Manipulating Biochemical Pathways in Rice

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ABSTRACT

The brown planthopper, *Nilaparvata lugens*, is a pest of rice in tropical regions. Its direct feeding results in loss in yield and plant death ("hopper burn"). Several compounds that stimulate insect attraction have been detected in rice plants colonised by *N. lugens*, including 1,2-dimethoxybenzene or veratrole. Electrophysiological studies and high-resolution gas chromatography have identified veratrole as an attractant of *N. lugens*. Veratrole is a product of salicylic acid, a derivative of the phenyl propanoid pathway. Salicylic acid is decarboxylated to catechol, a step which is encoded by salicylate hydroxylase. Catechol is subsequently methylated to veratrole, which is released as a volatile compound from rice leaves.

Mature scutellum-derived rice calli from (*Oryza sativa*) cv.Taipei 309 were transformed, using microprojectile bombardment, with pROB5 containing the *hpt* gene conferring resistance to the antibiotic hygromycin and pSLJ7307 carrying the *nahG* gene derived from *Pseudomonas putida* and coding for the enzyme salicylate hydroxylase. Following selection on hygromycin-containing medium, 17 independent transgenic rice plants were regenerated from >3600 bombarded calli, with a transformation frequency of 0.47%.

Transgenic plants were confirmed by RT-PCR. Plant lines were classified as high expressors (10 lines) and low expressors (7 lines) depending on salicylate hydroxylase production. All transgenic lines exhibited higher enzyme activity than wild-type plants. Transgenic plants produced had altered metabolism for antioxidant enzymes such as catalase, ascorbate peroxidase and superoxide dismutase and reactive oxygen species such as hydrogen peroxide.

Plants unable to accumulate salicylic acid exhibited delayed transcription of pathogenesis related genes and may therefore be compromised in their ability to respond to pathogen attack and mechanical wounding.

Enhanced veratrole production was corroborated using gas chromatography of volatiles released from transgenic undamaged and mechanically damaged plants. Bioassays indicated that *N. lugens* were more attracted to high expressing plants than to wild-type plants, making more visits to areas containing transgenic rice leaves than areas containing non-transformed leaves and spending longer in these areas. Manipulating the production of veratrole by enhancing salicylate hydroxylase activity has therefore modified attraction of the *N. lugens* for high expressing *nahG* positive rice plants.

CONTENTS

ABBREVIATIONS

2,6-INA	2,6-dichloroisonicotinic acid
2,4-D	2,4-dichlorophenoxyacetic acid
AA	medium for cell suspensions (see Appendix)
ANOVA	analysis of variance
AsA	ascorbic acid
ACS	ascorbate
AO	ascorbate oxidase
APX	ascorbate peroxidase
BAP	6-benzylaminopurine
bar	bialaphos resistance gene
BG	plants regenerated from calli subjected to microprojectile bombardment with uncoated gold
bp	base pairs
BPH	brown planthopper
Bph/bph	resistance genes to brown planthopper
BSA	bovine serum albumin
CAMV35S	cauliflower mosaic virus promoter
cat	chloramphenicol acetyl transferase gene
cata/catb	catalase gene
CAT	catalase enzyme
cDNA	complementary DNA recovered from RNA i.e.without introns
CIAP	calf intestinal alkaline phosphatase
cm	centimetre
C2/C12/C14	plants recovered from Taipei 309 calli maintained in culture but not subjected to transformation
cv.	cultivar
d	day
dATP	deoxy-adenisone triphosphate
dCTP	deoxy-cytidine triphosphate
dGTP	deoxy-guanosine triphosphate
dTTP	deoxy-thymidine triphosphate
dUTP	deoxy-uridine triphosphate

d.f.	degrees of freedom
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DTT	dithiothreitol
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FDA	fluorescein diacetate
G	grams
g	acceleration due to gravity
GC	gas chromatography
GC/MS	gas chromatography mass spectrometry
GNA	snowdrop lectin protein
GR	glutathione reductase
GSH	γ-glutamylcysteinyl glycine
GST	glutathione S transferase
gus	β-glucuronidase gene
GUS	β-glucuronidase protein
h	hours
ha	hectare
HPLC	high performance liquid chromatography
hpt	hygromycin phosphotransferase gene
IRRI	The international rice research institute Manila Philippines
JA	jasmonic acid
kb	kilobase
kD	kilodalton
KI	Kovat's index
kV	kilovolts
l	litre
LBA4404	strain of *Agrobacterium tumefaciens*
LB medium	Luria Bertani medium
LS	Linsmaier and Skoog (1965) medium
Ltd.	Limited Company
m	metre

M	molar
MAPK	mitogen activated protein kinase
MDA	monodehydroascorbate
MDAR	monodehydroascorbate reductase
mg	milligram
min	minutes
ml	millilitre
µl	microlitre
mM	millimolar
µM	micromolar
mm	millimetres
mRNA	messenger RNA
ms	mass spectrometry
MS	Murashige and Skoog (1965) medium
mV	millivolt
µV	microvolt
NAA	1-napthaleneacetic acid
NAD	nicotine adenine dinucleotide
nM	nanomolar
nm	nanometres
n	haploid number
nahG	the gene coding for salicylate hydroxylase in *Pseudomonas putida*
NOS	a 3' terminator from *Agrobacterium tumefaciens*
*npt*II	neomycin phosphotransferase gene
PA	peak area
PB1	Pusa Basmati 1 rice cultivar
pBBR1MCS	plasmid carrying the *virG54D* gene
pMOGB22	plasmid carrying the *gus* and *bar* genes
PMOG1/PMOG3	plants regenerated from calli transformed with *A. tumefaciens* LBA4404 containing pBBR1MCS, pSLJ7321::ROB5 and pMOGB22
pr1	pathogenesis related gene 1
pr5	pathogenesis related gene 5
pbz	pathogenesis gene inducible by paclobutrazole

PS1	photosystem 1
pSLJ7307	plasmid carrying the *nahG* gene for microprojectile bombardment
pSLJ7321	plasmid carrying the *nahG* gene for *Agrobacterium*- mediated transformation
pACH25	plasmid carrying the genes for *gus* and *bar*
pROB5	plasmid carrying the *hpt* gene
PCR	polymerase chain reaction
pH	the potential for hydrogen a logarithmn of the reciprocal of hydrogen ion concentration i.e. $\log_{10}(1/H^+)$
PMSF	phenyl methane sulphonyl flouride
pp	pages
PR	pathogenesis related
psi	pound per square inch
PVP	polyvinyl pyrrolidene
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT	retention times
RT-PCR	reverse transcription followed by polymerase chain reaction
SA	salicylic acid
SAH	salicylate hydroxylase
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	seconds
SIPK	salicylic acid induced protein kinase
SOD	superoxide dismutase
SSC	buffer made with salt (NaCl 750mM) and sodium citrate (75mM)
t	tonnes
T309	Taipei 309 rice cultivar
TAE	Tris acetate EDTA (see Appendix)
TBE	Tris borate EDTA (see Appendix)
T-DNA	transfer DNA
TEMED	N, N, N, tetramethylenthylenediamine

T1	progeny of initial transformants
T2	progeny of T1 plants
TE	tris EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
TritonX-100	octylphenolpoly(ethyleneglycolether)
tRNA	transfer RNA
UK	United Kingdom
USA	United States of America
u.v.	ultra violet
V	volts
Vir	virulence protein
vir	virulence gene
VIRG	plants regenerated from calli transformed with *A. tumefaciens* LBA4404 containing pBBR1MCS and pSLJ7321::ROB5
virG54D	*virG* gene responsible for initiating the signal cascade to enable *Agrobacterium*- mediated gene delivery
vol	volume
v/v	volume to volume
Vcm^{-2}	volts per centimetre squared
WIPK	wound induced protein kinase
w:v (w/v)	weight to volume
X-gluc	5-bromo-4-chloro-3-indolyl-D glucuronic acid
%	percentage
^{0}C	degrees Celsius
<	less than
>	greater than
=	equals
Ω	resistance
λ	lambda

CHAPTER 1. GENERAL INTRODUCTION

1.1 IMPORTANCE AND YIELD OF RICE

Rice (*Oryza sativa* L.) is one of the most important food crops, since it provides the staple diet for over a third of the worlds population. In excess of 120,000 varieties of rice exist, which date back 7,000 years (White, 1994). The increasing world population has promoted research to increase rice production. The International Rice Research Institute (IRRI), established in 1962, generated dwarf plants with a shorter growing period, enabling multiple cropping while the use of high tillering semi-dwarf varieties led to an increase in yield from 2-3 tha^{-1} to 8-10 tha^{-1} (Virimani and Sharma, 1993). World rice production has more than doubled in the past 30 years from 257 million t in 1966 to 560 million t in 1998. Ninety one percent of the total crop, (> 500 million t) is produced in Asia, where it provides a fundamental component of the diet of 3.1 billion people. World population is predicted to increase to 8.5 billion by the year 2025 and, consequently, world rice production will also have to increase by 70% (Paroda, 1998). Recently, 10-20% yield increases have been reported from conventional crossing of the cultivated rice *Oryza sativa* with *O. rufipogon, O. glaberrima* or *O. barthii* with improvements being attributed to heterosis (McCrouch, 1999).

1.2 MORPHOLOGY AND GROWTH OF RICE

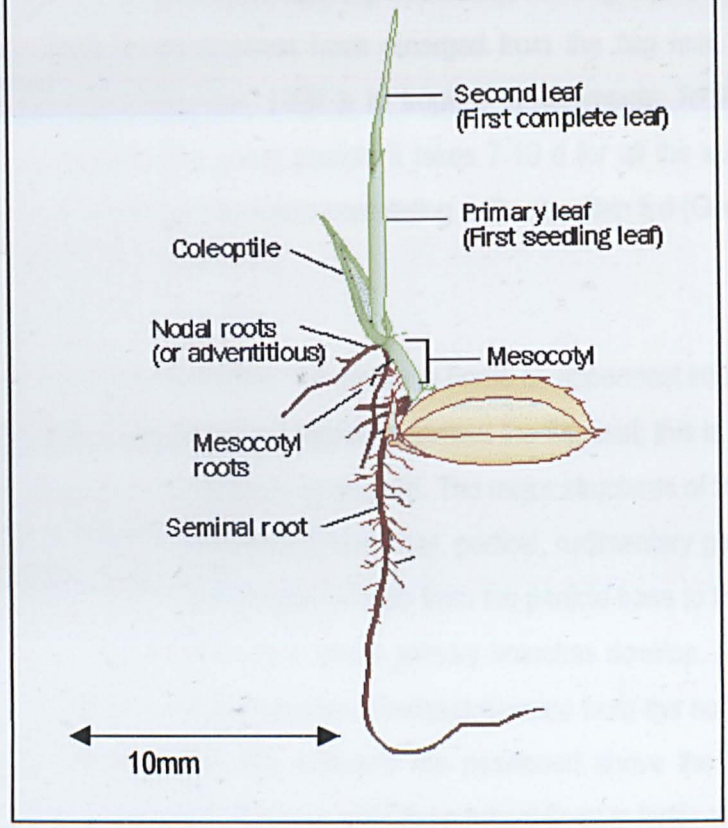
Rice *Oryza sativa* belongs to the phylum Angiospermae, class Monocotyledonae, family *Gramineae* sub family *Oryzoidae* and from the tribe *Oryzeae*. It is an annual grass with a round hollowed jointed stem with a culm, flat sessile leaf blades and terminal panicles (Grist, 1986). The growth duration of the plant is 3-6 months, depending on the variety and the environment, during which time, it completes vegetative and reproductive growth phases. The vegetative phase is subdivided into germination, early seedling growth, and tillering; the reproductive phase constitutes the time before and after heading. Potential grain yield is determined primarily before heading. However, the final yield, based on the amount of starch that fills the spikelets, is determined after heading. Hence, agronomically it is convenient to regard the life history of rice in terms of three growth phases namely, vegetative, reproductive and ripening. A 120-day variety, when planted in a tropical environment, is about 60 d in the vegetative phase, 30 d in the reproductive phase and 30 d in the ripening phase (Nanda, 2000).

