1* being on average shorter (mean of 12 cm) than the equivalent set of *SnNCED1* homozygotes (mean of 14 cm). This difference in height was maintained throughout the trial period, and the set of *SlNCED1 not-neo* paired control homozygotes were still significantly ($P<0.05$) shorter (mean of 96 cm) than the equivalent set of *SnNCED1 not-neo* homozygotes (mean of 110 cm). The selected *not-neo* homozygotes for *SnNCED1* were the tallest of the genotypes overall, and

significantly (P<0.01) taller than both sets of paired *not-galaneo6 SlNCED1* and *SgNCED1* homozygotes (which had means of 87 cm and 92 cm respectively). The paired sets of *not-galaneo6* homozygotes were also found to be significantly (P<0.05) shorter than the equivalent paired sets of *SlNCED1* and *SnNCED1*derived from *not-galaneo1* homozygotes (which had means of 108 cm and 110 cm respectively).

Table 3.3.7 The initial height at the start of the 30 day trial period and the final height (cm), mean internode length (cm) and mean total leaf area (cm^2) at the end of the trial period: Three sets of paired homozygous F_2 plants derived from backcross four lines: *S. lycopersicum x S. neorickii* (*not-neo*$_{BC4}$ *x* self), *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$ *x* self) and *S. lycopersicum x* (*S. galapagense x S. neorickii*)*6* (*not-galaneo6*$_{BC4}$ *x* self): F_2 individuals selected to be homozygous for either the wild species *SnNCED1* or *SgNCED1* allele were compared to control plants from the same F_2, selected to be homozygous for the *SlNCED1* allele. The 'between pairs of homozygotes' independent *t*-test (1-tailed) *P* values are given and significant (P<0.05) values marked with *. Overall ANOVA *P* values are also given, in this case values with a different letter are significantly (*=P<0.05, **=P<0.01) different according to *post hoc* test LSD (n = 4 ± S.E.).

SEGREGATING F_2 POPULATION	SELECTED GENOTYPES OF F_2 PLANTS	INITIAL HEIGHT (cm)	FINAL HEIGHT (cm)	INTERNODE LENGTH (cm)	TOTAL LEAF AREA (cm^2)
not-neo$_{BC4}$ *x* self	*SlNCED1* *SlNCED1*	12.05 ± 0.40	96 ± 5.12 ^{a*}	4.28 ± 0.27 ^{a*c}	3007.23 ± 366.18
	SnNCED1 *SnNCED1*	14.38 ± 0.69	110.25 ± 4.17 ^{b*}	4.1 ± 0.22 ^{abcd}	3377.46 ± 245.98
t-test *P*		0.014*	0.037*	0.318	0.146
not-galaneo1$_{BC4}$ *x* self	*SlNCED1* *SlNCED1*	11.5 ± 0.76	108 ± 4.74 ^{b*}	4.45 ± 0.06 ^{a*c}	3877.09 ± 249.97
	SnNCED1 *SnNCED1*	12.5 ± 1.04	109.5 ± 2.66 ^{b*}	4.18 ± 0.13 ^{ac*}	3951.26 ± 636.95
t-test *P*		0.241	0.396	0.055	0.046
not-galaneo6$_{BC4}$ *x* self	*SlNCED1* *SlNCED1*	12.38 ± 1.05	87 ± 1.83 ^{a**}	3.73 ± 0.13 ^{b*c}	3667 ± 118.71
	SgNCED1 *SgNCED1*	11.38 ± 0.38	91.75 ± 3.57 ^{a**}	3.6 ± 0.19 ^{b*d*}	3624.88 ± 175.22
t-test *P*		0.202	0.141	0.302	0.425
ANOVA *P*		0.187	0.001	0.028	0.844

There were no significant differences between the paired sets of homozygotes with regard to mean internode length (Table 3.3.7), but there were overall differences

between the various genotypes. The set of *not-neo SlNCED1* homozygote and the set of *not-galaneo1 SlNCED1* homozygotes had significantly (P<0.05) longer internodes (means of 4.3 cm and 4.5 cm respectively) than both sets of paired *SlNCED1* and *SgNCED1* homozygotes derived from *not*-galaneo6 (means of 3.7cm and 3.6 cm respectively). The set of *not-galaneo1 SnNCED1* homozygotes was also found to have significantly (P<0.05) longer internodes (mean of 4.2 cm) than the set of *not-galaneo6 SgNCED1* homozygotes (mean of 3.6 cm). Additionally, it should be noted that there were no differences found between the genotypes in their mean total leaf area (Table 3.3.7).

At the end of the 30 d trial period, it was also found that there were no significant (P>0.05) differences in the leaf carotenoid concentrations (Table 3.3.8) found between any of the paired sets of homozygotes or overall.

Table 3.3.8 The mean leaf carotenoid content (μg g^{-1} FWt) at the end of the 30 d experimental period. Six paired homozygous backcross four lines *S. lycopersicum x S. neorickii* (*not-neo*$_{BC4}$), *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$) and *S. lycopersicum x* (*S. galapagense x S. neorickii*)*6* (*not-galaneo6*$_{BC4}$): Three sets of paired homozygous F_2 plants derived from backcross four lines: *S. lycopersicum x S. neorickii* (*not-neo*$_{BC4}$ *x* self), *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$ *x* self) and *S. lycopersicum x* (*S. galapagense x S. neorickii*)*6* (*not-galaneo6*$_{BC4}$ *x* self): F_2 individuals selected to be homozygous for either the wild species *SnNCED1* or *SgNCED1* allele were compared to control plants from the same F_2, selected to be homozygous for the *SlNCED1* allele. Both 'between pairs of homozygotes' independent *t*-test (1-tailed) and overall ANOVA probability (*P*) values are given (n = 4 ± S.E.).

HOMOZYGOUS LINE	GENOTYPE	THE MEAN LEAF CAROTENOID CONTENT (μg g^{-1}FWt)						
		Lutein	Zeaxanthin	Violaxanthin	9'-cis-neoxanthin	All-trans-neoxanthin	β-Carotene	Total
not-neo$_{BC4}$	*SlNCED1* *SlNCED1*	75.27 ± 1.24	1.68 ± 0.18	0.54 ± 0.1	10 ± 0.56	1.4 ± 0.11	50.46 ± 4.05	139.08 ± 4.76
	SnNCED1 *SnNCED1*	75.36 ± 8.04	1.55 ± 0.36	0.72 ± 0.26	10.58 ± 1.2	1.23 ± 0.16	45.58 ± 5.39	135.01 ± 13.32
t-test *P*		0.496	0.378	0.277	0.338	0.333	0.248	0.395
not-galaneo1$_{BC4}$	*SlNCED1* *SlNCED1*	77.55 ± 7.02	1.82 ± 0.6	0.82 ± 0.13	10.81 ± 1.17	1.21 ± 0.13	56.41 ± 8.6	148.66 ± 17.27
	SnNCED1 *SnNCED1*	81.57 ± 8.9	1.36 ± 0.31	1.29 ± 0.29	12.86 ± 1.35	1.51 ± 0.13	49.59 ± 7.52	148.17 ± 16.85
t-test *P*		0.368	0.261	0.110	0.148	0.079	0.286	0.492
not-galaneo6$_{BC4}$	*SlNCED1* *SlNCED1*	77.18 ± 8.63	1.43 ± 0.54	0.6 ± 0.13	10.47 ± 1.26	1.18 ± 0.15	49.11 ± 4.19	139.96 ± 13.92
	SgNCED1 *SgNCED1*	80.80 ± 8.74	1.35 ± 0.21	0.88 ± 0.15	11.45 ± 0.72	1.21 ± 0.08	60.14 ± 6.44	155.84 ± 14.07
t-test *P*		0.389	0.447	0.100	0.262	0.421	0.101	0.227
ANOVA *P*		0.985	0.948	0.124	0.518	0.419	0.609	0.903

3.4 DISCUSSION

3.4.1 The Wild Species Preliminary Data

The introgression of the *S. galapagense* and *S. neorickii NCED1* alleles (*SgNCED1* and *SnNCED1*) into the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig, described in this chapter, was part of a joint Nottingham/Warwick-HRI DEFRA funded research programme involving the ten wild species (13 accessions) most

closely related to the commercial tomato. The introgression of the *SgNCED1* allele was initiated using the F_1 progeny of an interspecific hybrid cross between *S. galapagense* and *S. neorickii* (abbreviated to *galaneo*). This method was adopted because, as previously mentioned (section 3.2.1) *S. galapagense* flowers unreliably and has very dormant seed which will only germinate (in the laboratory) after partial degradation of the testa with bleach. The *galaneo*$_{F1}$ seed and plants were not affected by these traits, and were easier to use in the backcrossing programme. The introgression of the *SnNCED1* allele was achieved both directly from the pure parental *S. neorickii* (abbreviated to *neo*) and from the *galaneo*$_{F1}$ progeny in order to allow possible novel water stress phenotypes that may have arisen from the crossing of these two geographically isolated species, to be observed.

Preliminary HPLC-PDA analysis of some of the main carotenoids revealed that the control line, *S. lycopersicum* cv. Alisa Craig, contained significantly (3x and 1.8x) more all-*trans*-neoxanthin (Table 3.3.1) than both the *galaneo*$_{F1}$ and *neo* progeny. Additionally *galaneo*$_{F1}$ contained significantly (3x) less violaxanthin in its leaves than the WT. In the WT control, the relative abundance of violaxanthin and the relationship between 9′-*cis*-neoxanthin and all-*trans*-neoxanthin presented here concur with the previous findings of Parry *et al*., (1990). They reported that in tomato leaves violaxanthin made up 49.9% of the total epoxycarotenoid content, while 9′-*cis*-neoxanthin represented 46.6% and all-*trans*-neoxanthin represented 0.85% (Parry *et al.*, 1990; Taylor *et al.*, 2005). There were no significant differences found between the three lines with regard to the other carotenoids/xanthophylls analysed. Concurrent with previous findings in the leaves of most higher plant species, β-carotene and lutein were the most abundant non-oxygenated carotenoid and oxygenated carotenoid (i.e. xanthophyll) respectively (Salisbury and Ross, 1992), with lutein being about 30% more abundant than β-carotene in the three genotypes. The two ‘end’ products of the xanthophyll ‘cycle’, zeaxanthin and

violaxanthin, were found in lower amounts, with the zeaxanthin pools being typically very low in the absence of high light stress (Horton *et al.*, 1996; Niyogi, 1999).

There were no significant differences in *NCED1* mRNA or $[ABA]_{leaf}$ in the unstressed leaves of the three genotypes, and the apparently broad range of $[ABA]_{leaf}$ found, 192 ng g^{-1} FWt WT to 396 ng g^{-1} FWt in $galaneo_{F1}$ were reasonably similar to previously published values (Thompson *et al.*, 2000a; Thompson *et al.*, 2004; Thompson *et al.*, 2007a; Thompson *et al.*, 2007b) and not unexpected since the environmental stress history of each glasshouse grown plant was not tightly controlled. A detached leaf wilt experiment was performed to asses the short term response of *NCED1* mRNA production and $[ABA]_{leaf}$ in each genotype. The $galaneo_{F1}$ plants showed a highly significant rapid increase ($P<0.01$) in ABA concentration over the four hours of dehydration (Figure 3.3.2), which was correlated with a relatively rapid and significant increase ($P<0.05$) in *NCED1* mRNA abundance (Figure 3.3.1), concurrent with the results presented in Thompson *et al*., (2000a) who reported increases in *NCED1* mRNA levels when detached tomato leaves were dehydrated. In the WT, *NCED1* mRNA steadily increased by 30% over the four hour treatment ($P<0.05$) and this was coupled with a less substantial increase in $[ABA]_{leaf}$ which was consistent with the slower $[ABA]_{leaf}$ increase obtained for WT in Thompson *et al*., 2000a. The final $[ABA]_{leaf}$ attained by the WT dehydration stressed leaves was much lower than the 1000 ng g^{-1} FWt reported for 90% FWt dehydration for four hours (Thompson *et al.*, 2000a). Perhaps the treatment was not severe enough to illicit a full response and over a longer time period a greater increase in $[ABA]_{leaf}$ would have been observed.

In *neo* leaves *NCED1* mRNA and $[ABA]_{leaf}$ only showed a slight increase over the treatment period, and remained below that of the WT throughout the treatment. This could indicate that *neo* has other mechanisms of water conservation in response to short-term dehydration stress e.g. physiological adaptations or other drought responsive genes, which result in a slower induction of *NCED1* mRNA production and a much slower

increase in $[ABA]_{leaf}$. In contrast the *galaneo*$_{F1}$ progeny appeared to have a more rapid increase in *NCED1* mRNA from a lower basal level. This possibly different responsiveness could indicate an altered promoter with slightly different properties. It has been reported (Harrison, L. unpublished findings), that unlike the coding sequences of the different tomato wild species *NCED1* alleles, the adjacent promoter sequences show very little DNA sequence homology and therefore may function differently when introgressed into the commercial tomato background.

3.4.2 The Backcrossing Programme

The introgression of *SgNCED1* and *SnNCED1* alleles into the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig was initiated with a cross to a *S. lycopersicum* line homozygous for the ABA deficient mutant allele *notabilis* (*not*), which has been characterised as a null mutation in the commercial tomato *NCED1* gene (Burbidge *et al.*, 1999). This allowed easy phenotypic selection for the replacement of the non-functional *SlNCED1 not* allele (*SlNCED1*^{not}) with the functional *SlNCED1*, *SgNCED1* or *SnNCED1* allele. The *galaneo*$_{F1}$ progeny, *neo* and the WT *S. lycopersicum* were crossed to *not* to create three F_1 lines, named *not-galaneo*$_{F1}$, *not-neo*$_{F1}$ and *not*-WT$_{F1}$ respectively. Six individual *not-galaneo*$_{F1}$ plants were backcrossed to *not* to give a realistic probability of retaining both the *SgNCED1* and *SnNCED1* alleles since, as previously mentioned in section 3.3.3, the probability that one of the alleles would be completely missing was only one in sixty four. In addition, the *not-neo* line and *not*-WT were also backcrossed to the *not* mutant.

The leaf carotenoid content was analysed at each backcross and, as might be expected during the initial stages of a backcrossing programme, there were significant perturbations between the various lines and generations for the carotenoids analysed. Some variation in carotenoid content between subsequent generations would appear to be inevitable due to substantial changes in environmental conditions as successive

generations of plants were grown at different times of year. High light intensities result in increased total xanthophyll content in comparison to low light, and a reduction in the ratio of α-carotene-derived lutein to β-carotene-derived zeaxanthin and violaxanthin (Hirschberg, 2001). The *not*-WT was used a control to assess differences between the crosses in each generation, and although at each generation significant differences between the lines were found for various carotenoids analysed, they are described in the appropriate sub-sections in section 3.3 and will not be discussed in detail herein.

The $[ABA]_{leaf}$ of the glasshouse grown plants was also analysed during the initial *not* backcross generations but, as previously stated, the water stress history of these plants was not known or precisely controlled and this factor may have accounted for the differences in $[ABA]_{leaf}$ observed. Despite this, the use of the WT control allowed general comparisons between the lines to be made, the individual $[ABA]_{leaf}$ findings, and any significant differences between the lines are described in the appropriate sub-sections in section 3.3 and will not be discussed in detail herein. These preliminary leaf carotenoid and [ABA] findings were used to aid the selection of *not-galaneo*$_{BC2}$ lines for continuation to the BC3 generation, which were subsequently shown to contain the same wild species *NCED1* allele.

3.4.3 Identification of the *NCED1* Alleles

As a result of work carried out on a joint Nottingham/Warwick-HRI DEFRA funded research programme, the DNA sequences of a number of *NCED1* alleles from wild tomato species became available (Harrison, E., personal communication). This allowed CAPS markers to be designed to distinguish the two wild species, *SnNCED1* and *SgNCED1* alleles, from the *SlNCED1* allele (including the non-functional *SlNCED1*^{not} allele).

As previously mentioned (sections 3.3.5 and sections 3.3.7) the *SgNCED1* sequence, when aligned with either the *SlNCED1* or the *SnNCED1* allele, did not show

any potential CAPS restriction endonuclease cutting sites, i.e. any predicted CAPs cutting sites identified were present in the other sequence. The CAPs markers were designed in the ORF (open reading frame) of the sequence, in which there is a conserved lack of introns in the tomato *NCED1*, as well as in the other rapidly responding (to dehydration stress) *NCED* homologues of other plant species (Tan *et al.*, 2003; Thompson *et al.*, 2004; Taylor *et al.*, 2005). Introns are usually less conserved between species and as such are a major source of sequence differences. The lack of introns in *NCED1* undoubtedly made the identification of CAPs cutting sites within the *SgNCED1* sequence more difficult than it would have otherwise been. It has been suggested that the conserved lack of introns in the rapidly responding *NCED* genes of the multigene family may facilitate rapid gene expression in response to stress (Taylor *et al.*, 2005).

CAPs markers were used to identify which lines carried which wild species *NCED1* allele, and the leaf carotenoid and [ABA] data were also used to aid selection of specific lines. From this, two lines originally derived from the *S. galapagense x S. neorickii* interspecific F_1 hybrid cross, one known to carry the *SgNCED1* allele and one known to carry the *SnNCED1* allele were chosen. These along with the line originally derived from the pure *S. neorickii* parent, were backcrossed to the fourth generation followed by selfing to produce lines homozygous for the respective *NCED1* alleles. It should be reiterated at this point that in order to eliminate the mutated *SlNCED1*^{not} allele from the programme, and to confer TMV resistance to the lines, the recurrent parent was changed from *not* to Tm2a. These TMV resistant plants have essentially the same genetic background of *S. lycopersicum* cv. Alisa Craig and would not have interrupted the introgression process.

3.4.4 Gravimetric Water Use Efficiency

The results of a 30 d gravimetric trial did not reveal any significant effect of either the *SgNCED1* or *SnNCED1* allele on whole plant water use efficiency compared to

the *SlNCED1* allele. Plants homozygous for either the *SgNCED1* or the *SnNCED1* allele were found to produce the same amount of above ground biomass, and also to transpire the same amount of water over the trial period, as did equivalent plants homozygous for the *SlNCED1* selected from the same segregating lines (i.e. paired homozygotes with a similar genetic background, as explained in section 3.3.9). There were also no significant differences between the paired homozygotes with regard to the total leaf area or the leaf carotenoid/xanthophyll concentrations.

The paired homozygous lines *SgNCED1:SgNCED1* and *SlNCED1:SlNCED1* (derived from the *galaneo6* plant, which carried the *SgNCED1* allele) were found to have shorter internodes, a mean of 3.6 cm and 3.7 cm respectively than the other lines and were found to be significantly ($P<0.05$) shorter than both the *SlNCED1* homozygous lines derived from the pure *S. neorickii* wild species and *not-galaneo1*$_{F1}$ plant (carrying a copy of the *SnNCED1* allele) which had mean internode lengths of 4.3 cm and 4.5 cm respectively (Table 3.3.7). The paired homozygous plants derived from the *not-galaneo6* parent were also significantly ($P<0.01$) shorter than the other lines at the end of the 30 d trial period despite the genotypes having similar initial starting heights (apart from the *SlNCED1* homozygote derived from the pure *S. neorickii* wild species, discussed below).

The *S. galapagense* species has very short internodes (1.5 cm – 3.5 cm long; Peralta *et al.*, 2008) and the *not-galaneo6* plants appear to still be segregating for this characteristic. The *NCED1* allele and the locus influencing the length of plant internodes do not appear to be tightly linked however, since if the locus for having shorter internodes was tightly linked to the *SgNCED1* allele the plants homozygous for *SgNCED1* would have had significantly shorter internodes than the paired plants homozygous for the *SlNCED1* allele, which were derived from the same segregating line. There was no significant difference between the paired homozygous plants with regard to the mean internode length or final plant height and therefore these two loci do not appear

to be closely linked. Further backcrossing would therefore be expected to have removed this phenotype from the line if sufficient time had been available.

On the other hand, the *SnNCED1* homozygote derived from the pure *S. neorickii* wild species was significantly taller than the paired *SlNCED1* homozygote derived from the same segregating line, both at the start and at the end of the trial period (Table 3.3.7). This may indicate that the *S. neorickii* propensity for tallness (internodes recorded as 2 – 4.5 cm long; Peralta *et al.*, 2008) is tightly linked to the *SnNCED1* allele. However, this phenotype was not observed in the *not-galaneo1* line which was also carrying the *SnNCED1* allele, although this could have been replaced by the *S. galapagense* allele during the interspecific cross. These observations and comments on the inheritance of the height differences are very preliminary, and repeated measurements and further investigations would be required to test for any real effects.

3.5 CONCLUSIONS & FUTURE WORK

It can be concluded from the introgression of the two tomato wild relative alleles (*SgNCED1* and *SnNCED1*) into the cultivated tomato (*S. lycopersicum* cv. Alisa Craig) genetic background that these two alleles do not function to improve whole plant WUE when compared to the *SlNCED1* allele. A phylogenic tree of the protein sequences of the *NCED1* alleles of ten wild relatives of tomato (13 different accessions) revealed that the *SgNCED1* falls into the same clade as the cultivated *SlNCED1* allele (clade 1), while the *SnNCED1* falls into clade 2, which is the least divergent of the six different clades from clade 1 (Harrison E. unpublished findings). Therefore other tomato wild species *NCED1* alleles, which are more divergent from the *SlNCED1* allele and currently under investigation, may be expressed differently enough to impact on whole plant WUE when introgressed in to the cultivated tomato background.

Alternatively, it is also possible that natural variation in the functioning of *NCED1* to the extent that will have an impact on stomatal functioning and whole plant

water use may not be present in the wild tomato species. Plants in the wild have generally evolved to conserve cellular water, as opposed to conserving soil water. Conserving soil water, although beneficial in agriculture, is less likely to be beneficial in the natural environment since any water not used by one plant, would then be available to any competing neighbouring plants. The use of the wild relatives of tomato for conferring improved water use efficiency by other methods/traits is still a relatively unexplored resource that will be discussed in more detail in section 5.2.4.While the existence of a naturally occurring *NCED1* allele that functions to increase ABA biosynthesis in unstressed plants cannot be ruled out at this stage in the research programme, the goal of producing tomatoes that conserve water in the soil for future use may only be attainable by transgenic means.

CHAPTER 4:

A TRANSGENIC ROOTSTOCK APPROACH TO MANIPULATING ABA BIOSYNTHESIS

4.1 INTRODUCTION

This approach involved the development of a transgenic line, for use as a rootstock, that produced increased amounts of ABA via the overexpression of constructs encoding three key enzymes in the ABA biosynthesis pathway (see section 1.3 for ABA biosynthesis and section 1.9 for the three key enzymes). The underlying hypothesis was that this transgenic rootstock would potentially provide sufficient extra root sourced ABA to the non-transgenic scion, in order to detectably reduce stomatal conductance, thereby increasing crop water use efficiency.

The use of grafting to improve plant vigour and crop quality is not a new horticultural technique and is widely practiced in tree and vine crop production. The grafting of herbaceous vegetable crops, is an established practice in Asia (Lee and Oda, 2003). In 1990 the percentage of grafted plants for the production of Solanaceous and Cucurbitous fruit bearing vegetables, such as aubergines (*Solanum melongena*), cucumber (*Cucumis sativus*), tomato, and various melons, was 59% of the vegetable production in Japan and 81% in Korea (Lee, 1994; Rivero *et al.*, 2003). Despite this, vegetable production using grafted seedlings of Cucurbitous and Solanaceous crops only started to gain worldwide popularity from the 1990s, and its use is increasing throughout Europe, the USA and the Middle East (Lee and Oda, 2003). For example, in Italy the number of grafted vegetable plants increased from 4 to 14 million between 1997 and 2000 (Leonardi and Romano, 2004), in Spain in 2004 grafted tomato and watermelon

plants represented 40% and 50% respectively of total commercial plant production (Leonardi and Romano, 2004), while in the Netherlands, in 2008 approximately 75% of commercially grown greenhouse tomatoes were grafted onto high vigour rootstocks (Venema *et al.*, 2008).

The general objective in using these rootstocks was to promote scion growth and development in order to increase yield and fruit quality. However, they have also been selected to be resistant to a wide range of soil borne fungal, bacterial, viral and nematode diseases. Disease resistance is often difficult to breed directly into commercial cultivars due to close genetic linkage to other traits, such as fruit size, which makes grafting an effective means of conferring disease resistance without impacting on other desirable traits. Researchers have recommended more widespread use of grafted plants as an alternative to methyl bromide (a soil fumigant which is being phased out of use) to manage soil borne disease (Besri, 2005; Davis *et al.*, 2008). In tomato, the most commonly available commercial rootstocks are interspecific hybrids of *S. lycopersicum* and a *Solanum* wild species (Cortada *et al.*, 2009). For example; Beaufort, Maxifort and Multifort from De Ruiter seeds, HeMan from Syngenta Seeds, and Brigeor from Gautier Seeds are all interspecific hybrids of *S. lycopersicum* x *S. habrochaites* (formerly *L. hirsutum*). These rootstocks confer increased vigour and resistance to a number of common soil borne fungal, bacterial, viral and nematode diseases including; Tomato Mosaic Virus (ToMV), *Fusarium* wilt (*Fol0*, *Fol1*; *For*), corky root (*PI*), *Verticillium* wilt (*Va*, *Vd*) and the root knot neamatode genus *Meloidogyne* (*Ma*, *Mi*, *Mj*).

The benefit of grafting is now being expanded in order to enhance nutrient uptake (Rivero *et al.*, 2004) and to increase resistance to abiotic stresses such as flooding (Black *et al.*, 2003; Yetisir *et al.*, 2006), soil salinity (Fernandez-Garcia *et al.*, 2002; Estan *et al.*, 2005), temperature extremes (Rivero *et al.*, 2003) and drought (White and Castillo, 1989; Bhatt *et al.*, 2002). The Asian Vegetable Research and Development Centre (AVRDC) has recommend the use of aubergine rootstocks grafted to tomato

scions when flooding or water-logged soils are expected (Black *et al.*, 2003). The specified rootstocks are not only resistant to damage caused by flooding but also to bacterial wilt and other root borne diseases (Black *et al.*, 2003).

High or low temperature tolerant rootstocks could be used to extend the growing season, which could result in a better yield and greater economic stability throughout the year (Rivero *et al.*, 2003). Due to their low temperature tolerance, fig leaf gourd (*Curcurbita ficifolia*) and bar cucumber (*Sicos angulatus*) are used as rootstocks for commercial cucumber production and several studies have shown that these rootstocks improve vegetative growth and early yield at suboptimal temperatures (e.g. Bulder *et al.*, 1991). Venema *et al.,* (2008) suggested that grafting could also broaden the temperature optimum of elite tomato cultivars; a cold-tolerant high altitude accession (LA 1777) of the wild tomato species *S. habrochiates* was identified as a valuable germplasm tool for improving the cold tolerance of existing commercial rootstocks (Venema *et al.*, 2008). Research exploring the use of grafting to increase drought tolerance by White & Catilli (1998) in common bean (*Phaseolus vulgaris*) and by Bhatt *et al.,* (2002) in tomatoes, indicated that grafting could be a valuable tool for improving drought tolerance in these crops.

Methods for increasing ABA biosynthesis in non-photosynthetic tissues such as roots might be expected to be different from those in photosynthetic tissues, because roots contain much lower carotenoid levels (between 0.03% - 0.27%) compared to light grown leaves (Parry and Horgan, 1992). NCED (9′-*cis*-epoxycarotenoid dioxygenase; section 1.3.3) is an important rate limiting enzyme of ABA biosynthesis in both roots and leaves (Thompson *et al.*, 2000a; Qin and Zeevaart, 2002). However, in roots it is likely that the supply of xanthophyll precursors required by NCED will be limiting to any overall increase in ABA synthesis (Thompson *et al.*, 2007b). In roots it has been reported that dehydration and the overexpression of *SlNCED1* synergistically increased their ABA content (Thompson *et al.,* 2007b). It will probably be necessary to combine the

overexpression of constructs encoding NCED with the overexpression of constructs encoding other rate limiting enzymes further upstream in the ABA biosynthesis pathway, in order to commit xanthophylls to ABA production, and to increase the concentration of root sourced ABA (Taylor *et al.*, 2005).

β-Carotene hydroxylase (BCH) is a potentially rate-limiting enzyme acting upstream of NCED in the ABA biosynthesis pathway (see section 1.3.2). It has been observed that in response to water stress *SlBCH2* (*CrtR-b2*) is up-regulated in roots and leaves (Thompson *et al.*, 2007b; Sonneveld *et al.*, unpublished data) and this dehydration response of *SlBCH2* (*CrtR-b2*) in roots provided some new evidence that multiple steps of xanthophyll synthesis are up-regulated during stress-induced ABA accumulation in this tissue (Thompson *et al.*, 2007b; Sonneveld *et al.*, unpublished data). As previously mentioned (section 1.9.2) when *AtchyB,* (*Arabidopsis* a β-carotene hydroxylase gene) was over expressed in *Arabidopsis* substantial increases in epoxycarotenoids were reported, notably a 2-fold increase in the amount of leaf all-*trans*-violaxanthin, which was not accompanied by any fall in β-carotene levels (Davison *et al.*, 2002). Similar increases in leaf all-*trans*-violaxanthin were also observed in transgenic tomato lines constitutively overexpressing *SlBCH2*/*CrtR-b2* (Sonneveld *et al.*, unpublished data).

Interestingly, the overexpression of *SlBCH2* in transgenic tomatoes appears to confer a small but significant increase in leaf ABA content and transpiration efficiency (TE_p) (Sonneveld *et al.*, unpublished data). Double transgenic tomato lines, which simultaneously overexpress *BCH2* and *NCED1* have synergistically (as opposed to additively) higher levels of ABA in the leaves than either of the single transgenic parental lines (Sonneveld *et al.*, unpublished data). In addition, these double transgenic lines resulted in a greater than additive increase on whole plant TE_p, without an adverse effect on plant growth (Sonneveld *et al.*, unpublished data). This research demonstrated that limiting stomatal conductance, via increased ABA biosynthesis, in tomatoes at least, may be a viable method for improving crop water use efficiency.

A third enzyme, PSY (phytoene synthase; section 1.3.1) has been shown to restrict the supply of carotenoid precursors potentially available to sustain increased ABA biosynthesis in roots (Fray *et al.*, 1995; Taylor *et al.*, 2005; Li *et al.*, 2008). In support of this, in maize and rice *PSY3* expression has been found to be a key regulator of carotenoid biosynthesis in roots under drought and salt stress (Li *et al.*, 2008; Welsch *et al.*, 2008). Notably, it has been observed that double transgenic tomato plants simultaneously overexpressing both *SlNCED1* and *SlPSY1* have synergistically higher root ABA than either of the single transgenic parental lines (Jones, 2008).

It therefore appears likely that the simultaneous overexpression of constructs encoding PSY, BCH and NCED should greatly increase the flux of carotenoid precursors through each of these three 'bottle necks' in the ABA biosynthesis pathway in non-photosynthetic tissue, and would be expected to result in a substantial increase in root ABA biosynthesis. This triple transgenic rootstock line could be grafted to non-transgenic commercial cultivar scions to potentially provide extra root sourced ABA to the shoot, which may be sufficient to reduce stomatal conductance and increase crop water use efficiency. This is a unique 'semi-GM' approach, which may circumvent some of the objections made against the use of genetically modified crops, while still reaping the benefits of transgenic technology.

4.2 MATERIALS & METHODS

4.2.1 Plant Material

A number of transgenic tomato (*Solanum lycopersicum L.* cv. Alisa Craig) lines, overexpressing ABA biosynthetic genes and used in this research programme, are listed in Appendix I Table 7.1.1 with their genotype and the parental lineage. The single construct transgenic lines, sp5 and sp12 constitutively overexpress a *SlNCED1* ORF driven by the Gelvin Superpromoter (sp) and are homozygous for their transgene insertion loci (Thompson *et al.*, 2007a). The BCH12 single transgenic line contains a

CaMV35S-driven *SlCrtR-b2* (BCH2) ORF construct and is homozygous for the T-DNA insertion locus. The double construct transgenic lines, G28 and G29, are homozygous for the CaMV35S-driven *SlCrtR-b2* (BCH2) ORF found in line BCH12; and are simultaneously homozygous for either the sp12 (in the case of G28) or sp5 (in the case of G29) transgene insertions involving the Gelvin Superpromoter driven *SlNCED1* ORF transgene. The double construct transgenic line, known as MJ8, is homozygous for the sp5 transgene insertion based on the Gelvin Superpromoter driven *SlNCED1* ORF and also segregates for T-DNA insertions containing the 35S::*SlPSY1* construct from the transgenic tomato line Z171D4A, as described in Fray *et al.,* (1995). The triple transgenic lines were created by crossing the double transgenic lines G29 and MJ8, which essentially retained the homozygous sp5 genetic background common to both parents; the breeding pedigree is represented diagrammatically in Appendix VII in section 7.7. The Tm2a line, homozygous for the tomato mosaic virus (TMV) resistance gene (*Tm-2*^{a}*/Tm-2*^{2} locus AF536201; Lanfermeijer *et al.*, 2003), in the cultivated tomato background *S. lycopersicum* cv. Ailsa Craig (GCR267) was used as the control.

4.2.2 Establishing Seedlings in Sterile Culture Conditions

Seeds were surface sterilized for 10 min in 70% ethanol (Fisher Scientific, Leicestershire, UK), followed by a rinse with sterile distilled water (SDW); they were then further sterilized for 20 min in 25% sodium hypochlorite solution (Fisher Scientific, Leicestershire, UK, Cat No. S/5040/21), and finally rinsed ten times with SDW. Sterilized seeds were germinated on sterile culture medium; Murashige and Skoog (MS) mineral salts (Duchefa Biochemie, Melford Laboratories Ltd, Suffolk, UK) plus 8 gl^{-1} agar (Sigma-Aldrich, Dorset, UK) in 9 cm Petri dishes (Sterilin Ltd, Caerphilly, UK). Genotypes with high ABA concentrations in the seed were pre-treated with 1 mgl^{-1} norflurazon solution (Chem Service Inc, West Chester, USA), which was added to the culture media to break dormancy. The seeds were germinated in a growth room and

incubated horizontally at 24°C with a 16h photoperiod, PFD ~80 μmol $m^{-2}s^{-1}$. Once the radicle had just started to emerge each germinating seed was rinsed in SDW to remove residual norflurazon (if necessary), and transferred to a sterile magenta pot containing the same culture medium without norflurazon to allow further seedling establishment in the light, under the environmental conditions previously described.

4.2.3 Isolated Root Tissue Culture Conditions

After between two to three weeks of growth in magenta pots (as described above) ~3 cm sections of the primary root tips of each seedling were removed under sterile conditions and placed into 250 ml conical flasks containing 125 ml of liquid root culture medium (Appendix II in section 7.2.1) containing the antibiotic Co-Amoxiclav (TEVA UK limited, Castleford, UK) at a final concentration of 150 mgl^{-1} to inhibit infection. Flasks were incubated in the dark at 22 °C on an orbital shaker at 60 rpm for a six week period. Arrested root growth was prevented, when necessary, by sub-culture of the roots into fresh liquid medium. The root material (enough to obtain at least 150 mg DWt) was then transferred into 7 ml Bijous (Fisher Scientific, Leicestershire, UK), and freeze-dried for 48 h in a freeze-dryer (CHRIST, Freeze Drying Solutions, Shropshire, UK) at -90°C.

4.2.4 Establishment of Cuttings

Cuttings of established plants were rooted by removal of ~5 cm long side shoots. A clean diagonal cut was made across the end, and the shoot inserted into wet rockwool plugs. The cuttings were kept in a propagator at high humidity for about two weeks until roots were established.

4.2.5 Establishment of plants on sand for root analysis

Young plants from magenta pots or rooted cuttings were transferred to 9 cm pots containing Horticultural silversand (Eden Park Ltd, Kent, UK) on top of a sheet of Whatman® filter paper (No.1 qualitative) to prevent the sand from seeping out of the

bottom of the pot. Pots were placed on top of Florimat capillary matting (Flowering plants Ltd., Buckinghamshire, UK), and were watered with modified Hewitt's solution, N optimal (Hewitt and Smith, 1975) (Appendix II in Section 7.2.2) fully equilibrated to ambient glasshouse temperature. After around five weeks growth the roots were washed free of all the sand, removed from the main stem, harvested in 7 ml Bijous and flash frozen in liquid nitrogen.

4.2.6 Grafting

Grafting of tomato plants should ideally take place at the third to fourth true leaf stage (stem diameter 1.5-2 mm) which was usually around 2-3 weeks after germination. Generally staggered sowing was necessary to offset differences in germination and growth rate between genotypes. Since some of the different transgenic ABA overexpressing lines exhibited delays in both germination and seedling establishment, the seeds of each genotype were sown (with/without norflurazon pre-treatment; Appendix I Table 7.1.2) at staggered times to attempt to synchronise plant development at the time of grafting (Appendix I Table 7.1.3). The graft unions were made between hypocotyls just below the cotyledonary node. Silicon tubing, with an internal diameter similar to the stem diameters of the plants to be grafted, was used to hold the scion and root stock stems together while the graft union formed. The first step in the grafting process was to cut a suitable length of tubing (~2 cm), then the scion providing plant was cut just below the cotyledons at an angle of around 45° and the stem slipped halfway into the length of tubing. The stem of the rootstock providing plant was cut under the cotyledons at an angle of around 45° and the scion stem, with the length of tubing was slipped onto the rootstock stem base, so that the two cut stems aligned. The plants were immediately covered with transparent plastic bags, the ends of which were secured under the pots, and left to establish.

Once an individual plant had started recover i.e. stopped wilting, the plant could be slowly acclimatised to the growing conditions. Initially one corner of the plastic bag was cut off to create a small opening and two days later the other corner of the bag was similarly removed and the plant left to acclimatise for two days. After this period the top of the bag was cut completely open, and the plant left to acclimatise for a further two days, after which the remains of the bag were removed. Once the graft union had begun to cause the silicone tubing to bulge out, the tubing was carefully removed using a razor blade.

4.2.7 Carbon Isotope Analysis

The total plant lamina (oven dried for two days) was ground using an apex rotary mill (Apex Construction, Leicestershire, UK), and a sub-sample was further ground with a ball mill (Glen Creston, London, UK) and sieved to produce 0.1 mm particle size. The samples were analysed at Australian National University for $^{13}C/^{12}C$ and $^{18}O/^{16}O$ and expressed relative to Pee Dee Belemnite and Vienna Standard Mean Ocean Water to give $\delta^{13}C$ and $\delta^{18}O$ values respectively (Farquhar *et al.*, 1997; Barbour and Farquhar, 2000).

4.2.8 Xylem Sap Collection

The plant shoot was removed by cutting approximately 2 mm below the cotyledons. A delivery tube (approximately 15 cm long and covered in aluminium foil to prevent light induced isomerization of the ABA in the exudate) was attached to the stump. Once the exudate had run through the collection tube it was allowed to drip for 30 min before collection began to prevent contamination. The exudate emerging over the following one or two hours was allowed to drip into a 30 ml universal tube which was kept in a polystyrene cup of ice. The collection tube was sealed into the universal with parafilm to prevent contamination and evaporation. The whole apparatus was kept in the dark by covering with aluminium foil. After the collection period the samples were flash

frozen in liquid nitrogen and stored at -70°C. Before xylem sap ABA analysis was carried out, as described in section 4.2.9, the sample volume was measured to determine the xylem sap flow rate.

