Genetic Manipulation of Glutathione Levels in Lettuce: Crop Performance and Resistance to Tipburn

by

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CONTENTS PAGE

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	Amino acids
ABA	Abscisic acid
ADP	Adenosine diphosphate
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
AOS	Active oxygen species
As	Arsenic
ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
b.p.	Base pair
BSA	Bovine serum albumin
Ca	Chlorophyll a
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
$CaNO_3$	Calcium nitrate
CAT	Catalase
CaMV	Cauliflower mosaic virus
CAX	Cation exchangers
Cb	Chlorophyll b
Cd	Cadmium
cDNA	Copy deoxyribonucleic acid
Cl^-	Chloride ion
CLSM	Confocal laser scanning microscopy
cm	Centi-metre
CO_2	Carbon dioxide
CTAB	Cetyltrimethyl ammonium bromide
cv(s).	Cultivar(s)
CYS	Cystine (oxidised form of cysteine)
CYSH	Cysteine
d	Day
DIG	Digoxigenin-labeled
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DW	Dry weight
e	Epidermal layer
Ed(s).	Editor(s)
EDTA	Ethylendiaminetetraacetic acid
et al.	*et alia* (Latin; and others)
Fe	Iron
$FeCl_3$	Iron chloride
FW	Fresh weight
g	Gramme
GAE	Gallic acid equivalent
GC	Guanadine cytosine
GFP	Green fluorescent protein
GLU	Glutamate

GLY	Glycine
GR/GORI/*gor*I	Glutathione reductase
GSH	Reduced glutathione
GSHI/*gsh*I	γ-glutamylcysteine synthase
GSHII/*gsh*II	glutathione synthase
GSSG	Oxidised glutathione
h	Hour
H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
i.e.	*id est* (Latin; that is)
K^+	Potassium ion
k b.p.	Kilo base pair
KCl	Potassium chloride
kg	Kilogramme
K_2HPO_4	Dipotassium hydrogen orthophosphate
KH_2PO_4	Potassium dihydrogen orthophosphate
l	Litre
LB	Luria Broth
LEA	Late embrogenesis abundant protein
Ltd.	Limited Company
luc	Firefly luciferase
m	Metre
M	Molar
MDA	Malondialdehyde
mg	Milli-gramme
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
min	Minute
mg	Milli-gramme
mM	Milli-molar
ml	Milli-litre
Mn^{2+}	Manganese ion
mm	Milli-metre
mRNA	Messenger ribo nucleic acid
MS0	Murashige & Skoog (1962) basal medium
n	Number of replicates
Na^+	Sodium ion
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
N/A	Not applicable
NAA	α-naphthaleneacetic acid
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NH_4^+	Ammonium ion
nm	Nano-metres
nM	Nano-moles
NO_3^-	Nitrate ion

NPK	Nitrogen, phosphorous, potassium
*npt*II	Neomycin phosphotransferase
$^1O_2$	Singlet oxygen
O_2	Molecular oxygen
$O_2^{\cdot-}$	Superoxide anion radical
O_2^{2-}	Peroxide anion
$^{\cdot}OH$	Hydroxyl free radical
OX	Oxidation
Pb	Lead
PBS	Phosphate buffered saline
PC	Phytochelatin
PCR	Polymerase Chain Reaction
pH	Hydrogen potential
phgpx	Phospholipid hydroperoxide-dependant glutathione peroxidase
Pm	Palisade mesophyll
pp	page(s)
PTGS	Post-transcriptional gene silencing
PVP	Polyvinylpyrrolidone
qRT-PCR	Quantitative real-time PCR
RED	Reduction
RH	Relative humidity
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SARS-CoV	Severe acute respiratory syndrome coronavirus
Sm	Spongy mesophyll
SOD	Superoxide dismutase
SSC	Sodium citrate, sodium chloride buffer
T_0	Regenerated plant from tissue culture
$T_{1,2,3}$	Progeny derived from self-pollination of T_0, T_1, T_2 generation plants
TAE	Tris acetate EDTA buffer
TBA	Thiobarbituric acid
tDNA	Transfer deoxyribo nuceic acid
TE	Tris EDTA buffer
TGS	Transcriptional gene silencing
UV	Ultra-violet
v	Vascular bundle
V	Volt
VIGS	Virus-induced gene silencing
v/v	Volume to volume ratio
wk	Week
w/o	Without
w/v	Weight to volume ratio
x g	Multiple of gravity
Zn^{2+}	Zinc ion
ρM	Pico-molar

µg	Micro-gramme
µl	Micro-litre
µM	Micro-molar
°C	Degrees Celsius
α	Alpha
β	Beta
γ	Gamma
λ	Lambda
<	Less than
%	Percent
=	Equals

ABSTRACT

The four lettuce (*Lactuca sativa*) cvs. Evola, King Louie, Pic and Robusto were transformed with the binary expression construct pAFQ70.1, using *Agrobacterium tumefaciens* strain AGL1. The construct carried the glutathione metabolic genes *gsh*I, *gsh*II, *phgpx* and *gor*I. Both the *gsh*I and *gsh*II genes were fused to sequences encoding the pea glutathione reductase transit peptide (*grtp*), and were intended to influence glutathione synthesis and metabolism in the chloroplasts.

Reverse transcriptase PCR analysis of cvs. King Louie, Pic and Robusto T_0 transformants revealed that expression of the transgenes followed a varied pattern. These variations were most likely due to post-transcriptional gene silencing created by the presence of strong promoters, homology with endogenous plant genes and presence of multiple genes in a single vector. The presence of the transgenes, *gsh*I, *gsh*II, *phgpx*, and *gor*I, in cv. King Louie T_3 homozygous lines was confirmed by genomic DNA dot blots and Southern blots.

It was hypothesized that transformants would have an increased glutathione pool in the chloroplasts and thus would be able to withstand the damaging effects of active oxygen species generated by environmental stresses. Leaves of transgenic homozygous T_3 cv. King Louie lines were shown to have a 2-fold greater glutathione concentration than their respective azygous counterparts, although total antioxidant activity was similar in all lines. However, this did not result in enhanced stress tolerance, with the homozygous lines exhibiting no physiological or morphological advantage compared to the azygous and wild-type lines when grown under saline stress (150 mM NaCl).

Glasshouse trials during the summer of 2005 determined the susceptibility of cv. King Louie T_3 homozygous, azygous and wild-type lines to the foliar, stress related disorder tipburn. Wild-type plants grown both under calcium deficient conditions at the University of Nottingham and in a trial at Elsoms Seeds Ltd., Spalding, UK, had a reduced incidence of tipburn compared to transgenic plants of the homozygous and azygous lines. Macroscopic of tipburnt leaves revealed the condition formed sporadically, with small dark sunken necrotic spots spreading along the leaf margin restricting leaf expansion. Microscopic transverse sections of tipburnt leaves showed total collapse, disintegration and necrosis of the leaf structure.

CHAPTER 1 : GENERAL INTRODUCTION

1.1 Lettuce

Lettuce (*Lactuca sativa*) is one of the most widely grown salad crops in the USA, Europe and Australia, with a global production of 22 million tonnes in 2005 (Website 1). In the USA, lettuce production has an estimated value of $1.6 billion per annum, with crisphead types accounting for 90% of sales (Website 2). Sales of individual 'head' crops have been declining due to the increased popularity of prepared salad mixes containing a variety of vegetables, combined with sachets of salad dressing and croutons. This form of salad production has also proved popular with the fast-food industry and institutions such as hospitals and schools. Other uses of lettuce include the production of nicotine-free cigarettes and the isolation of sesquiterpene lactones from the milky sap, for use in medicine (Ryder, 1999).

1.1.1 Botany and morphology

Lettuce belongs to the largest dicotyledonous plant family, the *Asteraceae* (*Compositae*) (Hunter and Burritt, 2002). There are approximately 100 species of *Lactuca*, although only *L. serriola, L. saligna* and *L. virosa* share any sexual compatibility with *L. sativa*. The chromosome number of lettuce is most commonly n = 9, although n = 8 and n = 17 have been found (Ryder, 1999). Lettuce leaves are arranged spirally in the form of a rosette around a short stem. The plant has a tap root of up to 60 cm with lateral roots growing mainly in the upper soil levels. Leaf colour varies from light to dark green with anthocyanin sometimes being present. Lettuce appearance varies depending on the combination of leaf colour, shape and folding. When plants enter maturation, the stem elongates and branches to form an inflorescence. Each floret is a single, yellow ray type and due to the anthers and stigmas developing at identical times, the plant is primarily a self-fertilising species. Pollination by thrips and solitary bees sometimes occurs, resulting in approximately 1% sexual crossing (Ryder, 1999). Lettuce can be classified into seven morphological types based on their shape and growth, namely (i) Crisphead, (ii) Loose leaf, (iii) Butterhead, (iv) Romaine, (v) Latin, (vi) Stem and (vii) the Oilseed Group (Ryder 1999; de Vries 1997).

(i) Crisphead – Plants typically produce firm spherical heads composed of large cup-shaped leaves. Modern cvs. yield heads weighing 500 – 1000 g. This type of lettuce is sometimes incorrectly called "iceberg", which belongs to the subtype Batavia (Website 3; Ryder, 1999).

(ii) Loose leaf – Most commonly grown in home gardens. Leaves are rumpled, lobed and frilly, forming a flattened or open rosette. They range in colour from shades of green through to a reddish-bronze (Website 3).

(iii) Butterhead – Originating in Europe, this lettuce has loosely folded open-heads with soft, thin leaves. Most are dark green, sometimes purple-red tinged and have creamy yellow interiors (Website 3).

(iv) Romaine – This type is also known as cos or Roman. Plants have elongated leaves forming a cylindrical shaped head that is compact at maturity. Leaf colour ranges from yellow to dark green, though the inner leaves are creamy pale. Heads can weigh up to 750 g (Website 3; Ryder, 1999).

(v) Latin – Plants are a cross between the butterhead and cos types, forming loose heads with oval leaves (Ryder, 1999).

(vi) Stem – Referred to as asparagus lettuce or celtuce, a form of lettuce bred for its young fleshy stems (Deppe, 1993).

(vii) Oilseed Group – These types grow rapidly through the rosette stage and bolt early. The seeds are pressed for edible oils (Ryder, 1999).

1.1.2 Production and value

Lettuce is grown throughout the world in both the temperate and sub-tropical areas, although it is most easily produced under mild temperatures of 18 – 25 °C on neutral, well-irrigated soils. In the USA, planting takes the form of direct drilling of seeds while in Europe transplanting of seedlings is more common due to the higher crop prices making it economical. Most lettuce is harvested at 60 – 65 days although 110 – 120 days is common during winter periods (Website 4; Ryder, 1999). Lettuce is either harvested as whole heads which are cut and trimmed by hand in the field, or collected as mesclun, a mixture of young, small salad greens throughout the growing season. In order to guarantee maximum shelf life, lettuce heads must be transported from the field to a cooling plant as quickly as possible. A vacuum cooler allows lettuce to be cooled rapidly from 26 °C to 1 °C within 15 – 30 min. (Website 4 and 5). The lettuce heads are finally trimmed, washed and wrapped in film. The type of

film is important because it must be semi-permeable, allowing exchange of O_2 and CO_2, escape of excess moisture to prevent rotting and protection from damage during shipping. Final transportation is usually in refrigerated trailers at 3 – 4 °C (Ryder, 1999; Website 4).

More than 90% of the USA's lettuce is grown in California and Arizona, with Texas, Michigan, Ohio, New Mexico and Florida producing the remainder. Europe's largest producers are the UK, France, Spain, Italy, Germany and the Netherlands (Table 1.1). Lettuce production in the UK is based mainly in Kent, Lincolnshire and the Thames Valley, with growing aimed at Crisphead (75%), Butterhead (15%) and Romaine (10%) types. Outdoor production of lettuce in Northern Europe is confined to the summer months, with the crop being cultivated under glass at other times. Production in Australia is worth more than $43 million, while Japan produces 520,000 tonnes of lettuce worth $1.5 billion (Ryder, 1999).

Table 1.1: Total lettuce production for the leading European producers (Website 1).

Country	Area (1000 hectares)	Production (1000 tonnes)
Spain	33.600	920
Italy	21.300	846.8
France	13.500	526
UK	7.500	135
Germany	5.900	200
Netherlands	2.300	73

1.1.3 Nutritional value

Lettuce contains many useful vitamins, minerals, fibre and a considerable amount of water. Nutritional value varies depending on leaf colour, position of the leaf and morphological type. Romaine and loose leaf types are generally more nutritious than the butterhead and crisphead types. Lettuce is ranked at about twenty sixth in comparison to other fruits and vegetables in terms of its contribution to human diet. In the USA, it follows tomato and orange with respect to bulk consumption. The average nutritional values of lettuce are summarised in Table 1.2.

Table 1.2: Nutritional values of lettuce. Values are for 100g FW (Website 4).

Nutrient	Value (mg 100 g^{-1} FW)
Dietary fibre	1500
Protein	1000
Fat	400
Potassium (K)	240
Phosphorous (P)	27
Calcium (Ca)	23
Vitamin C	15
Sodium (Na)	9
Iron (Fe)	0.9
Zinc (Zn)	0.2
Vitamin B6	0.07
Vitamin E	0.05

1.1.4 Lettuce breeding

Constant demand by consumers and retailers for high quality fresh produce available throughout the year has resulted in intensive lettuce breeding aimed at improving pest and disease resistances, manipulation of leaf shape and colour together with improved succulence and flavour.

Each lettuce flower consists of many florets which open and become fertilised on the same day. Flower structure ensures self-fertilisation by dehiscing pollen as the stigma emerges from the anther sheath. The most common method used by plant breeders to ensure cross hybridisation involves washing pollen off the stigmas, after the pollen has been shed but before the stigma becomes receptive (Deppe, 1993). Nagata (1992) published an improved lettuce emasculation procedure which ensured 98% hybridisation compared to 25 - 75% hybridisation using conventional techniques. The method involved selecting the flowers buds to be pollinated, clipping all floret parts level with the top of the involucre and washing the exposed flower with a spray of water to remove any pollen adhering to the bud. The flower was sprayed every 10 min. to wash away any remaining pollen from when the stigma emerged to it becoming fully extended and receptive. Before cross pollinating

the flower, excess water was removed by gently blowing through a glass rod or straw (Nagata, 1992).

Related wild species of lettuce represent a significant untapped genetic resource (Beharav *et al.,* 2006). However, lettuce is only sexually compatible with a few of the species within the genus *Lactuca.* These include *L. serriola, L. saligna* and *L. virosa*, of which it crosses freely with *L. serriola.* The most commonly exploited traits include resistance to downy mildew (Beharav *et al.,* 2006), lettuce mosaic virus (LMV) (Ryder, 2002), corky root disease (Mou and Bull, 2004), *Fusarium* wilt (Tsuchiya *et al.*, 2004) and root knot nematode (Gomes *et al.*, 2000).

Downy mildew caused by the fungal pathogen *Bremia lactucae* is one of the most destructive lettuce diseases worldwide. More than 40 resistance genes (*Dm* genes) have been identified in lettuce that confer resistance to *B. lactucae.* However, a genetic host/pathogen relationship is formed, resulting in race-specific resistance to the *Dm* genes and resistant pathotypes of *B. lactucae* in the major lettuce growing regions (Grube and Ochoa, 2005). Screening for new *Dm* genes represents the main focus of lettuce breeding programmes. Beharav *et al.* (2006) screened 1027 wild genotypes of *Lactuca* (*L. serriola, L. saligna, L. aculeata*) using *B. lactucae* isolates with known *Dm* gene resistance. They identified 83 lines with possible new resistance potential. Grube and Ochoa (2005) used an alternative approach by screening the existing lettuce cvs. Grand Rapids and Iceberg for sources of unique resistance. Their study identified the possibility of unique resistance alleles due to plants from both cvs. maintaining field resistance, despite the presence of variation within the pathogen populations.

Screening for lines resistant to the corky root rot Gram negative bacterium *Rhizomonas suberifaciens* has focused on the use of marker-assisted breeding. The only known resistance gene, *cor*, can be detected using restriction fragment length polymorphism and single nucleotide polymorphism markers (Moreno-Vazquez *et al.*, 2003). Dufresne *et al.* (2004) indicated that fluorescence resonance energy transfer would offer a faster, more accurate form of selection especially for use in high-throughput screening.

Improved nutrient status through increased carotenoid content (Mou, 2005), reduction of nitrate accumulation capacity under low light conditions (Reinink, 1992) and improved flavanoid content in inner leaves of commercial head cvs. (Hohl *et al.*, 2001), are also relevant to this crop.

1.1.5 Lettuce tissue culture

Lettuce tissue culture and regeneration of adventitious shoots is considered to be highly genotype dependent (Hunter and Burritt, 2002). Early studies investigated and optimised tissue culture parameters, including type and concentration of growth regulators, media composition, light and temperature (Sasaki 1975, 1979a,b,c, 1982; Xinrun and Conner, 1992). Ampomah-Dwamena *et al.* (1997) screened 22 lettuce genotypes belonging to different morphological groups for their shoot regeneration response on Schenk and Hildebrandt (SH; 1972) medium supplemented with 3% (w/v) sucrose, 0.1 mg l^{-1} indoleacetic acid (IAA), 0.5 mg l^{-1} kinetin and 0.05 mg l^{-1} zeatin. They used Elf Bronze Mignonette as the standard genotype, allowing them to detect variations between experiments and to rank the genotypes. They did not find any statistical correlation between callus index and shoot index or between tissue culture performance and morphological grouping. Genotypes with good shoot regeneration included Bambino and Iceberg (Crisphead types), Cobham Green and Sweet Butter (Butterhead types), Simpson Elite (Leaf type), and Rosalita and Paris White (Cos types). Although the type and composition of tissue culture media and environmental parameters varies between studies, successful shoot regeneration can be accomplished using Murashige and Skoog (MS; 1962) medium with 3% (w/v) sucrose, 0.04 mg l^{-1} α-naphthalene acetic acid (NAA), 0.5 mg l^{-1} benzylamino purine (BAP) and semi-solidified with 0.8% (w/v) agar at pH 5.8. An incubation temperature of 23 ± 2°C with a 16 h photoperiod (50 μmol m^{-2} s^{-1}, Daylight fluorescent tubes) is satisfactory (Curtis *et al.*, 1994).

Hunter and Burritt (2004, 2005) investigated the influence of light quality on organogenesis and concentrations of endogenous polyamines (PA) in lettuce cotyledon explants grown under different light quality. They germinated seeds of the lettuce cvs. Bambino, Greenway, Red Coral and Red Oak Leaf in the dark or under white, red or blue light, and cultured the cotyledon explants on shoot inducing media for 28 days under white light. Germination in the dark reduced shoot numbers, blue light inhibited shoot production, while red light promoted shoot regeneration or had no effect on regeneration compared to the controls. Explants cultured under white or red light accumulated more PAs during shoot primordia production than those cultured under blue light. Polyamines from blue light cultures also contained more insoluble conjugates compared to explants from white or red light cultures which contained a greater quantity of soluble conjugates. Their results suggest that

phytochrome and cryptochrome play important roles in shoot regeneration and organogenesis in lettuce and that polyamines are involved in the formation of shoot primordia.

The tissue culture environment can also be used for the testing of disease resistance. Mazier *et al.* (2004) demonstrated the potential to screen LMV resistances in lettuce *in vitro*. Ten lettuce cvs. were used, there being 3 butterhead, 5 crisphead and 2 accessions of a related species, *L. virosa*. Cultures were inoculated with natural LMV isolates, as well as green fluorescence protein (GFP)-tagged recombinant virus isolates. Screening results showed good correlation between resistance of the whole plant and in tissue culture. The method also allowed large numbers of plants to be screened in a reduced space while maintaining quarantine regulations.

1.1.6 Protoplast culture and regeneration of somatic hybrids

Production of somatic hybrids from protoplast fusion offers a method of avoiding the pre- and post-zygotic barriers often related with sexual hybridisation. Desirable characteristics of wild *Lactuca* species can be introgressed into *L. sativa* which would normally be impossible using standard breeding methods (Maisonneuve *et al.*, 1995). Reports of lettuce somatic hybrids include those between *L. sativa* and *L. virosa* (Matsumoto, 1991), *L. sativa* and *L. virosa*, *L. tatarica* or *L. perennis* (Maisonneuve *et al.*, 1995), and *L. sativa* and *L. tatarica* or *L. perennis* (Chupeau *et al.*, 1994).

Matsumoto (1991) regenerated somatic hybrids between *L. sativa* and *L. virosa* following protoplast electrofusion. Hybrid selection was based on inactivation of *L. sativa* with 20 mM iodoacetamide and the inability of *L. virosa* protoplasts to divide in the culture conditions used. Hybrids were identified using isoenzyme analysis and displayed intermediate foliar morphology, normal flower morphology and all plants were sterile. Chupeau *et al.* (1994) created a 'universal-hybridiser' lettuce line from the cv. Ardente, heterozygous for kanamycin resistance, and the cv. Girrelle, heterozygous for a recessive albinism marker. Protoplasts derived from immature plantlets were fused with *L. tatarica* and *L. perennis* to produce 9 plants. Hybrid status was confirmed using random amplified polymorphic DNA (RAPD) analysis and on morphological traits. Maisonneuve *et al.* (1995) produced vigorous somatic hybrids between *L. sativa* and *L. virosa* with resistance to leaf aphid,

powdery mildew and bacterial rot. Hybrids between *L. sativa* and *L. tartarica* were resistant to downy mildew.

1.1.7 Introduction of agronomically important genes into lettuce by *Agrobacterium*

The use of recombinant DNA transformation technology has allowed specific characteristics of lettuce to be altered for agronomic use. The bialaphos resistance (*bar*) gene was introduced into the lettuce cv. Evola by *A. tumefaciens* mediated transformation (Mohapatra *et al.* 1999). Stable expression of the resistance gene was observed both in the T_1 and T_2 plant generations. Resistance was observed in seedlings cultivated in medium containing 5 mg l^{-1} glufosinate ammonium and glasshouse-grown plants sprayed with 300 mg l^{-1} of the herbicide. Nagata *et al.* (2000) produced 6 glyphosate resistant transgenic plants of the lettuce cv. South Bay. Plants were transformed with *A. tumefaciens* carrying a plasmid with a gene encoding the enzyme 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS). Twenty one d-old glasshouse transgenic plants were sprayed with glyphosate concentrations ranging from 0 – 35.84 kg ha^{-1}. All control plants died at 0.55 kg ha^{-1}, while transgenic lines were able to grow normally at concentrations of the herbicide up to 17.92 kg ha^{-1}.

Reduction of nitrate accumulation in winter grown lettuce leaves has been a target for genetic manipulation that has met with limited success. Curtis *et al.* (1999) introduced the nitrate reductase (*nia*2) gene into the lettuce cvs. Cortina, Evola, Flora and Luxor. Transgenic status was confirmed by nitrate reductase enzymatic assay and by Southern hybridisation. However, the transgenic plants did not appear to show any reduction in nitrate content compared to the wild-type plants. Dubois *et al.* (2005) transformed the lettuce cv. Jessy with the identical *nia*2 gene for nitrate reductase under the control of cauliflower mosaic virus 35S promoter. They suggested the lack of expression was due to the presence of endogenous nitrate reductase mRNA inducing gene silencing.

Research has also been directed towards improving the foliar composition and shelf-life of cultivated lettuce. Sun *et al.* (2006) cloned the miraculin gene from the West African shrub *Richadella dulcifica* and introduced it into the lettuce cv. Kaiser by *A. tumefaciens* transformation. Transgenic plants expressing the gene actively accumulated the sweet enhancing protein, miraculin. Improvement of tocopherol composition in the lettuce cv. Chongchima was attempted by Cho *et al.*

(2005). They used a gene encoding γ-tocopherol methyltransferase from *Arabidopsis thaliana* to increase enzyme activity and conversion of γ-tocopherol to the more potent α form. Improvement of plant element content represents an important step in the improvement of human nutrition, especially in developing countries. Macro- and micro-elements play important roles in enzyme activity, organ function and general health. Accumulation of increased zinc content was investigated by Zuo *et al.* (2002) in the cv. Salinas 88. The mouse metallothionein mutant β-cDNA was inserted using *A. tumefaciens*-mediated transformation. The concentration of zinc in transgenic plants was up to 400 $\mu g\ g^{-1}$ dry weight, significantly more than in wild-type plants. Goto *et al.* (2000) transformed the cv. Green Leaf with a plasmid containing a CaMV 35S promoter-soybean ferritin cDNA and the kanamycin (*npt*II) resistance gene. Transgenic plants contained 1.2 – 1.7 times greater iron contents than wild-type plants. A plant weight gain of 27 – 42% was observed in transgenic plants during the early developmental stages, as was an increased rate of photosynthesis. McCabe *et al.* (2001) inserted the *ipt* gene for the enzyme isopentenyl phosphotransferase, which is involved in biosynthesis of plant cytokinins. The cv. Evola was transformed with this gene under the control of the senescence specific SAG12 promoter from *A. thaliana*. Transgenic plants displayed retardation of leaf senescence and exhibited normal leaf morphology, head diameter and leaf and root fresh weights.

Modification of plant tolerance to biotic and abiotic stress is possibly the single most important area of plant genetic research. Resistance to *Sclerotinia sclerotiorum,* a plant pathogenic fungus that causes stem rot, was introduced into lettuce by Dias *et al.* (2006). Transgenic lettuce of the cv. Veronica was produced by *A. tumefaciens* mediated transformation with a plasmid containing the decarboxylase gene (*oxdc*) from the genus *Flammulina*. Thirty-four regenerated plants contained the gene of interest, which segregated in the T_1 generation in a typical Mendelian fashion. Resistance to the fungal pathogen *S. sclerotiorum* was confirmed using a leaf disc assay. Curtis *et al.* (1996) introduced the *rolAB* gene into the lettuce cv. Lake Nyah. Transgenic plants exhibited extensive root development, the result of an increased auxin content. Similar results were obtained by Kim and Botella (2004) in lettuce plants transformed with the ethylene mutant receptor *etr1-1* under the control of the senescence specific SAG12 promoter. Regenerating explants showed abnormal properties, with extensive root formation occurring immediately from the leaf explants and slow development of shoots. Niki *et al.* (2001) introduced the

pumpkin gibberellin (GA) 20-oxidase gene into the lettuce cv. Vanguard. Single gene copy was confirmed by Southern blot analysis, and transgenic plants segregated in a Mendelian fashion, suggesting the transgene was stable and dominant. Plants exhibited dwarf morphology and had reduced concentrations of GA_1 and GA_4, but increased concentrations of GA_{17} and GA_{25}. Genetic manipulation of drought and saline tolerance in lettuce represents an important aim for the future of intensive agriculture with excessive irrigation and poor drainage causing increased soil salinisation. Park *et al.* (2005a) introduced the late embryogenesis abundant (*lea*) gene from *Brassica napus* into lettuce by *A. tumefaciens* mediated transformation. Transgenic plants showed increased growth under salt stress and water deficient conditions than wild-type plants. After 10 d growth in 100 mM NaCl, transgenic lettuce plants weighed 2.5 g while the control plants were 0.3 g. The LEA protein may stabilise membranes, protecting them from osmotic damage. Overexpression of the *A. thaliana ABF3* gene in the lettuce cv. Chongchima was reported by Vanjildorj *et al.* (2005). The transgene encoded a transcription factor for the expression of abscisic acid (ABA) responsive genes. Plants had greater tolerance to drought and cold stress, were morphologically normal and set seed.

Use of plants to synthesize pharmaceutically important recombinant proteins has received much attention in recent years. The technology is still in its infancy, but could potentially be scaled up to produce high value recombinant proteins. Negrouk *et al.* (2005) utilised lettuce to produce the pharmaceutically important humanized IgG1 k anti-tissue factor antibody (hOAT). Commercially obtained lettuce heads were vacuum infiltrated with *A. tumefaciens* and incubated at 20 - 26°C, with a 16 h photoperiod, for 3 - 4 d. This optimised protocol allowed 20 – 80 mg of functional antibody per kg of fresh lettuce leaf tissue in less than 1 wk. A similar study was undertaken by Joh *et al.* (2005) using expression of the β-glucoronidase (*gus*) gene as a marker of transformation. Lettuce leaf discs of the cvs. Hearts Delight and Green Forest were vacuum infiltrated with *A. tumefaciens* and incubated for 72 h at 22°C in continuous darkness. Production of the GUS protein was 0.16% based on DW of tissue. Incubation of leaf disks in continuous light resulted in more rapid protein synthesis, although the final protein content was not different from that in dark incubated samples.

1.1.8 Plastid transformation

Plastid transformation is a novel system that can circumvent many of the disadvantages associated with nuclear transformation. These advantages include:

1. Lack of gene silencing which is frequently observed in nuclear transformants (Kanamoto *et al.*, 2006).
2. Lack of positional effects because the transgene is inserted into a known region of the plastid genome between flanking sequences e.g. *trnA* and *trnI* genes (Lelivelt *et al.*, 2005).
3. High expression of foreign genes with 7000 – 8000 copies per cell (De Cosa *et al.*, 2001).
4. The ability of chloroplasts to form disulphide bonds and to fold human proteins will enable the production of pharmaceuticals and vaccines in plants (Daniell *et al.*, 2002).
5. Inheritance of genes can occur maternally due to the transgenes being contained within the plastid genome (Daniell *et al.*, 2002). However, evidence exists for biparental plastid inheritance in some species (Ji *et al.*, 2004).

Lelivelt *et al.* (2005) utilised polyethylene glycol-mediated transformation of protoplasts of the lettuce cv. Flora. The transformation vector targeted the *trnA - trnI* intergenic region of the lettuce plastome using an *aadA* gene for resistance to spectinomycin. Plants were fertile and homoplasmic, and were able to transmit the plastid-encoded genes to the T_1 generation. Maternal (seed) based gene transmission was confirmed by crossing the transgenic plants with male sterile lines to establish that the antibiotic resistance was not transmitted by pollen. Kanamoto *et al.* (2006) developed a micro-projectile bombardment plastid transformation system for the lettuce cv. Cisco. The transformation vector carried the *aadA* spectinomycin resistance gene and was targeted to the *rbcL* and *accD* plastome genes. Their results showed that one fertile transgenic plant was produced from each bombardment and that stable transgene expression was observed in the T_1 generation.

1.2 Tipburn

Tipburn is characterised as a necrotic disorder occurring on the margins of young developing leaves of vegetable crops. It mainly affects head forming leafy vegetables, including lettuce (*L. sativa*), white cabbage (*Brassica oleracea* var. *capitata*) and Chinese cabbage (*B. pekinensis*). Localised foliar calcium (Ca^{2+}) deficiency is regarded to be the prime cause of tipburn (Everaarts and Blom-Zandstra, 2001). Symptoms are usually restricted to the inner leaves and thus are not noticed until cropping. This means that selective picking cannot be carried out and whole fields are often abandoned (Ryder, 1999; Misaghi *et al.*, 1992). Annual losses from tipburn are £1.3 million in the UK (Website 2).

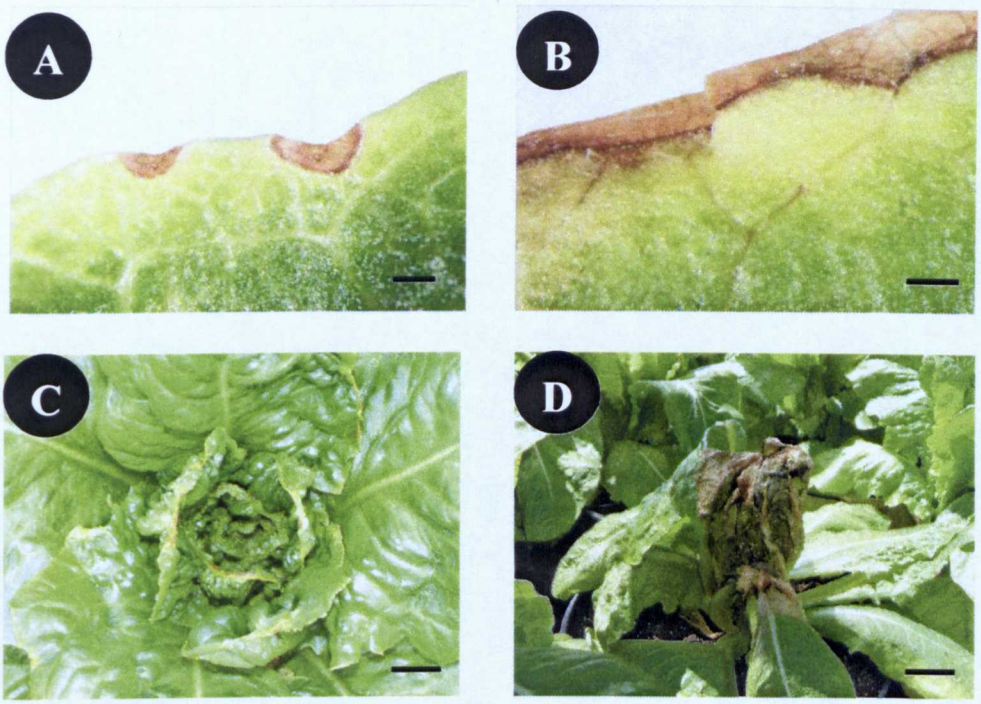
In addition to its effect in leafy vegetables, Ca^{2+} deficiency is thought to cause many crop disorders, such as blossom-end rot (BER) in tomato (*Lycopersicon esculentum*), kiwi (*Actinidia deliciosa*) and sweet pepper (*Capsicum annuum*), leaf necrosis in lily (*Lilium* sp) and bitter pit in apple (*Malus domestica*) (Saure, 2005). Translocation of Ca^{2+} to affected tissues occurs via the xylem, with a direct correlation between transpiration rate and Ca^{2+} delivery (White, 2001). However, Saure (2005) suggested Ca^{2+} deficiency is not due to lack of transport, but is actually a result of gibberellins restricting Ca^{2+} movement in order to maintain rapid fruit growth. Taylor *et al.* (2004) indicated that plant Ca^{2+} deficiency is caused by many factors such as accelerated growth rate, low water availability, low concentrations of soluble Ca^{2+} and high or low transpiration.

1.2.1 Symptoms and development of tipburn

Tipburn affects all forms of lettuce and usually occurs close to the time of harvest when leaves begin to bend inwards forming a head. Tipburn often forms sporadically, with plants exhibiting symptoms to full leaf membrane breakdown within days. Tipburn is characterised by small dark spots of collapsed and necrotic tissue along the leaf margins. This darkening is initially caused by loss of cell turgor and degeneration of organelle membranes. The sunken spots progress along the leaf edge, eventually forming and band of necrotic tissue which prevents further leaf expansion (Figure 1.1) (Ryder, 1999). In more severely affected plants the laticifers become ruptured and leakage of latex in to the surrounding tissue causes complete

collapse and necrosis of the leaf (Figure 1.2) (Collier and Tibbitts, 1982; Matyac and Misaghi, 1981).

Figure 1.1: Development of tipburn on lettuce leaves of cv. King Louie.



(A) Commencement of tipburn in lettuce with small dark sunken spots on the leaf margin. (B) Progression of tipburn along the leaf edge. (C) Tipburn preventing expansion of immature leaves. (D) Severe tipburn causing necrosis of the lettuce head, resulting in a fungal infection. Bars = 1 mm (A; B), 2.5 cm (C) and 3 cm (D).

1.2.2 Factors influencing the incidence of tipburn

1.2.2.1 Insufficient foliar calcium

Uptake of Ca^{2+} by plants is closely linked to the transpiration stream, which exerts a pull on the soluble calcium fraction from the soil. Calcium uptake is stimulated by NO_3^- and depressed by NH_4^+, K^+, Na^{2+}, Mg^{2+} and low soil pH (Park *et al.*, 2005b; Taylor *et al.*, 2004; Collier and Tibbitts, 1982). Several studies have suggested Ca^{2+} deficiency is the prime cause of tipburn due to its role in maintaining membrane stability, cell integrity and plant structure (Montanaro *et al.*, 2006; Park *et al.*, 2005b; Saure, 2005; Pressman *et al.*, 1993). Collier and Tibbitts (1982) suggested that lack of Ca^{2+} probably causes loss of membrane integrity which develops into tipburn. Rosen (1990) confirmed that unaffected leaves of cauliflower had 2 – 5

times more Ca^{2+} than tipburnt leaves of the same physiological age. Barta and Tibbitts (1986) used atomic absorption spectroscopy to demonstrate that tipburnt leaves had less Ca^{2+} (0.63 mg g^{-1} DW) than control plants (1.48 mg g^{-1} DW). Chang and Miller (2005) found that Ca^{2+} concentrations in necrotic tissues of lily were 6-fold lower than in normal leaves, while reduced tomato fruit Ca^{2+} concentrations resulted in 5 times more BER (Taylor *et al.*, 2004). Misaghi and Grogan (1978) suggested that increased foliar concentrations of organic and amino acids chelate and prevent availability Ca^{2+} before tipburn develops.

Spraying Ca^{2+} salts directly on to young foliage can reduce tipburn damage significantly (Pressman *et al.*, 1993). Exogenous applications of 25 mM $CaCl_2$ and $CaNO_3$ to the lily cv. Star Gazer for 2 wks significantly suppressed tipburn symptoms from 18 (severely necrosed) to below 3 (almost unnoticeable) (Chang *et al.*, 2004). However, the effect of Ca^{2+} on tipburn is usually as a direct result of other environmental conditions e.g. humidity, temperature, soil water, and so cannot be considered meaningful without taking other factors into consideration.

1.2.2.2 Humidity

A direct connection can be drawn between humidity and tipburn incidence (Ciolkosz *et al.*, 1998; Saure, 1998; Barta and Tibbitts, 1986). Barta and Tibbitts (1986) enclosed young lettuce plants under polythene sheaths where relative humidity (RH) was 65%. After 4 d, 53% of the leaves showed tipburn compared to 1% on the control plants. High RH appears to induce tipburn in young and old leaves in both head forming and loose leaf cultivars (Saure, 1998). Hernandez *et al.* (2004) studied the effect of row cover on the quality of Chinese cabbage crops during a 3 year period. Calcium concentration was significantly greater in the outer leaves of plants grown in the open, while the opposite was true for inner leaves. They suggested that reduced foliar Ca^{2+} concentrations encouraged tipburn. Constant RH caused a higher incidence of tipburn than low relative humidity in *Eustoma grandiflorum,* with tipburned leaves always having less foliar Ca^{2+} (Islam *et al.*, 2004). A 2 year study concentrating on collard (*B. oleracea*) production in the USA concluded that high humidity during summer months was the primary reason for tipburn (Mylavarapu *et al.*, 2005). High RH is thought to inhibit Ca^{2+} movement in the xylem by reducing transpiration (Everaarts and Blom-Zandstra, 2001; Barta and Tibbitts, 1986). Taylor *et al.* (2004) and McLaughlin and Wimmer (1999) found

direct correlations between high RH and reduced Ca^{2+} uptake. Nelson *et al.* (2003) studied hydroponic forcing in tulip (*Tulipa gesneriana*) cvs. They reported that Ca^{2+} uptake and accumulation were significantly less at 82% RH compared to 42% RH. A recent study found that blowing air directly onto lettuce meristems increased transpiration, allowing 3 times more light, 5°C higher temperature optimum and elevated CO_2 to be used without any affect on tipburn incidence. Over a 23 d period, plants grown under these conditions produced a 4-fold increase in biomass accumulation compared to controls (Frantz *et al.*, 2004).

1.2.2.3 Soil nutrients

It was concluded by Dickinson (1977) that high soil fertility, especially high nitrogen, causes very severe tipburn in susceptible cultivars of cabbage. This view has always been considered the general rule especially for high nitrate fertilisers in humid conditions (Saure, 1998). Magnusson (2002) found that large applications of mineral fertilisers to Chinese cabbage increased the occurrence of internal tipburn, while the use of green mulch resulted in slower growth but prevented internal tipburn. Conversely, some studies have found that increasing soil nitrate concentrations had the opposite effect on tipburn incidence. Rosen (1990) showed that a 3-fold increase in nitrate fertiliser did not significantly affect the incidence of tipburn, while Vavrina (1993) reported tipburn to decrease linearly with increasing nitrate concentrations. Mylavarapu *et al.* (2005) indicated that to maintain tipburn-free crops, an optimum supply of nutrients including S, Zn, and P must be used. Studies on leaf-tip scorch in the cut flower *Protea eximia* revealed that symptoms were linked to decreased foliar concentrations of Fe and increased concentrations of Na and Mn. Mass spectrometer analysis of plant leaves indicated no association between leaf-tip scorch and NO_3^- (Cramer, 2004). Both Taylor *et al.* (2004) and Park *et al.* (2005b) found that reduced uptake of calcium in tomato was caused by increased soil concentrations of Mg, Na and K, and low soil pH.

1.2.2.4 Light

Acceleration of the progress and the extent of tipburn is increased under high light intensity and by extended photoperiods (Wissemeier and Zuhlke, 2002; Saure, 1998). Because light is considered to be one of the primary factors influencing plant growth and development (Dorais *et al.*, 1990), tipburn incidence is likely to be

affected. An extensive study by Gaudreau *et al.* (1994) found lengthening photoperiod in glasshouse crops using supplementary lighting produced 270% more biomass accumulation in 30% less time than under natural light. High light also induced more tipburn, which appeared to affect different cultivars at different times of the year. Montanaro *et al.* (2006) grew kiwi plants under both full light and shade conditions. Shade treatment caused a 50% reduction in Ca^{2+} accumulation compared to exposed plants. They proposed that light induces biosynthesis of auxin protecting phenols which in turn decrease auxin degradation which causes increases in Ca^{2+} accumulation. Tibbitts *et al.* (1985) analysed the vascular turgor pressure of lettuce and found that pressure fluctuations created by reduced irradiance levels caused laticifer rupture and tipburn injury. The findings demonstrate the importance of environmental interactions with plant genotype.

1.2.2.5 Temperature

Temperature appears to promote Chinese cabbage tipburn very extensively during the summer months in sub-tropical and tropical regions (Saure, 1998). Tipburn severity can be enhanced in crops grown under polythene where temperatures are approximately 7°C above the ambient temperature (Misaghi *et al.*, 1992). The effect of temperature usually causes tipburn of the inner leaves resulting in break down of membranes followed by secondary pathogen attack rendering the crop useless. Thus, increased temperatures of 28 – 37°C are used to test for tipburn susceptibility (Saure, 1998). Tipburn resulting from temperature appears to be more prominent in harvested heads during storage. Ceponis *et al.* (1985) showed that from 1972 – 1985, 54% and 42% of iceberg lettuce shipments from Arizona and California had tipburn, respectively. However, modern food production has overcome this through cooling of the crop to 10 – 15 °C during transport and storage (Misaghi *et al.*, 1992). Even though the evidence linking temperature to tipburn looks promising, no direct link has been established between temperature and tipburn (Saure, 1998).

1.2.2.6 Active oxygen species

Programmed cell death (PCD), or apotosis, is a genetically defined process triggered by plant growth regulators in response to pathogen attack, environmental stresses or the initiation of senescence (Beligni *et al.*, 2002; de Jong *et al.*, 2002; Fath

et al., 2001). Both plants and animals appear to share the same morphological and biochemical features associated with PCD. Initiation of PCD occurs through the production of active oxygen species (AOS) (Section 1.3) by the mitochondria. Programmed cell death may be part of the tipburn reaction, creating a physical barrier of dead tissue preventing secondary pathogen entry through weakened tissues. The lack of AOS inhibiting antioxidant compounds could be due to rapid dehydration causing restraint of nutrient availability within the leaves (Concetta de Pinto, 2002). Aktas *et al.* (2005) found that Mn, Zn and Ca salts inhibited AOS production, thus preventing the spread of BER in sweet pepper fruits. Casado-Vela *et al.* (2005) used a proteomic approach to study the causes of BER in tomato fruits. They identified differences in number and expression of several proteins, namely those participating in the ascorbate-glutathione cycle and pentose phosphate pathway. They suggested these biochemical pathways scavenge AOS, restraining the spread BER to the whole fruit.

1.3 Free radicals and active oxygen species

Free radicals are defined as atoms or molecules which contain an unpaired electron (Benson, 1990). They are generally unstable and will react with non-radicals to produce a new radical in a self-propagating chain reaction called a 'cascade'. The most damaging free radicals are those derived from stable molecular oxygen (O_2) in metabolic processes by the gain of electrons or realignment of electron spins. They are collectively termed active oxygen species (AOS) and comprise of the superoxide anion radical ($O^{\cdot}_2^{-}$), the peroxide anion (O_2^{2-}), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Benson, 1990; Halliwell and Gutteridge, 1989).

In order to appreciate the formation of free radicals it is important to understand the role of electrons in molecular bonds. Electrons are able to spin about their own axes (characterised by quantum spin number: ±½) and to confer stability they must be paired in opposite spins (↑↓). When a molecular bond is broken under normal conditions, the electrons split heterolytically:

$$A{:}B \rightarrow A{:} + B \text{ or } A + B{:}$$

Conversely, when free radicals are produced, electrons are split homolytically:

$$A{:}B \rightarrow A\cdot + \cdot B$$

Almost all free radical reactions in biological systems involve the formation of oxyradicals. Molecular oxygen has two unpaired electrons that exhibit parallel spins i.e. spin in same direction (↑↑). This formation is called a triplet ground state, allowing oxygen to behave like a magnet when placed in the presence of an external magnetic field (Benson, 1990). In order to achieve a singlet state i.e. electrons spin in opposite directions, there must be absorption of sufficient energy to weaken the electron state or the electrons in parallel spin must enter bond sharing. Both types of reaction are important in biological systems, for example catalysis of 4 electrons from cytochrome C to oxygen in respiratory electron transport chains. Transfer of electrons to oxygen can also lead to production of the $O_2^{\cdot -}$ which, in turn, reacts with H_2O_2 in the presence of iron (Fe) to produce the hydroxyl free radical (·OH), one of the most reactive molecules in chemistry. It is estimated that for every 25 oxygen molecules used in respiration, 1 free radical is produced (Banerjee *et al.*, 2003).

1.3.1 Antioxidants

There are, however, many naturally occurring compounds, which function to protect against the potentially harmful effects of AOS. These substances, termed antioxidants, can be defined simply as chemical compounds or substances that inhibit oxidation (Basu, 1999). Several low molecular mass substances may function as antioxidants in plants and include catalase, superoxide dismutase (SOD), vitamin E, carotenoids and reduced glutathione (GSH) (Mateos *et al.*, 2003; Basu, 1999).

1.3.1.1 Vitamin E

Vitamin E (α-tocopherol) is found in both plant and animal tissues and is a powerful lipophilic antioxidant. Vitamin E is typically found in all green parts of the plant, while tocotrienol, which differs due to the degree of saturation on the hydrophobic prenyl side chains, is found in seeds (Munne-Bosch and Alegre, 2002). The phytyl chain and a chromanon ring of vitamin E allows it to become embedded in biological membranes, quenching $^1O_2$ and maintaining membrane integrity (Wrona *et al.*, 2003; Munne-Bosch and Alegre, 2002; Schmitz-Eiberger and Noga, 2001; Landvik, 1997; Benson, 1990). Alkoxyl and peroxyl radicals are reduced to

chromanoxyl, a weaker and less efficient lipid peroxidation radical, by vitamin E which in turn is oxidised to its quinone form. Ascorbate can reduce the α-tocopherol quinone form back to α-tocopherol.

1.3.1.2 Carotenoids

The carotenoids are plant pigments that have roles as colourants, antioxidants and hormone precursors, and are an essential part of the photosynthetic apparatus. The most abundant carotenoids are β-carotene, lycopene, lutein and zeaxanthin, although there are more than 700 carotenoids in total (Howitt and Pogson, 2006). They are highly abundant and accumulate in nearly all plant tissues, especially the chloroplasts, where they quench triplet chlorophyll and free radical propagating lipids (Acworth *et al.*, 1997; Benson, 1990; Sommerburg *et al.*, 2003). The efficiency of carotenoids to quench AOS is related to the number of conjugated double bonds that they are able to donate. For example, dodecapreno-β-carotene (15 double bonds) is more effective than septapreno-β-carotene (8 double bonds) (Cantrell *et al.*, 2002).