1.2.1 Vegetative phase

Germination and seedling development occur after dormancy has been broken. The seed absorbs adequate water and is exposed to a temperature of 10 - 40 ^{0}C. Under aerated conditions the seminal root is the first to emerge from the embryo, and this is followed by the coleoptile (Figure 1.1).

Under anaerobic conditions, the coleoptile is the first to emerge, with the roots developing when the coleoptile has reached the aerated regions of the environment. If the seed develops in the dark, a short stem (mesocotyl) develops, which lifts the crown of the plant to just below the soil surface. After the coleoptile emerges it splits and the primary leaf develops. The second leaf is differentiated into sheath, blade, ligule and auricle whilst the primary leaf has no blade, the stem has many nodes and is enveloped by the leaf sheath (Grist, 1986). Each stem or plant of rice has nodes and internodes. The internodes vary in length depending on the variety and environmental conditions, but generally increase from the lower to upper part of the stem (Grist, 1986). Each upper node bears a leaf and a bud, which can grow into a tiller. The number of nodes varies from 13 to 16 with only the upper 4 or 5 separated by long internodes. Under rapid increases in water level some deepwater rice varieties can also increase the lower internode lengths by over 30 cm each. The leaf blade is attached at the node by the leaf sheath, which encircles the stem. Where the leaf blade and the leaf sheath meet is a pair of clawlike appendages, called auricles, which encircle the stem. Coarse hairs cover the surface of the auricle. Immediately above the auricle is a thin, upright membrane called the ligule (Grist, 1986). The tillering stage commences as soon as the seedling is self-supporting and has five leaves and generally terminates at panicle initiation. This first tiller develops between the main stem and the second leaf from the base of the plant. Subsequently, when the 6th leaf emerges, the second tiller develops between the main stem and the 3rd leaf from the base. Primary tillers emerge from the main stem and may generate secondary tillers, which may in turn generate tertiary tillers. These are produced in a synchronous manner. Although the tillers remain attached to the plant, at later stages they are independent because they produce their own roots. Varieties and races of rice differ in tillering ability. Environmental factors also affect tillering including spacing, light, nutrient supply, and cultural practices.

Figure 1.1
Rice seedling development
http://www.riceweb.org



The vegetative phase is characterised by active tillering, a gradual increase in plant height and leaf emergence at regular intervals. Tillers that do not bear panicles are called ineffective tillers. The number of ineffective tillers is a closely examined trait in plant breeding, since it is undesirable in irrigated varieties, but sometimes an advantage in rainfed lowland varieties where productive tillers or panicles may be lost due to unfavourable conditions.

1.2.2 Reproductive phase

The reproductive growth phase involves culm elongation (which increases plant height), a decline in tiller number, emergence of the flag leaf (the last leaf), booting, heading, and flowering of the spikelets. Panicle initiation is the stage about 25 d before heading when the panicle has grown to about 1 mm in length and can be recognised visually following stem dissection (Grist, 1986).

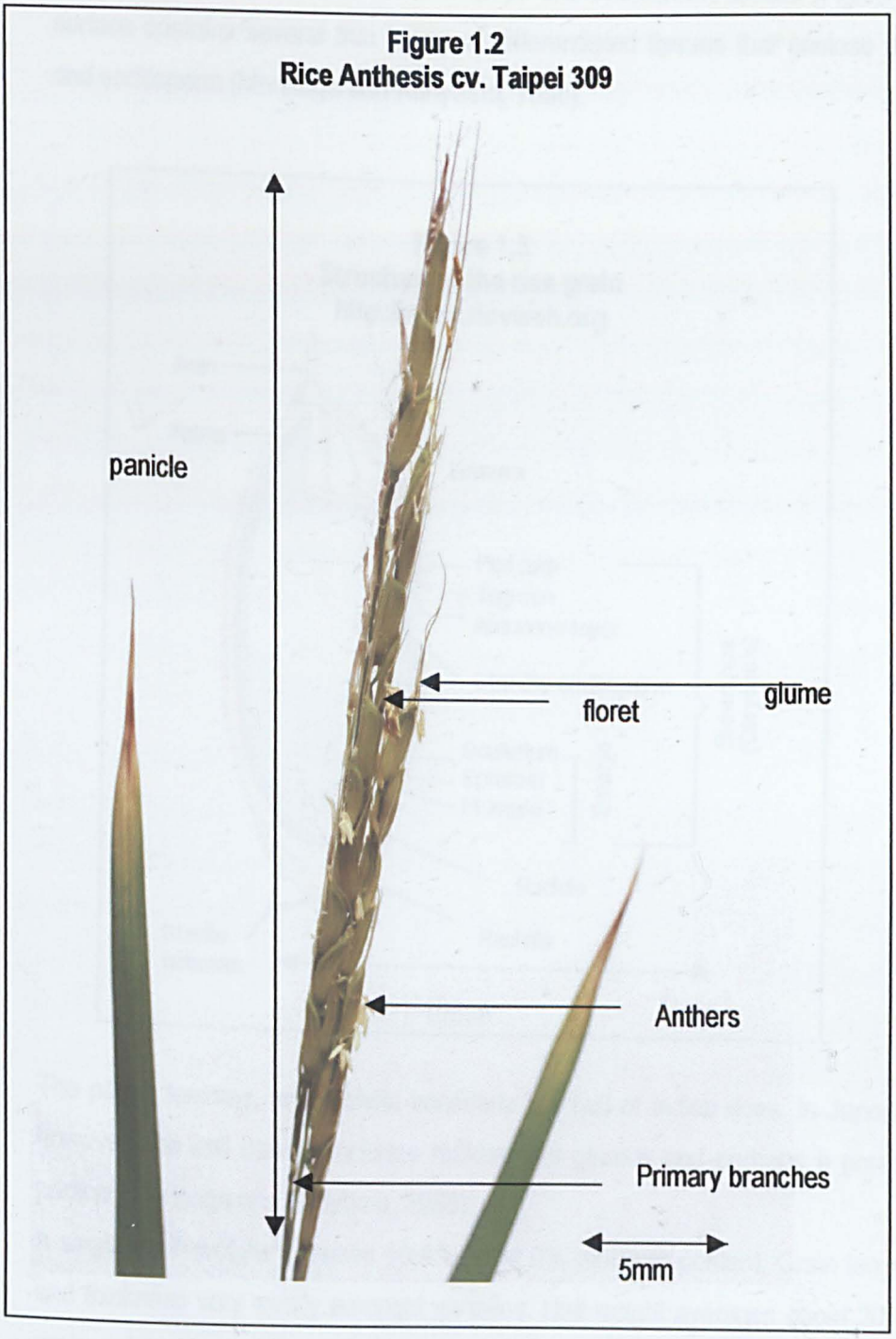
Spikelet anthesis begins with panicle heading, with 10-14 d for the rice crop to complete heading because there is variation in panicle development amongst tillers of the same plant and amongst plants in the same field. Agronomically, heading is usually defined as the time when 50% of the panicles have emerged from the flag leaf. Anthesis normally occurs between 1000 and 1300 h in tropical environments; fertilisation is completed within 6 h. Within the same panicle it takes 7-10 d for all the spikelets to complete anthesis; with individual spikelets completing anthesis within 5 d (Grist, 1986).

1.2.3 Anthesis

Rice plants produce branched panicles. Spikelets are borne on uppermost internodes of the culm. The uppermost leaf below the panicle is termed the flag leaf; this is important in carbohydrate synthesis and influences grain yield. The major structures of the panicle are the base, axis, primary and secondary branches, pedicel, rudimentary glumes and the spikelets (Figure 1.2). The panicle axis extends from the panicle base to the apex; it has 8-10 nodes at 2 - 4cm intervals from which primary branches develop. Secondary branches develop from the primary branches. Pedicels develop from the nodes of the primary and secondary branches. The spikelets are positioned above the branches. Each spikelet consists of 3 florets. However, only the terminal floret is fertile whilst the 2 lower florets are reduced to sterile sack-like lemmas. Inside the 2 glumes are the lemma and palea of the fertile floret. The pistil contains a single celled ovary and ovule. The six stamens are supported on slender filaments Inside the lemma and palea. The two glumes open for a short time and the anthers dehisce (Grist, 1986).

Rice is normally self-pollinated; cross-pollination is rare with rice being highly homozygous (Grist, 1986). The ovary gradually develops after fertilisation into the caryopsis, leading to the mature seed.

Figure 1.2
Rice Anthesis cv. Taipei 309

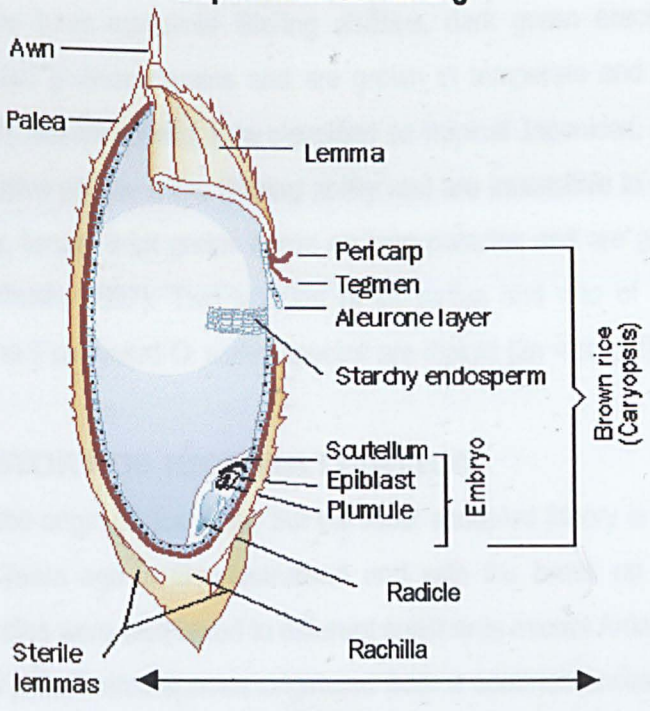


1.2.4 Ripening

Ripening may be subdivided into milky, dough, yellow-ripe, and maturity stages. These terms are based primarily on the texture and colour of the developing grains. The length of ripening varies among varieties from about 15 to 40 d. Rice varieties differ in growth duration, with some photoperiod insensitive varieties maturing in less than 80 d from

seed sowing to seed maturity. The rice grain (caryopsis) is enclosed by a husk (lemma and palea) and mainly consists of embryo and endosperm tissues (Figure 1.3). The surface contains several thin layers of differentiated tissues that enclose the embryo and endosperm (Morinaga and Kuriyama, 1958).