4.2.9 ABA analysis – Xylem Sap

The xylem sap samples were kindly analysed by M.H. Bennett at Imperial College London. The samples were filtered using a 0.45 micron filter (Millex® syringe-driven filter unit, Millipore Ltd., Watford, UK). A 900 µl aliquot was transferred to a 2 ml Amber screw top vial (Agilent Technologies, West Lothian, UK) and sealed with a PTFE septa cap (Agilent Technologies, West Lothian, UK). A 5 ng standard was added to each 100 µl of sample. Samples were then analysed by HPLC-electrospray ionisation/MS-MS as described in Forcat *et al.* (2008) using an Agilent 1100 HPLC coupled to an Applied Biosystems Q-TRAP 2000 (Applied Biosystems, California, USA). This standard operating procedure was slightly modified as follows: Chromatographic separation was carried out on a solid core column (Ascentis® Express C18 10cmx2.1mmx2.7µm, Sigma-Aldrich, Dorset, UK) at 35°C. The solvent gradient and step table used have been listed in Appendix V Table 7.4.3 Analysis of the compounds was based on appropriate Multiple Reaction Monitoring (MRM) of ion pairs for labelled and endogenous ABA using the mass transitions ABA 263>153 and ABA IS 269>159 (with a dwell time of 150ms) (Forcat *et al.*, 2008).

4.2.10 ABA analysis – Isolated Root Cultures

Freeze dried isolated root culture samples were sent for ABA and metabolite analyses at the NRC Plant Biotechnology Institute, Saskatoon, Canada. A 100 µl aliquot of deuterated internal standard mixture, at a concentration of 0.2 pg μl^{-1}, was added to a known mass (~ 120 mg) of homogenized root tissue. The internal standards were *d*3-DPA, *d*5-ABA-GE, *d*3-PA, *d*4-7′-OH-ABA, *d*3-neoPA, *d*4-ABA, and *d*4-*trans*-ABA; they were prepared according to Abrams *et al*. (2003) and Zaharia *et al*. (2005). Quality

control standards were prepared by adding either 100 µl or 30 µl of a mixture of the non-deuterated analytes of interest, each at a concentration of 0.2 pg µl-1, to 100 µl of the deuterated internal standard mix. For all samples, 3 ml of isopropanol:water:glacial acetic acid (80:19:1, v/v) was then added, and they were then agitated in the dark for 24 h at 4ºC. Samples were centrifuged and the supernatant was dried under vacuum, reconstituted in 100 µl of acidified methanol, adjusted to 1 ml with acidified water, and then partitioned against 2 ml hexane. After 30 min, the aqueous layer was dried, reconstituted as before and then loaded onto equilibrated Oasis HLB cartridges (Waters, Mississauga, ON, Canada). The cartridges were washed with acidified water, and then the metabolites were eluted with acetronitrile:water:glacial acetic acid (30:69:1). The eluates were dried and then reconstituted in 40% methanol (v/v) containing 0.5% acetic acid and 0.1 pg μl^{-1} of each of *d*6-ABA and *d*2-ABA-GE as recovery standards. Analysis was performed on a UPLC/ESI-MS/MS utilizing a Waters ACQUITY UPLC system, equipped with a binary solvent delivery manager, and a sample manager coupled to a Waters Micromass Quattro Premier XE quadrupole tandem mass spectrometer via a Z-spray interface. MassLynx™ and QuanLynx™ (Micromass, Manchester, UK) were used for data acquisition and data analysis was as described by Ross *et al*. (2004).

4.2.11 Nested PCR

To identify homozygous TMV resistance lines two sets of primer pairs were designed to differentiate between the Tm-2 susceptible allele and the resistant Tm-2^2 (also known as Tm-2^a) allele (Lanfermeijer *et al.*, 2003; Lanfermeijer *et al.*, 2005). To detect the susceptible allele a PCR (section 2.4) was run using the primer pair; Tm2a_F and Tm2a_Sus_R1 (Table 7.3.1). To detect the resistance allele a PCR was run using the primer pair; Tm2a_F and Tm2a_Res_R1. The amplified product was then further amplified in a nested PCR. A 1 µl aliquot of the PCR product was diluted in 49 µl SDW, a 1 µl aliquot of this diluted PCR product was used as the DNA template in a PCR

reaction using the Tm2a_Res_NF and Tm2a_Res_NR primer pair. The cycling conditions and primer sequences are listed in Appendix III Table 7.3.4 and Table 7.3.1, respectively. The PCR products were visualised by gel electrophoresis as described in section 2.4.

4.2.12 Quantitative Real Time PCR (qPCR)

Quantitative PCR (qPCR) was used to determine the zygosity of transgenic plants (German et al., 2003) by comparing the amplification of the transgene with the amplification of a reference gene. Usually an endogenous homozygous single copy gene is used a reference gene. In the case of transgenic insertions of unknown copy number, the amplification of the parental genotypes was compared to that of the offspring to determine zygosity of the T-DNA insertion locus, regardless of transgene copy number at that locus. TaqMan probes and unmodified oligonucleotide primers were designed for the DNA sequence encoding the reference gene i.e. tomato vacuolar invertase (Elliot et al., 1993), SlBCH2, and SlPSY1 (Sequences listed in Table 7.3.1). The dual labelled TaqMan probe for the DNA encoding tomato vacuolar invertase (VI) was labelled at the 5' end with the fluorescent reporter dye HEX (hexachlorofluorescein) and at the 3' end with a Black Hole Quencher ™ (BHQ™). The probes for SlBCH2 and SlPSY1 DNA were labelled at the 5' end with the reporter dye FAM (fluorescein) and at the 3' end with a BHQ™ (The probe sequences are listed in Appendix III Table 7.3.2). A master mix was prepared as described in (Appendix IV) so that each well would contain 10 µl 2x Sensi Mix No Ref (Quantace Ltd., Bioline, London, UK), 6 pmol of each primer, 2 pmol of each probe, 1 µl of sample DNA (5 ng μl^{-1}) in a final volume of 20 µl. Three technical replicates were performed for each sample, along with no template controls (NTC's) and negative controls (DNA from the appropriate plant not containing the transgene). All real time PCR was carried out on a Stratagene® Mx3005P™ machine with the thermal cycle profile: 95ºC 10 min; 42 cycles of 95ºC for 15 seconds and 60ºC for 60 seconds.

The threshold cycle (ct) at which each sample reached the threshold fluorescence level for both the transgene and the endogenous reference gene was determined for each sample. These ct values were used in Equation 5, where: *Ct ref* and *Ct tra* are the threshold cycles of the endogenous reference gene and the transgene of the plants to be genotyped respectively; *Ct refp* and *Ct trap* are the threshold cycles of the endogenous reference gene and the transgene in the parental plants respectively (German *et al.*, 2003). When DNA from both homozygous and heterozygous plants was included in the assay, copy number could be estimated using Equation 5.

Equation 5 $$2^{(Ct\ ref - Ct\ tra)} / 2^{(Ct\ refp - Ct\ trap)}$$

4.2.13 Southern Blotting and Hybridisation

Southern Blots were kindly performed by S. Awan at Warwick University. Genomic DNA was prepared from 150 - 170 mg of young fully expanded tomato leaves using the DNA extraction procedure described in Section 2.3. DNA (5-6 µg) was digested for 6h with the restriction enzymes; *BamH*I, *EcoR*I (incubation temperature 37^{o}C) and *Sma*I (incubation temperature.25^{o}C). Digested DNA was run on 0.8% agarose gel overnight and photographed using a UV-transilluminator to determine if the genomic DNA had been completely digested. The DNA was depurinated using 0.25M HCl and transferred to positively charged Nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) using 0.4M NaOH for 16hrs. The membrane containing single stranded DNA was incubated for 30 min at 120^{o}C to fix the DNA. Prehybridisation was carried out in a rotary incubator in a 10 cm diameter glass tube at 65^{o}C for up to 6 hours, in 30 ml of prehybridisation solution containing 2 X SSPE and 0.1% (w/v) sodium dodecyl sulphate (SDS) and 40 µgml^{-1} denatured Herring sperm DNA.

The DNA to be used as a probe was amplified from pT7T3 plasmid using SlPsy1 For1 and SlPsy1 Rev2 primers (Appendix III Table 7.3.1) and PCR cycling conditions listed in Appendix III Table 7.3.4. The probe for hybridisation was prepared

using a Random Prime DNA Labelling kit (GE Healthcare labs, Buckinghamshire, UK) and standard manufacturer's protocol was followed. Hybridisation was carried out for 16-24 hours.

The hybridised blot was washed three times using the 2 X SSPE buffer (2XSSPE,0.1% SDS w/v) followed by three washes using 1 X SSPE (1XSSPE, 0.5%SDS) all for 15 minutes at 65°C. The blots were incubated for up to one week against Phosphor screens. The images were developed using a Phosphorimager (Molecular Dynamics, Amersham Biosciences, New Jersey, USA) and analysed using ImageQuant TL software.

4.3 RESULTS

4.3.1 The Triple Transgenic Line Pedigree

In order to create a triple construct transgenic line that simultaneously and constitutively overexpresses *SlNCED1*, *SlBCH2* and *SlPSY1*, two previously assembled double construct transgenic lines were used as the starting parental genotypes (the transgenic tomato lines used in this research programme are listed in Appendix I Table 7.1.1). The first parental genotype, known as G29, was already homozygous for an *sp::SlNCED1* T-DNA insertion locus associated with the single construct transgenic line known as sp5 (Thompson *et al.*, 2000b) and a *35S::SlCrtRb2/BCH2* T-DNA insertion locus associated with the single construct transgenic line known as BCH12 (Thompson *et al.*, 2007b). The second double parental genotype, known as MJ8, was also homozygous for the sp5 T-DNA insertion locus and segregated for a number of T-DNA insertions of a *35S:SlPSY1* construct (Fray *et al.*, 1995).

The pedigree of the triple construct transgenic lines, which can be found in Appendix VII, will be briefly outlined below. The criteria used to select chosen lines will be explained more thoroughly at appropriate points throughout the chapter. Initially a sample of nine individual MJ8 plants, designated A – I, (from seventeen initial plants A-

Q) were crossed with G29 and seed from three of these crosses; MJ8A x G29, MJ8D x G29, and MJ8H x G29 successfully germinated following norflurazon treatment and were analysed further. The F_1 progeny were all homozygous for the *sp::SlNCED1* T-DNA insertion locus from the sp5 line, heterozygous for *35S::SlCrtRb2/BCH2* T-DNA insertion locus from the BCH12 line and, based on their morphology/biochemical phenotype (see below) must have contained at least one copy of the *35S::SlPSY1* construct. A selected group of five individual F_1 plants; H-15$_{F1}$, H-16$_{F1}$, H-18$_{F1}$, H-20$_{F1}$ and H-22$_{F1}$ were the first to be selfed and taken through to the F_2 generation (Appendix VII) after successful germination following norflurazon treatment. Nine individual F_2 plants; H-15-1$_{F2}$, H-15-22$_{F2}$, H-16-2$_{F2}$, H-18-15$_{F2}$, H-20-12$_{F2}$, H-20-18$_{F2}$, H-22-8$_{F2}$, H-22-22$_{F2}$, and H-22-24$_{F2}$ were selected from a range of F_2 plants to be selfed and taken through to the F_3 generation after successful germination following norflurazon treatment. The F_2 and F_3 plants were subject to qPCR genotyping for both of the segregating constructs i.e. *35S::SlCrtRb2/BCH2* and *35S::SlPSY1*. Three individual F_3 plants; H-22-8-8$_{F3}$, H-22-22-6$_{F3}$ and H-22-22-7$_{F3}$, were selected from the F_3 plants found to be homozygous for the *SlBCH2* T-DNA from the BCH12 line and at least one *SlPSY1* transgene insertion locus inherited from MJ8. The phenotype of the F_3 generation of plants appeared relatively stable, this lack of apparent segregation for the *SlPSY1* transgene indicated that their F_2 parent had been homozygous for this insertion locus. These three triple construct transgenic lines, thought to be homozygous for *SlNCED1*, *SlBCH2* and *SlPSY1* transgene insertion loci were selfed and taken through to the F_4 generation after successful germination following norflurazon treatment (Appendix VII). This phenotypically stable group of three F_4 triple construct transgenic lines were subsequently analysed for their capacity, when used as a transgenic rootstock, to alter stomatal behaviour in non-transgenic scions to improve whole plant WUE.

4.3.2 The Transgenic Lines Containing Two Constructs

The roots of all sand grown MJ8 plants subjected to HPLC-PDA analysis were found to have accumulated lycopene (to varying degrees), which was completely undetectable in either WT or G29 roots (Figure 4.3.1). Therefore the presence/absence of lycopene in these root samples represents an almost qualitative, rather than quantitative, biochemical test for the presence/absence of the *SlPSY1* transgene derived from line Z171D4A (Fray *et al.*, 1995). The concentrations of lycopene, β-carotene and other carotenoids found in the roots of the three individual MJ8 plants (A, D, and H), from which the F_1 progeny (see pedigree in Appendix VII for more details) were derived, are displayed in Table 4.3.1. The unique accumulation of lycopene in the roots, together with a substantially increased β-carotene content, was due to the overexpression of the *SlPSY1* transgene derived from line Z171D4A (Fray *et al.*, 1995).

Plants of the G29 transgenic line (containing two constructs, Appendix I Table 7.1.1) appeared to accumulate slightly more β-carotene in their roots than WT, Tm2a roots (Figure 4.3.1); although this difference was not found to be statistically significant ($P>0.05$, Table 4.3.1). This possible increase in the substrate of the BCH2 enzyme, which is overproduced as a result of the expression of the transgene derived from line BCH12 (Thompson *et al.*, 2007b), could possibly be due to compensatory feedback regulation of earlier steps in the carotenoid/ABA biosynthesis pathway. The roots of all of the MJ8 plants analysed were found to have greatly increased β-carotene levels, ranging from 2.5 μg g^{-1} DWt to 36.6 μg g^{-1} DWt, compared to both Tm2a roots, with a mean of 0.5 μg g^{-1} DWt, and G29 roots, with a mean of 0.9 μg g^{-1} DWt (Figure 4.3.1; Table 4.3.1). This is presumably due to the effect of *SlPSY1* overexpression, which increased root lycopene concentration, and resulted in increased xanthophyll flux through both the α- and β-branches of the ABA biosynthesis pathway (Table 4.3.1). The increased β-carotene accumulation in MJ8 plant roots should provide an excess of substrate for the additional

BCH2 enzyme, produced in the triple construct transgenic lines, as a result of overexpression of the *SlBCH2* transgene.

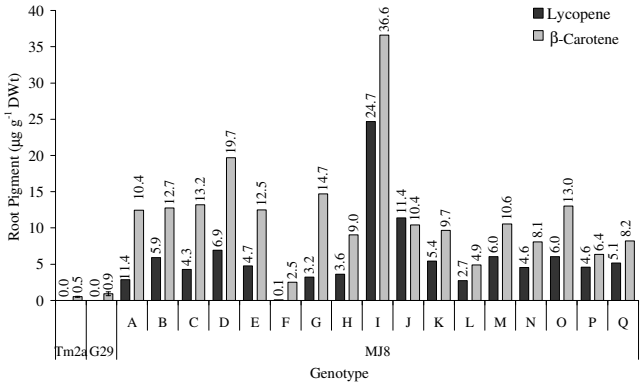

Figure 4.3.1 The accumulation of lycopene and β-carotene (μg g^{-1} DWt) in the roots of sand grown cuttings of: the WT, Tm2a line; the G29 double construct transgenic line constitutively overexpressing *SlBCH2* and *SlNCED1* (n = 8 ± S.E.) and individual sand grown cuttings of individual MJ8 plants constitutively overexpressing *SlPSY1* and *SlNCED1*.

Table 4.3.1 lists the composition of the main xanthophylls and other carotenoids in the roots of WT, G29 and the individual MJ8A, MJ8D and MJ8H plants. The roots of the MJ8 transgenic plants accumulated a much greater total amount of carotenoids than either Tm2a or G29 roots; mainly due to the substantially increased amounts of β-carotene and lycopene (Figure 4.3.1 and Table 4.3.1), which had the effect of reducing the contribution of lutein to 1% of the total carotenoids.

The total amount of xanthophylls in G29 roots (1 μg g^{-1} DWt) appeared to be slightly lower (although the difference was not statistically significant $P>0.05$) than in Tm2a roots (1.5 μg g^{-1} DWt) mainly due to a reduction in 9′-*cis*-neoxanthin (Table 4.3.1). This could be explained by the increased production of the NCED1 enzyme which is likely to cause a reduction in the amount of its substrate. The MJ8 roots accumulated less total xanthophylls (~ 0.6 μg g^{-1} DWt) than either Tm2a or G29 roots (Table 4.3.1), mainly accounted for by reduced amounts of the neoxanthin isomers. These isomers appeared to be present at lower concentrations than in G29, presumably because MJ8 plants did not overproduce BCH2 which, in the case of G29, appeared to cause an

increase in precursor flux through the β-branch of the pathway to maintain β-xanthophyll levels, which would otherwise be used up by the increased carotenoid cleavage activity of the NCED1 enzyme.

Table 4.3.1 The root carotenoid content (μg g^{-1} DWt) and percentage composition of the sand grown cuttings of: WT, Tm2a line, and the G29 transgenic line constitutively overexpressing *SlBCH2* and *SlNCED1*, independent *t* test probability (*P*) values are given (n = 8 ± S.E.). The root carotenoid content of single sand grown cuttings of individual MJ8 transgenic plants A, D and H constitutively overexpressing *SlPSY1* and *SlNCED1* are also included.

LINE	XANTHOPHYLL CONTENT(μg g^{-1} DWt) (% OF TOTAL CAROTENOIDS ANALYSED)						OTHER CAROTENOIDS (μg g^{-1} DWt) (% OF TOTAL CAROTENOID CONTENT ANALYSED)		
	Lutein	Zeaxanthin	Violaxanthin	9′-cis-neoxanthin	All-trans-neoxanthin	Total	β-carotene	Lycopene	Total
Tm2a	0.18 ± 0.04 (9)	0.75 ± 0.02 (4)	0.12 ± 0.02 (6)	0.50 ± 0.25 (25)	0.61 ± 0.10 (30)	1.48 ± 0.23	0.51 ± 0.07 (26)	0 (0)	2.0 ± 0.26
G29	0.23 ± 0.04 (12)	0.74 ± 0.02 (4)	0.14 ± 0.03 (7)	0.15 ± 0.04 (8)	0.44 ± 0.12 (23)	1.03 ± 0.24	0.90 ± 0.31 (47)	0 (0)	1.93 ± 0.43
P	0.229	0.488	0.303	0.094	0.163	0.100	0.121		0.453
MJ8A	0.13 (1)	0.14 (1)	0.10 (1)	0.06 (<1)	0.10 (1)	0.54	12.44 (79)	2.85 (18)	15.83
MJ8D	0.21 (1)	0.16 (1)	0.13 (<1)	0.07 (<1)	0.18 (1)	0.76	19.70 (72)	6.94 (25)	27.38
MJ8H	0.23 (2)	0.12 (1)	0.09 (<1)	0.06 (<1)	0.01 (<1)	0.50	9.04 (69)	3.64 (28)	13.18

In MJ8 plants (Table 4.3.1) the increased NCED1 enzyme activity appeared to deplete the pool of neoxanthin isomers. Additionally, MJ8 roots had lower amounts of zeaxanthin compared to both Tm2a and G29 roots. The apparently reduced concentrations of the β-xanthophylls situated downstream of the BCH2 enzyme highlights the potential problem of a BCH 'bottle neck' in the ABA biosynthesis pathway. This idea of a 'bottle neck' at this point is also supported by the (previously mentioned) increased concentration and percentage composition of β-carotene in MJ8 roots, which accounted for ~73% of the total carotenoids analysed, compared to Tm2a

and G29 roots; where β-carotene represented 26% and 47% of the total root carotenoids respectively (Figure 4.3.1 and Table 4.3.1).

The MJ8 plants exhibited other phenotypes associated with constitutive *PSY1* overexpression; they had a reduced growth habit compared to Tm2a as previously described for plants constitutively overproducing the PSY1 enzyme (Fray *et al.*, 1995). Additionally, the immature fruit of tomatoes is usually green, whereas the immature fruit of the MJ8 line was pale peach in colour (Figure 4.3.2) as previously described for tomatoes overexpressing the PSY1 enzyme (Fray *et al.*, 1995; Fraser *et al.*, 2007).

A **B**

Figure 4.3.2 Photographs illustrating the difference in immature fruit colour: **A**; WT, Tm2a line showing green coloured immature fruit and; **B**, MJ8, double transgenic construct line (constitutively overexpressing *SlNCED1* and *SlPSY1*) showing pale peach coloured immature fruit.

4.3.3 The Triple Transgenic Line Containing Three Constructs – The F_1 Generation

The roots of each of the triple transgenic F_1 progeny from the MJ8 x G29 cross (see pedigree in Appendix VII for more details) accumulated detectable levels of lycopene (Figure 4.3.3) to varying degrees, ranging from 0.04 μg g^{-1} DWt to 8.1 μg g^{-1} DWt. Detection of this compound in root tissue indicated the presence of at least one copy of a T-DNA insertion locus, carrying one or more copies of the *PSY1* construct, in all plants assessed.

The mean concentrations and percentage compositions of the main xanthophylls and other carotenoids found in the roots of the triple transgenic F_1 progeny from three of the MJ8 x G29 crosses (A_{F1}, MJ8A x G29; D_{F1}, MJ8D x G29; and H_{F1}, MJ8H x G29) are presented in Table 4.3.2. It should be noted that an ANOVA was not

performed on this data set because of the very different sample sizes caused by the H_{F1} sample number being 28, which would skew the results even using an appropriate *post hoc* test e.g. Hochberg's GT2. This difference in sample sizes is a result of this data being extracted from every triple transgenic F_1 plant (that successfully germinated and grew large enough to have a cutting taken) for the main purpose of identifying the absence/presence of root lycopene. The other main carotenoids/xanthophylls analysed are presented in Table 4.3.2 for completeness and can be generally compared using the relative percentage composition.

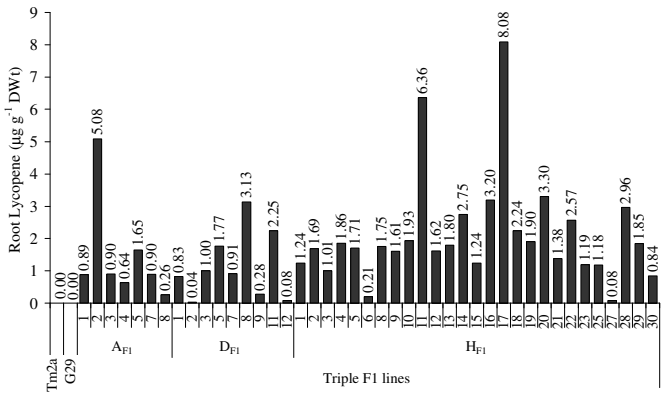

Figure 4.3.3 The accumulation of lycopene (µg g^{-1} DWt) in the sand grown roots of cuttings from: WT, Tm2a line; the G29 transgenic line constitutively overexpressing *SlBCH2* and *SlNCED1* (n=10 and 8 respectively); and individual F_1 progeny of three of the MJ8 transgenic line (constitutively overexpressing *SlPSY1* and *SlNCED1*) x G29 crosses: A_{F1}, MJ8A x G29; D_{F1}, MJ8D x G29 and H_{F1} MJ8H x G29.

The mean root lycopene concentrations (µg g^{-1} DWt) found in the F_1 progeny (A_{F1} mean of 1.5 µg g^{-1} DWt; D_{F1} mean of 1.1 µg g^{-1} DWt, and H_{F1} mean of 2.1 µg g^{-1} DWt) of each cross was lower than that found in the parental MJ8 plants (Table 4.3.1; MJ8A mean of 2.9 µg g^{-1} DWt, MJ8D mean of 6.9 µg g^{-1} DWt, and MJ8H mean of 3.6 µg g^{-1} DWt). Despite this reduction, possibly due to heterozygosity of the T-DNA insertion loci, it is interesting to note that in D_{F1} and H_{F1} plants the percentage of total carotenoids analysed represented by lycopene (27% and 29% respectively) was very similar to the 25% and 28% lycopene composition found in the individual parental MJ8D

and MJ8H plants. However, in A_{F1} plants lycopene made up a mean of 30% of the total carotenoids analysed, whereas in the parental MJ8A individual plant lycopene made up 18% of the total, but this apparent difference may be simply due to random variation.

Table 4.3.2 The root carotenoid content (μg g^{-1} DWt) and percentage composition in the sand grown cuttings of: WT, Tm2a line; and the G29 transgenic line constitutively overexpressing *SlBCH2* and *SlNCED1*. Independent *t* test probability (*P*) values are given (n = 10 and 8 ± S.E. respectively). The root carotenoid content of sand grown cuttings of individual F_1 progeny of three of the MJ8 (constitutively overexpressing *SlPSY1* and *SlNCED1)* x G29 crosses: A_{F1}, MJ8A x G29; D_{F1}, MJ8D x G29 and H_{F1}, MJ8H x G29. (n = 7, 9, 28 ± S.E. respectively).

LINE	XANTHOPHYLL CONTENT(μg g^{-1} DWt) (% OF TOTAL CAROTENOIDS ANALYSED)						OTHER CAROTENOIDS (μg g^{-1} DWt) (% OF TOTAL CAROTENOID CONTENT ANALYSED)		
	Lutein	Zeaxanthin	Violaxanthin	9'-*cis*-neoxanthin	All-*trans*-neoxanthin	Total	β-carotene	Lycopene	Total
Tm2a	0.23 ± 0.11 (14)	0.11 ± 0.36 (6)	0.16 ± 0.03 (8)	0.28 ± 0.02 (13)	0.64 ± 0.08 (31)	1.49 ± 0.22	0.59 ± 0.13 (28)	0.00 (0)	2.07 ± 0.33
G29	0.31 ± 0.08 (14)	0.08 ± 0.02 (4)	0.19 ± 0.03 (8)	0.21 ± 0.03 (9)	0.47 ± 0.11 (21)	1.25 ± 0.22	0.94 ± 0.37 (43)	0.00 (0)	2.20 ± 0.50
P	0.457	0.252	0.298	0.033	0.109	0.233	0.167	n/a	0.42
A_{F1}	0.37 ± 0.07 (8)	0.12 ± 0.02 (2)	0.50 ± 0.10 (10)	0.30 ± 0.04 (6)	0.86 ± 0.14 (17)	2.14 ± 0.28	1.31 ± 0.16 (27)	1.47 ± 0.62 (30)	4.93 ± 0.94
D_{F1}	0.19 ± 0.03 (4)	0.11 ± 0.02 (3)	0.33 ± 0.05 (8)	0.39 ± 0.08 (9)	0.83 ± 0.13 (20)	1.85 ± 0.24	1.21 ± 0.30 (29)	1.14 ± 0.35 (27)	4.19 ± 0.70
H_{F1}	0.33 ± 0.04 (5)	0.18 ± 0.01 (3)	0.64 ± 0.06 (9)	0.39 ± 0.02 (6)	1.29 ± 0.12 (18)	2.84 ± 0.20	2.10 ± 0.19 (30)	2.06 ± 0.32 (29)	6.99 ± 0.58

The roots of the three triple transgenic F_1 lines accumulated more β-carotene (means of 1.3, 1.2 and 2.1 μg g^{-1} DWt; Table 4.3.2) than the double transgenic G29 line (mean of 0.9 μg g^{-1} DWt) and the WT Tm2a line (mean of 0.6 μg g^{-1} DWt). The F_1 progeny had much lower root β-carotene contents than those found in the parental MJ8 lines (Table 4.3.1.) which is likely to be at least in part due to the presence of additional amounts of BCH2 enzyme in the triple transgenic F_1 progeny. These F_1 plants would be

expected to have used more of the β-carotene substrate than the MJ8 parental lines, which lack the additional BCH2 enzyme. Due to the lower lycopene and β-carotene content the total amount of carotenoids found in A_{F1}, D_{F1} and H_{F1} plants (Table 4.3.2; means of 5μg g^{-1} DWt, 4 μg g^{-1} DWt and 7 μg g^{-1} DWt respectively) was less than that found in the parental (Table 4.3.1) MJ8A (16 μg g^{-1} DWt), MJ8D (27 μg g^{-1} DWt) and MJ8H plants (13 μg g^{-1} DWt). Nevertheless, the total carotenoid concentrations in the triple transgenic F_1 progeny were still greater than the total carotenoids found in both G29, with a mean of 2.2 μg g^{-1} DWt, and the Tm2a line which was found to accumulate 2.1 μg g^{-1} DWt total root carotenoids (Table 4.3.2).

In the same way that increases in root lycopene and β-carotene composition reduced the percentage of lutein in MJ8 plant roots to around 1% (Table 4.3.1) the percentage of lutein in the F_1 plants (Table 4.3.2; A_{F1} = 8%, D_{F1} = 4% and H_{F1} = 5%) was also much lower than the 14% lutein found in both G29 and Tm2a roots (Table 4.3.2). This apparent reduction in precursor flux through the α-branch in the carotenoid biosynthesis pathway, which terminates in lutein, to increased flux through the β-branch of the pathway leading to ABA (in the triple transgene F_1 plants compared to MJ8 plants) may be due to feedback regulation responding to increased use of substrate by additional BCH2 enzyme activity in these F_1 plants.

Another way in which the root carotenoid profiles of the F_1 progeny differed from those of the MJ8 parental lines was that they had a greater concentration of total root xanthophylls (Table 4.3.2). This increase was mainly due to an increase in the neoxanthin isomers (especially all-*trans*-neoxanthin) which accumulated to mean amounts of 1.2 μg g^{-1} DWt, in A_{F1} and D_{F1} and 1.7 μg g^{-1} DWt in H_{F1} plant roots, while MJ8A, MJ8D and MJ8H plants respectively accumulated only 0.16, 0.25 and 0.07 μg g^{-1} DWt of the neoxanthin isomers. This may be due to the aforementioned increased flux through the β-branch of the carotenoid biosynthesis pathway leading towards ABA.

The triple transgenic F_1 plants showed a considerable variation in plant height, as can be seen in Figure 4.3.4 where the mean internode lengths ranged from 3 cm to 9 cm. The mean internode length was plotted against the root lycopene content in the sand grown cuttings taken from the same plants; the regression analysis indicated that the mean internode length of the F_1 plants was not correlated with the amount of root lycopene (Figure 4.3.4). This would appear to indicate that plants of dwarfed stature do not have to be selected in order to achieve maximum root lycopene accumulation. Additionally, the triple transgenic F_1 plants displayed varying degrees of leaf chlorosis (Figure 4.3.5) due to reduced chlorophyll content as previously described for plants overexpressing *PSY1* (Fray *et al.*, 1995).

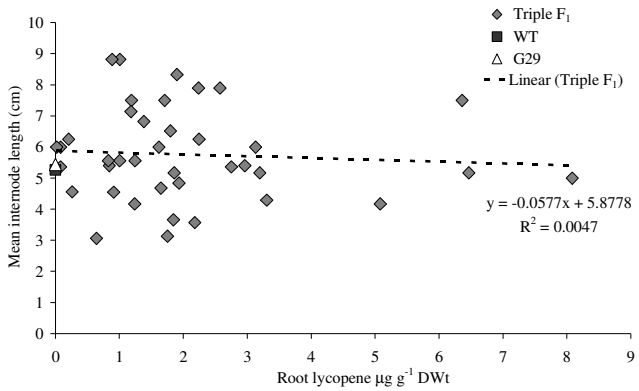

Figure 4.3.4 The relationship between the mean internode length (cm) and lycopene accumulation (µg g^{-1} DWt). The mean internode length of triple transgenic F_1 progeny (diamond); WT, Tm2a line (square); and the transgenic G29 line (triangle), taken at 1.5 m overall plant height, is plotted against the root lycopene concentration of sand grown cuttings taken from the same plants. The dashed line is the regression line, the equation of the line and R^2 value is given.

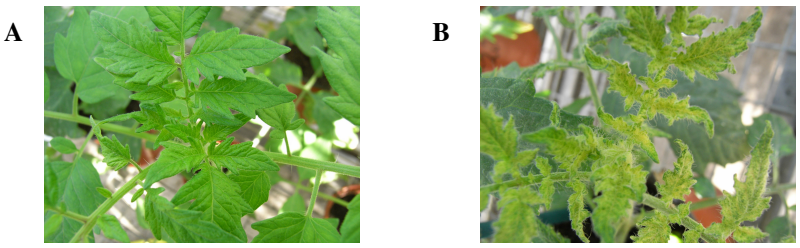

Figure 4.3.5 Photographs showing chlorosis of the shoot apex. **A;** WT, Tm2a line showing healthy green leaves; **B**; Triple transgenic F_1 plant, showing an extreme example of leaf chlorosis.

The early pigmentation of immature fruit phenotype mentioned previously, which resulted in the immature fruit of MJ8 plants being a pale peach colour (Figure 4.3.2), was not observed in the triple F_1 plants. This may be due to the lack of homozygosity of any of the T-DNA insertions for the *35S::PSY1* construct. The ripe fruit of the triple F_1 lines matured to orange rather than the typical, WT Tm2a red colour at full ripeness (Figure 4.3.6). This ripe fruit phenotype (i.e. orange rather than red) has been previously associated with the constitutive overexpression of the *35S::SlCrtRb2/BCH2* construct (Balasubramanian, 2007).

A  **B** 

Figure 4.3.6 Photographs illustrating the difference in mature fruit colour: **A**; WT, Tm2a red coloured ripe fruit; **B**, Triple transgenic F_1 orange coloured ripe fruit.

Five individual H_{F1} plants: H-15_{F1}, H-16_{F1}, H-18_{F1}, H-20_{F1} and H-22_{F1}, which represented a range of root lycopene concentrations and plant internode lengths (Table 4.3.3) were selected to take forward to the F_2 generation.

Table 4.3.3 The five triple transgenic F_1 plants chosen to take forward to the F_2 generation. The mean internode length and root lycopene concentration of sand grown cuttings taken from the individual H-15_{F1}, H-16_{F1}, H-18_{F1}, H-20_{F1} and H-22_{F1} plants.

TRIPLE F_1 PLANT	MEAN INTERNODE LENGTH (cm)	ROOT LYCOPENE CONCENTRATION (µg g^{-1} DWt)
H-15_{F1}	5.6	1.2
H-16_{F1}	5.2	3.2
H-18_{F1}	7.9	2.2
H-20_{F1}	4.3	3.3
H-22_{F1}	7.9	2.6

4.3.4 The Triple Transgenic Line Containing Three Constructs – The F_2 Generation

The triple transgenic F_2 generation of plants were segregating for both the *35S::SlCrtRb2/BCH2* T-DNA insertion locus derived from the single construct transgenic line known as BCH12, and one or more *35S::SlPSY1* T-DNA insertion loci derived from the Z171D4A line (Fray *et al.*, 1995). As a result of this segregation not all of the roots of the triple transgenic F_2 progeny accumulated lycopene (Figure 4.3.7), and those that did accumulated it to varying degrees, ranging from 0.05 μg g^{-1} DWt to 21.9 μg g^{-1}DWt. The root β-carotene content (Figure 4.3.7) also varied greatly, ranging from 0.09 μg g^{-1} DWt to 59.49 μg g^{-1} DWt.

The early pigmentation of the immature fruit phenotype (Figure 4.3.2) was observed again in some plants of this F_2 generation. Some of the immature fruit showed varying degrees of pigmentation, appearing yellow or very pale green, sometimes almost white in colour. Additionally, the fruit of the triple transgenic F_2 plants matured to either red or varying degrees of orange (Figure 4.3.6). These perturbations in fruit pigmentation are presumably dependent on the combined zygosity of each individual plant for the *SlBCH2* and *SlPSY1* T-DNA insertions.

Nine F_2 plants were selected to take forward to the F_3 generation based on both fruit colour phenotype and the root lycopene and β-carotene data. Selected plants had fruit that matured to an orange colour, which was associated with the presence of the *SlBCH2* transgene and immature fruit that was peach coloured (as opposed to, green or any of the other variations of pigmentation) which was associated with the *SlPSY1* transgene. From the F_2 plants with these fruit phenotypes nine individual F_2 plants were selected which represented a range of root lycopene concentrations from 0.32 μg g^{-1} DWt to 18.23 μg g^{-1} DWt. The mean concentrations and percentage compositions of the main

xanthophylls and other carotenoids of the nine individual F_2 plants that were selected to take through to the next generation are presented in Table 4.3.4.

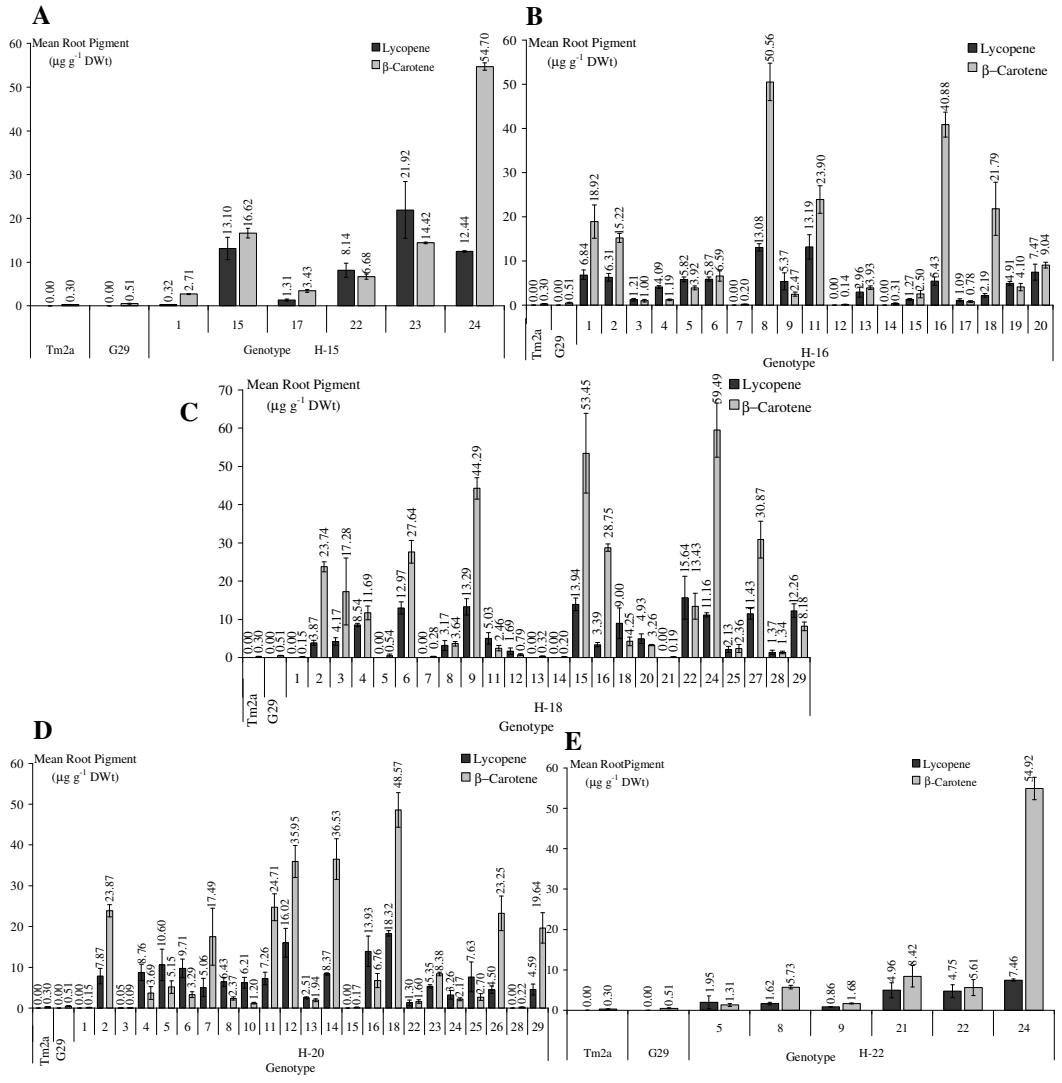

Figure 4.3.7 The accumulation of lycopene and β-carotene (µg g $^{-1}$ DWt) in the roots of sand grown cuttings of: WT, Tm2a line; G29 double construct transgenic line constitutively overexpressing *SlBCH2* and *SlNCED*; The F_2 progeny of **A,** H-15; **B**, H-16; **C,** H-18; **D**, H-20; **E** H-22 (n = 3 ± S.E.).