1.3.1.3 Superoxide dismutase

Superoxide dismutase (SOD) is a general name given to families of metalloenzymes, which contain either Mn, Cu, Fe or Zn (Benson, 1990). Distribution of these enzymes appears to be in all the main subcellular locations that produce AOS, including chloroplasts, mitochondria, peroxisomes, apoplasts and the cytosol (Alscher *et al.*, 2002). Superoxide dismuatse catalyses the breakdown reaction (Benson, 1990):

$$SOD_{OX} + O^{\cdot}_2{}^{-} \rightarrow SOD_{RED} + O_2$$
$$2H^+ + SOD_{RED} + O^{\cdot}_2{}^{-} \rightarrow SOD_{OX} + H_2O_2$$

1.3.1.4 Catalase

Catalase (CAT) is a haeme-containing enzyme that catalyses the dismutation of H_2O_2 into H_2O and O_2 (Acevedo *et al.*, 2001), and is located in the peroxisomes/glyoxysomes of the cell (Willekens *et al.*, 1997). Genes encoding CAT are expressed differentially as the plant develops and are able to respond to exogenous applications of abscisic and salicylic acids (Acevedo *et al.*, 2001). The importance of CAT in plant AOS scavenging was demonstrated by Mohammed *et al.*

(2003), who transformed tomato plants with a catalase gene, *katE*, from *Escherichia coli*. Leaf discs from transgenic plants remained green when treated with 1 mM paraquat for 24 h compared to wild-type plants which showed bleaching. Acevedo *et al.* (2001) reported that CAT deficient plants developed foliar lesions followed by death under high light conditions.

1.4 Glutathione

The tripeptide glutathione (γ-glutamylcysteinylglycine) (GSH) is one of the most important antioxidants in both plant and animal systems. It is the largest source of non-protein reduced sulphur in all organisms with good relative stability and high water solubility. It has vital intracellular and extracellular functions, including the detoxification of AOS, influencing gene expression and signal transduction pathways, altering cell redox state and regulation of gene transcription and transport of hormones (Mullineaux and Rausch, 2005; Jones, 2002; Foyer and Noctor, 2001; Tausz, 2001; Hagen *et al.*, 1990b; Wierzbicka *et al.*, 1989).

Cellular concentrations of GSH vary depending on environmental and seasonal factors, which in turn link several key processes such as mitosis and root development (May *et al.*, 1998) (Table 1.3). The importance of GSH on plant function can be observed in the *A. thaliana rml1* mutant that is GSH deficient, plants are absent of root development, have small shoots and can survive only in tissue culture with exogenously supplied GSH (May *et al.*, 1998).

Table 1.3: Effect of environmental and stress factors on the concentration of GSH and related enzymes and metabolites. Responses are shown as an increase (+) or decrease (-) in the concentration of the relevant metabolite. Abbreviations are CYSH, cysteine; GR, glutathione reductase; GSH, glutathione; GSSG, oxidised glutathione.

Stress factor	Response	Plant tissue	Reference
Sun exposure	+ GR	*Cryptomeria japonica* leaves	Han *et al.*, 2004
UV radiation	- GSH:GSSG	Sunflower cotyledons	Costa *et al.*, 2004
Pathogens	+ GSH and CYSH	Tomato xylem	Cooper and Williams, 2004
Drought	+GR, - GSH	Rice	Boo and Jung, 1999
Chilling	+ GR and GSH	Tomato fruit	Malacrida *et al.*, 2006
Salinity	+ GR	Barley	Kim *et al.*, 2005
Heavy metals	+ GSH:GSSG	Spruce cells	Schroder *et al.*, 2003

1.4.1 Chemistry of glutathione

Glutathione functions to protect enzymes and proteins from oxidative degradation by donating 1 of its thiol groups, and thus exists in both reduced (GSH) and oxidised (GSSG) forms. The redox relationship is written as:

$$2GSH \longleftrightarrow GSSG + 2H^{+} + 2e^{-}$$

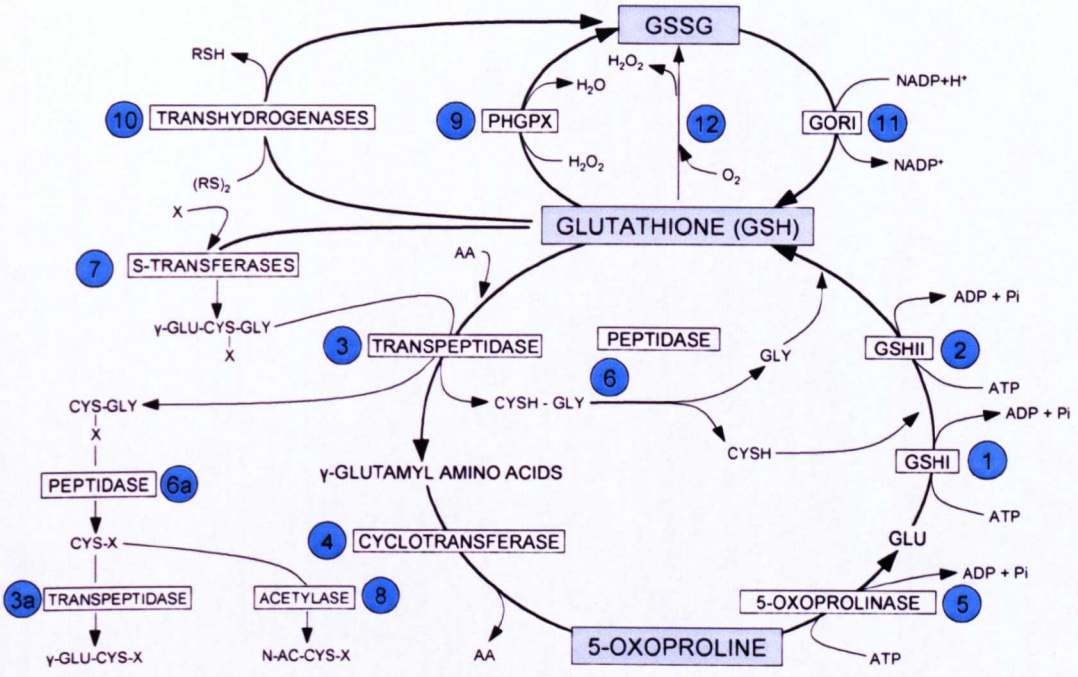
The product of this reaction is GSSG containing a disulphide bridge, which in high concentrations can be toxic to cells. Glutathione can work in association with ascorbate to detoxify H_2O_2 in the chloroplasts (Benson, 1990). Unlike other redox influencing compounds, both the GSH:GSSG ratio and GSH concentration affect cellular redox potential. For example, mobilisation of foliar GSH to the roots for heavy metal detoxification results in a reduction of leaf cellular redox without affecting the ratio of GSH to GSSG (Mullineaux and Rausch, 2005).

1.4.2 Glutathione biosynthesis

The synthesis of GSH in plants occurs through the chloroplasts, using an identical set of reactions to those of mammalian cells known as the γ-glutamyl cycle (Wonisch and Schaur, 2001; Noctor *et al.*, 1998) (Figure 1.2). Glutathione is

synthesized *de novo* in two ATP-dependent steps which are driven by the enzymes γ–glutamylcysteine synthetase (GSHI) (Reaction 1) and glutathione synthetase (GSHII) (Reaction 2). Reaction 1 is feedback inhibited by GSH (Noctor and Foyer, 1998; Meister and Anderson, 1983). The breakdown of GSH is catalysed by γ–glutamyl transpeptidase (Reaction 3) which transfers the γ–glutamyl group from GSH to amino acid acceptors e.g. glutamine, cystine (CYS) and methionine. The majority of transpeptidase is present on the outer cell membrane while GSH is mainly intracellular. For reaction 3 to occur, GSH is transported across the cell membrane to react with γ–glutamyl transpeptidase. The γ–glutamyl amino acids formed in the reaction are then transported back into the cell (Meister and Anderson, 1983). Cyclotransferase (Reaction 4) converts γ–glutamyl amino acids to 5-oxoproline (Wonisch and Schaur, 2001). Glutamate is synthesized from 5-oxoproline by 5-oxoprolinase (Reaction 5) in an ATP dependent reaction. The cysteinylglycine produced in reaction 3 is split by dipeptidase (Reaction 6) into cysteine (CYSH) and glycine for reactions 1 and 2 (Meister and Anderson, 1983). Derivatives of GSH are formed by S-transferases (Reaction 7) which in turn react with transpeptidase (Reaction 3) to remove the γ–glutamyl moiety. The remaining cysteinylglycines are cut by dipeptidase (Reaction 6a) to yield S-substituted CYSHs which either undergo N-acetylation (Reaction 8) or transpeptidation (Reaction 3a) (Meister and Anderson, 1983). Detoxification of H_2O_2 and other peroxides occurs by GSH using its redox-active sulphydryl group resulting in its convertion to its oxidised form, GSSG, through catalysis by glutathione peroxidase (PHGPX) (Reaction 9). Transhydrogenase enzymes facilitate cellular homeostatic control of GSH content (Reaction 10). Conversion of GSH to GSSG can also occur by extracellular reaction with O_2 to create H_2O_2 (Reaction 12). Finally, glutathione reductase (Reaction11) (GORI) mediates the conversion of GSSG to GSH using NADPH (Wonisch and Schaur, 2001; Meister and Anderson, 1983).

Figure 1.2: The glutathione cycle (adapted from Meister and Anderson, 1983). Abbreviations are AA, amino acids; CYS, cystine; CYSH, cysteine; GLU, glutamate; GLY, glycine; GSHI, γ–glutamylcysteine synthetase; GSHII, glutathione synthetase; GSSG, oxidised glutathione; PHGPX, glutathione peroxidase; GORI, glutathione reductase.



1.4.3 Plant functions of glutathione

1.4.3.1 Light

Active oxygen species in the chloroplasts are generated by light energy which stimulates the electron transport. Concentrations of GSH are modulated to match light régime and light intensity. Tausz *et al.* (1999) observed that needles of *Pinus ponderosa* exposed to the sun had a significantly greater ratio of GSH:GSSG indicating that GSH is readily oxidised by AOS. Chloroplasts in leaves of pea (*Pisum sativum*) grown in high light conditions had greater concentrations of GR compared to leaves grown in the shade (Gillham and Dodge, 1987). Han *et al.* (2004) found that leaves of the gymnosperm *Cryptomeria japonica* had more GR in the winter compared to the summer. They believed this was to counterbalance reduction in enzyme activity caused by reduced temperatures. Reduced presence of soluble antioxidants from the ascorbate-glutathione cycle was observed in apple fruits grown in the shade compared to the sun (Ma and Cheng, 2004).

Ultra violet (UV) radiation, particularly UV-B, can stimulate the formation of AOS both in the chloroplasts and also at the cell surface (Tausz, 2001). Costa *et al.* (2002) investigated the antioxidant reaction of sunflower (*Helianthus annuus*) cotyledons subjected to UV-B. They observed increased oxidative damage, reduced chlorophyll content and a significantly reduced GSH:GSSG ratio. However, Helsper *et al.* (2003) found no change in antioxidant capacity of leaves and petals from *Rosa hybrida* and *Fuchsia hybrida* radiated with UV-A. Their results indicated that plant protection from UV originates from absorption of irradiation rather than from scavenging AOS.

1.4.3.2 Defence

Infection by pathogens induces the production of several signals involved in the activation of resistance mechanisms in both the local tissues and on a systemic level. Evidence has shown that salicylic acid (SA), jasmonic acid (JA) and ethylene are the main signals for systemic resistance (Foyer and Noctor, 2001; Rao and Davis, 1999; Srivastava and Dwivedi, 1998). Srivastava and Dwivedi (1998) studied the cellular responses of pea seedlings after SA treatment. They found GSH content increased, GSSG content decreased, while the GSH:GSSG ratio increased. They proposed that GSH protects cells from increased concentrations of H_2O_2 produced as a result of the inhibition of CAT by SA. Clarke *et al.* (2002) also found elevated GR and peroxidase activity in plants treated with SA and JA prior to virus attack. Although the exact mechanism for GSH synthesis under pathogen attack is unknown, it is apparent that JA increases levels of RNA encoding enzymes for GSH synthesis while not influencing concentrations of GSH (Xiang and Oliver, 1998). Cooper and Williams (2004) noticed transient increases in GSH and CYSH concentrations in tomato xylem in response to fungal and bacterial pathogens. Glutathione and CYSH may also act as donors of elemental sulphur, the only inorganic phytoalexin produced in higher plants.

1.4.3.3 Drought

In order for plants to avoid desiccation from drought conditions, they must maintain a minimum level of cellular water, known as homoiohydricity. The result of this effect is closure of stomata, which in turn restricts CO_2 uptake and increases oxidative stress in illuminated chloroplasts (Tausz, 2001). Synthesis and cellular

concentrations of GSH have been found to be dependent on the length of drought and type of plant involved. Drought stressed wheat have been shown to have greater GR activity than well-irrigated plants (Smirnoff, 1993). Boo and Jung (1999) found that osmotically induced drought stress in rice cultures (*Oryza sativa* L.) produced an increase in GR concentrations while GSH was reduced.

Desiccation (poikilohydric) tolerant plants like mosses and lichens offer a better insight into the role of GSH during drought. In order for these plants to resume normal growth after a period of desiccation, large concentrations of reduced antioxidants must be available to protect proteins and photo-systems from oxidation (Tausz, 2001). Upon hydration of the moss *Tortula rutalis*, marked increases in oxidised GSH were found (+ 50%) (Dhindsa, 1991) while the resurrection plant *Boea hygroscopica* showed increased GSH concentrations (Navari-Izzo *et al.*, 1997). Kranner *et al.* (2002) studied the survival mechanisms during desiccation and subsequent rehydration of the woody shrub *Myrothamnus flabellifolia*. Desiccation produced considerable increases in zeaxanthin, while GSH and ascorbate became more oxidised. Rehydration triggered synthesis of GSH and ascorbate, reduction of their oxidised forms, and rapid production of α-tocopherol and carotenoids.

1.4.3.4 Low temperature

Low temperature and chilling induces two main types of stress in plants. Firstly, the combination of high light with reduced temperatures leads to a slowing of the Calvin cycle, but a continuation in electron transport. This effect leads to overproduction of AOS and leads to photoinhibition (Garcia-Plazaola *et al.*, 1999). Secondly, the formation of ice crystals, which remove water from cells and cause structural damage. Active oxygen species contribute to protein damage and diminish antioxidant concentrations (Tausz, 2001). Plants exposed to mild chilling show increased presence of GSH and its precursor CYSH (Kocsy *et al.*, 2001). Several studies have observed increases in GSH and GR during cold treatment (Malacrida *et al.*, 2006; Kocsy *et al.*, 2001; Hodges *et al.*, 1996). Malacrida *et al.* (2006) suggested that the antioxidant responses of tomato fruit during chilling could be mediated by CAT and GR. Garcia-Plazaola *et al.* (1999) provide contrasting results, whereby GSH concentrations did not increase in the leaves of *Quercus ilex* during winter, but GR concentrations increased in sun-exposed leaves during autumn.

1.4.3.5 Salinity

Plant exposure to salt stress causes osmotic damage as well as toxic effects created by large concentrations of Na^+ and Cl^-, which inhibit nutrient uptake (Avsian-Kretchmer *et al.*, 1994). Results from a study by Tsai *et al.* (2004) suggested that Cl^- ions were responsible for increased GSH concentrations in rice (*Oryza sativa*) seedlings. Barosso *et al.* (1999) reported that genes encoding cytosolic *O*-acetylserine(thiol)lyase, a key enzyme in CYSH synthesis, increased greatly in *A. thaliana* after salt treatment. The observation supported the concept that sulphurous compounds are precursors to protective antioxidants. Sairam *et al.* (2002) found long-term salinity stress in wheat caused decreases in water content, chlorophyll and carotenoids, while H_2O_2 and GR increased. Activities of GR and other antioxidant enzymes increased in barley (*Hordeum vulgare*) 1 d after salt treatment (Kim *et al.*, 2005), while synthesis of GPX was induced in salt-sensitive cells of orange (*Citrus sinensis* L. Osbeck) after salt exposure (Avsian-Kretchmer *et al.*, 1994). A study by Mittova *et al.* (2004) also showed a decreased GSH content in salt stressed wild tomato (*L. pennellii*).

1.4.3.6 Heavy metals

Phytochelatins (PCs) are simple peptides containing glutamate (GLU), CYSH and glycine (GLY) in the structure (γ-Glu-Cys)n-Gly where n is between 2 and 11 (Xiang *et al.*, 2001; Cobbett and Goldsborough, 2000; Salt *et al.*, 1997). Phytochelatins are derived from GSH and represent a major detoxification mechanism for both essential and non-essential transition metals (Clemens, 2006; Rauser, 2001; Salt *et al.*, 1997). Cuypers *et al.* (1999) found that enzymes involved in the ascorbate-glutathione pathway increased after heavy metal application. Synthesis of PCs is controlled by the enzyme PC synthase which appears to be activated by the presence of metals. Research has indicated that the induction time of PC synthase is directly related to the metal involved and its concentration in the plant (Cobbett and Goldsborough, 2000; Cuypers *et al.*, 1999). The importance of GSH in heavy metal detoxification was demonstrated by Schroder *et al.* (2003), who grew spruce (*Picea abies*) cells in cultures containing Cd, As and Pb soil eluates. Glutathione was the first to react to the presence of heavy metals, especially in Cd treated plants, where GSH and GSSG increased to 50 – 200% above that in control plants. Koprivova *et al.* (2002) tested the effects of over-expression of genes for

GSH synthesis in poplar (*Populus alba*) plants. Cadmium accumulation was greater in transgenic plants, particularly the young leaves which accumulated 2.5 – 3 times more Cd than the leaves of control plants. Their data shows that poplar plants with enhanced GSH content may be of use for the remediation of polluted soils.

1.5 Thesis Objectives

Lettuce is, globally, one of the major leafy vegetables, and is thus an obvious target for the development of unique traits, such as those for improved shelf-life, disease resistance and crop performance. The aim of this study was to transform commercial lettuce cvs. with genes that manipulated the biosynthesis and metabolism of GSH in the chloroplasts. Transformants were evaluated for gene insertion and expression, combined with assessments for tolerance to saline stress and tipburn resistance.

The first objective of the study was to assess the callus production and shoot regeneration efficiency of *Agrobacterium tumefaciens* transformed explants of the lettuce cvs. Evola, King Louie, Pic and Robusto (Chapter 2). The cv. Evola was previously identified to have good tissue culture responsiveness and transformation efficiency (Curtis *et al.*, 1994). Cultivar Evola has been transformed to express the *bar* gene for bialaphos resistance, the *nia*2 gene for the enzyme nitrate reductase and the *ipt* gene for the enzyme isopentenyl phosphotransferase, which is involved in biosynthesis of plant cytokinins (McCabe *et al.*, 2001; Curtis *et al.*, 1999; Mohapatra *et al.*, 1999). The cvs. King Louie, Pic and Robusto were supplied by Elsoms Seeds Ltd, Spalding, UK and were chosen for use in this study because of their commercial value and their susceptibility to the foliar condition tipburn.

The ease, at which *A. tumefaciens* infects and integrates T-DNA sequences into the lettuce genome (Curtis *et al.*, 1994), provided the assumption that a large percentage of the putative transformants would express the genes of interest (Chapter 3). Transgene integration and expression in the T_0 putative transformants of cvs. King Louie, Pic and Robusto were initially evaluated using polymerase chain reaction (PCR). The presence of the kanamycin sulphate resistance gene (*npt*II) and firefly luciferase gene (*luc*) adjacent to the left and right T-DNA border sequences, respectively, enabled easy identification of transgenic plants. Reverse transcriptase-

PCR (RT-PCR) was performed on PCR positive plants to confirm expression of the transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I. Production of homozygous lines and assessment of transgene expression was performed in the T_1 and T_2 generations of cv. King Louie. Use of DNA dot blots and Southern blots provided absolute confirmation of transgene integration in the cv. King Louie T_3 lines.

It was hypothesized that the transformed plants would have an increased resistance to AOS, a result of a sustained elevated GSH content in the chloroplasts. In turn, this was expected to improve plant post-harvest performance and resistance to abiotic stress. Homozygous, azygous and wild-type lines of cv. King Louie were subsequently evaluated for maintenance of shelf-life and resistance to saline stress (Chapter 4). The shelf-life assay was designed to yield data on the role of GSH on the maintenance of chlorophyll concentrations and also to assess its relation to leaf soluble protein and reducing sugar content. The saline stress assessments were aimed at comparing differences in cellular response of inner and outer leaves of control and saline (150 mM NaCl) grown plants. The basic aim of these studies was to assess whether an increased GSH concentration in the chloroplasts produced a significant crop advantage that could be measured on the cellular level.

Resistance to the foliar condition tipburn was evaluated in cv. King Louie homozygous, azygous and wild-type lines (Chapter 5). The assessments consisted of growing plants under Ca^{2+} deficient conditions at The University of Nottingham, and under glasshouse conditions at Elsoms Seeds Ltd., Spalding, UK. The role of both studies was to determine if an increased GSH chloroplast content could provide an improved resistance to tipburn, a stress related condition, on the whole plant level. Tipburnt and non-tipburnt leaves were also analysed using macroscopic and microscopic techniques. The aim of this research was to provide a pictorial guide showing the developmental stages of tipburn and to compare leaf structure changes in control and tipburnt leaves.

The relationship of the experimental data to the current literature and within the wider scientific context was discussed (Chapter 6). Proposals were made, concerning the role of new technologies and how they could be applied to further scientific understanding of lettuce transformation and gene expression, salinity tolerance and the causes of tipburn.

CHAPTER 2 : LETTUCE TRANSFORMATION

2.1 Introduction

Tissue culture represents an important step in the genetic manipulation and improvement of lettuce, with several agronomically important genes having now been introduced into commercial lettuce cvs. (refer to Chapter 1, Section 1.1.8 for further information). *A. tumefaciens*-mediated transformation remains the most common form of gene insertion due to the ease of regenerating shoots from leaf explants and the fact that *A. tumefaciens* readily infects lettuce (Curtis *et al.*, 1994). The regeneration of adventitious shoots from leaf explants of this crop is considered to be highly genotype dependent, although there is no link to the morphological groupings of lettuce cvs. (Hunter and Burritt, 2002; Ampomah-Dwamena *et al.*, 1997). Genetic variations such as chromosome number, outbreeding and inherited traits appear to be the major influence on tissue-culture response. In addition to transformation, the generation of genetically variable somaclonal variant plants from cotyledons and first true leaves of the cvs. Salad Bowl, Lobjoits Cos and Pennlake was reported by Brown *et al.* (1986). Such variant traits included more vigour and earlier flowering, changes in chlorophyll content, abnormal leaf shapes and reduced susceptibility to the pathogens LMV and *B. lactucae*. Engler and Grogan (1984) observed similar results with plants regenerated from mesophyll protoplasts of the lettuce cv. Climax. Aberrant phenotypes included variation in foliar pigmentation and shape, dwarfism and increased vigour. Plant regeneration from protoplasts of a wild-type lettuce species, *L. perennis* (Webb *et al.*, 1994), and somatic hybridisation between cultivated lettuce (*L. sativa*) and a wild relative, *L. virosa,* has also been documented (Matsumoto, 1991). Examples of lettuce protoplast regenerated plants and somatic hybrids are covered in Chapter 1, Section 1.1.6.

2.2 Aims and Objectives

The main objective of this experiment was to transform the lettuce cvs. Evola, King Louie, Pic and Robusto with the binary vector pAFQ70.1 carrying the genes *npt*II, *luc, gsh*I, *gsh*II, *phgpx* and *gor*I. *Agrobacterium tumefaciens* mediated-

transformation was utilised for this experiment due to its low transgene copy number insertion into the host genome and would ensure the stable integration of the T-DNA fragment (Ke *et al.*, 2001). Callus induction and shoot regeneration efficiency in transformed and non-transformed explants of the cvs. King Louie, Pic and Robusto, in relation to the cv. Evola was evaluted. The secondary aims were to identify the cv. which regenerated shoots the most rapidly and to identify whether any obvious morphological variations such as those caused by somaclonal variation had occurred in the regenerants.

2.3 Materials and Methods

2.3.1 Source of plant materials

Lettuce seeds (*L. sativa*) of the Romaine cvs. King Louie, Pic and Robusto, were supplied by Elsoms Seeds Ltd., Spalding, UK. Seeds of the cv. Evola were originally supplied by Leen de Mos ('s-Gravenzande, P.O. Box 54-2690 AB, The Netherlands), but for this study came from a selfed and isogenic T_{10} generation line maintained in the Plant Sciences Division, University of Nottingham. Seeds were stored at room temperature (RT) in the dark. The cv. Evola was chosen as a standard genotype because of its good tissue culture responsiveness and transformation efficiency (Curtis *et al.*, 1994) and was therefore suitable for comparison with the performances of other cvs. The cvs. King Louie, Pic and Robusto were selected for their susceptibility to tipburn (Chapter 5).

Plants grown *in vitro* were derived from seeds that were surface sterilised in 10% (v/v) Domestos-bleach solution (Lever Fabérgé, Port Sunlight, UK) for 20 min, and washed thoroughly (3 rinses) with sterile purified (reverse-osmosis) water. Seeds (20 seeds/9 cm Petri dish) were germinated on full-strength semi-solid MS0 medium (Appendix 8.1.1). Petri dishes (Barloworld Scientific Ltd., Stone, UK) were sealed with Nescofilm (Azwell Inc., Osaka, Japan) and maintained at 24°C with a 16 h photoperiod [50 μmol m^{-2} sec^{-1}, daylight fluorescent illumination (58 W 135, Phillips Electronics UK Ltd., Guildford, UK)].

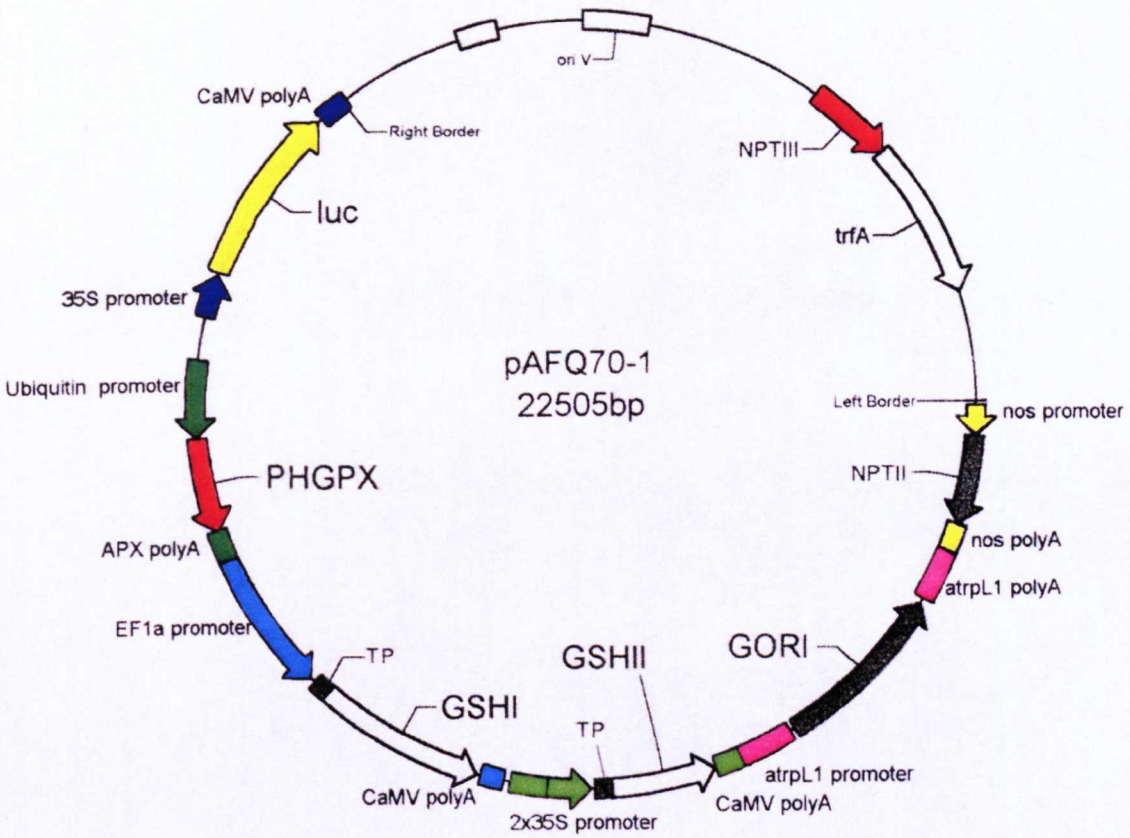
2.3.2 Transformation vector

The binary vector pAFQ70.1 (John Innes Centre, Norwich Research Park, Norwich) (Creissen *et al.*, 1995) contained the metabolic genes γ-glutamylcysteine synthase (*gsh*I; from *E. coli*), glutathione synthase (*gsh*II; *E. coli*), phospholipid hydroperoxide-dependant glutathione peroxidase (*phgpx*; from *P. sativum*) and plastidial glutathione reductase (*gor*I; *P. sativum*). Both the *gsh*I and *gsh*II genes were fused to sequences encoding the chloroplast pea glutathione reductase transit peptide (*grtp*), with the intent of influencing GSH synthesis and metabolism in the chloroplasts. The *gsh*1 gene was under the control of a weak promoter, EF1α, while the *gsh*II gene was under the control of a strong promoter, CaMV 35S. The reason for this was, to enable a differential expression of γ-glutamylcysteine synthase and glutathione synthase. Role of the construct was to provide transformed plants with an elevated GSH content and produce enhanced GSH cycling. The importance of GSH in relation to plant stress is discussed in Chapter 1, Section 1.4. (Creissen *et al.*, 1995) (Figure 2.1). The plasmid also carried a neomycin phosphotransferase (*npt*II) gene next to the left T-DNA border and a firefly luciferase (*luc*) gene adjacent to the right border. These genes served as selectable markers during transformation (Chapter 3, Section 3.4.1). The binary expression construct was introduced into *A. tumefaciens* strain AGL1 by triparental mating (Ditta *et al.*, 1980). *A. tumefaciens* strain AGL1 has been previously used for the successful transformation of lettuce (Garratt, 2002). The binary vector pAFQ70.1 was also introduced into *E. coli* strain DH5α for the production of plasmid stocks.

2.3.3 Culture of *Agrobacterium tumefaciens* and *Escherichia coli*

Cultures of *A. tumefaciens* were initiated from -80°C glycerol stocks, which were streaked onto semi-solid Luria Broth (LB) (Appendix 8.1.2) containing 50 mg l^{-1} kanamycin sulphate (Melford Laboratories Ltd., Ipswich, UK) and maintained in a dark incubator (28°C) for 5-6 d. One day prior to transformation, a loop of bacterial colonies were transferred to 50 ml of liquid LB in a 100 ml Erlenmeyer flask containing 25 mg l^{-1} kanamycin sulphate. A reduced concentration of kanamycin sulphate was used due to the increased availability of the antibiotic in the liquid medium. The flask was incubated on a horizontal rotary shaker (150 rpm, 28°C) overnight in the dark. *E. coli* cultures were grown under identical conditions but at 37°C.

Figure 2.1: The binary vector pAFQ70.1 (John Innes Centre, Norwich Research Park, Norwich) (Creissen *et al.*, 1995). Abbreviations are *gsh*I, γ-glutamylcysteine; *gsh*II, glutathione synthase; *phgpx,* phospholipid hydroperoxide-dependant glutathione peroxidase; *gor*I, glutathione reductase; *npt*II, neomycin phosphotransferase; *luc*, firefly luciferase; TP, transit peptide.



2.3.4 Transformation of explants

Whole adult leaves from 14 d-old *in vitro* grown lettuce seedlings (of all cvs.) were scored on their abaxial side. Leaves were immersed (5 sec) in the *A. tumefaciens* liquid culture, blotted dry on sterile filter paper (Whatman International Ltd., Maidstone, UK) and transferred to 9 cm Petri dishes (10 leaves/dish) containing 20 ml of full-strength semi-solid MS0 medium only. After 2 d co-cultivation under growth room conditions (Section 2.2.1), explants were transferred to 9 cm Petri dishes containing full strength semi-solid MS0 medium supplemented with 0.5 mg l^{-1} benzylaminopurine (BAP), 0.04 mg l^{-1} naphthalenacetic acid (NAA) (shoot regeneration medium) plus 50 mg l^{-1} kanamycin

monosulphate (Curtis *et al.*, 1994), 100 mg l^{-1} cefotaxime (Claforan; Sanofi-Aventis, Guildford, UK) and 500 mg l^{-1} carbenicillin (Melford Laboratories Ltd.) and sealed with Nescofilm (Azwell Inc., Osaka, Japan). Control explants of the same cvs. were immersed in LB without *A. tumefaciens* for the same time period and grown on shoot regeneration medium both with- and without antibiotic selection. Regenerating callus explants were sub-cultured every 14 d. When shoots reached ~1 cm in length, they were transferred to 175 ml powder round jars (Beatson Clarke, Rotherham, UK) (4 shoots per jar) containing 50 ml full strength semi-solid MS0 medium, without antibiotics, for rooting.

Callus induction and shoot regeneration efficiency of the non-transformed and transformed lettuce leaf explants were evaluated at 2 wk intervals post initiation. The measurements were recorded as the percentage of explants producing callus and the percentage of explants producing more than 1 shoot on shoot regeneration medium. The tissue culture of non-transformed and transformed lettuce explants was performed in duplicate. In total, explants of the transformed lines were cultured in 35 Petri dishes (10 leaves/dish) while the non-transformed control lines with and without were cultured in 30 and 20 Petri dishes (10 leaves/dish), respectively. Callus induction and shoot regeneration data was analyzed using analysis of variance (ANOVA) on Microsoft Excel (Office 2003; Microsoft Ltd., Reading, UK). Analysis of variance (P) values indicated the probability of obtaining the results by chance, where $P = < 0.05$ was significant, $P = < 0.01$ was highly significant and $P = < 0.001$ was very highly significant.

2.3.5 Transfer of plants to the glasshouse

One hundred and eighty six rooted putative transformed plants (Chapter 3, Section 3.3) were transferred to 9 cm diameter plastic pots (Richard Sankey Ltd., Bulwell, UK) filled with 3:1 (v/v) John Innes No. 3 compost (Scotts Company Ltd., Ipswich, UK) and perlite (William Sinclair Horticulture Ltd., Lincoln, UK). Plants were covered with clear polythene bags to prevent water loss and allow plant development. After 7 d the top corner of each bag was cut and removed to allow the plants to acclimatise for another 7 d. Growth conditions were 24°C with a 16 h photoperiod (light intensity at noon was approximately 610 µmol m^{-2} s^{-1}). The T_0

putative transformants exhibited normal morphology, were self-pollinated and allowed to set seed.

2.4 Results

2.4.1 Callus induction of control and transformed lettuce explants

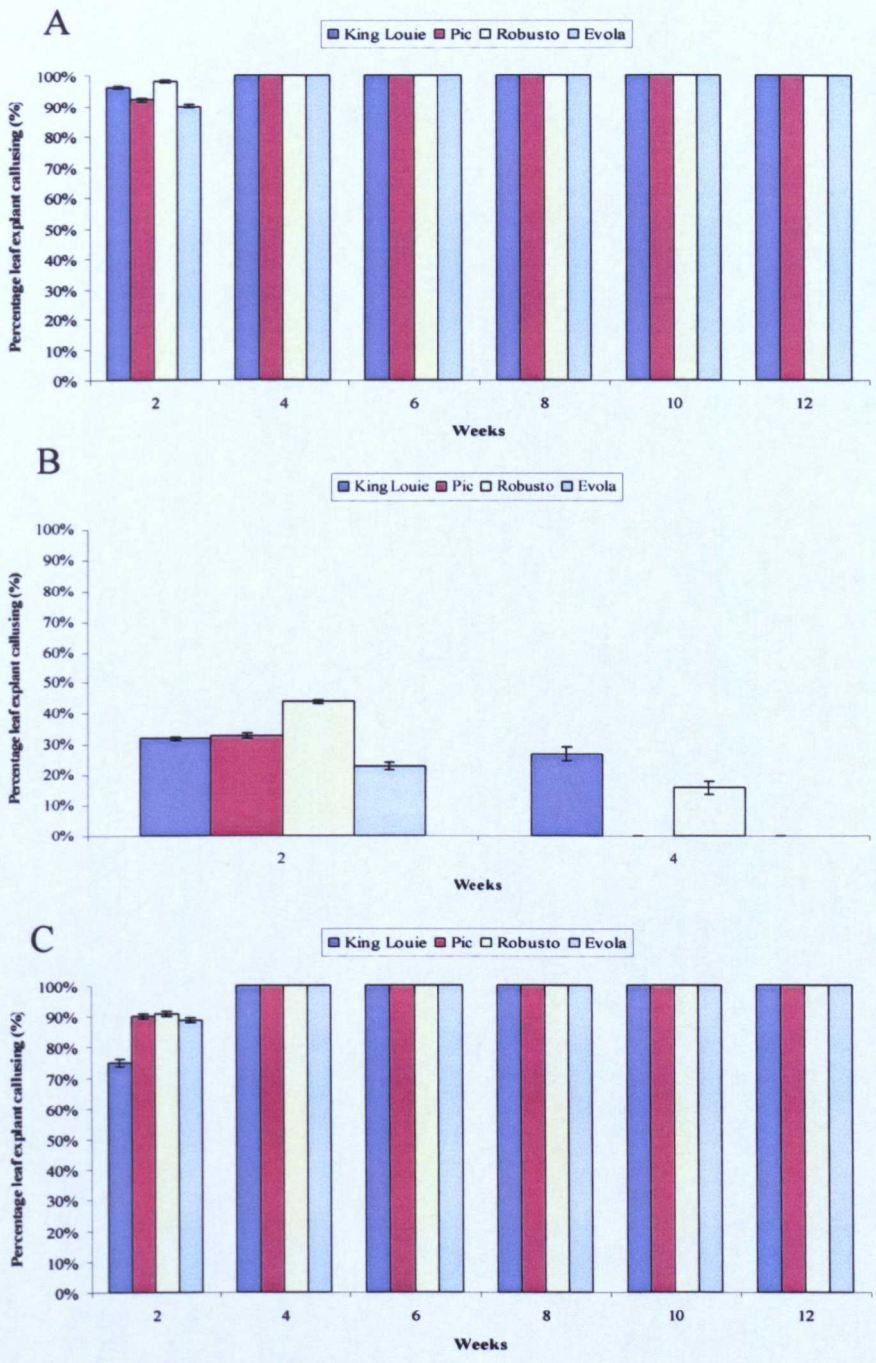
Callus induction in the controls on shoot regeneration medium but without antibiotics (Figures 2.2 A, 2.4 – 2.7) (Appendix 8.3.1, Tables 8.2 and 8.3) reached 100% in explants by wk 4. At wk 2, cv. Robusto had the highest callus induction percentile frequency followed by cvs. King Louie, Pic and Evola (98%, 96%, 92%, 90%, respectively). Data for cvs. King Louie and Robusto were statistically significant compared to the cv. Pic (P = 0.01 and 0.0005, respectively) and for cv. King Louie compared to cv. Evola (P = 0.0001). Callusing in explants treated for transformation (Figure 2.2 C) at wk 2 was greatest for cv. Robusto (91%) followed by cvs. Pic (90%), Evola (89%) and King Louie (75%), none of the results though were significantly different. One hundred percent callusing of explants had occurred by wk 4. Controls with the 3 antibiotics (Figure 2.2 B) produced little callusing by wk 2. However, cvs. King Louie and Pic were statistically significantly from cv. Evola (P = 0.0005 and 0.0006, respectively). At wk 6, all cultures were necrotic and did not exhibit organogenesis.

2.4.2 Shoot regeneration of control and transformed lettuce explants

Control callus tissues grown on shoot regeneration medium without antibiotics (Figure 2.3 A, 2.4 – 2.7) (Appendix 8.3.1, Tables 8.2 and 8.3) initiated shoot regeneration by wk 4, with the cv. Evola exhibiting the greatest number of totipotent calli (29%) followed by cvs. Robusto, Pic and King Louie (24%, 23%, 10%, respectively). Data for cv. Evola was significantly greater than cvs. Pic and Robusto (P = 0.03 and 0.05, repectively). At wk 6, cvs. Pic and Robusto exhibited a significantly higher percentage of explants with shoots when compared to cv. Evola (P = 0.0001 for both), as did cv. Evola compared to cv. King Louie (P = 0.05). At wk 8, all cultures of cvs. Robusto and Pic had undergone organogenesis, only cv. King Louie produced significantly more totipotent calli than cv. Evola (P = 0.01). All cvs. had produced 100% of shoot-producing calli by wk 12. None of the putatively

transformed tissues on selection medium (Figure 2.3 B) reached 100% shoot initiation by wk 12, but cv. Evola was the first to initiate shoots (wk 4), with the other cvs. following at wk 6. The cv. Pic gave a significantly higher proportion of explants with shoots (P = 0.03) at wk 8 (19%) than cv. King Louie (12%). Cultivar King Louie was also statistically greater than cv. Robusto (5%) (P = 0.001) but not cv. Evola (4%). But by wk 10 cv. King Louie emerged as the best cv. in terms of overall shoot regeneration. Cultivars King Louie and Pic were statistically significantly superior at producing shoots by wk 10 compared to cvs. Evola (P = 0.0001 and 0.0005, respectively) and Robusto (P = 0.008 and 0.01, respectively). However, by wk 12, cv. King Louie had produced the highest proportion of regenerating calli (62%) with cv. Evola the least efficient (51%), only cv. King Louie was statistically significant compared to cv. Robusto (P = 0.02).

Figure 2.2: Tissue culture of lettuce leaf explants: callus induction efficiency for cvs. King Louie, Pic, Robusto and Evola.



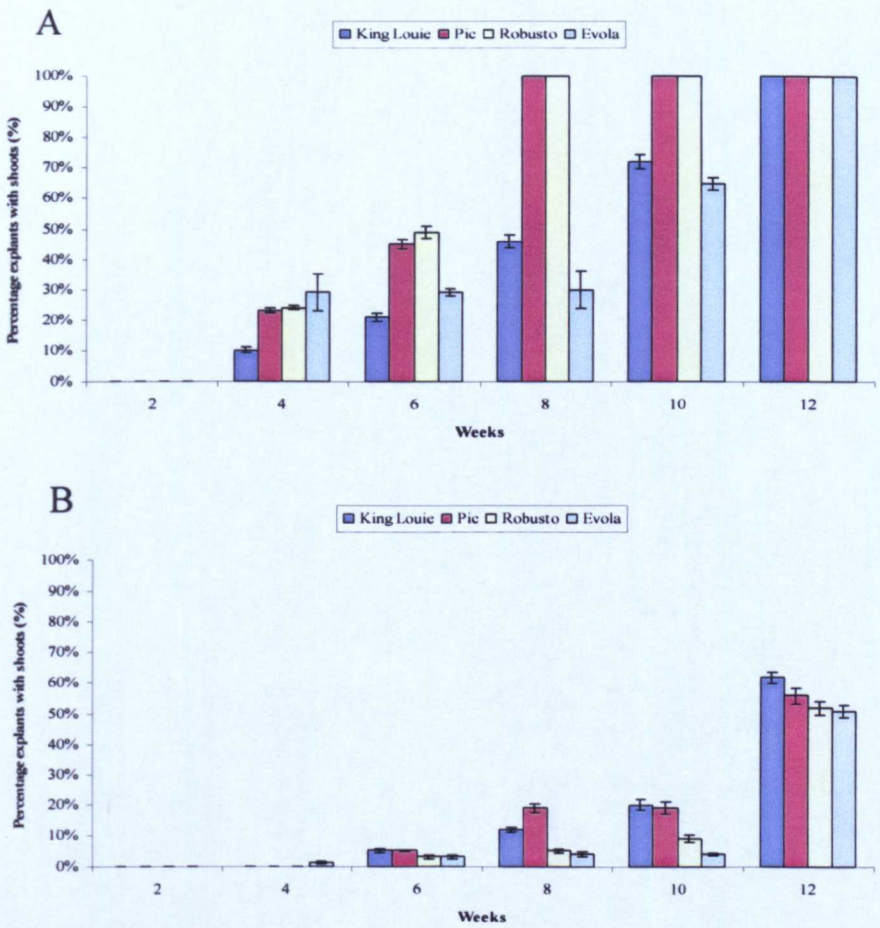
(A) Data for the non-transformed control lines grown on shoot regeneration medium without antibiotics.

(B) Data for the non-transformed control lines grown on shoot regeneration medium with antibiotics.

(C) Data for the transformed lines grown on shoot regeneration medium with antibiotics.

n = 20 (A, B) and n = 30 (C); error bars represent S.E.M.

Figure 2.3: Tissue culture of lettuce leaf explants: shoot regeneration efficiency for cvs. King Louie, Pic, Robusto and Evola.

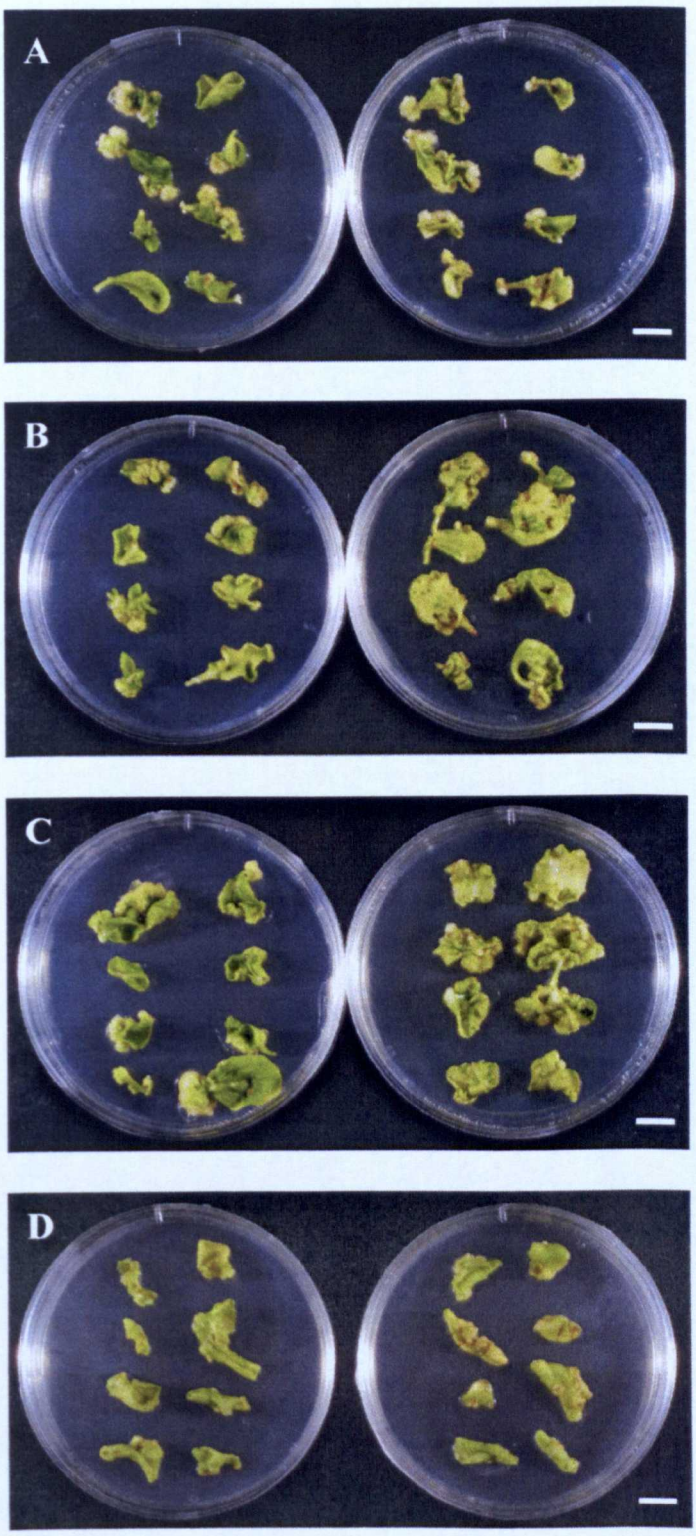


(A) Data for the non-transformed control lines grown on shoot regeneration medium without antibiotics.