Figure 1.3
Structure of the rice grain
http://www.riceweb.org



The palea, lemmas, and rachilla constitute the hull of indica rices. In Japonica rices, however, the hull usually includes rudimentary glumes and perhaps a portion of the pedicel (Morinaga and Kuriyama, 1958).

A single grain weighs between 10-45 mg at 0% moisture content. Grain length, width, and thickness vary widely amongst varieties. Hull weight averages about 20% of total grain weight (Grist, 1986). The embryo consists of an embryogenic axis with a plumule and a radicle. The scutellum and the epiblast enclose the plumule, whilst the coleoptile encloses the young leaves (Grist, 1986).

1.3 CLASSIFICATION OF RICE

Cultivated species are divided into 3 types Indica, Japonica, also called Sinica (Grist, 1986) and Javanica (Morinaga and Kuriyama, 1958). The types of rice are classified on the basis of demographic races, different geographical origins, plant morphology and environmental adaptation. The different types vary in their agronomic characters such as grain size, shape, morphology and temperature sensitivity. Indica rices grown in the tropics and sub-tropics are tall with large numbers of tillers, pendant leaves and long slender non-glutinous grains that remain separated.

Japonica rices have moderate tillering abilities, dark green erect leaves, produce shorter rounded glutinous grains and are grown in temperate and subtropical zones (Khush, 1997). Javanica rices, now classified as tropical Japonicas, are tall plants with a long vegetative phase, a low tillering ability and are insensitive to photoperiod. They produce long, broad, thick grains borne on long panicles and are grown in Indonesia, and Java (Khush, 1997). Two species of *O. sativa* and one of *O. glaberrima* are cultivated. The 2 cultivated *O. sativa* species are diploid (2n =2x =12) (Oka, 1991).

1.4 HISTORY OF RICE CULTIVATION

Theories of the origin of rice differ, but the most accepted theory is that rice originated 130 million years ago in Gondwanaland and with the break up of Gondwanaland, different species were distributed to different continents except Antartica (Chang, 1976; Khush, 1997). Cultivated species originated from a common ancestor, probably in the foothills of the Himalayas from which they spread to Western and Northern India and then South to Sri Lanka, where rice was a major crop as long ago as 1000 BC. It was brought to Greece and the neighbouring countries of the Mediterranean by 324 BC, possibly by soldiers returning from the campaigns of Alexander the Great, although it did not become an established crop in Europe until the 15th or 16th century. Rice also spread from India to Madagascar and to East and West African countries. The crop was brought to North and South America after European colonisation (Khush, 1997).

1.5 PESTS AND DISEASES OF RICE

Many species of organisms inhabit rice fields. For example, some 500 species of arthropods (insects and spiders) may appear in a rice field in a given season, but only a very few are a potential threat to the crop. Most are beneficial or have no obvious effect,

and include a wide range of predatory and parasitic natural enemies that contribute to keeping in check the insect pest organisms (Paroda, 1998).

Species of potentially harmful organisms (Table 1.1) include insects, pathogens, molluscs, and rodents. Some are herbivores that feed on the rice plant, others are parasitic disease organisms. Although only a few pest species cause sufficient yield reductions to require intensive control measures, they are frequently cited as major constraints to production. Potential losses of up to 55% before harvest have been estimated, but these estimates often represent the worst case or highest levels of loss with actual losses being much less. There have been serious outbreaks of insects such as *Nilaparvata. lugens* (Brown planthopper, BPH) and diseases such as blast, tungro virus, and sheath blight over large areas of cultivation (http://www.riceweb.org).

Rice is susceptible to pests and diseases (Table 1.1), which are major factors in yield loss. From 1965-1995, the number of insects classified as major pest status rose from 3 to 13. Planthoppers assumed major pest status after the green revolution. The advent of monoculture cropping of high yielding varieties with a narrow genetic base together with the excessive use of synthetic nitrogenous fertilisers, the absence of crop diversification and double cropping in rice systems accentuated insect and disease problems. In South East Asia, it has been estimated that over 600 million $US has been lost annually due to the insect attack with more that two thirds of the loss due to *N. lugens* and yellow stemborer (*Scirpophaga incertulas*) (Nanda, 2000). In 1975, *N. lugens* infested nearly 80% of the rice fields in the Republic of Korea. Crop loss in affected fields reached 20-30%. *N. lugens* destroyed 200,000 ha of rice between 1975 and 1980. In Indonesia, a virus disease destroyed 100,000 ha between 1972 and 1975 in South Sulawesi alone. In the early 1900s, insects and diseases caused widespread famine in Japan and China (Paroda, 1988). Calamities such as these encouraged crop protection approaches that emphasised eradication and prevention. Those approaches caused the use of agricultural pesticides to increase dramatically.

Although insect pest and disease resistance genes have been introduced into rice cvs., pesticide use has not declined. Pesticides are often uneconomical and they may disrupt the ecological balance of pests and their natural enemies. *N. lugens* outbreaks are often a direct consequence of insecticide use, which wipes out predators that regulate *N. lugens* and sometimes stimulate increased fecundity in surviving females (Nanda.2000). Besides often causing the pest problems, agricultural chemicals may pose a serious threat to humans and the environment.

Modern approaches to crop protection rely on integrated pest management rather than control or eradication. In this approach, a species is considered a pest only when it reaches numbers that can reduce yield. Factors, such as natural enemies, that prevent pests from increasing are emphasised. Pesticides are used as a last resort to bring abnormal pest densities down when crop loss is expected to exceed the cost of treatment. Rice cvs. that are resistant to major pests are grown, as these cvs. do not need prophylactic treatment to control the insects or diseases to which they are resistant (http://www.riceweb.org Jan 2003).

Table 1.1 Major pests and diseases of rice

Stem borers
Yellow stem borer (*Scirpophaga incertulas*), White stem borer (*Scirpophaga innotata*), Striped stem borer (*Chilo suppressalis*), Dark-headed rice borer (*Chilo polychrysus*)
Defoliators
Rice leaffolders (*Cnaphalocrocis medinalis*), Rice caseworm (*Nymphula depunctalis*)
Leafhoppers
Green leafhopper (*Nephotettix virescens, N. nigropictus, N. parvus, N. cincticeps*)
Planthoppers
Brown planthopper (*Nilaparvata lugens*), Whitebacked planthopper (*Sogatella furcifera*)
Rice bugs
Malayan black rice bug (*Scotinophara coarctata*), Rice grain bug (*Leptocorisa oratorius*)
Viral diseases and their vectors
Rice tungro (*Nephotettix virescens, N. nigropictus*), Ragged stunt (*Nilaparvata lugens*)
Bacterial diseases
Bacterial blight (*Xanthomonas oryzae pv. Oryzae*)
Fungal diseases
Blast (*Pyricularia oryzae Cav.*), Sheath blight (*Rhizoctonia solani, Thanatephorus cucumeris*)

Nilaparvata lugens, Hemiptera Delphacidae sub-order Homopetera is a small brownish piercing sucking insect that attacks rice in all stages of growth from the seedling stage to the milky grain stage (Figure 1.4). This insect is a devastating pest of rice in tropical and temperate regions. Its feeding blocks phloem vessels and results in loss in yield and plant death ("hopper burn") with 100% yield loss (Sögawa and Cheng, 1979). The pest also transmits grassy stunt and ragged stunt viruses, reducing plant height and the leaf area with a corresponding reduction of photosynthesis and a 50-90% loss in yield (Nanda, 2000).

1.6 *NILAPARVATA. LUGENS*

1.6.1 Morphology of *N. lugens*

The adult has two forms; the macropterous form is 4.0-4.5 mm in length (Figure 1.4) and the shorter winged bachypterous form (3.3-3.5 mm).

Figure 1.4
Nilaparvata lugens
(adult macropterous form)

The eggs are laid as a mass in the tissue of the lower parts of rice plants mainly in the leaf sheaths and blades with each female producing 550-600 eggs (Suenaga, 1965). In the tropics eggs hatch after 7-11 d. Although migration of large numbers of *N. lugens* has been reported into temperate areas, the insect does not winter north of the Tropic of Cancer (Riley *et al.*, 1987). Five nymphal stages each last 10-15 d depending on the temperature with the adult living approximately 28 d. High population densities during the nymphal stages increase the number of macropterous adults (Murata, 1930), as does the deterioration in quantity and quality of food. Macropterous adults have the

ability to travel long distances (Watnabe *et al.*, 1988). The female progeny of such adults may develop into brachypterous adults due to low population density and favourable food supply (Kisimoto, 1956).

Second to fifth instar nymphs and adults aggregate and feed on leaf sheaths at the base of rice plants. The insects penetrate the plants to a depth of 0.7mm with modified mandibles and maximillae, which constitute the stylet. Feeding removes assimilates, blocks phloem vessels and reduces the photosynthetic rate, leading to yellowing of older leaf blades. This loss of chloryphyll extending to all parts of the plant above the ground is followed by dehiscence and abscission of foliage (Watnabe and Kitagawa, 2000). A single adult female removes 2mg or more of sugar per day from a host plant (Sögawa, 1982).

Several factors influence the potential of *N. lugens* to become a major pest. The high fecundity of insects, their tolerance to overcrowding and the ability of the adults to disperse together with their adaptability to different rice cvs., has contributed to the increase in this pest (Miyata, 1989). The production of photoperiod insensitive rice cvs. led to multiple crops being produced annually. The increase in cropping intensity of cvs. with a narrow genetic base and closer spacing of plants in the field assist the carry over of pests. The intensive use of fertilisers and irrigation systems increased production of more susceptible rice varieties (Masri, 1995). The use of chemical pesticides killed bio-control agents whilst stimulated the reproduction of *N. lugens* at sub-lethal doses (Nanda, 2000), disrupting the ecological balance of natural enemies. All of these factors have contributed to the rise in status of *N. lugens* as a major pest of rice.