Table 4.3.4 displays the full array of the root carotenoids analysed from cuttings taken from the selected F_2 plants for completeness; an ANOVA was performed but *post hoc* test differences are not displayed to prevent over complicating an already full table of quite variable lines. One of the main points to note is that the selected triple transgenic F_2 plants contained at least 1.6x (H-15$_{F2}$) and at most 29x (H-18-15$_{F2}$) more

total carotenoids than the G29 transgenic line, mainly due to the previously mentioned increases in lycopene and β-carotene.

Table 4.3.4 The root carotenoid content (μg g^{-1} DWt) and percentage composition in the sand grown cuttings of: the WT, Tm2a line; the G29 transgenic line; and nine individual triple transgenic F_2 plants (n=3 ± S.E.).

LINE	XANTHOPHYLL CONTENT(μg g-1 DWt) (% OF TOTAL CAROTENOIDS ANALYSED)						OTHER CAROTENOIDS (μg g-1 DWt) (% OF TOTAL CAROTENOID CONTENT ANALYSED)		
	Lutein	Zeaxanthin	Violaxanthin	9′-*cis*-neoxanthin	All-*trans*-neoxanthin	Total	β-carotene	Lycopene	Total
Tm2a	0.25 ± 0.14 (17)	0.09 ± 0.03 (7)	0.21 ± 0.07 (14)	0.20 ± 0.02 (13)	0.40 ± 0.08 (28)	1.16 ± 0.29	0.30 ±0.02 (20)	0.00 ± 0.00 (0)	1.45 ± 0.30
G29	0.28 ± 0.12 (12)	0.09 ± 0.01 (4)	0.29 ± 0.07 (12)	0.29 ± 0.05 (13)	0.85 ± 0.17 (37)	1.81 ± 0.19	0.51 ± 0.05 (22)	0.00 ± 0.00 (0)	2.32 ± 0.12
H-15-1_{F2}	0.35 ± 0.06 (9)	0.24 ± 0.05 (7)	0.03 ± 0.01 (1)	0.08 ± 0.03 (2)	0.03 ± 0.003 (1)	0.72 ± 0.13	2.72 ± 0.09 (72)	0.32 ± 0.01 (8)	3.76 ± 0.23
H-15-22_{F2}	0.41 ± 0.01 (3)	0.21 ± 0.06 (1)	0.1 ± 0.01 (1)	0.14 ± 0.018 (1)	0.18 ± 0.05 (1)	1.04 ± 0.01	6.68 ± 0.6 (42)	8.14 ± 1.62 (51)	15.86 ± 1.95
H-16-2_{F2}	0.31 ± 0.05 (1)	0.22 ± 0.003 (1)	1.26 ± 0.06 (5)	0.32 ± 0.03 (1)	2.21 ± 0.1 (9)	4.31 ± 0.12	15.22 ± 1.02 (59)	6.31 ± 0.87 (24)	25.84 ± 0.29
H-18-15_{F2}	0.25 ± 0.1 (<1)	0.15 ± 0.2 (<1)	0.35 ± 0.06 (1)	0.12 ± 0.02 (<0)	0.79 ± 0.16 (1)	1.66 ± 0.34	53.45 ± 10.4 (77)	13.94 ± 1.62 (20)	69.04 ± 11.7
H-20-12_{F2}	0.16 ±0.02 (<1)	0.17 ± 0.33 (<1)	0.18 ± 0.04 (<1)	0.08 ± 0.01 (<1)	0.39 ± 0.08 (1)	0.96 ± 0.18	35.95 ± 3.93 (68)	16.01 ± 3.49 (30)	52.93 ± 7.2
H-20-18_{F2}	0.23 ± 0.11 (<1)	0.21 ± 0.12 (<1)	0.06 ± 0.04 (<1)	0.15 ± 0.04 (<1)	0.58 ± 0.06 (1)	1.46 ± 0.02	48.57 ± 4.25 (71)	18.32 ± 0.65 (27)	68.36 ± 4.83
H-22-8_{F2}	0.14 ± 0.03 (2)	0.22 ± 0.06 (2)	1.13 ± 0.22 (11)	0.28 ± 0.06 (3)	1.00 ± 0.32 (10)	2.87 ± 0.52	5.73 ± 0.45 (56)	1.62 ± 0.32 (16)	10.22 ± 0.71
H-22-22_{F2}	0.16 ± 0.01 (1)	0.19 ± 0.01 (1)	1.70 ± 0.51 (12)	0.21 ± 0.06 (2)	1.1 ± 0.38 (8)	3.35 ± 0.94	5.61 ± 1.97 (41)	4.75 ± 1.57 (35)	13.7 ± 2.64
H-22-24_{F2}	0.32 ± 0.05 (<1)	0.18 ± 0.01 (<1)	0.62 ± 0.06 (1)	0.18 0.01 (<1)	1.17 ± 0.08 (2)	2.45 ± 0.20	54.92 ± 2.79 (85)	7.46 ± 0.27 (12)	64.84 ± 3.23
P	0.520	0.053	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001

This increase in root carotenoid accumulation, in some F_2 plants resulted in these roots appearing more orange (Figure 4.3.8) compared to the roots of other genotypes. Another notable finding from the data displayed in Table 4.3.4 is that the amount of total xanthophylls analysed in the triple transgenic roots varies from 0.72 µg g^{-1} DWt in plant H-15-1_{F2} to 4.31 µg g^{-1} DWt in plant H-16-2_{F2}, this range includes the total xanthophyll content found in both the WT, Tm2a line (1.16 µg g^{-1} DWt) and the transgenic G29 line (1.81 µg g^{-1} DWt).

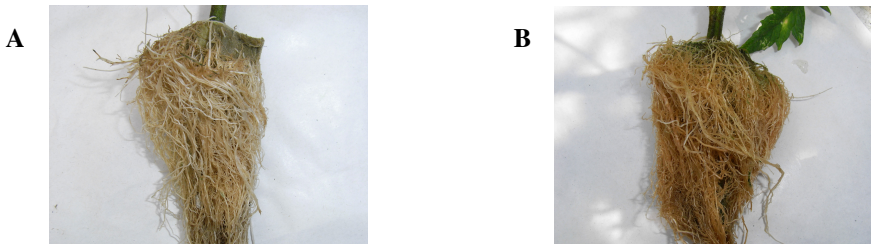

Figure 4.3.8 Photographs of the washed roots of sand grown cuttings illustrating the difference in root pigmentation: **A**, WT, Tm2a line typically coloured roots; **B**, an example of triple F_2 plant showing the more orange appearance due to increased pigmentation.

Additionally, the triple F_2 plants also displayed a range of the other 'typical' PSY1 overexpressor traits; including varying severities of leaf chlorosis and shorter internodes. The mean internode lengths of the F_2 progeny of the chosen F_1 lines is shown in Figure 4.3.9, the parental F_1 individual mean internode lengths are also given. This demonstrated that although the triple transgenic F_2 progeny had significantly shorter internodes than both the WT ($P<0.01$) and transgenic G29 ($P<0.01$), there was no difference ($P>0.05$) in mean internode length between the triple lines. This (Figure 4.3.9) also demonstrated that the dwarfed internode length phenotype was not directly heritable in a simple way, since the parental F_1 internodes did not appear to influence the mean F_2 internode lengths.

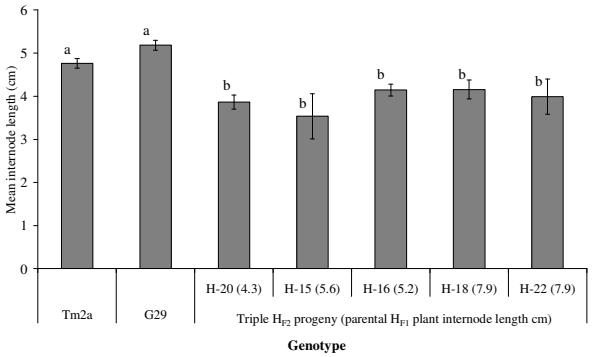

Figure 4.3.9 The mean internode length (cm) of the: WT, Tm2a line; G29 transgenic line; triple transgenic F_2 selfed progeny of the H-20$_{F1}$, H-15$_{F1}$, H-16$_{F1}$, H-18$_{F1}$ and H-22$_{F1}$ plants taken at 1 m overall height The mean internode length of the individual parental triple F_1 transgenic plant is given in brackets, and presented in order, shortest to tallest. (n = 20, 20, 22, 4, 18, 24, 5 ± S.E. respectively) Bars marked with a different letter are significantly different (P<0.01) from each other as determined by LSD *post hoc* test.

At this point the F_2 population was screened for zygosity of the *35S::SlCrtRb2/BCH2* transgene and the *35S::SlPSY1* transgene using quantitative real time PCR (qPCR). Two sets of primer pairs and associated TaqMan probes were available from A.J. Thompson (personal communication). One pair of primers (BCH2_For and BCH2_Rev Appendix III) and TaqMan probe (BCH2 Taq Probe Appendix III, technical details in section 4.2.12) was used for detection of the *35S::SlCrtRb2/BCH2* transgene, and the other pair of primers (VI_For and VI_Rev, Appendix III) and TaqMan probe (VI TaqMan Probe Appendix III, technical details in section 4.2.12), was used for detection of tomato vacuolar invertase (VI) (Elliot *et al.*, 1993) as an endogenous reference gene. A third primer pair (PSY1_For and PSY1_Rev) and TaqMan probe (PSY1 Taq probe) were designed using Primer express v1.5 software (Applied Biosystems, CA, USA) to detect the *35S::SlPSY1* transgene using the sequence from the pBDH5ST transformation vector (R.G. Fray, personal communication; Fray and Grierson, 1993) which was used to transform the transgenic *SlPSY1* overexpressing parental ZI7ID4 line (Fray *et al.*, 1995). A schematic diagram of the primer pair (Appendix III) and TaqMan probe (Appendix III, technical details in section 4.2.12) along with part of the pBDH5ST vector sequence are presented in Figure 4.3.10.

```
GACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATGTGGAAAAAGAAGACGTTCCAACCACGT
                                                                >>>>>
CTTCAAAGCAAGTGGATTGATGTGATATCTCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTC
>>>>>>>>>>>>>>>    ******************************
GCAAGACCCTTCCTCTATATAGGAAGTTCATTTCATTTGGAGAGGACAGGGTACCCGGgqatccccatt

CTCTAGTGGGAATCTACTAGGAGTAATTTATTTTCTATAAACTAAGTAAAGTTTGGAAGGTGACAAAAA

GAAAGACAAAAATCTTGGAATTGTTTTAGACAACCAAGGTTTTCTTGCTCAGAATGTCTGTTGCCTTGT
                                              <<<<<<<<<<<<<<<<<<<<
TATGGGTTGTTTCTCCTTGTGACGTCTCAAATGGGACAAGTTTCATGGAATCAGTCCGGGAGGGAAACC

GTTTTTTTGATTCATCGAGGCATAGGAATTTGGTGTCCAATGAGAGAATCAATAGAGGTGGTGGAAAGC
```

Figure 4.3.10 A schematic diagram of the qPCR PSY1 primer pair and TaqMan probe used to detect the presence of the *35S::SlPSY1* transgene. The sequence is a fragment of the pBDH5ST transformation vector: Italicised sequence, part of the cauliflower mosaic virus 35S promoter (CaMV35S); underlined sequence, multiple cloning sites from the pDH5S vector (uppercase) and the pT7T3TOM5 vector (lowercase), obtained from R.G. Fray (personal communication). The 20 bp forward (>>>) and reverse (<<<) primers were designed to amplify a 272 bp PCR product, which included part of the CaMV35S promoter, to avoid amplifying the endogenous *PSY1* transgene. The 32 bp TaqMan probe (***) was designed to target part of the sequence between the primers.

The zygosity of the F_2 plants that had not died prematurely, due to TMV infection or other causes, was determined using the threshold cycle values obtained from qPCR (as described in section 4.2.12) in Equation 5 (section 4.2.12) as described in German *et al*., (2003). The results for the *SlBCH2* transgene are presented in ranked order in Figure 4.3.11. Ranking the values in this way (Figure 4.3.11) allowed the F_2 plants to be divided, generally into three genotypes: azygous (++) for the transgene which have no value, as there was no transgene present to amplify; heterozygous (B+) for the transgene, which group around the value one; and homozygous (BB) for the transgene, which group around the value two, which is consistent with the ranking values presented in German *et al.,* (2003). The F_2 progeny segregated in an expected 1:2:1 Mendelian F_2 ratio χ^2 (d.f. = 2, n = 71) = 2.52, P>0.05 (N.B. the results were grouped into two in order to perform the test).

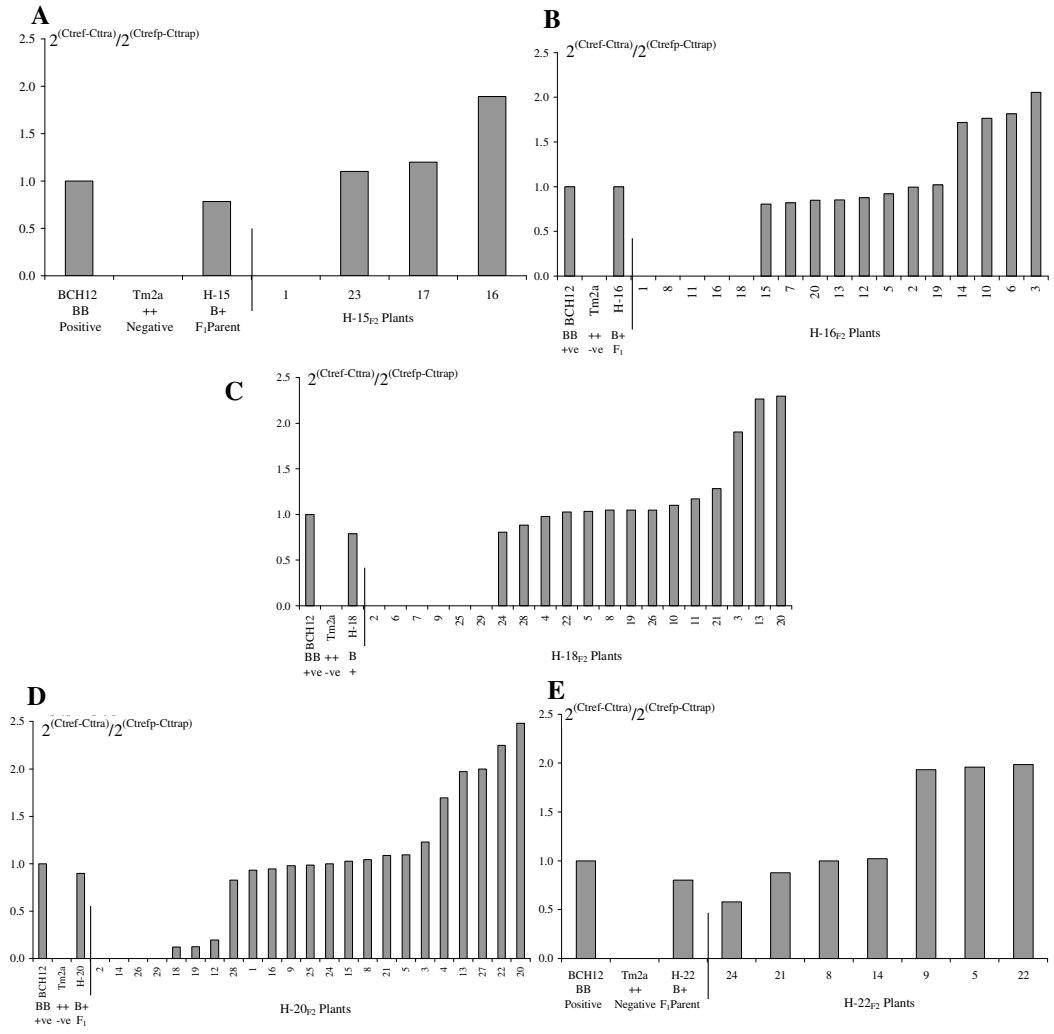

Figure 4.3.11 Estimation of *35S::SlCrtRB2/BCH2* zygosity in the F_2 progeny of the triple transgenic F_1 plants. The BCH12 line, homozygous for the transgene (BB) was used as a positive control (+ve) the $2^{(Ctref-Cttra)}$ value was divided by itself to give one. The Tm2a line was used as an untransformed azygous (++) negative control (-ve). The triple F_1 plants were known to be heterozygous (B+) for the transgene because the transgene donor parent, G29 was BB. In this case BCH12 was used as the parental $2^{(Ctrefp-Cttrap)}$ value. These are plotted with the zygosity values for the F_2 progeny of five F_1 plants: **A**, H-15_{F1}; **B**, H-16_{F1}; **C**, H-18_{F1}; **D**, H-20_{F1}; **E**, H-22_{F1}. Ctref and Ctra are the critical threshold cycles of the endogenous reference gene and the transgene; Ctrefp and Cttrap are the threshold cycles of the reference and transgenes in the parental plant.

The results of the estimated zygosity of the F_2 plants for the *SlPSY1* transgene are presented in ranked order in Figure 4.3.12. The F_2 plants would have been segregating for an unknown number of T-DNA insertions, because the parental MJ8 transgene donor was potentially segregating for a number of different T-DNA insertions. This ranked data could be divided, albeit not as distinctly as the previous set, into plants

that were azygous (++) for the transgene which have no value, as there was no transgene present to amplify; heterozygous (P+) for the transgene, which group around the value one; and homozygous (PP) for the transgene, which group around the value two, which is consistent (as previously mentioned) with the ranking values presented in German *et al.,* (2003).

These two sets of data were combined and ranked, first for *SlBCH2* zygosity values and then for the *SlPSY1* values. This combined ranking for seven of the nine previously chosen F_2 plants is presented in Table 4.3.5, plants H-15-22_{F2} and H-18-15_{F2} were not included because these plants had died before samples for DNA extraction were collected. From Table 4.3.5 it can be seen that each of the previously phenotype selected F_2 plants were classified as homozygous for the *SlPSY1* T-DNA insertion loci present, the zygosity of the *SlBCH2* transgene on the other hand ranged from azygous (H-15-1) to homozygous (H-22-22).

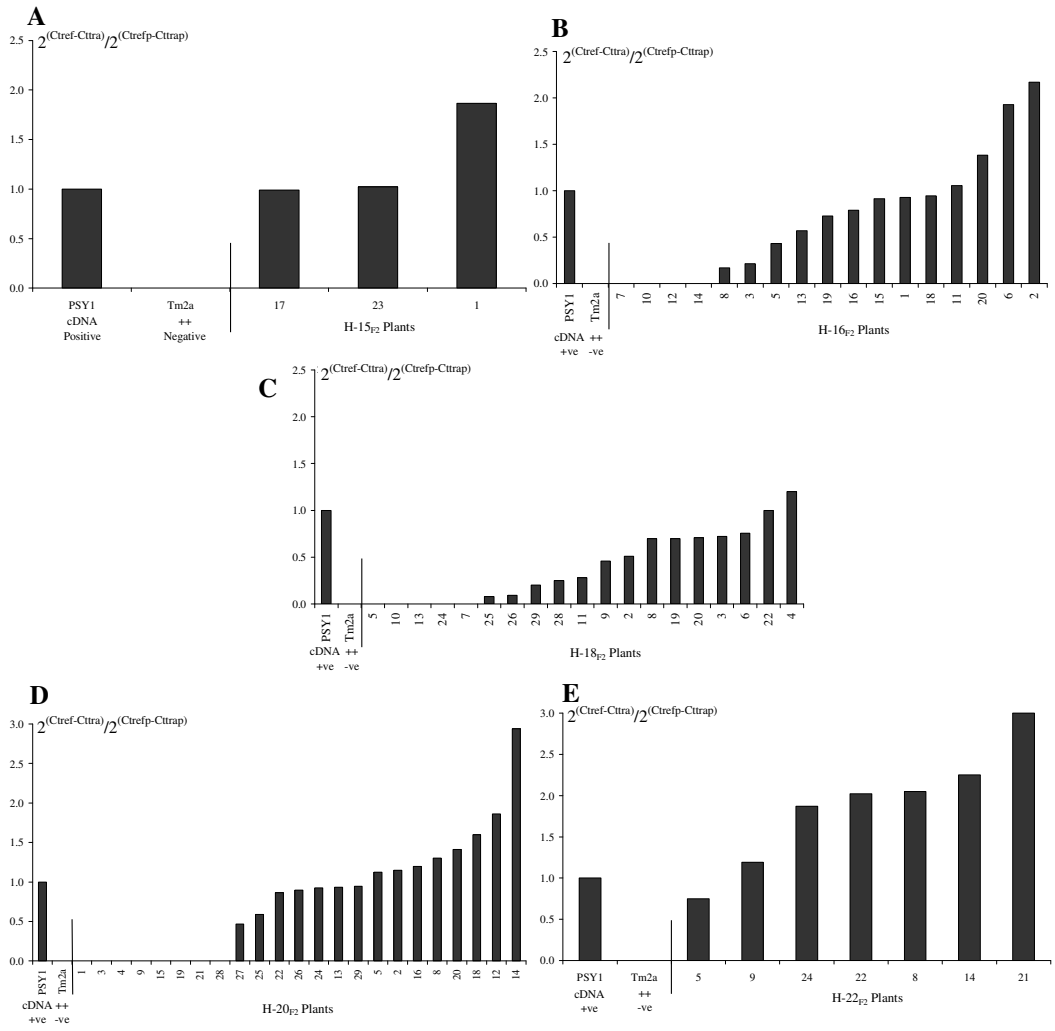

Figure 4.3.12 Estimation of *35S::SlPSY1* zygosity of the F_2 progeny of triple transgenic F_1 plants. *PSY1* cDNA derived from the transgene construct (courtesy of R.G. Fray) was used as a positive control (+ve) the $2^{(Ctref-Cttra)}$ value was divided by itself to give one. The Tm2a line was used as an untransformed azygous (++) negative control (-ve). The F_1 plants were segregating for an unknown number of copies of the transgene (P?) because the transgene parent, MJ8 was (P?). These are plotted with the zygosity values for the F_2 progeny of five F_1 plants: **A**, H-15_{F1}; **B**, H-16_{F1}; **C**, H-18_{F1}; **D**, H-20_{F1}; **E**, H-22_{F1}. Ctref and Ctra are the critical threshold cycles of the endogenous reference gene and the transgene; Ctrefp and Cttrap are the threshold cycles of the reference and transgenes in the parental plant.

Table 4.3.5 The estimated *SlBCH2* and *SlPSY1* zygosity of seven individual H_{F2} plants. The plants have been ranked first for *SlBCH2* and then for *SlPSY1* zygosity; from 0, azygous to 2, homozygous. Ctref and Ctra are the critical threshold cycles of the endogenous reference gene and the transgene; Ctrefp and Cttrap are the threshold cycles of the reference and transgenes in the heterozygous parental plant.

F_2 PLANT	*SlBCH2* $(2^{(Ctref-Ctra)}/2^{(Ctrefp-Ctrap)})$	*SlPSY1* $(2^{(Ctref-Ctra)}/2^{(Ctrefp-Ctrap)})$
H-15-1	0	1.9
H-20-18	0.1	1.6
H-20-12	0.2	1.9
H-22-24	0.6	1.9
H-22-8	1	2.1
H-16-2	1	2.2
H-22-22	2	2

4.3.5 The Triple Transgenic Line Containing Three Constructs – The F_3 Generation

The germination rates of the triple F_3 seed of the nine chosen F_2 plants following norflurazon treatment is shown in Figure 4.3.13, where it can be seen that four of the lines (H-15-22, H-16-2, H-22-8, and H-22-22) started to germinate after two days of imbibition on 1 mg g^{-1} norflurazon, but only three (H-15-22, H-16-2, H-15-1) had achieved 50% germination after four days of imbibition. Seven of the nine lines had achieved 80% germination after six days of imbibition. The H-20-12 and H-22-22 seeds achieved only 66% and 70% germination respectively after ten days of imbibition.

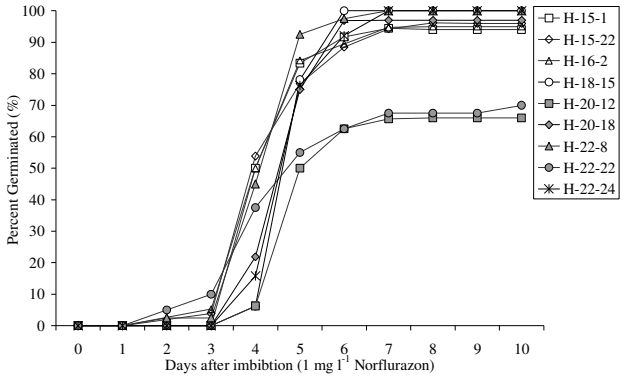

Figure 4.3.13 The germination rate (%) of the transgenic F_3 seed of the nine selected H_{F2} plants. The seeds were imbibed with 1 mg l^{-1} norflurazon solution until germination, i.e. the radicle tip was visibly emerging from the testa. n = 36, 52, 38, 32, 32, 32, 32, 40, 40, 38 from top to bottom in the key.

Three Triple F_3 lines (H-22-8, H-22-22 and H-22-24) were chosen from the nine lines previously selected and sown, with the intention of eventually selecting just one line. The six lines that were not chosen were discarded because they were either: azygous for the *BCH2* transgene, as was the case for the H-15-1_{F3} progeny; or indicated that they were TMV susceptible, as was the case for the H-20-12_{F3} and H-20-18_{F3} progeny; or due to the lack of genotyping data, as was the case for the H-15-22_{F3} and H-18-15_{F3} progeny. The final three F_3 lines were selected from the remaining four because under glasshouse assessment each plant within a line appeared similar, indicating that the various phenotypic traits were becoming heritably stable and the plants appeared relatively TMV free. It is interesting to note that the final three lines were each derived from the same parental H-22_{F1} plant.

The mean internode lengths of the three selected H-22-8_{F3}, H-22-22_{F3} and H-22-24_{F3} plants are presented in Figure 4.3.14, along with the parental F_2 individual mean internode lengths. The double transgenic line G29 was found to have the tallest ($P<0.01$) mean internode length (5.1 cm), the triple transgenic F_3 progeny H-22-22_{F2} plant was found to have the shortest mean internode length of 4.21 cm, which was significantly ($P<0.01$) shorter than G29, Tm2a (mean of 4.7 cm), and the F_3 progeny of the H-22-8_{F2} plant (mean of 4.7 cm). The triple transgenic F_3 progeny of the H-22-24_{F2} plant, with a mean internode length of 4.5 cm was only significantly shorter than G29.

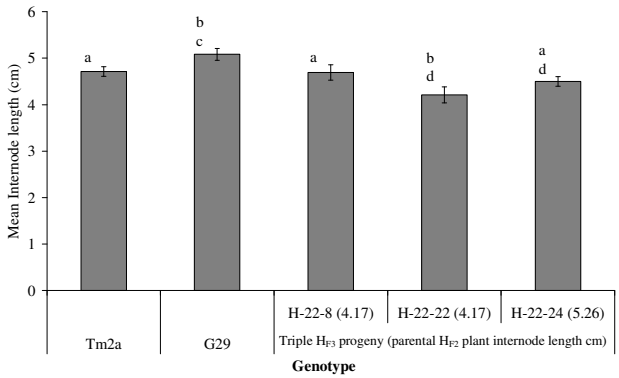

Figure 4.3.14 The mean internode length (cm) of the: WT, Tm2a line; G29 transgenic line; triple transgenic F_3 selfed progeny of the H-22-8_{F2}, H-22-22_{F2}, and H-22-24_{F2} plants taken at 1 m overall height The mean internode length of the parental triple F_2 transgenic plant (in brackets) and the lines presented in internode length order, shortest to tallest. (n = 16, 16, 14, 14, 16 respectively ± S.E.) Bars marked with a different letter are significantly different (P<0.01) from each other as determined by LSD *post hoc* test.

The F_3 generation displayed the previously mentioned *SlPSY1* overexpressor traits of shoot chlorosis (Figure 4.3.5), early pigmentation of immature fruit (Figure 4.3.2) and mature fruit that ripened to orange (Figure 4.3.6). It should be noted at this point that the 'PSY1' phenotype appeared relatively stable amongst the F_3 sample of plants. This lack of apparent segregation for the *SlPSY1* transgene further indicated that their F_2 parent had been homozygous for this insertion locus. At this point the F_3 plants were in turn subjected to qPCR genotyping for both the *35S::SlCrtRb2/BCH2* construct (Figure 4.3.) and the *35S::SlPSY1* construct (Figure 4.3.16). It can be seen in Figure 4.3., as found previously in section 4.3.5, that the H-22-8_{F3} and H-22-24_{F3} progeny could be divided, generally, into three genotypes: azygous (++) for the transgene which have no value, as there was no transgene present to amplify; heterozygous (B+) for the transgene, which group around the value one; and homozygous (BB) for the transgene, which group around the value two, which is consistent with the ranking values presented in German *et al.,* (2003). Since the H-22-22_{F2} parent was previously found to be homozygous (Table 4.3.5) for the *SlBCH2* transgene, the progeny were expected to be homozygous and therefore group around the value one. There were not enough samples to perform a χ^2 test on lines H-22-8_{F3} and H-22-$24_{F3,}$ but the segregation ratios appeared to be skewed

towards the azygous, with only one homozygous plant being identified from both parental lines. The F_2 plants were previously found to be homozygous (Table 4.3.5) for at least one *SlPSY1* transgene insertion locus, therefore (as previously explained) each of the H-22-22$_{F3}$ progeny were expected to be homozygous and the zygosity values group around the value one as seen in Figure 4.3.16.

Three individual triple transgenic F_3 plants, H-22-8-8$_{F3}$, H-22-22-6$_{F3}$ and H-22-22-7$_{F3}$ were selected from the F_3 plants, for being homozygous for both transgenes and showing TMV resistance, to be selfed and taken through to the F_4 generation.

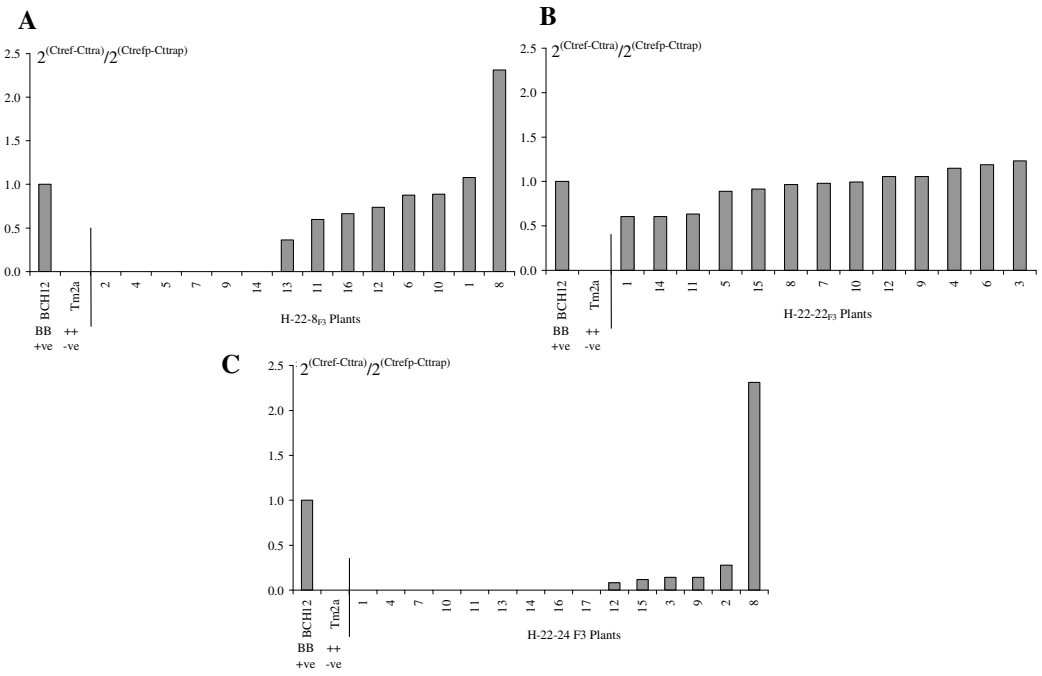

Figure 4.3.15 Estimation of *35S::SlCrtRb2/BCH2* zygosity in the F_3 progeny of the triple transgenic H_{F2} plants. The BCH12 line, homozygous for the transgene (BB) was used as a positive control (+ve) the $2^{(Ctref-Cttra)}$ value was divided by itself to give one. The Tm2a line was used as an untransformed azygous (++) negative control (-ve). These are plotted along with the zygosity values of the F_3 progeny of three previously genotypes H_{F2} plants: **A**, H-22-8$_{F2}$ previously estimated as heterozygous (B+); **B**, H-22-22$_{F2}$ previously estimated as BB therefore the results are expected to group around the value one; **C**, H-22-24$_{F2}$ previously identified as B+. Ctref and Ctra are the critical threshold cycles of the endogenous reference gene and the transgene; Ctrefp and Cttrap are the threshold cycles of the reference and transgenes in the parental plant.

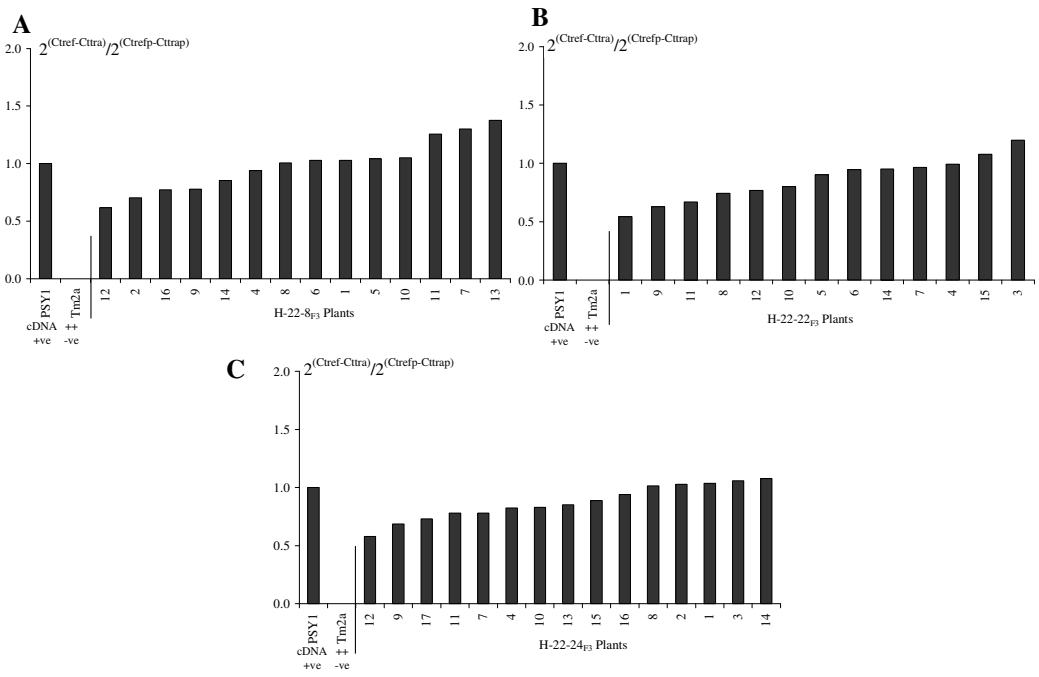

Figure 4.3.16 Estimation of *35S::SlPSY1* zygosity in the F_3 progeny of the triple transgenic H_{F2} plants: *PSY1* cDNA derived from the transgene construct (courtesy of R.G. Fray) was used as a positive control (+ve) the $2^{(Ctref-Cttra)}$ value was divided against itself to give one. The Tm2a line was used as an untransformed azygous (++) negative control (-ve). These are plotted with the zygosity values of the F_3 progeny of three previously estimated as homozygous (PP; for an unknown number of T-DNA insertions of the *35S::SlPSY1* construct) F_2 plants: **A**, H-22-8_{F2}; **B**, H-22-22_{F2}; **C**, H-22-24_{F2}. Ctref and Ctra are the critical threshold cycles of the endogenous reference gene and the transgene; Ctrefp and Cttrap are the threshold cycles of the reference and transgenes in the parental plant.

The mean concentrations and percentage compositions of the main xanthophylls and other carotenoids of the three F_3 lines that were selected to take through to the F_4 generation are presented in Table 4.3.6. It can be seen that H-22-8-$8_{F3,}$ H-22-22-6_{F3} and H-22-22-7_{F3} contain significantly ($P<0.01$) greater amounts of the root carotenoids analysed, (means of 20.4 µg g^{-1} DWt, 12.8 µg g^{-1} DWt and 17 µg g^{-1} DWt) compared to both the WT, Tm2a line, with a mean of 1.8 µg g^{-1} DWt and the G29 line, with a mean of 2.2 µg g^{-1} DWt total root carotenoids. As with previous generations this difference was mainly due to increased concentrations of lycopene and β-carotene. Lines H-22-8-8_{F3} and H-22-22-7_{F3} accumulated significantly ($P<0.01$) more β-carotene (means of 9.1 and 6.3 µg g^{-1} DWt respectively) than H-22-22-6_{F3} (mean of 5.2 µg g^{-1} DWt) and

all three triple transgenic lines accumulated much more β-carotene than either G29 or Tm2a (means of 0.8 and 0.6 μg g^{-1} DWt respectively). The Tm2a and G29 lines also had significantly (P<0.05) less root zeaxanthin, both had means of 0.1 μg g^{-1} DWt, than H-22-8-8_{F3} and H-22-22-6_{F3}, which had mean zeaxanthin concentrations of 0.6 and 0.5 μg g^{-1} DWt. H-22-22-7_{F3} contained 0.3 μg g^{-1} DWt zeaxanthin, which was not found to be a significantly different (P>0.05) amount from the other lines.

Compared to the previous generation (Table 4.3.4) the H-22-8-8_{F3} progeny contained 2x more total carotenoids analysed, (20.4 μg g^{-1} DWt) than the parental H-22-8_{F2} plant (10.2 μg g^{-1} DWt). This increase was mainly due to an increase in lycopene concentration from a mean of 1.62 μg g^{-1} DWt in H-22-8_{F2} to a mean of 9 μg g^{-1} DWt in the H-22-8-8_{F3} progeny. The mean total carotenoid content found in the roots of H-22-22-6_{F3} (Table 4.3.6) was similar to the total amount found in the H-22-22_{F2} parent plant (Table 4.3.4) with means of 12.8 and 13.7 μg g^{-1} DWt respectively. The H-22-22-7_{F3} progeny (Table 4.3.6) contained more total carotenoids (17 μg g^{-1} DWt) than the amount found in the H-22-22_{F2} individual parent plant (Table 4.3.4) which had a mean of 13.7 μg g^{-1} DWt total carotenoids analysed, due to an increase in the concentration of β-carotene from 5.61 μg g^{-1} DWt in H-22-22_{F2} to 6.3 μg g^{-1} DWt in the H-22-22-7_{F3} progeny.