(B) Data for the transformed lines grown on shoot regeneration medium with antibiotics.

n = 20 (A) and n = 30 (B); error bars represent S.E.M.

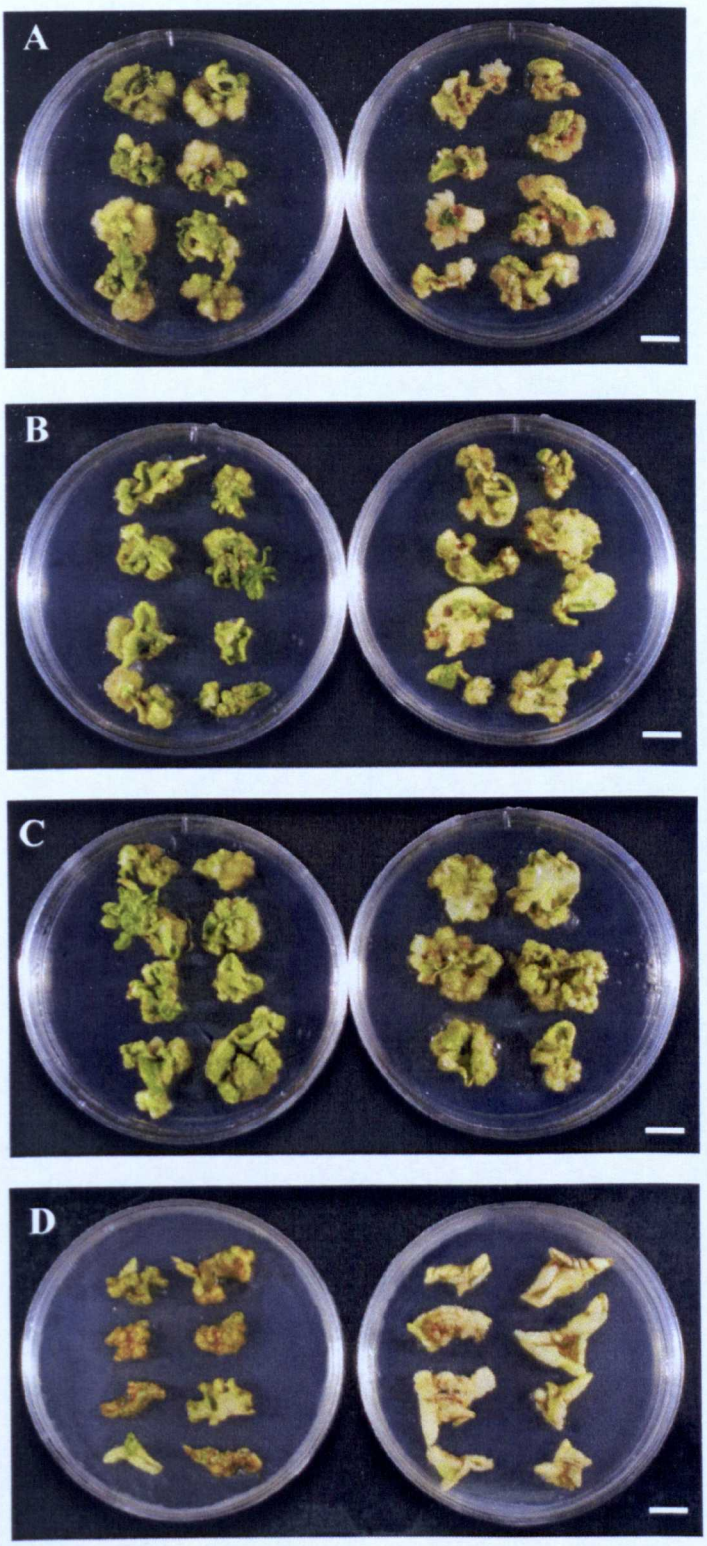
Figure 2.4: Tissue culture of lettuce leaf explants at 2 wks: callus induction and shoot regeneration for cvs. King Louie, Pic, Robusto and Evola.



The cvs. (A) King Louie, (B) Pic, (C) Robusto and (D) Evola.

Non-transformed controls (left) and transformed explants (right) on MS0 shoot regeneration medium. Bars = 1 cm.

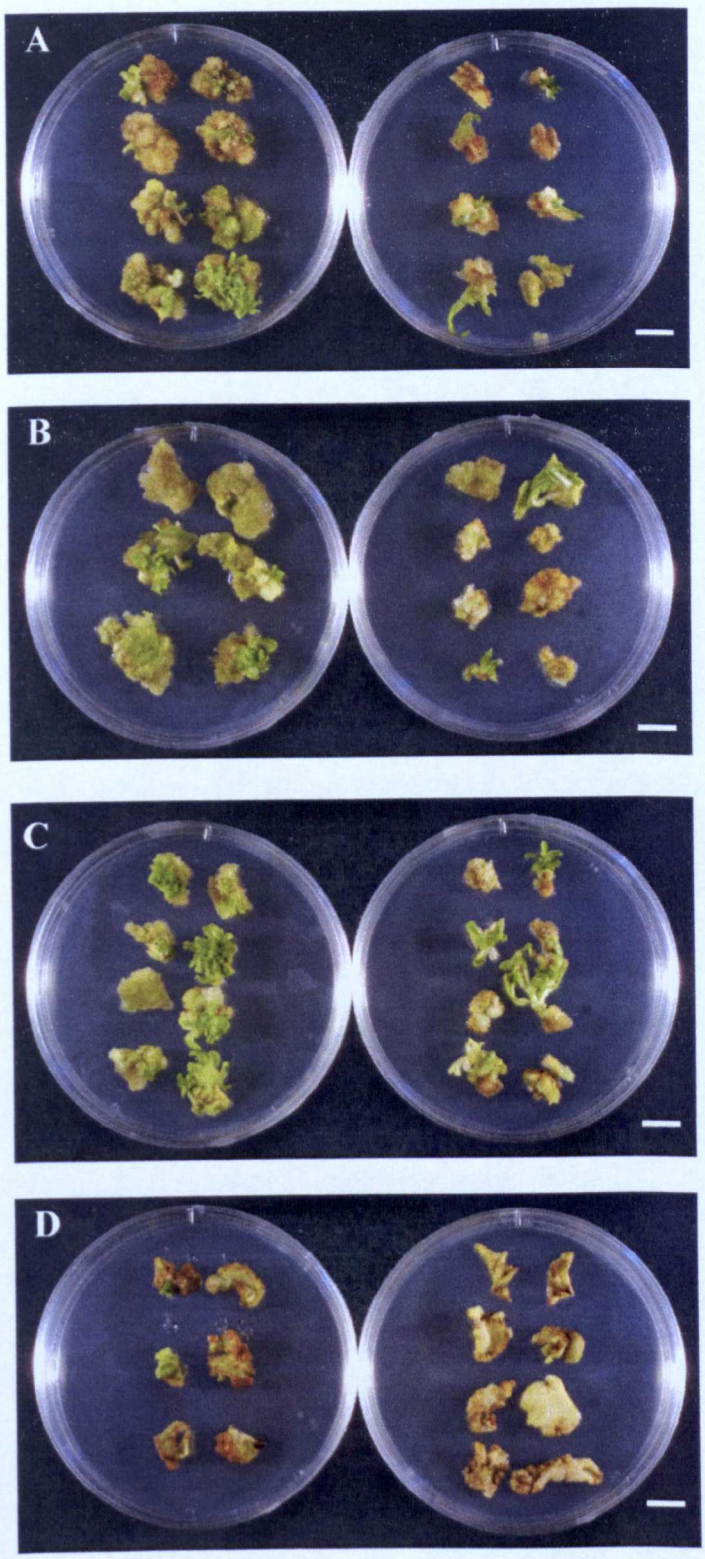
Figure 2.5: Tissue culture of lettuce leaf explants at 4 wks: callus induction and shoot regeneration for cvs. King Louie, Pic, Robusto and Evola.



The cvs. (A) King Louie, (B) Pic, (C) Robusto and (D) Evola.

Non-transformed controls (left) and transformed explants (right) on MS0 shoot regeneration medium. Bars = 1 cm.

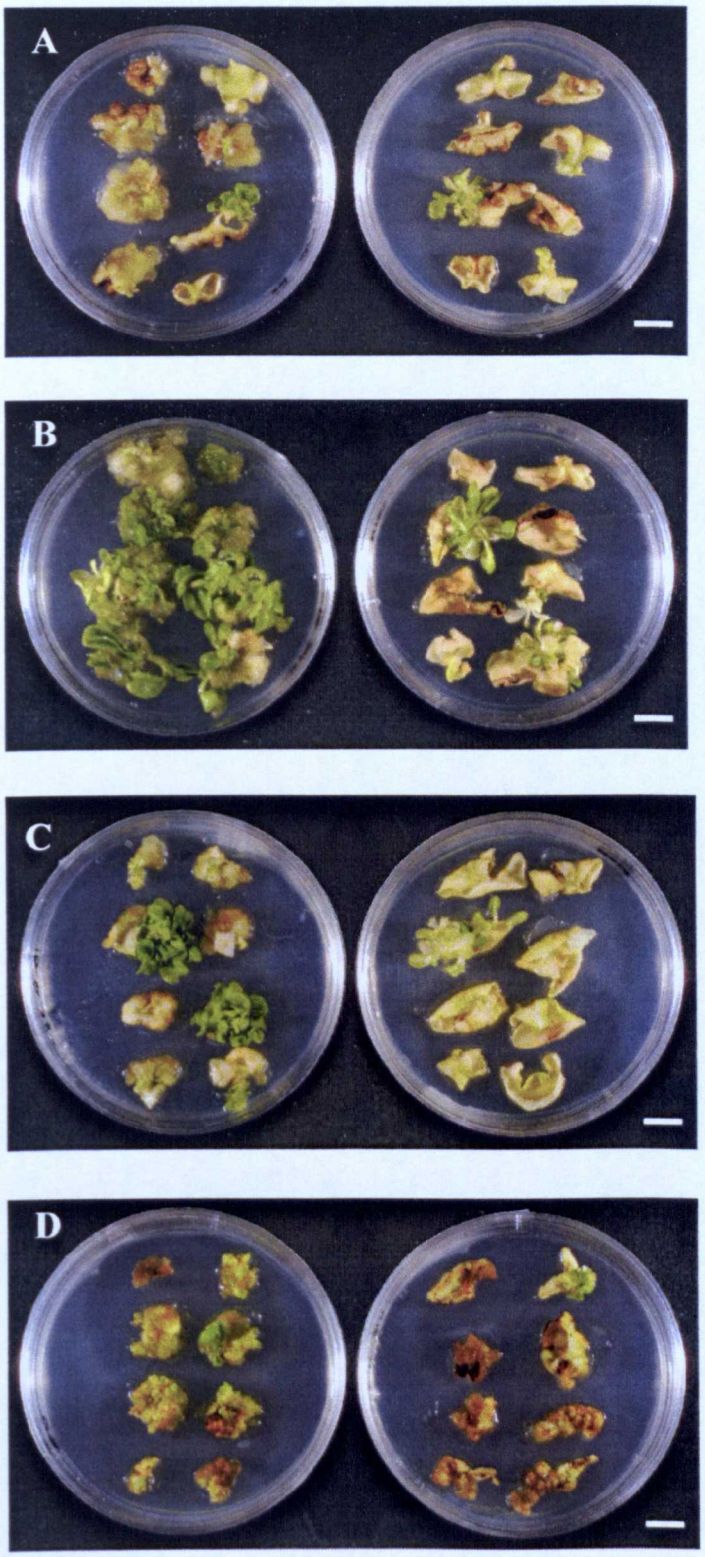
Figure 2.6: Tissue culture of lettuce leaf explants at 6 wks: callus induction and shoot regeneration for cvs. King Louie, Pic, Robusto and Evola.



The cvs. (A) King Louie, (B) Pic, (C) Robusto and (D) Evola.

Non-transformed controls (left) and transformed explants (right) on MS0 shoot regeneration medium. Bars = 1 cm.

Figure 2.7: Tissue culture of lettuce leaf explants at 8 wks: callus induction and shoot regeneration for cvs. King Louie, Pic, Robusto and Evola.



The cvs. (A) King Louie, (B) Pic, (C) Robusto and (D) Evola.

Non-transformed controls (left) and transformed explants (right) on MS0 shoot regeneration medium. Bars = 1 cm.

2.5 Summary

Data involving the lettuce explants treated for transformation showed that cv. Robusto produced the greatest number of callusing explants at wk 2 followed by the cvs. Pic, Evola and King Louie cultured on the shoot regeneration medium with antibiotics. Control cultures, grown without antibiotics, followed a similar pattern with again the cv. Robusto giving the largest percentage of callusing tissues. Growth though was more rapid due to a lack of antibiotic-based selection pressure. Shoot regeneration was most efficient for cvs. Robusto and Pic in controls but this pattern did not follow with transformed explants where cv. Robusto was the least successful.

These results correlate with those of Xinrun and Conner (1992), who found marked differences occurred in callus initiation and shoot regeneration in different lettuce cvs. They also identified that hyperhydricity could impair shoot regeneration in some cvs. such as Flora. The use of "nurse" cell suspensions of *Nicotiana plumbaginifolia* was identified to stimulate shoot regeneration from cultured cotyledons of the cv. Cobham Green (Michelmore *et al.*, 1987). An alternative to semi-solid media based batch cultures was demonstrated by Teng *et al.* (1993), who studied the regeneration of lettuce plants from cells cultured in liquid medium in a 2 l bioreactor. Growth parameters such as foaming, the wall effect of the culture vessel, aeration and dissolved oxygen were studied in relation to cell growth and differentiation. They found that sieving the liquid inoculum to remove single cells and debris prevented foaming and damage to the cultures, enabling shoot regeneration comparable to that of cells in batch culture in 125 ml flasks.

Explants of the cv. Evola were initially highly responsive to the tissue culture medium (Curtis *et al.*, 1994), although they became less efficient in later weeks. Such cultures contained oxidised phenolic compounds as indicated by a yellow/green media colouration linked to a blackening and necrosis of explant tissues. Methods of overcoming this condition can include more frequent sub-culturing and use of activated charcoal- or polyvinylpyrrolidone- (PVP) containing media as employed successfully for *Brassica campestris* transformation and to absorb such phenolic compounds (Guo and Pulli, 1996). Controls (all cvs.) with antibiotic selection pressure did not survive beyond 4 wks, indicating that kanamycin monosulphate at 50 mg l^{-1} was appropriate as the key selective pressure agent for the preferential selection of putative lettuce transformants.

This experiment demonstrated that lettuce tissue culture is highly genotype dependent (Hunter and Burritt, 2002). However, this study has shown that modern, commercially valuable cvs. of lettuce respond to tissue culture and can be easily transformed. Several recent studies have also established that new lettuce cvs. can be successfully tissue cultured and transformed including cv. Veronica (Dias *et al.*, 2006), cvs. Hearts Delight and Green Forest (Joh *et al.*, 2005), cv. Chongchima (Cho *et al.*, 2005; Vanjildorj *et al.*, 2005), cv. Salinas 88 (Zuo *et al.*, 2002) and cv. Vanguard (Niki *et al.*, 2001). The tissue culture environment can also be utilised for the testing of disease resistance and the rescue of immature seed embryos from unique lettuce hybrid genotypes. Mazier *et al.* (2004) used tissue culture to simply and efficiently assess the resistance of explant-derived shoots and *in vitro*-grown seedlings of lettuce cvs. to LMV. Outstanding correlation was found between those lettuce cvs. already known to be LMV resistant and their resistance following virus inoculation of *in vitro*-grown material. Maisonneuve *et al.* (1995) used *in vitro* embryo rescue to overcome sexual incompatibility between *L. sativa* and wild *Lactuca* species. The technique successfully produced vigorous hybrid plants between *L. sativa* and seven accessions of *L. virosa*.

Of the 186 putative transformed plants transferred to the glasshouse, none were observed to be somaclonal variants. Somaclonal variation can take the form of both morphological and physiological traits such as early flowering habit, increased chlorophyll concentration compared to its parent and increased tolerance to pests and diseases (Brown *et al.*, 1986). Somaclonal variation is generally observed as morphological differences between individuals, and in some cases 30-40% of regenerated plants may exhibit at least one variant character (Daub, 1986). Causes of somaclonal variation include changes in chromosome complement, structure and DNA sequence caused by tissue culture environment stress, interference with the normal cell cycles and chromosome breakage and rearrangement (Cassells and Curry, 2001).

Putative T_0 generation transformants of cvs. King Louie, Pic and Robusto were subsequently evaluated for integration and expression of the transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I using the molecular techniques PCR and RT-PCR. Plants of cv. King Louie T_1 and T_2 lines were also assessed for transgene expression

using RT-PCR. Transgene integration and copy number were confirmed in cv. King Louie T_3 lines by dot blot and Southern blot (Chapter 3).

CHAPTER 3 : MOLECULAR ANALYSIS OF TRANSGENIC LETTUCE

3.1 Introduction

Agrobacterium tumefaciens has enabled an innovative approach to plant genetic transformation due to its low transgene copy number insertion into the host genome and the simplicity of the method (Ke *et al.*, 2001). However, utilisation of constitutive promoters and transgenes has resulted in the occurrence of gene silencing (Zhang and Ghabrial, 2006; Bastar *et al.*, 2004; Curtis *et al.*, 2000; McCabe *et al.*, 1999; Aida and Shibata, 1996; van Blokland *et al.*, 1994). Gene silencing occurs naturally in plants where it can defend against invasive DNA sequences, such as transposons and retroviruses (Chan *et al.*, 2004). It is essential for normal development and also regulates endogenous gene expression, for example, seed coat pigmentation in soybean (*Glycine max*) is controlled by homology-dependent silencing of chalcone synthase (*chs*) genes (Senda *et al.*, 2004).

In transgenic plants, gene inactivation falls into two groups; transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). Transcriptional gene silencing is associated with methylation of cytidine groups of promoter sequences, and local chromatin remodelling (Park *et al.*, 1996; Finnegan *et al.*, 1998). The two forms of DNA methylation, *cis*-inactivation and *trans*-inactivation, work using similar mechanisms whereby transgenes are inactivated due to the presence of multiple or homologous endogenous gene copies (Matzke and Matzke, 1995). Mutants of *A. thaliana* have yielded valuable information on the molecular components of TGS. The mutants *ddm1* and *hog1* reduce DNA methylation, while *sil1* and *mom1* reactivate genes without changing their methylation state (Scheid and Paszkowski, 2000). The *ddm1* mutant encodes a protein with high similarity to the SW12/SNF2 chromatin remodelling proteins, suggesting that the form of chromatin is essential for TGS (Morel *et al.*, 2000). This particular mutant also exhibits an abnormal morphological phenotype and may indicate that TGS regulates endogenous sequences (Scheid and Paszkowski, 2000).

PTGS occurs when transgenes and promoters remain active, but the mRNA is degraded (Chicas and Macino, 2001; Vaucheret and Fagard, 2001). Often referred as co-suppression or quelling in fungi, it will degrade RNAs encoded by both transgene

and homologous endogenous gene(s). Three models for PTGS have been proposed. The first is based on a threshold model whereby RNA from highly transcribed single copy transgene exceeds a critical concentration, activating RNA degrading mechanisms (Vaucheret *et al.*, 2001; Praveen *et al.*, 2005). The second type is triggered when transgene copies are inserted as inverted repeats, resulting in double stranded RNA (dsRNA) that cause the phenomenon of RNA interference (RNAi) (Vaucheret *et al.*, 2001; Kerschen *et al.*, 2004). Gene silencing induced by RNAi can be transmitted by grafting silenced stocks onto unsilenced plants expressing the corresponding transgene, suggesting a diffusible molecule propagates *de novo* post-transcriptional silencing through the plant (Palauqui *et al.*, 1998). The third model, virus-induced gene silencing (VIGS) causes an RNA-mediated defence mechanism initiated by virus vectors containing sections of the host genome (Faivre-Rampant *et al.*, 2004). The result is RNA-dependent RNA polymerases producing dsRNA using RNA as a template (Vaistij *et al.*, 2002). It has been reported that VIGS is an effective way of assessing gene function in *Solanum* species through the use of tobacco rattle virus (Brigneti *et al.*, 2004) and potato virus X (Faivre-Rampant *et al.*, 2004). Zhang and Ghabrial (2006) used bean pod mottle virus based vectors to study gene expression and VIGS in soybean.

3.2 Aims and Objectives

The primary aim of this chapter was to analyse the T_0 putative transformants of cvs. King Louie, Pic and Robusto for integration and expression of the transgenes. Putative transformants were initially screened using PCR for the *npt*II and *luc* marker genes. Since the marker genes flanked the genes of interest, the latter was assumed to be present in the transgenic plants. PCR positive T_0 plants and T_1 and T_2 generation lines of cv. King Louie were further analysed for expression of the transgenes using RT-PCR. It was hypothesized that the genomes of cv. King Louie T_3 homozygous lines would contain a single copy of each transgene, a result of transformation by *A. tumefaciens*. Dot blot and Southern blot were used to test this assumption and also provide confirmation of the presence of the transgenes. Production of T_3 generation homozygous and azygous lines of cv. King Louie was also carried out.

3.3 Materials and methods

3.3.1 DNA extraction

Prior to DNA extraction a 1 cm^2 disc of T_0 generation lettuce leaf of cvs. King Louie, Pic and Robusto (Chapter 2, Section 2.3.5) was excised using the lid of a sterile 1.5 ml microfuge tube and immediately frozen in liquid nitrogen. If required, the sample could be stored long-term at -80°C. The Genelute Plant Genomic Miniprep Isolation Kit (Sigma-Aldrich, Gillingham, UK) was used to isolate pure DNA; the manufacturer's guidelines were followed throughout. Samples were stored at -20°C until needed. The Genelute Plasmid Miniprep Isolation Kit (Sigma-Aldrich) allowed the binary vector pAFQ70.1 to be isolated from *E. coli* strain DH5α (Chapter 2, Section 2.3.3).

3.3.2 RNA extraction and cDNA synthesis

RNA was extracted from PCR positive T_0 generation plants of lettuce cvs. King Louie, Pic and Robusto (Chapter 2, Section 2.3.5) and T_1 and T_2 generation plants of cv. King Louie (Section 3.3.8). Leaf discs (100 mg FW) were placed in 1.5 ml microfuge tubes and immediately frozen in liquid nitrogen. In order to reduce contamination and degradation of the RNA, sterile RNase free pipette tips were used and Micro-Touch latex gloves (Ansell Healthcare, Brussels, Belgium) were worn throughout all procedures. The SV Wizard Plus RNA isolation kit (Promega UK, Southampton, UK) was used to isolate and purify the RNA, with the manufacturer's guidelines being followed throughout. Each sample produced 40 µl of RNA solution that was frozen at -20°C. The RNase free DNase kit (Promega) was used to remove any contaminating DNA from the RNA samples. Conversion of the isolated RNA to cDNA was performed using the Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Buckinghamshire, UK), using random nonamers (Sigma-Aldrich).

3.3.3 Amplification and separation of DNA

Two µl of isolated template DNA was added to a PCR reaction tube containing 10 µl of Red-Taq ready mix (Sigma-Aldrich), 6 µl of sterile purified water and 1 µl of each primer (MWG Biotech, Ebersberg, Germany) (Table 3.1, Figure 3.1). Primers were supplied at a concentration of 100 ρmol and diluted to a 20

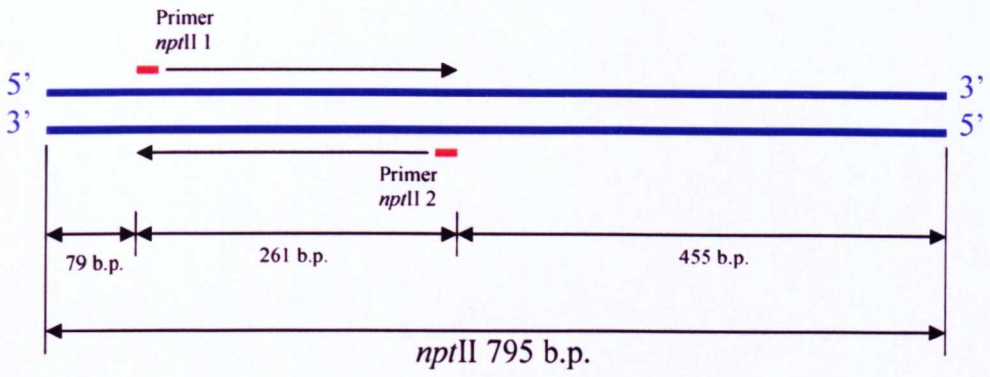
pmol with sterile purified water prior to use. PCR was performed using the Techne Flexigene thermal cycler (Techne, Cambridge, UK) using the conditions: 4 min initial denaturation (94°C), followed by 35 cycles of [40 sec denaturation (94°C), 40 sec annealing (see Table 3.1), 40 sec extension (72°C)], and a 10 min final extension (72°C). The PCR samples, including a negative (wild-type DNA) and positive control (pAFQ70.1 plasmid) (Chapter 2, Figure 2.1) and 100 bp molecular marker (ØX174 RF DNA *HAE* III; AB Gene, Cambridge, UK), were loaded onto a 1.5% (w/v) SeaKem LE agarose (BioWhittaker, Rockland, USA) gel made with 0.5 x TAE (Appendix 8.2.1) and run for 1.5 h at 100 V in a horizontal gel unit (Flowgen Instrument Ltd., Sittingbourne, UK). Gels were stained with ethidium bromide (0.5 $\mu g\ ml^{-1}$) for 10 min on an orbital shaker and visualised on a UV fluorescence imaging system (Syngene, Cambridge, UK).

Table 3.1: Details of the primer sequences used for testing transgenic plants. Abbreviations are, GC, guanidine cytosine; b.p., base pair.

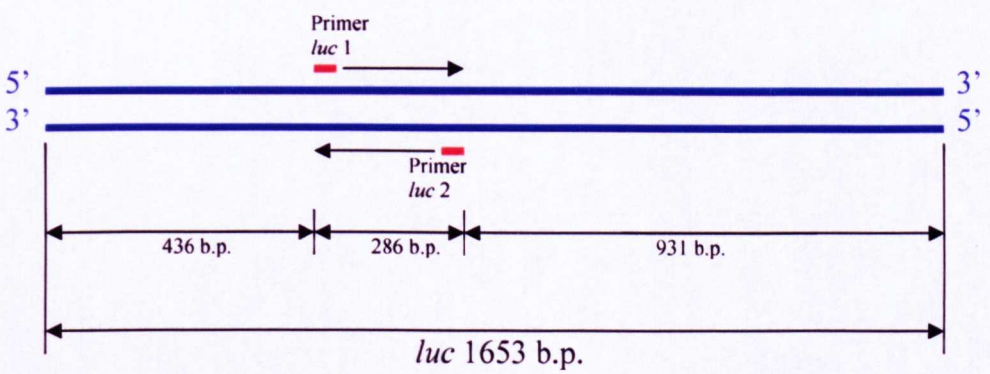
Primer	Sequence 5' to 3'	GC content (%)	Annealing temperature (°C)	Product size (b.p.)
*npt*II 1	AGACAATCGGCTGCTCTGAT	50	55	261
*npt*II 2	ATACTTTCTCGGCAGGAGCA	50		
luc 1	ATGTAAACAATCCGGAAGCG	45	53	286
luc 2	TTTTCCGTCATCGTCTTTCC	45		
*gsh*I 1	GAAAACCGAGTGCGGTATGT	50	53	270
*gsh*I 2	GTAACGCGTTTTGGACGAAT	45		
*gsh*II 1	TACCGCCTGGTTCTCTGACT	55	53	231
*gsh*II 2	ATGGCTGGCAGGTAATTTTG	45		
phgpx 1	AGCAAATTCAAGGGGAAGGT	45	53	296
phgpx 2	AAGCCCCCAGAACTTGATTT	45		
*gor*I 1	GGATGGAGATATGACAGTGA	45	50	482
*gor*I 2	CCTTAGTGATAGCTACAGGA	45		

Figure 3.1: Images showing the binding sites of the *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I primers on their respective genes.

- *npt*II



- *luc*



- *gsh*I

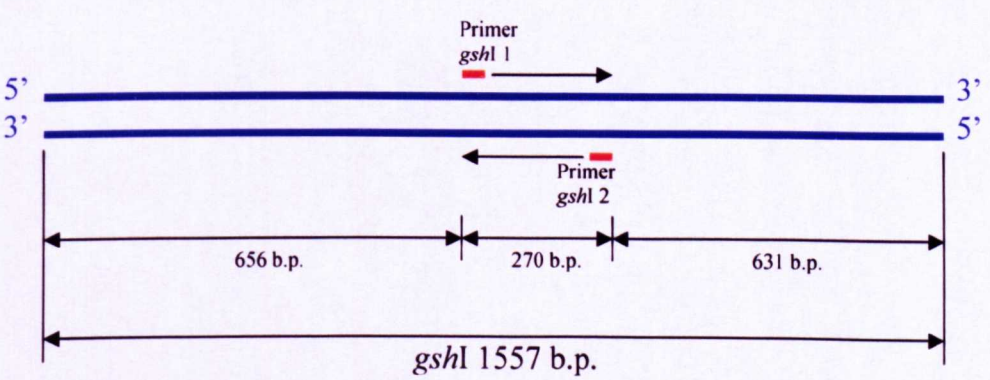
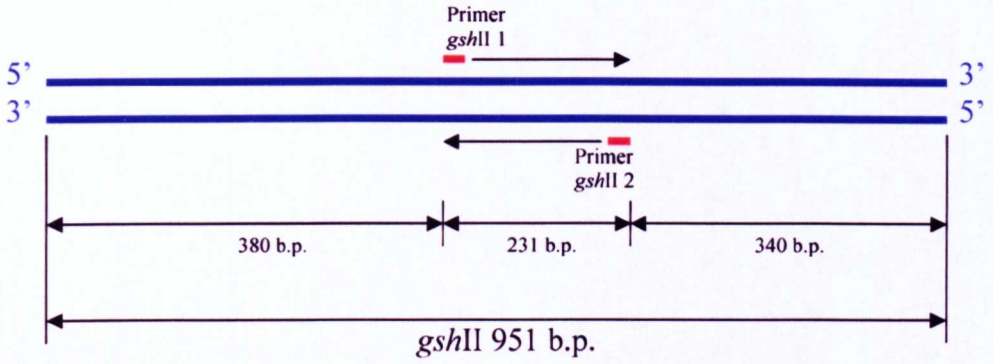
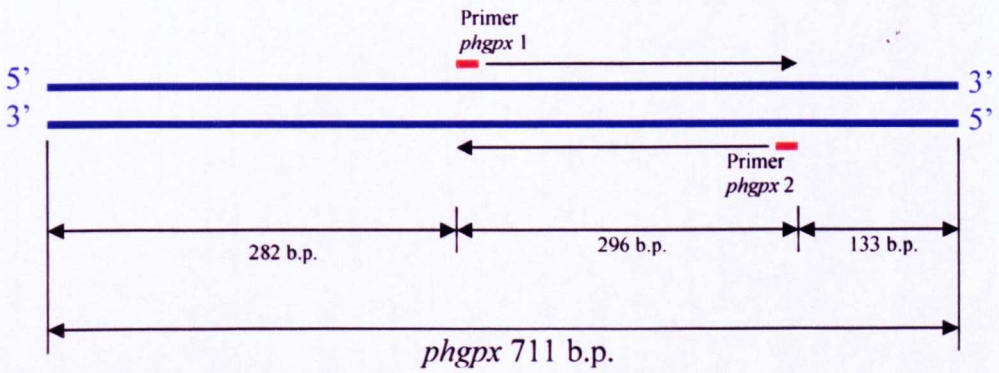


Figure 3.1 continued.

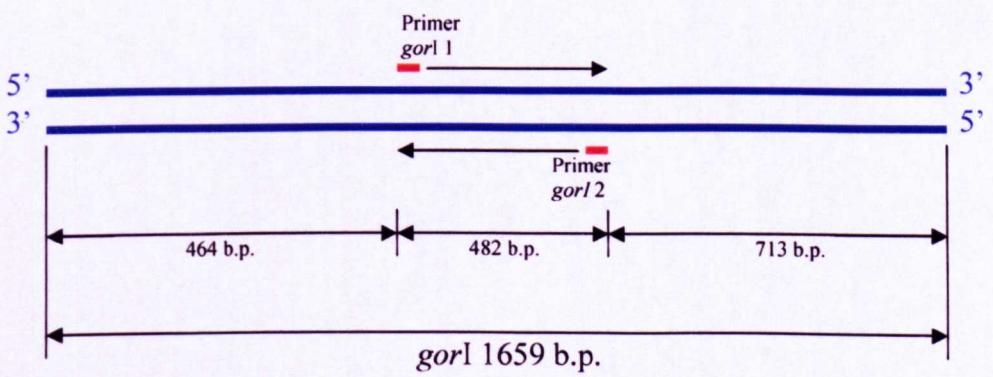
- *gsh*II



- *phgpx*



- *gor*I



3.3.4 CTAB extraction of genomic DNA

Total genomic DNA was isolated from the T_3 homozygous lines 32.4, 43.17 and 44.2 and the wild-type line (Chapter 4, Section 4.3.1) using a modified cetyltrimethylammonium bromide (CTAB) method of Michiels *et al.* (2003). Lettuce leaves (2 g) were frozen in liquid nitrogen and ground in a chilled mortar and pestle. The leaf powder was added to 30 ml of CTAB extraction buffer (Appendix 8.2.2), homogenised and incubated at 60°C for 30 min. One volume of chloroform : isoamyl alcohol (24:1) (v:v) was added to the extract, vortexed thoroughly and centrifuged at 2,500 x g for 10 min at RT. The upper aqueous phase was transferred to a clean tube and the chloroform : isoamyl alcohol extraction step repeated. The aqueous phase was mixed with 2/3 volume of isopropanol, inverted 2 – 3 times and the nucleic acids precipitated overnight at -20°C. The samples were centrifuged at 2,500 x g for 15 min at RT, the supernatants removed and the pellets washed in 70% (v/v) ethanol. After washing, the pellets were air dried overnight. The nucleic acid pellets were resuspended in 1 ml TE buffer (Appendix 8.2.3) and incubated with RNase A (Sigma-Aldrich) (10 μg ml^{-1}) at 45°C for 30 min. One volume of phenol was added and the samples mixed thoroughly and centrifuged at 2,500 x g for 10 min at RT. The aqueous phase was removed and the extraction process repeated with phenol : chloroform : isoamyl alcohol (25:24:1) (v:v) and chloroform : isoamyl alcohol (24:1) (v:v). Two volumes of 100% ethanol were added to each sample, incubated on ice for 5 min and centrifuged at 10,000 x g for 5 min at RT. The supernatant was discarded and the pellet air dried for ~1 h. The DNA pellet was resuspended in 200 μl TE buffer and stored at -20°C.

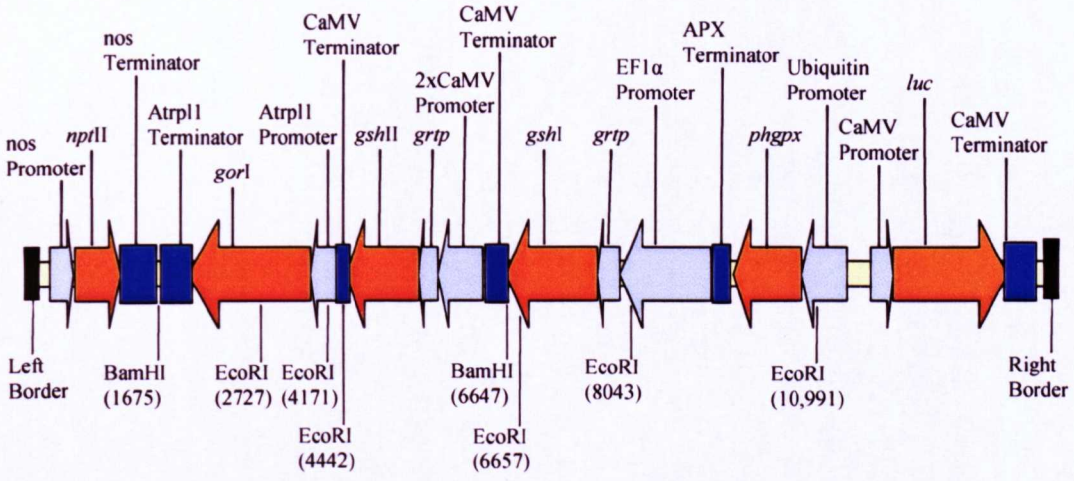
The quantity and quality of the genomic DNA was determined using a Nano Drop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The DNA quantified was mixed with a solution of sodium acetate (3 M, pH 5.2) and dextran (10 mg ml^{-1}) in a ratio of 6:1 (v:v) and precipitated by adding 3 volumes of 100% ethanol and incubating for 20 min at -20°C. The precipitated DNA was centrifuged at 10,000 x g for 5 min at RT, air dried overnight and resuspended in TE buffer at a concentration of 1μg μl^{-1}.

3.3.5 Genomic DNA dot blot analysis

Seven μg of DNA was diluted serially to give concentrations of 1 μg μl^{-1}, 0.5 μg μl^{-1}, 0.25 μg μl^{-1}, 0.125 μg μl^{-1}, denatured at 95°C for 10 min, followed by 5 min

on ice. Seven µl of each dilution was pipetted onto dots circled on Hybond N^+ nylon membrane (Roche Diagnostics GmbH, Penzberg, Germany) and UV crosslinked (Syngene) for 30 sec. Cultivar King Louie wild-type DNA and pAFQ70.1 plasmid were used as negative and positive controls. Prepared nylon membranes were subsequently probed for the presence of the transgenes (Section 3.3.7).

Figure 3.2: The binary vector pAFQ70.1 T-DNA displaying the BamHI and EcoRI cutting locations.



pAFQ70.1 T-DNA
14,558 b.p.

3.3.6 Southern blot analysis

3.3.6.1 Restriction enzyme digest of genomic and plasmid DNA

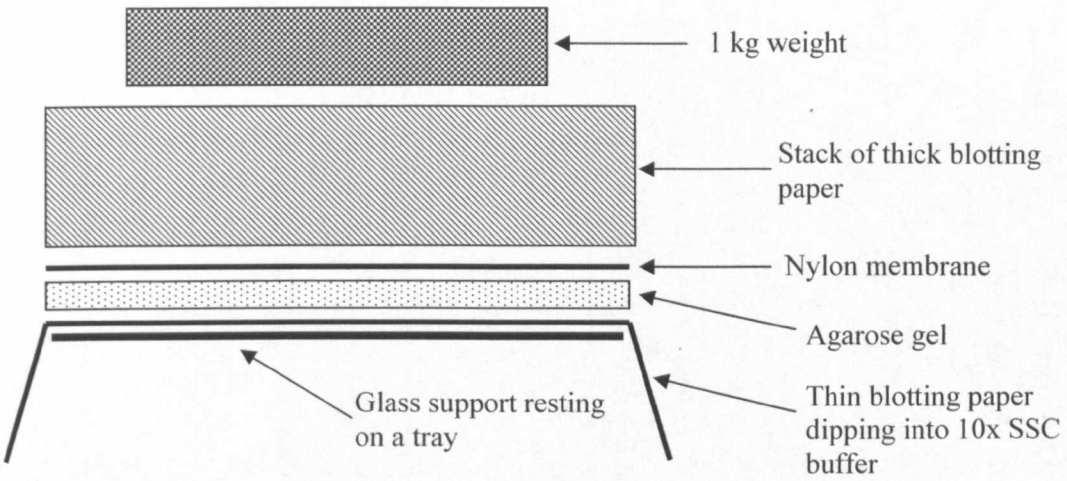
Ten µg of DNA from each plant line and 0.5 µg of the pAFQ70.1 plasmid (Sections 3.3.1 and 3.3.4) were cut with the restriction enzymes BamHI and EcoRI (Promega) in a 100 µl reaction for 16 h at 37°C. The restriction enzymes BamHI and EcoRI were used for the detection of *gsh*I, *gor*I and *gsh*II, *phgpx*, respectively. Cutting the pAFQ70.1 plasmid with BamHI and EcoRI yielded DNA fragments of 17,533 b.p., 4972 b.p. and 14,241 b.p., 2948 b.p., 2215 b.p., 1444 b.p., 1386 b.p., 271 b.p., respectively (Figure 3.2). Cultivar King Louie wild-type DNA and the pAFQ70.1 plasmid were used as negative and positive controls, respectively. The restriction digested DNA and a DIG labelled 21 k b.p. molecular marker (Roche)

were separated using a 0.8% (w/v) SeaKem LE agarose gel made with 0.5 x TAE and run for 14 h at 35 V in a horizontal gel unit. The agarose gel was stained with ethidium bromide (0.5 μg ml^{-1}) for 20 min on an orbital shaker and visualised on a UV fluorescence imaging system to confirm the restriction and separation of the fragments (Figure 3.4).

3.3.6.2 Transfer of restriction enzyme digested DNA to a nylon membrane

The agarose gel was bathed in 250 mM HCl for 5 min at RT to depurinate the digested DNA and aid the transfer of large DNA fragments to the nylon membrane. The gel was then submerged in denaturation solution (Appendix 8.2.4) for 30 min at RT. The gel was rinsed with purified water and submerged in neutralisation solution (Appendix 8.2.5) for 30 min at RT. The denaturation and neutralisation processes were performed on an orbital shaker. The agarose gel was placed on top of thin blotting paper (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) dipping into 10 x SSC buffer (Appendix 8.2.6). The Hybond N^+ nylon membrane (Roche) was then placed on top of the agarose gel and covered with a stack of thick blotting paper (Bio-Rad Laboratories Ltd.) and a 1 kg weight. This caused the denatured DNA fragments to be transferred from the agarose gel to the nylon membrane by capillary action (Figure 3.3). The apparatus was incubated at RT for 12 – 16 h. After blotting was complete, the DNA was cross linked to the nylon membrane using UV light for 30 sec (Syngene). Prepared nylon membranes were subsequently probed for the presence of the transgenes (Section 3.3.7).

Figure 3.3: The Southern blot apparatus.



3.3.7 Hybridisation and detection of the transgenes

PCR products of the respective genes were labelled using the PCR DIG probe synthesis kit (Roche) (Figure 3.5), according to the manufacturer's instructions. Pre-hybridization was carried out for 1.5 h in DIG Easy hyb buffer (Roche) at 37°C. The probe (60 µl) was denatured at 95°C for 10 min followed by 5 min on ice, then added to 30 ml DIG Easy hyb buffer and the membrane incubated overnight at 37°C. Following hybridization, the membrane was washed in twice in 2 x wash solution [2 x SSC (Appendix 8.2.6) and 0.5 g l^{-1} SDS] for 5 min at room temperature, washed twice in 0.5 x wash solution [0.5 x SSC (Appendix 8.2.6) and 0.5 g l^{-1} SDS] at 65°C for 5 min and finally washed once in washing buffer (Appendix 8.2.7) for 2 min at RT. The membrane was incubated in 70 ml of 1% (w/v) blocking solution (Roche) for 1 h at RT, followed by 30 ml of blocking solution containing anti-dig alkaline phosphatase enzyme (Roche) at a concentration of 1 : 20,000 (v:v) and incubated for 30 min at RT. The membrane was washed 2 times in washing buffer for 15 min at RT. After the washing, the membrane was incubated in 20 ml of detection buffer (Appendix 8.2.8) containing 5 drops of CDP-star (Roche) and incubated for 5 min to equilibrate the membrane for detection. The presence of bound probe to the membrane was detected using Kodak Biomax X-ray film (Kodak, Cedex, France) after 12 - 24 h at RT.

3.3.8 Segregation analysis of cv. King Louie T_1 and T_2 transformed lines

Self-pollinated seed from cv. King Louie T_0 transgenic lines (Chapter 2, Section 2.3.5) was surface sterilised in 10% (v/v) Domestos-bleach solution (Chapter 2, Section 2.3.1) and germinated on 20 ml aliquots of full strength semi-solid MS0 medium (Appendix 8.1.1) containing kanamycin sulphate (200 mg l^{-1}) (Garratt, 2002); 20 seeds/9 cm Petri dish. One hundred seeds from each line were germinated at 24°C with a 16 h photoperiod (50 µmol m^{-2} sec^{-1}, daylight fluorescent illumination). Seedlings were scored 14 d after germination and categorised as resistant (green leaves, branched roots) or sensitive (bleached leaves, stunted roots) (Figure 3.6). Resistant T_1 generation plants derived from the T_0 lines 32, 43 and 44 were maintained and the seeds segregated into T_2 homozygous, heterozygous and azygous lines. Twelve plants from the T_2 homozygous lines 32.4, 43.17, and 44.2, and 12 plants from the T_2 azygous lines 32.9, 43.16, and 44.12, were grown for T_3 seed.

3.3.9 Statistics

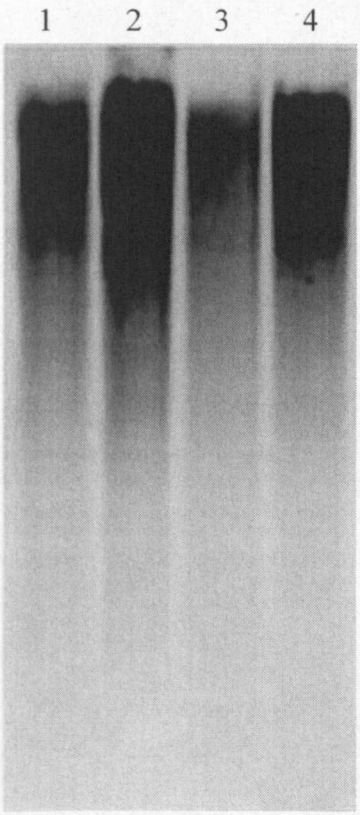
In total, 186 T_0 putative transformants, 98 of cv. King Louie, 56 of cv. Robusto and 32 of cv. Pic, were analysed by PCR for the selectable marker transgenes *npt*II and *luc*. PCR results were presented as combined data for the 3 cvs. Ninety eight PCR positive T_0 transformants were further analysed by reverse transcriptase PCR (RT-PCR) for expression of the transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I. RT-PCR results were presented as the frequency of expression of single transgenes and groups of genes for both plants of individual cvs. and as combined data for the 3 cvs. Five plants from each cv. King Louie T_1 generation line, 32, 43 and 44, and 12 plants from each T_2 generation line 32.4, 43.17 and 44.2 were analysed by RT-PCR for inheritance and expression of the transgenes. Histograms did not show error bars for S.E.M. because experiments were not repeated. Data was analysed using Microsoft Excel.

3.4 Results

3.4.1 PCR analysis of T_0 putative transformants

PCR was used to assay putatively transformed shoots for the flanking marker transgenes, *npt*II and *luc* (Figure 3.7) (Appendix 8.3.2, Table 8.4). In total, 143 of the 186 T_0 putative transformants contained both marker transgenes (77% of plants). The individual marker transgenes, *npt*II and *luc*, were found in 17 and 16 of the 186 T_0 putative transformants, respectively (Figure 3.8). Only 10 plants, 5% of the 186 T_0 putative transformants, lacked either transgene. Statistical analysis of the PCR data confirmed no significant difference in the presence of *npt*II and *luc* in cvs. King Louie, Pic and Robusto. Putative transformants were regenerated under antibiotic (kanamycin sulphate) selection for 12 wks to ensure a minimum number of non-transformed regenerants were assayed by PCR.