1.6.2 Chemical control of insects

Until the 19th century, whale oil was applied as a spray to control *N. lugens*. However, in 1840, mineral oil was used with the addition of pyrethrum and nicotine (Suenaga and Nakatsuka, 1958). Benzene hexachloride was employed in Japan in 1949 against both *N. lugens* and striped stemborer (*Chilo suppressalis*) until resistance was detected in 1967 (Miyata, 1989). This chemical was banned in 1971 after it was found in the milk of cows that had consumed rice straw (Nagata, 1982). Organophosphates such as parathion, soon replaced pyrethrum. Between 1967 and 1969, resistance developed to several insecticides such as carbaryl, malathion and fenitrothion. The International Rice Research Institute (IRRI) used diazinon for 10 successive rice crops. However, control

declined in 1969. In 1975 *N. lugens* infested nearly 80% of rice fields in the republic of Korea. Crop loss in affected fields reached 20-30%, *N. lugens* destroyed 200,000 hectares of rice between 1975 and 1980. IRRI recommendations for *N. lugens* control 2001 included the use of buprofezin, deltamethrin, cartap, fipronil, carbaryl and isoprocarb (http://www.irri/tropicrice/insect/control.html, 2001). The resistance of *N. lugens* to carbamates and organophosphates was reported in Taiwan (Miyata, 1989). Pyrethroid resistance has subsequently been reported in Taiwan (Dai and Sun, 1984). Insect resistance and insect resurgence is therefore a serious problem in rice management. Stringent regulations that govern environmental pollution and limitations on the use of insecticides due to mammalian toxicity mean it is unlikely that new insecticides will be developed to replace those to which *N. lugens* is resistant.

1.6.3 Biological control of brown planthopper

Predators, parasites and pathogens have been used to reduce the pest population since there are more than 200 natural enemies of *N. lugens* (Ooi, 1988). Predators of eggs, nymphs and adults are more efficient at controlling pest infestations than parasitoids (Ooi, 1988; Hinckley, 1963). The most common predator is the mirid bug, (*Cytorhinus lividipennis*) which feeds on all stages of *N. lugens* but primarily on eggs. The bug is widely distributed in many countries where *N. lugens* occurs (Ooi, 1988; Hinckley, 1963). At least 16 species of spider have been identified as preying on *N. lugens,* while other predators include various species of coccinellids (Ooi, 1988; Kenmore 1980). Egg, nymphal and adult parasitism all occur. Parasitism of egg stages is higher than parasitism of other stages and occurs widely in Thailand (Miura *et al.*, 1979), in Sri lanka (Otake *et al.*, 1976), in The Philippines (Pena and Shepard, 1986), and in Malaysia (Ooi, 1988).

1.6.4 Breeding for resistance to *N. lugens* in rice

There are two basic types of resistance, monogenic complete race-specific resistance and polygenic incomplete race-nonspecific resistance. Partial resistance is quantitative and may be equated to field resistance. IRRI has identified five dominant and five recessive genes for resistance to *N. lugens*, these genes and cvs. released with these genes are listed in Table 1.2.

Given a choice insects prefer susceptible varieties for alighting, shelter and oviposition possibly due to semiochemical involvement. More widespread and distinct physiological

resistance involves antibiotic effects of the resistant plant on the insect pest, resulting in reduced nymphal survival, poor growth and development, weakened adults and low build-up of pest populations through generations.

Table 1.2 Resistance genes and cultivars containing them

Gene	Cv./variety
Bph1	Mudgo
bph2	ASD7
Bph3	Rathu Heenati
bph4	Babawee
bph5	ARC10550
Bph6	Swarnalatha
bph7	T12
bph8	Chin saba
Bph9	Balamawee
Bph10	IR65482-4-136-2-2

In 1973, IRRI released the variety IR26, which contained the dominant resistance gene *Bph1*. There was an immediate and dramatic reduction of *N. lugens* numbers in all countries where IR26 was planted. Within two years, the single gene resistance of *Bph1* was overcome and massive outbreaks of *N. lugens* were reported on IR26 and other rice varieties containing the *Bph1* gene (Gallagher, 1988). In 1976, IR36 and other varieties containing the recessive resistance gene *bph2* were released, with subsequent widespread planting. Resistance broke down in 1985 in Indonesia and in 1986 in Thailand (Kenmore, 1991). Supan 60, IR56 and other varieties containing the dominant resistance gene *Bph3* were planted in Indonesia in 1982. Since then, *N. lugens* populations in Indonesia have adapted and are able to develop on these rice varieties. The cycle of high intensity rice production and resultant insect outbreaks threatens food security. Therefore, gene pyramiding is the preferred option to develop insect resistant rice varieties.

1.6.5 Genetic manipulation approaches

Other approaches to contain *N. lugens* include the use of snowdrop lectin (GNA) protein genetically engineered into rice. Since this protein has been shown to bind to the insect mid-gut producing an anti-feedant effect resulting in decreased insect survival developed from an inability to assimilate nutrients. Planthopper nymphs avoided the genetically modified plants. However, tissue-specific resistance resulted in less pronounced avoidance that took longer to develop (Tang *et al.*, 2001; Fiossac *et al.*, 2000). Novel genes have been inserted into rice to confer resistance against viral diseases. Successful transformations include a gene encoding for the coat viral protein of ragged stunt virus used to genetically engineer rice plants (Upadhyaya *et al.*, 1994), though this does not reduce incidences of hopper burn (Zhou *et al.*, 1999). Proteinase trypsin inhibitor from soybean has been engineered into rice plants and shown to confer resistance to *N. lugens* (Lee *et al.*, 1999). Whilst the expression of wheat germ agglutinin in rice has been shown to reduce insect honeydew excretion and produce anti-metabolic effects towards first and third instar nymphs (Brar and Khush, 1997).

1.7 IDENTIFICATION OF CHEMICAL ATTRACTANTS

Some plants as part of their metabolism produce behaviour modifying chemicals generally termed semiochemicals. It has long been recognised that these chemicals are detected by insects and used for host plant localisation in order to feed or oviposit (Carde, 1984). Plant volatiles are a complex mixture of compounds and only a proportion of these chemicals may be recognised by the insect (Visser, 1986). Many plants are only suitable for hosts at a particular stage of their development. Volatile cues from plants may involve the production of different plant volatile compounds or change in concentration of chemicals already present. Some plant volatiles are specific, whilst others are common to many green leaf plants. The 'green odour' of plants is largely derived from 8 volatile compounds (Hartanaka, 1993), composed mainly of 6 carbon aldehydes such as (*E*)-2- hexanal and 6 carbon alcohols such as (*Z*)-3-1-hexen-1-ol and are synthesized in green leaves from α-linolenic and linoleic acids. The non-directional dispersal flight of an insect may be converted into active host search behaviour once specific host volatile information is perceived (Carde, 1984). Extraction, distillation and air entrainment have all been used to collect and identify volatiles released from plants (Guerin *et al.*, 1982). Changes in amounts of volatiles released can increase with increasing stress stimuli (Kimmerer and Kozlowski, 1982).

1.7.1 Volatiles in rice

Chemicals released from the rice cv. 'Nipponbare' and isolated using steam distillation included 27 volatile chemicals, namely 14 esters, 7 carbonyl compounds and 5 alcohols (Obata *et al.*, 1983). Air entrainment and thermal desorption of 2 rice cvs. (Mars, PI 346833) identified 28 volatiles with hexanal, (*E*)-2-hexanal, (*Z*-*Z*) and (*E*-*E*)-2-4-heptadienal comprising more than 50% of the compounds released (Hernandez *et al.*, 1989). Compounds collected from the headspace of rice plants have been identified as the alcohols 1-heptanol, *Z*-2-heaxan-1-ol and linalool, aldehydes such as benzaldehyde, hexanal and (*E*)-2-hexanal, ketones acetophenone, β-ionone, 2-nonenone, tridecanone and 4-oxoisophorone, terpenes such as α-pinene, esters such as amyl acetate, methyl benzoate and methyl salicylate and other monoterpenes and sequiterpenes (Ramachandran, 1990; Hernandez *et al.*, 1989; Obata *et al.*, 1983; Masri, 1995). Masri (1995) identified 1-2,dimethoxybenzene or veratrole as a volatile of rice.

1.7.2 Olfactory orientation of insect

N. lugens has been shown to be attracted to the volatile semiochemicals from plant extracts (Saxena and Pathak, 1979). The insects flew towards intact fresh rice plants as well as extracts of the plants. However, antennectomised planthoppers showed no response to rice plants or to extracts suggesting that olfactory receptors located on antennae were responsible for detecting the stimuli (Obata, 1986). Rice volatiles were extracted using steam distillation from rice plants resistant or susceptible to *N. lugens* infection in order to examine attraction, settling, feeding and oviposition responses. Gas chromatography revealed more than 30 peaks within different rice cvs., of the susceptible cv. TN1 (Taichung native 1), and the resistant cvs. PTB 33, ASDY, ARC 6650 Rathu, Heenati and Mudgo (Saxena and Okech, 1985). Most peaks were common to all varieties, with peaks unique to some. More females of *N. lugens* settled and fed on tillers of the susceptible rice cv. TN1 sprayed with its own extract, than on TN1 sprayed with extracts of either ARC 6650 or PTB 33 (Khan and Saxena, 1988). Liu *et al.* (1984) demonstrated both qualitative and quantitative differences in volatiles released from rice plants susceptible to white backed planthopper (*Sogatella furchifera*). These authors suggested that volatiles released by rice plants functioned both as

attractants and repellents, which influenced host plant selection by this insect. Identification of compounds in rice facilitating attraction for planthoppers is imperative if progress is to be made in insect resistance coupled with examination of the responses of *N. lugens* to both resistant and susceptible cvs. of rice.

Insect antennograms linked to gas chromatography (Masri, 1995) have identified a range of volatiles that produced a significant response in *N. lugens*, the most potent of which are summarised in Table 1.3.

Table 1.3 Chemical attractants of *N. lugens*

Chemical	**Maximal response measured as a percentage of response to standard 1µg hexanol**
(*E*)-2-hexen-1-ol	120
Hexanal	170
4-oxoisophorone	240
α-pinene	240
Methyl benzoate	160
Methyl salicylate	180
Veratrole	240

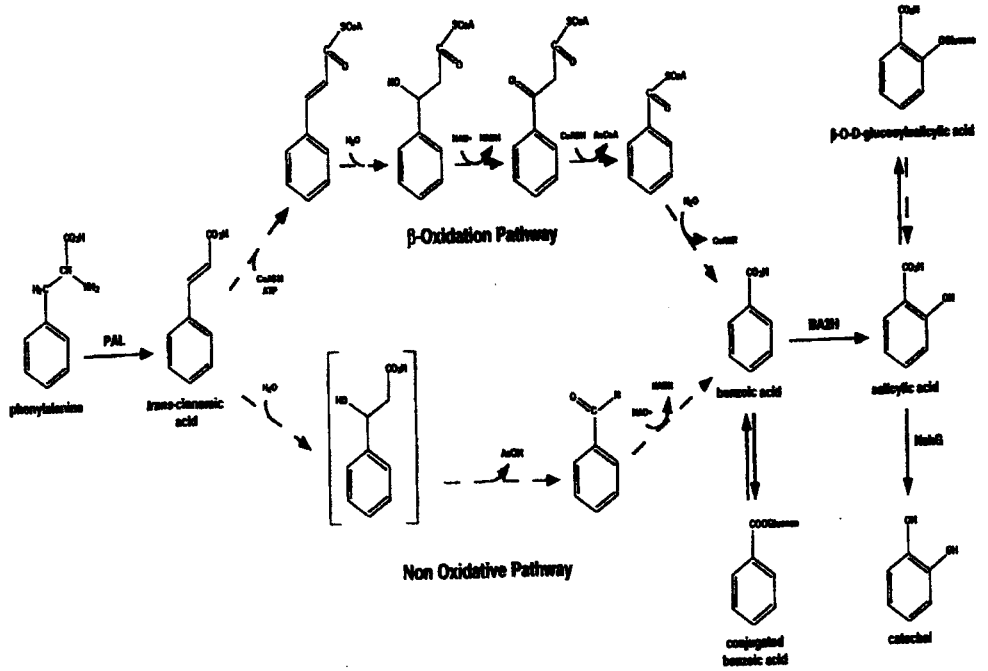
The same process has been used to identify other attractant semiochemicals in rice plants colonised by *N. lugens,* including salicylic acid, catechol and linalool (Masri, 1995).