Table 4.3.6 The root carotenoid content (μg g^{-1} DWt) and percentage composition in the sand grown cuttings of: WT, Tm2a line; the G29 transgenic line constitutively overexpressing *SlBCH2* and *SlNCED1*; three triple transgenic H-22-8-8_{F3} H-22-22-6_{F3} and H-22-22-7_{F3} lines constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. Values marked with a different letter are significantly (*P<0.05, **P<0.01) different from each other according to LSD *post hoc* test (n = 3 ± S.E.).

LINE	XANTHOPHYLL CONTENT (μg g^{-1} DWt) (% OF TOTAL CAROTENOIDS ANALYSED)						OTHER CAROTENOIDS (μg g^{-1} DWt) (% OF TOTAL CAROTENOID CONTENT ANALYSED)		
	Lutein	Zeaxanthin	Violaxanthin	9′-cis-neoxanthin	All-trans-neoxanthin	Total	β-carotene	Lycopene	Total
Tm2a	0.26 ± 0.02 (14)	0.11^{a*} ± 0.02 (6)	0.15 ± 0.03 (8)	0.23 ± 0.02 (13)	0.48 ± 0.06 (27)	1.23 ± 0.08	0.58^{ac**} ± 0.15 (32)	0.00^{a**} ± 0.00 (0)	1.80^{a} ± 0.21
G29	0.26 ± 0.01 (12)	0.10^{a*} ± 0.02 (4)	0.21 ± 0.04 (9)	0.25 ± 0.03 (11)	0.67 ± 0.14 (30)	1.49 ± 0.23	0.75^{ac**} ± 0.15 (34)	0.00^{a**} ± 0.00 (0)	2.24^{a} ± 0.27
H-22-8-8_{F3}	0.27 ± 0.02 (1)	0.55^{b*} ± 0.13 (3)	0.47 ± 0.10 (2)	0.36 ± 0.11 (2)	0.56 ± 0.19 (3)	2.22 ± 0.20	9.13^{bd**} ± 0.88 (45)	9.02^{b**} ± 1.21 (44)	20.36^{b} ± 1.48
H-22-22-6_{F3}	0.38 ± 0.09 (3)	0.54^{b*} ± 0.13 (4)	0.41 ±0.15 (3)	0.28 ± 0.07 (2)	0.63 ± 0.15 (5)	2.24 ± 0.57	5.19^{bc**} ± 1.74 (41)	5.34^{b**} ± 1.78 (42)	12.78^{b} ± 3.98
H-22-22-7_{F3}	0.24 ± 0.07 (1)	0.30^{ab*} ± 0.05 (2)	0.51 ± 0.20 (3)	0.24 ± 0.08 (1)	0.58 ± 0.23 (3)	1.86 ± 0.62	6.34^{bd**} ± 1.72 (37)	8.81^{b**} ± 2.12 (52)	17.00^{b} ± 4.33
P	0.362	0.017	0.221	0.686	0.934	0.362	0.002	0.001	0.002

4.3.6 The Triple Transgenic Line Containing Three Constructs – The F_4 Generation

The germination rates of the triple transgenic F_4 seed of the three chosen F_3 plants following norflurazon treatment is plotted in Figure 4.3.17, where it can be seen that H-22-22-7_{F4} seed germinated at a faster rate than the other two lines, with 25% of seed germinating three days after imbibition with norflurazon. Over 80% of the seed had germinated (88%) five days after imbibition, and 93% had germinated seven days after imbibition. The seeds from plant H-22-8-8_{F4} had the slowest germination rate, 5% of seeds germinated after three days of norflurazon treatment and 80% had germinated six days after, and a final 93% had germinated eight days after imbibition. Seeds from plant

H-22-22-7_{F3} achieved the lowest germination rate, 89%, starting with 9% germinating three days after imbibition, and over 80% of seed had germinated (84%) after six days of norflurazon treatment.

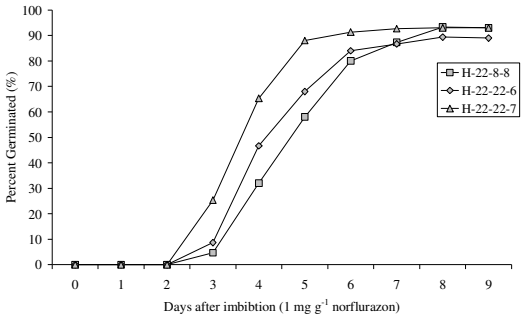

Figure 4.3.17 The germination rate (%) of the triple transgenic F_4 seed of the three selected F_3 plants. The seeds were imbibed with 1 mg l^{-1} norflurazon solution until germination, i.e. the radicle tip was visibly emerging from the testa, n = 150.

A southern analysis (kindly performed by S. Awan at Warwick University) of genomic DNA extracted from one F_4 plant from each of the triple transgenic lines; H-22-8-8-3_{F4}, H-22-22-6-11_{F4} and H-22-22-7-8_{F4}, along with the WT, Tm2a line is displayed in Figure 4.3.18. The presence of the intact transgene (pBDH5ST transformation vector, Fray and Grierson, 1993; Fray *et al.*, 1995) is expected to be indicated by the presence of a 2.25 Kbp band when the DNA is cut with *EcoR*I (R.G. Fray personal communication). This was confirmed using the DNASTAR© Lasergene 8 SeqBuilder programme (DNASTAR Inc., Madison, USA) which revealed two cutting *EcoR*I cutting sites, one at either end of the T-DNA sequence; (the first at sequence position two, resulting in two bands of 1 bp, and 2236 bp in length, the other at position 2238 which result in two bands of 2238 bp and 5 bp in length. This would result in the one visible large band, the smaller fragments would not be expected from plant genomic DNA). It can be seen in Figure 4.3.18 that DNA from each of the three triple transgenic plants, digested with *Eco*RI and probed for *SlPSY1* (889 bp probe prepared from the pT7T3TOM5 vector using the LePSY1_For1 and LePSY11_Rev2 primer pair, Appendix III, Table 7.3.1) resulted in a

hybridising band of ~2 Kb (as expected), which was not present in the untransformed Tm2a control.

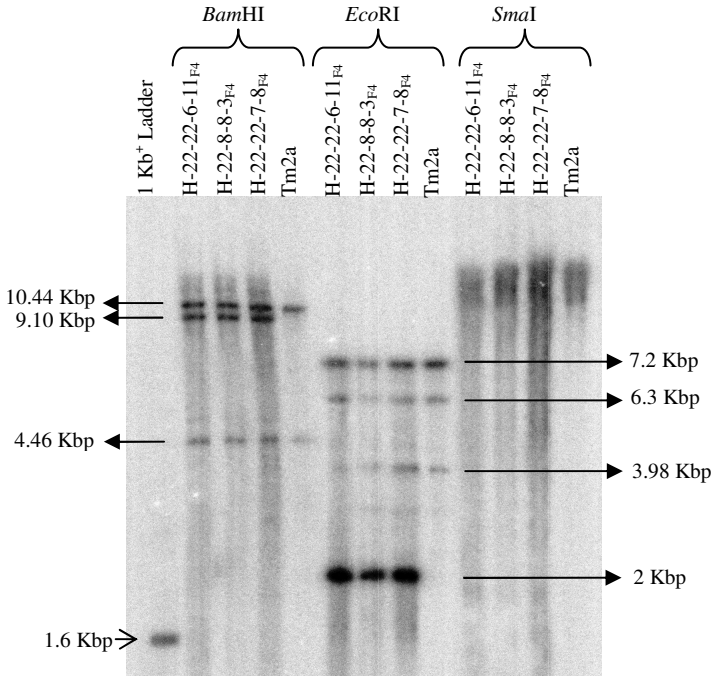

Figure 4.3.18 A Southern blot of genomic DNA from three triple transgenic F_4 plants and the WT, Tm2a line, digested with either, *BamH*I, *Eco*RI, or *Sma*I and probed for *SlPSY1* (889 bp probe prepared from the pT7T3TOM5 vector). Bands present in the triple transgenic plants that are not present in Tm2a are expected to be from the transgene. A 1.6 Kbp marker from the 1Kb+ DNA ladder is visible. Tm2a genomic DNA digested with *Eco*RI resulted in three endogenous bands of 7.2, 6.3 and 3.98 Kbp. The presence of the 2 Kbp band in the DNA from each of the transgenic plants cut with *Eco*RI but not in Tm2a indicates the presence of the intact transgene. Tm2a genomic DNA digested with *BamH*I resulted in two endogenous bands of 10.44 and 4.46 Kbp, the presence of the 9.1 Kbp band in each of the three transgenic plants cut with *BamH*I indicates the presence of the transgene. An indication of the number of transgene loci is given by the number of unique hybridising bands present in the transgenic plants compared to Tm2a. *Sma*I did not completely digest the DNA.

The *BamH*I enzyme is predicted to cut only once within the T-DNA sequence (R.G. Fray personal communication) confirmed by DNASTAR© Lasergene 8 SeqBuilder programme (DNASTAR Inc., Madison, USA) which revealed one *Bam*HI cutting site at sequence position 542, giving two T-DNA fragments of 541 bp and 1701 bp. Therefore, the number of additional bands seen in the transgenic DNA compared to Tm2a DNA gives an indication of the number of T-DNA insertion sites (further confirmation of this

could have been obtained using *Sma*I digestion but the DNA was not completely digested). Genomic DNA from each of the triple transgenic plants had only one additional hybridised band of 9.1 Kbp, compared to the two bands (10.44 and 4.46 Kbp) observed in Tm2a (Figure 4.3.18) which indicated that the three triple transgenic lines contained only one *35S::SlPSY1* T-DNA insertion event.

The F_4 progeny of each line were then tested for their potential susceptibility to TMV infection by assessing plants for the presence/absence of the dominant *Tm-2*^{2} (also known as *Tm-2*^{a}) resistance and recessive *tm-2* susceptible alleles (Lanfermeijer *et al.*, 2003). One PCR assay was designed to identify the susceptible *tm-2* allele (accession number AF536199) and another PCR, together with a subsequent nested PCR were designed to identify the *Tm-2*^{2} resistance allele (accession number AF536201). The two allele sequences were aligned using BLASTn (Zhang *et al.*, 2000) to identify sequence differences and then a sub-section of the sequence, selected for containing a high number of sequence differences, was entered into the Primer3 programme (Rozen and Skaletsky, 2000). A schematic diagram of the three primer pairs (Appendix III, Table 7.3.1): Tm2a_F, Tm2a_Sus_R1 designed to identify the *tm-2* allele; Tm2a_F, Tm2a_Res_R1 designed to identify the *Tm-2*^{2} allele, and the subsequent nested primer pair, Tm2a_Res_NF and Tm2a_Res_NR is shown in Figure 4.3.19.

The results from the PCR assay for the *tm*-2 allele can be seen in Figure 4.3.20. As expected, TMV susceptible *not* mutant DNA resulted in a 274 bp band, whereas the TMV resistant Tm2a line DNA did not produce a band. The individual H-22-8-8$_{F3}$ plant must have been homozygous (TT) for the *Tm-2*^{2} allele, since none of the F_4 progeny were found to be positive for the *tm-2* allele. The H-22-22-6$_{F3}$ and H-22-22-7$_{F3}$ plants on the other hand must have been heterozygous (Tt) since some, but not all, of the plants were found to be positive for the *tm-2* allele. The results of the nested PCR assay for the *Tm-2*^{2} allele can be seen in Figure 4.3.21; as expected the TMV resistant Tm2a line DNA resulted in a 168 bp band whereas the *not* mutant DNA did not. The suggestion

that plant H-22-8-8$_{F3}$ was homozygous (TT) for the *Tm-2*^{2} allele is confirmed in Figure 4.3.21, since all of the F_4 progeny were found to be positive for the *Tm-2*^{2} allele. All but one of the F_4 progeny of the heterozygous (Tt) H-22-22-6$_{F3}$ and H-22-22-7$_{F3}$ plants were positive for the *Tm-2*^{2} allele. The individual genotypes, derived from comparing the results from both PCR assays, of the F_4 plants is summarised in Table 4.3.7. The segregation ratios for the F_4 progeny of the heterozygous H-22-22-6$_{F3}$ and H-22-22-7$_{F3}$ plants were skewed slightly towards the *Tm-2*^{2} allele (sample size too small to perform a χ^2 test).

```
                    Tm2a_F
                    >>>>>>>>>>>>>>>>>>>>
Tm-2²  8281  TTGAACCGAGGTTGTTGCACCGATTGATCAATTTAAGAAAACTGGGTATACTGGGAGTGT  8340
Alignment    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tm-2   2221  TTGAACCGAGGTTGTTGCACCGATTGATCAATTTAAGAAAACTGGGTATACTGGGAGTGT  2280
                                                Tm2a_Res_NF
                                                >>>>>>>>>>>>>>>>>>
Tm-2²  8341  CCAATTCTACCGTTAAGATGTTATCAATATTTAGCCCTGTGCTTAAGGCGCTGGAGGTTC  8400
Alignment    |||||||| |||||||||| |||||||| || | |||||||||  ||||||||  ||||||
tm-2   2281  CCAATTCAACCGTTAAGATATTATCAACATGTCGCCCTGTGCCAAAGGCGCTAAAGGTTC  2340

Tm-2²  8401  TGAAGCTCAG-TTTTTCCAGTGACCCGAGTGAACAAATAAAGTTGTCATCGTATCCACAT  8459
Alignment    |||||||||| |||||| ||||| |||||||| |||||||| |||||||| |||||| |
tm-2   2341  TGAAGCTCAGGTTTTTC-AGTGATCCGAGTGAGCAAATAAACTTGTCATCCTATCCAAAA  2399

Tm-2²  8460  ATTGCTAAGTTGCATTTGAATGTTAACAGAACAATGGCCTTGAACTCTCAATCATTTCCT  8519
Alignment    |||| |||||||||||||||||| |||||||||| |||||||||||| || |||| |||
tm-2   2400  ATTGTTAAGTTGCATTTGAATGTTGACAGAACAATAGCCTTGAACTCTGAAGCATTCCCT  2459
                          Tm2a_Res_NR                    Tm2a_Res_R1
              <<<<<<<<<<<<<<<<<<<<<<<<   <<<<<<<<<<<<<<<<<<<<<<<<<<<<<<
Tm-2²  8520  CCAAATCTCATCAAGCTTACTCTAGCCTACTTTAGTG-TAGACCGTTATATACTGGCAGT  8578
Alignment    |||||| | |||||||||||||||| | || ||||| || ||||| ||| | ||||||||||
tm-2   2460  CCAAATATTATCAAGCTTACTCTTGTCTGCTTTA-TGGTAGACAGTTGTCTACTGGCAGT  2518
                               <<<<<<<<<<<<<<<<<<<<<<<<<<<
                                            Tm2a_Sus_R1
Tm-2²  8579  ACTTAAGACATTTCCCAAATTAAGAAAACTTAAAATGTTCATCTGCAAGTATAATGAAGA  8638
Alignment     |||||||||| |||||||||||||||||||||||| |||||||||||||||||||||||
tm-2   2519  GCTTAAGACATTACCCAAATTAAGAAAACTTAAAATGGTCATCTGCAAGTATAATGAAGA  2578
```

Figure 4.3.19 A schematic diagram of the primers used to identify the *Tm-2*^{2} resistance and *tm-2* susceptible alleles. A 357 bp section of the aligned 9781 bp *Tm-2*^{2} allele and 2761 bp *tm-2* allele are shown: | indicates sequence similarity, whereas gaps indicate sequence differences. The 20 bp Tm2a_F (forward, >>>>) primer was designed to work with both: the 24 bp Tm2a_Sus_R1 (reverse, <<<<) primer to amplify a 274 bp PCR product from the *tm-2* allele, and the 27 bp Tm2a_Res_R1 primer to amplify a 288 bp product from the *Tm-2*^{2} allele. Identification of the *Tm-2*^{2} allele requires the use of an additional nested PCR primer pair; the 18 bp Tm2a_Res_NF and 24 bp Tm2a_Res_NR primer pair amplify a 168 bp PCR product from the 274 bp product produced by the Tm2a_Res primer pair.

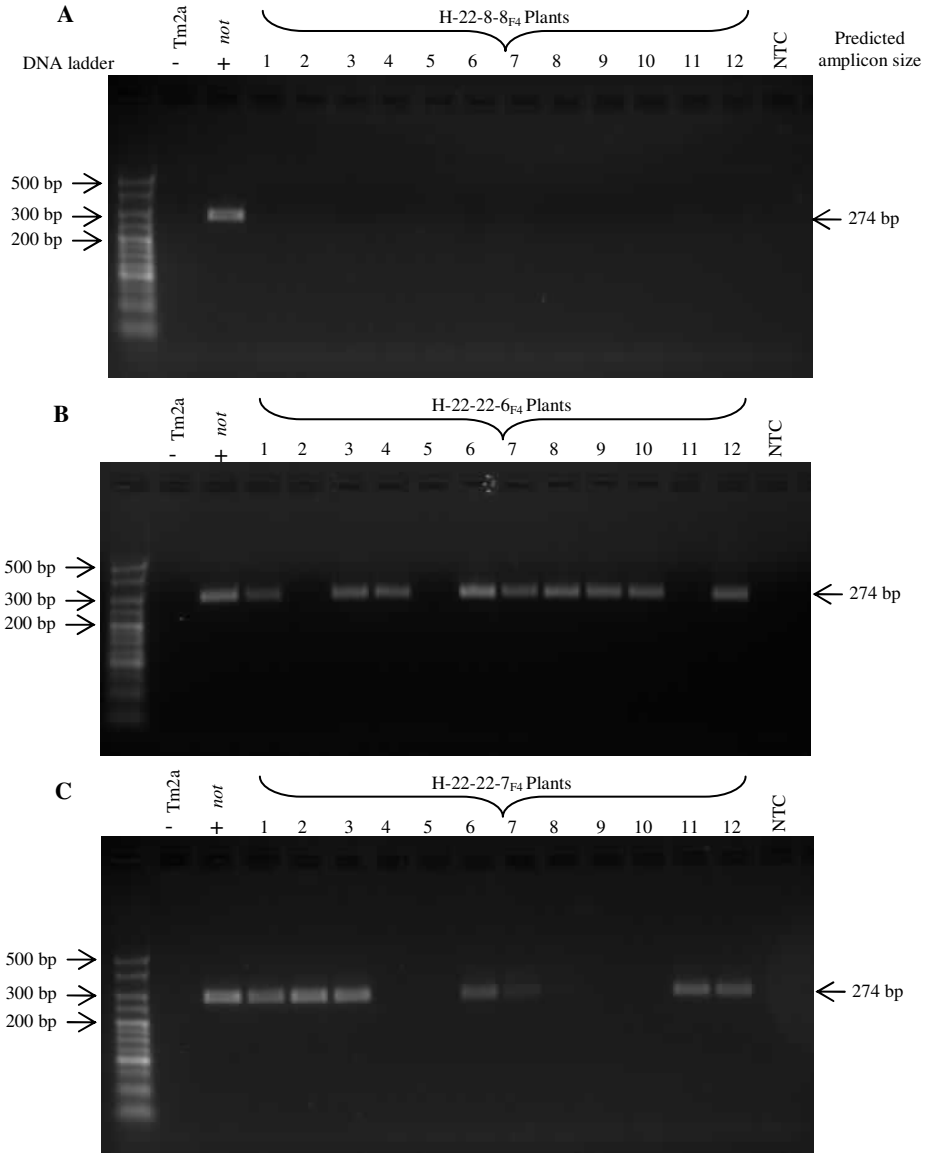

Figure 4.3.20 Gel electrophoresis images used to identify the TMV (tomato mosaic virus) susceptible *tm-2* allele. The primers amplify a predicted 274 bp amplicon (2.5% agarose gel).-, negative control, the TMV resistant Tm2a line; +, positive control, the TMV susceptible *notabilis* (*not*) mutant; NTC, no template control; Lanes **1-12**: **A**) H-22-8-8_{F4} plants 1-12; **B**) H-22-22-6_{F4} plants 1-12; **C**) H-22-22-7_{F4} plants 1-12.

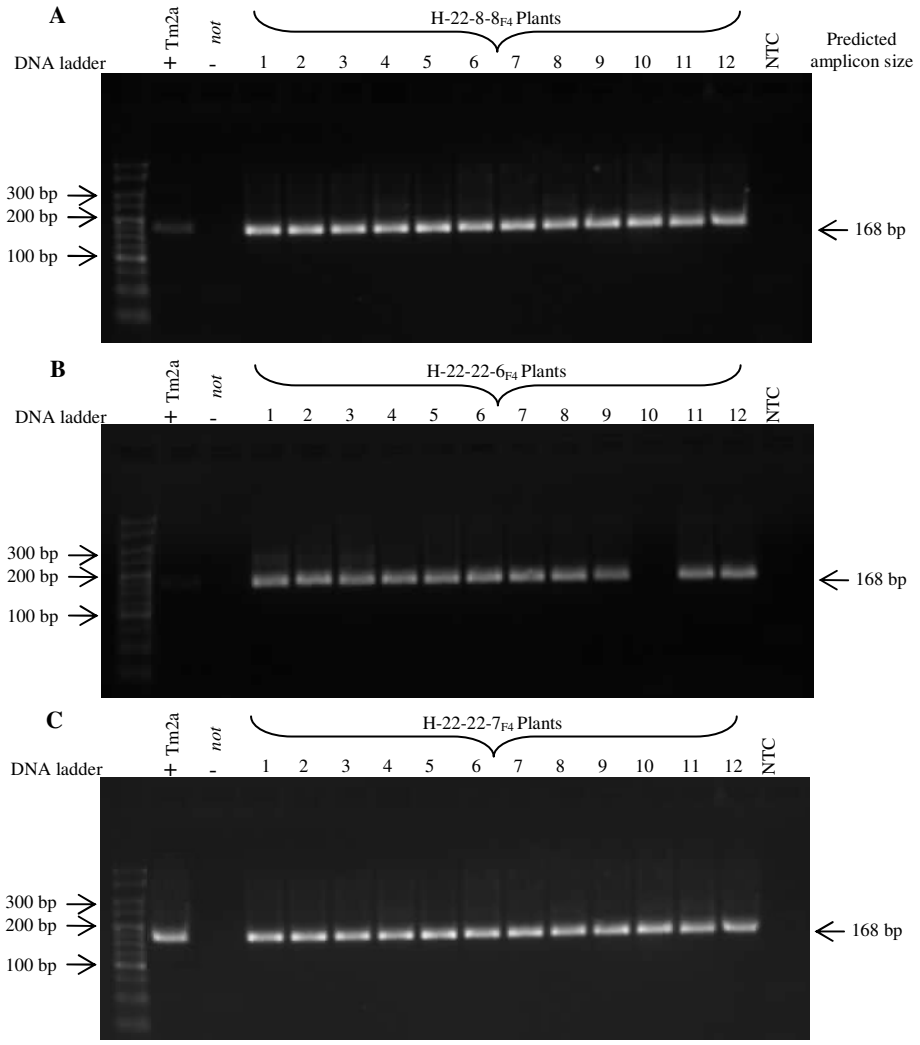

Figure 4.3.21 Gel electrophoresis images used to identify the TMV (tomato mosaic virus) resistance *Tm-2*^{2} allele. The nested primers amplify a predicted 168 bp amplicon from the initial 288 bp *Tm-2*^{2} PCR amplicon (2.5% agarose gel).+, positive control, the TMV resistant Tm2a line; -, negative control, the TMV susceptible *notabilis* (*not*) mutant; NTC, no template control; Lanes **1-12**: **A**) H-22-8-8$_{F4}$ plants 1-12; **B**) H-22-22-6$_{F4}$ plants 1-12; **C**) H-22-22-7$_{F4}$ plants 1-12.

Table 4.3.7 A summary of the *Tm-2*^{2} 'T' and *tm-2* 't' allelic genotype and zygosity (homo, homozygous; hetero, heterozygous) of 12 F_4 plants derived from the individual triple transgenic plants: H-22-8-8$_{F3}$, H-22-22-6$_{F3}$ and H-22-22-7$_{F3.}$

F_4 PLANT	LINE					
	H-22-8-8		H-22-22-6		H-22-22-7	
	Genotype	Zygosity	Genotype	Zygosity	Genotype	Zygosity
1	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'
2	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'
3	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'
4	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'
5	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'
6	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'
7	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'
8	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'
9	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'
10	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*tm-2* *tm-2*	Homo 'tt'	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'
11	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'
12	*Tm-2*^{2} *Tm-2*^{2}	Homo 'TT'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'	*Tm-2*^{2} *tm-2*	Hetero 'Tt'
TOTALS	Homo 'tt' = 0 Hetero 'Tt' = 0 Homo 'TT' = 12		Homo 'tt' = 1 Hetero 'Tt' = 8 Homo 'TT' = 3		Homo 'tt' = 0 Hetero 'Tt' = 7 Homo 'TT' = 5	

The mean internode lengths of the three selected H-22-8-8$_{F4}$, H-22-22$_{F4}$ and H-22-24$_{F4}$ are presented in Figure 4.3.22 along with the parental F_3 individual mean internode length. The transgenic line G29 was found to have the tallest mean internode length, at 5.1 cm significantly (P<0.05) taller than H-22-22-7$_{F4}$ and H-22-8-8$_{F4}$ (means of 4 and 4.5 cm respectively). The triple transgenic line H-22-22-7$_{F4}$ was found to have the shortest mean internode length, significantly (P<0.05) shorter than the Tm2a, G29 and H-22-22-6$_{F4}$ internode lengths (means of 4.7, 5.1 and 4.7 cm respectively).

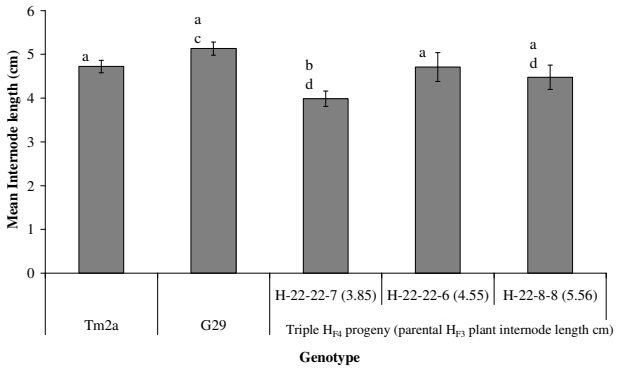

Figure 4.3.22 The mean internode lengths (cm) of the: WT, Tm2a line; G29 transgenic line; triple transgenic F_4 selfed progeny of the H-22-8-8_{F3}, H-22-22-6_{F3}, and H-22-22-7_{F3} plants taken at 1 m overall height The mean internode length of the parental triple F_3 transgenic plant (in brackets) and the lines presented shortest to tallest (n = 11 ± S.E.). Bars marked with a different letter are significantly different (P<0.05) from each other as determined by LSD *post hoc* test.

The F_4 generation also displayed the 'typical' *SlPSY1* overexpressor traits of shoot chlorosis, a typical triple transgenic F_4 plant shoot apex is shown in Figure 4.3.23, along with the shoot apex of a WT Tm2a plant. Additionally, the early pigmentation of immature fruit phenotype was prevalently now that of the pale green almost white colour (mentioned in section 4.3.4) and, as can be seen in Figure 4.3.24 the mature fruit ripened to an orange colour and was much smaller when compared to Tm2a fruit.

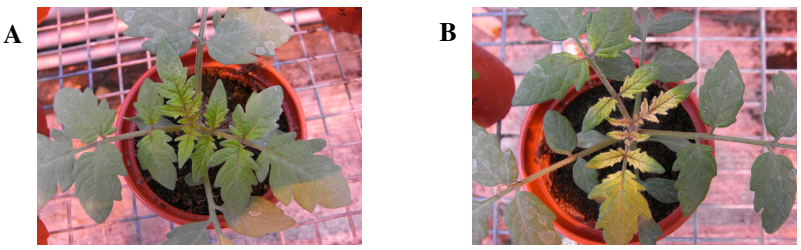

Figure 4.3.23 Photographs of the shoot apex of young plants of. **A;** WT, Tm2a line, showing typical green leaves; and **B**; A typical triple transgenic F_4 plant, showing typical leaf chlorosis.

Figure 4.3.24 A photograph comparing the colour and size of the ripe fruit. **A**) The WT, Tm2a line with red coloured ripe fruit; **B**) Typical triple transgenic F_4 with smaller orange coloured ripe fruit.

4.3.7 The Triple Transgenic Line Containing Three Constructs – Used as a Rootstock

The three selected triple transgenic lines, H-22-8-8, H-22-22-6 and H-22-22-7, constitutively overexpressing and homozygous for: *sp::SlNCED1*, *35S::SlCrtRb2/BCH2*, and *SlPSY1* were analysed for their capacity, when used as a transgenic rootstock, to alter stomatal behaviour in a non-transgenic scion, in order to potentially improve whole plant WUE. The three triple transgenic lines were grafted (as described in section 4.2.6) as rootstocks to WT Tm2a line scions, along with Tm2a/Tm2a and Tm2a/G29 (scion/rootstock) controls. These grafted plants were then subjected to a 30 d gravimetric water use trial. As can be seen in Table 4.3.8 there were no significant differences (P>0.05) in WUE, as measured by whole plant transpiration efficiency (TE_p) between the grafted plants. Despite this the control Tm2a self grafts had the lowest mean TE_p of 3.6 g DWt kg H_2O^{-1} and the Tm2a/H-22-8-8 plants had the highest mean TE_p of 4.5 g DWt kg H_2O^{-1}. The component measurements (Table 4.3.8) from which TE_p was calculated also revealed no significant overall differences between the genotypes: TE_P is based on two components, the amount of biomass accumulated and the amount of water transpired over the experimental period. It should be noted that the above ground biomass produced during the 30 d trial was determined as the difference between the initial biomass (g DWt) at the start of the trial and the final biomass at the end of the trial. The different genotypes all had a similar biomass (Table 4.3.8) at the start of the experimental period.

Table 4.3.8 The effect of the three triple transgenic lines as rootstocks on whole plant transpiration efficiency (TE_P, g DWt kg H_2O^{-1}) determined gravimetrically over a period of 30 days. The Tm2a/Tm2a (scion/rootstock) self grafts were used as the control; Tm2a/G29 grafted plants were used to test any additional effect of the third transgene against the two transgenes present in the double transgenic G29 line constitutively overexpressing *SlBCH2* and *SlNCED1*; Tm2a/H-22-8-8, Tm2a/H-22-22-6 and Tm2a/H-22-22-7 grafted plants with the triple transgenic rootstocks constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. The above ground biomass production (g DWt) during the trial period was determined as the difference between the initial and final above ground biomass (g DWt). Overall ANOVA probability (*P*) values are given (n = 7 ± S.E.).

Graft Combination (Scion/Rootstock)	Initial Biomass (g DWt)	Biomass Produced (g DWt)	Total Transpiration (Kg H_2O)	TE_P, (g DWt kg H_2O^{-1})
Tm2a/Tm2a	2.20 ± 0.18	32.14 ± 3.95	8.73 ± 0.66	3.61 ±0.22
Tm2a/G29	2.10 ± 0.26	29.33± 2.74	8.00 ± 0.48	3.68 ± 0.29
Tm2a/H-22-8-8	1.68 ± 0.22	38.63 ± 2.79	8.71 ± 0.40	4.47 ± 0.32
Tm2a/H-22-22-6	2.54 ± 0.24	33.69 ± 2.26	8.63 ± 0.36	3.99 ± 0.40
Tm2a/H-22-22-7	2.28 ± 0.24	31.96 ± 2.86	8.45 ± 0.57	3.77 ± 0.19
P	0.141	0.278	0.842	0.265

The genotypes produced similar amounts of above ground biomass over the 30 d period (Table 4.3.8), ranging from a minimum mean of 29.3 g DWt in Tm2a/G29 to 38.6 g DWt in Tm2a/H-22-8-8. The different graft combinations also transpired similar amounts of water (kg H_2O, Table 4.3.8) over the 30 d trial period; which ranged from a minimum mean of 8 kg H_2O in Tm2a/G29 plants to a maximum mean of 8.7 Kg H_2O in Tm2a self grafts. As shown in Table 4.3.9, the Tm2a/H-22-8-8 plants were significantly ($P<0.05$) shorter (43.8 cm) at the start of the experimental period than all but the Tm2a/G29 (mean of 47.1 cm) grafts. The Tm2a self grafts were the tallest at the start of the trial, mean of 53 cm. There were no significant differences between the graft combinations with regard to the mean internode length (cm) or the mean leaf area (cm^2) of the third, fourth, fifth and sixth leaves at the end of the trial period (Table 4.3.9).

Table 4.3.9 The initial height at the start of the 30 day trial period and the final height (cm), mean internode length (cm) and mean total leaf area of the third, fourth fifth and sixth leaves (cm^2) at the end of the trial period: The Tm2a/Tm2a (scion/rootstock) self grafts were used as the control; Tm2a/G29 grafted plants were used to test any additional effect of the third transgene against the two transgenes present in the double transgenic G29 line constitutively overexpressing *SlBCH2* and *SlNCED1*; Tm2a/H-22-8-8, Tm2a/H-22-22-6 and Tm2a/H-22-22-7 grafted plants with the triple transgenic rootstocks constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. Overall ANOVA probability (*P*) values are given, values marked with a different letter are significantly ($P<0.05$) different from each other as determined by LSD *post hoc* test (n = 7 ± S.E.).

GRAFT COMBINATION (SCION/ROOTSTOCK)	INITIAL HEIGHT (cm)	FINAL HEIGHT (cm)	INTERNODE LENGTH (cm)	LEAF AREA (cm^2)
Tm2a/Tm2a	53.00 ± 2.59^{a}	149.67 ± 5.64	4.63 ± 0.19	435.31 ± 44.466
Tm2a/G29	47.14 ± 2.68^{ab}	147.31 ± 6.84	4.52 ± 0.19	445.20 ± 36.89
Tm2a/H-22-8-8	43.79 ± 2.11^{b}	152.93 ± 2.97	4.68 ± 0.12	493.73 ± 37.61
Tm2a/H-22-22-6	52.57 ± 2.01^{a}	146.71 ± 9.08	4.47 ± 0.27	490.47 ± 42.04
Tm2a/H-22-22-7	52.86 ± 2.68^{a}	152.29 ± 2.58	4.64 ± 0.13	469.98 ± 42.28
P	0.035	0.927	0.921	0.797

The ABA content in the root exudates is shown in Table 4.3.10, the variation between the biological samples was quite high so the data was log transformed before analysis. This revealed that the Tm2a self grafts had significantly less ($P<0.05$) root exudate [ABA] (3.6 n*M*) than the graft combinations with transgenic rootstocks, including G29 (13.8 n*M*). The root exudate [ABA] was found to be similar in the grafted plants with transgenic rootstocks, but greatest in the Tm2a/H-22-8-8 graft combination, (48.3 n*M*) and lowest in Tm2a/H-22-22-7 (12.7 n*M*). The root exudate flow rate of Tm2a/H-22-8-8 grafts was found to be significantly ($P<0.01$) faster, at 2.3 ml h^{-1} than all other graft combinations apart from Tm2a/H-22-22-6, which had an exudate flow rate of 1.65 ml h^{-1}. The Tm2a/G29 and Tm2a/H-22-22-7 graft combinations had the slowest flow rates at 0.88 ml h^{-1} and 0.89 ml h^{-1} respectively, while the control Tm2a self graft had a mean exudate flow rate of 1.2 ml h^{-1}.

Table 4.3.10 The root exudate flow rate (ml h^{-1}) and ABA concentration (n*M*) of the grafted plants at the end of the 30 d gravimetric water use trial period: The Tm2a/Tm2a (scion/rootstock) self grafts were used as the control; Tm2a/G29 grafted plants were used to test any additional effect of the third transgene against the two transgenes present in the double transgenic G29 line constitutively overexpressing *SlBCH2* and *SlNCED1*; Tm2a/H-22-8-8, Tm2a/H-22-22-6 and Tm2a/H-22-22-7 grafted plants with the triple transgenic rootstocks constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. Overall ANOVA probability (*P*) values are given, values marked with a different letter are significantly (**P*<0.05,***P*<0.01) different from each other as determined by LSD *post hoc* test (n = 4 ± S.E.).

GRAFT COMBINATION (SCION/ROOTSTOCK)	ROOT EXUDATE FLOW RATE (ml h^{-1})	LOG[ROOT EXUDATE ABA CONCENTRATION (n*M*)]
Tm2a/Tm2a	$1.21 \pm 0.20^{a**}$	$1.10 \pm 0.11^{a*}$
Tm2a/G29	$0.88 \pm 0.25^{ac**}$	$1.70 \pm 0.09^{b*}$
Tm2a/H-22-8-8	$2.29 \pm 0.37^{bd**}$	$2.02 \pm 0.26^{b*}$
Tm2a/H-22-22-6	$1.65 \pm 0.15^{ad**}$	$1.75 \pm 0.21^{b*}$
Tm2a/H-22-22-7	$0.89 \pm 0.12^{ac**}$	$1.65 \pm 0.09^{b*}$
P	0.003	0.021

The $\delta^{13}C$ values of the total leaf area of the grafted plants at the end of the gravimetric trial period are displayed in Figure 4.3.25, the Tm2a/H-22-8-8 graft combination had a significantly (*P*<0.05) larger i.e. less negative $\delta^{13}C$ value (mean of -30.9), than the other transgenic graft combinations but not significantly different from the Tm2a self graft, which had a mean $\delta^{13}C$ of -31.3.

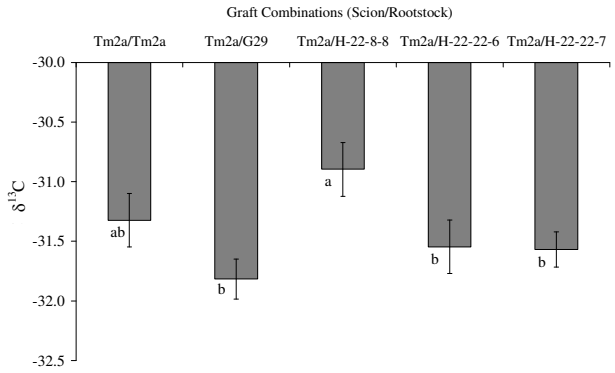

Figure 4.3.25 The δ ^{13}C values of the total leaf area of the grafted plants at the end of the 30 d gravimetric trial period. The Tm2a/Tm2a (scion/rootstock) self grafts were used as the control; Tm2a/G29 grafted plants were used to test any additional effect of the third transgene against the two transgenes present in the double transgenic G29 line constitutively overexpressing *SlBCH2* and *SlNCED1*; Tm2a/H-22-8-8, Tm2a/H-22-22-6 and Tm2a/H-22-22-7 grafted plants with the triple transgenic rootstocks constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. Overall ANOVA probability (*P*) values are given, bars marked with a different letter are significantly ($P<0.05$) different from each other as determined by LSD *post hoc* test (n = 7 ± S.E.).