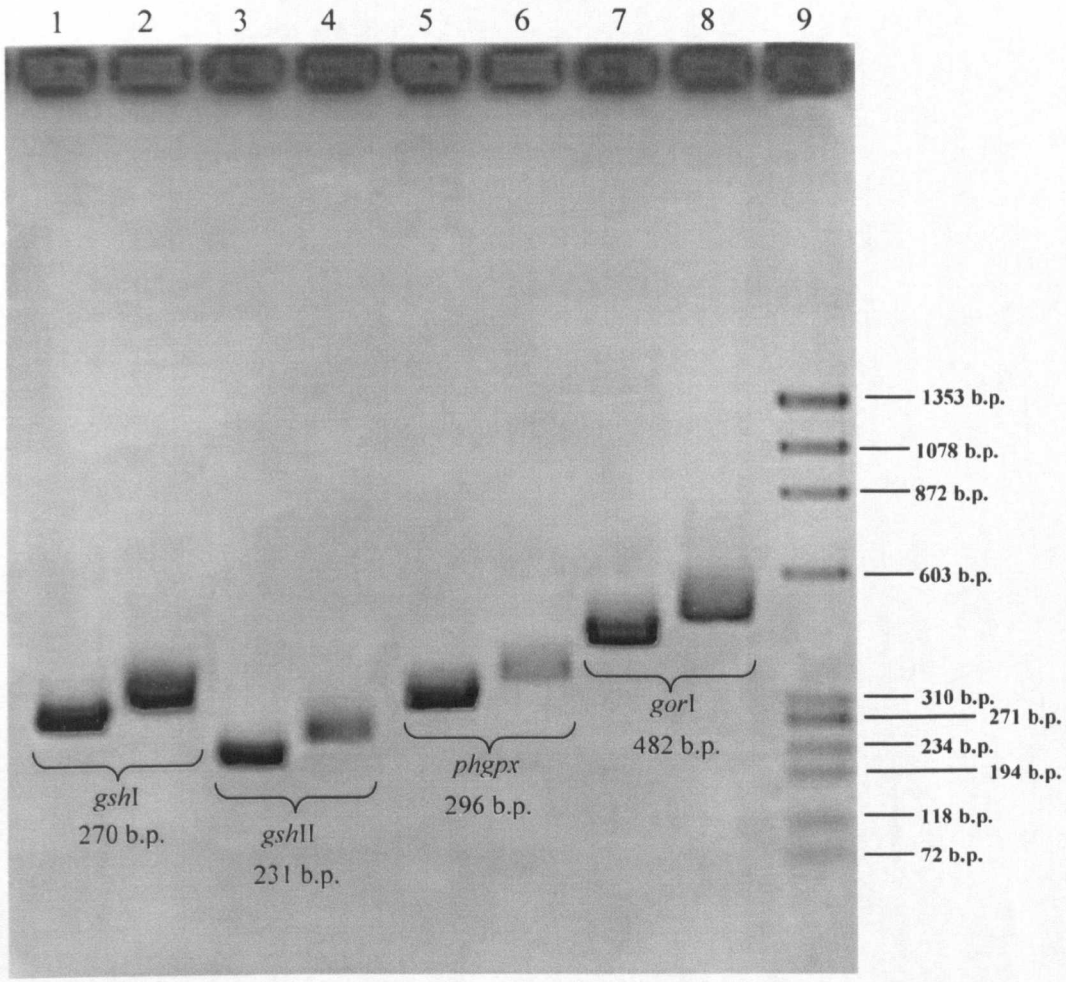
Figure 3.4: Example of restriction enzyme digests of cv. King Louie wild-type and T_3 homozygous line genomic DNA with the restriction enzyme BamHI.



Lane 1: Cultivar King Louie wild-type DNA.

Lanes 2, 3, 4: Cultivar King Louie T3 line 32.4, 43.17 and 44.2 DNA, respectively.

Figure 3.5: DIG labeled PCR probes for the genes *gsh*I, *gsh*II, *phgpx* and *gor*I.

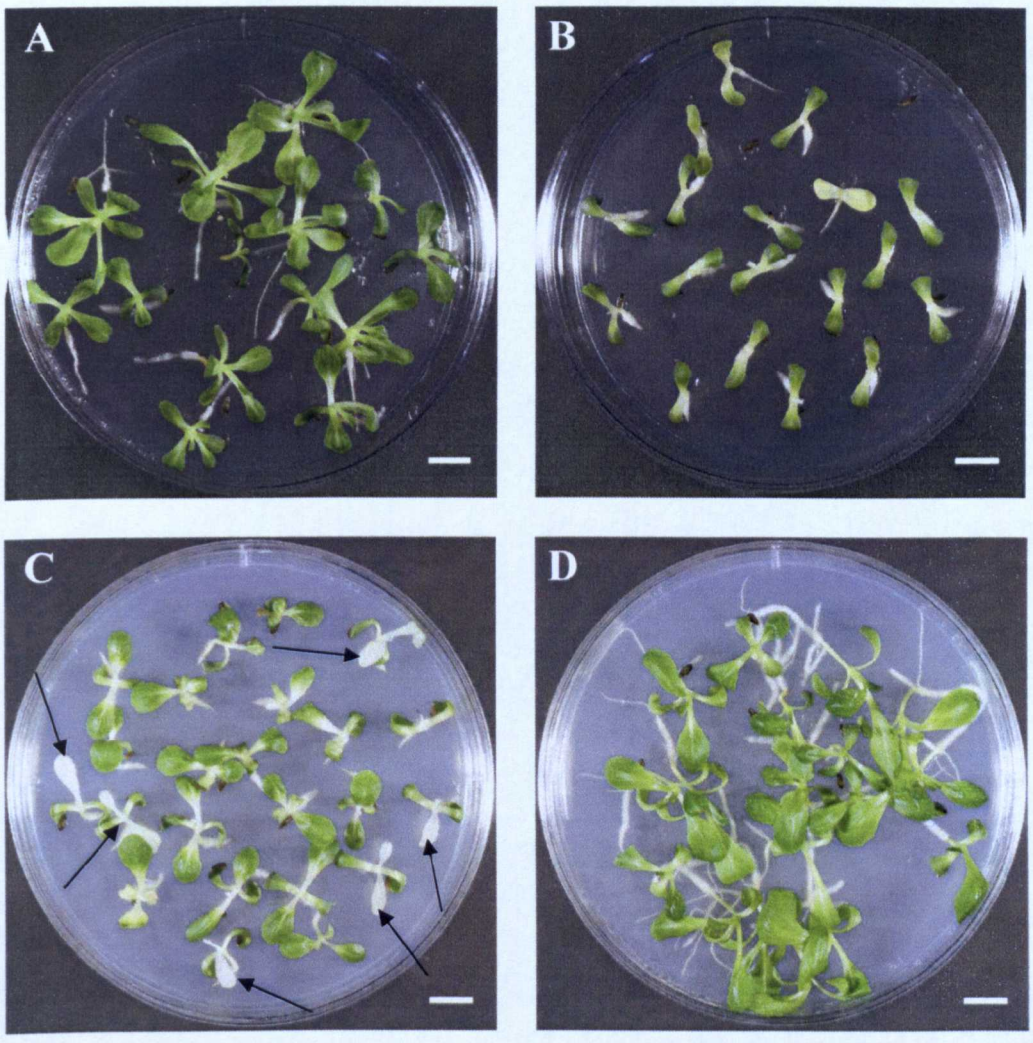


Lanes 1, 3, 5, 7: PCR product.

Lanes 2, 4, 6, 8: DIG labelled PCR product.

Lane 9: 100 b.p. molecular marker.

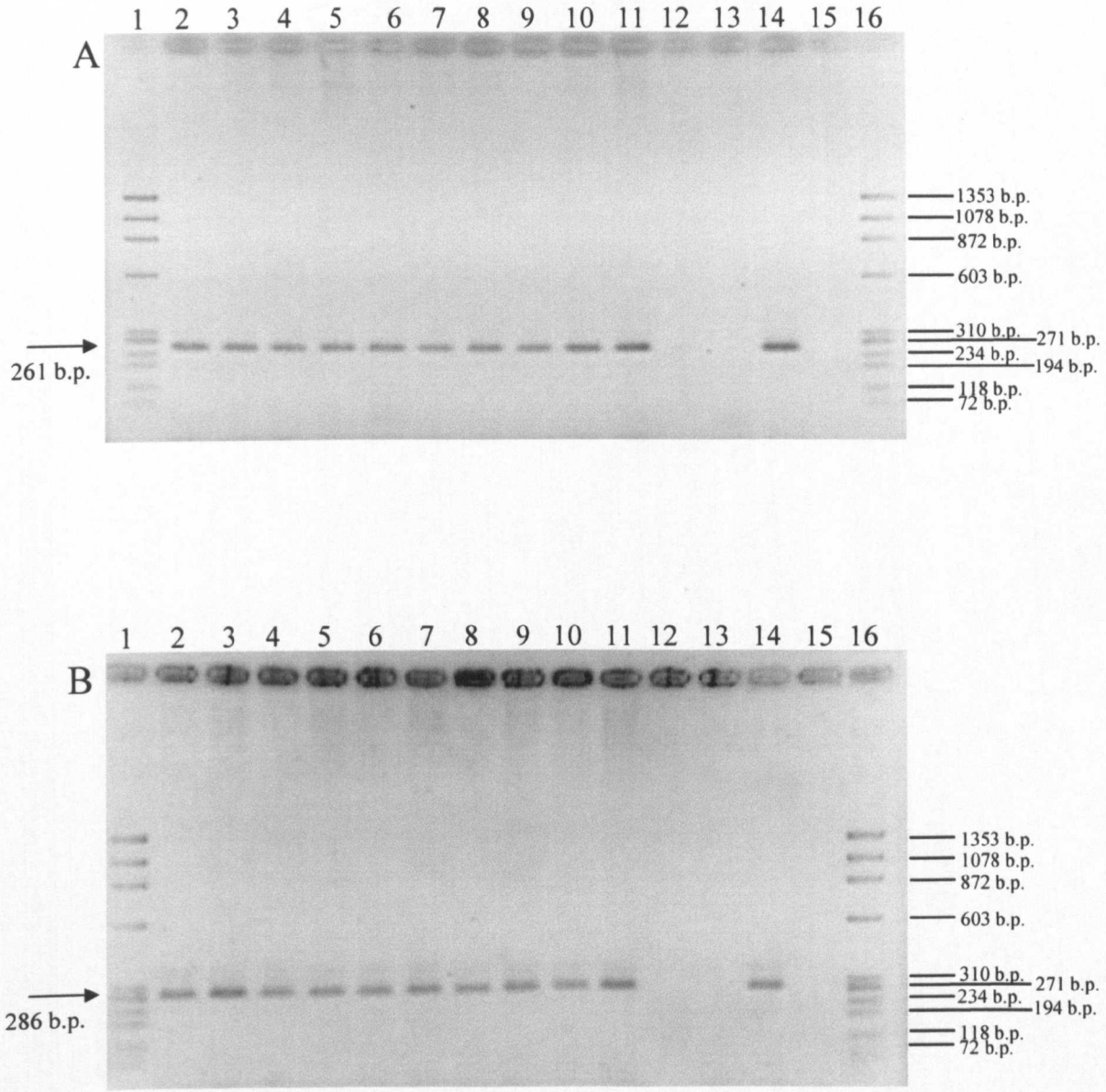
Figure 3.6: Example of cv. King Louie homozygous, heterzygous and wild-type seeds growing on MS0 medium containing 200 mg l^{-1} kanamycin sulphate after 2 wks.



The cv. King Louie wild-type line on (A) MS0 medium only and (B) MS0 medium containing 200 mg l^{-1} kanamycin sulphate.

A cv. King Louie (C) heterozygous T_1 line and (D) homozygous T_1 line on MS0 medium containing 200 mg l^{-1} kanamycin sulphate. Arrows indicate kanamycin sulphate sensitive plants. Bars = 1cm.

Figure 3.7: Example of PCR analysis for *npt*II and *luc* transgenes in putatively transformed T_0 lines of cv. King Louie.



(A) PCR analysis for *npt*II and (B) PCR analysis for *luc*.

Lanes 1, 16: 100 b.p. molecular marker.

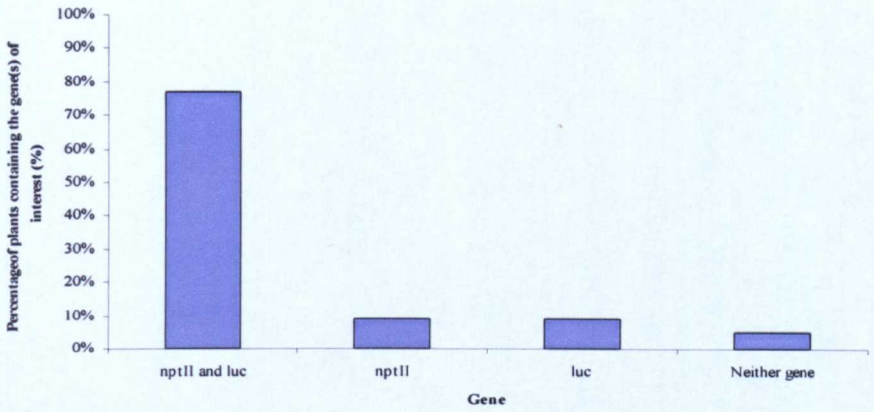
Lanes 2-11: Putatively transformed plants with *nptII and luc* gene.

Lane 12, 15: Water control.

Lane 13: Cultivar King Louie wild-type negative control.

Lane 14: pAFQ70.1 plasmid positive control.

Figure 3.8: PCR data indicating the distribution of the selectable marker transgenes *npt*II and *luc* in T_0 putative transformants.



Results expressed as combined data from the cvs. King Louie, Pic and Robusto. n = 186.

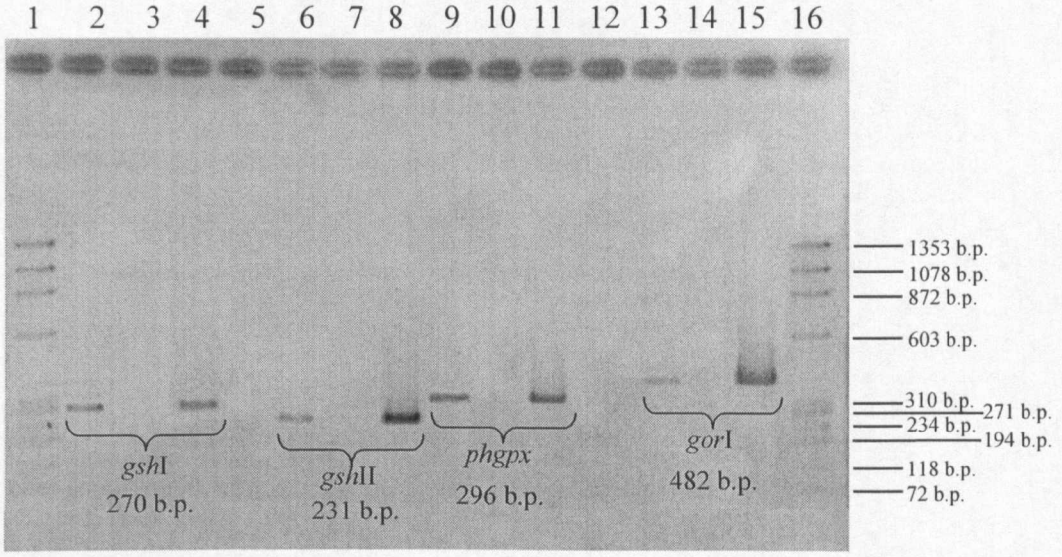
3.4.2 RT-PCR analysis of T_0 putative transformants

RT-PCR analysis of the PCR positive plants of cvs. King Louie, Pic and Robusto showed that distribution of the expressed transgenes followed a varied pattern (Figures 3.9 – 3.13) (Appendix 8.3.2, Table 8.5 and 8.6). The transgene *npt*II was expressed in the most plants followed by *gsh*I, *gsh*II, *phgpx*, *luc* and *gor*I respectively, based on combined data from the cvs. King Louie, Pic and Robusto. Analysis of cv. King Louie transformants found that *npt*II and *gsh*I were the most expressed transgenes, while *luc*, *gsh*II, *phgpx* and *gor*I were expressed in fewer plants. Cultivars Pic and Robusto followed similar trends to each other, with the transgenes *luc* and *gor*I expressed in the least number of plants (22% and 9%, 4% and 4% of plants, respectively). The number of expressed transgenes appeared to follow a normal distribution, based on combined data from the cvs. King Louie, Pic and Robusto. These data showed that 2 transgenes were most commonly expressed (22% of plants). Cultivars Pic and Robusto had few plants expressing 5 or 6 transgenes, while plants of cv. King Louie did not follow this pattern and had more plants expressing all 6 transgenes (34% of plants) than other combinations. Only 4% of all screened plants did not express any transgene.

3.4.3 RT-PCR analysis of cv. King Louie T_1 and T_2 lines

Five randomly selected plants from each T_1 cv. King Louie line of 32, 43 and 44, were screened using RT-PCR (Figure 3.14) (Appendix 8.3.2, Table 8.7). Data from each line indicated a varied transgene expression pattern. All plants expressed the transgenes *npt*II, *gsh*I, *gsh*II and *gor*I. The transgenes *luc* and *phgpx* were not expressed in 3 and 1 plants, respectively based on combined data from the lines 32, 43 and 44. Plants of line 44 expressed all transgenes. Twelve T_2 generation plants from each cv. King Louie line, 32.4, 43.17 and 44.2, were assayed using RT-PCR (Figure 3.15) (Appendix 8.3.2, Table 8.7). The transgene, *luc,* was only expressed in 11% of plants based on combined data from the lines 32.4, 43.17 and 44.2. The remaining transgenes were expressed in almost all plants with the exception of line 44 which had low expression of *gor*I (25% of plants).

Figure 3.9: Example of RT-PCR analysis for *gsh*I, *gsh*II, *phgpx* and *gor*I transgenes in PCR positive transformed T_0 lines of cv. King Louie.



Lanes 1, 16: 100 b.p. molecular marker.

Lanes 2, 6, 9, 13: PCR positive transformed T_0 lines of cv. King Louie.

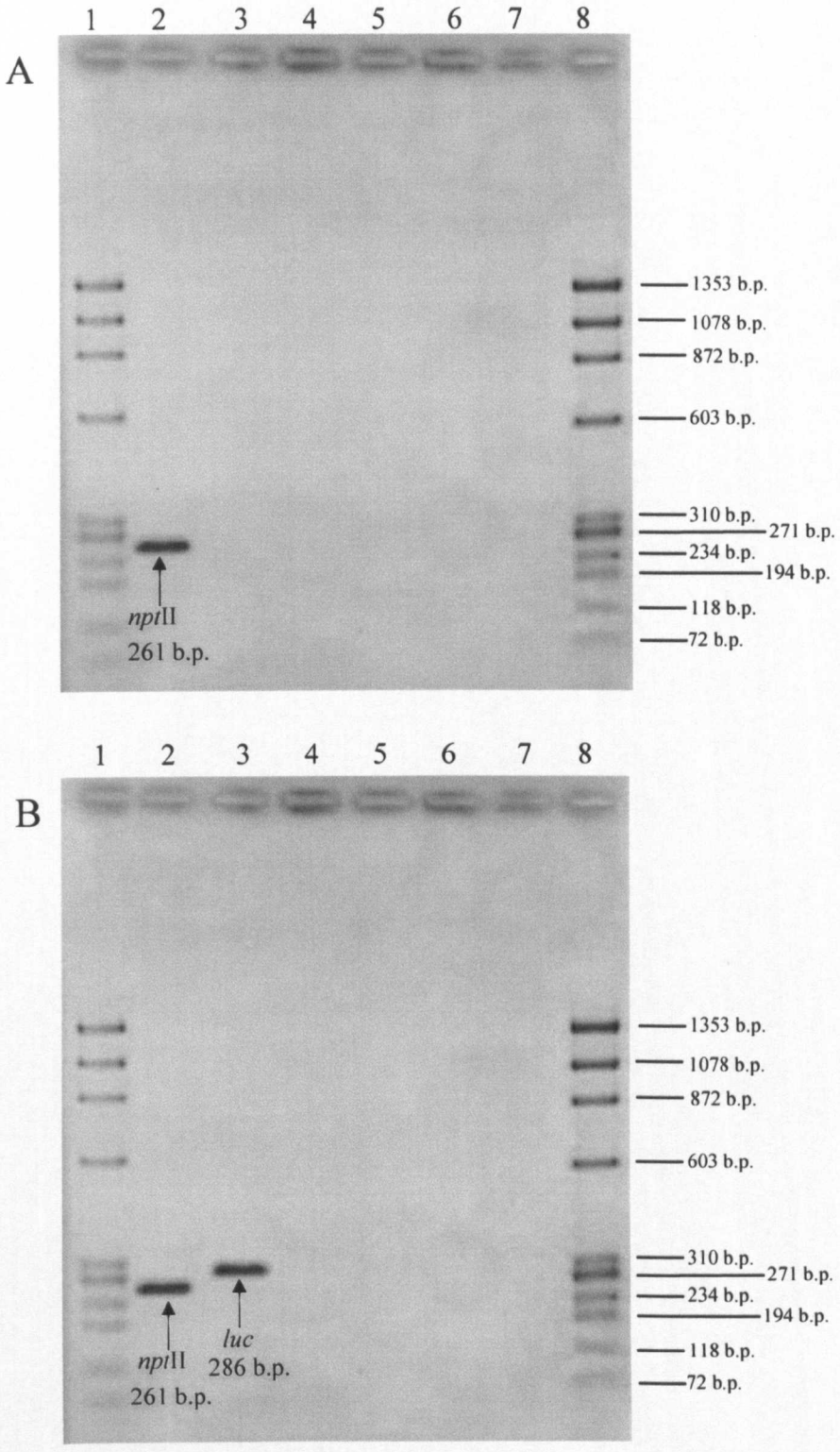
Lanes 3, 7, 10, 14: Cultivar King Louie wild-type negative control.

Lanes 5, 12: Water control.

Lanes 4, 8, 11, 15: pAFQ70.1 plasmid positive control.

RT-PCR product sizes: *gsh*I (270 b.p.), *gsh*II (231 b.p.), *phgpx* (296 b.p.), *gor*I (482 b.p.).

Figure 3.10: Example of RT-PCR analyses for the transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I in PCR positive transformed T_0 lines of cv. King Louie.

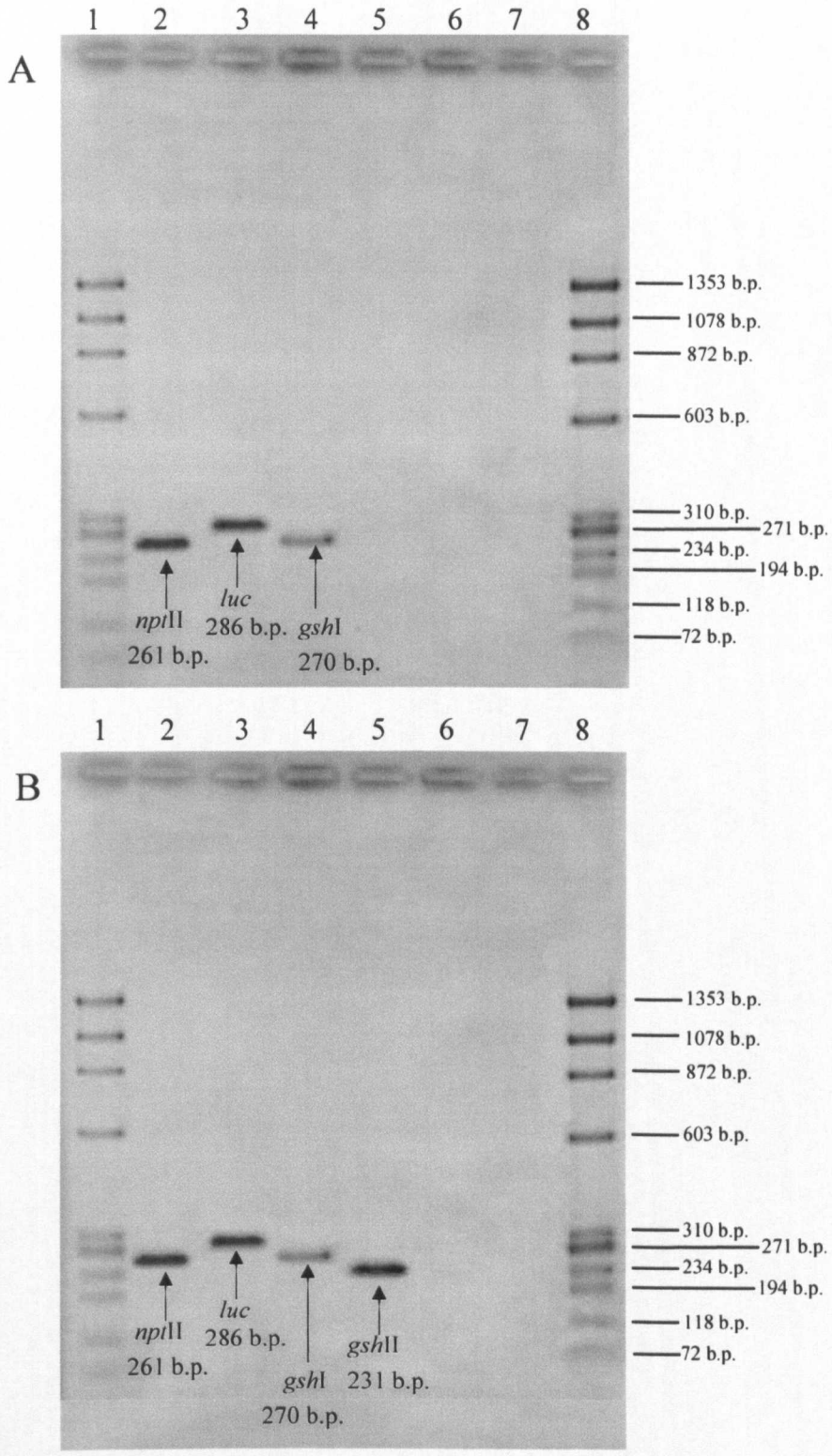


RT-PCR analyses showing PCR product bands for (A) *npt*II and (B) *npt*II, *luc*.

Lanes 1, 8: 100 b.p. molecular marker.

Lanes 2, 3: RT-PCR positive transformed T_0 lines of cv. King Louie.
RT-PCR product sizes: *npt*II (261 b.p.), *luc* (286 b.p.).

Figure 3.10 continued.

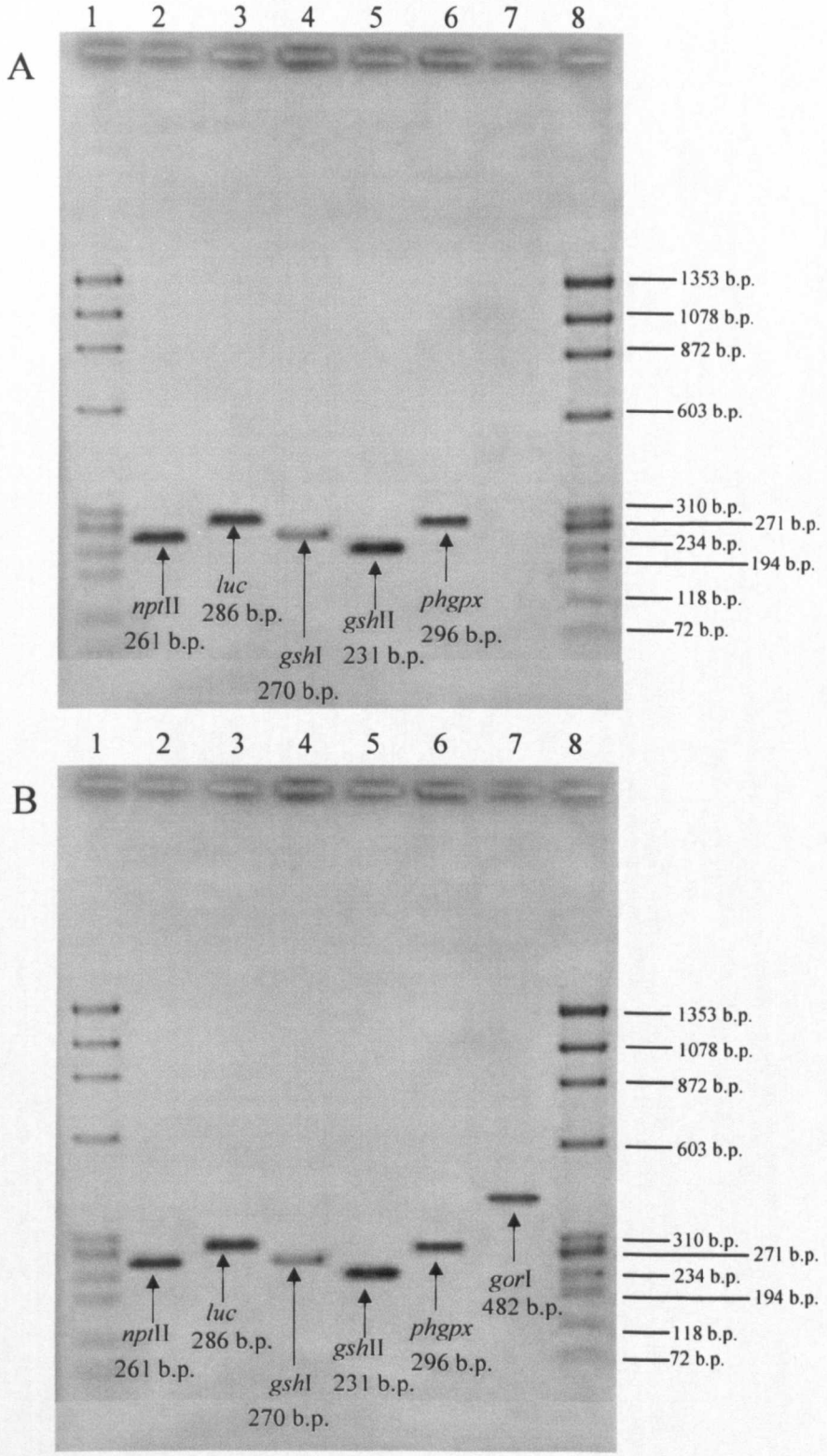


RT-PCR analyses showing PCR product bands for (A) *npt*II, *luc*, *gsh*I and (B) *npt*II, *luc*, *gsh*I, *gsh*II.

Lanes 1, 8: 100 b.p. molecular marker.

Lanes 2 - 5: RT-PCR positive transformed T_0 lines of cv. King Louie.
RT-PCR product sizes: *npt*II (261 b.p.), *luc* (286 b.p.), *gsh*I (270 b.p.), *gsh*II (231 b.p.).

Figure 3.10 continued.

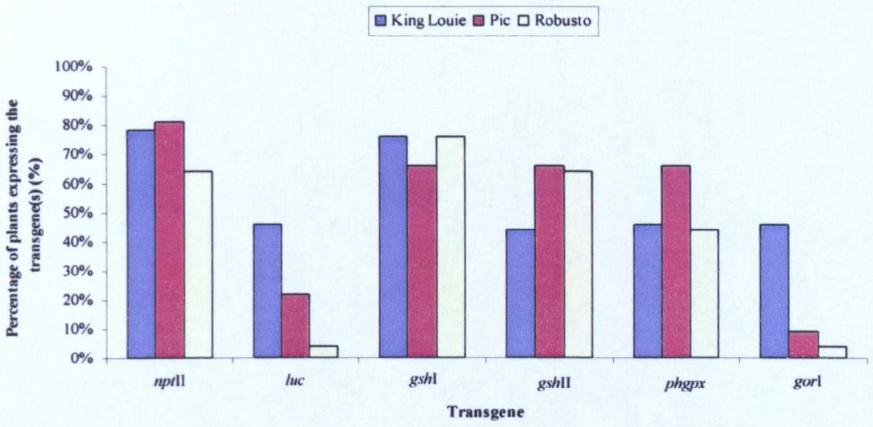


RT-PCR analyses showing PCR product bands for (A) *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and (B) *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx*, *gor*I.

Lanes 1, 8: 100 b.p. molecular marker.

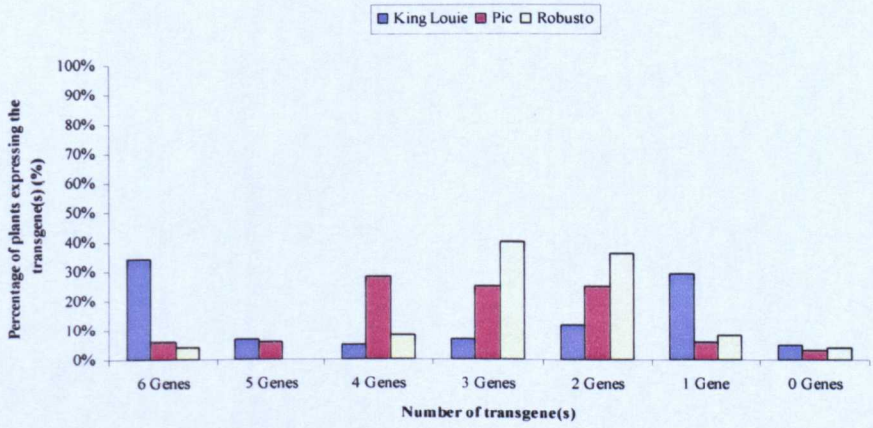
Lanes 2 - 7: RT-PCR positive transformed T_0 lines of cv. King Louie.
RT-PCR product sizes: *npt*II (261 b.p.), *luc* (286 b.p.), *gsh*I (270 b.p.), *gsh*II (231 b.p.), *phgpx* (296 b.p.), *gor*I (482 b.p.).

Figure 3.11: RT-PCR data indicating the distribution of the expressed transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I in T_0 putative transformants of cvs. King Louie, Pic and Robusto.



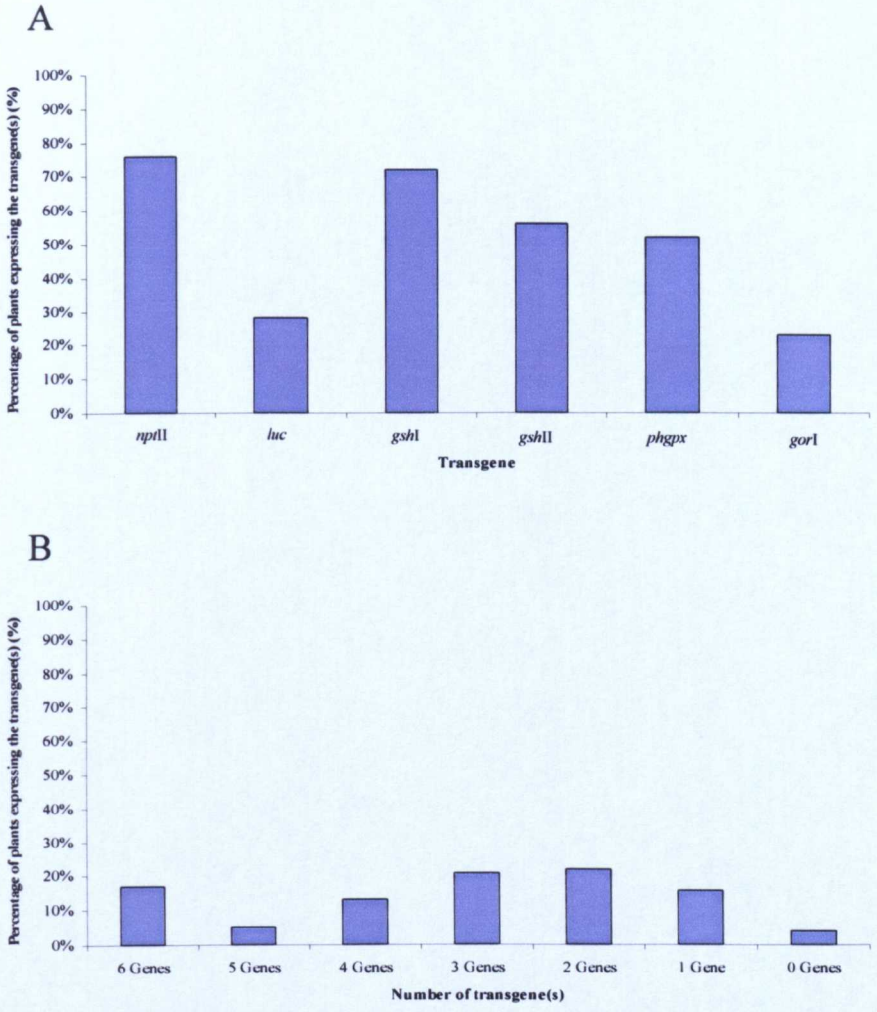
Data expressed as the frequency of occurrence of single genes.
n = 98.

Figure 3.12: RT-PCR data indicating the distribution of the number of expressed transgenes in T_0 putative transformants of cvs. King Louie, Pic and Robusto.



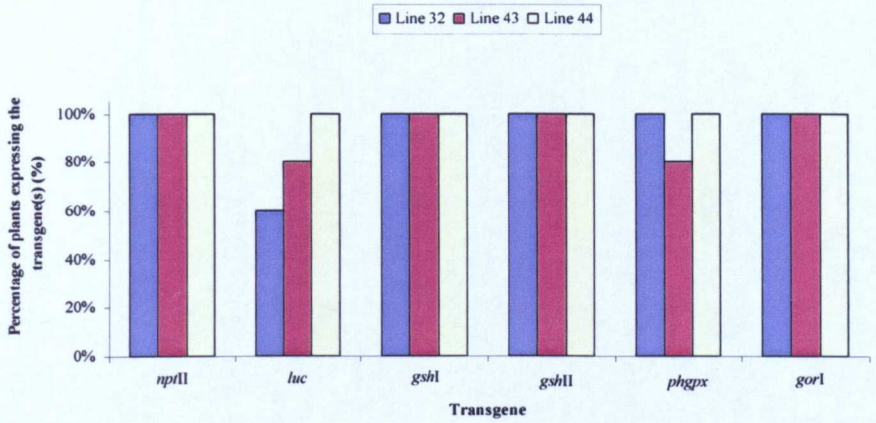
Data expressed as the frequency of occurrence of groups of genes.
n = 98.

Figure 3.13: RT-PCR data indicating the distribution of the expressed transgenes in T_0 putative transformants.



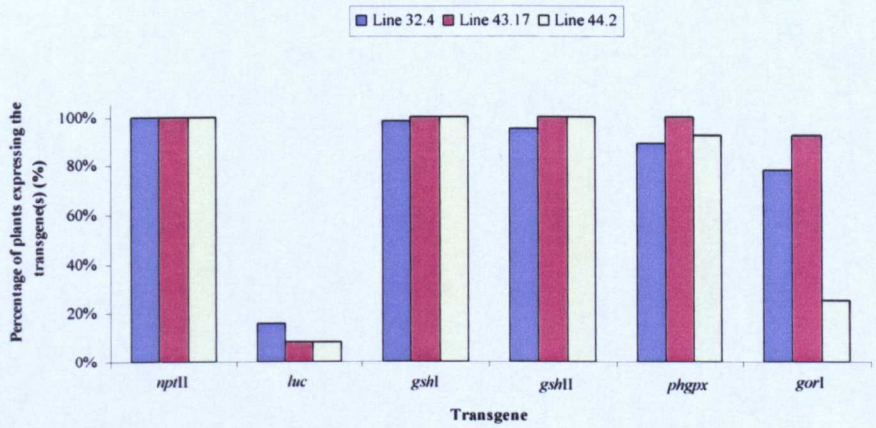
Results expressed as combined data for the frequency of occurrence of (A) single genes and (B) groups of genes from the cvs. King Louie, Pic and Robusto. n = 186.

Figure 3.14: RT-PCR data indicating the distribution of the expressed transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I in cv. King Louie T_1 lines of 32, 43 and 44.



Data expressed as the frequency of occurrence of single genes.
n = 5.

Figure 3.15: RT-PCR data indicating the distribution of the expressed transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I in cv. King Louie T_2 lines of 32.4, 43.17 and 44.2.



Data expressed as the frequency of occurrence of single genes.
n = 12.

3.4.4 Identification of cv. King Louie homozygous lines

Kanamycin sulphate resistance data from the cv. King Louie T_1 generation seed allowed identification of lines which exhibited the Mendelian 3:1 gene segregation ratio. Twenty kanamycin sulphate resistant plants from the lines 32, 43, 44 were selected to produce T_2 lines (Table 3.2).

None of the cv. King Louie T_2 lines were azygous (reverted to wild-type), although there was a 3:1 ratio of heterozygotes to homozygotes based on combined segregation data from the T_2 lines. One homozygous line was maintained from each of the cv. King Louie T_2 lines, 32, 43 and 44. From each homozygous line, 12 plants were transferred to the glasshouse and allowed to set seed. Because none of the T_2 lines were azygous, suitable plants were selected from heterozygous T_1 lines and grown for seed.

Table 3.2: Kanamycin sulphate segregation data of cv. King Louie T_1 lines.

Line	Percentage kanamycin sulphate resistant plants (%)
32	75
42	67
43	84
44	74
50	73

3.4.5 Dot blot and Southern blot analysis of cv. King Louie T_3 homozygous lines

Before dot blot and Southern blot analysis commenced, presence of the transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I in cv. King Louie T_3 homozygous lines was confirmed using PCR analysis (Figure 3.16). Dot blot analysis of pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie allowed initial detection of the transgenes. The homozygous T_3 lines of cv. King Louie, 32.4, 43.17 and 44.2, showed positive signals for the transgenes in DNA concentrations ranging from 1 μg μl^{-1} to 0.125 μg μl^{-1} (Figures 3.17 – 3.20).

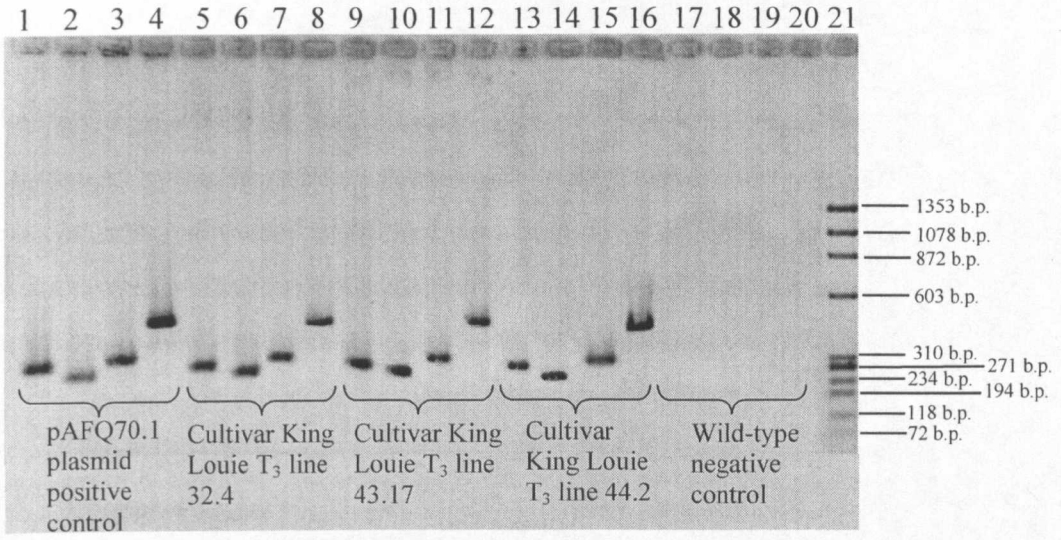
Further analysis by Southern blotting allowed the detection of transgene copy number (Figures 3.21 – 3.24, Table 3.3). The gene *gsh*I was present in lines 32.4,

43.17 and 44.2 in 2, 2 and 3 DNA copies, respectively. DNA fragments that were positive for the DIG-labelled probe ranged from 22 k b.p. to 18 k b.p. The probe successfully bound to the 17,533 b.p. plasmid fragment cut by the BamHI restriction enzyme. The gene *gsh*II was present in few DNA copies in lines 43.17 and 44.2 (2 copies for both), yet was present 9 times in line 32.4. All lines showed 2 distinct bands of approximately 21 k b.p. and 18 k b.p. in size. Line 32.4 was the exception, with 7 gene inserts ranging from 15 - 2 k b.p. in size. The EcoRI restriction enzyme cut the plasmid T-DNA fragment more frequently than BamHI, resulting in a smaller (2,215 b.p.) positive band for *gsh*II. Results for the *gor*I gene showed that the lines 32.4, 43.17 and 44.2 had 1, 1 and 2 copies of the transgene present. Lines 32.4 and 43.17 produced a DNA fragment identical in size (4,972 b.p.) to the plasmid. Line 44.2 produced 2 fragments of 15 k b.p. and 8.6 k b.p. in size. The gene *phgpx* did not test positive in any of the homozygous lines and therefore copy number could not be determined. The plasmid positive control was detected as a 2,948 b.p. fragment. In both the dot blot and Southern blot analyses, the cv. King Louie wild-type line did not test positive for any of the transgenes.

Table 3.3: Summary of analysis of T-DNA integration of the T_3 homozygous lines of cv. King Louie transformed with the transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I. Abbreviation, N/A, not applicable.

Line	Transgene			
	*gsh*I	*gsh*II	*phgpx*	*gor*I
32.4 homozygous	2	9	N/A	1
43.17 homozygous	2	2	N/A	1
44.2 homozygous	3	2	N/A	2

Figure 3.16: PCR analysis for the genes *gsh*I, *gsh*II, *phgpx* and *gor*I in cv. King Louie T_3 lines 32.4, 43.17 and 44.2. Cultivar King Louie wild-type DNA and the pAFQ70.1 plasmid were used as negative and positive controls, respectively.



Lanes 1, 5, 9, 13: *gsh*I PCR product (270 b.p.).

Lanes 2, 6, 10, 14: *gsh*II PCR product (231 b.p.).

Lanes 3, 7, 11, 15: *phgpx* PCR product (296 b.p.).

Lanes 4, 8, 12, 16: *gor*I PCR product (482 b.p.).

Lane 21: 100 b.p. molecular marker.

Figure 3.17: Dot blot for the transgene *gsh*I in pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie.

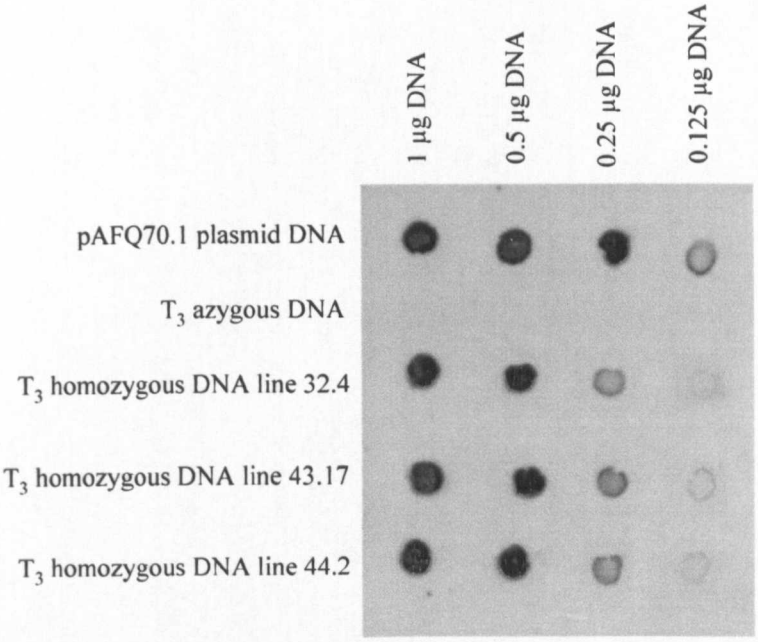
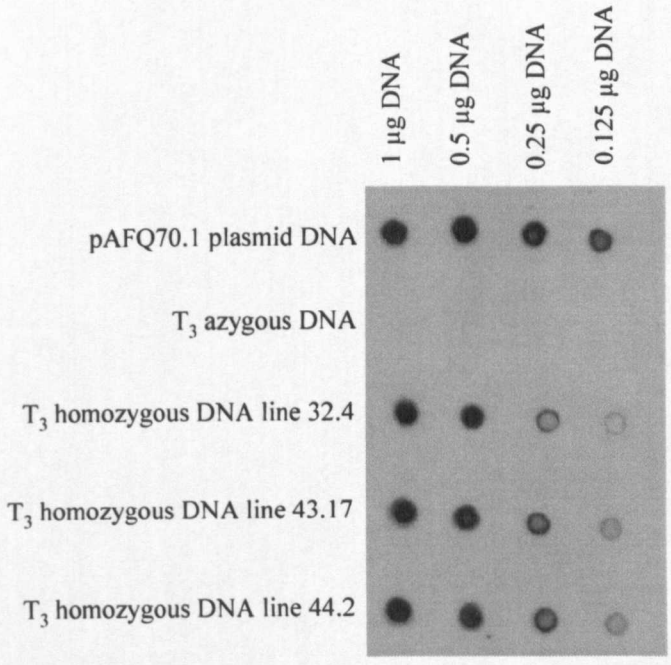


Figure 3.18: Dot blot for the transgene *gsh*II in pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie.