1.7.3 Putative biosynthesis of veratrole

Veratrole is most likely a product of salicylic acid, a derivative of the phenyl propanoid pathway. Phenylalanine is converted to *trans*-cinnamic acid using the enzyme phenylalanineammonia lyase, followed by conversion to benzoic acid using a β-oxidation pathway (Ryals *et al.*, 1996). Hydroxylation at the second carbon position results in salicylic acid (Figure 1.5). Shoots of rice plants converted ^{14}C cinnamic acid to benzoic acid and then to salicylic acid (Silverman *et al.*, 1995). Salicylic acid is oxidatively decarboxylated to catechol, a step encoded by the enzyme salicylate hydroxylase, a product of the *nahG* gene.

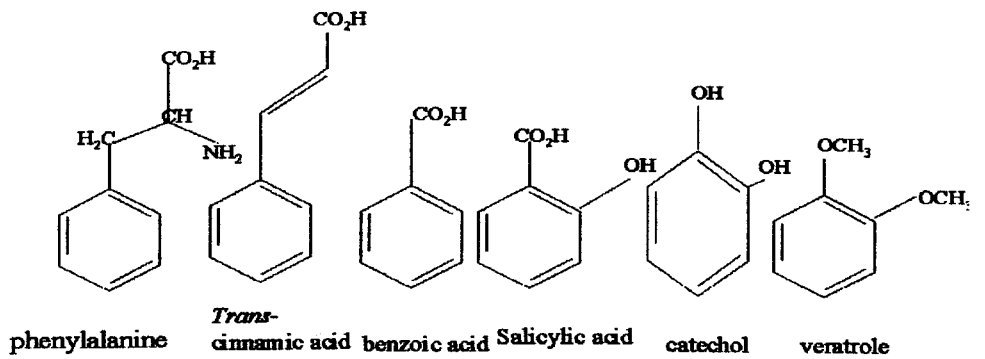
Figure 1.5

Phenyl propanoid pathway



Catechol is then methylated to veratrole or 1,2-dimethoxybenzene (Ryals *et al.*, 1994) (Figure 1.6), which is released as a volatile and is an attractant for *N. lugens* (Masri, 1995).

Figure 1.6
Phenyl propanoid pathway



Salicylic acid is also methylated to produce methyl salicylate, another volatile that attracts *N. lugens*. In addition, plants that are unable to accumulate salicylic acid have been shown to be unable to exhibit systemic acquired resistance. The mechanisms by

which systemic acquired resistance is initiated are diverse, complex and incompletely understood, although salicylic acid has been shown to be crucial in the initiation of plant defence genes.

1.8 OBJECTIVES OF THE PRESENT RESEARCH

- These experiments aim to verify whether Microprojectile and *Agrobacterium*-mediated transformation of rice with the *nahG* gene, derived from *Pseudomonas putida* and encoded for salicylate hydroxylase, could enhance expression of the salicylate hydroxylase enzyme resulting in increased veratrole production and may result in a "trap crop" for *N. lugens*.

- Rice is a major food crop and is severely damaged by *N. lugens*. Therefore, this work has social and economic potential. Taipei 309 is a rice cv. that is relatively straightforward to cultivate in tissue culture and readily regenerates from tissue culture. Taipei 309 has been transformed by microprojectile bombardment (Al-Forkan, 2000) and by *Agrobacterium*-mediated transformation (Azhakanandam, 1999). The protocols for transformation of this rice cv. were in place, which facilitated transformation of this rice cv.

- Transformation of rice using an *Agrobacterium*-mediated tertiary/ternary transformation system developed by De fits *et al.*(2000) for use with *Cathranthus roseus* could indicate whether this system is appropriate for use with rice. This system could be used to transfer two distinct binary T-DNAs simultaneously enabling transfer of a selectable marker on a separate plasmid to the gene of interest, facilitating loss of the selectable marker by segregation in the T1 and T2 generations.

- Reactive oxygen species involved in plant metabolism in addition to plant wounding and defence responses, are dependent on the concentrations of chemicals such as hydrogen peroxide and salicylic acid. Therefore, transgenic plants produced may have altered metabolism for antioxidant enzymes and reactive oxygen species.

- Salicylic acid has been implicated as essential in the signal pathway for the expression of plant defence genes to enable the initiation of systemic acquired resistance (Malamy *et al.*, 1990; Métraux *et al.*, 1990) and the octadecanoid pathway (Leon *et al.*, 2001) associated with plant wounding. Plants unable to accumulate salicylic acid may therefore be compromised in their ability to respond to pathogen attack and mechanical wounding.

- Gas chromatography could be used to quantify the volatiles released from intact and mechanically damaged rice plants.
- Screening of plant leaf material for insect resistance or enhanced insect attraction in bioassays could confirm whether rice plant over-expressing salicylate hydroxylase resulted in modified insect attraction, in terms of the time the insect spent with each plant line and the rapidity of the insect response.

CHAPTER 2. BIOLISTIC TRANSFORMATION OF RICE

2.1 INTRODUCTION

Biolistics was derived from the term biological bolistics, also called microprojectile bombardment. Microprojectile bombardment, involves particles of gold or tungsten of a suitable size (0.4-1.2μm) being coated in DNA and delivered at high velocity into cells. These cells can be regenerated into whole plants (Cao *et al.*, 1991; Davey *et al.*, 2000). The technique has provided the ability to introduce one or more genes of agronomic importance into target plants. The technique combines the relative ease of DNA introduction into cells with an efficient regeneration protocol from callus or explants (Davey *et al.*, 2000). This avoids extensive periods of cell suspension culture and the use of protoplasts. Microprojectile transformation bypasses any host specificity associated with *Agrobacterium*-mediated gene delivery, is cv. and species independent, simple to perform and transgenes can be introduced into any tissue of any plant genotype (Tyagi, 1999).

Gold is preferred, as it is chemically inert. Totipotent cells from a variety of tissues and organs have been used as targets for transformation. Christou *et al.* (1991) originally applied this technology to immature embryos of Indica and Japonica cvs. of rice, which led to the recovery of fertile transgenic plants at high frequencies. Amongst the four major cereals, rice has been the easiest to manipulate in terms of initiation and establishment of dedifferentiated callus and suspension cultures from explants (Christou, 1997).

2.1.1 History of biolistic transformation of rice

Originally, rice immature embryos were isolated from glasshouse-grown plants for particle bombardment. Transient activity from the introduced genes was observed 24 h later. Christou (1992) stated that up to 50% of bombarded explants were capable of developing transformed embryogenic callus with the potential to regenerate plants. Transient GUS activity has been found to increase with increasing amounts of DNA. In later experiments, improvements in the procedure increased transformation frequencies and resolved problems associated with tissue-specific expression, recovery of multiple transformation events and stable inheritance of introduced genes (Christou, *et al.*,1991; Christou 1992; Christou and Ford, 1995; Christou 1997). Plant regeneration ability was

strongly genotype-dependent and a significant factor in the transformation of Indica rice cvs. (Li *et al.*, 1993). Zhang *et al.* (1996) regenerated transgenic plants from bombarded embryogenic suspensions of elite Indica rice cvs. They commented that this genotype and environment independent transformation system using regenerable embryogenic suspensions might be used routinely for the introduction of useful genes into elite rice cvs. This work complemented the immature embryo and mature seed-based systems described and provided additional flexibility for the use of bombardment for rice transformation.

Microprojectile bombardment has been used to transform embryogenic calli derived from mature seed (Khanna *et al.*, 1997; Tang *et al.*, 1999). Mature embryos from seeds are available in large quantities throughout the year and, unlike cell suspension cultures, do not require such laboratory precision. Khanna *et al.* (1997) transformed scutella-derived calli from mature embryos of elite Indica cvs. using microprojectile delivery of a plasmid encoding the *bar* gene. From 2000 IR64 calli processed, 350 resistant calli survived and 150 plants were regenerated. Forty plants were selected for molecular analysis, of which 17 were found to be transgenic when tested by PCR and Southern analysis.

Transforming DNA is maintained in transgenic plants by integration into the nuclear genome. The best evidence for such integration is the observation of predicted Mendelian segregation ratios in subsequent sexual generations. Segregation analysis from this and the other systems have shown that the vast majority of loci of multiple integration events are genetically linked. Direct gene transfer often produced fragmented and rearranged multiple transgenic integrations at a single genetic locus (Christou, 1992). In experiments in which multiple genes were introduced into rice plants utilising one construct, co-expression frequencies of all genes were shown to be dependent on the nature and orientation of the promoters and the number of genes present on the transforming plasmid. It can be concluded that the molecular and genetic properties of transgenic rice plants derived through particle bombardment experiments are very similar to those of plants obtained through alternative direct DNA transfer procedures.

In rice, the co-integration frequency of transgenes on co-integrate vectors is close to 100%, with 73 to 80% co-expression of the selectable marker gene and gene(s) of interest. The majority of transgenic rice plants recovered through particle bombardment have 1- 3 and, less frequently, up to 10 copies of the transgene per haploid genome

(Christou, 1997). Biolistic transformation enables rice tissues to be transformed with a number of plasmids simultaneously. For example, Chen *et al.* (1998) co-transformed rice with 13 plasmids each carrying a gene.

2.1.2 Aims of the present research

The experiments aimed to optimise the transformation of the Japonica rice cv. Taipei 309 by co-bombardment with pAHC25 carrying the *gus* and *bar* genes and pROB5 carrying the *hpt* gene. Optimisation of the technique was followed by transformation of Taipei 309 with the *nahG* gene using co-bombardment with pSLJ7307 carrying the *nahG* gene encoding for salicylate hydroxylase and pROB5 carrying the *hpt* gene encoding for hygromycin phosphotransferase. The probability that the constructs fragment during preparation of the microprojectiles for bombardment or during the bombardment is proportional to the size of the plasmid. This can result in a higher percentage of fragments of DNA integrating into the plant genome, leading to a lower transformation efficiency and disruption of the metabolism and/or morphology of transformed plants (Southgate *et al.*, 1995; Marchant and Southgate, 1996). Therefore, it has been recommended that microprojectile bombardment be carried out using plasmids smaller than 15 kilobases. It was decided in the present work to restrict pSLJ7307 and, following extraction of the fragments containing the gene of interest, to use these fragments in microprojectile bombardments.