Overall, the results from this 30 d gravimetric trial indicated that the three triple transgenic lines were possibly having a slight, albeit not statistically significant, negative effect on the whole plant water use (i.e. reducing it) when grafted to non-transgenic WT scions. This trial was conducted during the winter months of November/December, which was not ideal as the light levels were low, despite supplementary lighting. Additionally, due to a glasshouse heating failure, the plants were exposed to low night time temperatures (~12°C) for ten days. Therefore, a second grafting experiment focusing on the best performing triple transgenic line, H-22-8-8 was carried out during May/June.

As well as the Tm2a/H-22-8-8 graft combination; Tm2a self grafts (Tm2a/Tm2a) were used as the WT control and transgenic line sp5 self grafts (sp5/sp5) as a positive control. The transgenic sp5 line (section 4.2.1 and Appendix I Table 7.1.1) constitutively overexpresses *SlNCED1* and has been previously shown to have a higher water use efficiency than WT, Tm2a plants (Thompson *et al.*, 2007a). The grafted plants were subjected to a 30 d gravimetric water use trial. It can be seen in Table 4.3.11 that the

sp5 self grafts had a significantly (P<0.05) greater WUE, mean TE_p of 4.0 g DWt kg H_2O^{-1}, than both the control Tm2a self grafts (mean TE_p of 2.8 g DWt kg H_2O^{-1}) and the Tm2a/H-22-8-8 plants (mean TE_p of 3.0 g DWt kg H_2O^{-1}). There was no statistically significant (P>0.05) difference between the WUEs of the Tm2a self grafts and the Tm2a/H-22-8-8 grafts.

Table 4.3.11 The effect of the triple transgenic line H-22-8-8 as a rootstock on whole plant transpiration efficiency (TE_P, g DWt kg H_2O^{-1}) determined gravimetrically over a period of 30 days. The Tm2a/Tm2a (scion/rootstock) self grafts were used as a control; the sp5/sp5 self grafts were used as a positive transgenic control, the sp5 line constitutively overexpresses *SlNCED1*; Tm2a/H-22-8-8 grafted plants with the triple transgenic rootstock constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. The above ground biomass production (g DWt) during the trial period was determined as the difference between the initial and final above ground biomass (g DWt). Overall ANOVA probability (P) values are given, values marked with a different letter are significantly different (P<0.01) from each other as determined by LSD *post hoc* test (n = 10 ± S.E.).

GRAFT COMBINATION (SCION/ROOTSTOCK)	Initial Biomass (g DWt)	Biomass Produced (g DWt)	Total Transpiration (Kg H_2O)	TE_P, (g DWt kg H_2O^{-1})
Tm2a/Tm2a	1.87 ± 0.07	54.40 ± 1.27[a]	19.56 ± 0.49	2.79 ± 0.07[a]
sp5/sp5	1.83 ± 0.37	74.00± 1.28[b]	18.77 ± 0.42	3.95 ± 0.07[b]
Tm2a/H-22-8-8	1.78 ± 0.08	54.41 ± 2.42[a]	18.30 ± 0.67	2.99 ± 0.11[a]
P	0.966	<0.001	0.259	<0.001

The component measurements (Table 4.3.11) from which TE_p was calculated (as previously mentioned TE_P is based on two components, the amount of biomass accumulated and the amount of water transpired over the experimental period) revealed that the different graft combinations transpired similar (P>0.05) amounts of water over the trial period, ranging from 18.3 Kg H_2O in the Tm2a/H-22-8-8 graft to 19.6 Kg H_2O in the sp5 self grafts. Therefore the difference in WUE between the graft combinations resulted from the sp5 self grafts producing significantly (P<0.01) more above ground biomass, 74 g DWt than both the Tm2a self grafts and the Tm2a/H-22-8-8 graft combination, which both produced almost identical mean above ground biomasses of 54.4g DWt. As previously mentioned, the above ground biomass produced during the 30 d trial was determined as the difference between the initial biomass g DWt at the start of

the trial and the final biomass at the end of the trial. The different genotypes all had a similar biomass (Table 4.3.11) at the start of the experimental period.

It can be seen in Table 4.3.12 that the sp5 self grafts had significantly (P<0.01) longer mean leaf length, 33.8 cm, compared to both the Tm2a self grafts (mean leaf length of 28.1 cm) and the Tm2a/H-22-8-8 grafted plants (mean of 30.8 cm), which was expected from previously reported findings (Thompson *et al.*, 2007a). The sp5 self grafts also had a significantly (P<0.01) longer mean internode length, of 3.8 cm compared to the Tm2a self grafts and the Tm2a/H-22-8-8 plants (which had mean internode lengths of 3.3 cm and 3.6 cm respectively). There was no significant difference (P>0.05) in the heights of the plants at the end of the trial period, which ranged from 105 cm in the sp5 self grafts to 108 cm in the Tm2a self grafts. Additionally there was no significant difference found between the rootstock stem diameters (measured just below the graft union) which ranged from 1.3 cm in the Tm2a self grafts to 1.4 cm in the sp5 self grafts (Table 4.3.12).

Table 4.3.12 The initial height at the start of the 30 day trial period and the final height (cm), mean internode length (cm), mean leaf length (cm) and mean stem diameter measured below the graft union (cm), at the end of the trial. The Tm2a/Tm2a (scion/rootstock) self grafts were used as a control; the sp5/sp5 self grafts were used as a positive transgenic control, the sp5 line constitutively overexpresses *SlNCED1*; Tm2a/H-22-8-8 grafted plants with the triple transgenic rootstock constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. Overall ANOVA probability (P) values are given, values marked with a different letter are significantly different (P<0.05) from each other as determined by LSD *post hoc* test (n = 10 ± S.E.).

GRAFT COMBINATION (SCION/ROOTSTOCK)	INITIAL HEIGHT (cm)	FINAL HEIGHT (cm)	INTERNODE LENGTH (cm)	LEAF LENGTH (cm)	STEM DIAMETER (cm)
Tm2a/Tm2a	26.25 ± 2.02	107.50 ± 1.83	3.32 ± 0.05^{a}	28.11 ± 0.75^{a}	1.25 ± 0.023
sp5/sp5	22.75 ± 2.50	105.40 ± 1.11	3.84 ± 0.11^{b}	33.77 ± 1.16^{b}	1.36 ± 0.05
Tm2a/H-22-8-8	26.75 ± 1.44	105.69 ± 2.42	3.45 ± 0.07^{a}	30.81 ± 1.42^{ab}	1.31 ± 0.028
P	0.358	0.692	<0.001	0.006	0.125

As previously mentioned, the Tm2a self grafts and the Tm2a/H-22-8-8 grafts produced almost identical amounts of above ground biomass (Table 4.3.11) over the 30 d experimental period, which meant that the daily water use data of these two graft

combinations could be directly compared and explored in more detail. The mean daily water use of the two graft combinations is displayed in Figure 4.3.26. It should be noted that during the 30 d trial period (May/June) their were two high temperature peaks, where temperatures reached ~28°C, which translated to glasshouse temperatures of at least 35°C: the first peak was over days 14 to 16 of the trial, and the second was over days 27 to 29 of the trial. During the second peak, in order to prevent the stress experienced by the plants during the first peak, supplementary water was given to all of the plants (accounted for in all calculations). With hindsight it would have been better to give supplementary water at an earlier stage of the trial (i.e. from day 10 to day 30) in order to prevent the plants from ever experiencing stress. The high temperature peaks coincide with the peaks in daily water used and are clearly visible in Figure 4.3.26 A.

Figure 4.3.26 A, shows the daily fluctuations in water use of the two graft combinations; the mean daily water use of the Tm2a/H-22-8-8 plants was found to be significantly (P<0.05) less than the Tm2a self graft plants on eight days out of 30 (27%) which were at the beginning (before the onset of water stress) and end of the trial period (after the addition of supplementary water to allow recovery from stress). On the other 22 days the water consumption of the Tm2a/H-22-8-8 plants was at the same level as that of the Tm2a self grafted controls, but never exceeded them.

Figure 4.3.26 B, shows the water saved by the H-22-8-8 rootstock on a daily basis; the mean daily water consumed by the control Tm2a self graft plants was used as the base line which was subtracted from the individual daily water use of the Tm2a/H-22-8-8 plants. The bars below the line (negative values for water used) represent a water saving by the triple transgenic rootstock, and any bars above the baseline would therefore reveal increases in water use by the Tm2a/H-22-8-8 plants compared to the Tm2a/Tm2a control. It can clearly be seen in Figure 4.3.26 B, that on days where there was a large difference in water use it was always the Tm2a/H-22-8-8 plants which used less water

than the Tm2a self grafts. The Tm2a/H-22-8-8 plants used, on average, less water on 26 days out of 30 (86% of the trial period) and is therefore unlikely to be a random difference between the two groups of plants. The Tm2a/H-22-8-8 plants on average only used more water on three days (16 - 18, 10% of the trial period), which followed the first high temperature peak. The statistically significant daily water use data presented in Figure 4.3.26 is persuasive towards a negative effect of the triple transgenic rootstock on whole plant water use without negatively affecting vegetative above ground biomass accumulation. Although, this water saving was only detectable on days where none of the plants experienced water stress.

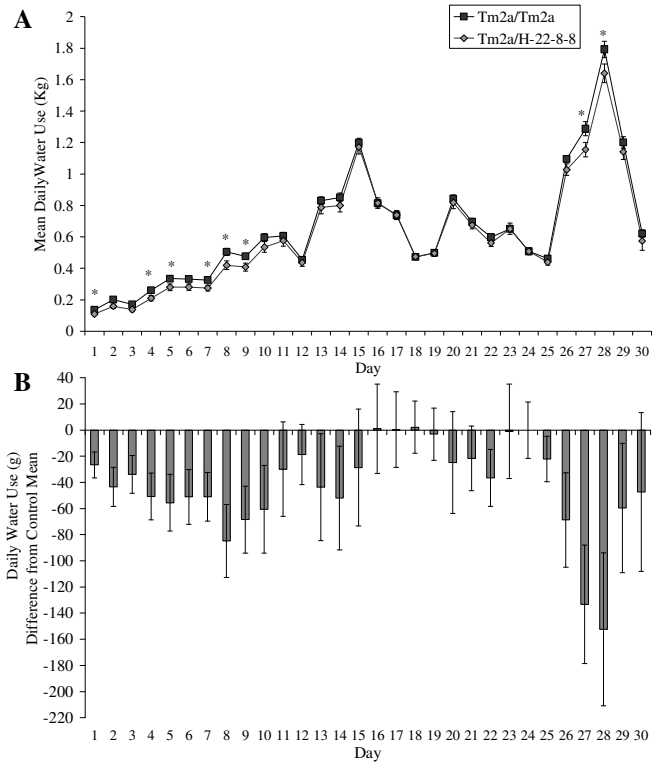

Figure 4.3.26 The daily water use of the Tm2a/Tm2a (scion/rootstock) and Tm2a/H-22-8-8 grafted plants, which produced almost identical amounts of biomass (54.4 g DWt) over the 30 d experimental period: **A**, the mean daily water use (Kg) of each graft combination; means marked with * are significantly different (P<0.05) according to Dunnett t-test. **B**, The mean daily water use (g); the mean daily water use of Tm2a/Tm2a control plants (baseline, 0) was subtracted from the daily water use of each individual Tm2a/H-22-8-8 plant (n=10 ± S.E.).

As seen in Table 4.3.13, there was no significant difference (P>0.05) found between the root exudate ABA concentrations from the Tm2a/Tm2a and Tm2a/H-22-8-8

plants from this second trial, means of 19.7 n*M* and 20.8 n*M* respectively. As reported previously (Thompson *et al.*, 2007a; Thompson *et al.*, 2007b) the root exudate ABA concentration taken from sp5/sp5 self grafts was significantly (P<0.01) higher, at a mean concentration of 50.7 n*M*, than the other two graft combinations. Additionally, as expected, sp5 self grafted plants were found to have a significantly (*P*<0.01) higher exudate flow rate of 7.8 ml h^{-1} compared to that of 4.4 ml h^{-1} in Tm2a/H-22-8-8 plants and 3.6 ml h^{-1} in Tm2a self grafts, which were not found to be significantly different from each other. It should be noted that the $\delta^{13}C$ data from this second water use trial has not yet been returned from the Australian National University laboratory at the time of writing.

Table 4.3.13 The root exudate flow rate (ml h^{-1}) and ABA concentration (n*M*) of the grafted plants at the end of the 30 d gravimetric water use trial period: The Tm2a/Tm2a (scion/rootstock) self grafts were used as the control; the sp5/sp5 self grafts were used as a positive transgenic control, the sp5 line constitutively overexpresses *SlNCED1*; Tm2a/H-22-8-8 grafted plants with the triple transgenic rootstock constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. The above ground biomass production (g DWt) during the trial period was determined as the difference between the initial and final above ground biomass (g DWt). Overall ANOVA probability (*P*) values are given, values marked with a different letter are significantly different (*P*<0.01) from each other as determined by LSD *post hoc* test (n = 5 ± S.E.).

GRAFT COMBINATION (SCION/ROOTSTOCK)	ROOT EXUDATE FLOW RATE (ml h^{-1})	ROOT EXUDATE ABA CONCENTRATION (n*M*)
Tm2a/Tm2a	3.64 ± 0.43^{a}	19.74 ± 2.12^{a}
sp5/sp5	7.84 ± 0.81^{b}	50.72 ± 4.53^{b}
Tm2a/H-22-8-8	4.42 ± 0.92^{a}	22.82 ± 6.93^{a}
P	0.004	0.001

The photographs of the graft union in Figure 4.3.27 highlight the degree of pigmentation due to high concentrations of β-carotene and lycopene (Table 4.3.14) in the stem and roots of the triple transgenic H-22-8-8 line compared to the green WT, Tm2a line.

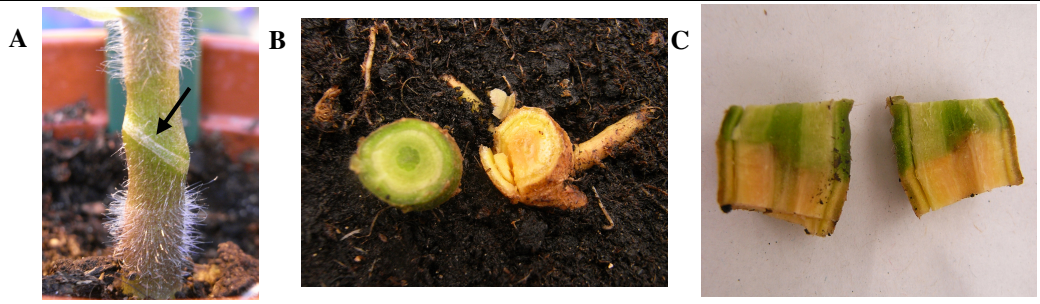

Figure 4.3.27 Photographs of the graft union: **A,** showing the graft union scar (indicated by arrow) just after the union has healed and removal of the silicon tubing (Tm2a self graft plant); **B** and **C** show the graft union between Tm2a/H-22-8-8 (scion/rootstock) illustrating the orange pigmentation in the triple transgenic rootstock: **B,** horizontal section through the stem above the graft union (**left**) showing the green coloured Tm2a section of the stem, horizontal section through the stem below the graft union (**right**) showing the orange coloured rootstock stem base and roots; **C**, transverse section through the stem section containing the graft union, revealing the difference in pigmentation at the graft union between the lower orange coloured triple transgenic H-22-8-8 rootstock stem section and the upper green coloured Tm2a scion stem section.

4.3.8 The Triple Transgenic Line Containing Three Constructs – Isolated Root Cutures

The mean concentrations and percentage compositions of the main xanthophylls and other carotenoids from isolated root cultures of: the triple transgenic lines, H-22-8-8, H-22-22-6 and H-22-22-7, the double transgenic line G29, the single transgenic lines, sp5 and BCH12 and the WT Tm2a line are displayed in Table 4.3.14. It should be noted that the growth of these isolated root tissue cultures was not closely monitored during the week prior to harvest and it is considered likely that some genotypes, i.e. G29 may have experienced a period of stress due to sub-optimal growing conditions just before extraction, which may have unequally affected the carotenoid (Table 4.3.14) and ABA (Figure 4.3.28) concentrations obtained from some of these root cultures; therefore they should be considered as preliminary. Ideally, it would have been better to have monitored the individual growth rates every few days throughout the entire experimental period, to more precisely identify optimal times for harvest of each sample to avoid the possibility that any of the roots were exhausting their nutrient supply. Unfortunately, in the present case, the roots grew more rapidly than expected during a

short period of annual leave and all the samples had to be harvested on the day of return to the laboratory.

Table 4.3.14 The carotenoid content (μg g^{-1} DWt) and percentage composition in the isolated root systems grown in tissue culture of: WT, Tm2a line; the BCH12 transgenic line constitutively overexpressing *SlBCH2*; the sp5 transgenic line, constitutively overexpressing *SlNCED1*; the G29 transgenic line constitutively overexpressing *SlBCH2* and *SlNCED1*; three triple transgenic H-22-8-8_{F4} H-22-22-6_{F4} and H-22-22-7_{F4} lines constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1*. Values marked with a different letter are significantly (**P*<0.05, ***P*<0.01) different from each other according to [1]LSD or [2]Games-Howell *post hoc* test (n = 5 ± S.E.).

Genotype	Xanthophyll Content (μg g^{-1} DWt) (% of Total Carotenoids Analysed)						Other Carotenoids (μg g^{-1} DWt) (% of Total Carotenoid Content Analysed)		
	Lutein[1]	Zeaxanthin[2]	Violaxanthin[2]	9'-*cis*-neoxanthin[2]	All-*trans*-neoxanthin[2]	Total[2]	β-carotene[2]	Lycopene[2]	Total[2]
Tm2a	0.02^{a**} ± 0.003 (11)	0.01^{a**} ± 0.001 (6)	0.003 ± 0.001 (4)	0.05^{a**} ± 0.003 (28)	0.05^{a*} ± 0.005 (29)	0.13^{a**} ± 0.009	0.04^{a**} ± 0.005 (22)	0.00 ± 0.000 (0)	0.17^{a**} ± 0.009
BCH12	0.02^{a**} ± 0.003 (5)	0.02^{a**} ± 0.001 (4)	0.02 ± 0.009 (7)	0.13^{bc**} ± 0.002 (35)	0.13^{ab*} ± 0.039 (34)	0.32^{ac**} ± 0.044	0.05^{ac**} ± 0.004 (14)	0.00 ± 0.000 (0)	0.37^{ac**} ± 0.047
sp5	0.02^{a**} ± 0.002 (14)	0.01^{a**} ± 0.001 (8)	0.01 ± 0.007 (9)	0.02^{bd**} ± 0.002 (14)	0.05^{a*} ± 0.020 (37)	0.11^{a**} ± 0.031	0.03^{ad**} ± 0.001 (19)	0.00 ± 0.000 (0)	0.14^{ad**} ± 0.030
G29	0.03^{a**} ± 0.004 (5)	0.01^{a**} ± 0.003 (2)	0.07 ± 0.036 (12)	0.08^{ac**} ± 0.008 (14)	0.34^{ab*} ± 0.130 (61)	0.53^{abcd} ± 0.177	0.03^{a**} ± 0.002 (6)	0.00 ± 0.000 (0)	0.56^{a**} ± 0.180
H-22-8-8	0.07^{b**} ± 0.004 (1)	0.21^{b**} ± 0.029 (3)	0.03 ± 0.009 (<1)	0.11^{abcd} ± 0.036 (2)	0.21^{b*} ± 0.026 (3)	0.64^{bc**} ± 0.81	1.03^{be**} ± 0.115 (17)	4.46 ± 0.311 (73)	6.13^{b**} ± 0.387
H-22-22-6	0.08^{b**} ± 0.006 (1)	0.19^{b**} ± 0.010 (3)	0.04 ± 0.019 (1)	0.18^{bc**} ± 0.013 (3)	0.27^{b*} ± 0.038 (4)	0.76^{bd**} ± 0.029	1.05^{ae**} ± 0.285 (17)	4.54 ± 0.475 (71)	6.35^{b**} ± 0.617
H-22-22-7	0.09^{b**} ± 0.008 (1)	0.21^{b**} ± 0.22 (3)	0.02 ± 0.003 (<1)	0.16^{bc**} ± 0.018 (2)	0.30^{ab*} ± 0.084 (4)	0.77^{bc**} ± 0.108	1.58^{be**} ± 0.130 (21)	5.26 ± 0.36 (69)	7.61^{b**} ± 0.374
P	<0.001	<0.001	0.180	<0.001	0.011	<0.001	<0.001	<0.001	<0.001

Table 4.3.14 contains a vast array of data which is presented for completeness; one main point to note is the constant and significantly larger total amounts (*P*<0.01) of carotenoids detected in the triple transgenic isolated roots (average of 6.7μg g^{-1} DWt) i.e. over 10x greater than in any of the other genotypes. This was mainly due, as in previous

generations, to the accumulation of large amounts of lycopene and β-carotene, which in this experiment accounted for ~71% and ~18% respectively of the total carotenoids in the triple transgenic roots.

The non-transgenic control (Tm2a) roots were found to have almost 40x less carotenoids than the triple transgenic roots, at just 0.17 μg g^{-1} DWt. The total amounts of carotenoids found in the sp5, BCH12 and G29 isolated roots, which had means of 0.14, 0.37 and 0.56 μg g^{-1} DWt respectively were not significantly different from these 'control' levels. However it is important to note that, sp5 isolated roots, which constitutively overexpress *SlNCED1* alone accumulated significantly less (P<0.01) β-carotene (mean of 0.03 μg g^{-1} DWt) than BCH12 roots, which had a mean of 0.05 μg g^{-1} DWt, and constitutively overexpress *SlBCH2*. The Tm2a control had a mean β-carotene content of 0.04 μg g^{-1} DWt which was between that of sp5 and BCH12. The additional β-carotene in BCH12 may be due to feedback regulation at earlier steps in the pathway responding to the increased BCH2 enzyme activity by up-regulating production of the β-carotene substrate. The reduction in β-carotene in sp5 could partly be due to the excess NCED1 enzyme cleaving β-xanthophylls and thereby depleting the β-carotene pool.

The triple transgenic isolated roots also produced significantly (P<0.01) increased amounts of the xanthophylls (0.72 μg g^{-1} DWt on average), compared to Tm2a, sp5 and BCH12. This consisted of increased amounts of all the xanthophylls analysed, except violaxanthin which accumulated to similar levels in the isolated roots of all genotypes analysed. The Tm2a and sp5 genotypes accumulated similar amounts of the xanthophylls analysed, means of 0.13 μg g^{-1} DWt and 0.11 μg g^{-1} DWt respectively, which was around 6x less than the total amounts of xanthophylls found in the triple transgenic lines. The BCH12 and G29 genotypes accumulated reasonably similar amounts of total xanthophyll, means of 0.32 and 0.53 μg g^{-1} DWt respectively, which

were respectively around 2x and 1.3x less than the total amounts of xanthophylls found in the triple transgenic lines respectively.

Notably BCH12 accumulated significantly (P<0.01) more 9′-*cis*-neoxanthin (mean of 0.13 μg g^{-1} DWt) than the non transgenic Tm2a roots (mean of 0.05 μg g^{-1} DWt). This appears to indicate increased precursor flux through the pathway due to the increased BCH2 enzyme activity, which resulted in an accumulation of 9′-*cis*-neoxanthin downstream. In contrast, the sp5 roots accumulated significantly less (P<0.01) 9′-*cis*-neoxanthin than the Tm2a and BCH12 roots, presumably due to the increased NCED1 enzyme activity depleting the substrate pool. In the absence of the *SlNCED1* transgene the ratio of neoxanthin isomers is approximately 1:1, as observed in the Tm2a and BCH12 isolated root cultures. On the other hand, the presence of the *SlNCED1* transgene appears to alter this ratio to approximately 1:2 9′-*cis*-neoxanthin:all-*trans*-neoxanthin, reflecting the depletion of the 9′-*cis*-neoxanthin substrate for the NCED1 enzyme.

Another point of interest in Table 4.3.14, is that the triple transgenic roots contained significantly (P<0.01) increased amounts of lutein (~0.08 μg g^{-1} DWt) and zeaxanthin (~0.20 μg g^{-1} DWt) compared to the other four genotypes, which all had similar lutein and zeaxanthin contents, averaging 0.02 and 0.01 μg g^{-1} DWt respectively. This increase in the triple transgenic lines is presumably due to the additional PSY1 enzyme activity increasing precursor flux through both the α- and β-branches of the pathway.

The ABA content of the isolated root cultures is shown in Figure 4.3.28 and it should be noted that the variation between the biological samples was high, so the data was log transformed before statistical analysis. Perhaps surprisingly, in view of the differences in β-branch precursor pools noted earlier, the isolated roots of the triple transgenic H-22-8-8, double transgenic G29 and single transgenic sp5 lines all appear to have produced similar total amounts of *cis*-ABA within their root systems. The sp5, G29

and H-22-8-8 isolated roots all had significantly (P<0.01) greater amounts of *cis*-ABA, 426 ng g^{-1}DWt, 365 ng g^{-1} DWt and 464 ng g^{-1} DWt respectively, than the WT Tm2a and transgenic BCH12 roots which accumulated *cis*-ABA in amounts that were around 7x lower, means of 60 ng g^{-1} DWt and 55 ng g^{-1} DWt respectively. The distinguishing feature of the former three genotypes (sp5, G29 and H-22-8-8) is that they carried that *SlNCED1* transgene, whereas the latter two genotypes (WT Tm2a and BCH12) did not.

In all of the genotypes containing the *SlNCED1* transgene (i.e. H-22-8-8, G29 and sp5) the ABA metabolites analysed were also found in significantly (P<0.01) greater quantities than those found in the roots of the WT, Tm2a and single transgenic BCH12 line which did not have this construct. This appears to be a general trend, although it should be noted that not all of the ABA metabolites analysed were detected in all of the genotypes. DPA, derived from the 8′-hydroxylation pathway via PA, was present in similar, but significantly lower, amounts (means of 26 ng g^{-1} DWt and 37 ng g^{-1} DWt respectively) in Tm2a and BCH12 than in the roots of sp5, G29 and H-22-8-8 (means of 480 ng g^{-1} DWt, 253 ng g^{-1} DWt and 738 ng g^{-1} DWt respectively). The ABA glucose conjugate ABA-GE was also present in all of the genotypes, with the Tm2a and BCH12 genotypes having the same mean (21 ng g^{-1} DWt) which was significantly lower than the corresponding means for sp5, G29 and H-22-8-8 (216 ng g^{-1} DWt, 319 ng g^{-1} DWt and 620 ng g^{-1} DWt respectively). The fact that the roots of the triple transgenic line (H-22-8-8) produce the highest amounts of both DPA and ABA-GE (738 and 620 ng g^{-1} DWt respectively) is interesting to note, but due to low sample number and high variability, these apparent increases were not statistically significant.

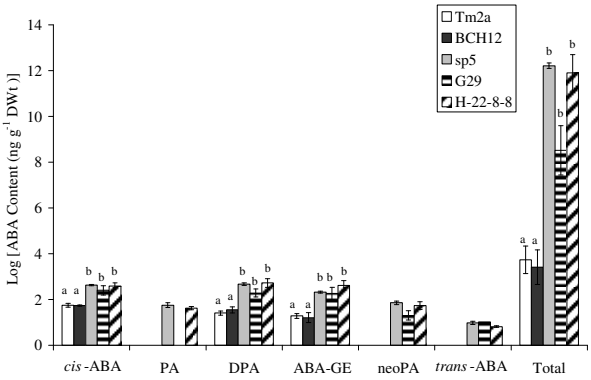

Figure 4.3.28 The effect of single, double and triple *SlNCED1*, *SlBCH2* and *SlPSY1* overexpression on the ABA and ABA metabolite content of isolated root systems grown in tissue culture. The root cultures of: the WT, Tm2a line, the transgenic BCH12 line overexpressing *SlBCH2*; the transgenic sp5 line overexpressing *SlNCED1*; the double transgenic G29 line overexpressing *SlBCH2* and *SlNCED1*; and the triple transgenic H-22-8-8 line overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1* were analysed for *cis*-ABA, phaseic acid (PA), dihydrophaseic acid (DPA), ABA-glucose ester (ABA-GE), *neo*phasic acid (neoPA) and *trans*-ABA. Statistical treatment was by ANOVA, and bars marked with a different letter are significantly (P<0.001) different from each other as determined by *LSD* post hoc test (n = 4 ± S.E). N.B. For DPA, Tm2a and BCH12, n = 2, for ABA-GE, and for BCH12 n= 3 as some of the replicates fell below the detection level.

PA, the cyclised form of 8′-hydoxyl ABA was only detected in sp5 and H-22-8-8 isolated roots, with means of 62 ng g^{-1} DWt and 44 ng g^{-1} DWt respectively. NeoPA, the equivalent cyclised form of 9′-hydroxyl ABA, was found in similar amounts in sp5 and H-22-8-8 isolated roots, but was additionally detected in G29 roots (means of 75 ng g^{-1} DWt, 71 ng g^{-1} DWt and 28 ng g^{-1} DWt respectively). Finally, it appears that the *trans*-ABA isomer (which can be reversibly produced by the exposure of *cis*-ABA to UV light; although this seems unlikely to have happened in root cultures) was also detected in the roots of sp5, G29 and H-22-8-8 lines in similar amounts, 10 ng g^{-1} DWt, 11 ng g^{-1} DWt and 7 ng g^{-1} DWt respectively. The 7′-hydroxyl ABA catabolite was not present at detectable levels in any of the isolated root samples.

The ABA isomer and catabolite data presented in Figure 4.3.28 only distinguishes between two statistically significant categories of genotype (in contrast to the root culture carotenoid data in Table 4.3.14) the two groups are those in which the *SlNCED1* transgene is present, i.e. sp5, G29 and H-22-8-8, which accumulate

significantly greater amounts of ABA and ABA catabolites (with the exception of PA, which was not detected in G29) and those genotypes in which where the *SlNCED1* transgene is absent (i.e. Tm2a and BCH12).

4.4 DISCUSSION

4.4.1 The Parental Transgenic Lines – Altering Carotenoid Profiles

A true breeding triple transgenic line, that simultaneously and constitutively overexpresses *SlNCED1*, *SlBCH2* and *SlPSY1*, was created with the purpose of increasing carotenoid precursor flux through to ABA in roots. The triple line was generated from a cross between two double transgenic lines (MJ8 and G29) and throughout the breeding process the roots of sand grown cuttings were analysed by HPLC-PDA to assess their carotenoid profiles. It should be noted that freeze drying roots (as was carried out in this study) has been previously reported to cause some isomerization of neoxanthin, resulting in a reduction in the amount of all-*trans*-neoxanthin compared to 9′-*cis*-neoxanthin. The all-*trans*:9′-*cis* neoxanthin isomer ratio was reported to be 4.7:1 in frozen roots and 0.7:1 in freeze dried roots (Parry *et al.*, 1992). This should be born in mind for any future comparison of findings in this work to findings from tissues that were not freeze dried before analysis.

The double transgenic parental line MJ8 (Appendix I Table 7.1.1) constitutively overexpressing *SlPSY1* and *SlNCED1* was found to accumulate large amounts of lycopene (Figure 4.3.1). This was a novel feature, as lycopene was not detectable in the roots of either the WT Tm2a line or the G29 double transgenic line (Appendix I Table 7.1.1, constitutively overexpressing *SlBCH2* and *SlNCED1*). This lycopene accumulation appears to be a direct result of PSY1 enzyme overproduction and this observation is in agreement with published findings. The constitutive overexpression of *PSY1* constructs in transgenic tomato plants resulted in a visible increase in root

pigmentation and an accumulation of lycopene in vegetative tissues, which were not normally detectable in WT controls (Fray *et al.*, 1995; Jones, 2008). There was also a vast increase in β-carotene concentration in MJ8 roots compared with both G29 and WT roots, which may be due to increased cyclisation as a consequence of the high lycopene levels stimulating the rate of flux through the β-branch of the carotenoid biosynthesis pathway. This appears to have saturated the capacity of the non-transgenic BCH enzymes, creating a 'bottle neck' causing β-carotene to accumulate to excess levels. The total amount of carotenoids analysed in MJ8 sand grown roots was ~9x more than that found in both WT and G29 roots: predominantly comprised of β-carotene (~73 % of the total carotenoids) and lycopene (~24 % of the total carotenoids). It should be noted that this 3:1 ratio was reversed to a 1:3 ratio in the triple transgenic roots (~18% and ~71%). This difference appears likely to reflect increased use of β-carotene as a substrate for the excess BCH2 enzyme in the triple transgenic line. Fray *et al*., (1995) also reported an increase in total carotenoid levels (14%) in the vegetative tissues of *SlPSY1* overexpressing tomatoes. In this context it is interesting to note that an increase in total carotenoid content, involving a substantial rise in β-carotene content, was also reported in transgenic potato tubers overexpressing a construct containing a bacterial phytoene synthase gene (Ducreux *et al.*, 2005).

MJ8 roots also appeared to have severely depleted pools of 9′-*cis*-neoxanthin (0.06 μg g^{-1} DWt) and all-*trans*-neoxanthin (0.1 μg g^{-1} DWt) compared to WT Tm2a roots (0.5 μg g^{-1} DWt 9′-*cis*-neoxanthin and 0.6 μg g^{-1} DWt all-*trans*-neoxanthin). This reduction in neoxanthin isomers in MJ8 roots is likely to be due to increased activity of NCED1, which uses 9′-*cis*-neoxanthin as a substrate. This is supported by the root carotenoid profile of isolated sp5 roots in tissue culture (Table 4.3.14) which were also found to have significantly (P<0.01) reduced amounts of 9′-*cis*-neoxanthin (0.02 μg g^{-1} DWt) compared to WT Tm2a roots (0.05 μg g^{-1} DWt). Depletion of neoxanthin in

response to the overexpression of an NCED enzyme was also observed in tomato leaves in transgenic tomatoes overexpressing an *rbcS3C::SlNCED1* construct (Tung *et al.*, 2008). It should be noted that although the isolated roots in tissue culture accumulated approximately 10x less carotenoids compared to sand grown roots from an intact plant, the relative percentage compositions of the carotenoid profile in the control Tm2a roots were reassuringly similar, making it possible to make comparisons between the data sets.

The tissue cultured roots (Table 4.3.14) of the BCH12 line (Thompson *et al.*, 2007b), overexpressing only the *SlBCH2* gene, accumulated significantly ($P<0.01$) greater amounts of 9′-*cis*-neoxanthin (0.13 μg g^{-1} DWt), than either Tm2a roots (0.05 μg g^{-1}) or sp5 roots (0.02μg g^{-1}). The BCH12 roots in tissue culture also accumulated almost an order of magnitude more violaxanthin (0.02 μg g^{-1} DWt) compared to Tm2a roots (0.003 μg g^{-1} DWt). This was probably due to the higher rate of conversion of β-carotene to zeaxanthin causing a subsequent increase in the flux of β-xanthophylls through the pathway. Two epoxidation reactions are involved in the conversion of zeaxanthin to violaxanthin via the intermediate antheraxanthin and these are both catalysed by zeaxanthin epoxidase (ZEP) (Marin *et al.*, 1996; North *et al.*, 2007). Similar increases in leaf violaxanthin content have been reported for this transgenic tomato line (Sonneveld *et al.*, unpublished data) and equivalent transgenic lines in *Arabidopsis* (Davison *et al.*, 2002). Therefore, it does not appear to be necessary to upregulate the ZEP catalysed step in the pathway.

Perhaps more surprisingly the isolated roots of the BCH12 line accumulated significantly more β-carotene (mean of 0.05 μg g^{-1} DWt) than the sp5 roots (mean of 0.03 μg g^{-1} DWt), with the amount found in non-transgenic Tm2a roots being intermediate between the two (0.04 μ g g^{-1}DWt). This appears to indicate that there is a feedback mechanism in relation to BCH enzyme activity, which appears to automatically prevent depletion of the β-carotene pool by up-regulating earlier steps in the pathway. This result

is again consistent with findings in transgenic *Arabidopsis* plants (Davison *et al.*, 2002) overexpressing an *Arabidopsis* β-carotene hydroxylase (*chyB*) where increases in epoxycarotenoids, predominantly violaxanthin, were observed in leaves with no accompanying fall in β-carotene. Feedback mechanisms presumably operate in both of these species to maintain pool sizes of this important compound at optimum levels.

A carotenoid/xanthophyll profile of isolated roots overexpressing only *SlPSY1*, such as from the Z171D4A tomato line (Fray *et al.*, 1995) would have been valuable to provide a more complete isolated root culture data set (Table 4.3.14). It seems likely, based on other work overexpressing the PSY1 enzyme in tomato (Fray *et al.*, 1995; Fraser *et al.*, 2002; Fraser *et al.*, 2007), potato (Ducreux *et al.*, 2005; Diretto *et al.*, 2007), *Arabidopsis* (Lindgren *et al.*, 2003) and oilseed rape (Shewmaker *et al.*, 1999), that lycopene, lutein and β-carotene would all become more abundant. Ideally this set of root culture data would also have included roots from the MJ8 double transgenic lines, previously analysed and reported to have increased lycopene and β-carotene levels compared to Tm2a and sp5 roots, but the analysis was not reported in its entirety (Jones, 2008). Despite this, the carotenoid profiles of sand grown roots of this genotype (MJ8) are available for comparison with Tm2a and G29 roots to reveal characteristic differences in the root carotenoid profile (Table 4.3.1).

However, the carotenoid profiles of isolated G29 roots grown in tissue culture are not entirely consistent with those previously obtained from sand grown G29 roots; unlike those of the Tm2a roots which are consistent. This discrepancy in the G29 roots grown in tissue culture may be due to these samples experiencing nutritional stress due to sub-optimal growing conditions in the period immediately prior to harvest (as previously mentioned) and ideally these tissue cultures would be repeated. The impact of combining the overexpression of *SlBCH2* and *SlNCED1* on the carotenoid profile was previously discussed in relation to the sand grown G29 roots. Generally (observed from the four sets of sand grown cuttings) it appeared that G29 roots accumulated slightly more β-carotene

(~0.78 μg g^{-1} DWt) than Tm2a roots (0.5 μg g^{-1} DWt) and less 9′-*cis*-neoxanthin (~0.23 μg g^{-1} DWt) than Tm2a roots (~ 0.3 μg g^{-1} DWt). These differences are most likely to be due to the combined overproduction of the BCH2 and NCED1 enzymes. It might have been expected that the concentration of neoxanthin isomers in G29 would be slightly higher than in sp5, because of the increased BCH2 activity would have prevented excessive depletion of the β-xanthophylls due to the increased conversion of β-carotene to zeaxanthin, which in turn would be converted to violaxanthin and the neoxanthin isomers.

Previously it has been found that the sp5 (Thompson *et al.*, 2007a), BCH12, G29 (Balasubramanian, 2007; Sonneveld *et al.*, unpublished data) and MJ8 (Jones, 2008) genotypes all produced increased concentrations of ABA in leaves and roots, with the double transgenic lines producing more ABA than the single transgenic lines. The isolated root culture data presented herein (Figure 4.3.28) revealed that the sp5 and G29 transgenic lines accumulated significantly more root ABA than BCH12 and the WT, control Tm2a isolated roots. This data will be discussed in more detail in section 4.4.3 of this chapter discussion, when it is compared with data from the triple transgenic line.

The excessive accumulation of β-carotene in MJ8 roots, along with the reduced concentrations of the β-xanthophylls situated downstream of the BCH2 enzyme, supports the idea of a 'bottle-neck' at this point in the ABA biosynthesis pathway in roots, which made the creation of a triple transgenic line, additionally expressing *SlBCH2*, a plausible strategy for increasing root ABA production.