Positive control, pAFQ70.1 plasmid DNA; negative control, cv. King Louie wild-type DNA; T_3 homozygous lines of cv. King Louie, 32.4, 43.17 and 44.2. DNA present in 1 µg, 0.5 µg, 0.25 µg, 0.125 µg concentrations.

Figure 3.19: Dot blot for the transgene *phgpx* in pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie.

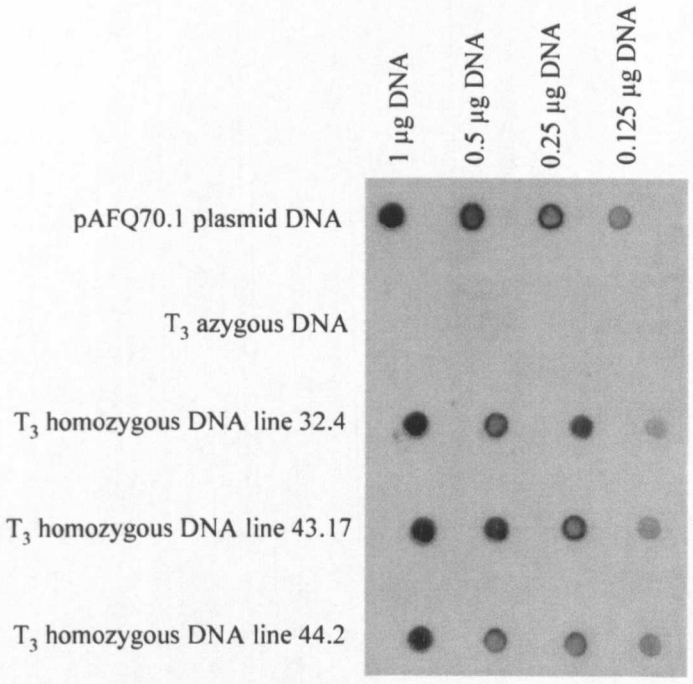
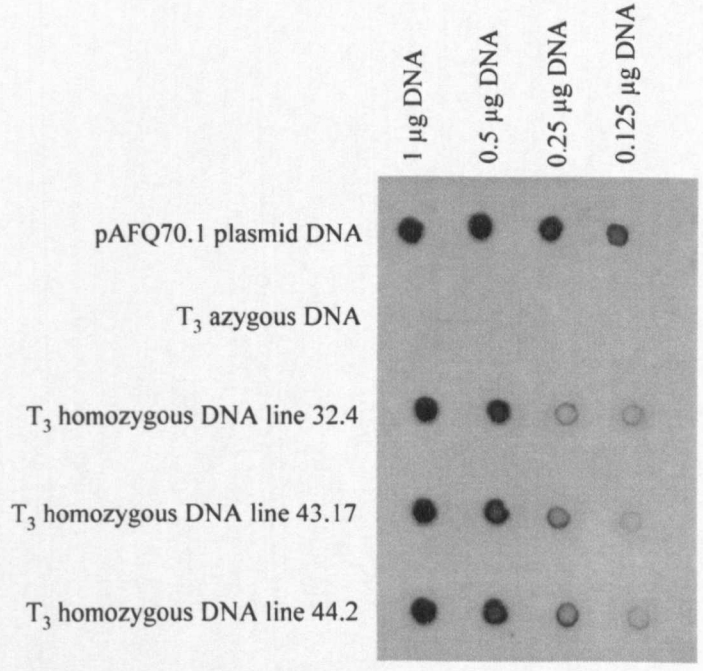


Figure 3.20: Dot blot for the transgene *gorI* in pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie.



Positive control, pAFQ70.1 plasmid DNA; negative control, cv. King Louie wild-type DNA; T_3 homozygous lines of cv. King Louie, 32.4, 43.17 and 44.2. DNA present in 1 µg, 0.5 µg, 0.25 µg, 0.125 µg concentrations.

Figure 3.21: Southern blot for the transgene *gsh*I in pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie.

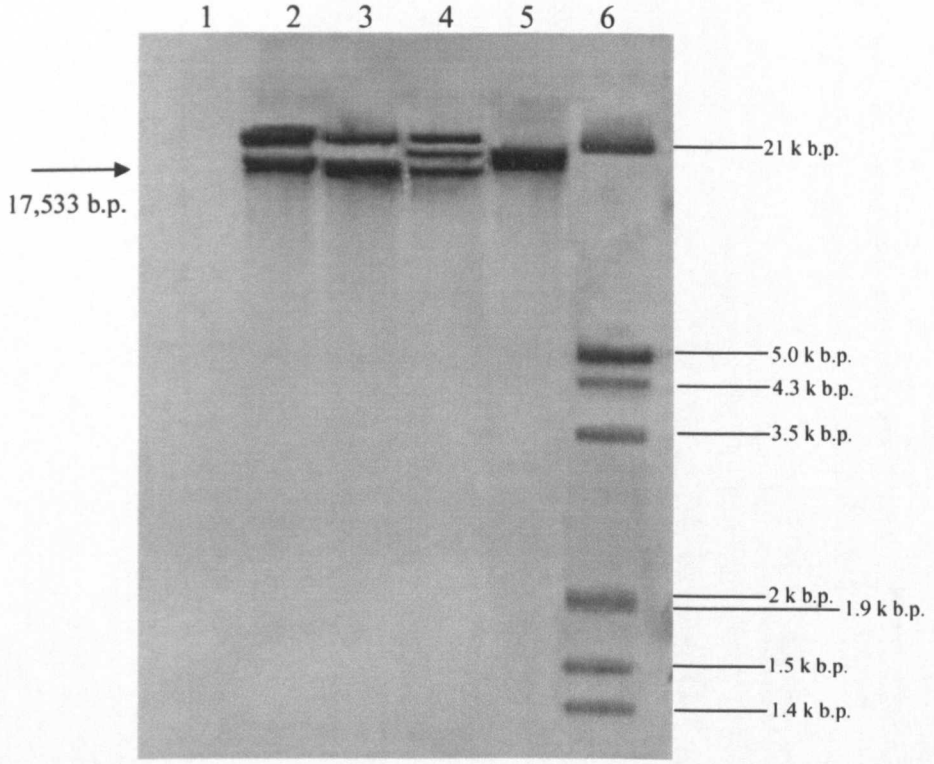
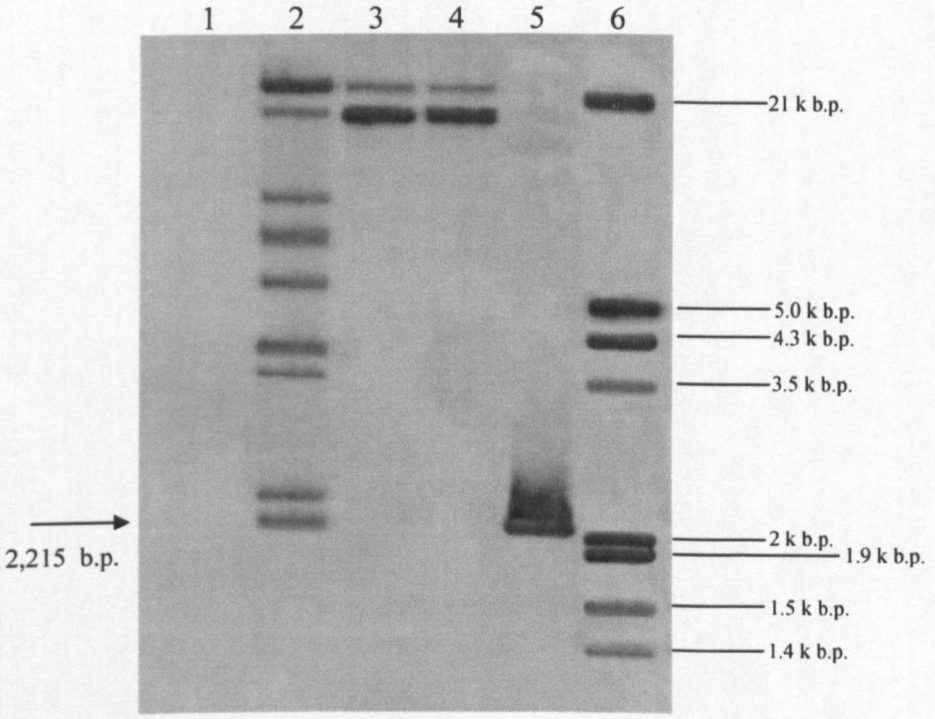


Figure 3.22: Southern blot for the transgene *gsh*II in pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie.



Lane 1: Cultivar King Louie wild-type negative control. Lanes 2 – 4: Cultivar King Louie T_3 homozygous lines 32.4, 43.17 and 44.2, respectively. Lane 5: pAFQ70.1 plasmid positive control. Lane 6: DIG labelled molecular marker.

Figure 3.23: Southern blot for the transgene *phgpx* in pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie.

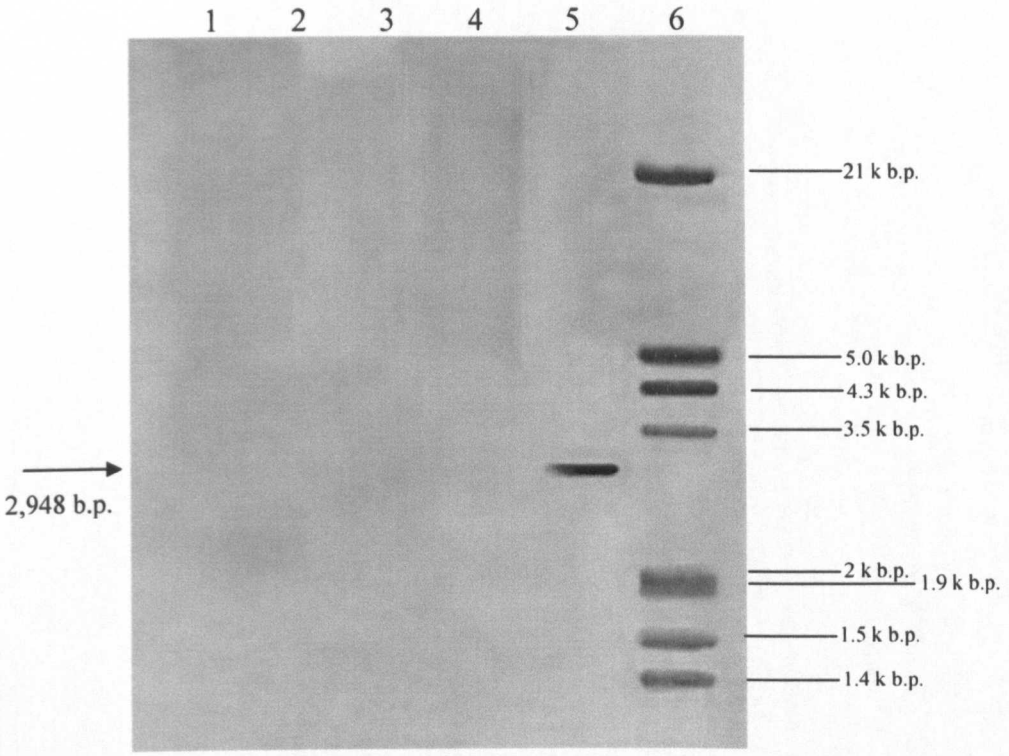
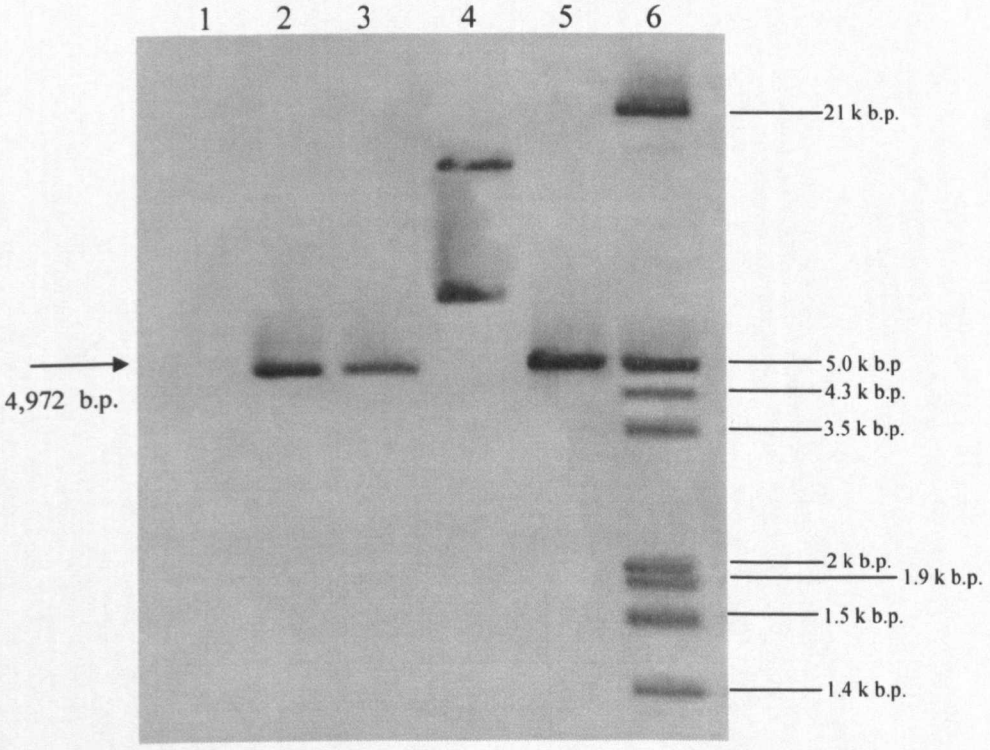


Figure 3.24: Southern blot for the transgene *gorI* in pAFQ70.1 transformed homozygous T_3 lines of cv. King Louie.



Lane 1: Cultivar King Louie wild-type negative control. Lanes 2 – 4: Cultivar King Louie T_3 homozygous lines 32.4, 43.17 and 44.2, respectively. Lane 5: pAFQ70.1 plasmid positive control. Lane 6: DIG labelled molecular marker.

3.5 Summary

3.5.1 PCR analysis of T_0 putative transformants

The large number of PCR positive T_0 regenerant plants was most likely due to the constant selective pressure of kanamycin sulphate in the tissue culture medium. The presence of single marker transgenes in transgenic plants may be explained by truncations to the inserted DNA fragments. However, this does not mean that the transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I, were not present. The few plants lacking marker transgenes was almost certainly a result of non-transformed 'escapees' avoiding kanamycin selection. Haldrup *et al.* (2001) showed that the xylose isomerase gene (*xyl*A) from *Thermoanaerobacterium thermosulfurogenes* and *Streptomyces rubiginosus* allowed a more efficient selection of transformants than the *npt*II gene. Xylose isomerase allows transgenic plants to utilise xylose as a carbohydrate source and to out grow non-transformed plants. Enzyme activity in plants transformed with the *xyl*A gene, is 5 to 25-fold greater than that of plants transformed with the *npt*II gene.

3.5.2 RT-PCR analysis of T_0 putative transformants

RT-PCR analyses of the T_0 generation plants indicated that *npt*II was the most expressed transgene. The 2nd and 3rd most commonly expressed transgenes, *gsh*I and *gsh*II, respectively, originated from *E. coli* and had little homology with endogenous plant genes. The 4th and 6th expressed transgenes, *phgpx* and *gor*I, respectively, originated from pea and could have created conflicts with endogenous genes (sequence identity) leading to down-regulation of mRNA (Stam *et al.*, 1997). This does not explain why the transgene *luc,* which originated from firefly (*Lucidota atra*) and had little homology with endogenous plant genes, was expressed in few plants. The presence of a cauliflower mosaic virus (CaMV) 35S promoter driving the *luc* transgene might explain this predicament (Vaucheret *et al*, 2001) but does not clarify why the *gsh*II transgene, which was driven by 2 x CaMV 35S, being expressed in many plants. It could be possible that oxidative stress within the tissue culture environment influenced the frequency of gene silencing or exerted selective pressure for genes that could detoxify AOS (Halliwell, 2003; Meza *et al.*, 2001). The reduced expression of the transgene *gor*I could be explained by the promoter, *atrpL*I,

which originated from *A. thaliana*, exhibits particularly weak transcription and thus is not proof of gene silencing (Dr. G Creissen, personal communication, 27/09/2004).

Combined RT-PCR data showed the cvs. Pic and Robusto had few plants expressing 5 or 6 transgenes, this could be based on the reduced expression of *luc* and *gor*I, or that the large number of cv. King Louie plants expressing 6 transgenes was due to a cv. specific response. A very small percentage of plants expressed no transgenes whatsoever, possibly a result of gene insertion into heavily methylated and heterochromatic chromosomal regions leading to inactivation of the DNA (Stam *et al.*, 1997). The normal distribution patterns of transgene expression may have resulted from spontaneous triggering of PTGS. This can occur from highly transcribed single transgene copies producing RNA above a 'threshold concentration' (Vaucheret *et al.*, 2001; Praveen *et al.*, 2005). The presence of multiple genes in a single vector may have amplified this unexpected result. It is also possible for these variations to be due to the presence of several strong promoters, although this is unlikely because the binary vector, pAFQ70.1, contained various promoter types to avoid transcription conflicts (Chicas and Macino, 2001).

An alternative hypothesis to gene silencing is recombination of the transgenes. The pAFQ70.1 construct was designed to contain transgenes with little or no sequence homolgy to endogenous lettuce genes. However, studies have established that T-DNA transformed into plant host genomes can result in alterations of DNA sequences of endogenous genes such as base changes and gene replacement, and are termed homologous recombination events (Iida and Terada, 2005; Li *et al.*, 2004a). Homologous recombination is the process by which a transgene can replace an endogenous gene that has a similar sequence, and thus can disrupt specific gene function. This effect may also be enhanced when multiple transgene copies are present. Integration of transgene DNA into plants by homologous recombination has been shown to inactivate important genes or lead to unpredictable transgene expression (Vergunst and Hooykaas, 1999). However, current literature has indicated that genetic recombination mainly occurs in plants transformed by particle bombardment (Choffnes *et al.*, 2001; Svitashev *et al.*, 2000).

Few binary constructs containing 5 genes or more have been transformed into plants due to the technical difficulties associated with assembling complex plasmids. Most standard transformation vectors will have few restriction sites, limiting the number of genes that can be inserted, without inefficient partial digests or blunt-end

cloning (François *et al.*, 2002). Goderis *et al.* (2002) utilised a single binary vector to integrate 6 transgenes into *A. thaliana* using *A. tumefaciens*-mediated floral dip transformation. The 6 genes consisted of 2 reporter genes, 2 herbicide resistance genes and 2 genes encoding anti-fungal proteins. Each gene utilised a different promoter to avoid transcription conflicts and gene silencing. Transformation efficiency was comparable to that of other floral dip studies and plants had a transgene expression similar to that of those transformed with single-gene constructs. Segregation analysis indicated that use of multi-gene vectors does not result in T-DNA insertions at multiple loci. Cao *et al.* (2004) used *A. tumefaciens*–mediated transformation to insert a single construct containing 5 genes. Genetic analyses confirmed that 90% of transgenic plants contained all the genes of interest, and that all transgenes were stably co-expressed.

The alternative to a single, multi-gene transformation event is to perform the re-transformation of transgenic plants or co-transform plants with different plasmid constructs (François *et al.*, 2002). Hird *et al.* (2000) transformed tobacco (*Nicotiana tabacum*) with the pathogenesis-related β-1,3-glucanase gene under the control of the *A. thaliana* A9 tapetum-specific promoter. Expression of the transgene caused the degradation of the β-1,3-glucan callose microsporogenous cell walls, resulting in male sterility. Re-transformation of the transgenic plants with the β-1,3-glucanase gene in the antisense orientation resulted in undetectable concentrations of the β-1,3-glucanase enzyme in the anthers. The advantage of re-transforming a transgenic plant is that it allows the maintenance of an elite genotype. However, for every transformation event a different selectable marker gene is required. The most common selectable marker genes are neomycin phosphotransferase (*npt*II) for kanamycin sulphate resistance, hygromycin phosphotransferase (*hpt*) for hygromycin B resistance, phosphinothricin acetyl transferase (*bar*) for bialaphos resistance and β-glucuronidase (*gus*) for histochemical analysis of gene expression (François *et al.*, 2002).

Wu *et al.* (2002) transformed rice with 9 different plasmids using particle bombardment. They produced 66 transgenic rice lines, 11 of which carried all the transgenes. Genetic analysis confirmed the transgenes were integrated into the same locus of the rice genome, indicated by a 3:1 gene segregation ratio. Their results confirmed that multiple genes could be transformed into rice simultaneously, and that the expression of a transgene does not interfere with the expression of another.

Radchuk *et al.* (2005) transformed *A. thaliana* with 3 distinct *A. tumefaciens* strains carrying different plasmid constructs. PCR analysis revealed that 9.5% of transformants contained transgenes from all 3 constructs. Southern blot analysis also confirmed that the different transgenes integrated into the same locus in a single plant genome. Northern blot analysis of the T_1 lines showed that transgene expression and mRNA concentration was not influenced by gene copy number.

The results from these studies show that multiple transgenes can be successfully integrated and expressed in plants by either single or multiple transformation events. All studies have indicated that gene expression is not influenced by other transgenes. This suggests that the gene silencing observed in the T_0 lettuce lines was a result of conflicts with endogenous genes and also due to the presence of viral promoters.

3.5.3 RT-PCR analysis of cv. King Louie T_1 and T_2 lines

Analysis of cv. King Louie T_1 lines, 32, 43 and 44, indicated no particular pattern in transgene expression that would correspond to the events in the T_0 lines. All plants in the T_1 lines expressed the marker transgene *npt*II due to the presence of kanamycin sulphate in the MS0 germination medium. The transgenes *luc* and *phgpx* were expressed in fewer plants of the T_1 lines than other genes. Analysis of the RT-PCR data suggested random silencing of the transgenes had occurred. Data from cv. King Louie T_2 lines, 32.4, 43.17 and 44.2, indicated the transgene *luc* was expressed in the least number of plants. DNA methylation caused by presence of the CaMV 35S promoter is the most probable cause of the *luc* silencing (Vaucheret *et al*, 2001). Expression of the remaining transgenes appeared to be randomly distributed among the 3 lines and did not show any link with silencing of *luc*. All lines had reduced expression of *gor*I, particularly line 44.2 where only a quarter of plants expressed the transgene compared to almost all plants in the lines 32.4 and 43.17. It is possible that the reduced expression could be caused by the weak *atrpL*I promoter. Expression of transgenes did follow similar trends to those in the T_0 lines, suggesting that similar silencing events were occurring.

3.5.4 Inheritance of transgene expression from T_0 to the T_2 lines of cv. King Louie

This study has shown that loss of expression of the transgenes *luc* and *gor*I from the T_0 to the T_2 lines of cv. King Louie was almost certainly due to TGS or

PTGS. The only drawback with gene silencing causing the reduced inheritance of expression of *luc* and *gor*I is that the binary vector, pAFQ70.1, contained the genes *gsh*I and *gsh*II, which were expressed in a relatively large number of T_2 plants. This may suggest that transgene silencing was dependent on homology with endogenous genes or a result of promoter inactivation (Chicas and Macino, 2001; Vaucheret *et al*, 2001). Loss of gene expression between the T_1 and T_3 seed generations of lettuce was observed by McCabe *et al.* (1999b). McCabe *et al.* (1999b) reported a significant loss of expression of the herbicide resistance transgene, *bar*, when placed under the CaMV 35S promoter compared to the plastocyanin promoter from pea (*petE*). Only 2.5% of T_0 CaMV 35S-bar plants transmitted herbicide resistance to the T_3 seed generation compared to 97% of *petE*-bar plants. It could be expected that the number of plants expressing the transgenes, *npt*II, *gsh*I, *gsh*II and *phgpx,* would decrease through the plant generations. The lack of kanamycin sulphate selection would almost certainly cause a reduction in the number of plants expressing *npt*II in subsequent generations. Use of the de-methylating chemical 5-azacytidine and Northern blotting would help to identify whether gene silencing was as a result of DNA methylation or degradation of mRNA (McCabe *et al.*, 1999a).

3.5.5 Identification of cv. King Louie homozygous lines

The large number of cv. King Louie T_0 putative transformants combined with good transgene expression, particularly *luc* and *gor*I, meant that it was suitable for further studies. Cultivar King Louie T_1 segregation data showed that seeds derived from the lines 32, 43 and 44 contained on average 78% kanamycin sulphate resistant plants. A study by Wu *et al.* (2002) transformed rice with 9 different transgenes, showed that the genes were integrated into the same locus of the rice genome, indicated by a 3:1 segregation ratio. This result confirms that the transgenes were almost certainly integrated at the same locus of the lettuce T_1 line genomes. The lettuce lines also possessed little variation in segregation ratio, which usually indicates a multiple transgene copy number (Subr *et al.*, 2006). The lack of cv. King Louie azygous lines in the T_2 populations was almost certainly a result of the strong selective pressure exerted by the kanamycin sulphate. However, this did not inhibit the selection of azygous plants from the T_1 heterozygous populations.

3.5.6 Dot blot and Southern blot analysis of cv. King Louie T_3 homozygous lines

The PCR and dot blot analyses clearly indicated the presence of the transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I in the T_3 homozygous lines of cv. King Louie. These data combined with RT-PCR results for gene expression and GSH assays (Chapter 4, Section 4.4.2.7) confirmed the transgenes were integrated and expressed in the homozygous lines.

The Southern blot analyses were not as successful as the dot blots, with presence of the transgene *phgpx* not detected in any of the homozygous lines. The most likely reason for this, was the probe not hybridising to the genomic DNA because of transgene methylation or too greater stringency conditions employed when the hybridisation membrane was washed. Due to the T-DNA cutting locations of the BamHI and EcoRI restriction enzymes, it was not possible to confirm the presence of *phgpx* by the detection of another transgene.

Although the detection of the transgenes *gsh*I and *gsh*II required the use of the restriction enzymes BamHI and EcoRI, respectively, the genomic transgene copies were relatively the same size. The 3 homozygous lines had 2 - 3 distinct bands ranging from 18 - 22 k b.p., and may have consisted of several transgene copies present in uncut DNA. Results for the transgene *gsh*II in line 32.4 demonstrated this idea, which had a copy number of 9. However, the *gsh*I probe would have hybridised to fragments containing the junction between the right border of the T-DNA and the plant genomic DNA. This would result in a large DNA fragment on the Southern blot (McCabe *et al.*, 1999b). The presence of large transgene copy fragments also suggests a poor enzyme restriction and can be rectified by use of double digestions, although this results in a greater loss of genomic DNA (McCabe, 1997). It is also possible that restriction enzyme digestion of plant genomic DNA may be impaired by the presence of methyl groups or show some form of preferential cutting (Website 6). Treatment of the homozygous line seedlings with 5-azacytidine prior to DNA extraction, may alleviate the presence of methylation groups and enable improved restriction enzyme cutting (McCabe *et al.*, 1999a). Both BamHI and EcoRI are referred to as a '6-cutter', in that they need to recognize an exact sequence of 6 nucleotides long, this may result in a lower frequency of potential cutting sites in the lettuce genome.

Detection of the gene *gor*I in the homozygous lines was more successful than for the other transgenes. Both lines 32.4 and 43.17 showed a single transgene copy,

which appeared to be the same size fragment as that from the pAFQ70.1 plasmid (4,972 b.p.). Due to the location of the BamHI cutting sites on the pAFQ70.1 plasmid, it was possible to confirm the presence of the *gsh*II transgene in the lines 32.4 and 43.17. Line 44.2 showed 2 copies of the transgene *gor*I as T-DNA inserts of 15 k b.p. and 8.6 k b.p. A lower frequency and specificity of cutting by BamHI in the genome of line 44.2 may have resulted in a larger DNA fragment containing the transgene *gor*I. Angel *et al.* (1993) reported that the efficiency of 6-cutter enzymes was influenced by the presence of increased DNA polymorphisms like those found in lettuce.

Previous studies that have tested lettuce transformants for transgene copy number have recorded mixed results. Several reports have confirmed that T_0 transformants typically contain a single transgene insert (Vanjildorj *et al.*, 2005; McCabe *et al.*, 2001; Niki *et al.*, 2001; Curtis *et al.*, 1999). However, some studies have produced contradictory results. McCabe *et al.* (1999b) and Mohapatra *et al.* (1999) analysed 25 T_1 lettuce transformants for the *bar* gene, and found 1 to 3 copies present in each plant. Further analysis revealed the presence of an extra T-DNA insert, which was thought to be due to a more efficient transformation, a result of more virulence genes in the construct. They also determined that a high T-DNA copy number was one of the main reasons for transgene silencing (Mohapatra *et al.*, 1999). Sun *et al.* (2006) found as many as 7 T-DNA inserts present in 36 independent transformants. Goto *et al.* (2000) determined copy number in self-pollinated T_1 progenies derived from 8 independent T_0 transformants. Southern blotting confirmed 1 - 2 bands were present in all plants examined, and were a result of at least 1 or 2 intact cDNAs becoming integrated into the lettuce genome.

Presence of multiple copies of the transgenes in the homozygous lines was almost certainly due to segregation and rearrangement of the T-DNA insert. This may indicate that the T-DNA insert was not intact in the T_0 lines, and may have become heavily rearranged and recombined by the T_3 generation (McCabe *et al.*, 1999a).The possibility for multiple transgene copies would potentially increase in correlation with the size of the T-DNA insert transformed into the host genome. The Southern blot results disprove the hypothesis, in that most of the homozygous lines appeared to have 2 or more copies of the transgenes, when they were expected to only have single inserts. This was almost likely due to the large T-DNA insert and the fact that T_3 plants were analysed. Even though the presence of multiple

transgenes was unwanted, variations in their size and number ensured the homozygous lines were essentially independent. The cv. King Louie wild-type control DNA confirmed that endogenous genes involved in the synthesis and metabolism of GSH did not share a high degree of sequence homology with the transgenes. This was confirmed by the fact that the DIG-labelled probes for the transgenes did not cross-hybridise with the endogenous genes.

The advantage of using of dot blots to initially determine the presence of the transgenes in the T_3 homozygous lines was that the technique was relatively easy to perform. Dot blots also allowed the determination of conditions required for Southern blotting, such as probe concentration, stringency conditions and membrane exposure times. This was essential, as the Southern blotting protocol consisted of many stages with a large potential for human error. For example, the *gor*I Southern blot was performed after the *gsh*I and *gsh*II blots, and demonstrated the improvements in the technique of preparing DNA restriction enzyme digestions.

Expression of the transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I was hypothesized to enhance chloroplast GSH biosynthesis and metabolism. In turn, this was expected to improve plant post-harvest performance and resistance to abiotic stress. Cultivar King Louie T_3 homozygous, azygous and wild-type lines were subsequently assessed for post-harvest performance, resistance to saline conditions and tolerance to the foliar condition tipburn. Post-harvest performance was assessed by a shelf-life assay, while the saline stress assessments compared plant cellular response of inner and outer leaves of control and saline grown plants (Chapter 4). Tolerance to tipburn, a stress related foliar condition, was evaluated by growing plants under Ca^{2+} deficient conditions and under normal glasshouse conditions. The cellular changes occurring in tipburnt and non-tipburnt leaves were also analysed using macroscopic and microscopic techniques (Chapter 5).

CHAPTER 4 : ANALYSIS OF CROP PERFORMANCE

4.1 Introduction

Abiotic stresses in crops represent some of the most serious world-wide problems for agriculture, they include salinity, drought, and extreme temperature responses and, in turn, have major impacts on plant growth, development and production. The most common result of abiotic stress is a restriction of water uptake and in the case of salinity, specific ion toxicity (Arbona *et al.*, 2003). Cellular water deficit stimulates the production of AOS such as $O^{\cdot}_2^{-}$, $^1O_2$ and H_2O_2 (Benson, 1990; Halliwell and Gutteridge, 1989). Large concentrations of AOS cause lipid peroxidation, membrane damage and enzyme inactivation (Benson *et al.*, 1992; Davies, 1987). To counter the effects of AOS, plants produce an array of antioxidant compounds and enzymes that include CAT, SOD, GSH and carotenoids (Mateos *et al.*, 2003). Catalase has a primary role in the decomposition of H_2O_2 to H_2O in the peroxisomes of the cell. Superoxide dismutase is a general name given to families of metalloenzymes, which catalyse the breakdown reaction of $O^{\cdot}_2^{-}$ to H_2O_2. Glutathione can work in association with ascorbate to detoxify H_2O_2 in chloroplasts [Chapter 1, Section 1.4, Benson (1990)]. The carotenoids are highly effective antioxidants and radical scavengers, although their efficiency is related to the number of conjugated double bonds the substance is able to donate (Cantrell *et al.*, 2002) (refer to Chapter 1, Section 1.3.1 for further background).

Analysis and characterisation of genes involved in signalling and biochemical pathways in stressed plants has helped identify potential genes with stress-specific responses (Winicov, 1998). These genes encode osmoprotectants, specific transcription factors, ethylene biosynthesis enzymes and chaperones such as the boiling-stable, homo-oligomeric, SP1 protein (Altman, 2003; Stearns and Glick, 2003; Winicov, 1998). Over-expression of these genes may also alter and extend fruit ripening, leaf shelf-life and flower wilting by inhibiting the synthesis and accumulation of phenylalanine ammonia lyase and oxidation of phenolic compounds which discolour and lead to senescence in crops (Beltran *et al.*, 2005; Choi *et al.*, 2005; Saltveit, 2004; Stearns and Glick, 2003).

4.2 Aims and Objectives

The aims and objectives of this chapter were to assess the homozygous, azygous and wild-type lines of cv. King Louie for post-harvest performance in the form of a shelf-life assay and to test for resistance to saline stress. The shelf-life assay was designed to test the ability of leaf cells to resist senescence and degradation of chlorophyll content, and was based on methods of Garratt (2002). The salinity trial was aimed at assessing the role of GSH in plant tolerance to abiotic stress. This was determined by measuring cellular metabolites and antioxidant concentrations in the inner and outer leaves of control and saline grown plants. The secondary role of the salinity trial was to verify whether over expression of the transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I produced an increase in GSH concentration in the tissues of the homozygous lines. Plants were assayed after 2 wks, to allow a view into the cross-section of cellular events that were occurring.

4.3 Materials and Methods

4.3.1 Shelf-life assessments of cv. King Louie T_3 homozygous, azygous and wild-type lines

Lettuce seeds of cv. King Louie T_3 homozygous (containing and expressing the pAFQ70.1 transgenes) lines 32.4, 43.17, 44.2, azygous (reverted to wild-type) lines 32.9, 43.16, 44.12 and the wild-type line (Chapter 3, Section 3.3.8) were germinated in half-size plastic seed trays (H. Smith Plastics Ltd., Wickford, UK) (50 seeds/tray) containing John Innes Seed Compost (Scotts Company Ltd.). Fourteen d-old seedlings were transferred to 9 cm diameter plastic pots filled with 3:1 (v:v) John Innes No. 3 compost and perlite. Five plants of each line were grown under controlled room conditions at 24°C with a 16 h photoperiod (50 μmol m^{-2} sec^{-1}, daylight fluorescent illumination). At 6 wks, dark green mature leaves were removed from the middle sections of all 5 plants per line and cut into leaf discs (1 cm^2) using a cork borer. Leaf discs were floated on 20 ml aliquots of sterile purified water in 9 cm Petri dishes sealed with Nescofilm and incubated for 0, 2, 5, 7, 10, 14, 18 and 21 d. under the conditions described above. Six leaf discs were floated in each Petri dish, with 3 replicates. Three leaf discs (100 mg FW) from each replicate were washed in purified water, blotted dry and flash frozen in liquid nitrogen. Leaf discs were ground

to a fine powder using a micropestle (Anachem Ltd., Luton, UK) and stored at -80°C until needed. Ground leaf tissue of the 3 leaf discs were first analysed for soluble protein content (Section 4.3.3), with the homogenised leaf pellet from this assay subsequently used to determine chlorophyll and carotenoid concentrations (Section 4.3.4). The remaining 3 leaf discs were analysed for glucose and fructose content in a combined assay (Section 4.3.5).

4.3.2 Plant growth requirements for saline stress assessments of cv. King Louie T_3 homozygous, azygous and wild-type lines

Fourteen d-old seedlings of cv. King Louie T_3 homozygous, azygous and wild-type plants (Section 4.3.1) were transferred to 9 cm diameter plastic pots (Richard Sankey Ltd.) filled with 50:50 (v:v) perlite and vermiculite (William Sinclair Horticulture Ltd., Lincoln, UK) and acclimatised for 5 wks to ensure a sufficient leaf FW for the subsequent analyses. Glasshouse conditions were ~25°C with an approximate 16 h photoperiod (light intensity at noon was approximately 1000 μmol m^{-2} s^{-1}). The experiment was run for 2 wks and consisted of 2 groups, control [nutrient solution only (Appendix 8.2.9)] and saline [nutrient solution containing NaCl (8.76 g l^{-1})], with plants watered every 2 d. Sodium chloride concentrations ranging from 5.84 – 11.68 g l^{-1} (100 – 200 mM) are frequently used to assess salinity tolerance and response in many plant species (Kim *et al.*, 2005; Park *et al.*, 2005a). Both groups had 4 plants of each homozygous and azygous line, and 7 plants of the wild-type line. The greater number of wild-type plants provided an improved comparison when evaluating results from the trial. The experiment was duplicated and data from both trials combined. On completion of each experiment, 5 – 10 inner (youngest) and outer (oldest) leaves were removed from each plant, flash frozen in liquid nitrogen and ground to a fine powder in a chilled mortar and pestle. The ground leaves of the individual plants were placed in sterile 30 ml plastic universal tubes and stored at -80°C until needed. Leaf tissues were quantified for soluble protein, chlorophyll and carotenoid, glucose and fructose, phenolic compounds and GSH concentration. Plant antioxidant activity and the extent of lipid peroxidation were also determined (Sections 4.3.3 – 4.3.9).

4.3.3 Soluble protein quantification

Soluble proteins were extracted by adding 500 µl of ice-cold protein extraction buffer (Appendix 8.2.10) (Jordi *et al.*, 1996) to 100 mg FW of ground leaf tissue (Sections 4.3.1 and 4.3.2) and vortexing for 30 sec at RT. Samples were centrifuged at 10,000 x g for 10 min at RT. A 5 µl aliquot of supernatant was added to 995 µl of 20% (v/v) Bradford's dye protein reagent (Bio-Rad Laboratories Ltd.) (Bradford,1976) in a 1.5 ml polystyrene cuvette (Sarstedt Ltd., Beaumont Leys, UK) and inverted 4-5 times. Soluble protein was quantified using the λ–Bio UV Spectrophotometer (Perkin Elmer, Beaconsfield, UK) at 595 nm. A BSA standard curve was used to determine the concentration of soluble proteins in the cuvette.

4.3.4 Determination of chlorophyll and carotenoids

One ml of ice-cold 80% (v/v) acetone was added to 100 mg FW of ground leaf tissue (Sections 4.3.1 and 4.3.2), vortexed for 30 sec and centrifuged at 10,000 x g for 2 min at RT. The supernatant was transferred to a 1.5 ml polystyrene cuvette and fluorescence measured at 663 nm, 647 nm and 470 nm using the λ–Bio UV Spectrophotometer. Chlorophylls a, b and total carotenoids were calculated according to Lichtenthaler (1987) with results equivalent to µg chlorophyll/carotenoids ml^{-1} 100 mg^{-1} FW leaf.

Chlorophyll a (C_a): $12.25\ A_{663} - 2.79\ A_{647}$

Chlorophyll b (C_b): $21.50\ A_{647} - 5.10\ A_{663}$

Total chlorophyll: $C_a + C_b$

Total carotenoids: $(1000\ A_{470} - 1.82\ C_a - 85.02\ C_b) / 198$

4.3.5 Glucose and fructose quantification

One hundred mg FW of ground leaf tissue (Sections 4.3.1 and 4.3.2) was freeze-dried for 24 h (Alpha 2 LD; Martin Christ GmbH, Osterode am Harz, Germany). Freeze-dried leaf tissue was shaken in 10 ml of 80% (v/v) ethanol and the soluble fraction centrifuged at 5,000 x g for 10 min at RT. Glucose and fructose concentrations were determined by reacting 500 µl of supernatant with 2.5 ml of fresh anthrone (150 mg anthrone in 100 ml concentrated H_2SO_4) whereby the mixture was incubated for 5 min at 100°C for glucose and 30 min at 40°C for fructose (Halhoul and Kleinberg, 1972). One ml of reacted sample was transferred to

a 1.5 ml polystyrene cuvette and absorbance determined at 625 nm using the λ–Bio UV Spectrophotometer. A standard curve was used to determine the concentration of glucose/fructose in the cuvette.

4.3.6 Determination of ferric-reducing antioxidant activity of lettuce leaf isolates

This assay determined the total plant antioxidant activity by its ferric reducing ability, and was based on the methods of Benzie and Strain (1996). One ml of phosphate buffered saline (PBS) solution (Appendix 8.2.11) was added to 500 mg FW of ground leaf tissue (Section 4.3.2), vortexed for 30 sec and centrifuged at 10,000 x g for 5 min at RT. A 100 µl aliquot of supernatant was added to 900 µl of ferric-reducing antioxidant activity assay reagent (Appendix 8.2.12) in a 1.5 ml polystyrene cuvette, inverted 4-5 times and incubated for 4 min at RT. Absorbance was measured at 593 nm using the λ–Bio UV Spectrophotometer. Values were expressed as µM equivalents of iron II sulphate.

4.3.7 Determination of phenolic compounds

Total phenolic content was determined using the Folin-Ciocalteu method (Kang and Saltveit, 2002). One ml of HEPES buffer (Appendix 8.2.13) was added to 500 mg FW of ground leaf tissue (Section 4.3.2), vortexed for 30 sec and centrifuged at 10,000 x g for 5 min at RT. A 100 µl aliquot of supernatant was added to 500 µl of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich) and 400 µL 7.5% (w/v) Na_2CO_3 in a 1.5 ml polystyrene cuvette. Cuvettes were inverted 4-5 times and incubated for 30 min at RT. Absorption was measured at 765 nm using the λ–Bio UV Spectrophotometer with total phenolic content expressed as gallic acid equivalents (GAE).

4.3.8 Determination of the extent of lipid peroxidation

This assay is based upon the reaction of thiobarbituric acid (TBA) with aldehyde breakdown products such as malondialdehyde (MDA) (Benson *et al.*, 1992). A 50 µl aliquot of HEPES supernatant (Section 4.3.7) was added to 500 µl of purified water and 500 µl of TBA reagent (Appendix 8.2.14). Samples were boiled for 25 min followed by 5 min on ice to stop the reaction. One ml of reacted sample was transferred to a 1.5 ml polystyrene cuvette and the net absorbance determined by subtracting the non-specific absorbance at 600 nm from the specific absorbance

value at 532 nm using the λ–Bio UV Spectrophotometer. Concentration of MDA was relative to net absorbance.

4.3.9 Glutathione (GSH) quantification

Quantification of GSH was based upon the methodology of Griffith (1980). One ml of 5% (w/v) sulphosalycylic acid was added to 500 mg FW of ground leaf tissue (Section 4.3.2), vortexed for 30 sec and centrifuged at 10,000 x g for 5 min at RT. A 40 µl aliquot of supernatant in duplicate was used for the determination of total GSH. The supernatants were transferred to a 96 well polystyrene microtitre plate (Sarstedt Ltd.), to which 160 µl of GSH assay reagent (Appendix 8.2.15) was added to each well and incubated for 5 min at 30°C. Prior to analysis, 50 µl of glutathione reductase (262 units l^{-1}) was pipetted into each well and absorbance read at 405 nm over 1 min using the Tecan Sunrise 96 well plate UV Spectrophotometer (Tecan UK Ltd., Theale, UK). A standard curve was used to determine the concentration of GSH (reduced form) in the plate.

4.3.10 Statistics

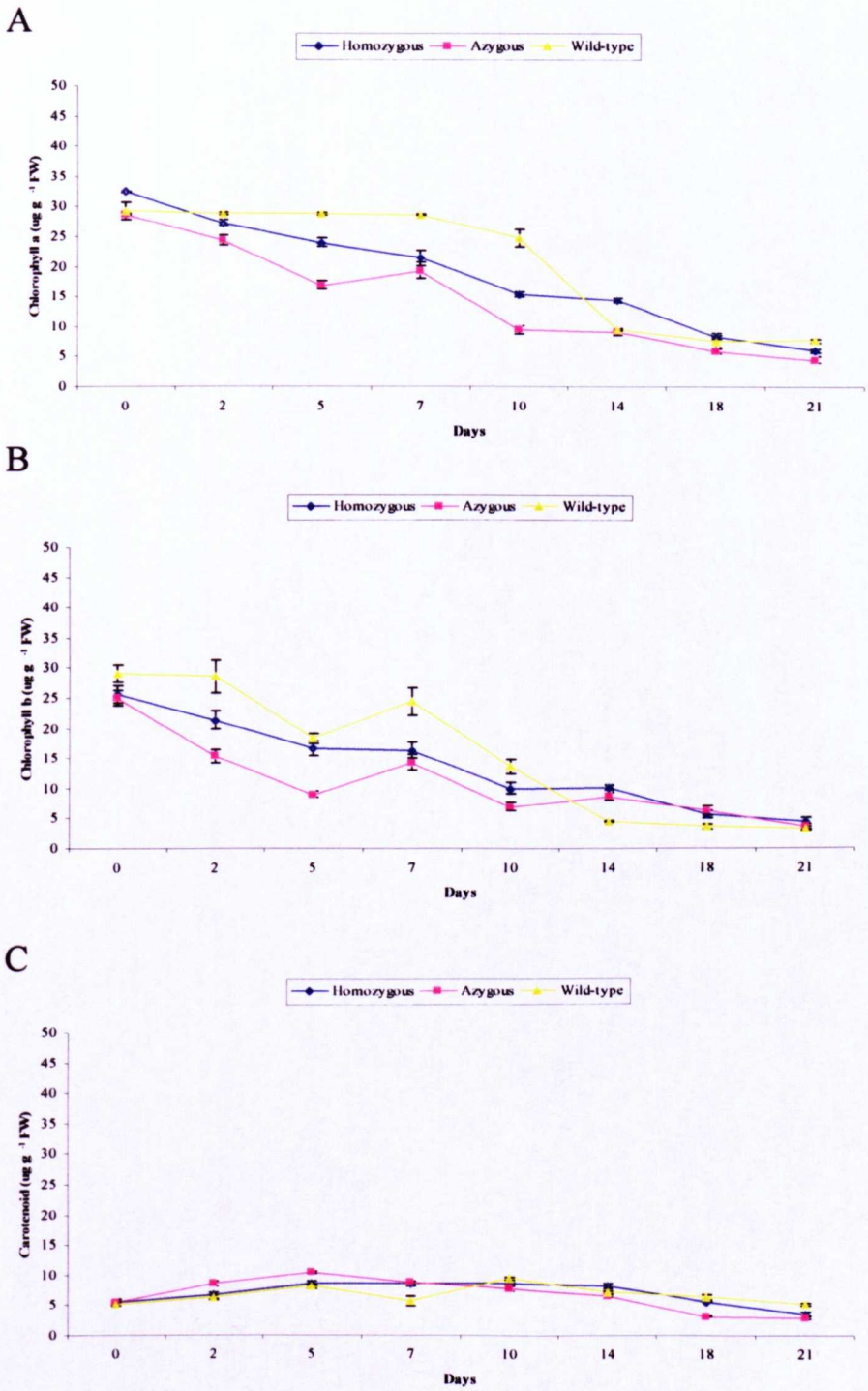
Shelf-life and saline stress assessment results were presented as average results for the 3 homozygous (32.4, 43.17, 44.2) and for the 3 azygous lines (32.9, 43.16, 44.12). Shelf-life assessment results were presented as the concentration of the appropriate compound for the homozygous lines, the azygous lines and the wild-type line during a 21 d period. Saline stress assessment results were presented as the concentration of the appropriate compound for the inner and outer leaves of control and saline-treated plants of the homozygous lines, the azygous lines and the wild-type line. Statistical analysis of the results was carried out using ANOVA on Microsoft Excel. Analysis of variance (P) values indicated the probability of obtaining the results by chance, where $P = < 0.05$ was significant, $P = < 0.01$ was highly significant and $P = < 0.001$ was very highly significant.