Bombardment with intact plasmids was compared to bombardment with linear fragments of plasmids and to *Agrobacterium*-mediated transformation. The techniques used and the results are described in Sections 2.2 and 2.3.

2.1.3 Optimisation of microprojectile bombardment

Several factors have to be considered in order to optimise the transformation of tissues using biolistics. These include the use of calcium chloride and spermidine to aid adherence of the DNA to microprojectiles, the nature of the microprojectiles and the choice of DNA construct and explant tissue (Davey *et al.*, 2000). In addition to these factors, the physical bombardment parameters, such as the flight distance of the particles in the instrument, the helium pressure and the vacuum conditions, affect the depth of particle penetration and degree of tissue damage (Christou, 1992). Poor donor plant conditions influence transformation efficiency, in addition to environmental factors,

such as humidity and temperature (Christou and Ford, 1995; Christou *et al.*,1995). Chen *et al.* (1998) reported protocols for consistent, large-scale production of transgenic rice plants using biolistics, while Christou (1997) showed that scutella-derived calli were most competent for transformation.

2.1.4 Regeneration of plants from cultured tissues

Factors, such as genotype, explant, hormonal composition of the medium, carbohydrate source and water stress-inducing treatments determine the efficiency of plant regeneration from callus. Plant regeneration via somatic embryogenesis has been reported in many rice varieties. Plants obtained from somatic embryos are uniform and genetically stable (Vasil, 1995; Chowdhury *et al.*, 1993). Somatic embryos arise directly or indirectly from single cells, are non-chimeric in nature and possess a root-shoot meristem, which is critical for survival *ex vitro*. Both Azhakanandam (1999) and Al-Forkan (2000) found that plant regeneration was more influenced by genotype than the subspecies used, composition of the plant growth regulators or the composition of agarose in the medium.

2.1.5 Analysis of putative transgenic plants

Initially, assessments (GUS assays, PCR, RT-PCR, Southern analysis, northern analysis and protein gels) were made of the transgenic status of plants regenerated following transformation. Later, plant morphology was assessed with growth, chlorophyll and protein analyses. Reproductive viability was assessed with pollen viability and seed germination assays. The transgenic status of the progeny was assessed using PCR and RT-PCR.

ß-glucuronidase, encoded by the *uidA* locus, is a hydrolase that catalyses the cleavage of a wide cv. of ß-glucuronides many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates. The ß-glucuronidase gene has been cloned and sequenced and encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions, the *gus* gene. Minute quantities of tissue can be assayed for GUS activity using histochemical methods. GUS concentrations have been measured in isolated single cells of transformed plants (Stomp, 1992). Different cell types within plants are expected to have differing metabolic activity with corresponding differences in the rates of transcription and translation. Different cell types present in each organ contribute in various ways to the

patterns of gene expression and each organ consists of different proportions of these cell types. Cleavage of 5-bromo-4-chloro-3-indolyl-β-D-glucoside occurs at a mildly acidic pH (5.5-6.0; Stomp, 1992). Staining due to cleavage can be intensified by the presence of peroxidases ubiquitous in plant tissues. Unbuffered or weakly buffered Sectioned or damaged plant tissues could have a pH of 5.5-6.0 due to leakages from cell vacuoles. Endogenous GUS activity present in some tissues was minimised if substrate solution was buffered with 0.1 molar sodium phosphate pH7.0 or if the tissues were heated for 5-10 min at 60^{0}C (Doby, 1965). Histochemical GUS staining has been used for a variety of purposes, usually to calculate the frequency of GUS positive blue spots as a measure of expression of transient gene integration and as a tool for promoter studies designed to identify DNA sequences (Jefferson *et al.*, 1987).

2.1.5.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows the amplification of a defined region of DNA. The technique is able to detect the presence of a defined region within the genome. PCR involves DNA synthesis from two oligonucleotide primers that act as sites for initiation by DNA polymerase and, therefore, define the limits of the template DNA that will be copied (Mullis and Falcona, 1987). The primers are complementary to regions of known sequence on opposite strands of the template DNA and are extended in the presence of the four deoxynucleotides (dATP, dGTP, dCTP and dTTP). Specific buffer conditions are maintained so that the DNA between the two primer binding sites becomes amplified during each cycle of the PCR (McPherson and Møller, 2000).

2.1.5.2 Reverse transcription coupled to polymerase chain reaction

The discovery of reverse transcriptase, an RNA dependent DNA polymerase, found in retrovirus infection, catalysed the synthesis of a proviral DNA from the virion's RNA genome at the onset of the infection process (Frohman *et al.*, 1988). The primary *in vitro* use of reverse transcriptase is in the production of complementary DNA sequence libraries where they are used to copy RNA into the first strand of the cDNA product to be cloned. Similarly, reverse transcriptase can be used in the first step of a two enzyme process, reverse transcription coupled to PCR. In this process, reverse transcriptase is used to generate the RNA-DNA hybrids which can be used as a DNA template required for themostable DNA polymerases (Watson *et al.*, 1996). Reverse transcriptase resembles other DNA polymerases requiring a primer for synthesis. A primer may be

oligo dTTPs, which hybridise to the polyA tail of eukaryotic mRNAs. However, these are inefficient when used with a long mRNA (Ausubel *et al.*, 1993). Random hexamers can be used as primers. These prime syntheses from internal mRNA sites or a sequence-specific primer may be used which hybridises to a known sequence within the mRNA. Some reverse transcriptases possess intrinsic 3' and/or 5' exo-ribonuclease RNaseH which degrades the RNA template after the first strand of cDNA is produced. The absence of RNaseH may result in the production of a longer cDNA (Sambrook *et al.*, 1989).

2.1.5.3 Southern blotting

Southern blotting, developed by E.M. Southern in 1975, has become a powerful tool for analysing gene structure. Genomic DNA is cut with one or several restriction enzymes and the resultant fragments separated by size on an agarose gel. The gel is then overlaid with a membrane and a flow of buffer set up through the gel and membrane, causing DNA fragments to be transferred from the gel onto the membrane where they are bound using ultraviolet light. A labelled probe specific for the gene under study is then hybridised to the DNA molecules on the filter. A probe may consist of purified RNA, a cloned cDNA, a labelled PCR product or a short synthetic oligonucleotide. If the probe was labelled using P^{32}, autoradiography of the membrane will result in a pattern of bands indicating the number and size of DNA fragments complementary to the probe (Watson *et al.*, 1996). Alternatively, the probe may be labelled with digoxigenin and visualised using the chemiluminescent substrate disodium-2-chloro-5-(methoxyspo{1,2-dioxetane-3,2-(5-chloro)trichloro[3.3.1^{37}]decan}-4-yl)-1-pentylphosphate.

The development of increasingly sensitive chemiluminescent substrates and the ability to label probes with digoxigenin (DIG) using PCR, has resulted in non-radioactive Southern analysis becoming the preferred method in many laboratories (Garratt *et al.*, 2001).

Microprojectile bombardment has several disadvantages, including little control over both the position of the DNA inserted into the recipient genome and the number of copies of the gene inserted (Repellin *et al.*, 2001). Therefore, it is necessary to assess the growth and morphology of plants derived using microprojectile bombardment as they may exhibit morphological abnormalities and aberrant growth patterns resulting from genomic changes (Tyagi *et al.*, 1999).

2.1.5.4 Assessment of plant morphology

Plant growth has traditionally been measured by sampling, drying, weighing and analysing the dried material. In contrast, the infra red gas analyser produces instant non-destructive measurements of growth. A continuous stream of air of specific carbon dioxide and water concentration is passed through a chamber containing known area of intact leaf. Photosynthesis and transpiration by the enclosed leaf tissue depletes the carbon dioxide in the air passing through the chamber and enriches it with water vapour relative to the air entering the chamber. Measurements are taken and expressed as the difference between the reference and analysis concentrations. The apparatus calculates the net rate of photosynthesis, stomatal conductance and transpiration. Chlorophyll content is an indication of the ability of the plant to photosynthesise and can be calculated from the absorbance at 470nm, 647nm and 663nm of plant leaf extract dissolved in a suitable solvent.

2.2 MATERIALS AND METHODS

2.2.1 Tissue Culture

Seeds of *Oryza sativa* cv. Taipei 309 (Japonica) were provided by The Plant Sciences Division (University of Nottingham, UK). These seeds were utilised for genetic transformation in addition to tissue culture.

Basal medium for the induction and maintenance of callus was based on that used by Azhakanandam (1999). This consisted of LS (Linsmaier and Skoog, 1965) supplemented with 2.5mgl^{-1} (2,4-D), 30gl^{-1} sucrose and semi-solidified with 0.4% (w:v) Sea Kem Le agarose (FMC Bioproducts, Rockland, ME, USA), pH 5.8. The medium was prepared by mixing equal volumes of sterilised double strength LS medium with 0.8% (w:v) aqueous molten agarose (40^{0}C). The medium was then dispensed as 20ml aliquots into 9cm Petri dishes (Bibby-Sterillin, Stone, UK).

2.2.1.1 Callus induction and maintenance from mature seed

The method was based on that detailed by Azhakanandam (1999). However, callus initiation with seed of Taipei 309 required 21 d rather than 14 d. Batches of 100 seeds were dehusked, surface sterilised by immersion in 50% (w:v) 'Domestos' bleach (Faberge, UK) for 30 min, followed by 3-5 washes with sterile reverse osmosis water. Seeds were placed on media with 10 seeds per dish. The Petri dishes were sealed with Nescofilm (Bando Chemical Co. Kobe Japan) and maintained in the dark for 21 d at 28 ±1^{0}C in order to initiate callus. The percentage of embryogenic and non-embryogenic callus was calculated based on the number of inoculated seeds that produced callus.

After 21 d, calli were dissected from any shoots, roots and remains of embryo. Both embryogenic and non-embryogenic calli were transferred to new medium and maintained for a further 14d in the dark at 28±1^{0}C. Subculture of tissues was carried out at 14d intervals. Only embryogenic calli were transferred and maintained in the dark at 28±1^{0}C. Any cultures exhibiting browning, organogenesis or slow growth were discarded. After 35d of culture, 8 calli were transferred into cell suspension medium.

2.2.2 Microprojectile bombardment

Medium for microprojectile bombardment was based on a protocol devised by Al-Forkan (2000). This consisted of LS medium supplemented with 2.5mgl^{-1} 2,4-D, 30gl^{-1} sucrose and 0.4M mannitol, semi-solidified with 0.4% (w:v) Sea Kem Le agarose, pH

5.8. The medium was prepared by mixing equal volumes of sterilised double strength LS medium with 0.8% (w:v) aqueous molten agarose (40^{0}C). The mixture was dispensed as 10 ml aliquots in 5cm Petri dishes.