4.4.2 The Triple Transgenic Line Containing Three Constructs – The Shoot, Fruit and Root Phenotype

The triple transgenic lines manifested a number of abnormal phenotypes that had previously been associated with the overexpression of *SlPSY1* and *SlBCH2*. Firstly, the young leaves at the shoot apex of the triple transgenic plants exhibited severe

chlorosis giving them a mottled/speckled appearance (Figure 4.3.23), which has been previously identified as symptomatic of tomatoes constitutively overexpressing *SlPSY1* (Fray *et al.*, 1995). This leaf phenotype appeared to result from homozygosity for at least one *SlPSY1* transgene insertion locus and was observed in the MJ8 double transgenic lines (herein and in Jones, 2008). However it should be noted that this phenotype has not been observed in heterozygotes generated at various times during the crossing programme. This 'severe chlorosis' phenotype was first reported by Fray *et al.,* (1995) for the *SlPSY1* overexpressing line, who speculated that the reduction in chlorophyll may be due to a reduction in phytol which is required for chlorophyll formation and is formed from the same GGPP substrate pool as phytoene. It was proposed that the increased conversion of GGPP to phytoene by PSY1 diverts the GGPP substrate away from use in other branches of the plastidial (MEP) terpenoid biosynthesis pathway, thereby affecting chlorophyll biosynthesis and GAs (Fray *et al.*, 1995).

The dwarf phenotype, previously reported to be associated with *PSY1* overexpression (Fray *et al.*, 1995), was also observed in MJ8 plants and in some of the triple transgenic lines. In the triple transgenic lines it should be noted that the mean internode length (used as a measure of height) was not correlated with root lycopene content. This appears inconsistent with the relationship reported for the single transgenic *SlPSY1* overexpressor, where the most severely stunted transformants were found to have the most substantially increased phytoene and lycopene levels, concurrent with reduced levels of GAs (Fray *et al.*, 1995). However, a number of different T-DNA insertion loci contributed to the overall phenotype of the transgenic lines described in Fray *et al.,* (1995), some of which have been lost while 'breeding' the triple transgenic lines.

The immature fruit of some progenitors of the triple transgenic lines developed pigmentation very early in development and appeared a pale peach colour (Figure 4.3.2), although in the finally selected triple transgenic lines the immature fruit appeared a very pale, almost white colour. This early carotenoid pigmentation/premature chlorophyll loss

phenotype was previously reported to be associated with the constitutive overexpression of *SlPSY1* in tomatoes; Fray *et al*., (1995) observed that the fruit of transgenic tomatoes overexpressing *SlPSY1* produced lycopene much earlier in development than is normal. Fraser *et al.* (2007) examined this phenotype in more detail and reported that the changes in carotenoid composition in the fruit were associated with the development of chromoplast-like structures earlier than is usual. The immature fruit of the final selected triple transgenic lines all appeared to lack chlorophyll compared to the typical green coloured immature fruit phenotype. This premature loss of chlorophyll may be associated with an alteration in plastid morphology/development. However, further investigation of this aspect of fruit phenotype was beyond the scope of the present study.

The roots (Figure 4.3.8) and the basal parts of the stems (Figure 4.3.27) of the triple transgenic lines appeared orange in colour, probably due to the accumulation of β-carotene and lycopene. This was consistent with the observations of the MJ8 parental lines (Jones, 2008) and the single *SlPSY1* overexpressing line (Fray *et al.*, 1995). It can be speculated that the changes in plastid morphology and functioning observed in immature fruit of *SlPSY1* overexpressing tomatoes by Fraser *et al.,* (2007) may also be occurring in the plastids of the pigmented roots and stems of these plants. It would be interesting to examine the plastid structure of these pigmented tissues, and to explore how the relationship between this change in plastid morphology/development towards more chromoplast-like structures is directly related to the overexpression of the *SlPSY1* gene. It would be interesting, but also beyond the scope of the present study, to explore these alterations in relation to links between the plastidial developmental pathway and the carotenoid biosynthesis pathway (of which the conversion of GGPP to phytoene is the first committed step).

The overexpression of *SlBCH2* in transgenic tomatoes surprisingly resulted in the mature fruit ripening to orange rather than red, due to reduced lycopene and total carotenoid levels compared to WT Tm2a fruit (Balasubramanian, 2007; Sonneveld *et al.*,

unpublished data). This orange fruit phenotype was maintained constantly through the breeding programme and was therefore conferred to the triple transgenic line (Figure 4.3.24). The triple transgenic fruit carotenoid profiles have not been analysed to date, and it would be interesting to investigate the impact of the simultaneous overexpression of the three carotenoid/ABA biosynthesis enzymes on the fruit pigment profiles throughout development and ripening. The reduced size of the mature fruit of the triple transgenic lines indicates that the fruit development, maturation and ripening processes have been substantially altered/affected by the transgenes. The precise cause/s of this, which may or may not directly involve the increased plant ABA content, require further investigation.

The sand grown roots of the triple transgenic lines (Table 4.3.6) produced a minimum of ~9x more total carotenoids (ranging from a mean of 12.8 μg g^{-1} DWt in H-22-22-6_{F3} to a mean of 20.4 μg g^{-1} DWt in H-22-8-8_{F3}) compared to the WT Tm2a plants which had a mean of only 1.8 μg g^{-1} DWt. This relationship was also observed in the roots grown in tissue culture, where the triple transgenic roots accumulated ~ 39x more total carotenoids than the corresponding Tm2a roots. These increases were mainly due to substantial increases in the levels of lycopene and β-carotene; the lycopene content, which in isolated triple transgene roots accounted for ~71% of the total carotenoids and in the sand grown roots ranged from 5.3 μg g^{-1} DWt (42% of the total carotenoids analysed) in H-22-22-6_{F3} to 9 μg g^{-1} DWt (52% of the total carotenoids analysed) in H-22-8-8_{F3}. The amount of lycopene obtained from these triple transgenic lines appeared to be even greater than that previously found in sand grown roots of cuttings from the parental double transgenic MJ8H plant (3.6 μg g^{-1} DWt, 28 % of the total).

The β-carotene levels in the isolated roots of the triple genotypes were ~30x higher than those found in Tm2a roots grown in tissue culture. In the sand grown roots of the three triple transgenic lines, β-carotene levels ranged from 5.2 μg g^{-1} DWt (41% of the total) in H-22-22-6_{F3} to 9.1 μg g^{-1} DWt (45% of the total) in H-22-8-8_{F3}, which was

~12x greater than the amount of β-carotene found in the WT Tm2a roots (only 0.58 μg g^{-1} DWt, although this still represented around 30% of a much smaller total). The triple transgenic roots accumulated (on the whole, average 6.9 μg g^{-1} DWt) a little less β-carotene than the MJ8H parental plant 9 μg g^{-1} DWt, but β-carotene accounted for ~41% of the total carotenoids analysed in the triple roots compared to 69% in the double transgenic parent. This altered composition is associated with a 4x increase in xanthophyll accumulation in the triple transgenic lines, which had a mean of ~2 μg g^{-1} DWt xanthophylls, compared to the MJ8H parental plant which accumulated only 0.5 μg g^{-1} DWt xanthophylls. This difference was mainly due to an increase in the β-ring xanthophylls from less than 1% of the total in the MJ8H plant up to a maximum of 5% in the triple transgenic plants. This is likely to be due to the increased activity of the extra transgene encoded BCH2 enzyme, using the additional β-carotene substrate which also accumulated in the MJ8 double transgenic lines, to produce increased flux through the β-branch of the ABA biosynthesis pathway, as initially hypothesized. The increase in these carotenoid pools and the apparently increased flux through the biosynthetic pathway would be expected to have resulted in an increase in root ABA accumulation (discussed further at the end of section 4.4.3).

4.4.3 The Triple Transgenic Line Containing Three Constructs – As a Rootstock

Two gravimetric water use trials were conducted to determine whether or not using the triple transgenic lines as rootstocks, results in sufficient additional root synthesised ABA to detectably affect stomatal behaviour in the non-transgenic scion and, to increase whole plant water use efficiency. The first of these trials, involved the use of rootstocks from all three triple transgenic lines, grafted to WT, Tm2a scions; i.e. Tm2a/H-22-8-8 (scion/rootstock), Tm2a/H-22-22-6, and Tm2a/H-22-22-7, along with Tm2a/Tm2a self grafts and Tm2a/G29 controls. It had been previously shown that the

G29 double transgenic line, despite producing increased amounts of ABA (Sonneveld *et al.*, unpublished data), does not produce enough of a root-sourced ABA signal to improve whole plant WUE when used as a rootstock grafted to a Tm2a scion (R.C. Smeeton, personal communication).

The results of this 30 day gravimetric water use trial, despite not producing statistically significant differences between the graft combinations (Table 4.3.8), did appear to indicate that the triple transgenic line H-22-8-8 might be having a slight negative effect on whole plant water use; the Tm2a/H-22-8-8 graft combination had the highest mean TE_p value of 4.5 g DWt Kg H_2O^{-1} compared to the Tm2a self graft mean of 3.6 g DWt Kg H_2O^{-1}. Additionally, the Tm2a/H-22-8-8 graft combination had the highest (least negative) mean $\delta^{13}C$ value of -30.9, indicating a higher WUE, which was significantly ($P<0.05$) greater than the Tm2a/G29, Tm2a/H-22-22-6 and Tm2a/H-22-22-7 graft combinations, but not significantly greater than the Tm2a self graft which had a mean $\delta^{13}C$ of -31.3.

The root exudate flow rate from detopped root systems of the Tm2a/H-22-8-8 graft combination was significantly ($P<0.01$) greater (mean flow rate 2.3 ml h^{-1} than all the other combinations, including the Tm2a self grafts mean flow rate 1.2 ml h^{-1}). The Tm2a/H-22-8-8 plants also had the highest mean xylem sap ABA concentration ($[ABA_{xyl}]$ = 48.3 n*M*) of the plants analysed, which was similar to the other grafts with transgenic rootstocks (H-22-22-6, H-22-22-7 and G29). These were all significantly ($P<0.05$) higher ABA levels than that detected in xylem sap from the Tm2a self grafts, which had a mean $[ABA_{xyl}]$ of only 3.6 n*M*. It should be noted that the collection of xylem sap from roots via exudation has been reported to overestimate xylem hormone concentration because the solute concentration increases as the xylem flow rate declines (Munns, 1985; Else *et al.*, 1995; Schurr and Schulze, 1995; Dodd, 2005). Since root exudation rate increases with root ABA concentration (Nagel *et al.*, 1994) differences in

[ABA_{xyl}] between the Tm2a rootstocks and the transgenic ones producing more ABA, would be minimised. This is because the sap flow from the higher ABA roots should be faster, thereby decreasing [ABA_{xyl}], as pointed out by Dodd *et al*., (2009). Therefore the higher exudate [ABA_{xyl}] detected in the transgenic double and triple rootstocks, compared to that found in the Tm2a self grafts, probably underestimates the differences in [ABA_{xyl}] as the xylem sap exits from the root system in the intact plant.

The second gravimetric water use trial focused on the previously best performing triple transgenic line from the first trial (i.e. H-22-8-8), and was conducted at a more ideal time of year with regard to temperature, light and levels of water use (i.e. late May/early June as opposed to the less ideal late November/early December period). The trial involved the use of the Tm2a/H-22-8-8 graft combination along with Tm2a self grafts as the WT control and sp5 self grafts as a positive control. The sp5 transgenic line (constitutively overexpressing *SlNCED1*) had been previously reported to have a significantly higher WUE than Tm2a plants (Thompson *et al.*, 2007a). The results presented herein are congruent with these published findings, in that the sp5 self grafts had significantly ($P<0.05$) greater TE_p (4 g DWt Kg H_2O^{-1}) than the Tm2a self grafts (2.8 g DWt Kg H_2O^{-1}). There was no significant difference in TE_p between the Tm2a self grafts and Tm2a/H-22-8-8 grafts (3 g DWt Kg H_2O^{-1}). It should be noted that the improved WUE of the sp5 self grafts was due to the fact that they produced significantly more biomass (74 g DWt) over the 30 d trial period, compared to the Tm2a self grafts and the Tm2a/H-22-8-8 plants, which produced almost identical amounts of biomass to each other (54.40 g DWt and 54.41 g DWt, respectively). The sp5 self grafts were also found to have significantly longer mean internodes than the Tm2a scions and significantly longer leaf lengths than the Tm2a self grafts, which was consistent with the increased petiole length of the sp5 plants (compared to WT) previously reported by Thompson *et al*. (2007).

The total amount of water transpired by the Tm2a/H-22-8-8 plants (18.30 Kg H_2O) was lower than that used by the Tm2a self grafts (19.56 Kg H_2O), but this was not statistically significant overall, due to high levels of variation from day to day throughout the experimental period. However, it is important to note that from similar initial biomasses these two graft combinations produced almost identical amounts of biomass over the 30 day trial period. As a consequence of this the water use of the Tm2a self grafts and the Tm2a/H-22-8-8 plants could be compared on a daily basis, with the not unreasonable assumption that the rate of biomass production was matched between these two combinations on a daily basis, as well as over the 30 day trial period as a whole. The Tm2a/H-22-8-8 plants used significantly less water than the Tm2a self grafts on the most 'stress free' days of 30 day trial period. When the mean daily water use of the Tm2a self grafts was used as a baseline measure to subtract from each individual Tm2a/H-22-8-8 plants daily water use it became apparent that any significant differences in water use were always associated with the Tm2a/H-22-8-8 plants using less water than the control Tm2a self grafts. The Tm2a/H-22-8-8 plants (on average) used less water than the control Tm2a self grafts on 26 days of the 30 day trial, if there was no real difference between these two groups then this data would have been random. These differences in daily water use are supportive of the hypothesis that the H-22-8-8 triple transgenic rootstock is capable of having a positive water saving effect by restricting stomatal opening and reducing whole plant water use in the non-transgenic scion, without having a negative impact on above ground biomass production.

There was no significant difference between the Tm2a self grafts and Tm2a/H-22-8-8 plants with regard to the root exudate [ABA] (means of 19.7 n*M* and 22.8 n*M* respectively) or flow rate (3.6 ml h^{-1} and 4.4 ml h^{-1}) in the second trial, although in both cases Tm2a/H-22-8-8 once again had the greater mean value. The sp5 self grafts were found to have significantly greater root exudate [ABA] (mean of 50.7 n*M*) and flow rate, (mean of 7.8 ml h^{-1}) than both the Tm2a self grafts and the Tm2a/H-22-8-8 plants. It is

interesting to couple these results with the findings that the isolated tissue culture grown roots (Table 4.3.14) of both sp5 and H-22-8-8 genotypes had similar root ABA concentrations [ABA_{root}] and that these were significantly greater than the [ABA_{root}] found in isolated Tm2a roots. It can be speculated from this that the significantly greater [ABA_{xyl}] and exudate flow rate found in the sp5 self grafts compared to the Tm2a/H-22-8-8 plants is probably due to the recirculation of ABA from the sp5 shoot, to the root system, and back up to the shoot via xylem sap. This also reflects the previously mentioned findings that the root exudation rate increases with root ABA concentration (Nagel *et al.*, 1994).

The lack of a significant difference between the [ABA_{xyl}] of the Tm2a self grafts and the Tm2a/H-22-8-8 plants, considering that the H-22-8-8 isolated root cultures produced significantly more ABA than the Tm2a isolated roots could also be in part due to a recirculation effect. It could be speculated that the extra ABA produced by the H-22-8-8 rootstock is initially transported via the xylem sap to the Tm2a scion, where it enters a physiologically active pool and can therefore explain the modest restriction of stomatal opening. However, the total amount of shoot ABA has not been elevated sufficiently to increase the ABA recirculated back to the root system much above that of a normal Tm2a scion.

Studies of root systems grown in tissue culture avoid the complications of recirculation of ABA between shoot and root systems. The isolated roots of the sp5, G29 and H-22-8-8 genotypes not only had significantly more *cis*-ABA than the WT Tm2a and BCH12 lines, but also had significantly more ABA metabolites. The presence of large amounts of various products of ABA catabolism indicates that ABA was being synthesized excessively in some of the transgenic roots and then being further metabolized to physiologically inactive compounds. Both DPA, from the major ABA 8'-hydroxylation pathway and the main ABA conjugate (ABA-GE) were detected in all five genotypes. Additionally PA was detected in the isolated roots of sp5 and H-22-8-8, which

further implies that the 8′-hydroxylation pathway, starting with the hydroxylation of ABA to form PA and its subsequent reduction to DPA, was strongly activated in all of the transgenic lines which overexpressed *SlNCED1*. The detection of PA in these roots is consistent with previously published reports where increases in ABA content of stressed transgenic *Arabidopsis* plants overexpressing *AtNCED3* resulted in increased levels of PA (Iuchi *et al.*, 2001; Priest *et al.*, 2006). These results were also consistent with published findings that the overexpression of bean *PvNCED1* in transgenic tobacco (*N. Plumbaginifolia*) plants resulted in increases in PA.

Additionally neoPA, a product of the recently discovered 9′ hydroxylation pathway of ABA catabolism, was detected in sp5, G29 and H-22-8-8 roots. NeoPA has previously been detected in drought stressed barley and oilseed rape seedlings (Zhou *et al.*, 2004), and its detection in the roots of the tomato transgenic lines is consistent with the idea that multiple pathways of ABA catabolism have been activated in these genotypes in response to their increased production of ABA. When the ABA *cis* and *trans* isomer concentrations are grouped together with the ABA conjugate (ABA-GE) and the various ABA hydroxylation catabolites measured, it can be concluded that the isolated roots of the transgenic sp5, G29 and H-22-8-8 lines produced significantly and substantially greater quantities of ABA compared to the isolated roots of BCH12 and Tm2a. The common factor here appears to be the presence or absence of the *SlNCED1* transgene, emphasising its key role in the control of ABA biosynthesis.

4.4.4 Conclusions & Future Work

It can be concluded from these results that the simultaneous and constitutive overexpression of *SlNCED1*, *SlBCH2* and *SlPSY1* in transgenic tomato lines increases flux through the carotenoid biosynthesis pathway leading to increased ABA biosynthesis. The potential value of these triple transgenic lines as a ‘water-saving’ rootstock remains to be fully established: the Tm2a/H-22-8-8 (scion/rootstock) plants used significantly less

water than the Tm2a self grafts on some individual days, mainly during the first week of the 30 day trial period. However for 73% of the trial period when the more severe environmental conditions were allowed to result in water stress being experienced by all plants the level of water use by the Tm2a self grafts was restricted to the same extent as the Tm2a/H-22-8-8 grafts.

There is compelling evidence to suggest that if the water supplied constantly meets demand and soil moisture is always maintained at 'field capacity' significant water savings by the Tm2a/H-22-8-8 plants could have been detected on almost everyday of the trial period. It is likely that if the plants had not experienced water stress during the trial, i.e. watering more than once a day during hot weather, an overall difference in WUE would have been established. Allowing the plants to experience water stress would have triggered the Tm2a self grafts to synthesise additional stress induced ABA, which would have obscured any genetic differences between the graft combinations. The present data is suggestive but not conclusive and further testing of the rootstock is on-going.

It would also be interesting to repeat and extend the experiments on isolated root cultures, to confirm the current preliminary findings and to complete the data set for the individual and cumulative effect of each transgene on the root carotenoid biosynthesis pathway, and ABA biosynthesis and catabolism. Additionally, it is possible that both ABA and some ABA catabolites may have been exported from the root system to the culture media. In addition to testing the root tissue, the liquid tissue culture media should also be analysed for the ABA isomers and catabolites.

The triple transgenic genotypes did produce increased root sourced ABA, but this was quickly catabolised. However, it should be reiterated that the analyses from the isolated roots grown in tissue culture are from a highly artificial system and should be interpreted as indicative, as opposed to definitive in terms of total ABA export from root to shoot. With this in mind, an interesting/surprising feature from these results is that the sp5 roots appeared to produce as much ABA and ABA metabolites as the triple

transgenic H-22-8-8 roots. However, the carotenoid analysis from the same material suggested that more substrate was being made available to the NCED1 enzymes present in the H-22-8-8 roots than in sp5; implying a potentially greater flux through the pathway to ABA and its metabolites in the former case. One question would appear to be 'if more ABA was synthesized, where has it gone?' It could be a matter of export from isolated roots to the culture medium or perhaps sub-optimal growing conditions resulted in nutrient stress induced ABA production in some genotypes, obscuring the real differences between genotypes. The significant changes in stomatal behaviour, (indicated by reduced water use), on a daily basis in the Tm2a/H-22-8-8 (scion/rootstock) compared to Tm2a self grafts, suggests that the triple transgenic rootstocks must be exporting more ABA from the root systems to a biologically active pool of apoplastic ABA arriving at the stomata in the leaves.

The next step could be to create a transgenic rootstock that can produce a strong enough root-sourced ABA signal to influence whole plant WUE by artificial manipulation of the main ABA catabolism pathways. One method would be to overexpress a β-glucosidase which has been shown to hydrolyse ABA-GE back to ABA. The *Arabidopsis* β-glucosidase1 (AtBG1) enzyme activity has been reported to increase in response to drought stress, which quickly induces the release of biologically active ABA from the inactive pool of ABA-GE (Lee *et al.*, 2006). *AtBG1* or the equivalent tomato *AtBG1* homologues are possible targets for overexpression.

An alternative method to increase ABA levels would be to block the hydroxylation of ABA, through the creation of knock out lines. The most obvious candidate would appear to be the 8′-hydroxylase, from the major ABA hydroxylation pathway, which would prevent the formation of 8′-hydroxyl ABA thereby avoiding the accumulation of PA and DPA that was found in the isolated root cultures of the transgenic lines. Four *CYP707A* genes encoding ABA 8′-hydroxylases have been identified in tomato (Vriezen *et al.*, 2008; Nitsch *et al.*, 2009) and it is tentatively

suggested that the tomato homologue of the *AtCYP707A3*, which has been reported to be localised mainly in the vascular tissues and particularly responsive to drought (Okamoto *et al.*, 2009), might be a sensible target for silencing.

Increasing the pool of biologically active ABA in the transgenic roots by blocking the hydroxylation of ABA and also recovering ABA from the conjugated ABA-GE could generate a stronger root sourced ABA signal.

CHAPTER 5:

GENERAL DISCUSSION

5.1 RESEARCH SUMMARY

The work presented in this thesis involved two approaches to potentially improve the water use efficiency of tomatoes (*S. lycopersicum*) via manipulation of the ABA biosynthesis pathway. The first approach was part of a larger joint Nottingham University and Warwick-HRI (DEFRA funded) project to investigate the natural allelic variation of the *NCED1* gene in the group of wild species most closely related to the cultivated tomato. The results presented herein relate to the introgression of the *S. galapagense* and the *S. neorickii NCED1* alleles (*SgNCED1* and *SnNCED1* respectively) into the cultivated tomato genetic background, *S. lycopersicum* cv. Alisa Craig. This part of the programme involved four backcrosses, followed by selfing and the selection of F_2 progeny homozygous for either the respective wild species allele or the *SlNCED1* allele from the cultivated tomato, but segregating at random for other interspecies differences throughout the rest of the genome. These pairs of homozygous lines were subject to a 30 d gravimetric water use trial, which did not reveal any significant differences in the effect of these two wild species alleles on WUE compared to the appropriate control.

A phylogenic tree of the NCED1 protein sequences from ten wild species closely related to tomato (Harrison L. unpublished findings) revealed that the *SgNCED1* and *SnNCED1* alleles are not very divergent from each other. The *SgNCED1* allele falls into the same clade as the cultivated *SlNCED1* allele (clade 1), while *SnNCED1* was categorised into clade 2, which is the least divergent of the six identified clades from clade 1. The more divergent wild species *NCED1* alleles however may confer a difference, and investigations into these are currently ongoing. It has also been reported

(Harrison L. personal communication) that the wild species promoter sequences show very little homology (unlike the *NCED1* coding sequences mentioned earlier in section 3.4.1) and therefore may function differently when introgressed into the cultivated tomato genetic background.

It is also possible that natural variation in the expression of *NCED1* genes, to an extent that could have an impact on whole plant water use, may not be present in the wild species populations; plants tend to conserve cellular water to maintain water status rather than to conserve soil water, because of competition between plants for available soil water. The use of the wild tomato relatives as a resource for improving tomato water use efficiency by other methods/traits is discussed in section 5.2.4. While the existence of naturally occurring variation in the expression of *NCED1* alleles cannot be ruled out, this method of improving tomato water use efficiency, i.e. overexpressing *NCED1* genes to increase the concentration of ABA in order to restrict stomatal opening, may only be achievable by transgenic means.

The second approach to improve WUE in tomatoes described in this thesis, involved the creation of a genetically modified rootstock that constitutively overexpressed three key enzymes in the ABA biosynthesis pathway, such that it would produce sufficient ABA to improve the WUE of grafted non-GM scions. This 'semi-GM' approach may circumvent some of the objections made against the use of genetically modified crops since the transgenes would not be present in the edible crop or the sexually reproductive organs of the cropping plants, which would limit the risk of accidental transgene transfer. Additionally, due to the extreme shoot chlorosis phenotype and reduced growth rate, any vegetative growth from the rootstock would be extremely visible.

The triple transgenic line constitutively overexpressing *SlPSY1*, *SlBCH2* and *SlNCED1* was generated by crossing two double transgenic lines (the breeding pedigree is presented in Appendix VII). The first double transgenic line, G29 overexpressed two

key enzymes involved in ABA biosynthesis; the first, *SlNCED1*, has been previously identified as an important rate limiting enzyme for ABA biosynthesis (Thompson *et al.*, 2000b; Qin and Zeevaart, 2002). The isolated roots of the sp5 transgenic line, which overexpresses *SlNCED1*, were found to have increased [ABA] compared to WT roots, which was consistent with previous findings from this transgenic line (Thompson *et al.*, 2007b). Reduced amounts of the neoxanthin isomers were detected in the sp5 roots indicating that, as suggested by Taylor *et al*., (2005), the supply of the xanthophyll substrate required by NCED was limiting in roots. These results agreed with previous reports of the depletion of neoxanthin isomers in tomato plants more strongly overexpressing *SlNCED1* (Tung *et al.*, 2008). The second enzyme overexpressed in the double transgenic line (G29) is *SlBCH2*, which encodes an enzyme which acts upstream of NCED and has also been identified as potentially rate-limiting in the ABA biosynthesis pathway (Thompson *et al.*, 2007b; Sonneveld *et al.*, unpublished data). The tissue culture grown roots of the single transgenic line BCH12 (Thompson *et al.*, 2007b) overexpressing *SlBCH2*, had increased amounts of epoxyxanthophylls, notably 9′-*cis*-neoxanthin and violaxanthin and increased amounts of β-carotene. This was consistent with previous findings from this line (Sonneveld *et al.*, unpublished data) and published reports from *Arabidopsis* plants overexpressing an *Arabidopsis* β-carotene hydroxylase encoding construct (Davison *et al.*, 2002).

The combined overexpression of constructs encoding these two enzymes in the G29 double transgenic line was found to increase the supply of β-xanthophylls for use by the overproduced NCED1 enzyme, since the neoxanthin isomers were found in greater amounts when compared to sp5 roots. The isolated roots of the G29 line were also found to have increased [ABA] compared to the WT and BCH12 roots. The combined overexpression of these two genes has been previously reported to increase leaf ABA content and to confer a greater than additive increase on whole plant WUE (Sonneveld *et al.*, unpublished data). However, when G29 was grafted as a rootstock to a WT scion, any

increase in root sourced ABA was insufficient to improve WUE (R.C. Smeeton, personal communication).

The second double transgenic line MJ8, used to generate the triple transgenic line overexpressed *SlNCED1* and *SlPSY1* (Jones, 2008), although this line was segregating for a number of T-DNA insertion loci carrying an unknown number of copies of the *35S::SlPSY1* construct (Fray *et al.*, 1995). The *SlPSY1* enzyme has been previously shown to increase the supply of some of the carotenoid precursors required to sustain increased ABA biosynthesis in roots (Fray *et al.*, 1995; Taylor *et al.*, 2005; Li *et al.*, 2008). The roots of the MJ8 line were found to accumulate lycopene, which was not detectable in the roots of the WT Tm2a or G29 lines. This confirmed previous descriptions of this line (Jones, 2008) and agreed with published descriptions of transgenic tomato lines constitutively overexpressing *SlPSY1*. Root pigmentation and an accumulation of lycopene was detected in vegetative tissues, that was not detectable in WT controls (Fray *et al.*, 1995). There was also an accompanying large increase in β-carotene levels in the roots of MJ8 plants compared to WT Tm2a and G29 lines, which was presumably due to the cyclisation of some of the additional lycopene present. This appears to saturate the BCH enzymes, resulting in an accumulation of β-carotene. These findings are consistent with reports that the overexpression of constructs containing a bacterial phytoene synthase in transgenic potato plants resulted in an accumulation of lutein in the tubers (Ducreux *et al.*, 2005). The accumulation of β-carotene in the MJ8 line supports the idea of a 'bottle neck' at this point in the pathway. The MJ8 lines also appeared to have severely depleted pools of neoxanthin isomers presumably due to the overexpression of *SlNCED1*. Both the MJ8 double transgenic line and the single *SlPSY1* overexpressing lines were previously reported to have increased [ABA_{root}] (Jones, 2008), but as with the G29 double transgenic line, the increase in [ABA_{root}] was insufficient when grafted as a rootstock, to have an impact on stomatal behaviour in a non-transgenic WT scion (Jones, 2008).

Two gravimetric water use trials were conducted to assess the effect of the triple transgenic lines when used as rootstocks grafted to non-GM Tm2a scions. Overall the results were indicative of a positive effect of the triple transgenic rootstocks on WUE, although the results were not definitive. The first trial revealed that H-22-8-8 was the most effective of the three triple transgenic lines assessed, having in the highest mean TE_p and the highest (least negative) mean $\delta^{13}C$ value; both results being indicative of improved WUE, despite not being found to be statistically greater than the Tm2a self grafts. Additionally the Tm2a/H-22-8-8 (scion/rootstock) graft combination had a significantly greater root exudate flow rate, as measured from detopped root systems and a significantly higher [ABA_{xyl}] than the WT.

ABA is known to increase root hydraulic conductivity (Lp_r) (Glinka, 1980; Hose *et al.*, 2000) which has been previously suggested to be partly explained by the upregulation of aquaporin genes (Zhu *et al.*, 2005; Tardieu *et al.*, 2010). It can therefore be speculated that the increased [ABA_{xyl}] of the H-22-8-8 rootstock, compared to Tm2a self grafts, had resulted in a greater Lp_r, possibly due to an increase in aquaporins, which might explain the higher root exudate flow rate. Additionally, the increased [ABA_{xyl}] may also indicate that increased [ABA_{root}] is being exported from the root system to the Tm2a shoot. Increases in hydraulic conductivity have been reported to result in an increase in tissue water status (Parent *et al.*, 2009) and it is therefore likely that the Tm2a/H-22-8-8 plants may have had improved leaf water status.

The second trial focused on the Tm2a/H-22-8-8 graft combination, which was again found to have a slightly higher TE_p value than the Tm2a self grafts, although the difference was not statistically different over the whole 30 day experimental period. These two graft combinations however did produce almost identical above ground biomass over the trial period, which meant that the water use could be explored on a daily basis. The comparisons revealed that any significant differences in water use were always

associated with days during which the Tm2a/H-22-8-8 graft combination used less water than the Tm2a self grafts. The Tm2a/H-22-8-8 plants used less water on 26 days of the 30 day trial. The Tm2a/H-22-8-8 plants were again found to have a greater root exudate flow rate and [ABA_{xyl}] than the Tm2a self grafts, although the differences were not statistically significant from this second trial. However, it can be speculated that the extra ABA produced by the H-22-8-8 rootstock was transported to theTm2a scion, where it had a modest but consistent negative effect on whole plant water use.

Collectively the results from both of the gravimetric water use trials are persuasive of the H-22-8-8 triple transgenic line, when used as a root stock, having a small negative effect on whole plant water use by the non-transgenic Tm2a scion, without having a negative impact on biomass production.

The simultaneous overexpression of *SlPSY1*, *SlBCH2* and *SlNCED1* in triple transgenic tomato lines did apparently result in an increase in carotenoid precursor flux through the pathway. The tissue cultured roots of the triple transgenic line accumulated at least 12x more carotenoids than comparable isolated transgenic lines (i.e. G29, sp5 and BCH12) and around 39x more carotenoids than comparable isolated WT Tm2a roots. This increase was mainly accounted for by the accumulation of large quantities of lycopene and β-carotene. The previously mentioned depletion of the neoxanthin isomers was not evident in the triple transgenic lines. This was likely to be due to the additional overproduction of both the BCH2 and PSY1 rate limiting enzymes upstream of NCED1 which committed additional carotenoids and β-xanthophylls to ABA biosynthesis.

The isolated roots of the sp5, G29 and H-22-8-8 genotypes were not only found to have significantly more *cis*-ABA than the Tm2a and BCH12 lines, but also contained significantly more ABA catabolites. The accumulation of additional amounts of these biologically inactive ABA catabolites indicates that increased amounts of ABA had been initially synthesized, but was then further metabolized. Both DPA and ABA-GE were detected in each of the lines analysed (Tm2a, BCH12, sp5, G29 and H-22-8-8) but

in significantly greater quantities in the latter three. Additionally, the roots of the three transgenic lines containing the *SlNCED1* construct, i.e. sp5, G29 and H-22-8-8 also accumulated neoPA, and the *trans*-ABA isomer which were not detectable in the comparable WT Tm2a control or BCH12 roots. Additionally, PA was detected only in the isolated roots of sp5 and H-22-8-8. The detection of PA in transgenic plants with increased levels of ABA has also been reported in *Arabidopsis* plants overexpressing *AtNCED3* (Iuchi *et al.*, 2001; Priest *et al.*, 2006) and in tobacco (*N. Plumbaginifolia*) plants overexpressing bean *PvNCED1* (Qin and Zeevaart, 2002). NeoPA, a product of the 9′-hydroxylation pathway was also detected in the sp5, G29 and H-22-8-8 isolated roots.

The detection of these increased amounts of ABA catabolites, as well as increased amounts of the ABA isomers is consistent with the idea that these lines are producing increased amounts of ABA in the roots and that multiple ABA catabolism pathways have been activated in response, and may limit the ability of the triple gene approach to produce enough active ABA to influence the stomatal conductance of the shoot. It is interesting to note that the sp5 and H-22-8-8 roots appeared to produce equivalent amounts of ABA and ABA metabolites; whereas the carotenoid analysis indicated that there was an increase in precursor flux through the pathway to ABA in H-22-8-8 roots compared to sp5 roots. As mentioned in section 4.4.4 the isolated root culture analysis is not definitive and if more ABA was produced by the triple transgenic roots, it may have been exported to the culture media, or perhaps sub-optimal growing conditions/nutrient stress could have elevated ABA production in all genotypes, which could have obscured real differences between the genotypes.

The consistent but modest reduction in water use conferred by the H-22-8-8 rootstock compared to a WT rootstock is indicative that the triple rootstocks should be exporting more ABA to a biologically active pool of apoplastic ABA, which has a modest negative effect on stomatal conductance, without negatively impacting on biomass production.

5.2 AREAS OF FUTURE RESEARCH

5.2.1 Considerations from the Current Research Programme

Further testing of the effectiveness of the H-22-8-8 triple transgenic line, as a rootstock, on whole plant water use when grafted to a non-GM scion is on-going. Additionally, it would be valuable to repeat the analysis of isolated root cultures to confirm the preliminary findings, analyse the liquid culture media and to expand the genotypes tested to investigate more fully the individual and cumulative effect of each transgene; not only on ABA biosynthesis and catabolism, but also on carotenoid composition. Ideally, a single transgenic tomato line homozygous for the same *SlPSY1* T-DNA insertion present in the triple H-22-8-8 line would be created, since a southern blot indicated that H-22-8-8 has a single T-DNA insertion site. H-22-8-8 could be crossed to Tm2a and F_2 plants homozygous for just the *SlPSY1* insertion would be selected. The resulting line would be more ideal material than the original more complex Z171 parental line (Fray *et al.*, 1995). Additionally, an equivalent double transgenic line overexpressing *SlPSY1* and *SlNCED1* could be generated in a similar way by crossing to the sp5 line, which would be more comparable than the original parental MJ8 double transgenic line. These new genotypes would be the ideal plant material for investigating the alterations in the carotenoid/ABA and ABA catabolism pathways of overexpressing these three enzymes in various combinations.

The results presented herein indicate that the H-22-8-8 triple transgenic line does produce increased amounts of ABA which may have a modest but consistent effect on the WT scion WUE, although the observed effect was not statistically significant. The increased ABA appears to be rapidly catabolised in the roots; it may be possible to increase the amount of ABA transported from the rootstock to the WT scion in order to have a large effect on scion WUE. This could be done by targeting the ABA catabolism pathways for manipulation. One method would be to hydrolyse the excess ABA-GE pool

in the triple transgenic roots to release ABA via the overexpression of a β-glucosidase gene. It has been reported by Lee *et al.,* (2006) that the AtBG1 enzyme (ABA-GE specific *Arabidopsis* β-glucosidase1) can catalyse the release of biologically active ABA from ABA-GE. The identification of possible tomato *AtBG1* homologues for overexpression, or the direct use of *AtBG1* could further increase the pool of ABA sufficiently to generate a strong enough root sourced signal to have a statistically significant positive effect on whole plant WUE.

An alternative, or possibly accompanying method of recovering ABA which would otherwise be unavailable due to metabolism, would be to block the hydroxylation of ABA through the creation of knockout lines, e.g. by using RNAi constructs. The most obvious target for this approach would be the 8′-hydroxylase, from the major ABA hydroxylation pathway. Preventing the formation of 8′-hydroxyl ABA and the subsequent accumulation of PA and DPA, as was found in the isolated root cultures of the transgenic lines, may further increase formation of ABA-GE; but, as already mentioned, ABA can be recovered from ABA-GE (section 4.3.7). Four *CYP707A* genes encoding ABA 8′-hydroxylases have been identified in tomato (Vriezen *et al.*, 2008; Nitsch *et al.*, 2009), similar to the four found in *Arabidopsis* (Kushiro *et al.*, 2004; Saito *et al.*, 2004). Considering that the 8′-hydroxylases have been reported to have differential expression patterns (Okamoto *et al.*, 2009), and that *SlCYP707A1* and *AtCYP707A4* have been reported to have high homology (Nitsch *et al.*, 2009); it is tentatively suggested that the tomato homologue of the *AtCYP707A3* might be a sensible target for RNAi. Since it has been reported to be localised mainly in the vascular tissues and is particularly responsive to drought (Okamoto *et al.*, 2009).

A separate consideration for future research relates to the various abnormal pigmentation phenotypes exhibited in the root system, stems and fruit of the triple transgenic lines which have been associated with phytoene synthase overexpression. The immature fruit of the final homozygous triple transgenic lines appeared very pale/almost

white, while the immature fruit of earlier generations of the triple transgenic line, the double transgenic line MJ8 and the single *SlPSY1* overexpressing lines, appeared peach coloured. This peach colour phenotype has been previously reported to be due to an increase in lycopene accumulation not normally apparent at this stage of fruit development (Fray *et al.*, 1995; Fraser *et al.*, 2007). Fraser *et al.,* (2007) reported that both the carotenoid and chlorophyll content in the immature fruit of *PSY1* overexpressing tomato lines (immature was in this case defined as 11 ± 2 d after anthesis) had increased, with no alteration in their ratio. It was at the mature green stage (defined as 37 ± 1 d after anthesis) that there was a reduction in chlorophyll and an increase in carotenoids (Fraser *et al.*, 2007), these changes were associated with the development of chromoplast like structures earlier than is usual (Fraser *et al.*, 2007).