4.4 Results

4.4.1 Shelf-life assessments

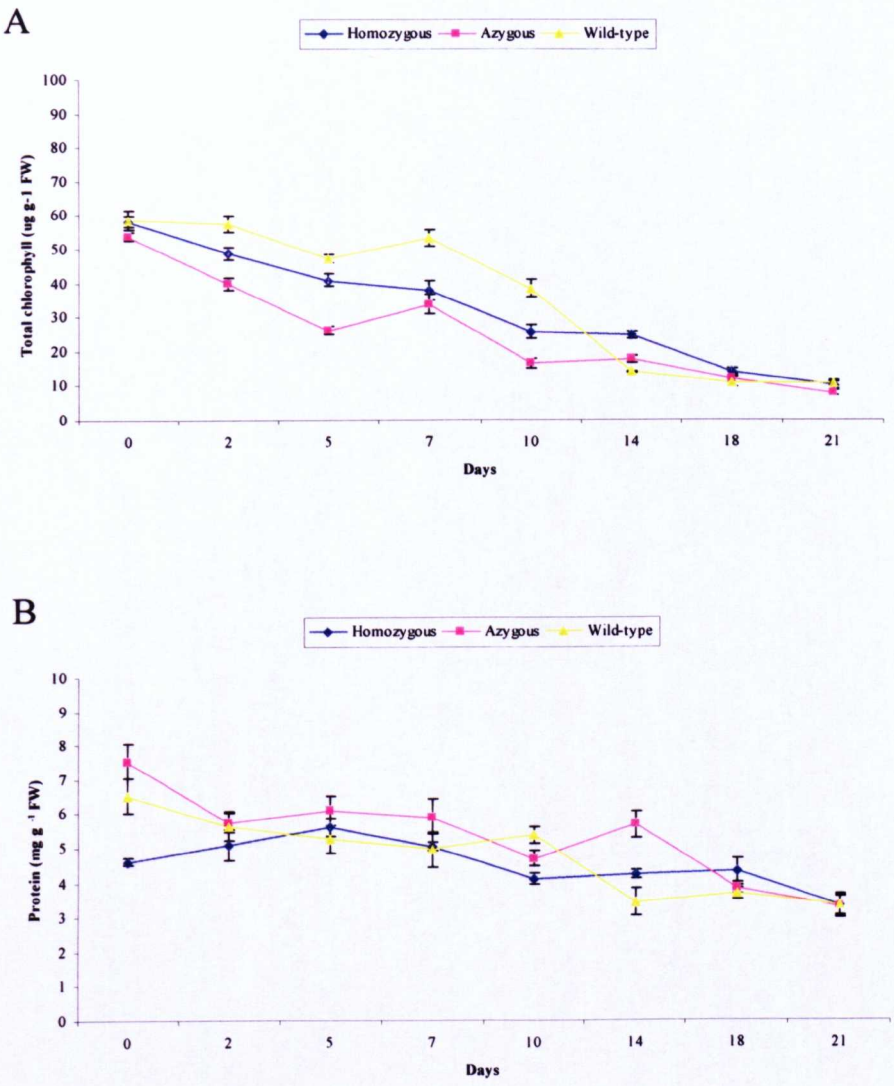
Concentration of total chlorophyll reduced in the homozygous, azygous and wild-type lines during the 21 d period, decreases of 47.63, 45.53, 47.58 μg g^{-1} FW, respectively (Figure 4.2 A) (Appendix 8.3.3.1, Table 8.10). Concentrations of chlorophyll a (Ca) were greater than chlorophyll b (Cb) by d 21 for the homozygous and azygous lines, the opposite occurred in the wild-type line (Figure 4.1 A, B) (Appendix 8.3.3.1, Tables 8.8 and 8.9). Soluble protein content appeared to follow a similar trend to total chlorophyll concentration during the 21 d period. The reduction in soluble protein content was less in homozygous lines than the wild-type and azygous lines (1.28, 3.18, 4.23 mg g^{-1}FW, respectively) (Figure 4.2 B) (Appendix 8.3.3.1, Table 8.12). Total carotenoid concentration initially increased in all lines from 0 to 5 d, but then decreased until 21 d (Figure 4.1 C) (Appendix 8.3.3.1, Table 8.11). Both glucose and fructose concentrations increased in the homozygous and azygous lines at 21d were greater than their starting content (181 and 568 mg g^{-1}FW and 100 and 56 mg g^{-1}FW, respectively) (Figure 4.3 A, B) (Appendix 8.3.3.1, Tables 8.13 and 8.14). However, glucose and fructose content decreased in the wild-type line over the 21 d, by 669 and 57 mg g^{-1} FW, respectively. No data was significantly different.

Figure 4.1: Chlorophylls a, b and total carotenoid concentrations (μg g^{-1} FW) in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period .



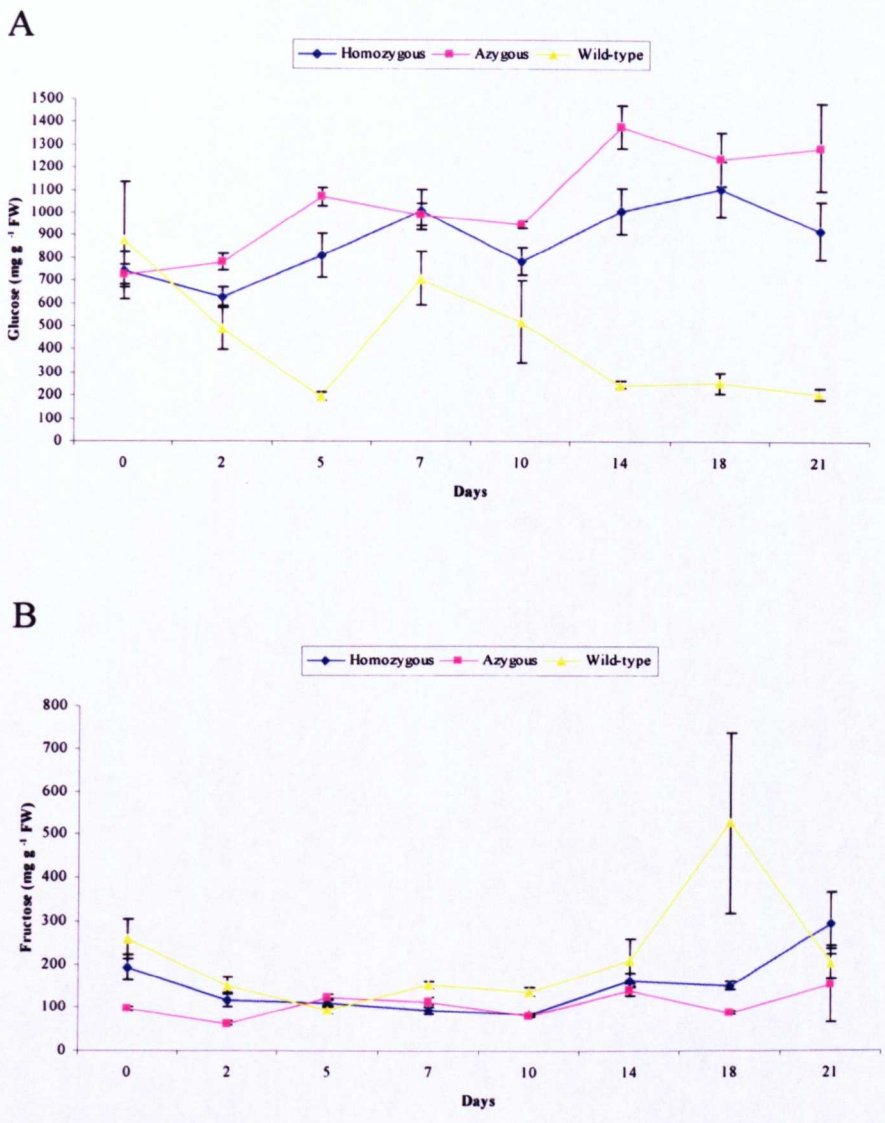
(A) Chlorophyll a, (B) chlorophyll b and (C) total carotenoid concentration data from the homozygous, azygous and the wild-type lines. n = 9; error bars represent S.E.M.

Figure 4.2: Total chlorophyll (μg g^{-1} FW) and soluble protein (mg g^{-1} FW) concentration in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period .



(A) Total chlorophyll and (B) soluble protein concentration data from the homozygous, azygous and the wild-type lines. n = 9; error bars represent S.E.M.

Figure 4.3: Glucose and fructose (mg g^{-1} FW) concentration in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period .



(A) Glucose and (B) fructose concentration data from the homozygous, azygous and the wild-type lines. n = 9; error bars represent S.E.M.

4.4.2 Saline stress assessments

4.4.2.1 Soluble protein quantification

Soluble protein concentration increased in inner leaves but decreased in outer leaves of the homozygous, azygous and wild-type lines grown under saline conditions compared to those grown under control conditions (Figure 4.4) (Appendix 8.3.3.2, Table 8.15). Soluble protein content significantly increased in the inner leaves of the homozygous and wild-type lines ($P = 0.003$ and 0.0001, respectively), and decreased in the outer leaves of the homozygous lines ($P = 0.03$). The wild-type line produced the greatest increase in inner leaf soluble protein content, comparing control and saline treated plants, followed by the homozygous and azygous lines (increases of 1.74, 1.34, 0.56 mg g^{-1} FW, respectively). Outer leaves of the azygous lines produced the greatest decrease in soluble protein concentration, comparing control and saline treated plants, followed by the wild-type and homozygous lines (decreases of 1.81, 1.74, 1.05 mg g^{-1} FW, respectively).

4.4.2.2 Chlorophyll and carotenoids contents

Concentrations of total chlorophyll and total carotenoids increased in inner leaves and decreased in outer leaves of the homozygous, azygous and wild-type lines grown under saline conditions compared to those grown under control conditions (Figures 4.5 and 4.6) (Appendix 8.3.3.2, Tables 8.16 - 8.19). The homozygous lines had a statistically greater total chlorophyll and total carotenoid concentration in the outer leaves of control and saline treated plants than did the azygous lines ($P = 0.004$ for both). Under control conditions, the ratio of Ca to Cb was greater in the inner leaves than the outer leaves of the homozygous, azygous and wild-type lines (Table 4.1). Inner and outer leaves of the homozygous, azygous and wild-type lines grown under saline treatments had a Ca:Cb ratio of 2.3:1, except for inner leaves of the azygous line which were 2.1:1.

Figure 4.4: Soluble protein concentration (mg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

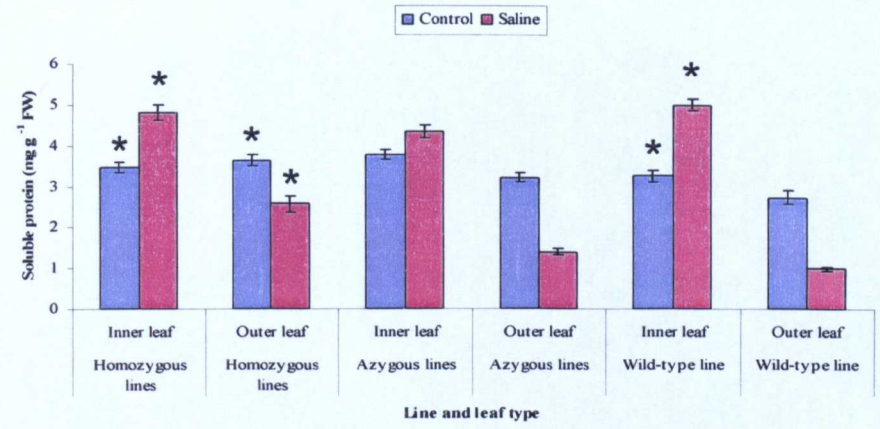


Figure 4.5: Total chlorophyll concentration (µg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

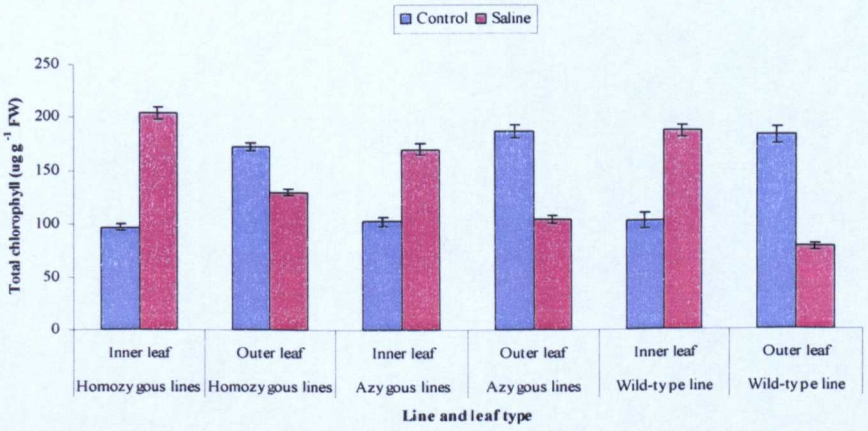


Figure 4.6: Total carotenoid concentration (µg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Asterisks indicate significantly different data between non–saline control and saline grown plants of the same line/leaf type. Average data for the homozygous lines (n = 24), the azygous lines (n = 24), and the wild-type line (n = 14); error bars represent S.E.M.

Figure 4.7: Glucose concentration (mg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

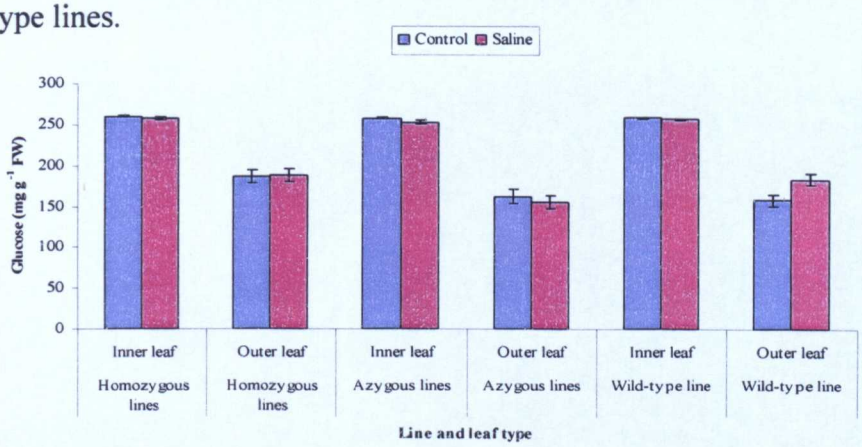


Figure 4.8: Fructose concentration (mg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

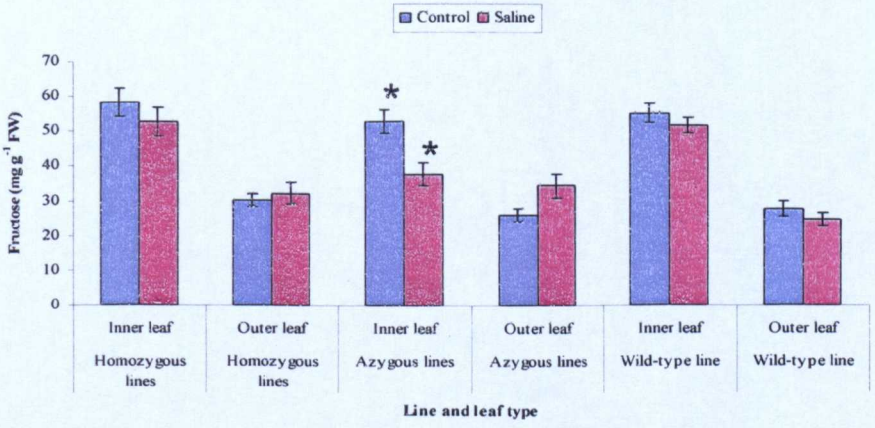
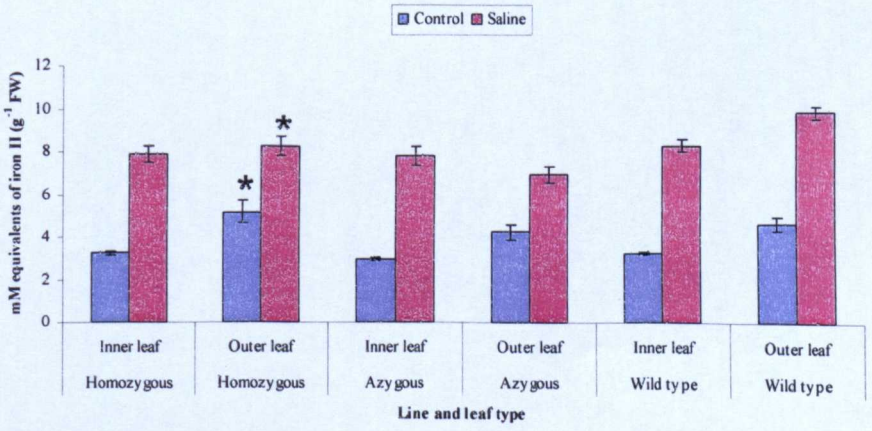


Figure 4.9: Equivalent iron II concentration (mM g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.



Asterisks indicate significantly different data between non–saline control and saline grown plants of the same line/leaf type. Average data for the homozygous lines (n = 24), the azygous lines (n = 24), and the wild-type line (n = 14); error bars represent S.E.M.

Figure 4.10: Total phenolic concentration (μg GAE g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

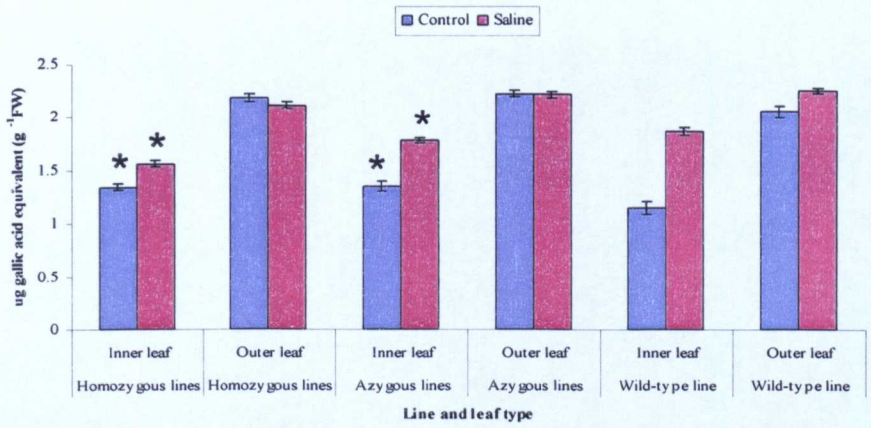
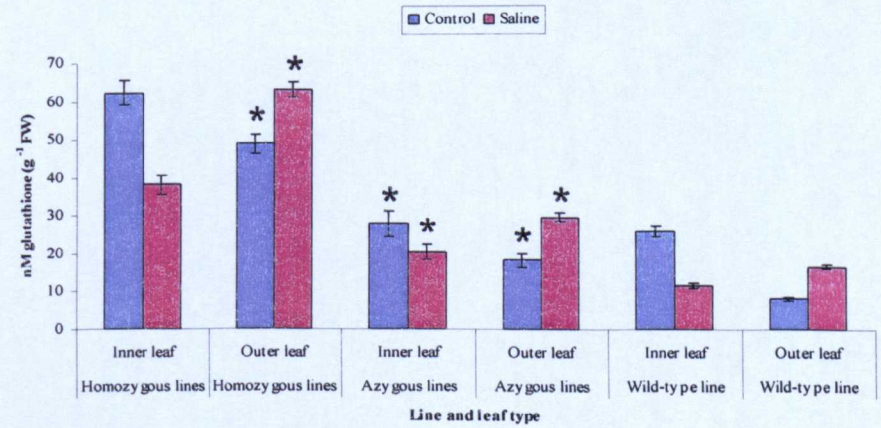


Figure 4.11: Lipid peroxidation net absorbance (g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Figure 4.12: Total glutathione concentration (nM g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.



Asterisks indicate significantly different data between non–saline control and saline grown plants of the same line/leaf type. Average data for the homozygous lines (n = 24), the azygous lines (n = 24), and the wild-type line (n = 14); error bars represent S.E.M.

Table 4.1: Chlorophyll a:b ratios in inner and outer leaves of cv. King Louie homozygous, azygous and wild-type lines grown under control and saline conditions.

Line	Leaf type	Control	Saline
		Ca:Cb ratio	Ca:Cb ratio
Homozygous	Inner leaf	2.5:1	2.3:1
Homozygous	Outer leaf	2.2:1	2.3:1
Azygous	Inner leaf	2.5:1	2.1:1
Azygous	Outer leaf	2.2:1	2.3:1
Wild-type	Inner leaf	2.5:1	2.3:1
Wild-type	Outer leaf	2.2:1	2.3:1

4.4.2.3 Glucose and fructose quantifications

There were no statistically significant increases or decreases in glucose concentration in the inner and outer leaves of the homozygous, azygous and wild-type lines grown under control and saline treatments (Figure 4.7) (Appendix 8.3.3.2, Tables 8.20 and 8.21). The largest decrease in glucose content occurred in the inner leaves of the azygous lines when comparing control and saline treatments (4.62 mg g^{-1} FW). The greatest increase in glucose concentration occurred in the outer leaves of the wild-type when comparing control and saline treatments (25.47 mg g^{-1} FW).

Fructose concentration decreased in the inner leaves of the homozygous, azygous and wild-type lines grown under saline treatments, compared to those grown under control treatments (reductions of 5.43, 15.13, 3.42 mg g^{-1} FW, respectively) (Figure 4.8). Outer leaf fructose concentration increased in the homozygous and azygous lines and decreased in the wild-type line when comparing control and saline treatments. Fructose concentration only significantly decreased in the inner leaves of saline treated azygous lines compared to control grown plants ($P = 0.01$).

4.4.2.4 Determination of ferric-reducing antioxidant activity of lettuce leaf isolates

Total antioxidant activity, measured as equivalent iron II concentration, increased by more than 2-fold in the inner leaves of the homozygous, azygous and wild-type lines when comparing control and saline treatments (Figure 4.9) (Appendix 8.3.3.2, Table 8.22). The wild-type line produced the greatest increase in inner leaf

total antioxidant activity, followed by the azygous and the homozygous lines (5.04, 4.88, 4.67 mM equivalent iron II g^{-1} FW, respectively). Total antioxidant activity also increased in outer leaves of the homozygous, azygous and wild-type lines grown under saline treatments compared to those under control conditions, but the gain was less substantial. Outer leaves of the wild-type line produced the largest increase in total antioxidant activity when comparing control and saline treated plants, followed by the azygous and homozygous lines (5.19, 3.06, 2.69 mM equivalent iron II g^{-1} FW, respectively). The only statistically significant increase in total antioxidant activity occurred in the outer leaves of the homozygous lines when comparing control and saline treatments ($P = 0.0006$).

4.4.2.5 Phenolic compound content in lettuce

Concentrations of total phenolic compounds, measured as gallic acid equivalents (GAE), increased in the inner leaves of the homozygous, azygous and wild-type lines when comparing control and saline treatments (Figure 4.10) (Appendix 8.3.3.2, Table 8.23). Inner leaves of the homozygous and azygous lines produced statistically significant increases ($P = 0.02$ and 0.0002, respectively) in concentration of phenolic compounds, when grown under saline conditions compared to those grown under control conditions (0.22 and 0.44 μg GAE g^{-1} FW, respectively). Conversely, inner leaves of the wild-type line produced the greatest increase in phenolic compound concentration when comparing control and saline treatments (0.72 μg GAE g^{-1} FW). Outer leaves of the homozygous and azygous lines produced a slight decrease in phenolic compound concentration (0.07 and 0.01 μg GAE g^{-1} FW, respectively), while an increase occurred in the wild-type line (0.2 μg GAE g^{-1} FW), when comparing control and saline treatments.

4.4.2.6 Determination of lipid peroxidation

Lipid peroxidation and presence of MDA increased in inner and outer leaves of the homozygous, azygous and wild-type lines when grown under saline treatments compared to control conditions (Figure 4.11) (Appendix 8.3.3.2, Table 8.24). Inner leaves of the homozygous and azygous lines produced similar net absorbance increases when comparing control and saline treatments (0.036 and 0.038 net absorbance g^{-1} FW, respectively). Outer leaves of control and saline grown homozygous, azygous and wild-type lines had a greater lipid peroxidation than their

respective inner leaves. Inner and outer leaves of the wild-type line had the greatest lipid peroxidation increases when comparing control and saline treatments (0.056 and 0.085 net absorbance g^{-1} FW, respectively). No results were statistically significant.

4.4.2.7 Glutathione quantification

Glutathione concentrations (Figure 4.12) in inner and outer leaves of control grown homozygous lines were more than 2-fold greater than their respective azygous counterparts, but not statistically significant (Appendix 8.3.3.2, Table 8.25). Inner leaves of the homozygous, azygous and wild-type lines produced a decrease in GSH concentration, when comparing control and saline treatments, while increases occurred in the outer leaves. The greatest decrease in GSH concentration occurred in inner leaves of the homozygous lines followed by the wild-type and azygous lines, when comparing control and saline treatments (24.26, 14.25, 7.44 nM g^{-1} FW, respectively). Outer leaves of the homozygous and azygous lines produced the largest increases in GSH concentration when comparing control and saline treatments (14.39 and 11.44 nM g^{-1} FW, respectively), and were both statistically significant ($P = 0.01$ and 0.02, respectively).

4.5 Summary

4.5.1 Shelf-life assessments

Loss of chlorophyll and protein from the leaf discs was expected because the tissues were almost certainly senescing, a result of ethylene biosynthesis by wounded cells (Choi *et al.*, 2005; de Jong *et al.*, 2002). The greater concentration of soluble proteins in leaf discs of the homozygous lines could due to over-expression of the genes *gsh*I, *gsh*II, *phgpx* and *gor*I, which are involved in the synthesis and metabolism of GSH. A study by Davey and Keulemans (2004) found connections between improved storage properties of apple fruits and the ability to maintain normal GSH pools. All lines exhibited similar carotenoid concentrations during the 21 d period, suggesting that the homozygous lines had little shelf-life advantage when compared to the wild-type and azygous lines. Increased concentration of

hexoses in the homozygous and azygous lines was probably a result of starch being metabolised into sucrose and eventually glucose and fructose.

4.5.2 Saline stress assessments

4.5.2.1 Soluble protein quantification

Loss of soluble protein from the outer leaves of saline-grown lines suggested senescence and subsequently cell death were responsible. Genes involved in salinity tolerance such as transcription factors also regulate leaf development and the onset of senescence (Munns, 2005). These reductions coincided with protein concentration increases in the inner leaves of the same lines. This was expected since the apical meristem will give rise to new leaves and inflorescences. The amino acid proline appears to the most important of the proteins, with studies indicating its role as an osmoprotectant by stabilizing membranes (Yamada *et al.*, 2005; Bernacchi and Furini, 2004; Arbona *et al.*, 2003). Yamada *et al.* (2005) demonstrated that petunia (*Petunia hybrida* cv. Mitchell) accumulated free proline in drought stress conditions, while Hartzendorf and Rolletschek (2001) reported a 200-fold increase in proline content in saline-treated *Phragmites australis* rhizomes. Park *et al.* (2005) transformed lettuce with the late embryogenesis abundant (LEA) protein gene. These transgenic plants exhibited enhanced growth compared to non-transformed plants under saline and water-deficit conditions. Results from Bernacchi and Furini (2004) confirmed that LEA proteins were expressed at higher concentrations in the cytoplasm of the resurrection plant (*Craterostigma plantagineum*) upon dehydration or after abscisic acid treatments. These studies support the notion that the homozygous lines were less stressed by the saline conditions.

4.5.2.2 Chlorophyll and carotenoid contents

Inner leaves of the homozygous, azygous and wild-type lines grown in control conditions had reduced total chlorophyll and total carotenoid concentrations compared to the outer leaves, the opposite occurred in saline-grown lines. This was most likely due to heading in the control plants, resulting in the inner leaves becoming etiolated. Plants grown in saline conditions were observed to have open heads with dark green inner and senescing outer leaves. Munne-Bosch *et al.* (2001) showed that drought stressed senescing leaves of sage (*Salvia officinalis*) had greater chlorophyll loss and reduced photosynthetic activity. Outer leaves of saline grown

homozygous lines had significantly more chlorophyll and carotenoids than their azygous counterparts, thus confirming their improved antioxidant status (Cantrell *et al.*, 2002). Loss of chlorophyll from water stressed plants has been reported by Upadhyaya and Panda (2004) in drought affected tea *(Camellia sinensis)* seedlings and Fedina *et al.* (2003) in barley (*Hordeum vulgare* cv. Alfa) seedlings grown in 150 mM NaCl. Percival (2005) successfully used chlorophyll fluorescence to quickly and reliably determine salt tolerance in woody perennials. The ratio of Ca to Cb was much greater in inner leaves than outer leaves of all control grown lines. Almost all inner and outer leaves of saline grown lines had a consistent Ca:Cb ratio of 2.3:1. Tas *et al.* (2005) grew lettuce cv. Longifolia in a closed hydroponic system under NaCl and $CaCl_2$ salinity. Although they found leaf chlorophyll concentration was unaffected, leaf Mg^{2+} concentrations did decrease. They reported that Cl^- ions from NaCl had a toxic effect and this probably caused the Ca:Cb ratio to be lowered in the salt-stressed leaves. Therefore, plants with increased concentrations of Ca, total chlorophyll and improved photosynthetic efficiencies grown under stress conditions would have improved antioxidant mechanisms and therefore potentially produce a normal yield (Masojidek *et al.*, 2000).

4.5.2.3 Glucose and fructose quantification

Only small variations in glucose concentration were observed in the inner and outer leaves of the homozygous, azygous and wild-type lines grown in control and saline treatments. However, fructose concentration did show more variation, with similar increases and decreases occurring to those of glucose content. These results did not correlate with existing research data on plant carbohydrate content. Studies have reported that hexose sugars are accumulated under drought and osmotic stress (Villadsen *et al.*, 2005; Zhu *et al.*, 2005; Cui *et al.*, 2004; Hartzendorf and Rolletschek, 2001; Al Hakimi *et al.*, 1995). Villadsen *et al.* (2005) demonstrated that cells of osmotically stressed barley leaves incubated in light and dark conditions produced identical increases in sugar content, suggesting that an increased rate of photosynthesis had no effect on sugar accumulation. Studies have shown that soluble sugars especially sucrose, glucose and fructose, play important roles in cellular osmotic adjustment, membrane stabilisation, and act as metabolite signalling molecules that activate specific hormone-crosstalk pathways related to the control of oxidative stress (Couee *et al.*, 2006; Bernacchi and Furini, 2004). The lack of sugar

accumulation in leaves of salt stressed plants could be explained by the fact that lettuce is moderately salt sensitive (Khah and Passam, 2005), with some studies indicating that responses to osmotic stress are also genotype-dependent (Al Hakimi *et al.*, 1995; Bolarin *et al.*, 1995). Kerepesi *et al.* (1998) reported that salt tolerant wheat (*Triticum aestivum*) genotypes accumulated greater soluble carbohydrate concentrations than sensitive ones. This raises the question of whether increased concentrations of specific sugars will result in plants being more salt tolerant, and if studying these changes will allow us to engineer plants accordingly.

4.5.2.4 Determination of ferric-reducing antioxidant activity of lettuce leaf isolates

This assay provided a simple measure of antioxidant activity based on the reduction of iron. Leaves of the homozygous, azygous and wild-type lines produced increases in antioxidant activity between non-saline control and saline treatments. Kang and Saltveit (2002) wounded Iceberg and Romaine lettuce leaves with heat-shock treatments and found that total antioxidant power increased by 42% and 39%, respectively compared to non-wounded controls. An increase in total antioxidant power in salt-treated plants was expected since several studies have reported strong evidence linking biotic and abiotic stress to increased synthesis of antioxidant enzymes and molecules (Kim *et al.*, 2005; Mittova *et al.*, 2004; Parida *et al.*, 2004; Tsai *et al.*, 2004; Arbona *et al.*, 2003; Muscolo *et al.*, 2003). A study by Neta *et al.* (2005) reported that pre-treating a salt-sensitive maize (*Zea mays*) genotype with 1 μM hydrogen peroxide in hydroponic culture for 2 d resulted in increased tolerance to a subsequent salt stress exposure. Both Khah and Passam (2005) and Sivritepe *et al.* (2005) demonstrated that NaCl priming treatments of lettuce (*L. sativa* cvs. Cristel and Juventa) and melon (*Cucumis melo* cvs. Hasanbey and Kirkagac) seed, respectively improved germination efficiency, and plant FW and DW.

4.5.2.5 Phenolic compound content in lettuce

The only statistically significant concentration increases in phenolic compounds occurred in the inner leaves of homozygous and azygous lines compared to control and all saline treatments. This was most probably to protect the young inner leaves from AOS. However, this result conflicts with a study by Nicolle *et al.* (2004) which stated that phenolic content in lettuce (*L. sativa folium*) accounted for more than 60% of the total antioxidant capacity. Considering that total antioxidant

power increased by more than 2-fold in the inner leaves of the homozygous, azygous and wild-type lines between control and saline treatments, a similarly large increase would be expected for the phenolic compounds. Lovelock *et al.* (1992) suggested the role of phenolic compounds was to provide protection from UV-B radiation in tropical mangroves (*Bruguiera parviflora*) naturally growing under saline conditions. Kang and Saltveit (2002) heat-shocked Iceberg and Romaine lettuce leaf tissues, which resulted in a 4-fold increase in phenolic content. They proposed that differences in concentration could be due to the presence of other absorptive compounds such as carotenoids, vitamin E and GSH.

4.5.2.6 Determination of lipid peroxidation

Increases in lipid peroxidation were observed in inner and outer leaves of the homozygous, azygous and wild-type lines grown in saline conditions when compared to plants grown under control conditions. Several studies on salinity and drought have found similar results, with MDA concentration increasing in greater levels of stress (Mittova *et al.*, 2004; Upadhyaya and Panda, 2004; Arbona *et al.*, 2003; El-baky *et al.*, 2003; Fedina *et al.*, 2003). Although none of the results were statistically significant, the inner and outer leaves of the azygous and wild-type lines appeared to show the largest increases in lipid peroxidation between control and saline treatments. A study by Amor *et al.* (2005) grew the perennial halophyte *Crithmum maritimum* under a concentration of 50 mM NaCl. Both root and shoot MDA concentrations were lower than control lines, this was related to enhanced activities of antioxidant enzymes. Parida *et al.* (2004) reported that concentrations of MDA remained unchanged in mangroves treated with NaCl due to high concentrations of antioxidant enzymes. These results could indicate that lipid peroxidation was reduced in the homozygous lines because of increased synthesis of GSH.

4.5.2.7 Glutathione quantification

The massive increases in GSH concentration between the homozygous and azygous lines was due to the over-expression of the genes *gsh*I, *gsh*II, *phgpx* and *gor*I (Creissen *et al.*, 1999). The young inner leaves of saline treated plants had reduced concentrations of GSH compared to those of control plants. Similar results have been observed in drought and salt stressed plants (Sharma and Dubey, 2005; Mittova et al., 2004; Parida *et al.*, 2004; Upadhyaya and Panda; 2004). However, Di

Baccio *et al.* (2004) reported GSH content in sunflower (*Helianthus annuus*) leaves decreased with the application of 10% seawater but increased with 20% seawater. The outer leaves of the homozygous, azygous and wild-type lines produced increases in GSH concentration between control and saline treatments; this may have resulted from senescence or have been a reaction to toxic Cl^- ions (Tsai *et al.*, 2004). The homozygous, azygous and wild-type lines grown in control treatments had greater GSH concentrations in the young, inner leaves than the older, outer leaves. This was probably because the young tissues were more prone to damage by AOS than the older senescing leaves. Although the homozygous lines had a greater GSH content in the inner and outer leaves than the azygous and wild-type lines, the total antioxidant status was similar. This suggests that concentrations of other antioxidants were reduced to compensate for the increased GSH content. Kanwischer *et al.* (2005) reported that an α-tocopherol deficient mutant of *A. thaliana* (*vte1*) had increased ascorbate and GSH concentrations. Plants over expressing the same gene had reduced ascorbate and GSH contents.

The cv. King Louie T_3 homozygous, azygous and wild-type lines that were assessed for resistance to abiotic stresses as described in this chapter, were consequently evaluated for resistance to tipburn. Trials based at The University of Nottingham and Elsoms Seeds Ltd., Spalding, UK, were designed to assess the incidence of tipburn in these lines when grown under Ca^{2+} deficient and glasshouse conditions. Macroscopic and microscopic observations of tipburnt leaves were also performed (Chapter 5).

CHAPTER 5 : INCIDENCE OF TIPBURN IN LETTUCE

5.1 Introduction

Tipburn is characterised as a necrotic disorder occurring on the margins of young developing leaves of vegetable crops. It mainly affects head forming leafy vegetables including lettuce, white cabbage and Chinese cabbage. Localised foliar Ca^{2+} deficiency is regarded to be the prime cause of tipburn (Everaarts and Blom-Zandstra, 2001; Corgan and Cotter, 1971) (refer to Chapter 1, Section 1.2 for further background).

A direct connection has been made between increased RH and the incidence of tipburn. High RH causes reduced water movement in the transpiration stream, limiting the available foliar Ca^{2+}, which increases tipburn. Barta and Tibbitts (1986) found that an artificially induced RH of 65% caused 53% of lettuce plants to develop tipburn after 4 d. Only 1% of the control-grown plants showed the condition. Frantz *et al.* (2004) eliminated tipburn in lettuce by blowing air directly onto the plants. In turn, this allowed a greater photosynthetic flux density, 2 to 3 times more than normally used for lettuce, elevated CO_2 concentrations and increased temperature resulting in a doubled edible yield when compared to control plants.

Studies have shown that the use of high nitrate fertilisers encourages fast, weak growth resulting in plants being more susceptible to tipburn (Saure, 1998; Dickson, 1977). Magnusson (2002) found that large applications of mineral fertilisers increased growth and occurrence of internal tipburn in Chinese cabbage. The use of green mulch as the only fertiliser, or with small amounts of mineral fertiliser resulted in slower plant growth and prevented the occurrence tipburn on internal leaves. Barta and Tibbitts (1986) used atomic absorption spectroscopy to demonstrate that tipburnt leaves had less Ca^{2+} (0.63 mg g^{-1} DW) than control-grown plants (1.48 mg g^{-1} DW). Spraying Ca^{2+} salts directly onto young foliage can also significantly reduce tipburn damage (Pressman *et al.*, 1993).

Calcium deficiency disorders such as BER and leaf scorch have also been extensively researched. Ho and White (2005) proposed that induction of BER in tomato is associated with Ca^{2+} deficiency in the distal portion of the fruit. They suggested that expanding cells within the fruit demanded so much Ca^{2+} that critical

apoplastic and cytoplasmic functions suffered, resulting in a loss of cellular integrity, weakening of cell walls and necrosis of the tissues. However, Saure (2005) suggested that gibberellins prevent Ca^{2+} translocation in order to maintain rapid fruit growth. Park *et al.* (2005b) demonstrated that tomato plants expressing the *A. thaliana* H^+/ cation exchangers (CAX) had increased fruit Ca^{2+} concentrations and extended shelf life. They studied plants expressing *sCAX1* and *CAX4* genes. They found that *sCAX1* had increased root vacuolar H^+/Ca^{2+} transport, increased fruit Ca^{2+} concentration and prolonged fruit shelf life, although the incidence of BER increased. The *CAX4* expressing plants had reduced Ca^{2+} concentrations and plant growth was not affected. Aktas *et al.* (2005) investigated the possibility that AOS contributed towards BER in sweet pepper (*Capsicum annuum*) plants grown under control or saline conditions. They found that salinity increased concentrations of AOS and NADPH oxidase in the pericarp of the pepper fruits that were most affected by BER. Their results suggest that generation of AOS may contribute to BER and that cations such as Mn^{2+}, Zn^{2+} and Ca^{2+} may alleviate BER symptoms in the fruit. Casado-Vela *et al.* (2005) used peptide mass fingerprinting analysis to show that proteins involved with the ascorbate–GSH and pentose phosphate pathways scavenged AOS in BER affected fruits, possibly restraining the spread of blackening to the whole fruit.

Experiments by Chang and Miller (2003) and Chang *et al.* (2004) evaluated the importance of Ca^{2+} in *Lilium* cv. Star Gazer. They found that daily applications of $CaCl_2$ and $CaNO_3$ sprays for 2 wk could significantly suppress tipburn symptoms. They also cultivated plants in reduced Ca^{2+} or Ca^{2+} free conditions and found that leaf necrosis symptoms were more prominent than in control plants. Chang and Miller (2005) studied upper leaf necrosis in *Lilium* cv. Star Gazer and found that Ca^{2+} concentration in necrosed leaves was nearly 6-fold lower than that of normal leaves. H8igh photosynthetic flux density, greater RH and reduced leaf Ca^{2+} concentration were identified by Islam *et al.* (2004) to cause an increased incidence of tipburn in plants of *Eustoma grandiflorum.*

5.2 Aims and Objectives

The main objective of the experiments in this chapter was to assess the homozygous, azygous and wild-type lines of cv. King Louie for resistance to the

foliar condition tipburn. A study by Garratt (2002) confirmed an enhanced tipburn tolerance in homozygous lines expressing the pAFQ70.1 transgenes. The role of these genes was to increase chloroplast antioxidant content and plants should therefore have an improved resistance to tipburn, a stress related disorder. Tipburn tolerance was assessed by growing plants under Ca^{2+} deficient conditions in the glasshouse at The University of Nottingham. Some studies have shown links between lack of Ca^{2+} and the occurrence of tipburn (Collier and Tibbitts, 1982; Rosen, 1990). Two trials based at Elsoms Seeds Ltd., Spalding, UK, were also intended to evaluate incidence of tipburn in plants grown under normal glasshouse conditions. Incidence of tipburn was determined according to a tipburn index (Frantz *et al.*, 2004), and took into account the severity of tipburn and the number of plants affected. Macroscopic and microscopic observations of control and tipburnt lettuce leaves were also performed. The aim of these observations was to expand current knowledge of the biological events leading up the development of tipburn and the cellular changes occurring in the leaves.

5.3 Materials and Methods

5.3.1 Tipburn trial under calcium deficient conditions in the glasshouse at Plant Sciences Division, University of Nottingham

Lettuce seeds of cv. King Louie T_3 homozygous (containing and expressing the pAFQ70.1 transgenes) lines 32.4, 43.17, 44.2, azygous (reverted to wild-type) lines 32.9, 43.16, 44.12 and the wild-type line (Chapter 3, Section 3.3.8) were germinated in half-size plastic seed trays (50 seeds/tray) containing John Innes Seed Compost. Fourteen d-old seedlings were transferred to 9 cm diameter plastic pots filled with 50:50 (v:v) perlite and vermiculite and acclimatised for 2 wk. Glasshouse conditions were ~25°C with a ~16 h photoperiod (light intensity at noon was approximately 1000 μmol m^{-2} s^{-1}) and plants were spaced ~25 cm apart. Plants were watered every 2 d with nutrient solution (Appendix 8.2.9) lacking $CaNO_3$. The experiment consisted of 15 plants of each homozygous line, each azygous line and the wild-type line.

At 4 and 5 wks after sowing, plants from all lines were scored for severity of tipburn. Incidence of tipburn was determined according to a tipburn index (Frantz *et*

al., 2004), the following equation was used: {[(L x 1) + (M x 3) + (S x 5)] x 100} / P x 5, where L was the number of plants with light tipburn, M was the number of plants with medium tipburn, S was the number of plants with severe tipburn, and P was the total number of plants (Figure 5.1). A tipburn index of 100% indicated that all plants had severe tipburn while an index of 20% indicated all plants had only minor symptoms.

5.3.2 Tipburn trials in the glasshouse at Elsoms Seeds Ltd., Spalding, UK

Lettuce seeds of cv. King Louie T_3 homozygous lines 32.4, 43.17, 44.2, azygous lines 32.9, 43.16, 44.12 and the wild-type line were germinated in 4 cm diameter peat propagation plugs (Fertil UK, Threapwood, UK) (2 seeds/plug). At 3 wks post-germination, seedlings were thinned to 1 plant per plug and transferred to 10 litre plastic pots filled with 6:1 (v:v) Levington M3 compost (Scotts Company Ltd.) and vermiculite. Plants were watered daily by drip feeder with tap water (300 – 400 ml per pot). Two trials were conducted by Mrs. S. Kennedy, Elsoms Seeds Ltd., during the summer of 2005, the first trial was run from 20/04/2005 to 16/06/2005, and the second trial was run from 23/06/2005 to 23/08/05. Average temperature and RH during the first trial at midday was 22°C (59% RH) and at midnight was 10°C (85% RH), and the second trial at midday was 25°C (52% RH) and at midnight was 15°C (82% RH). Photoperiod was ~16 h (light intensity at midday was approximately 1000 μmol m^{-2} s^{-1}) and plants were spaced 30 cm apart. Plants were organised into 3 rows, each consisting of 7 plots (1 plot for each line) of 28 plants (Figure 5.3). Plots were organised randomly among the rows, with identical layouts used for both trials (Figure 5.4).

Approximately 7 wks after sowing, plants from all lines were scored twice, 1 wk apart, for severity of tipburn. Incidence of tipburn was determined according to a modified tipburn index of Frantz *et al.* (2004), the following equation was used: {[(L x 1) + (L/M x 2) + (M x 3) + (M/S x 4) + (S x 5)] x 100} / P x 5, where L was the number of plants with light tipburn, L/M was the number of plants with light/medium tipburn, M was the number of plants with medium tipburn, M/S was the number of plants with medium/severe tipburn, S was the number of plants with severe tipburn, and P was the total number of plants (Figure 5.5). The tipburn index used in the trial at Elsoms Seeds Ltd., improved detection of minor changes in severity of tipburn present in large number of plants.

5.3.3 Data analysis and statistics

Results from the Ca^{2+} deficiency trial were presented as tipburn index for individual homozygous lines (32.4, 43.17, 44.2), azygous lines (32.9, 43.16, 44.12) and the wild-type line. Tipburn index was also presented as a combined average for the homozygous lines and the azygous lines. Results from both trials at Elsoms Seeds Ltd. were presented as tipburn index for individual homozygous, azygous, and wild-type lines in planting rows A, B, and C for the first and second tipburn severity scorings. Results for each trial were also presented as average tipburn index for the individual homozygous, azygous, and wild-type lines, combining data from rows A, B, and C, and both tipburn severity scorings. Results from the first, second, and both trials at Elsoms Seeds Ltd. were presented as average tipburn index for the homozygous, azygous and wild-type lines. Histograms of Figures 5.2 A, 5.6 A, B, and 5.7 A, B did not show error bars for S.E.M. because results were from 1 replicate. Statistical analysis of the results was carried out using ANOVA on Microsoft Excel. Analysis of variance (P) values indicated the probability of obtaining the results by chance, where $P = < 0.05$ was significant, $P = < 0.01$ was highly significant and $P = < 0.001$ was very highly significant.

5.3.4 Macroscopic observations of control and tipburnt lettuce leaves

Initial observations of 4 - 5 wk old whole leaves of glasshouse grown cv. King Louie (Section 5.3.1) were made using a Zeiss Stemi SV 6 stereomicroscope (Carl Zeiss Ltd., Welwyn Garden City, UK). Images were captured using a DC290 digital camera (Kodak) and Adobe Photoshop (Version 6.0.1; Adobe Systems UK, Uxbridge, UK).