Calli were placed in the centre of 5 cm Petri dishes, which were wrapped in aluminium foil and maintained at room temperature for 4 h. All microprojectile bombardments were conducted with the biolistic PDS-1000/He system (BIO-RAD™ Bio-Rad Laboratories California USA). After 4h (Zhang *et al.*, 1996), the calli were placed in the biolistic particle delivery system, 4th shelf down, 2.5cm below the stopping screen of the launch assembly. Helium pressure of 1350 psi was used to accelerate the particles (Chen *et al.*, 1998).

After gene delivery, calli were left on the same culture plates for 24 h before being transferred to 9cm Petri dishes each containing callus induction medium and maintained in the dark at 28±1^{0}C for 6 d (Zhang *et al.*, 1996). After 6d, a GUS assay was performed on selected calli to assess the percentage of tissues exhibiting transient GUS expression. Calli were transferred to LS2.5 medium with the addition of 50mgl^{-1} hygromycin for selection where they were maintained for 60 d with subculture every 14 d.

2.2.2.1 Concentration of antibiotic for selection of transformants

The optimum concentration of hygromycin was established that resulted in maximum viability of transformed embryogenic calli with the minimum survival of non-transformed calli. One hundred-ml aliquots of LS2.5 medium were prepared containing hygromycin at 40mgl^{-1} - 80mgl^{-1} in increments of 10mgl^{-1}. Medium was semi-solidified with the addition of an equal volume of molten 0.4% agarose (w:v) and 20ml aliquots were pipetted into 9cm Petri dishes. Putatively transformed calli were placed onto the culture medium at a density of 10 calli per plate, with 4 replicates. Likewise non-tranformed calli were placed on culture medium at each hygromycin concentration (again with 4 replicates). Calli were subcultured after 14d. The viability of the tissues was investigated after 28d and the results plotted as a kill curve.

2.2.2.2 DNA Isolation for bombardment

Plasmid AHC25 with the *gus* reporter gene and the *bar* gene as the selectable marker, was used in conjunction with pROB5 containing the *hpt* hygromycin phosphotransferase gene with the CAMV35S promoter and NOS terminator. In other

experiments, pSLJ7307 containing the *nahG* gene derived from *Pseudomonas putida,* encoding for the enzyme salicylate hydroxylase that degrades salicylic acid to catechol, was used to transform scutellum-derived rice calli together with pROB5. Dr C. Andras (University of Nottingham) supplied pROB5 and pAHC25. Plasmids SLJ7307 and ROB5 were grown in *Escherichia coli* and extracted using Wizard™ Miniprep kit (Promega, USA). In order to restrict the genes of interest from the plasmids 50µl of plasmid, 1 µl of BSA (New England Biolabs, Beverley, USA), 10 µl of 10X buffer (New England Biolabs), 1 µl of *Hin*dIII restriction enzyme (New England Biolabs) and 38 µl of sterile deionised water were mixed together and incubated at 37^{0}C for 1 h.

This mixture was visualised against a 1kb ladder (New England Biolabs) using a 1% (w/v) agarose gel in 0.5 TBE buffer with 30µl per well and the addition of 2µl of loading buffer, 2 h, 50 Vcm^{-2}(see appendix). The appropriate fragment was excised from the gel after staining with ethidium bromide. The DNA was extracted using a QIA quick gel extraction kit (Qiagen Ltd., Crawley, UK.). A 5µl sample of the recovered fragment was subjected to gel electrophoresis (1µl loading buffer) using a 1%(w/v) agarose gel in 0.5 TBE buffer 2 h at 50 Vcm^{-2} in order to quantify the DNA. After quantification, these fragments were used for microprojectile bombardment. It was also decided to ligate the isolated *Hin*dIII fragment into pROB5. Fifty µl of pROB5 were restricted using *Hin*d III as detailed above. The 5' ends were dephosphorylated to prevent self ligation of the vector, followed by purification of the vector DNA and ligation of the insert and vector following a standard protocol (Promega, 1996). The putatively ligated plasmid was used to transform competent *E. coli.* Samples were retained from the vector, insert and ligated plasmid. These were visualised using gel electrophoresis [2% (w/v) agarose gel in 0.5 TBE buffer, 6µl per well, run for 2 h at 75 Vcm^{-2}].

2.2.2.3 Preparation of microprojectiles

Gold micro-carriers were coated with plasmid DNA using the protocol detailed by Marchant and Southgate (1996). Gold microrojectiles (0.4-1.2µm; supplied by Heraeus GmbH, Karlsruhe, Germany) were prepared by adding 1ml of absolute ethanol to 60mg particles followed by vortexing for 2 min. The mixture was centrifuged (1 min, 10,000 r.p.m.) and the supernatant removed. These steps were repeated twice and 1ml of sterile reverse osmosis water was added. The supernatant was finally removed and 1ml of sterile reverse osmosis water added. Gold stock suspensions were stored at 4^{0}C until required. The biolistic apparatus and laminar airflow cabinet were surface sterilised

by spraying 70% (v/v) ethanol. The macrocarrier holders, macrocarriers, stopping screens and forceps were soaked in 70% (v/v) ethanol for at least 1h and dried in the laminar airflow cabinet.

2.2.2.4 Microprojectile coating and biolistic bombardment

The suspension containing the microprojectiles was vortexed for 5 min. In order to prepare for 6 bombardments, 50µl of microprojectile stock solution (60mgml^{-1}) was dispensed into a sterile microfuge tubes. Under continuous vortexing, 5µl of plasmid DNA (1mgml^{-1}), 50µl of 2.5M $CaCl_2$ and 20µl of spermidine (Sigma) (free base, tissue culture grade) were added. This mixture was vortexed for 3 min and centrifuged for 10 sec. The supernatant was removed and the microprojectiles resuspended in 250µl of absolute ethanol. After a 10 sec centrifuge pulse, the supernatant was removed and the particles resuspended in 75µl of absolute ethanol by vortexing briefly. Ten µl of the freshly coated microprojectiles was pipetted into the centre of an inverted macrocarrier and allowed to dry. The biolistic device was loaded with the first macrocarrier and a rupture disc of 1350 psi was fitted to the rupture disc-retaining cap (Khanna *et al.*, 1997). The Petri dish containing calli for bombardment was placed on the 4th shelf from the top of the apparatus prior to bombardment. The rupture pressure was noted for each bombardment.

2.2.3. Plant regeneration

Calli producing somatic embryos were transferred to regeneration medium, but with the agarose content reduced to 0.4% (w/v) and incubated in the light 16 h photoperiod (47µE m^{-2} s^{-1}, Cool White Florescent tubes; Thorn EMI Ltd, Hayes, UK) at 26±1^{0}C. Shoot regeneration frequency was recorded after 25 d as the percentage of scutellum derived calli producing 1 or more shoots. Shoots 2cm in height were detached from individual calli and transferred to 40ml aliquots of multiplication medium [MSB2 medium with the addition of 2mgl^{-1} BAP, 50gl^{-1} sucrose, 0.5mgl^{-1} NAA, semi-solidified using 0.25% (w/v) Phytagel] in 175 ml capacity screw capped glass jars for 20-25d. If the plants had not rooted by the end of this period, they were transferred to root induction medium for 7-10d [MS with 30gl^{-1} sucrose and 1.5mgl^{-1} NAA semi-solidified with 0.25% (w:v) Phytagel, pH6.8; autoclaved at 121^{0}C for 20 min]. Regenerated plants were maintained in this medium. Fifty percent of each line of rooted regenerated transformed

and non-transformed somatic embryo-derived plants were transferred to plastic pots and maintained in the glasshouse.

2.2.3.1 Transfer from tissue culture

Regenerated plants, each approx 5-7 cm in height were removed from medium (either MSB2N or MS1.5N) and their roots washed with tap water. Plants were transferred to 7.5cm diam. plastic pots each containing a mixture 6:6:6:1 (v/v) of perlite (Silvaperl Ltd. Gainsborough, UK), Vermiculite (Silvaperl Ltd), Levington M3 compost (Fisons plc. Ipswich, UK) and John Innes No. 3 compost (J. Bentley, Barton-on-Humber, UK) and well watered. Plants and pots were covered with a plastic bag and transferred to a controlled environment room with a temperature of 27±1^{0}C and a 16 h photoperiod (180µE m^{-2} s^{-1}, Cool White Florescent tubes). Seed-derived plants were also grown alongside those from mature scutellum callus. After 14 d, the corners of the bag were removed and after another 14 d, the bags were removed. Leaf samples were collected for enzyme analysis.

2.2.3.2 Transfer of regenerated plants to the glasshouse

Thirty d later, the plants were transferred into 12.5cm diameter pots each containing a mixture 1:1:1:6 (v/v) of Perlite, Vermiculite, Levington M3 compost and John Innes No. 3 compost and well watered. Plants were fed weekly with Maxicrop tomato food with sequestered iron and seaweed (Levington) at a dilution of 30ml to 5 litres of water (total Fe 2.0%, P 2.2%, K 5.6%). Plants were transferred to the glasshouse with a temperature of 30±1^{0}C and natural light supplemented by a 16 h photoperiod (180µE m^{-2} s^{-1}, cool white florescent tubes). The glasshouse floor was soaked twice daily to increase the relative humidity and promote seed production. Plant leaf samples were removed to extract DNA for PCR, RT-PCR and Southern analysis to confirm the molecular status of the plants. Samples were also taken for chlorophyll analysis.

2.2.4 Analysis of putative transgenic plants

2.2.4.1 Histochemical GUS assay

Histochemical assays were carried out in the GUS reaction mixture detailed in Table 2.1 below and incubated overnight.

Table 2.1 GUS assay	
10ml final volume	Chemical
5ml	0.2M phosphate buffer (0.2M NaH_2PO_4) A & 0.2M Na_2HPO_4) B. (39ml of A was mixed with 61ml of B.)
3.69ml	Sterile distilled water
200 µl	0.5 M EDTA disodium salt
10 µl	0.1% (v/v)Triton T-X100
500 µl	10mM K3 (potassium ferricyanide, 16mg in 5ml phosphate buffer)
500 µl	10mM K4 (potassium ferrocyanide, 21mg in 5ml phosphate buffer)
5mg	X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucoside)

Calli were placed in the wells of $10cm^{-2}$ 25 compartment dishes (Bibby Sterilin) with 3-5 calli per well; 500 µl of stain solution was added to each well. Dishes were wrapped in Al foil to exclude light and incubated at 37°C for 16-24h. Fixed or unfixed tissues were placed in just enough staining reagent to cover the tissues. Blue colour may appear after 10 min with certain promoters, but may take several h to develop in other cases (Jefferson *et al*, 1987). A negative control was included in the assay to check for endogenous GUS activity. A histochemical GUS assay for transient expression was carried out 6 d after transformation. GUS-positive spots were counted and the percentage GUS positive tissues calculated. The assay was repeated 42 d after transformation to confirm gene integration into the recipient plant genome.