The symptoms of *PSY* overexpression described in papers by Fray *et al*., (1995), Shewmaker *et al.,* (1999) and Fraser *et al*., (2007) are similar to the leaf chlorosis, very pale immature fruit and reduced height phenotype of the triple transgenic lines observed in the present study. The overproduction of phytoene synthase, as initially suggested by Fray *et al.,* (1995), causes the GGPP substrate to be preferentially used in the synthesis of carotenoids at the expense of other MEP plastidial pathways. These include the phytol synthesis pathway, which is used in the formation of chlorophyll and the GA synthesis pathway. It has been reported by Shewmaker *et al.,* (1999) that the seed specific overexpression of a bacterial phytoene synthase (*crtB*) in *B. napus* transformants resulted in a reduction of other plastidial isoprenoids that are derived from GGPP e.g. tocopherols and chlorophyll. The plastid structure in these transgenic seeds was also reported to be altered; the typical thylakoid structure found in chloroplasts was not evident, instead a membrane bound inclusion body was described. It was suggested that this is a mechanism to sequester carotenoids instead of carotenoid binding proteins, which are not normally present in developing seeds (Shewmaker *et al.*, 1999).

Fraser *et al*., (2007) speculated that the effect on plastid transition was due to the alteration in metabolite composition, rather than the overexpression of a plastid localised protein. This is supported by research involving the *high-pigment 3* (*hp3*) tomato mutant, which has an increased number of plastids with larger compartments. The *hp3* mutation is impaired in ZEP functioning and has 75% lower ABA levels than normal (Galpaz *et al.*, 2008). It is suggested that the plastid phenotype is the result of plastid division since increased mRNA levels of *FtsZ*, a gene involved in plastid division, were also detected. This allows increased carotenoid biosynthesis and increases the capacity for carotenoid storage. An increase in plastid number and plastid compartment size and fruit lycopene levels was also observed in the ABA deficient tomato mutants, *flacca* and *sitiens*, (Galpaz *et al.*, 2008). This work indicates that there is an inverse relationship between ABA and plastid number (Galpaz *et al.*, 2008; Giuliano *et al.*, 2008). As a result of their findings Galpaz *et al.,* (2008) suggested that the larger plastid compartments in the *hp3* ABA deficient mutant are probably a result of increased plastid division, which allows increase carotenoid biosynthesis and creates increased carotenoid storage. Galpaz *et al.,* (2008) suggest a regulatory circuit which connects a carotenoid/xanthophyll derivative that influences carotenoid biosynthesis via the process of plastid division.

Two other *hp* tomato mutants accumulate increased amounts of carotenoids, notably lycopene, in their fruits and have increased plastid compartment size; *hp1* (Reynard, 1956) encodes an *Arabidopsis* homologue of UV-Damaged DNA binding protein1 (DDB1) (Lieberman *et al.*, 2004; Liu *et al.*, 2004) and *hp2* (Soressi, 1975) which encodes an orthologue of *Arabidopsis* de-etiolated1 (DET1) (Mustilli *et al.*, 1999). In *Arabidopsis* DDB1 has been shown to interact with DET1 (Schroeder *et al.*, 2002) to regulate aspects of photomorphogenesis, and presumably function in plastid development. It is interesting to note that the *hp1* mutant has higher phytoene synthase activity in the plastids of ripe fruit (Cookson *et al.*, 2003). An additional relevant mutant to note is the *Orange* (*Or*) gene mutation in cauliflower (*Brassica oleracea*), which

confers the accumulation of high levels of β-carotene in tissues usually devoid of carotenoids, and is associated with triggering the differentiation of proplastids, or other non-coloured plastids, into chromoplasts for carotenoid accumulation/sequestration (Lu *et al.*, 2006).

Similar changes in plastid morphology and development may also be occurring in the roots, stems and fruit of the triple transgenic lines. It would be interesting to examine the plastids in the developing fruit and the roots of the triple lines (and possibly the 'ideal' double and single transgenic tomato lines discussed earlier) with regard to the relationship between alterations in metabolite composition and plastid transition. It seems reasonable to expect that the vast increase in carotenoid content in the triple transgenic lines has triggered changes in the plastids. Future studies of these alterations in both the fruit and root tissues, may contribute to the understanding of the processes of plastid development and redifferentiation. Despite being well described, the molecular processes underlying these transitions are poorly understood (Pyke, 2009) but could provide additional approaches for improving carotenoid biosynthesis (Giuliano *et al.*, 2008) and possibly ABA biosynthesis.

A final consideration from this research that will be discussed herein, relates to the finding that drought stress can increase steady state *SlBCH2* mRNA levels in vegetative tissues e.g. leaves and roots (Sonneveld *et al.*, unpublished data). This appears to indicate, that the *SlBCH2* gene has a novel role in supplying the xanthophyll precursors required to sustain stress-induced ABA biosynthesis. Two BCH enzymes are known to exist in pepper (*Capsicum annum*) (Bouvier *et al.*, 1998) and *Arabidopsis* (Sun *et al.*, 1996; Tian and DellaPenna, 2001). Phylogenic analysis has revealed that the tomato *SlBCH2* (also known as *CrtR-b2*) gene is more similar to the pepper *CaBCH1* than it is to its tomato homologue *SlBCH1* (Galpaz *et al.*, 2006). *SlBCH2* and the pepper *CaBCH1* also map to the same chromosome (no. 3) of their respective Solanaceous genomes (Thorup *et al.*, 2002; Galpaz *et al.*, 2006). Additionally, gene structure has been

reported to be very similar in all genes encoding β-hydroxylases from tomato, pepper and *Arabidopsis* e.g. they have conserved intron splicing sites. Galpaz *et al*., (2006) have suggested that the β-carotene hydroxylase genes in these three species have evolved from the same ancestral gene. It would be interesting to investigate the possibility that the *CaBCH1* and one of the *Arabidopsis* β-carotene hydroxylase genes function like the *SlBCH2* gene, in being upregulated in response to drought in order to maintain the supply of xanthophyll precursors for stress-induced ABA biosynthesis.

It is interesting to note that at least one member of the *NCED* multigene family appears to be capable of responding very rapidly to leaf dehydration (section 1.9.1) in most plant species. A drought responsive gene encoding a PSY enzyme which functions to influence root carotenogenesis in members of the Poaceae has been identified recently (section 1.3.1). This, together with the identification of a drought responsive BCH gene in tomatoes, (and it is cautiously suggested, possible extension to the *BCH* gene family members in other plant species) further highlights the need to maintain carotenoid precursor supply to sustain stress induced ABA biosynthesis.

5.2.2 Exploring the Effects of Increased [ABA]

Transgenic plants that produce varying amounts of ABA are now available, ranging from the extreme e.g. the rbcS lines (Tung *et al.*, 2008) via the moderate e.g. the G29 line (Sonneveld *et al.*, unpublished data) and the sp5 line (Thompson *et al.*, 2000a) to the slight, e.g. the BCH12 line (Sonneveld *et al.*, unpublished data). This range of ABA overproducing genotypes will be useful in the investigation of the long term effects of elevated concentrations of endogenous ABA on a number of different physiological processes.

One area that could be investigated further using these lines is that of the influence of ABA on testa imposed dormancy, testa and cotyledon development during embryogenesis and post germination cotyledon expansion. As mentioned in section 1.5,

the WT tomato seed coat is normally five cells thick, whereas the ABA deficient *sitiens* (*sit*) mutant was found to have a thinner testa, consisting of just one cell layer. In the transgenic tomato plants described by Tung *et al.*, (2008), which overexpressed *SlNCED1* under the control of a green tissue specific promoter (rbcS), post germination cotyledon emergence from the surrounding testa was severely hampered. Reciprocal crosses between the transgenic and WT tomatoes indicated that the embryo genotype was solely responsible for this and, since imbibition with an aqueous norflurazon solution could not reverse this phenotype, it was concluded that increased embryonic ABA biosynthesis during seed development influenced testa development. It would be interesting to investigate this further, gathering data on the influence of high endogenous ABA concentrations on testa thickness, development during embryogenesis, cotyledon expansion and emergence. It has also been hypothesised that cotyledon size at germination is affected by ABA, and that this negatively influences the rate of growth during the seedling establishment phase. The slower early growth of transgenic plants that accumulate greater amounts of ABA may simply be a function of their reduced initial photosynthetic area, associated with their reduced small cotyledons (I.B. Taylor, and R.C. Smeeton personal communication).

Another aspect of the influence of ABA that would be interesting to investigate further using the various transgenic lines, is the effect of increased endogenous ABA on root architecture and growth rate. ABA has been previously implicated in the regulation of root growth and initiation (De Smet *et al.*, 2003; De Smet *et al.*, 2006). The application of exogenous ABA has been reported to maintain root growth under water deficit (Saab *et al.*, 1990). It has also been found to decrease the number of lateral roots (Guo *et al.*, 2009) resulting in a less dense but deeper root system, favourable for water uptake from deep soil layers (de Dorlodot *et al.*, 2007). In maize, the accumulation of ABA in roots in response to drought conditions was reported to stimulate primary root elongation (Sharp *et al.*, 1994). Additionally, ABA deficient and

ABA insensitive mutants of *Arabidopsis* have been found to have a reduced root biomass in drying soil compared to WT plants (Vartanian *et al.*, 1994). Tan *et al.,* (2003) hypothesised that ABA may have a dual function to either promote or inhibit lateral root development, and they suggested that a threshold may exist in root cells which provides a switch between these two opposite responses (Tan *et al.*, 2003). Comparing the root architecture, biomass and growth rates of the different transgenic tomato lines with varying degrees of endogenous [ABA] would be valuable towards understanding the complex roles of endogenous ABA in the morphology of root systems.

ABA is also known to influence important aspects of root physiology e.g. hydraulic conductivity (Lp_r) (Glinka, 1980; Hose *et al.*, 2000; Tardieu *et al.*, 2010). This may be partly explained by the upregulation of aquaporin genes by ABA (Zhu *et al.*, 2005; Tardieu *et al.*, 2010). Consistent with this, ABA deficient tomato mutants are known to have decreased Lp_r (Tal and Nevo, 1973) and in contrast ABA overproducing transgenic tomato plants have increased Lp_r (Thompson *et al.*, 2007a). This is congruent with the findings presented in section 4.3.7. It has relatively recently been demonstrated in maize (Parent *et al.*, 2009) and tobacco (*Nicotiana tabacum*) (Mahdieh and Mostajeran, 2009) that ABA can increase aquaporin activity and the hydraulic conductivity of both roots and shoots. This can result in increased tissue water status and, greater leaf elongation rates, at least in maize (Parent *et al.*, 2009). It would be interesting to also investigate whether, and the extent to which, the increased [ABA] in the various transgenic tomatoes lines described herein has affected aquaporin regulation.

Additional areas of possible future research involving the effect of increased endogenous [ABA], which will be mentioned in this discussion, involve assessing the effect of the reduced transpiration on tomato crop yield. This is a vital issue with regard to the potential commercial application of this method of increasing water use efficiency. Desired genotypes would ideally be able to maintain yield, while water input is significantly reduced. Crop yield is often positively related to the total amount of water

transpired. It is of course possible that reducing transpiration by increasing ABA to the point at which the reduced g_s will limit CO_2 assimilation (*A*), will inevitably reduce biomass production and adversely affect crop yield (Boyer, 1982; Condon *et al.*, 2004; Blum, 2005). However, it is also possible that there may be a degree of excessive non-productive transpiration under well watered conditions. As previously mentioned in section 1.1, computer simulation analyses, which placed a limit on the maximal transpirational rate in sorghum (*S. bicolor*) actually resulted in a slight yield increase over the 115 seasons included in the analysis, as well as reducing water use. The yield increase was mainly due to improved yield in dry, low-yielding years (Sinclair *et al.*, 2005). The yield increases associated with the limited maximal transpiration rate were associated with increased water use efficiency, because transpiration was reduced during periods when atmospheric demand (VPD) was highest, i.e. when water loss would be greatest. Additionally, a decline in g_s and transpiration is generally proportionally greater than the associated reduction in *A* (Jones, 1976; Hetherington and Woodward, 2003); an increase in TE_i is therefore often observed as g_s decreases. A result of this nonlinear relationship, as pointed out by Thompson *et al*., (2007a), is that a given reduction in g_s has a small effect on A at high g_s but at lower g_s the effect on A will be larger.

This was experimentally demonstrated by the shape of the A/g_s curve obtained using WT and a range of transgenic tomato lines overexpressing *SlNCED1* to varying extents (Thompson *et al.*, 2007a). These transgenic tomato lines did not show a significant reduction in overall biomass, despite exhibiting slightly reduced carbon assimilation. It was also reported (Thompson *et al.*, 2007a) that these plants allocated fewer resources to reproductive tissues, although this may have been an artefact due to increased flower abscission in the high ABA lines. These plants were not grown to crop harvest and no yield data was given. Other studies (Tung, 2007; R.C. Smeeton & I.B. Taylor, personal communication) using these lines, found that flower abscission was minimal/absent, and have not demonstrated a significant yield loss compared to WT

plants. It should be born in mind that these results are not definitive and further trials should be conducted into the effect of increased endogenous ABA on tomato fruit crop production on a commercial scale.

A related issue is that transpiration also has a cooling effect on leaves, and reducing transpiration by restricting the stomatal aperture may in some cases limit a plants tolerance to higher temperatures. This could theoretically have an adverse impact on the commercial application of such plants in countries with hotter climates. Breeding for high yields in hot environments has inadvertently led to selection of cotton and wheat varieties with higher transpiration rates (Radin *et al.*, 1994; Lu *et al.*, 1998). Since genetically modifying plants to have lower transpiration rates may lead to a reduced ability to tolerate high temperatures, it would be sensible to obtain experimental evidence on the extent of this effect. Computer models could be used to theoretically estimate the effects of specified reductions in g_s on leaf temperatures.

5.2.3 Fine Tuning the ABA Signal

Increasing [ABA] via the constitutive overexpression of ABA biosynthesis gene/s has been demonstrated to have a positive impact on WUE, but was also associated with some undesirable traits. These included delayed germination, slightly slower seedling establishment and can, with some constructs (e.g. Tung *et al*., 2008); result in leaf chlorosis and severely stunted growth. Promoter selection could offer greater temporal and spatial control over the expression of these ABA biosynthesis genes, hopefully conferring the positive saving on water use, while avoiding less desirable traits. One experimental approach that is currently being taken is to select promoters that will only target expression of *SlNCED1* to mature green tissues, thereby avoiding adverse effects on germination and early seedling establishment. A second approach currently under investigation, with a similar goal of overexpressing the *SlNCED1* gene to different levels and with different tissue specificity, involves the use of a transposable transgene

integrated at random positions throughout the genome. Current results appear to suggest that a promising candidate line has been generated using this approach (S. Awan & A.J. Thompson, personal communication).

Root specific promoters may also offer an alternative potentially valuable avenue of investigation. Root specific expression of the three key rate limiting ABA biosynthesis genes and/or ABA catabolism genes would avoid not only the undesirable effects of ABA on germination but circumvent the need for grafting, which can increase the cost of production and reduce growing time (the commercial application of grafting, including rootstock lines such as Beaufort and Brigeor, is discussed in section 4.1). It would also mean that seed could be made available to benefit all growers. One candidate root specific promoter is the recently characterised *SlREO* gene promoter (Jones *et al.*, 2008). This novel gene was reported to be highly expressed in the cortex of mature tomato roots and would be ideal for the strong and specific expression of ABA biosynthesis/catabolism genes in not only tomato roots but also the roots of other closely related Solanaceous species such as potatoes.

A further possibility for fine tuning the ABA signal is the recently expanding field of ABA transport and perception (section 1.6). Recent reports have revealed the importance of transporter driven uptake and export of ABA (Raghavendra *et al.*, 2010), implicating some ABC (ATP-binding cassette) proteins in stomatal responses and as ABA transporters (Kang *et al.*, 2010; Kuromori *et al.*, 2010). The recent identification of a unique class of ABA receptors the PYR/PYL receptor family, have been independently corroborated and are highly conserved in crop species (Melcher *et al.*, 2009); this has greatly contributed to the understanding of the molecular mechanisms involved in ABA signalling. These ABA transporters and receptors are prime targets for manipulation (Klingler *et al.*, 2010). This approach could involve either transgenic modification or more classical breeding methods, for increasing the transport of ABA or to increase stomatal sensitivity to the existing ABA signal, with the goal of enhancing drought and

other abiotic stress tolerance. Additionally, the discovery of pyrabactin, a selective ABA agonist of PYR/PYL proteins (Park *et al.*, 2009) could lead to the development of potential chemical strategies for regulating ABA receptor activity in crops (Cutler *et al.*, 2010).

5.2.4 Exploiting Natural Variation in Wild Tomato Species

Wild germplasm is a potentially valuable and sustainable resource for crop improvement (Zamir, 2001). It can be used to "enrich the genetic basis of cultivated plants with novel alleles that improve productivity and adaptation" (Gur and Zamir, 2004). Virtually all of the disease resistance genes present in commercial tomato varieties have originated from the wild species of tomato (Rick and Chetelat, 1995) and much genetic variation for the tolerance of abiotic stresses, such as cold tolerance and drought, exist in the wild species germplasm.

The non-transgenic approach described in this thesis, involved exploring the natural allelic variation of *NCED1*, which had been previously demonstrated to be a key rate limiting enzyme in ABA biosynthesis (Thompson *et al.*, 2000b) and that its overexpression could improve WUE (Thompson *et al.*, 2007a; as previously described in section 1.9 and 4.1). Exploring the natural variation of *NCED1* activity as means of improving WUE without resorting to the development of transgenic crops was a direct and targeted approach to exploiting the diversity of wild relatives of tomato. An alternative wider approach to improving WUE via classical breeding could have involved QTL (quantitative trait loci) mapping. Drought responses are extremely complex and drought tolerance is a quantitative trait in which numerous genes are involved. A number of tomato QTLs have now been identified and mapped for traits such as cold and salt tolerance, fruit size and quality; as well as for resistance to pests and pathogens (Lindhout, 2005).

Gur and Zamir (2004) reviewed tomato QTL data and results of QTL mapping studies from other species, and speculated that these studies indicate that it is unlikely that a single introgression will induce a striking improvement in a yield-associated phenotype (Gur and Zamir, 2004). However, it is suggested that pyramiding a number of independent introgressions in a single genotype, each with a positive effect on the desired trait, could be an effective strategy (Gur and Zamir, 2004). These authors created a tomato containing a pyramid of three independent yield-promoting genomic regions introduced from the drought-tolerant wild species *S. pennellii*. This tomato line was reported to yield 50% more than a market leading variety under wet and dry field conditions (Gur and Zamir, 2004). It is interesting to note that a QTL for $\Delta^{13}C$ has been detected in tomato, designated QWUE5.1, and marker assisted selection (MAS) is recommended to transfer QWUE5.1 into other cultivars (Xu *et al.*, 2008).

Studies comparing the drought response of *S. pennellii*, a self-incompatible species from dry environments, with *S. lycopersicum*, the self-compatible cultivated tomato, have shown that *S. pennellii* had the higher WUE in both water stressed and non-stressed conditions (Kebede *et al.*, 1994; Martin *et al.*, 1999). *S. pennellii* has succulent leaves and a shallow spreading root system (Rick, 1973), apparently, the increased WUE and ability to withstand drought of *S. pennellii* is primarily due to changes in leaf anatomy (smaller area and lower stomatal density) combined with altered stomatal behaviour that conserves water (Kebede *et al.*, 1994).

One recent study involved micro-array analysis of the differences between two drought tolerant *S. pennellii* introgression lines (ILs) with recurrent parent *S. lycopersicum* cv. M82. Approximately 400 genes were identified which were responsive to drought stress only in the drought tolerant lines (Gong *et al.*, 2010). These results indicate that these ILs contain drought tolerance QTLs. It will be interesting to see if any of these QTL's co-map with the *NCED1* gene locus on chromosome 7. An *NCED1*-

specific QTL could lead directly to discovery of a natural promoter variant, capable of elevating *NCED1* expression to the same extent as that achieved transgenically using the Gelvin Superpromoter (sp) (Thompson *et al.*, 2007a). This would offer the potential to contribute to improving crop water use without the necessity for genetic modification and the controversy surrounding it.

5.3 CONCLUSION

Two approaches towards improving the water use efficiency of tomatoes were outlined in this thesis. The first involved the investigation of the natural allelic variation of the *NCED1* gene in two wild tomato species, *S. galapagense* and *S. neorickii*, via the introgression of these divergent alleles into a cultivated tomato background, *S. lycopersicum* cv. Alisa Craig. This formed part of a larger joint DEFRA funded project between Nottingham University and Warwick-HRI. The effect of these two introgressed wild species *NCED1* alleles on WUE was assessed via a gravimetric water use trial, which found that these two wild species alleles did not function to improve whole plant water use efficiency.

The second approach involved the generation of a transgenic rootstock which constitutively overexpressed three key enzymes in the carotenoid/ABA biosynthesis pathway, with the objective of increasing root-synthesised ABA to sufficient quantities to improve whole plant WUE of non-GM scions. The constitutive simultaneous overexpression of *SlPSY1*, *SlBCH2* and *SlNCED1* resulted in increased carotenoid biosynthesis, indicated by the accumulation of large amounts of lycopene, β-carotene and xanthophylls in the roots, which appear to increase the supply of carotenoid and xanthophyll precursors to sustain increased root ABA biosynthesis. The different analyses from two 30 day gravimetric water trials, assessing the WUE of Tm2a/H-22-8-8 (scion/rootstock) plants against Tm2a self grafts, are suggestive that the triple transgenic rootstock conferred a modest but cumulative water saving.

CHAPTER 6:

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CHAPTER 7:

APPENDICES

7.1 APPENDIX I: TRANSGENIC TOMATO LINES

Table 7.1.1 A list of the transgenic tomato lines used herein and their T-DNA insertion loci relating to constructs involving the three ABA biosynthetic genes that are the focus of this work: + denotes the wild type allele (azygous for the transgene); a capital letter denotes the transgene insertion (i.e. N = *NCED1* transgene; B = *BCH2* transgene; P = *PSY1* transgene); ? indicates an unknown zygosity and number of insertion loci, but that the transgene was present.

TRANSGENIC LINE	T-DNA INSERTION LOCUS			PARENTAL LINES CROSSED (if applicable)
	CONSTRUCT	CONSTRUCT	CONSTRUCT	
	sp::SlNCED1	*35S::SlCrtRb2/BCH2*	*35S::PSY1*	
sp5	NN	++	++	-
BCH12	++	BB	++	-
G29	NN	BB	++	sp5 x BCH12
MJ8	NN	++	P?	sp5 x Z171D4A
Triple	NN	BB	PP	G29 x MJ8

Table 7.1.2 The imbibition protocol for transgenic lines requiring treatment with norflurazon. The norflurazon concentration (mgl^{-1}) and the duration of imbibition required before sowing to achieve uniform germination are listed in the table.

TRANSGENIC LINE	NORFLURAZON CONCENTRATION ($mg\ l^{-1}$)	DURATION (HOURS)
sp5	0.5	24
G29	1	96
Triple	1	~144*

*During development of the homozygous triple construct lines the seeds were imbibed with a 1 mgl^{-1} norflurazon solution until the radicle could be seen visibly emerging from the testa. The seed was then very carefully rinsed in water before sowing, this is the average imbibition duration and a specific trial is still required to establish the best imbibition duration for the Triple line.

Table 7.1.3 Sowing protocol for the transgenic lines. The advanced sowing of transgenic (± pre-imbibition with norflurazon) and WT seed to synchronise seedling development: for grafting at the third to fourth true leaf stage, at hypocotyl emergence from the soil and, at the end of the seedling establishment phase i.e. the fourth expanded leaf stage (soil emergence and establishment phase data courtesy of R.C. Smeeton).

LINE	ADVANCED SOWING (DAYS) OF SEED (± PREIMBIBTION) TO SYNCHRONISE DEVELOPMENT		
	With WT at hypocotyl soil emergence	For Grafting*[1] (between three and four true leaves)	With WT at the end of seedling establishment phase (fourth expanded leaf)
Tm2a	0	16-22	0
sp5	7	26-32	15
G29	4	26-32	16
Triple	*[2]	40-52	*[2]

*[1]Staggered sowings we used to synchronise plants for grafting; the days given are the recommended periods during which to stagger sowing before the intended grafting date. *[2]Trials to fully synchronise development of the recently obtained triple transgenic line with that of WT for hypocotyl hook emergence from the soil and at the end of the seedling establishment, phase have not been conducted at the time of writing.

7.2 APPENDIX II: BUFFERS AND SOLUTIONS

7.2.1 Root Culture Medium pH 5.7

COMPONETS	IN 1l
Half MS (Murashige and Skoog) mineral salts	2.2 g
3% Sucrose	15 g

Adjusted to pH 5.7 using 1M KOH (~ 200µl).

7.2.2 Hewitt's Solution N Optimal

(Hewitt and Smith, 1975)

ELEMENT	CONCENTRATION ($mMol.l^{-1}$)
Nitrogen (N)	12.3
Phosphorous (P)	1.33
Potassium (K)	6.2
Calcium (Ca)	3.03
Sodium (Na)	1.56
Magnesium (Mg)	1.49
Iron (Fe)	0.11
Chlorine (Cl)	0.34
Manganese (Mn)	0.01
Boron (B)	0.03
Zinc (Zn)	0.001
Copper (Cu)	0.001
Molybdenum	0.0004

STOCK SOLUTION	COMPONENT	IN 1l
1	Potassium nitrate (KNO_3)	121.2 g
2	Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	73.8 g
3	Calcium nitrate hydrated ($Ca(NO_3)_2 \cdot 4H_2O$	144.25 g
4	Sodium di-hydrogen orthophosphate dehydrate($NaH_2PO_4 \cdot 2H_2O$)	41.6 g
5	EDTA – disodium salt ($C_{10}H_{14}O_8N_2Na_2 \cdot 2H_2O$)	8.325 g
	Ferric chloride ($FeCl_3$)	3.625 g
6 A	Trace Element Concentrate	
	Copper sulphate ($CuSO_4 \cdot 7H_2O$)	0.475 g
	Borric acid (H_3BO_3)	3.725 g
	Maganese sulphate ($MNSO_4 \cdot 4H_2O$)	4.45 g
	Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	0.575 g
	Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$)	0.15 g
6 B	Trace Element Stock	
	6 A, trace element concentrate	400 ml

100 ml each of stock solutions 1 – 5 & 6B were mixed together and made up to 20 l with water.

7.2.3 1 M Tris HCL pH 8

121.1 g Tris base was dissolved in 800 ml sterile distilled water and adjusted to pH 8 by adding ~ 42 ml concentrated HCl (N.B. The solution was fully equilibrated to room temperature before adjusting pH). Solution was topped up to 1 l with sterile distilled water before autoclaving.

7.2.4 0.5 M Na_2EDTA pH 8

186.1 g of disodium EDTA·$2H_2O$ was added to 800 ml distilled water. The solution was stirred on a magnetic stirrer, and the pH adjusted to 8 with NaOH. The solution was then sterilized by autoclaving. (N.B. The disodium salt would not go into solution until the pH of the solution was adjusted to 8 by the addition of NaOH.)

7.2.5 2% CTAB Buffer

COMPONENT	IN 1l
2% CTAB (Hexadecyltrimethylammonium bromide)	10 g
1.4 M NaCl	40.91 g
20 mM EDTA	16 ml of 0.5M EDTA
100 mM Tris HCl (pH 8)	40 ml of 1M Tris HCl
2% PVP-40	10 g
1% 2-mercaptoethanol (added to the solution just before use)	2µl ml^{-1}

7.2.6 CTAB-DNA mini-prep Wash Buffer

COMPONENT	IN 1l
76% Ethanol	76 ml
10 mM ammonium acetate	10 ml of 1M

7.2.7 T.E. (10 x Tris EDTA)

COMPONENT	IN 100 ml
100 mM Tris-Cl pH 8	1 ml
10 mM EDTA pH 8	200 µl

+ 0.3 µg ul^{-1} RNaseA; 3µl of 10mg ml^{-1} stock was added to each 100 µl T.E. to be used.

7.2.8 RNaseA 10 mg ml^{-1} Stock

Prepared as per manufacture's instructions (Sigma-Aldrich, Dorset, UK, R5500) briefly; the enzyme was suspended in 10 mM sodium acetate buffer pH 5.2, and

heated to 100 ºC for 15 min. The solution was allowed to cool to room temperature, and adjusted to pH 7.4 using 0.1 Volume of 1M Tris-HCl pH 7.4. The solution was then divided into aliquots and stored at -20 ºC.

7.2.9 10 x TBE Buffer

COMPONENTS	AMOUNT (ml)
1 M Tris-HCl pH 8	90 ml
0.5 M Na_2EDTA	4 ml
Distilled Water	6 ml

7.2.10 Ethidium Bromide Loading Buffer

COMPONENT	AMOUNT (µl)
De-ionised formamide	1,000
5 M Sodium Chloride (NaCl)	40
100 mM EDTA	40
10 MG ML^{-1} ethidium bromide	40
SDW	330

7.2.11 Sodium Phosphate Buffer pH 6.5

Firstly, 500 ml of 1 M monobasic (NaH_2PO_4) and 1 L of 1 M dibasic (Na_2HPO_4) sodium phosphate were prepared in separate bottles. The dibasic sodium phosphate solution was then slowly added to 400 ml the monobasic solution until the pH of the monobasic solution reached 6.5.

7.2.12 1% (w/v) Denaturing Agarose Gel

COMPONENT	AMOUNT
Agarose	1 g
Distilled Water	90 ml
1 M Sodium Phosphate buffer pH 6.5	2 ml
Deionised formamide pH 7	8 ml

7.2.13 Gel Running Buffer for Northern Blot

COMPONENT	AMOUNT
Distilled water	593.5 ml
1 M Sodium phosphate pH 6.5	6.5 ml
Deionised formamide pH 7	20 ml

7.2.14 Prehybridisation Buffer for Northern Blot Analysis

All components except the salmon sperm and SDS were added to a 25 ml beaker, with a magnetic flea, and covered with cling film. This beaker was then placed in a larger beaker half filled with water, which was in turn placed on a heat stirrer. The mixture was heated and stirred for about 1 h until the dextran sulphate had been dissolved. The SDS and salmon sperm were then added.

COMPONENT	AMOUNT
Deionised formamide	10 ml
50 % Dextran Sulphate	2 g
5 mg ml $^{-1}$ denatured salmon sperm DNA[1]	0.8 ml
5 M Sodium Chloride	4 ml
Distilled Water	4 ml
20 % (w/v) SDS	2 ml

[1]Salmon sperm DNA was denatured by boiling for 5 min before adding to the mixture.

7.2.15 Linearised Template DNA

Plasmid pNCEaa.3 (10 µg) containing the complete coding region of *SlNCED1* (Burbidge *et al.*, 1997) was linearised overnight with *Xba*I. The digest was then made up to 100 µl with water and extracted twice with phenol/chloroform and once with chloroform to remove RNase A that may have been present in the plasmid preparation. The DNA was then precipitated with ethanol in the presence of 2.0 M ammonium acetate pH 5.4. Precipitated DNA was washed with 70% ethanol and dissolved in RNase-free water to give a final concentration of 0.5-1.0 µg µl^{-1}. RNA probes were synthesised from plasmid DNA (20 ng) by transcription with T7 RNA polymerase using a Rediprime kit (Amersham Biosciences, Buckinghamshire, UK).

7.2.16 Riboprobe (Antisense RNA Probe) Generation

The components were added in order to a 1.5 ml Eppendorf tube and incubated for 45 min at 37 ºC. Then 2 µl RNase free DNase (Promega, Wisconsin, USA) was added and the mixture incubated for 15 min at 37 ºC. Unincorporated nucleotides were removed using Sephadex G-50 'nick' column (Fizer Ltd (Promega), Surrey, UK); the column was

primed with 400 µl TE/1 % (w/v) SDS pH 8.0. The probe was transferred to the column and 400 µl TE/1 % (w/v) SDS pH 8.0 was added, another 400 µl TE/1 % (w/v) SDS pH 8.0 was added and both elutions were collected in the same 1.5 ml Eppendorf tube.

COMPONENT (IN ORDER)	AMOUNT (µl)
5x transcription buffer	4
Nuclease free water	4.5
10 mM ATP	2
10 mM GTP	2
10 mM CTP	2
100 µM UTP	2
Linearised template DNA	0.5
[^{32}P]-UTP 800 Ci mmol $^{-1}$	2.5
T7 Polymerase[1]	0.5

[1] Rediprime kit (Amersham Biosciences, Buckinghamshire, UK)

7.2.17 Phosphate Buffered Saline (PBS)

Firstly, 250 ml of 50 mM sodium di-hydrogen orthophosphate (A; 1.5 g) and 250 ml of 50 mM di-sodium orthophosphate (B; 1.77 g) were prepared. Solution B was then added to solution A until pH reached 6. The exact amount of B added was recorded so that sodium chloride could be added to the solution to a final concentration of 0.1M (1.34 g/ 300 ml)

7.2.18 ^{3}H-ABA Buffer

The (+)-*cis-trans*-[G-^{3}H]-ABA (Amersham Biosciences, Buckinghamshire, UK) arrives as 250 µl in ethanolic solution at an approximate concentration of 1.85 MBq mmol^{-1}. This was diluted 1:10 with distilled water and stored at -20 ^{o}C in 600 µl aliquots, which were to be used as the working stock solution. The ^{3}H-ABA buffer was prepared by adding 100 mg bovine γ-globulin (5 mg ml^{-1}; Fluka, Sigma-Aldrich, Dorset, UK) to 20 ml 100% PBS. For use in the assay, 150 µl ^{3}H-ABA was added to 20 ml ^{3}H-ABA buffer, and this could be stored at -20 ^{o}C and used as required.

7.2.19 MAC 252 Buffer

COMPONENT	AMOUNT
4 M Bovine Serum Albumin (BSA)	200 mg
20 mM polyvinylpyrrolidone (PVP)	160 mg
100 % PBS	40 ml

MAC 252 Buffer was stored at -20 ºC.

For use in the assay 200 µl MAC252 antibody (Brabaham Bioscience Technologies, Cambridgeshire, UK) was added to 20 ml of MAC 252 Buffer.

7.2.20 Saturated Ammonium Sulphate

Initially, 290 g of $(NH_4)_2SO_4$ was added to 348 ml water and heated to 50 ºC with continuous stirring to dissolve the salts. The solution was considered saturated when a layer of undissolved $(NH_4)_2SO_4$ formed with no turbidity present. The clear solution was then transferred to a fresh bottle, taking care not to disturb the precipitated layer.

7.3 APPENDIX III: PRIMERS, CAPS MARKERS & PROBES

Table 7.3.1 A list of the PCR primers used, with nucleotide sequences (5′-3′) VI, Vacuolar invertase; BCH2, β-carotene hydroxylase2; PSY1, phytoene synthase1.

PRIMER NAME	SEQUENCE (5′- 3′)
VI_For	CTGGGTCAAGTTCAAAGGCAAC
VI_Rev	CATTTTGTGGTCCGGTCCA
BCH2_For	GGAACTAGCGGAGGCTGAAA
BCH2_Rev	TTCGCAAGACCCTTCCTCTATATAA
PSY1_For	CCTTCGCAAGACCCTTCCTC
PSY1_Rev	CCCGGACTGATTCCATGAAA
LePSY1_For1	GGGACAAGTTTCATGGAATCAGTCCG
LePSY1_Rev2	CTTCATCGGATAGACCTGCCTGTGC
Tm2a_F	AGGTTGTTGCACCGATTGAT
Tm2a_Sus_R1	TGTCTACCATAAAGCAGACAAGAG
Tm2a_Res_R1	TGCCAGTATATAACGGTCTACACTAAA
Tm2a_Res_NF	GTGCTTAAGGCGCTGGAG
Tm2a_Res_NR	GGCTAGAGTAAGCTTGATGAGATT

Table 7.3.2 A list of the qPCR Taqman probes used, with nucleotide sequences (5′-3′) with the: fluorescent reporter dyes; FAM (fluorescein) and HEX™ (hexachlorofluorescein), and Black Hole Quencher1 ™ (BHQ1™) specified. VI, Vacuolar invertase; BCH2, β-carotene hydroxylase2; PSY1, phytoene synthase1.

PROBE NAME	SEQUENCE (5′- 3′)
VI Taq probe	HEX-CGGTTCTGGTTCCTCCACCCGG-3'BHQ1
BCH2 Taq probe	FAM-TCCGGCCGCCATGGACG-3'BHQ1
PSY1 Taq probe	FAM-TAGGAAGTTCATTTCATTTGGAGAGGACAGGG-3'BHQ1

Table 7.3.3 A list of the CAPS Marker primer sequences (5′-3′) used, with the specific restriction enzyme, and the target allele which is cut by the restriction enzyme. Schematic diagrams of the CAPS Markers are located in the main text; Figure 3.3.6 and Figure 3.3.7.

CAPS MARKER NAME: CAP_NCED1	SEQUENCE (5′- 3′)	RESTRICTION ENZYME	TARGET ALLELE	BUFFER & COMPOSITION
Neo_F	GGTGGTTTACGACAAGAACAAA	*Hinf*I incubate at 37ºC (MBI Fermentas)	*SnNCED1* in *Solanum neorickii*	Buffer R^+ 10 mM Tris-Hcl (Ph 8.5) 10 mM $MgCl_2$ 100 mM Kcl 0.1 mg BSA
Neo_R	ATTCCAGAGGTGGAAACAGAAA			
Gala_F	CACGATGAGAAAGAATGGAAATC	*Hind*III Incubate at 37°C (MBI Fermentas)	*SlNCED1* in *Solanum lycopersicum*	Buffer R^+ 10 mM Tris-Hcl (pH 8.5) 10 mM $MgCl_2$ 100 mM Kcl 0.1 mg BSA
Gala_R	CATTGGCGTTTATGAATGTTCC			

Table 7.3.4 A list of the different PCR cycling conditions for each specific primer pair listed in Appendix III

PRIMER PAIR	CYCLING CONDITIONS					
	Initial Denaturation	Denaturation	Annealing	Extension	No. of Cycles	Final Extension
VI BCH2 PSY1	95°C for 5 min	95°C for 30 sec	54°C for 45 sec	72°C for 60 sec	35	72°C for 10 min
Cap_NCED1_Neo Cap_NCED1_Gala	95°C for 5 min	95°C for 30 sec	52°C for 45 sec	72°C for 60 sec	35	72°C for 10 min
Tm2a	95°C for 5 min	95°C for 30 sec	50°C for 45 sec	72°C for 60 sec	35	72°C for 10 min
LePSY1	95°C for 2 min	95°C for 30 sec	54°C for 30 sec	72°C for 60 sec	30	72°C for 5 min

7.4 APPENDIX IV: MASTER MIX CONSTITUENTS FOR qPCR

CONSTIUENT		FINAL AMOUNT	AMOUNT PER WELL (µl)
Sensi Mix[1]		1 x	10
Reference gene	Forward Primer	6 pmol	1
	Reverse Primer	6 pmol	1
	Probe	2 pmol	1
Transgene	Forward Primer	6 pmol	1
	Reverse Primer	6 pmol	1
	Probe	2 pmol	1
H_2O		n/a	3

[1] Sensi Mix No Ref DNA Kit (Quantace, Bioline, London, UKCat No. QT505-05)

7.5 APPENDIX V: THE GRADIENT FLOW SOLVENT REGIME FOR HPLC

Table 7.5.1 Solvent regime for C_{30} (reverse) phase HPLC. Solvent A, 100% methanol; solvent B, 80% methanol 20% water; solvent C, 100% methyl-tert-butyl ether (MTBE). NB: needle wash, 95% methanol, 5% water; seal wash, 20% methanol, 80% water

TIME (min)	FLOW RATE (ml min^{-1})	% SOLVENT A	% SOLVENT B	% SOLVENT C
	1	95	5	0
12	1	95	5	0
13	1	80	5	15
45	1	30	5	65
60	1	95	5	0

Table 7.5.2 Solvent regime for adsorption (forward) phase HPLC. Solvent A, 100% hexane; solvent B, 60% hexane: 40% propan-2-ol. N.B. needle wash, 100% hexane; seal wash, 50% hexane/50% isopropanol

TIME (min)	FLOW RATE (ml min^{-1})	% SOLVENT A	% SOLVENT B
	1	100	0
7	1	80	20
11	1	80	20
23	1	50	50
28	1	10	90
30	1	90	10
33	0	100	0
38	0	100	0

Table 7.5.3 Solvent regime for ABA analysis by HPLC-electrospray ionisation/MS-MS. Solvent A, 94.9% water: 5% acetonitrile: 0.1% cholesterol hydroperoxide (Ch-OOH); Solvent B, 5% water: 94.9% acetonitrile: 0.1% cholesterol hydroperoxide (Ch-OOH).