5.3.5 Preparation and sectioning of control and tipburnt lettuce leaves

Small sections of 4 - 5 wk old non-tipburnt and tipburnt cv. King Louie leaf (Section 5.3.1), each approximately 2 mm by 10 mm, were incubated in phosphate buffered (pH 8.0) 3% (v/v) glutaraldehyde fixative (Appendix 8.2.16) overnight in the dark at 4°C. Leaf sections were washed twice in phosphate buffer (Appendix 8.2.17) at RT and dehydrated in ascending grades of ethanol (10%, 20%, 40%, 60%, 80%, and twice at 100%) (v/v) for at least 1 h each. Each leaf section was then placed in a 1 ml gelatine capsule (TAAB Laboratories Equipment Ltd., Reading, UK) filled with Technovit 7100 resin (Kulzer Histo-Technik, Heraeus Kulzer,

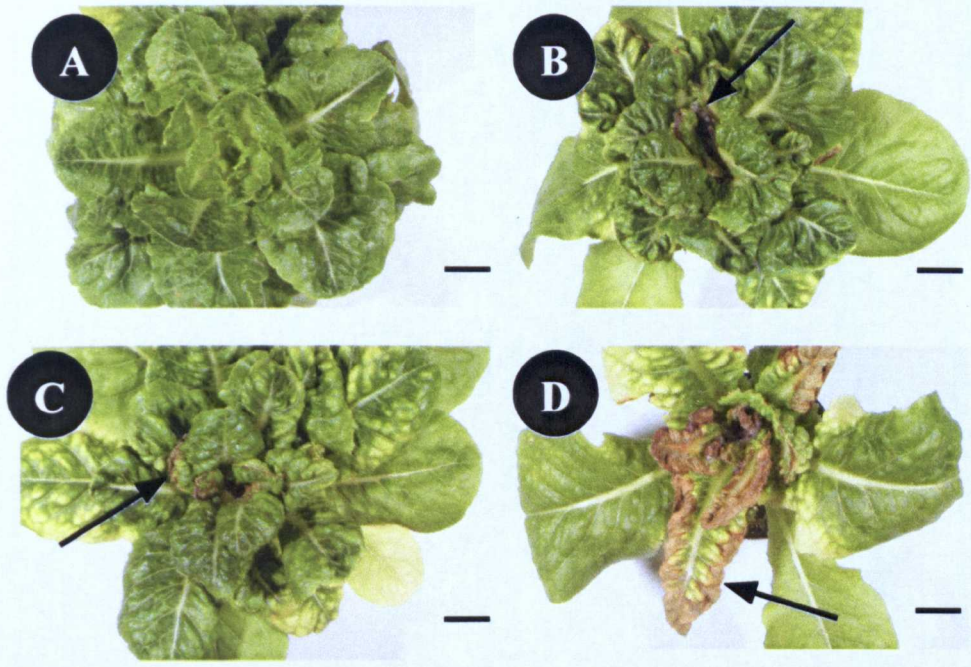
Hanau, Germany), oriented to allow transverse sectioning and cured overnight at RT. Resin blocks containing the leaf tissues were attached to sectioning blocks (Kulzer Histo-Technik) using Technovit 3040 impression resin (Kulzer Histo-Technik). Resin blocks were sectioned (5 – 10 μm thickness) using a Bright 5040 microtome (Bright Instrument Co Ltd., Huntingdon, UK) and the sections stained with 1% (w/v) toluidine blue (Appendix 8.2.18) for 10 sec at RT (Trump *et al.*, 1961). Sections were mounted on glass slides (Chance Propper Ltd., Warley, UK) using DPX mountant (Sigma-Aldrich), viewed using a Optiphot light microscope (Nikon UK Ltd., Kingston-upon-Thames, UK) and images captured using the ACT-1 computer program (Version 2.12; Nikon UK Ltd.).

5.4 Results

5.4.1 Tipburn trial under calcium deficient conditions in the glasshouse at Plant Sciences Division, University of Nottingham

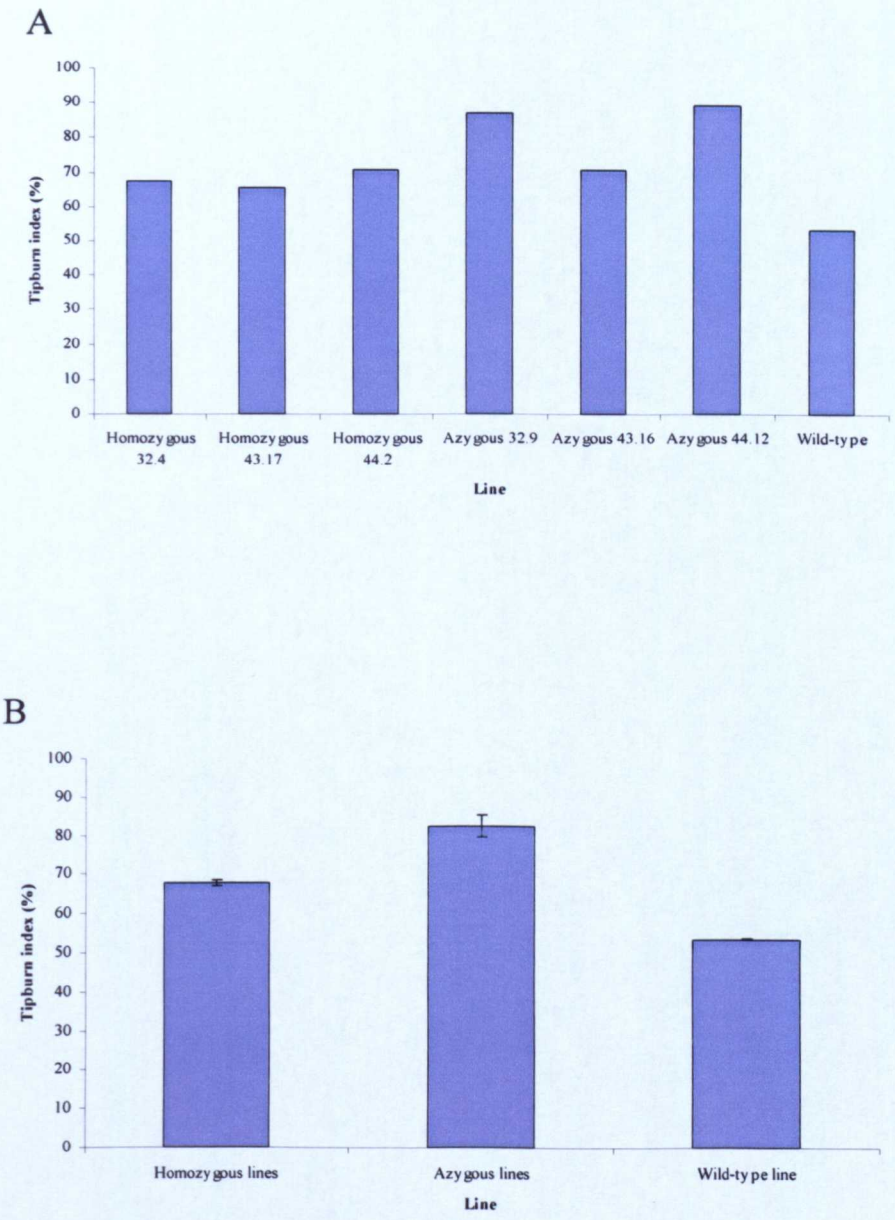
Incidence of tipburn in plants grown under Ca^{2+} deficient conditions was lowest in the wild-type line, followed by the homozygous and azygous lines (53%, 68%, 82% tipburn index, respectively) (Figure 5.2 B) (Appendix 8.3.4, Table 8.26). The incidence of tipburn was only significantly different between the homozygous and azygous lines ($P = 0.03$). These data did not indicate whether any of the homozygous lines were more or less tolerant to tipburn. However, the severity of tipburn was greater in the azygous lines 32.9 and 44.12 (87% and 89% tipburn index, respectively) than in line 43.16 (71% tipburn index) (Figure 5.2 A).

Figure 5.1: Cultivar King Louie showing different stages of the development of tipburn in the glasshouse calcium deficiency trial at Plant Sciences Division, University of Nottingham.



(A) No tipburn, (B) light tipburn, (C) medium tipburn and (D) severe tipburn. Arrows indicate tipburnt leaf; bars = 2cm.

Figure 5.2: Incidence of tipburn in cv. King Louie T_3 homozygous, azygous and wild-type lines grown under calcium deficient conditions in the glasshouse at Plant Sciences Division, University of Nottingham.



(A) Tipburn index for the individual homozygous (32.4, 43.17, 44.2), azygous (32.9, 43.16, 44.12) and wild-type lines. (B) Average tipburn index for the homozygous, azygous and wild-type lines. n = 15 (A, B wild-type line) and n = 45 (B homozygous lines/azygous lines); error bars represent S.E.M

5.4.2 Tipburn trials in the glasshouse at Elsoms Seeds Ltd.

Results from both trials based at Elsoms Seeds Ltd. (Figure 5.8 C) showed that average tipburn index for the homozygous lines was greater than the azygous and the wild-type lines (68%, 62%, 49% tipburn index, respectively). Conversely, the azygous line 43.16 had a lesser incidence of tipburn than the wild-type line in both scorings of the first trial (36% and 48%, 37% and 55% tipburn index, respectively) (Figure 5.6). Tipburn index in both scorings of the second trial was least in the wild-type line (43% and 62% tipburn index, respectively) (Figure 5.7). Incidence of tipburn increased in the second trial for the homozygous, azygous and wild-type lines, when compared to results from the first trial (increases of 15%, 14%, 6% tipburn index, respectively) (Figure 5.8 A, B). Furthermore, plants of the homozygous, azygous and wild-type lines appeared to have a reduced incidence of tipburn when grown in row B compared to those grown in rows A and C. Statistical analysis confirmed that average tipburn index was less in the azygous lines than the homozygous lines in the first scoring of the first trial and the second scoring of the second trial ($P = 0.003$ and 0.01, respectively).

Results from both scorings of the first trial (Figure 5.6 A, B) indicated no particular pattern in incidence of tipburn, when comparing data from the individual homozygous lines (Appendix 8.3.4, Tables 8.27 and 8.28). However, incidence of tipburn in the azygous lines was greater in line 32.9 than lines 44.12 and 43.16, respectively (Figure 5.6 C). In both scorings of the first trial, incidence of tipburn in row A was least in the homozygous line 32.4 and row B, the azygous line 43.16. For row C, the homozygous line 44.2 and the wild-type line had the least tipburn in the first scoring and the wild-type line had the least tipburn in the second scoring.

Results from both scorings of the second trial showed that incidence of tipburn was reduced in plants growing in row B compared to rows A and C (Figure 5.7 A, B) (Appendix 8.3.4, Tables 8.29 and 8.30). Conversely, plants of the homozygous line 32.4 had similar tipburn indices in rows A, B and C in both scorings, as did the wild-type line in the second scoring. None of the homozygous lines showed any particular trend in incidence of tipburn during the second trial. However, the azygous line 32.9 had the greatest incidence of tipburn in the second trial followed by the lines 44.12 and 43.16 (74%, 70%, 63% tipburn index, respectively). The wild-type line had the least tipburn in both scorings of the second trial (43% and 62% tipburn index, respectively).

Figure 5.3: Images from the first tipburn glasshouse trial at Elsoms Seeds Ltd.

(A) Image taken 13/05/05 at planting out and (B) image taken 09/06/05 during the first tipburn severity scoring. Rows A, B and C from left to right.

Figure 5.4: Planting layout for both tipburn glasshouse trials at Elsoms Seeds Ltd.

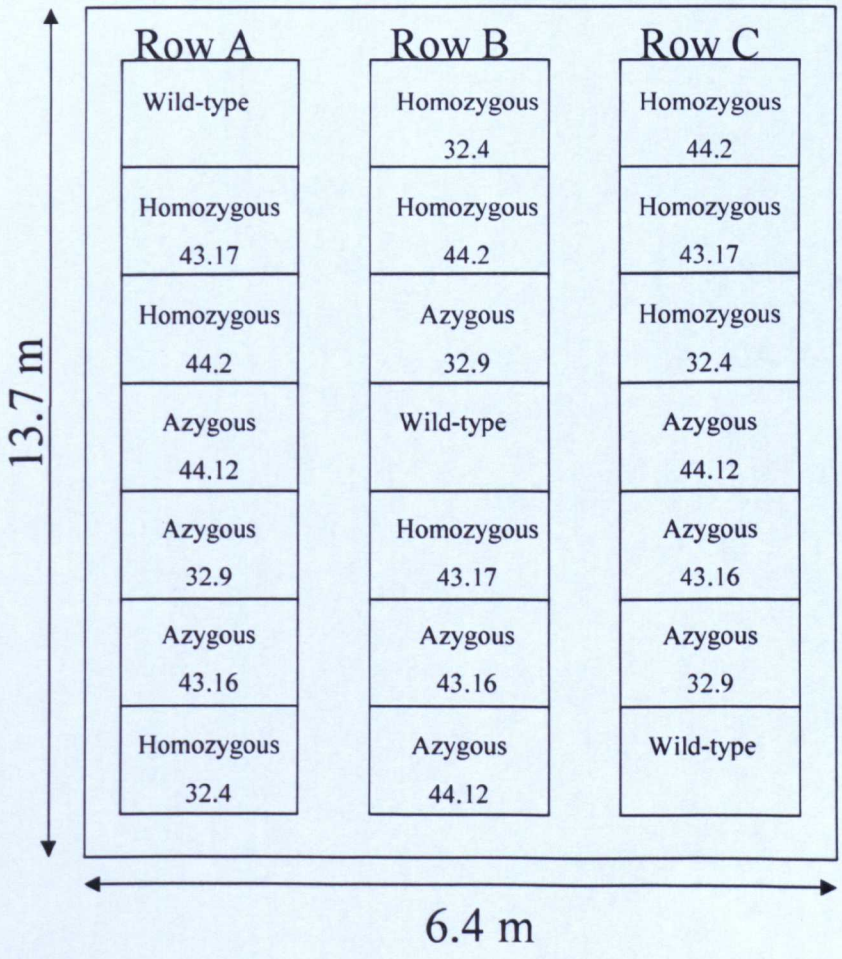
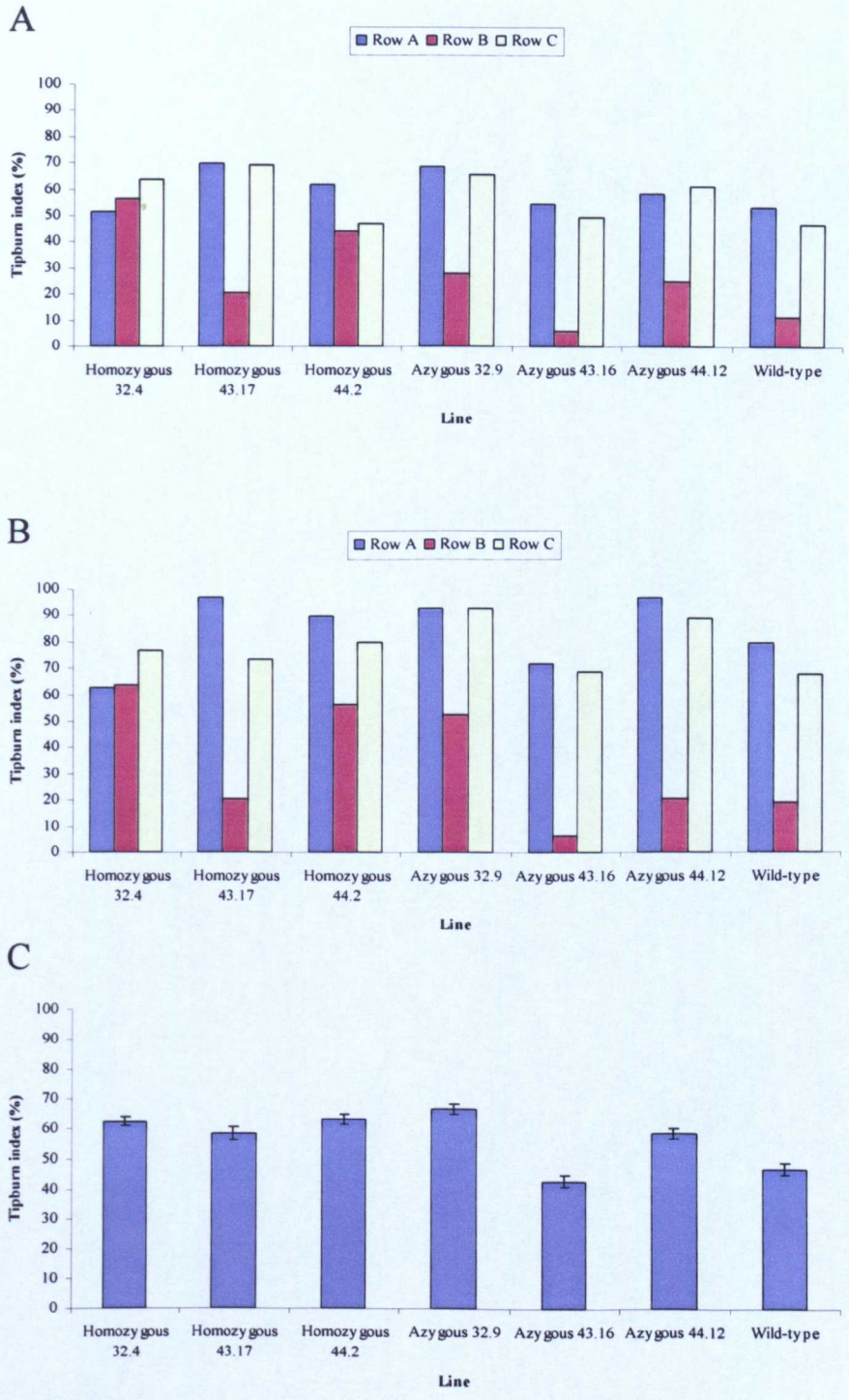


Figure 5.5. Cultivar King Louie showing different stages of the development of tipburn in the glasshouse trial at Elsoms Seeds Ltd.

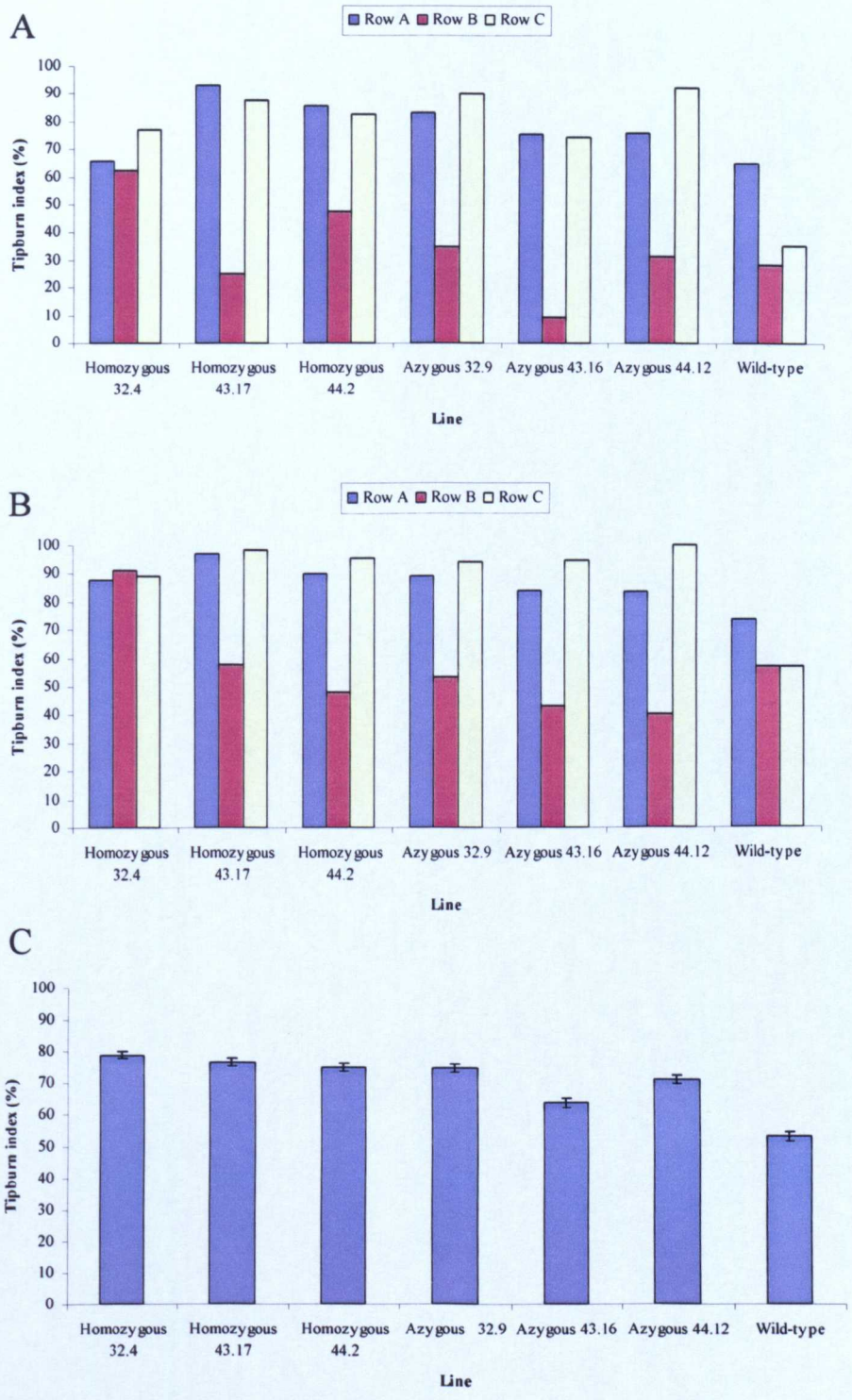
(A) No tipburn, (B) light tipburn, (C) light/medium tipburn, (D) medium tipburn, (E) medium/severe tipburn and (F) severe tipburn. Arrows indicate tipburnt leaf; bars = 5 cm.

Figure 5.6: Incidence of tipburn in cv. King Louie T_3 homozygous, azygous and wild-type lines grown in the first glasshouse trial at Elsoms Seeds Ltd.



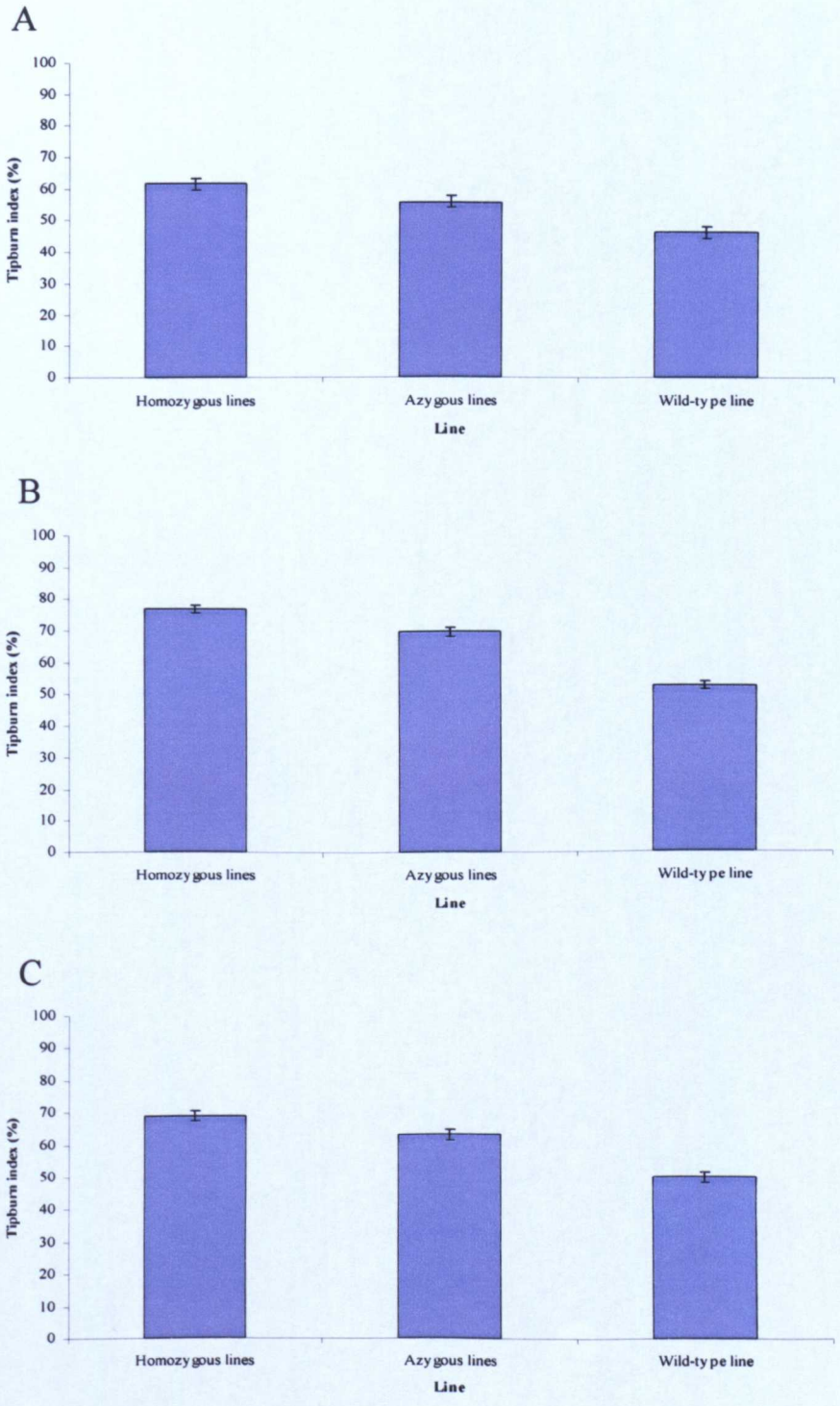
Tipburn index for the individual homozygous (32.4, 43.17, 44.2), azygous (32.9, 43.16, 44.12) and wild-type lines in rows A, B, and C for (A) the first scoring and (B) the second scoring. (C) Average tipburn index for the individual homozygous, azygous and wild-type lines combining data from rows A, B and C, and both scorings. n = 28 (A; B) and n = 168 (C); error bars represent S.E.M.

Figure 5.7: Incidence of tipburn in cv. King Louie T_3 homozygous, azygous and wild-type lines grown in the second glasshouse trial at Elsoms Seeds Ltd.



Tipburn index for the individual homozygous (32.4, 43.17, 44.2), azygous (32.9, 43.16, 44.12) and wild-type lines in rows A, B, and C for (A) the first scoring and (B) the second scoring. (C) Average tipburn index for the individual homozygous, azygous and wild-type lines combining data from rows A, B and C, and both scorings. n = 28 (A; B) and n = 168 (C); error bars represent S.E.M.

Figure 5.8: Incidence of tipburn in cv. King Louie T_3 homozygous, azygous and wild-type lines grown in both glasshouse trials at Elsoms Seeds Ltd.



Average tipburn index for the homozygous, azygous and wild-type lines in (A) the first trial and (B) the second trial. (C) Average tipburn index for the homozygous, azygous and wild-type lines, combining results from both trials. n = 504 (A/B homozygous and azygous lines), n = 168 (A/B wild-type line), n = 1008 (C homozygous/azygous lines), n = 336 (C wild-type line); error bars represent S.E.M.

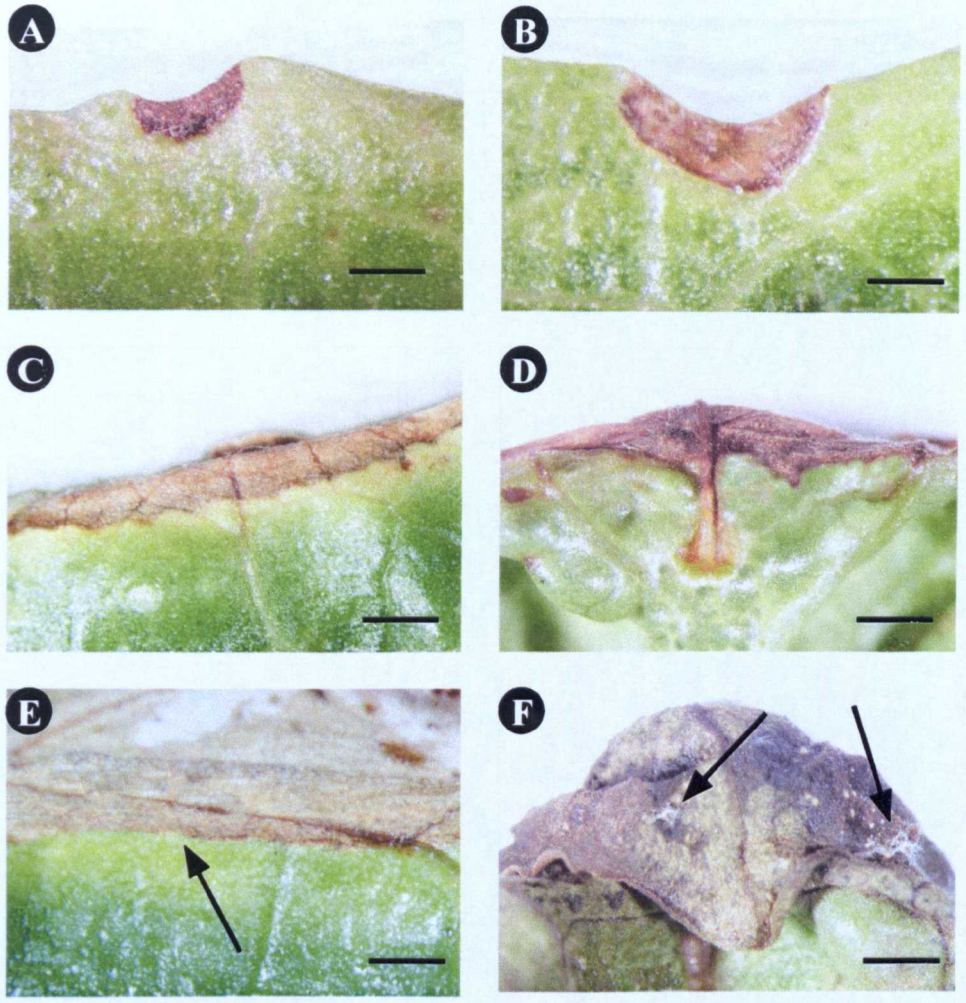
5.4.3 Macroscopic observations of control and tipburnt lettuce leaves

Mature leaves of the lettuce cv. King Louie are elongated ovate in shape, with the lower part of the leaf irregularly lobed. Tipburn injury commenced with small dark sunken spots on the leaf margin (Figure 5.9 A and B) which spread rapidly, resulting in a band of necrotic tissue along the entire leaf margin (Figure 5.9 C and D). It appeared that tipburn formed along a definite line on the surface of affected leaves, with no relation to the position of vascular bundles (Figure 5.9 E). Enclosed leaves were most prone to secondary pathogen infection (Figure 5.9 F).

5.4.4 Microscopic observations of control and tipburnt lettuce leaves

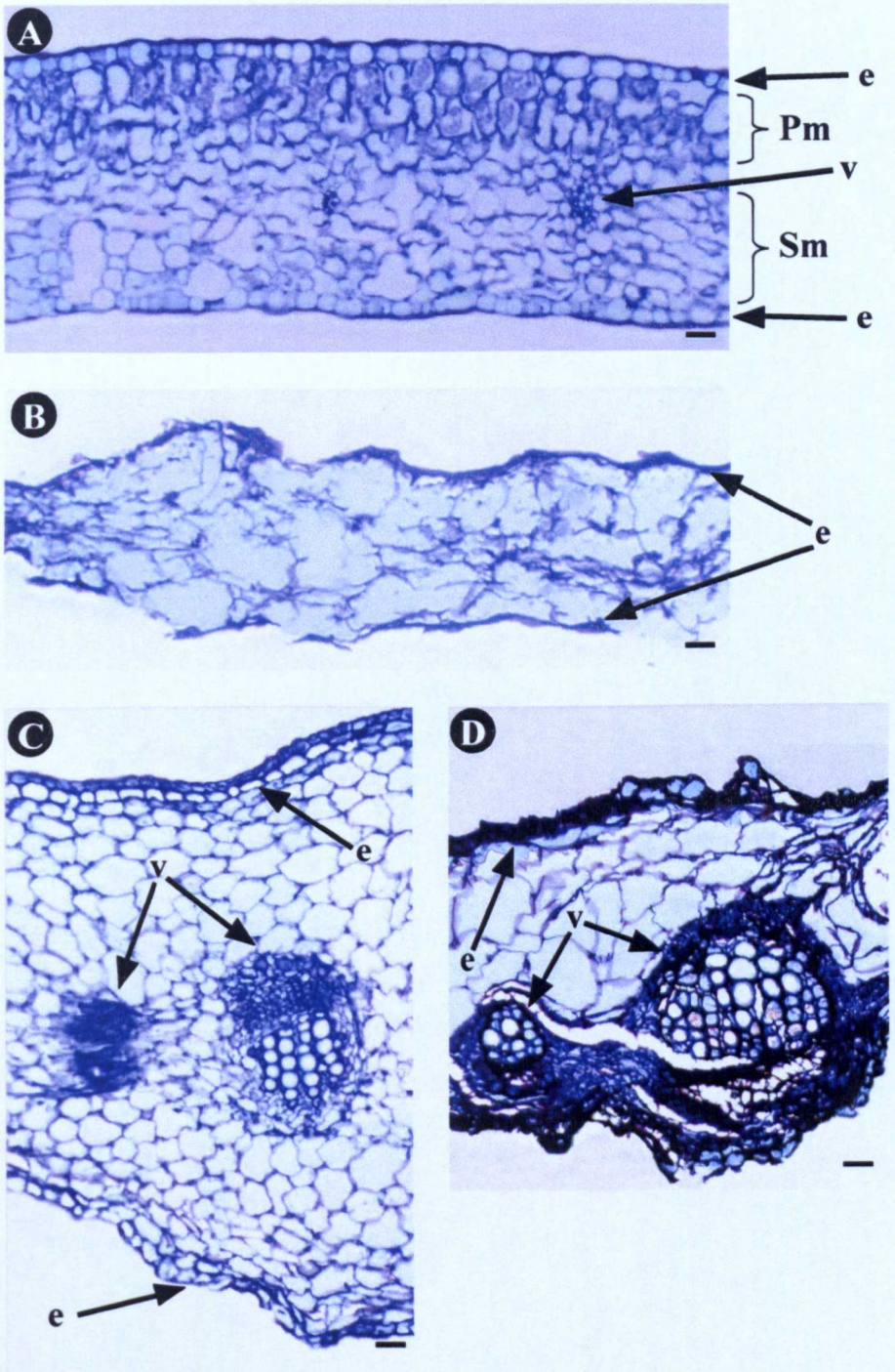
Transverse sections of control leaf showed healthy, turgid cells of the palisade and spongy mesophyll (Figure 5.10 A and C). Epidermal layers and vascular bundles were also clearly visible. The palisade cells were elongated and localised beneath the upper epidermis. The spongy tissues contained irregular shaped cells with large intercellular spaces. Chloroplasts and other organelles were stained by the toluidine blue. The regions of tipburnt leaves showed total disintegration and necrosis of the leaf structure (Figure 5.10 B and D). Fragmented cell walls were scattered throughout the leaf, although the vascular bundles were still clearly visible. The upper and lower epidermal layers appeared to have become dehydrated, and as result collapsed and detached from parts of the leaf. Chloroplasts and other organelles were not visible in the tissues.

Figure 5.9: Lettuce cv. King Louie whole leaves viewed with a stereomicroscope at various stages during the development of tipburn.



(A; B) Tipburn initiated with small dark sunken spots on the leaf margin. (C) Tipburn rapidly progressed along the leaf margin, (D) restricting leaf expansion. (E) Tipburn appeared to form along a definite line (arrow). (F) Secondary pathogen infection on a tipburnt leaf (arrows). Bars = 0.25 mm (A), 0.5 mm (B), 1 mm (E), 2 mm (C; D; F).

Figure 5.10: Lettuce cv. King Louie light micrographs of transverse sections of regions of control leaves and those showing tipburn.



(A; C) Sections through normal regions of leaf showing turgid, healthy cells with stained chloroplasts and organelles. (B; D) Sections through regions of tipburnt leaf showing total disintegration and necrosis of the intercellular leaf structure. Abbreviations are e, epidermal layer; Pm, palisade mesophyll; Sm, spongy mesophyll; v, vascular bundle. Bars = 50 μm (A; B; C; D).

5.5 Summary

5.5.1 Tipburn trial under calcium deficient conditions in the glasshouse at Plant Sciences Division, University of Nottingham

The aim of the trial at the University of Nottingham was to study the effect of Ca^{2+} deficient conditions on the growth and tipburn susceptibility of the homozygous, azygous and wild-type lines. Studies by Rosen (1990) and Barta and Tibbitts (1986) have linked lack of foliar Ca^{2+} to tipburn in lettuce. Calcium is also essential for cellular signalling, mediation of cytokinin activity and maintaince of cell wall integrity (Ho and White, 2005; del Amor and Marcelis, 2003; Pressman *et al.*, 1993). Increased cytosolic Ca^{2+} concentrations have been shown to alleviate plant stress and injury, and help cells to better survive (Gong *et al.*, 1998). Flego *et al.* (1997) found enhanced resistance to *Erwinia carotovora*, the cause of bacterial soft rot disease, in tobacco (*Nicotiana tabacum*) plants with high Ca^{2+} cell concentrations. However, a constant increased cellular concentration of Ca^{2+} may have a cytotoxic effect (Wang and Li, 1999). The fact that the homozygous lines had less tipburn than the azygous lines could be due to increased synthesis of GSH making them more stress tolerant (Casado-Vela *et al.*, 2005). Aktas *et al.* (2005) suggested that generation of AOS may contribute to BER in sweet pepper, while Casado-Vela *et al.* (2005) showed that proteins involved with the ascorbate–GSH and pentose phosphate pathways scavenge AOS in BER affected tomato fruits. Jiang and Huang (2001) studied the effect of Ca^{2+} application on antioxidant activities of heat stressed grass species. They found that application of Ca^{2+} increased water and chlorophyll content in heat stressed plants, whereas the water control had the opposite effect. Calcium treated plants also accumulated less MDA, a by-product of lipid peroxidation, than untreated plants. Suzuki *et al.* (2003) used the antimonite precipitation method to observe the presence of calcium precipitates in BER affected tomato fruits. The antimonite precipitation method involved the chemical modification of Ca^{2+} precipitates into calcium antimonite, which can be viewed using energy dispersive X-ray spectrometry. There were no visible Ca^{2+} precipitates on the plasma membranes of BER affected cells, but their concentration increased as distance from the affected cells increased. They concluded that BER was a result of Ca^{2+} deficiency causing cell membrane collapse. These studies demonstrate the

importance of Ca^{2+} in stress tolerance and its role in maintaining leaf chlorophyll and carotenoid content.

5.5.2 Tipburn trials in the glasshouse at Elsoms Seeds Ltd.

The results from both trials at Elsoms Seeds Ltd. confirmed the homozygous lines had the greatest incidence of tipburn in each trial. This was not expected because data from the Ca^{2+} deficiency trial showed the homozygous lines had a lesser incidence of tipburn than the azygous lines. The azygous line 32.9 was the most susceptible to tipburn, followed by the lines 44.12 and 43.16 based on results from both trials. This result followed an identical trend to the azygous lines grown in the Ca^{2+} deficiency trial and was almost certainly a result of background genetic variation. However, a similar trend should have been observed in the homozygous lines. It is quite possible that over-expression of transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I, in the homozygous lines may have upset this relationship.

Overall, incidence of tipburn was greater in plants grown in the second trial, because it ran later in the summer when the temperature and light levels were greater. Dorais *et al.* (1990) suggested that light was one of the primary factors influencing incidence of tipburn. Gaudreau *et al.* (1994) found the short- and long-d lettuce cvs. Karlo and Rosana, respectively, were affected by tipburn at different times of the year. Their results showed that plants of cv. Rosana gained less FW and were more susceptible to tipburn than plants of cv. Karlo. Jenni (2005) found that short periods of high temperatures could induce a greater occurrence of rib discolouration in lettuce compared to plants grown under cooler control conditions. The study identified that heat stressing plants during earlier growth phases lowered the incidence of the disorder in mature plants. These results suggest the importance maintaining favourable conditions to reduce the occurrence of tipburn and the potential use of stress pre-treatments to improve plant performance during the heading stage.

The reduced incidence of tipburn in plants growing in row B compared to rows A and C was almost certainly due to increased air circulation. Personal observations confirmed the glasshouse apex and roof ventilation were directly above plants growing in row B. A study by Frantz *et al.* (2004) demonstrated that blowing air directly onto the meristem region of lettuce plants almost eliminated tipburn. In turn, this allowed the use of a greater photosynthetic flux (1000 $\mu mol\ m^{-2}\ s^{-1}$), CO_2

concentration and temperature which increased leaf expansion rate and doubled yields. Therefore, plants growing in rows A and C would have been more stressed by the increased RH found in stagnant non-moving air, which has been linked to a greater incidence of tipburn (Ciolkosz *et al.*, 1998; Saure, 1998; Barta and Tibbitts, 1986). Studies have also shown that high light intensity and extended photoperiods, and an increased glasshouse temperature of 28 – 37°C can accelerate incidence of tipburn (Frantz *et al.*, 2004; Wissemeier and Zuhlke, 2002; Misaghi *et al.*, 1992; Dorais *et al.*, 1990).

5.5.3 Potential causes of the greater incidence of tipburn in the homozygous and azygous lines compared to the wild-type line

Incidence of tipburn was least in the wild-type lines in both the Ca^{2+} deficiency trial at the University of Nottingham and the glasshouse trials at Elsoms Seeds Ltd. Incidence of tipburn in the Ca^{2+} deficiency trial was less in the homozygous lines than in the azygous lines, the opposite occurred in the trials at Elsoms Seeds Ltd. A study by Garratt (2002) showed that homozygous lines of cv. Evola containing the expression construct pAFQ70.1, had a statistically lower incidence of tipburn than their azygous controls. However, the plants were grown under growth room conditions with constant light, temperature and air circulation. Tipburn was scored as either mild or severe, necrotic lesions less or more than 2 mm in diameter, respectively. Even though the homozygous lines had a lower incidence of tipburn in the Garratt (2002) study, the severity of the necrotic lesions were not comparable with those in the Ca^{2+} deficiency and Elsoms Seeds trials.

Results from the trials at The University of Nottingham and Elsoms Seeds Ltd. showed that incidence of tipburn was greater in the homozygous and azygous lines than the wild-type line. This might be a result of genetic changes, such as somaclonal variation in the tissue cultured T_0 generation lines, from which the homozygous and azygous lines were derived (Brown *et al.*, 1986). It has been suggested that somaclonal variation is heavily influenced by AOS, tissue culture protocol, over-exposure to auxins and the *in vitro* environment (Halliwell, 2003; Cassells and Curry, 2001; Scheid *et al.*, 1996). The potential for somaclonal variation often increases with sub-culture number, for example, lettuce transformants were maintained on selection medium for the longest time possible, to ensure a minimum number of non-transformed escapees were present. Genetically altered plants may

also arise from mutated explant cells and mutations during cell culture or morphogenesis (Cassells and Curry, 2001). This variation can be caused by a series of factors and include chromosomal abnormality, gene mutations and silencing/activation of genes and transposons (Kaeppler *et al.*, 2000). However, the idea that somaclonal variation only manifests negative effects on plant physiology and morphology is not true, these genetic variations have been shown to improve disease resistance and vigor (Brown *et al.*, 1986). Labra *et al.* (2004) compared genomic DNA changes between *A. thaliana* plants derived from floral dip transformation and plants produced by *in vitro* cell culture. They used AFLP (amplified fragment length polymorphism) analysis to show that transformation did not result in any genomic modifications compared to untreated control plants. They were also able to determine that genetic variation due to somaclonal variation was correlated with the stress imposed by the *in vitro* culture.

Other types of *in vitro* modification include the habituation of cultures, characterized by an irreversible decrease in regeneration capacity, and hyperhydricity, a waterlogged appearance of the tissues leading to necrosis of the apical meristem. Hyperhydric plants have been observed to have greater concentrations of H_20_2, MDA and a limited ascorbate-glutathione cycle (Joyce *et al.*, 2003). Presence of Ca^{2+} and growth regulators may also lead to cell signal interference and thus cause breakdown in cellular regulation. Schmulling *et al.* (1997) observed that plant gene expression, both on the transcriptional and post-transcriptional level, could be significantly altered in response to cytokinins. They found that relatively small variations in cytokinin content could produce large alterations in gene mRNA levels. This suggests that the *in vitro* cytokinins could have influenced whole plant gene expression and resulted in heritable gene modifications. It is true to say that the potential for genetic variations due to *in vitro* factors becomes greater, the longer plant tissues remain in culture.

Alternatively, silencing of the transgenes in the T_3 generation may have modified the GSH redox balance in the plastids. For example, there appeared to be more variation in incidence of tipburn between the individual homozygous lines than the azygous lines, in the trials at Elsoms Seeds Ltd. Creissen *et al.* (1999) transformed tobacco (*Nicotiana tabacum* cv. Samsun) plants with either the metabolic gene *gsh*I or *gsh*II. Plants expressing the *gsh*I transgene produced a 3-fold increase in foliar GSH content compared to non-transformed plants. However,

enhanced oxidative stress, manifested by light intensity-dependent necrosis was observed in plants grown under glasshouse conditions. Creissen *et al.* (1999) proposed that the oxidative damage was caused by a redox-sensing failure in the chloroplasts.

5.5.4 Macroscopic and microscopic observations of control and tipburnt lettuce leaves

The use of microscopy in this thesis gave a limited insight into the causes of tipburn. Macroscopic observations of tipburnt cv. King Louie leaves highlighted the unpredictability of the condition and the damage it can cause. Light micrographs of transverse sections of regions of tipburnt leaf showed complete cellular disintegration and necrosis. The formation of tipburn along the leaf margins was most likely due to localised Ca^{2+} deficiency. Collier and Tibbitts (1982) showed that lack of Ca^{2+} causes loss of membrane integrity in mesophyll cells which developed into cell collapse, necrosis and eventually tipburn. A study by Rosen (1990) showed that tipburnt leaves of cauliflower had up to 5-fold less Ca^{2+} than unaffected control leaves of the same physiological age. Cubeta *et al.* (2000) found that excessive K fertilisation caused an increased incidence of tipburn in cabbage. Calcium uptake by the roots is inhibited by metal ions such as K^+ and Na^+ (Taylor *et al.*, 2004). Studies have shown definite links between application of Ca^{2+} and the retardation of leaf necrosis and senescence in a wide range of plant species. Martin *et al.* (2007) found that the application $CaCl_2$ to Murashige and Skoog medium facilitated the recovery of more than 90% of banana (*Musa* species) shoots that exhibited necrosis. Chang *et al.* (2004) found that exogenous applications of Ca^{2+} could significantly suppress leaf tipburn and necrosis symptoms in lily. Cheour *et al.* (1992) found that senescence in cabbage leaf discs could be delayed by application of $CaCl_2$. They concluded that Ca^{2+} protected the lipid membranes from degradation. Calcium supply to the leaves is largely controlled by translocation in the transpiration stream, although presence of organic acids may preserve it in an insoluble chelated state. (Ryder, 1999).

Fornaseiro (2001) observed leaf margin necrosis in fluoride treated plants of *Hypericum perforatum.* Regions of tipburnt leaf were separated from healthy leaf by a very sharply marked reddish-brown line, identical to observations made in the present investigation with leaves of the lettuce cv. King Louie. Matyac and Misaghi (1981) found that leaves in advanced stages of tipburn development had cells with

degenerating plasmalemma and organelle membranes. Rupturing of the laticifers and leakage of latex in to the lettuce tissues has been observed to cause complete collapse and necrosis of tipburn affected leaves (Collier and Tibbitts, 1982; Matyac and Misaghi, 1981). This may have lead to the cellular disintegration and necrosis observed in tipburnt leaves of cv. King Louie. Suzuki *et al.* (2003) found that cell collapse in BER affected tomato fruits commonly occurred during the rapid-fruit-growth stage. Their analyses also revealed that Ca^{2+} precipitates were not present on the plasma membranes of BER affected cells. Fornaseiro (2001) found total collapse of tissues in fluoride affected leaf, although the epidermal layers remained largely intact. As with the Fornaseiro (2001) study, tipburnt cv. King Louie leaves were observed to have complete collapse of the palisade and spongy mesophyll, yet the epidermal cells remained undamaged.

The relationship of the results obtained in chapters 2-5 were subsequently discussed within a wider context and concluded. The possibilities for the use of new concepts and technology in future studies were also discussed and how this may enhance current knowledge (Chapter 6).

CHAPTER 6 : GENERAL DISCUSSION AND CONCLUSIONS

The transformation of the 4 lettuce cvs. Evola, King Louie, Pic and Robusto with an *A. tumefaciens* protocol of Curtis *et al.* (1994) proved to be reliable and efficient. The cv. Evola was chosen as a standard genotype with which to compare the performance of the cvs. King Louie, Pic and Robusto. However, cultures of cv. Evola proved to be less responsive to the tissue culture medium compared to the 3 Romaine cvs., resulting in reduced callus induction and shoot regeneration frequencies. Several factors can influence the growth of explants *in vitro*, and include osmotic shock and high ammonium ion concentration from the tissue culture media, unusual ratios of auxins to cytokinins and the accumulation of gases, particularly ethylene (Gaspar *et al.*, 2002; Kumar *et al.*, 1998). It has been suggested that these *in vitro* factors may act in the same way as an environmental stress, and may lead to signal interference and loss of cellular regulation (Joyce *et al.*, 2003). However, once an *in vitro* stress has been identified, alterations to growth parameters, culture vessel design and media components can be made, optimising explant tissue culture response.

The potential of explants to induce somatic embryogenesis and shoot regeneration is related to tissue culture age, presence of AOS and the method of transformation used. Tissue culture ageing, recalcitrance and somaclonal variation often occur in conjunction with DNA methylation and presence of AOS (Joyce *et al.*, 2003; Benson *et al.*, 1992). Latham *et al.* (2006) proposed that the probability for an aberrant phenotype increased with the level of genetic scrambling and DNA methylation. *Agrobacterium tumefaciens*-mediated transformation is frequently used in the creation of transgenic plants due to its single copy transgene insertion into the host genome. However, several studies have shown that deletions to the host genomic DNA occur at the transgene insertion site, and have ranged from a few nucleotides to 75,800 b.p. in size (Kumar and Fladung, 2002; Kaya *et al.*, 2000). Lee *et al.* (2004) investigated the integration of T-DNA by *A. tumefaciens*-mediated transformation in tobacco (*Nicotiana tabacum*). They sequenced the genomic DNA that flanked the transgenes, and observed abnormal integration patterns in many of the lines. Forsbach *et al.* (2003) analysed the T-DNA flanking sequences in 112 *A. thaliana* single copy transformants. The vast majority of lines exhibited DNA

rearrangements such as deletions, duplications and translocations in the sequences bordering the T-DNA insertion site. It is highly possible that the transformed explants from the 4 lettuce cvs. contained segments of superfluous DNA as well as unusual phenotypes, a result of the tissue culture environment. Using this knowledge, future transformation studies should concentrate on trying to understand the effect of genetic modifications that occur through tissue culture. For example, non-transformed controls and plants transformed with T-DNA containing selectable marker genes only should be regenerated *in vitro*. These plants could then be used as a control lines in crop trials and may be used to indicate any background genetic modifications that may have occurred *in vitro*. Johnston *et al.* (2005) used high performance liquid chromatography (HPLC) to study the extent of DNA methylation in tissue cultured explants of *Ribes ciliatum*. This technique would allow the characterisation of epigenetic changes that occur during *in vitro* culture, when explants are most susceptible to genetic change. However, HPLC methodologies for DNA methylation in plants are yet to be fully optimized.

It is highly possible that genetic changes occurring as a result of the tissue culture environment may have influenced the expression of the transgenes in the 3 lettuce cvs. King Louie, Pic and Robusto. Down *et al.* (2001) observed variations in gene expression in transgenic potato plants when grown under controlled environmental and glasshouse conditions. They suggested that variations in the stresses between these environments were strong enough to induce changes in DNA methylation, transgene inactivation and the accumulation of transgenic proteins (Down *et al.*, 2001).

The results from the RT-PCR analysis of the T_0, T_1 and T_2 cv. King Louie lines do correlate with those of other studies on gene expression in transgenic plants, for example high expression viral promoters have been shown to cause TGS. Curtis *et al.* (2000) found that *Solanum dulcamara* lines containing both single and multiple copies of the beta-glucuronidase (*gus*) gene driven by CaMV 35S showed loss of transgene expression. A knock on effect of the gene silencing was plants exhibiting leaf malformations and reductions in flower, fruit and seed production compared to non-silenced transgenic and non-transformed (control) plants. Aida and Shibata (1996) also observed identical transgene silencing events in *Kalanchoe blossfeldiana* plants transformed with the *gus* gene under the control of CaMV 35S. No clear

correlation was observed between copy number and gene silencing, and there was no change in phenotype.

Post-transcriptional gene silencing of transformed plants has also been observed in several studies. Metzlaff *et al.* (2000) introduced the chalcone synthase A gene into *Petunia* plants, which resulted in the degradation of the transgene mRNA. The transgene silencing also induced the loss of expression of endogenous chalcone synthase genes in *Petunia*, resulting in a white flower phenotype. Dubois *et al.* (2005) produced a population of 50 independent transgenic lettuce lines transformed with the endogenous *nia* gene for the enzyme nitrate reductase. They found that approximately one third of transformants exhibited bleaching of the leaves leading to the death of the plants, a result of gene silencing. Northern blot analysis confirmed degradation of mRNA was occurring, a result of PTGS during the early stages of plant development. However, Pang *et al.* (1996) utilised PTGS to an advantage, by over expressing the nucleocapsid protein gene from the tomato spotted wilt virus in lettuce. Homozygous lines expressing the transgene showed a uniform suppression of nucleocapsid protein accumulation and consequently high levels of virus resistance in silenced plants from an early developmental stage.

Plastid transformation systems probably offer the most successful route toward producing transgenic plants without gene silencing. Several recent studies focusing on the transformation of lettuce have shown successful transmission of expression to the T_1 generation, although this is due to insertion of the transgene into a known region of the plastid genome (Kanamoto *et al.*, 2006; Lelivelt *et al.*, 2005). Li *et al.* (2006) demonstrated the high transgene expression and ability of chloroplasts to fold human proteins by transforming lettuce plants to express the partial spike protein of severe acute respiratory syndrome coronavirus (SARS-CoV). Their results demonstrated that edible plants could act as bioreactors producing a safe oral recombinant subunit vaccine against SARS-CoV.

Although Southern blotting can provide an accurate determination of transgene copy number in the plant genome, the technique is time consuming and laborious (Li *et al.*, 2004b). Song *et al.* (2002) and Li *et al.* (2004b) adapted quantitative real-time PCR (qRT-PCR) to determine transgene copy number in transgenic maize and wheat, respectively. Both studies found significant correlation between qRT-PCR and Southern blot analysis. The fact that qRT-PCR offers the potential to simultaneously analyse hundreds of plants in a day, would allow

improved speed and detection of copy number in transgenic plants. Due to the difficulties observed with Southern blotting in this study, it would be sensible to utilise qRT-PCR in future transformation studies.

The transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I increased GSH content in the T_3 homozygous lines of cv. King Louie by more than 2-fold, when compared to their azygous controls. However, this did not result in enhanced antioxidant status, stress tolerance and crop performance. For example, saline stress assessments showed the homozygous lines had little advantage when compared to the azygous and wild-type control lines. Over expression of genes encoding GSH enzymes for the enhancement of plant stress tolerance was performed by Roxas *et al.* (1997). Tobacco seedlings had a 2-fold greater concentration of the enzymes GSH transferase and GSH peroxidase and were able to grow significantly faster than non-transformed control plants when exposed to salt stress (Roxas *et al.*, 1997). This suggests that the homozygous lines expressing the transgenes *gsh*I, *gsh*II, *phgpx* and *gor*I should have had enhanced growth and salt stress tolerance. This may have been undermined by the fact that the tobacco plants used by Roxas *et al.* (1997) were more salt tolerant than lettuce or that gene silencing may have interfered with the reduced/oxidised GSH balance in the chloroplast.

The findings from the saline stress assessments indicated that the salt treated plants showed marked increases in synthesis of AOS, GSH and antioxidants compared to plants grown under control conditions. The concentrations of soluble protein, chlorophyll and carotenoids increased in the inner leaves of salt treated plants, while the opposite occurred in the outer leaves. Content of the hexose reducing sugars glucose and fructose did not alter significantly in either control or saline treated plants. These results show that several compounds provide defensive roles in plant salt stress response (Sahi *et al.*, 2006). Several recent studies have provided an insight into what genes are transcribed in abiotically stressed plants. Gong *et al.* (2005) compared microarray data between salt stressed plants of *A. thaliana* and a salt-tolerant relative *Thellungiella halophila*. Transcript profiling of plants grown under 150 mM NaCl revealed that genes controlling ribosomal function, photosynthesis, cell growth, ABA pathways and osmolyte production were up-regulated. A similar study by Andjelkovic and Thompson (2006) analysed gene transcription changes in maize kernels 15 d after pollination in plants grown during

water- and salt-stress treatments. There was approximately a 2-fold increase in gene expression in stressed plants compared to those grown under control conditions. Up-regulated genes included ABA response binding factors and glycine- and proline-rich proteins. Studies by Miyama *et al.* (2006) and Ma *et al.* (2006) performed microarrays concentrating on the up-regulation of genes across the entire genome. Miyama *et al.* (2006) sequenced and analysed 14,842 expressed sequence tags (ESTs) from in the mangrove *Bruguiera gymnorrhiza*, and Ma *et al.* (2006) analysed 1,500 up-regulated genes in the roots and shoots of salt-stressed *A. thaliana* plants. However, the results of both these studies indicated that the vast majority of stress related genes encoded putative and hypothetical proteins. Until the exact role of these gene products is clarified and their relationship to salt tolerance is determined, then future transformation work in this area will be hindered.

Future studies on salt tolerance in lettuce should initially concentrate on use of microarrays to determine what new genes are being expressed in stressed plants and the relationship of these genes to those found in salt tolerant species. Through this research, key tolerance genes could be identified, cloned and transformed into lettuce. Current transformation studies have utilised single genes, often encoding betaines, amino acid derivatives which act as osmoprotectants, Na^+ transporters, ABA transcription factors and protein biosynthesis enzymes. Bhattacharya *et al.* (2004) introduced the bacterial *betA* gene for biosynthesis of glycinebetaine into cabbage, with transformants having a greater tolerance to saline stress compared to non-transformed controls. Zhang and Blumwald (2001) transformed tomato plants to overexpress a vacuolar Na^+/H^+ antiporter. Plants grown under 200 mM NaCl accumulated greater Na^+ concentrations in the leaves than the fruits. Both Park *et al.* (2005a) and Vanjildorj *et al.* (2005) transformed lettuce to express the late embryogenesis abundant protein and the *ABF3* gene, a transcription factor for the expression of ABA, respectively. Results from these studies proved that introduction of a single gene can significantly improve plant salinity tolerance.

However, transformation of plants with multiple tolerance genes or genes encoding transcription factors would offer the most successful route to producing salt tolerant plants. Molecular analysis of the homozygous plants in this study confirmed that 6 transgenes can be transformed into lettuce and stably expressed. Alternatively, marker assisted plant breeding programmes, utilising quantitative trait loci have been successfully used with tomato, rice and citrus. Enhanced characteristics included

improved seed germination, vegetative growth and fruit yield characteristics when plants were grown under saline conditions (Flowers and Flowers, 2005). However, successful phenotyping of large populations of plants requires soils with a constant salinity concentration (Cuartero *et al.*, 2006). Tissue culture represents another opportunity for crop improvement. Supplementation of tissue culture media with NaCl or osmoticants such as sorbitol and mannitol allows thousands of plants to be selected for unique tolerance traits within a restricted space and time (Dita *et al.*, 2006).

In contrast to plant transformation and breeding, seed priming is a relatively easy and low cost alternative. The technique involves the immersion of seeds in a solution that will stimulate metabolic activities without resulting in germination. The result, is a more uniform germination with improved plant stress tolerance (Iqbal and Ashraf, 2005). Studies have reported the use of H_2O_2, NaCl and polyamine pre-treatments to successfully enhance germination efficiency, growth and tolerance to saline soils in lettuce, melon, tomato and wheat (Khah and Passam, 2005; Iqbal and Ashraf, 2005; Neta *et al.*, 2005; Sivritepe *et al.*, 2005; Cano *et al.*, 1991). Barassi *et al.* (2006) studied the effect of inoculation of lettuce seeds with the nitrogen fixing soil bacterium *Azospirillum brasilense.* Germination efficiency and fresh and dry weights of plants grown in saline media was greater in inoculated seeds than control treated seeds. The fact that lettuce is relatively salt sensitive means that seed priming would offer a low cost, short-term alternative to plant breeding.

The results from the glasshouse trials at The University of Nottingham and Elsoms Seeds Ltd. have shown that lettuce transformed with transgenes influencing the synthesis and metabolism of GSH in the chloroplasts, have little advantage in reducing the incidence of tipburn. Even though each leaf contains many thousands of chloroplasts and that tipburn affects the leaves, this would suggest that the cause of the condition was not stress related. Due to the complexity of tipburn and associated disorders, it would be naïve to assume that the condition is purely a result of a direct foliar Ca^{2+} deficiency. Calcium has been shown to act as an important messenger ion, in both local and whole plant tissue responses (Medvedev, 2005). Calcium signalling stimuli include pathogen, osmotic, oxidative, heat and mechanical stress (Lecourieux *et al.*, 2006). Studies on plant and animal cells have indicated that variations in cytosolic Ca^{2+} concentration, known as a Ca^{2+} signature, act as a signalling switch.

Calcium signatures vary in cellular location, concentration, lag time and frequency. To understand the relevance of calcium concentration and signalling in relation to tipburn, it is important to be able to visualise its presence in affected tissues. Many advances have been made in the use of confocal laser scanning microscopy, energy-dispersive X-ray analysis and use of Ca^{2+} isotopes to view the presence of Ca^{2+} in specific plant cells (Busse and Palta, 2006; Broadhurst *et al.*, 2004; Stricker and Whitaker, 1999).

Confocal laser scanning microscopy (CLSM) and fluorescence ion imaging utilise the Ca^{2+} triggered photo-protein aequorin from the jellyfish *Aequorea*, and reporter dyes such as calcium green-1-dextran (Creton *et al.*, 1999; Xu and Heath, 1998). Xu and Heath (1998) used CLSM to investigate the role of cytosolic free Ca^{2+} in the hypersensitive response of disease-resistant cells of cowpea (*Vigna unguiculata*) to the cowpea rust fungus (*Uromyces vignae*). They found that Ca^{2+} concentrations slowly increased in epidermal cells as the fungal mycelia grew through the cell wall, but returned to a normal concentration when the fungus grew with the cell. Bhatla and Kalra (2004) visualised spatial and temporal Ca^{2+} concentration variations within the cytoplasm of plant cells. They identified that specific channels are able to selectively control Ca^{2+} influx and Ca^{2+} gradients in different cells of the same tissue. Use of CLSM could allow researchers to identify Ca^{2+} fluctuations in the different leaf cell types both prior to and after tipburn has occurred. However, these techniques are usually based on micro-injection of the reporter proteins/dyes into the target cells and can inadvertently induce signalling pathways. Alternatively, some researchers have transformed plants with genes that encode these reporter dyes. Both Allen *et al.* (1999) and Iwano *et al.* (2004) transformed *A. thaliana* with genes encoding the protein-based calcium indicator yellow cameleon 2.1. In both studies, the gene was successfully expressed, accumulated predominantly in the cytoplasm, and enabled accurate determination of the patterns and dynamics of cytosolic Ca^{2+} movement. Use of this construct could allow Ca^{2+} movement to be monitored in lettuce leaves throughout the growth and development stages leading up to tipburn.

Scanning electron microscopy/energy-dispersive X-ray analysis was used by Broadhurst *et al.* (2004) to determine region specific concentrations of Ni, Mn, and Ca in leaf trichomes of *Alyssum murale*. Energy-dispersive X-ray analysis is a tool that allows simultaneous non-destructive determination of the elemental composition

of a sample. Their data confirmed that Ca was strongly concentrated in the trichome rays and nodules, while Ni accumulated at the trichome pedicle and epidermal layer (Broadhurst *et al.*, 2004). Busse and Palta (2006) used the radioactive isotope ^{45}Ca to investigate Ca^{2+} translocation pathways in developing potato tubers (*Solanum tuberosum*). Plants were grown in split pots and nutrient solution containing ^{45}Ca was placed to allow uptake of the isotope through either the main root or the tuber/stolon areas. This study confirmed that Ca^{2+} translocation from the roots to the shoots occurred through the xylem. Once the ^{45}Ca ions had reached the leaf tissues, they became fixed, and were not able to be re-translocated by the phloem to other tissues.

A more simple and straightforward method of visualising Ca^{2+} uses the cuvette/microplate based fluorescence of isolated tissues. Individual cells or groups of cells are isolated, homogenised and reacted with the same Ca^{2+}-sensitive fluorescent dyes used in CLSM. A more broad result is obtained using this method than that of the microscopy techniques. However, high throughput equipment such as a fluorescence imaging plate reader can simultaneously measure Ca^{2+} signals in microplates with 96-, 384- and 1536-well formats (Monteith and Bird, 2005).

Currently, the occurrence of tipburn in commercially grown leafy vegetables varies between location, year, planting dates and presence of external factors (Saure, 1998). Past studies have made basic comparisons in incidence of tipburn between cvs. of the same species when grown under unnatural environmental conditions. Because of this, contradictions exist between the many studies and as a result, no definition of the cause of tipburn has been provided. Future tipburn trials should focus on studying the effect of combining different environmental conditions such as relative humidity, temperature, photoperiod and plant nutrition. Samples of tipburnt and non-tipburnt leaves could be analysed for variations in Ca^{2+} concentration both on the cellular and whole plant level. A molecular approach such as microarray could be used to determine the genetic changes occurring between tipburnt and non-tipburnt leaves. Assuming an appropriate gene is identified, its promoter could be tagged with GFP, transformed into lettuce and used to identify key physiological changes at the cellular level (Dixit *et al.*, 2006). A study by Wyatt *et al.* (2002) transformed A. *thaliana* to express the maize calreticulin gene fused to GFP under the control of a heat shock promoter. Calreticulin is a multi-functional protein that binds Ca^{2+} in the endoplasmic reticulum. The aim of their study was to increase the content of Ca^{2+} in the ER and, in turn, make plants utilise this reserve in times of

stress. Transformed plants showed delayed loss of chlorophyll when grown on Ca^{2+} depleted medium compared to controls transformed with GFP only. The presence of GFP allowed fluorescence imaging to confirm uniform expression of calreticulin in the seedling leaves and roots. Alternatively, lettuce could be transformed to accumulate greater concentrations of Ca^{2+} within the cytoplasm. Park *et al.* (2005b) demonstrated that fruit from tomato plants expressing the *A. thaliana* H^+/cation exchangers (*CAX*) accumulated more Ca^{2+} and had a prolonged shelf-life when compared to controls. However, plant morphology and incidence of BER were affected. A study by Kim *et al.* (2006) transformed potato to express the *A. thaliana* *CAX2B* gene. Although similar to the *CAX* gene used by Park *et al.* (2005b), *CAX2B* encodes a Ca^{2+}/H^+ antiporter. Transgenic potato tubers accumulated 50-65% more Ca^{2+} than wild-type tubers yet, did not accumulate any undesirable metals. They observed that the *CAX2B* potatoes retained stable transgene expression for 3 generations.

In conclusion, transformation of lettuce with the 6 transgenes, *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I does result in their stable integration into the host genome. Transformants containing the 4 genes of interest were observed to the T_3 generation. However, the presence of viral promoters and sequence homology between the transgenes and endogenous genes resulted in silencing events. Presence of the genes *gsh*I, *gsh*II, *phgpx* and *gor*I in cv. King Louie T_3 homozygous lines produced a 2-fold increase in GSH content in leaf tissues, compared to the azygous lines. Conversely, this did not result in any significant enhancement in plant growth under normal or stressed conditions. Trials at The University of Nottingham and Elsoms Seeds Ltd., Spalding, UK confirmed the occurrence of tipburn was least in the cv. King Louie wild-type line. Macroscopic and microscopic observations of tipburnt leaves showed that the condition resulted in the necrosis and disintegration of affected cells.

CHAPTER 7 : REFERENCES

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CHAPTER 8 : APPENDICES

8.1 Media preparation

8.1.1 MS0

MS0 semi-solid medium contained agar (7 g l^{-1}), sucrose (30 g l^{-1}) and Murashige and Skoog (1962) basal salts (4.41 g l^{-1}) (Table 8.1), and was made with purified (reverse-osmosis) water to pH 5.6 – 5.8. MS0 medium was autoclaved for 20 min at 121°C in either 175 ml powder round jars or 250 ml Duran flasks (Schott UK Ltd, Stafford, UK) for pouring Petri dishes.

Table 8.1: The chemical formulation of Murashige and Skoog basal salts.

Compound	Value (mg l^{-1})
$CaCl_2$	332.00
$CoCl_2.6H_20$	0.025
$CuS0_4.5H_20$	0.025
FeNaEDTA	36.70
H_3B0_3	6.20
KH_2P0_4	170.00
KI	0.83
$KN0_3$	1900.00
$MgS0_4$	181.00
$MnS0_4.H_20$	16.90
$Na_2Mo0_4.2H_20$	0.25
NH_4N0_3	1650.00
$ZnS0_4.7H_20$	8.60
Inositol	100.00
Nicotic acid	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
Glycine	2.00

8.1.2 Luria broth (LB)

LB contained tryptone (10 g l^{-1}), yeast extract (5 g l^{-1}), NaCl (10 g l^{-1}) and Bacto-agar (15 g l^{-1}), and was made with purified water to pH 7.0. LB was autoclaved for 20 min at 121°C in 175 ml powder round jars.

8.2 Buffers and solutions

8.2.1 TAE

TAE buffer (0.5 x strength) contained tris base (2.42 g l^{-1}), ethylenediaminetetraacetic acid (EDTA) (146 mg l^{-1}) and acetic acid (600 mg l^{-1}), and was made with purified water to pH 8.0.

8.2.2 CTAB extraction buffer

CTAB extraction buffer contained tris (12.1 g l^{-1}) made with purified water to pH 8.0. NaCl, (81.2 g l^{-1}), EDTA (5.845 g l^{-1}), 0.2% (v/v) β-mercaptoethanol and 2% (w/v) CTAB were added before use.

8.2.3 TE buffer

TE buffer contained tris (1.21g l^{-1}) and EDTA (292 mg l^{-1}), and was made with purified water to pH 8.0.

8.2.4 Denaturation solution

Denaturation solution contained NaCl (87.66 g l^{-1}) and NaOH (20 g l^{-1}) and was made with purified water.

8.2.5 Neutralisation solution

Neutralisation solution contained NaCl (175.32 g l^{-1}) and tris HCl (78.79 g l^{-1}) and was made with purified water.

8.2.6 SSC buffer

A 10 x SSC buffer contained NaCl, (87.65 g l^{-1}) and sodium citrate (44.1 g l^{-1}), and was made with purified water to pH 7.0. A 2 x and a 0.5 x SSC buffer was made by diluting the above solution 5-fold and 20-fold with purified water, respectively.

8.2.7 Washing buffer

Washing buffer contained maleic acid (11.61 g l^{-1}) and NaCl (8.76 g l^{-1}), and was made with purified water to pH 7.5. Polyoxyethylene (20) sorbitan monolaurate (Tween 20) [0.3% (v/v)] was added prior to use.

8.2.8 Detection buffer

Detection buffer contained tris HCl (15.7 g l^{-1}) and NaCl (5.84 g l^{-1}), and was made with purified water to pH 9.5.

8.2.9 Nutrient solution

Nutrient solution contained Sangral (1:1:1) fertiliser (400 mg l^{-1}) (20:20:20 NPK soluble fertiliser with trace elements; William Sinclair Horticulture Ltd.) and $CaNO_3$ (1.18 g l^{-1}) (reagent grade), and was made with purified water.

8.2.10 Protein extraction buffer

Protein extraction buffer contained tris HCl (9.45 g l^{-1}) made with purified water to ph 8.0. NaCl (29.22 g l^{-1}) and EDTA (2.92 g l^{-1}) were then added. Before use, β-mercaptoethanol (2.34 ml l^{-1}) and phenylmethylsulfonyl fluoride (17 mg l^{-1}) were added.

8.2.11 Phosphate buffered saline (PBS) solution

PBS solution consisted of potassium phosphate buffer (Section 8.2.15) diluted 1:20 (v:v) with purified water. To this solution, KCl (201 mg l^{-1}) and NaCL (8 g l^{-1}) were added.

8.2.12 Ferric reducing antioxidant activity assay reagent

The ferric reducing antioxidant activity assay reagent consisted of sodium acetate (24.6 g l^{-1}) made with purified water to pH 3.6. Before use, 2,4,6-tripyridyl-1,3,5-triazine (312 mg l^{-1}) and $FeCl_3$ (324 mg l^{-1}) were added.

8.2.13 HEPES buffer

HEPES buffer contained (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (11.9 g l^{-1}) made with purified water to pH 7.5. EDTA (292 mg l^{-1}), $MgCl_2 \cdot 6H_2O$ (1.01 g l^{-1}) and 0.2% (v/v) octylphenolpoly (ethyleneglycolether) (Triton X-100) [0.2% (v/v)] were added before use.

8.2.14 Thiobarbituric acid (TBA) reagent

Trichloroacetic acid (200 g l^{-1}) was made with purified water, to which TBA (5 g l^{-1}) was added.

8.2.15 Glutathione (GSH) assay reagent

The GSH assay reagent consisted of 7:1 (v:v) NADPH (250 mg l^{-1}) and 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) (2.37 g l^{-1}) made with 125 mM potassium phosphate buffer. Potassium phosphate buffer (Section 8.2.17) was diluted 1:1.5 (v:v) with purified water containing EDTA (1.46 g l^{-1}), and was adjusted to pH 7.5.

8.2.16 Glutaraldehyde fixative

Glutaraldehyde fixative contained 0.2 M potassium phosphate buffer (Section 8.2.15) and 3% (v/v) glutaraldehyde, and was adjusted to pH 7.0.

8.2.17 Potassium phosphate buffer

A 0.2 M potassium phosphate buffer consisted of 3.1:6.9 (v:v) KH_2PO_4 (27.21 g l^{-1}) and K_2HPO_4 (45.64 g l^{-1}) made with purified water to pH 8.0.

8.2.18 Toluidine blue stain

Toluidine blue stain was made by adding toluidine blue O (50 mg) to 50 ml of 0.2 M potassium phosphate buffer (Section 8.2.17) and 50 ml of purified water. Before use, the solution was diluted 1:1 (v:v) with purified water and adjusted to pH 6.8.

8.3 Raw data

8.3.1 Chapter 2 lettuce tissue culture data

Table 8.2: Tissue culture of lettuce leaf explants from wk 2 to wk 6: callus induction and shoot regeneration efficiency for the cvs. King Louie, Pic, Robusto and Evola. Abbreviation, w/o, without.

Lettuce cv. / culture type	Week 2		Week 4		Week 6	
	Callus (%)	Shoots (%)	Callus (%)	Shoots (%)	Callus (%)	Shoots (%)
King Louie transformed	75	0	100	0	100	5
Pic transformed	90	0	100	0	100	5
Robusto transformed	91	0	100	0	100	3
Evola transformed	89	0	100	1.13	100	3
King Louie control w/o antibiotics	96	0	100	10	100	21
Pic control w/o antibiotics	92	0	100	23	100	45
Robusto control w/o antibiotics	98	0	100	24	100	49
Evola control w/o antibiotics	90	0	100	29	100	29
King Louie control with antibiotics	32	0	27	0	0	0
Pic control with antibiotics	33	0	0	0	0	0
Robusto control with antibiotics	44	0	16	0	0	0
Evola control with antibiotics	23	0	0	0	0	0

Table 8.3 Tissue culture of lettuce leaf explants from wk 8 to wk 12: callus induction and shoot regeneration efficiency for the cvs. King Louie, Pic, Robusto and Evola.

Lettuce cv. / culture type	Week 8		Week 10		Week 12	
	Callus (%)	Shoots (%)	Callus (%)	Shoots (%)	Callus (%)	Shoots (%)
King Louie transformed	100	12	100	20	100	62
Pic transformed	100	19	100	19	100	56
Robusto transformed	100	5	100	9	100	52
Evola transformed	100	4	100	4	100	51
King Louie control w/o antibiotics	100	46	100	72	100	100
Pic control w/o antibiotics	100	100	100	100	100	100
Robusto control w/o antibiotics	100	100	100	100	100	100
Evola control w/o antibiotics	100	30	100	65	100	100
King Louie control with antibiotics	0	0	0	0	0	0
Pic control with antibiotics	0	0	0	0	0	0
Robusto control with antibiotics	0	0	0	0	0	0
Evola control with antibiotics	0	0	0	0	0	0

8.3.2 Chapter 3 PCR and RT-PCR data

Table 8.4: PCR data indicating the percentage of cv. King Louie, Pic and Robusto T_0 plants containing the selectable marker transgenes *npt*II and *luc*.

Lettuce cv.	Transgene(s) present			
	*npt*II only (%)	*luc* only (%)	*npt*II and *luc* (%)	No genes (%)
King Louie	6	12	77	5
Pic	16	6	75	3
Robusto	11	3	79	7
Total data	9	9	77	5

Table 8.5: RT-PCR data indicating the percentage of cv. King Louie, Pic and Robusto T_0 plants expressing the transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I.

Lettuce cv.	Transgene(s) expressed					
	*npt*II (%)	*luc* (%)	*gsh*I (%)	*gsh*II (%)	*phgpx* (%)	*gor*I (%)
King Louie	78	46	76	44	46	46
Pic	81	22	66	66	66	9
Robusto	64	4	76	64	44	4
Total data	76	28	72	56	52	23

Table 8.6: RT-PCR data indicating the number of expressed transgene(s) as percentage of cv. King Louie, Pic and Robusto T_0 plants.

Lettuce cv.	Number of transgene(s) expressed						
	All 6 genes (%)	Any 5 genes (%)	Any 4 genes (%)	Any 3 genes (%)	Any 2 genes (%)	Any 1 gene (%)	No genes (%)
King Louie	34	7	5	7	12	29	5
Pic	6	6	28	25	25	6	3
Robusto	4	0	8	40	36	8	4
Total data	17	5	13	21	22	16	4

Table 8.7: RT-PCR data indicating the percentage of cv. King Louie T_1 and T_2 plants expressing the transgenes *npt*II, *luc*, *gsh*I, *gsh*II, *phgpx* and *gor*I.

Lettuce cv. / generation	Transgene					
	*npt*II (%)	*luc* (%)	*gsh*I (%)	*gsh*II (%)	*phgpx* (%)	*gor*I (%)
King Louie line 32 / T_1	100	60	100	100	100	100
King Louie line 43 / T_1	100	80	100	100	80	100
King Louie line 44 / T_1	100	100	100	100	100	100
King Louie line 32.4 / T_2	100	16	98	95	89	75
King Louie line 43.17 / T_2	100	8	100	100	100	92
King Louie line 44.2 / T_2	100	8	100	100	92	25

8.3.3 Chapter 4 assay data

8.3.3.1 Shelf-life assays

Table 8.8: Chlorophyll a concentration (µg g^{-1} FW) in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period. Abbreviation, N/A, not applicable.

Plant type	Line	Days / chlorophyll a concentration (µg g^{-1} FW)							
		0	2	5	7	10	14	18	21
Homozygous	32.4	28.82	27.01	21.88	21.17	13.42	13.65	6.86	3.47
	43.17	28.42	25.61	21.89	14.21	9.06	10.58	10.80	5.63
	44.2	40.17	29.18	28.39	29.05	23.89	19.03	7.55	8.97
Azygous	32.9	29.25	22.28	16.45	16.78	5.79	6.76	6.02	3.95
	43.16	28.20	25.73	16.02	11.58	9.60	9.21	7.05	4.72
	44.12	28.68	25.03	18.27	29.83	13.00	11.36	4.58	4.98
Wild-type	N/A	29.29	28.84	28.81	28.71	24.84	9.63	7.45	7.65

Table 8.9: Chlorophyll b concentration (μg g^{-1} FW) in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period.

Plant type	Line	Days/ chlorophyll b concentration (μg g^{-1} FW)							
		0	2	5	7	10	14	18	21
Homozygous	32.4	29.08	24.88	11.16	15.89	10.27	9.05	3.91	1.46
	43.17	17.58	14.82	17.05	10.42	5.78	11.86	9.60	7.48
	44.2	30.15	24.51	21.83	22.69	14.09	9.78	3.73	4.32
Azygous	32.9	24.29	12.21	8.60	12.67	2.94	7.42	5.08	6.15
	43.16	20.45	15.72	8.10	6.56	9.01	9.38	9.33	2.57
	44.12	30.15	18.11	10.35	23.71	9.02	9.16	4.53	2.05
Wild-type	N/A	29.10	28.59	18.31	24.47	13.54	4.52	3.59	3.16

Table 8.10: Total chlorophyll concentration (μg g^{-1} FW) in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period.

Plant type	Line	Days/ total chlorophyll concentration (μg g^{-1} FW)							
		0	2	5	7	10	14	18	21
Homozygous	32.4	57.90	51.89	33.04	37.06	23.69	22.71	10.78	4.94
	43.17	46.01	40.44	38.94	24.63	14.84	22.45	20.40	13.11
	44.2	70.32	53.69	50.23	51.74	37.99	28.81	11.28	13.29
Azygous	32.9	53.54	34.49	25.06	29.45	8.74	14.18	11.10	10.10
	43.16	48.65	41.45	24.13	18.15	18.62	18.59	16.38	7.30
	44.12	58.83	43.15	28.62	53.54	22.03	20.53	9.11	7.04
Wild-type	N/A	58.39	57.43	47.13	53.18	38.38	14.16	11.04	10.81

Table 8.11: Total carotenoid concentration (μg g^{-1} FW) in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period.

Plant type	Line	Days/ total carotenoid concentration (μg g^{-1} FW)							
		0	2	5	7	10	14	18	21
Homozygous	32.4	3.79	5.05	10.07	9.14	8.24	8.83	4.76	2.65
	43.17	8.26	9.20	8.77	9.87	7.92	5.39	5.47	1.93
	44.2	4.50	5.68	6.71	6.42	9.20	10.09	6.52	5.99
Azygous	32.9	5.71	8.99	11.03	9.72	6.37	4.74	4.10	1.44
	43.16	7.05	8.78	9.87	9.44	6.11	5.98	2.49	3.17
	44.12	3.43	7.98	10.20	6.93	10.41	8.63	2.84	4.18
Wild-type	N/A	5.18	6.29	8.12	5.75	9.27	7.16	6.29	5.05

Table 8.12: Soluble protein concentration (mg g^{-1} FW) in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period.

Plant type	Line	Days / soluble protein concentration (mg g^{-1} FW)							
		0	2	5	7	10	14	18	21
Homozygous	32.4	4.16	5.66	5.49	4.89	4.07	3.70	3.45	3.42
	43.17	3.97	4.51	5.29	4.71	3.53	4.29	5.11	2.98
	44.2	3.98	5.06	5.98	5.54	4.66	4.68	4.32	3.57
Azygous	32.9	8.31	4.44	5.18	3.67	5.29	4.92	4.21	2.29
	43.16	3.95	5.10	3.99	4.77	3.12	3.92	3.18	2.64
	44.12	10.32	7.68	9.06	9.26	5.65	8.16	4.03	4.89
Wild-type	N/A	6.51	5.60	5.25	4.93	5.37	3.43	3.66	3.33

Table 8.13: Glucose concentration (mg g^{-1} FW) in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period.

Plant type	Line	Days/ glucose concentration (mg g^{-1} FW)							
		0	2	5	7	10	14	18	21
Homozygous	32.4	421.20	810.60	1327.80	780.6	979.8	1048.80	1723.20	1569.60
	43.17	1226.40	708.60	705.00	907.80	933.60	1592.40	1267.80	898.80
	44.2	591.54	367.32	394.08	1355.04	446.94	389.70	334.86	313.68
Azygous	32.9	907.20	823.80	1032.60	1200.00	982.20	1870.20	1905.60	1413.60
	43.16	604.80	860.40	953.40	921.60	941.40	951.00	1156.20	1271.40
	44.12	670.20	651.00	1225.80	859.80	921.00	1324.80	667.80	1202.00
Wild-type	N/A	876.06	485.82	194.76	710.22	521.16	246.30	255.36	207.96

Table 8.14: Fructose concentration (mg g^{-1} FW) in leaf discs of cv. King Louie homozygous, azygous and wild-type lines during a 21 d period.

Plant type	Line	Days/ fructose concentration (mg g^{-1} FW)							
		0	2	5	7	10	14	18	21
Homozygous	32.4	62.60	83.80	148.80	77.40	84.40	105.80	165.80	135.40
	43.17	127.60	43.20	71.60	65.40	68.60	164.20	107.10	113.20
	44.2	383.22	223.74	112.14	135.72	98.1	214.92	173.70	626.94
Azygous	32.9	91.60	77.00	138.00	166.98	87.20	178.00	91.80	190.20
	43.16	103.80	79.20	90.40	93.40	81.00	72.20	85.40	147.60
	44.12	94.00	30.00	141.80	85.80	79.20	168.80	85.60	1200.00
Wild-type	N/A	258.30	149.94	94.86	151.56	137.34	207.18	527.04	201.06

8.3.3.2 Saline assays

Table 8.15: Soluble protein concentration (mg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Soluble protein (mg g^{-1} FW)	Soluble protein (mg g^{-1} FW)
Homozygous	Inner leaf	32.4	3.32	4.56
		43.17	3.34	5.52
		44.2	3.72	4.32
Homozygous	Outer leaf	32.4	3.33	1.98
		43.17	3.38	3.64
		44.2	3.70	2.06
Azygous	Inner leaf	32.9	4.50	4.45
		43.16	2.88	4.46
		44.12	3.91	4.11
Azygous	Outer leaf	32.9	3.36	2.04
		43.16	2.67	1.34
		44.12	3.57	0.81
Wild-type	Inner leaf	N/A	3.26	5.00
Wild-type	Outer leaf	N/A	3.74	0.99

Table 8.16: Chlorophyll a concentration (μg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Chlorophyll a (μg g^{-1} FW)	Chlorophyll a (μg g^{-1} FW)
Homozygous	Inner leaf	32.4	62.90	147.22
		43.17	77.29	163.40
		44.2	67.23	119.06
Homozygous	Outer leaf	32.4	114.79	90.68
		43.17	111.14	97.81
		44.2	135.34	82.59
Azygous	Inner leaf	32.9	77.84	101.37
		43.16	66.25	120.22
		44.12	75.48	123.35
Azygous	Outer leaf	32.9	119.64	82.17
		43.16	119.17	68.77
		44.12	146.22	67.48
Wild-type	Inner leaf	N/A	73.47	131.28
Wild-type	Outer leaf	N/A	125.66	54.92

Table 8.17: Chlorophyll b concentration (μg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Chlorophyll b (μg g^{-1} FW)	Chlorophyll b (μg g^{-1} FW)
Homozygous	Inner leaf	32.4	25.27	62.47
		43.17	30.76	70.80
		44.2	27.41	50.95
Homozygous	Outer leaf	32.4	51.15	38.62
		43.17	49.85	42.57
		44.2	60.28	36.41
Azygous	Inner leaf	32.9	31.37	43.82
		43.16	27.02	67.45
		44.12	29.03	52.16
Azygous	Outer leaf	32.9	52.58	35.39
		43.16	55.00	29.53
		44.12	66.29	28.47
Wild-type	Inner leaf	N/A	28.86	55.67
Wild-type	Outer leaf	N/A	57.35	23.21

Table 8.18: Total chlorophyll concentration (μg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Total chlorophyll (μg g^{-1} FW)	Total chlorophyll (μg g^{-1} FW)
Homozygous	Inner leaf	32.4	88.17	209.69
		43.17	108.05	234.20
		44.2	94.64	170.01
Homozygous	Outer leaf	32.4	165.94	129.29
		43.17	161.00	140.38
		44.2	195.62	119.00
Azygous	Inner leaf	32.9	109.21	145.20
		43.16	93.27	187.67
		44.12	104.51	175.51
Azygous	Outer leaf	32.9	172.22	117.57
		43.16	174.17	98.30
		44.12	212.50	95.95
Wild-type	Inner leaf	N/A	102.34	186.96
Wild-type	Outer leaf	N/A	183.01	78.13

Table 8.19: Total carotenoid concentration (μg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Total carotenoid (μg g^{-1} FW)	Total carotenoid (μg g^{-1} FW)
Homozygous	Inner leaf	32.4	27.34	58.27
		43.17	31.55	67.36
		44.2	28.28	50.25
Homozygous	Outer leaf	32.4	51.00	41.88
		43.17	46.90	45.13
		44.2	61.08	40.79
Azygous	Inner leaf	32.9	32.78	42.68
		43.16	27.48	53.95
		44.12	32.12	48.41
Azygous	Outer leaf	32.9	52.13	38.65
		43.16	51.86	35.21
		44.12	58.80	31.08
Wild-type	Inner leaf	N/A	29.67	53.58
Wild-type	Outer leaf	N/A	53.56	28.45

Table 8.20: Glucose concentration (mg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Glucose (mg g^{-1} FW)	Glucose (mg g^{-1} FW)
Homozygous	Inner leaf	32.4	260.47	258.77
		43.17	260.34	254.36
		44.2	257.88	259.66
Homozygous	Outer leaf	32.4	198.24	146.21
		43.17	180.23	206.43
		44.2	182.52	213.30
Azygous	Inner leaf	32.9	259.56	248.95
		43.16	256.35	258.51
		44.12	258.93	253.52
Azygous	Outer leaf	32.9	165.79	144.84
		43.16	145.53	150.72
		44.12	174.61	172.67
Wild-type	Inner leaf	N/A	258.75	256.98
Wild-type	Outer leaf	N/A	158.43	183.90

Table 8.21: Fructose concentration (mg g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Fructose (mg g^{-1} FW)	Fructose (mg g^{-1} FW)
Homozygous	Inner leaf	32.4	65.88	57.59
		43.17	55.59	48.03
		44.2	53.10	52.65
Homozygous	Outer leaf	32.4	32.02	26.14
		43.17	29.29	36.27
		44.2	28.98	34.23
Azygous	Inner leaf	32.9	47.88	30.03
		43.16	47.67	42.47
		44.12	62.47	40.11
Azygous	Outer leaf	32.9	20.89	25.25
		43.16	26.93	34.80
		44.12	29.66	42.31
Wild-type	Inner leaf	N/A	55.02	51.60
Wild-type	Outer leaf	N/A	27.72	24.81

Table 8.22: Equivalent iron II concentration (mM g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Equivalent iron II (mM g^{-1} FW)	Equivalent iron II (mM g^{-1} FW)
Homozygous	Inner leaf	32.4	3160.28	7782.93
		43.17	3170.78	8761.06
		44.2	3341.04	7154.13
Homozygous	Outer leaf	32.4	3882.07	6703.55
		43.17	5780.80	8871.18
		44.2	5858.65	9152.43
Azygous	Inner leaf	32.9	2783.31	6951.31
		43.16	2865.84	8438.86
		44.12	3118.23	8020.93
Azygous	Outer leaf	32.9	4364.29	5884.38
		43.16	3181.25	8377.12
		44.12	5137.93	6509.35
Wild-type	Inner leaf	N/A	3160.78	8204.81
Wild-type	Outer leaf	N/A	4515.63	9700.89

Table 8.23: Total phenolic compound concentration (µg GAE g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Phenolic compounds (µg GAE g^{-1} FW)	Phenolic compounds (µg GAE g^{-1} FW)
Homozygous	Inner leaf	32.4	1.31	1.55
		43.17	1.47	1.52
		44.2	1.22	1.60
Homozygous	Outer leaf	32.4	2.20	2.15
		43.17	2.23	2.11
		44.2	2.09	2.08
Azygous	Inner leaf	32.9	1.26	1.79
		43.16	1.23	1.85
		44.12	1.54	1.70
Azygous	Outer leaf	32.9	2.35	2.03
		43.16	2.17	2.23
		44.12	2.17	2.38
Wild-type	Inner leaf	N/A	1.14	1.86
Wild-type	Outer leaf	N/A	2.06	2.26

Table 8.24: Lipid peroxidation net absorbance (g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			Lipid peroxidation net absorbance (g^{-1} FW)	Lipid peroxidation net absorbance (g^{-1} FW)
Homozygous	Inner leaf	32.4	0.072	0.101
		43.17	0.082	0.122
		44.2	0.074	0.115
Homozygous	Outer leaf	32.4	0.084	0.120
		43.17	0.087	0.132
		44.2	0.087	0.132
Azygous	Inner leaf	32.9	0.073	0.093
		43.16	0.068	0.121
		44.12	0.071	0.110
Azygous	Outer leaf	32.9	0.078	0.126
		43.16	0.079	0.133
		44.12	0.078	0.177
Wild-type	Inner leaf	N/A	0.061	0.117
Wild-type	Outer leaf	N/A	0.080	0.165

Table 8.25: Glutathione concentration (nM g^{-1} FW) in inner and outer leaves of control and saline grown cv. King Louie homozygous, azygous and wild-type lines.

Plant type	Leaf type	Line	Control	Saline
			GSH (nM g^{-1} FW)	GSH (nM g^{-1} FW)
Homozygous	Inner leaf	32.4	55.74	50.79
		43.17	64.91	30.43
		44.2	66.48	33.13
Homozygous	Outer leaf	32.4	48.54	77.73
		43.17	51.18	50.40
		44.2	47.08	61.83
Azygous	Inner leaf	32.9	22.44	28.85
		43.16	22.27	14.56
		44.12	38.47	17.43
Azygous	Outer leaf	32.9	20.92	35.32
		43.16	9.78	16.20
		44.12	23.68	37.18
Wild-type	Inner leaf	N/A	25.65	11.4
Wild-type	Outer leaf	N/A	7.95	16.38

8.3.4 Chapter 5 incidence of tipburn data

Table 8.26: Incidence of tipburn in cv. King Louie homozygous, azygous and wild-type lines grown without calcium in the glasshouse at the University of Nottingham.

Plant type	Line	Tipburn index (%)
Homozygous	32.4	67
	43.17	65
	44.2	71
	Homozygous average	68
Azygous	32.9	87
	43.16	71
	44.12	89
	Azygous average	82
Wild-type	N/A	53

Table 8.27: Incidence of tipburn in cv. King Louie homozygous, azygous and wild-type lines; the first scoring of the first trial at Elsoms Seeds Ltd.

Plant type	Line	Tipburn index (%)		
		Row A	Row B	Row C
Homozygous	32.4	51	56	63
	43.17	69	20	68
	44.2	61	44	47
	Homozygous average	60	40	59
Azygous	32.9	68	27	65
	43.16	54	5	49
	44.12	58	25	61
	Azygous average	60	19	58
Wild-type	N/A	53	11	47

Table 8.28: Incidence of tipburn in cv. King Louie homozygous, azygous and wild-type lines; the second scoring of the first trial at Elsoms Seeds Ltd.

Plant type	Line	Tipburn index (%)		
		Row A	Row B	Row C
Homozygous	32.4	62	63	77
	43.17	97	20	73
	44.2	89	56	80
	Homozygous average	83	46	76
Azygous	32.9	92	52	92
	43.16	71	6	68
	44.12	96	20	88
	Azygous average	86	26	83
Wild-type	N/A	79	19	67

Table 8.29: Incidence of tipburn in cv. King Louie homozygous, azygous and wild-type lines; the first scoring of the second trial at Elsoms Seeds Ltd.

Plant type	Line	Tipburn index (%)		
		Row A	Row B	Row C
Homozygous	32.4	65	62	77
	43.17	93	25	87
	44.2	85	47	82
	Homozygous average	81	44	82
Azygous	32.9	83	35	90
	43.16	75	9	74
	44.12	76	31	92
	Azygous average	78	25	85
Wild-type	N/A	65	28	35

Table 8.30: Incidence of tipburn in cv. King Louie homozygous, azygous and wild-type lines; the second scoring of the second trial at Elsoms Seeds Ltd.

Plant type	Line	Tipburn index (%)		
		Row A	Row B	Row C
Homozygous	32.4	87	91	89
	43.17	96	57	98
	44.2	90	48	95
	Homozygous average	91	65	94
Azygous	32.9	89	53	94
	43.16	83	43	94
	44.12	83	40	100
	Azygous average	85	45	96
Wild-type	N/A	73	56	56