STEP	TOTAL TIME (min)	FLOW RATE (µl min^{-1})	% SOLVENT A	% SOLVENT B
0	5	250	80	20
1	0	250	80	20
2	10	250	10	90
3	13	250	10	90
4	13.5	250	80	20
5	14	250	80	20

7.6 APPENDIX VI: CHROMATOGRAMS & ABSORBANCE SPECTRA OF THE CAROTENOIDS RESOLVED BY HPLC.

7.6.1 Example chromatograms of carotenoids resolved by HLPC

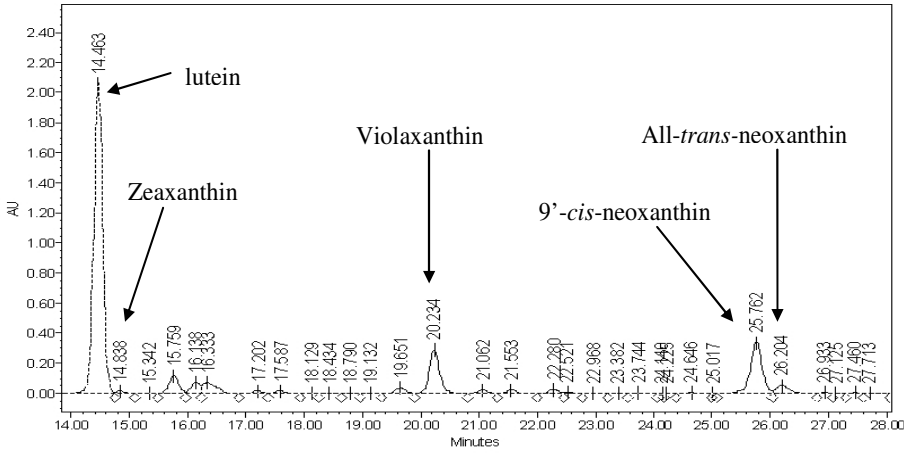


Figure 7.6.1 Example chromatograph of tomato (*Solanum lycopersicum* L. cv. Alisa Craig) leaf carotenoids separated on the adsorption phase column. The carotenoids investigated herein are labelled and the individual retention times (min) are shown. Au = absorbance units.

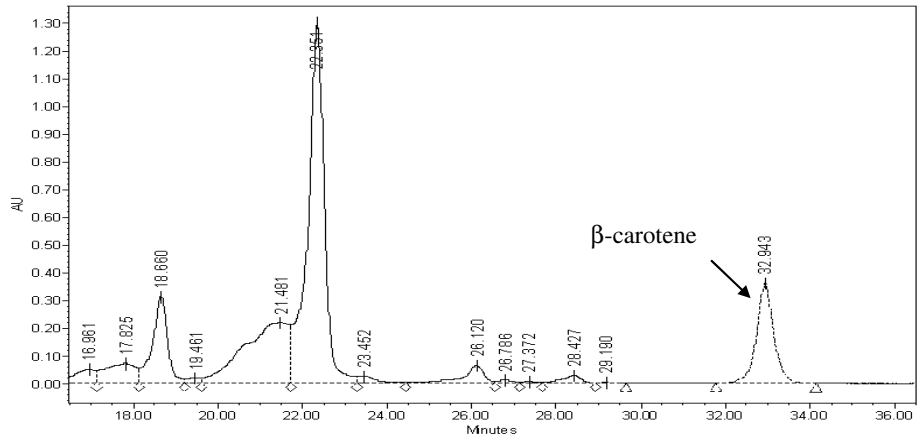


Figure 7.6.2 Example chromatograph of tomato (*Solanum lycopersicum* L. cv. Alisa Craig) leaf carotenoids separated on the C_{30} reverse phase column. β-carotene is labelled and the individual retention times (min) are shown Au = absorbance units.

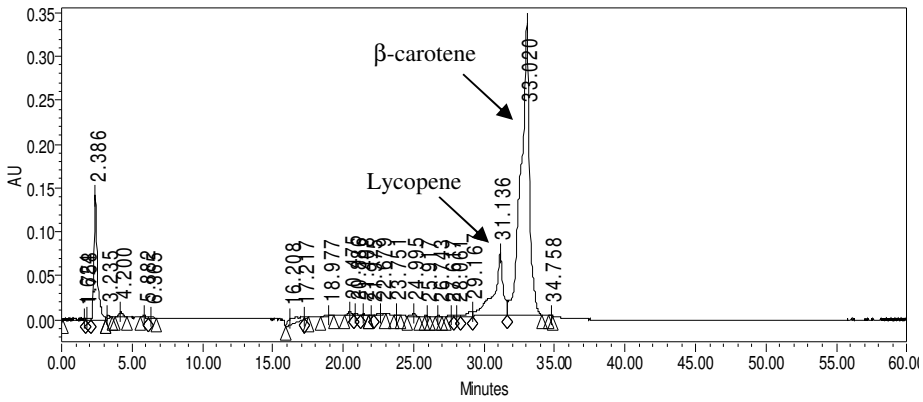


Figure 7.6.3 Example chromatograph of transgenic tomato (*Solanum lycopersicum* L. cv. Alisa Craig) line MJ8 root carotenoids separated on the C_{30} phase. Lycopene and β-carotene are labelled and the individual retention times (min) are shown.

7.6.2 Absorbance spectra (obtained from photodiode array detector) of the carotenoids resolved by HPLC

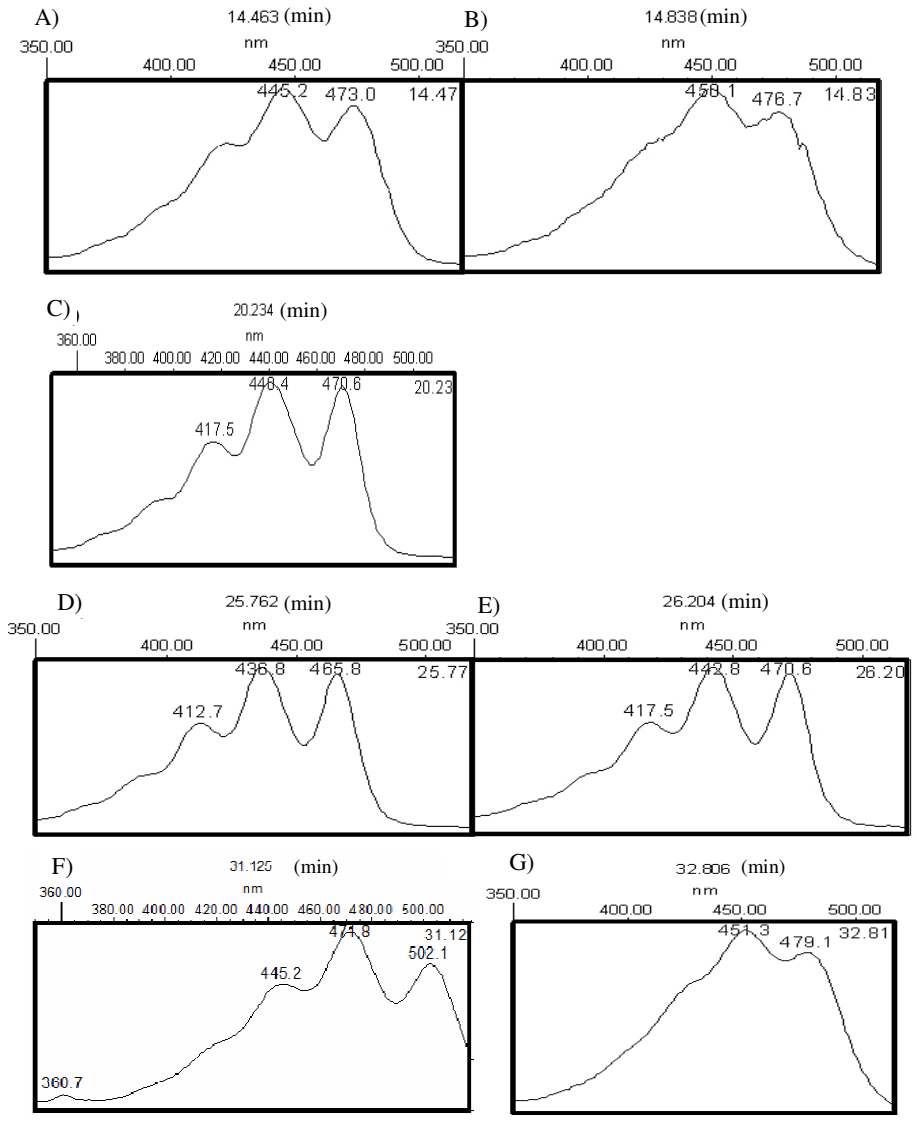
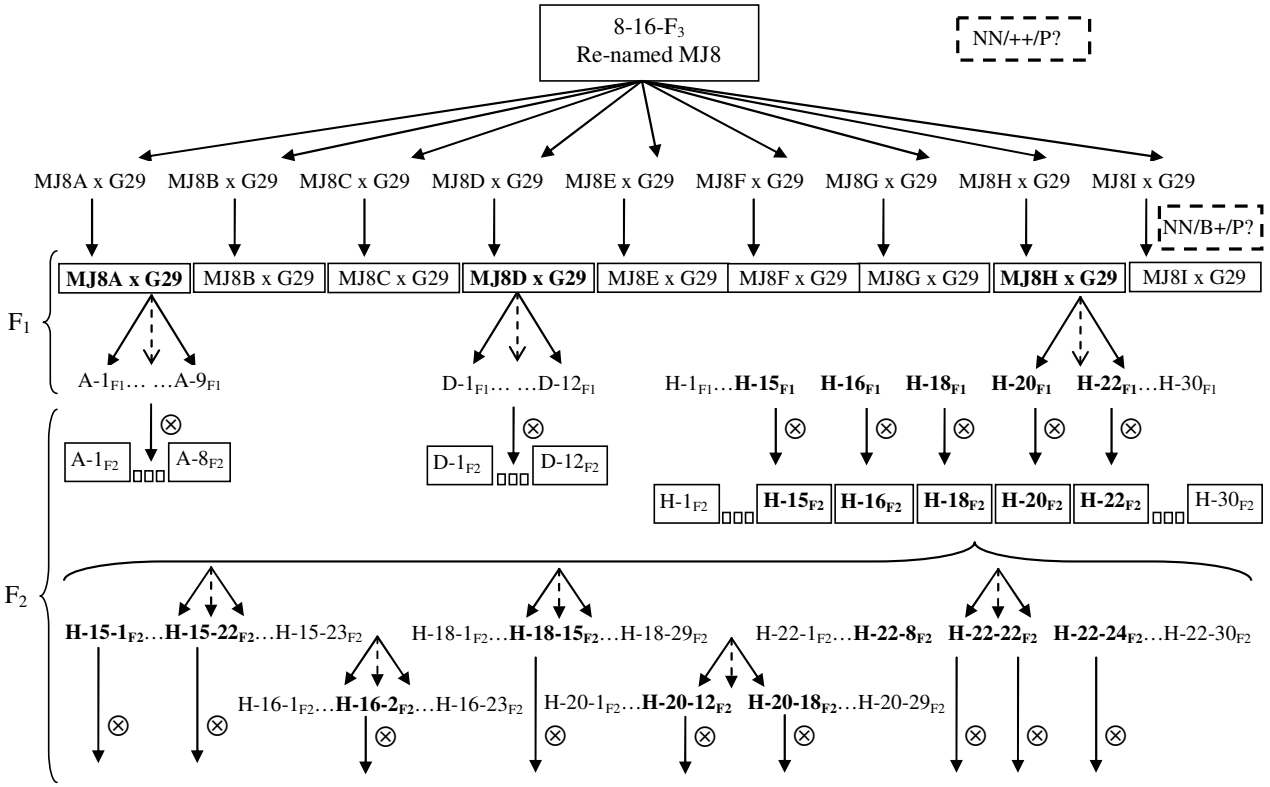


Figure 7.6.4 Typical absorbance spectra produced by tomato (*Solanum lycopersicum* L. cv. Alisa Craig) leaf carotenoids: resolved in the forward phase: A) lutein; B) zeaxanthin; C) violaxanthin; D) 9′-*cis*-neoxanthin; E) all-*trans*-neoxanthin (spectra were produced during a forward phase run). Leaf carotenoids resolved in the reverse phase: F) lycopene; G) β- carotene (spectra were produced during a reversed phase run). Each spectrum shows the λ maxima values and the retention time (min).

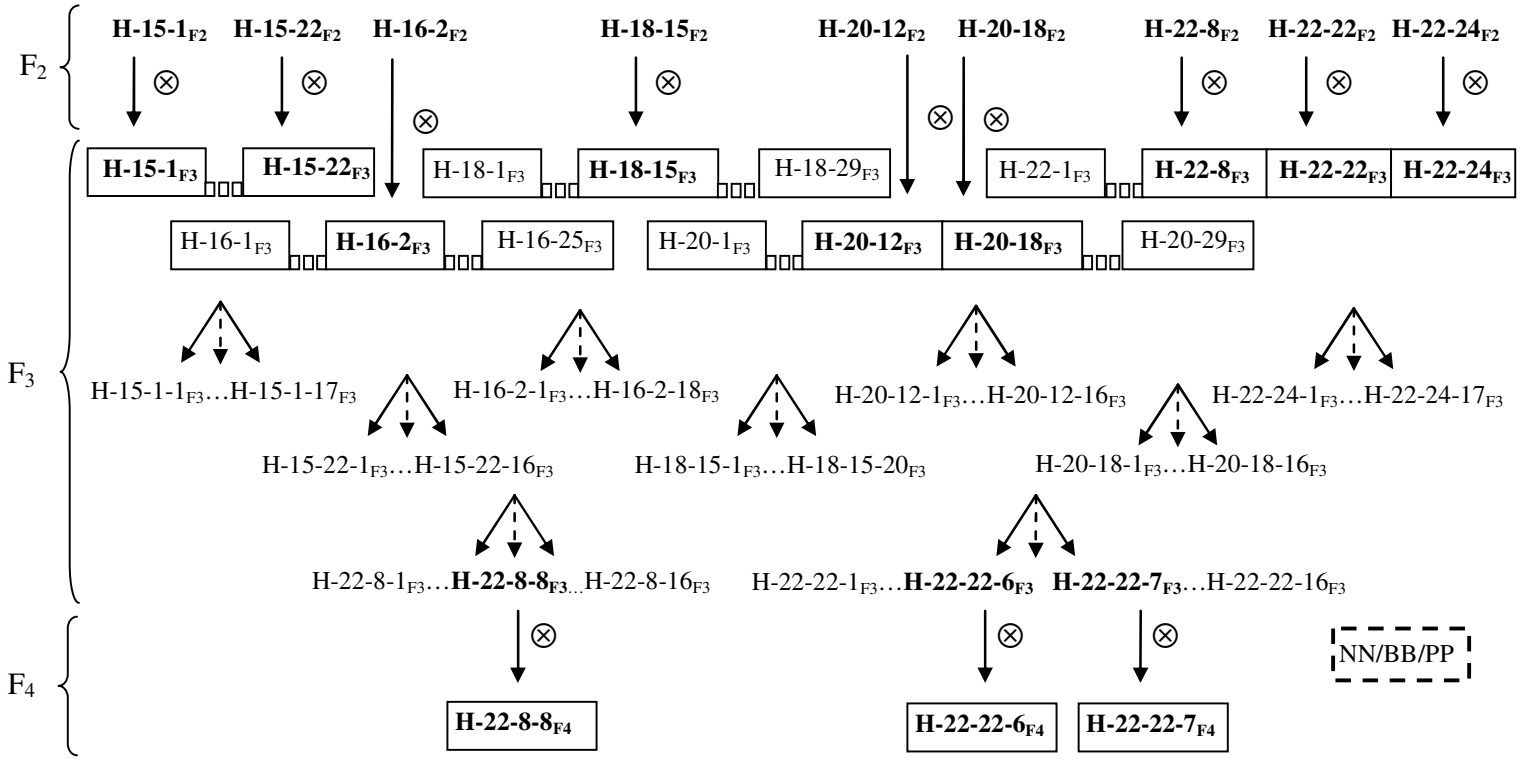
7.7 APPENDIX VII: THE TRIPLE LINE PEDIGREE

The diagram overleaf and continued on to the following page outlines the breeding pedigree of the triple construct transgenic lines produced and analysed in this research programme. The diagram starts with the initial set of crosses between individual plants of the double construct transgenic line MJ8 (homozygous for the *SlNCED1* T-DNA insertion locus associated with the single construct transgenic line known as sp5 and segregating for a number of T-DNA insertions of a *35S::SlPSY1* construct) and the double construct transgenic line G29 (homozygous for the same *SlNCED1* T-DNA insertion locus as in MJ8 but in this case also carrying *SlCrtRb2/BCH2* transgene inherited from BCH12). The diagram outlines the generations of plants derived from these initial crosses, focusing on selected individual plants, and ending with the F_4 generation of the three triple construct transgenic lines analysed in this research program i.e. H-22-8-8_{F4}, H-22-22-6_{F4} and H-22-22-7_{F4}. These three triple transgenic lines remained homozygous for the *SlNCED1* transgene inherited from sp5 and were verified using qPCR as homozygous for the *SlBCH2* transgene inherited from BCH12 and at least one *SlPSY1* transgene insertion locus inherited from MJ8.

In the diagram: Plants/seed packets in bold were selected to take to the next generation; ellipsis (…) represent multiple individual plants; solid line boxes represent seed packets; three small solid line boxes indicate multiple individual seed packets; a cross in a circle indicates a selfing event; dashed line boxes represent zygosity where: N = *SlNCED1* transgene; B = *SlBCH2* transgene; P = *SlPSY1* transgene; + = wild type allele; and ? = a transgene in an unknown number of copies present at an unknown number of T-DNA insertion loci.



In the diagram: Plants/seed packets in bold were selected to take to the next generation; ellipsis (…) represent multiple individual plants; solid line boxes represent seed packets; three small solid line boxes indicate multiple individual seed packets; a cross in a circle indicates a selfing event; dashed line boxes represent zygosity where: N = *SlNCED1* transgene; B = *SlBCH2* transgene; P = *SlPSY1* transgene; + = wild type allele; and ? = a transgene in an unknown number of copies present at an unknown number of T-DNA insertion loci.



7.8 APPENDIX VIII: THE NON-TRANSGENIC APPROACH

7.8.1 Allelic Identification of the Fourth Backcross

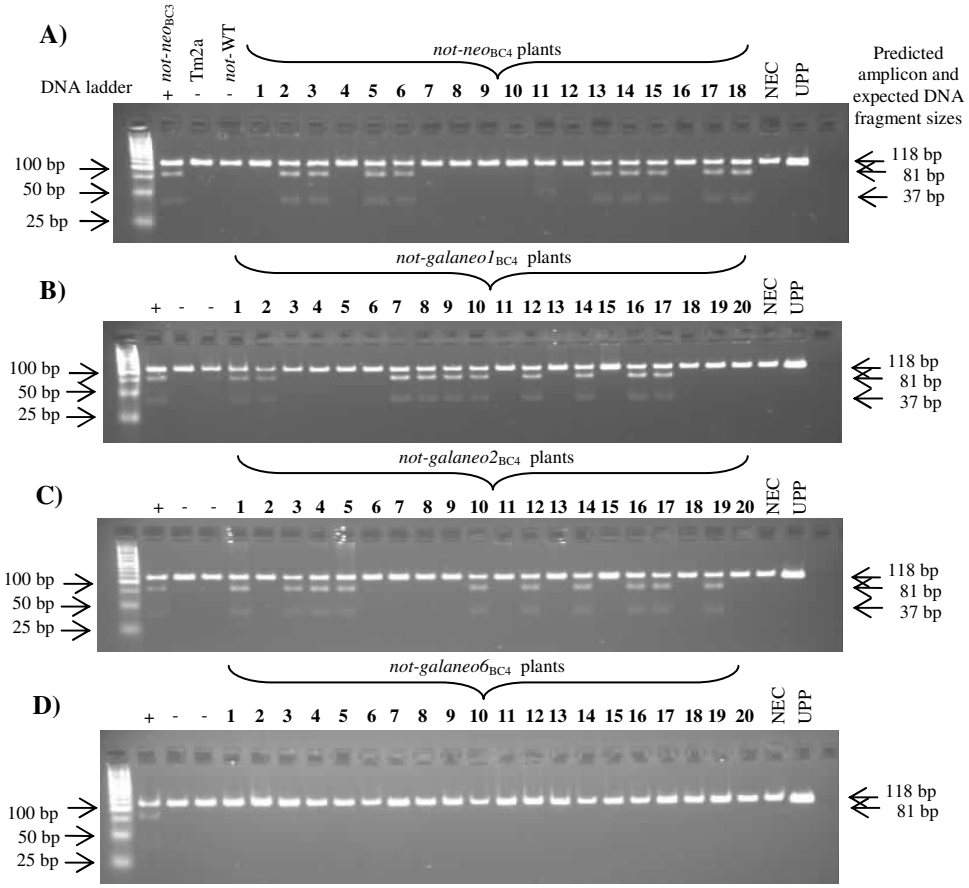


Figure 7.8.1 Gel electrophoresis images used to identify the *SnNCED1* allele in plants from the fourth backcross. The restriction enzyme digest of the predicted 118 bp *NCED1* amplicon with *Hinf*I, cleaves the *SnNCED1* allele to form two expected DNA fragments of 81 bp and 37 bp (4.5 % agarose gels). +, positive control, notabilis *x S. neorickii*$_{BC3}$; (*not-neo*$_{BC3}$); -, negative controls, *S. lycopersicum* line Tm2a, and *notabilis x S. lycopersicum* (*not*-WT); NEC, no enzyme control; UPP, undigested PCR product of *NCED1* amplicon using the *not-neo* DNA as a template; **A)** Lanes **1 – 18**, *not-neo*$_{BC4}$ plants 1 – 18; **B)** Lanes **1 – 20** *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$) plants 1 – 20: **C)** Lanes **1 – 20**, (*not-galaneo2*$_{BC4}$) plants 1 – 20: **D)** Lanes **1 – 20** (*not-galaneo6*$_{BC4}$) plants 1 – 20.

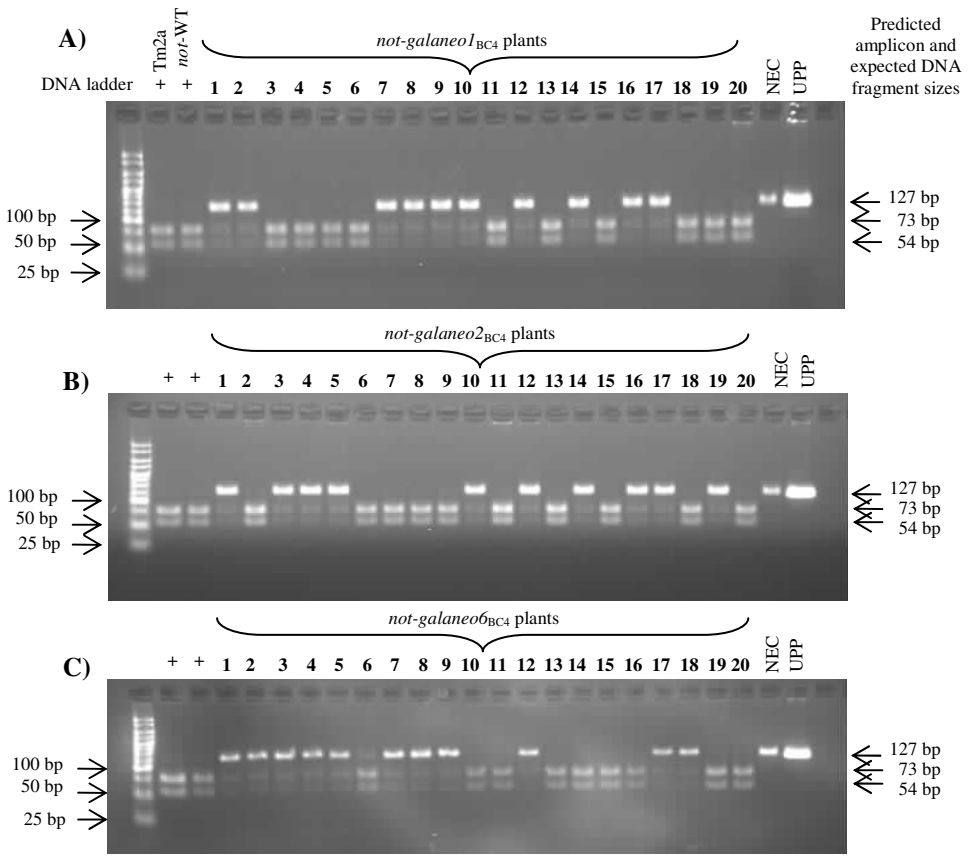


Figure 7.8.2 Gel electrophoresis images used to identify the *SlNCED1* allele in plants from the fourth backcross. The restriction enzyme digest of the predicted 127 bp *NCED1* amplicon with *Hind*III, cleaves the *S. lycopersicum NCED1* allele to form two expected DNA fragments of 73 bp and 54 bp (3.5% agarose gel). +, positive controls, *S. lycopersicum* line Tm2a, and *notabilis x S. lycopersicum* (*not*-WT); NEC, no enzyme control; UPP, undigested PCR product of *NCED1* amplicon using *not*-WT DNA as a template. **A)** Lanes **1 – 20**, *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$) plants 1 – 20: **B)** Lanes **1 – 20**, (*not-galaneo2*$_{BC4}$) plants 1 – 20: **C)** Lanes **1 – 20** (*not-galaneo6*$_{BC4}$) plants 1 – 20.

Table 7.8.1.a. A summary of the full *NCED1* allelic genotype and zygosity (homo, homozygous; hetero, heterozygous) of the individual plants derived from the BC4 lines: *notabilis x S. neorickii*$_{BC4}$; (*not-neo*$_{BC4}$), *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$), (*not-galaneo2*$_{BC4}$) and (*not-galaneo3*$_{BC4}$). Continued overleaf in Table 7.8.1.b.

PLANT	LINE							
	not-neo$_{BC4}$		*not-galaneo1*$_{BC4}$		*not-galaneo2*$_{BC4}$		*not-galaneo6*$_{BC4}$	
	Genotype	Zygosity	Genotype	Zygosity	Genotype	Zygosity	Genotype	Zygosity
1	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
2	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
3	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
4	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
5	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
6	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
7	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
8	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
9	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
10	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
11	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
12	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
13	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
14	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
15	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
16	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'

Table 7.8.1.b. continued from Table 7.8.1.a: A summary the *NCED1* allelic genotype of the BC4 generation of plants.

PLANT	LINE							
	not-neo$_{BC4}$		*not-galaneo1*$_{BC4}$		*not-galaneo2*$_{BC4}$		*not-galaneo6*$_{BC4}$	
	Genotype	Zygosity	Genotype	Zygosity	Genotype	Zygosity	Genotype	Zygosity
17	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
18	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
19	n/a	n/a	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
20	n/a	n/a	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
TOTALS	hetero = 9 homo = 9		hetero = 10 homo = 10		hetero = 10 homo = 10		hetero = 11 homo = 9	

7.8.2 Allelic Identification of Homozygous F_1 Plants from the Fourth Backcross

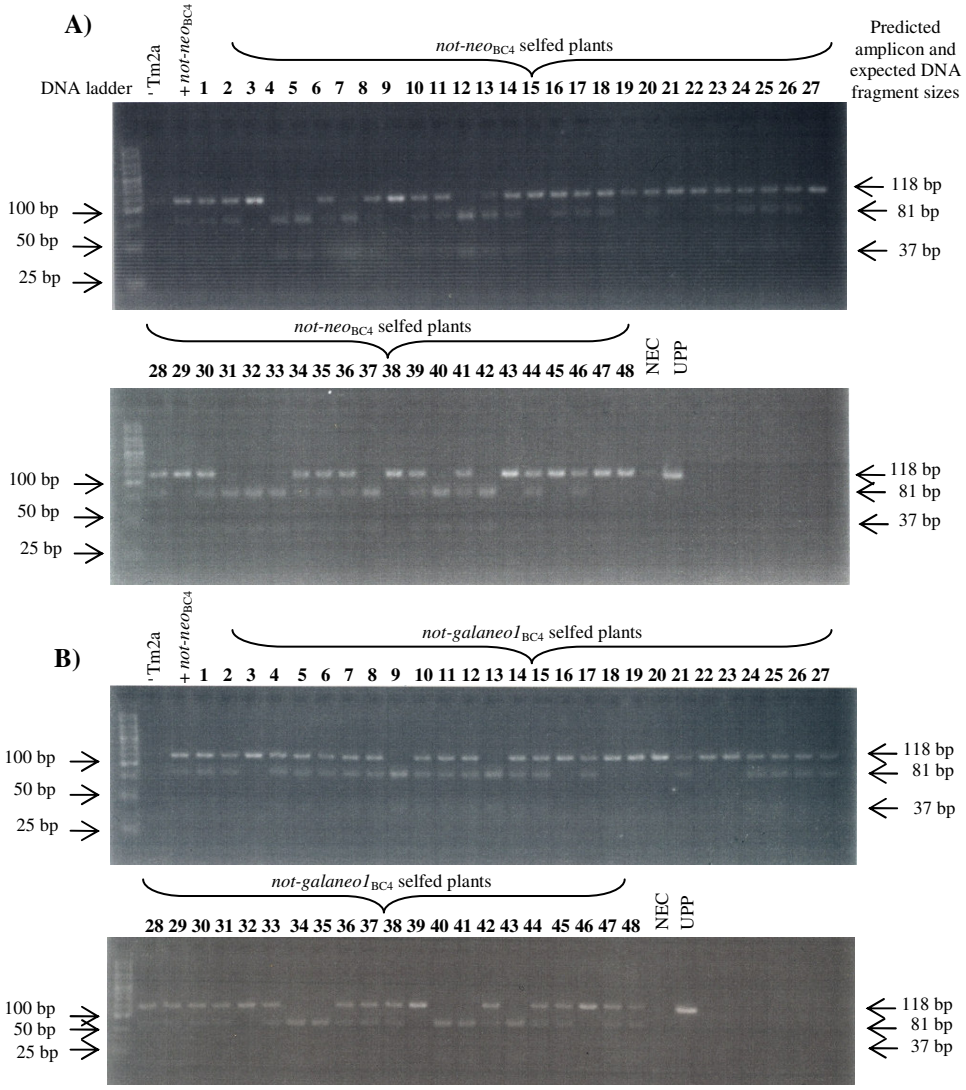


Figure 7.8.3 Gel electrophoresis images used to identify the *SnNCED1* allele in plants from the selfed fourth backcross. The restriction enzyme digest of the predicted 118 bp *NCED1* amplicon with *Hinf*I, cleaves the *SnNCED1* allele to form two expected DNA fragments of 81 bp and 37 bp (4.5 % agarose gels, 37 bp band sometimes too faint to be visible in re-prints). -, negative control, *S. lycopersicum* line Tm2a; +, positive control, *notabilis x S. neorickii*$_{BC4}$; (*not-neo*$_{BC4}$); NEC, no enzyme control ; UPP, undigested PCR product of *NCED1* amplicon using the *not-neo* DNA as a template; **A)** Lanes **1 – 48**, *not-neo*$_{BC4}$ selfed plants 1 – 48. **B)** Lanes **1 – 48**, *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$) selfed plants 1 – 48.

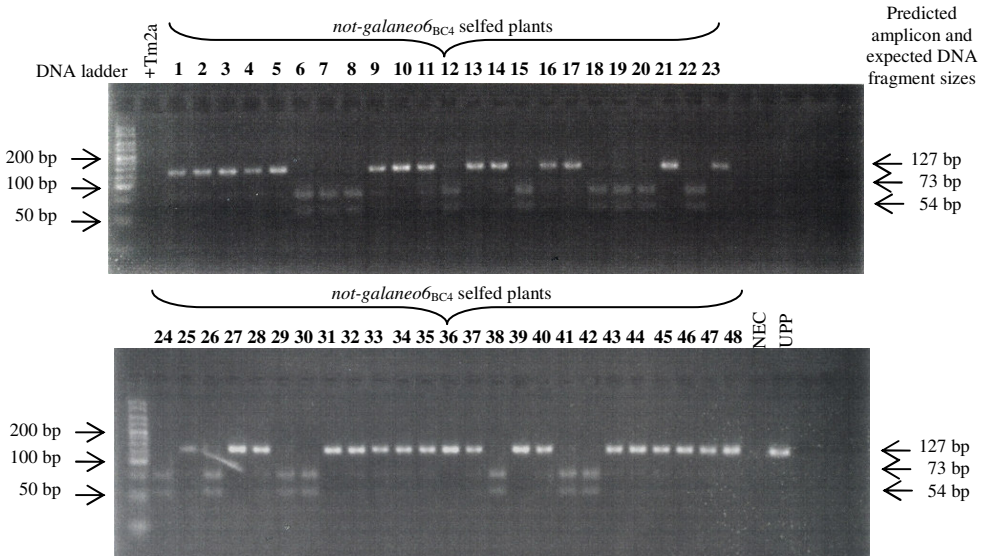


Figure 7.8.4 The gel electrophoresis images used to identify the *SlNCED1* allele in plants from the fourth backcross. The restriction enzyme digest of the predicted 127 bp *NCED1* amplicon with *Hind*III, cleaves the *SlNCED1* allele to form two expected DNA fragments of 73 bp and 54 bp (3.5% agarose gel). +, positive control, *S. lycopersicum* line Tm2a (not visible); NEC, no enzyme control; UPP, undigested PCR product (UPP) of *NCED1* amplicon using *not-galaneo6*$_{BC4}$ DNA as a template. Lanes **1 – 48** *S. lycopersicum x* (*S. galapagense x S. neorickii*)*6*$_{BC4}$ (*not-galaneo6*$_{BC4}$) selfed plants 1– 48.

Table 7.8.2.a. A summary of the full *NCED1* allelic genotype and zygosity (homo, homozygous; hetero, heterozygous) of the selfed BC4 generation of plants derived from the lines: *notabilis x S. neorickii*$_{BC4}$; (*not-neo*$_{BC4}$), *S. lycopersicum x* (*S. galapagense x S. neorickii*)*1* (*not-galaneo1*$_{BC4}$), (*not-galaneo2*$_{BC4}$) and (*not-galaneo3*$_{BC4}$). Continued overleaf in Table 7.8.2.b.

SELFED PLANT	**LINE**					
	not-neo$_{BC4}$		*not-galaneo1*$_{BC4}$		*not-galaneo6*$_{BC4}$	
	Genotype	Zygosity	Genotype	Zygosity	Genotype	Zygosity
1	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
2	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
3	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
4	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
5	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SgNCED1*	homo 'gg'
6	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
7	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
8	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
9	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SnNCED1*	homo 'nn'	*SgNCED1* *SlNCED1*	hetero 'gl'
10	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SgNCED1*	homo 'gg'
11	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
12	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
13	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SnNCED1*	homo 'nn'	*SgNCED1* *SgNCED1*	homo 'gg'
14	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
15	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
16	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'

Table 7.8.2.b. Continued from Table 7.8.2.a: A summary of the full *NCED1* allelic genotype and zygosity (homo, homozygous; hetero, heterozygous) of the selfed BC4 generation of plants continued overleaf in Table 7.8.2.c.

SELFED PLANT	**LINE**					
	not-neo$_{BC4}$		*not-galaneo1*$_{BC4}$		*not-galaneo6*$_{BC4}$	
	Genotype	Zygosity	Genotype	Zygosity	Genotype	Zygosity
17	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
18	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
19	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
20	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
21	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SgNCED1*	homo 'gg'
22	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'	*SlNCED1* *SlNCED1*	homo 'll'
23	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
24	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
25	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SgNCED1*	homo 'gg'
26	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
27	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SgNCED1*	homo 'gg'
28	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
29	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
30	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
31	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
32	*SnNCED1* *SnNCED1*	homo 'nn'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
33	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'

Table 7.8.2.c. Continued from Table 7.8.2.a: A summary of the full *NCED1* allelic genotype and zygosity (homo, homozygous; hetero, heterozygous) of the selfed BC4 generation of plants.

SELFED PLANT	LINE					
	not-neo$_{BC4}$		*not-galaneo1*$_{BC4}$		*not-galaneo6*$_{BC4}$	
	Genotype	Zygosity	Genotype	Zygosity	Genotype	Zygosity
34	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SnNCED1*	homo 'nn'	*SgNCED1* *SlNCED1*	hetero 'gl'
35	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SnNCED1*	homo 'nn'	*SgNCED1* *SlNCED1*	hetero 'gl'
36	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SgNCED1*	homo 'gg'
37	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
38	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
39	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SgNCED1*	homo 'gg'
40	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SnNCED1*	homo 'nn'	*SgNCED1* *SlNCED1*	hetero 'gl'
41	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SnNCED1*	homo 'nn'	*SlNCED1* *SlNCED1*	homo 'll'
42	*SnNCED1* *SnNCED1*	homo 'nn'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'
43	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SnNCED1*	homo 'nn'	*SgNCED1* *SlNCED1*	hetero 'gl'
44	*SnNCED1* *SlNCED1*	hetero 'nl'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
45	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
46	*SnNCED1* *SlNCED1*	hetero 'nl'	*SlNCED1* *SlNCED1*	homo 'll'	*SgNCED1* *SlNCED1*	hetero 'gl'
47	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
48	*SlNCED1* *SlNCED1*	homo 'll'	*SnNCED1* *SlNCED1*	hetero 'nl'	*SgNCED1* *SlNCED1*	hetero 'gl'
TOTALS	Homo 'll' = 13 Hetero 'nl' = 25 Homo 'nn' = 10		Homo 'll' = 10 Hetero 'nl' = 31 Homo 'nn' = 7		Homo 'll' = 15 Hetero 'gl' = 25 Homo 'gg' = 8	