2.2.4.2 DNA Extraction

DNA was extracted from putatively transformed and wild type rice leaves by CTAB extraction (Ausubel *et al.*, 1989 see Appendix). This was tested using the plasmid DNA as a control. The CTAB extraction protocol produced variable yields of DNA. Therefore DNA for PCR from leaf tissue was extracted using a GenElute Plant Genomic DNA kit (Sigma-Aldrich, Poole, UK.). For Southern analysis, 10µg of DNA was needed for each reaction (Garratt *et al.*, 2000) and, therefore, a DNA extraction protocol was used based on the method of Dellaporta *et al.* (1983, see Appendix).

Leaf tissue was collected, placed in a 50ml polypropylene tube (Alpha laboratories, Eastleigh, UK.) and frozen in liquid nitrogen. Tissues were ground using a pestle and mortar whilst still frozen, then replaced into the tube. Five g of ground tissue were used from each plant. Twelve ml of extraction buffer (0.1M Tris, 0.05M EDTA, 0.5M NaCl, 1% (w/v) PVP and 250µl β-mecaptoethanol) were added to each tube, followed by 1.6ml of 10% (w/v) SDS. The tubes were vortexed for 1 min, 4ml of 5M potassium acetate (pH 5.8) was added and each tube again vortexed for 30 sec before placing on ice for 30 min. Tubes were centrifuged (10,000g, 15 min) and the supernatant filtered through Miracloth (Calbio Corporation) into a clean falcon tube, isopropanol (0.7vol of the mixture) was added, and the mixture placed on ice for 20 min. Tubes were centrifuged (10,000g, 20 min) and the aqueous phase discarded. The pellet was washed with ice cold 80% ethanol and allowed to air dry.

The pellet was resuspended in 200µl of TE buffer (0.05M Tris, 0.01M EDTA pH 8.0) and 10µl of RNase (2mgml^{-1}) (Promega, Madison, USA) was added. The mixture was transferred to a microfuge tube and incubated at 37^{0}C for 30 min. The samples were centrifuged in a microfuge for 1 minute at 13,000rpm to pellet any particulate matter. The aqueous phase was removed to a fresh eppendorf and 0.7volumes of isopropanol added and 0.1 vol of 3M sodium acetate pH 5.2. The mixture was place on ice for 20 min and then centrifuged at 10,000g for 20 min. The aqueous phase was removed and discarded. The pellet was washed with cold 70% ethanol and allowed to air dry before being resuspended in 25µl of sterile distilled water. DNA was quantified using a spectrometer (Perkin Elmer) at 260nm and 280nm.

2.2.4.3 Polymerase Chain Reaction

The primer sequences were generated using the sequence from the n*ahG* gene published on the internet (Accession number X83926).

Forward Primer sequence : **ctc act ttt ccg gtg agg aa**

Reverse primer sequence : **aga gag ttg gtg gtc ggg atg**

Primer 3 programme was used to select primers. The product size was 196 base pairs and the optimum annealing temperature for the reaction was determined empirically using the following equation (McPherson and Møller, 2000).

T_m= [(number of G+C) X 4^{0}C +(number of A+T) X 2^{0}C

The alternative optimised annealing temperature +/- 2.5^{0}C (Wu *et al.*, 1991) was calculated using the formula:

$T_p = 22 + 1.46$ [(2 X number of G + C) = (number A + T)

The optimum annealing temperature was found to be 65^{0}C. The thermal cycler was therefore programmed to run 45 cycles with the following programme: 94^{0}C for 1 min, 65^{0}C for 45 sec, 72^{0}C for 1 min. The thermo-cycler was cooled to 4^{0}C for 5 min at the end of the programme. Initial experiments using 35 cycles, annealing time of 30 sec and a hot start were discontinued, as there was no amplification. The annealing time was therefore increased and the number of cycles increased to increase product (McPherson and Møller, 2000).

Table 2.2 Reaction mixture for polymerase chain reaction

Substance	Amount	Concentration
PCR 10X buffer (Promega)	25µl	as supplied
DNTPs	25µl	2 mM
Magnesium Chloride (Promega)	25µl	25mM
PVP	8.3µl	30%
Sterile distilled water	142µl to make 250µl of master mix which was then aliquoted as 25µl per tube	
DNA template	0.5µl	40ngµl^{-1}
Forward primer (MWG Biotech)	0.5µl	20mM
Reverse primer (MWGbiotech)	0.5µl	20mM

The reaction was first tested on samples of the construct once optimum conditions had been established. DNA was extracted from putatively transformed and wild-type rice leaves by CTAB extraction (Ausubel, 1990; Appendix) This was then tested using the plasmid DNA as a control.

2.2.4.4 RT-PCR

RNA was extracted from samples of leaf tissue frozen in liquid nitrogen and ground whilst still frozen. One hundred mg of tissue was used per sample. RNA was extracted

using an RNA mini kit (Promega) and following the instructions in the RNeasy mini handbook 06/2001 (available on-line at http://www.promega.com). To eliminate the possibility of DNA being present following elution of the RNA, RNase–Free DNase was used. (RQ1 Promega Madison USA). In the presence of magnesium ions, this cleaves each strand of DNA independently, the sites of cleavage distributed in a statistically random fashion (Promega Technical bulletin No 518). To each microfuge tube containing 50µl of RNA solution, 6 µl of 10X buffer was added and 1 µl of RNase-Free DNase. The mixture was incubated at 37^{0}C for 1 h. One µl of DNase stop solution was added to the mixture, which was then incubated at 65^{0}C for 10 min to inactivate the DNase. This mixture was then used for RT-PCR.

RT-PCR was carried out using the ABgene Reverse^{TM}1st strand synthesis kit using anchored oligonucleotides dTs for the synthesis of the first strand and MSB2 the positive control supplied with the kit to confirm that the kit worked. Ten µl of the RNA mixture was used in each reaction to this was added 2 µl of anchored oligo dTs. This was heated at 70^{0}C for 5 min and then cooled to 5^{0}C. The first strand was synthesised by the addition of first strand synthesis buffer, 5mM dNTP mix reverse transcriptase 25 units per µl and RNase inhibitor in a 1 : 2 : 1 : 1 ratio. This mixture was incubated at 42^{0}C for 1 h then the RTase was inactivated by incubation at 75^{0}C for 10 min. PCR was then immediately carried out (Section 2.2.4.3) using 5 µl of this reaction mixture as the DNA template and reducing the amount of sterile water correspondingly. Reaction parameters consisted of 35 cycles (94^{0}C 1 min, 60^{0}C 30 sec, 72^{0}C 1 min), followed by 1 cycle (72^{0}C for 3 min followed by 4^{0}C for 5 min). PCR was carried out in a Techne Genius machine using the programme described in Section 2.2.4.3.

2.2.4.5 Northern blots

RNA was extracted from plant leaf samples using the protocol described in Section 2.2.4.4. Following purification of the RNA sample, 5µl of RNA solution was quantified from each sample by spectrometry at 260nm in order to load the gel with an equal quantity of RNA. The whole process was carried out in a fume hood due to the use of formaldehyde and formamide. The gel was prepared with 1% agarose, 90ml sterile distilled water and 2ml of 1M sodium phosphate buffer (equal volumes of 2M disodium hydrogen orthophosphate and 2M sodium dihydrogen orthophosphate, pH 6.5). Just before the gel was poured 8ml of formaldehyde was added. The gel was run in running

buffer (593.5ml of sterile distilled water, 50ml of formaldehyde, 6.5ml of sodium phosphate buffer).

Samples were prepared by mixing RNA (4µl) to an equal volume of RNA loading dye (1000µl formamide, 550µl water, 330µl formaldehyde, 40µl of 0.5M EDTA pH 8.0, 40µl 10mgml^{-1} ethidium bromide, 40µl of 1M sodium phosphate buffer pH 6.5). Samples were heated at 65^{0}C for 15 min before snap freezing on ice and adding blue loading dye. Samples were run at 75 Vcm^{-2} for 1.5 h.

RNA was transferred to the membrane using the same protocol as for Southern analysis (Section 2.2.4.6) with the exception that denaturing and neutralisation steps were not performed and sodium phosphate buffer replaced the 20 X SSC used. Sodium phosphate buffer 0.25M (equal volumes of 0.5M disodium hydrogen orthophosphate, 0.5M sodium dihydrgogen orthophosphate, pH 6.5) was used to transfer the DNA from the gel to the membrane in the same protocol as for Southern analysis (Section 2.2.4.6). The position of the lanes was marked with pencil on the wrong side of the membrane. The positions of the two ribosomal band subunits were marked at the side of the membrane in pencil for reference and the top right hand corner was cut so that the membrane orientation could be confirmed on the X-ray film. The edges of the filter paper were sealed with Nescofilm to ensure that buffer passed through the gel and membrane. The apparatus was incubated overnight. The RNA was cross-linked to the membrane using a Stratalinker for 2 min.

Pre-hybridisation, hybridisation and post-hybridisation washes were carried out with the membrane inside a 200ml hybridisation tube rotating slowly to ensure all the membrane was covered. Hybridisation was carried out using a DNA probe, as there was insufficient time to develop an RNA probe.

Prehybridisation solution was made consisting of 4 ml 50% dextran sulphate solution, 1ml 20% SDS, 4ml of 5M sodium chloride, 10ml of 50% formamide and 1ml salmon sperm (5mg ml^{-1}) denatured by boiling for 5min and snap cooled on ice. Prehybridisation solution (10ml) was added to each membrane The membrane was incubated in a hybridisation oven at 38^{0}C for 2 h. Prehybridisation solution was discarded and replaced with 10ml of hybridisation solution. This comprised 4µl of linearised probe, which was denatured by boiling for 5 min then snap cooled on ice, and 4.5µl of nuclease free water. This mixture was added to the random prime labelling kit and labelled by the addition of 5µl of P^{32} UTP, the DNA was denatured by incubation at

100°C and added to the membrane with 10ml of prehybridisation solution. The membrane and hybridisation solution were incubated overnight at 38°C.

The membrane was washed for 10 min at 65°C in washing buffer 1 (5ml of 0.5% SSC, 10ml 0.5% SDS. 180ml sterile distilled water) and then washed for 10 min in washing buffer 2 (1ml of 0.1%SSC, 10ml of 0.5% SDS, 189ml sterile distilled water) at 38°C.

The wet membrane was immediately removed from the hybridisation tube, sealed and visualised. Standard X-ray film (Amersham) was used with a 1h to 7 d exposure. In membranes with high radioactivity a phosphoimager was used to expose the membranes.

2.2.4.6 Southern Analysis

2.2.4.6.1 Probe synthesis

Probe synthesis was carried out by PCR using the PCR Probe synthesis kit (Cat No 1 636 090; Roche Diagnostics, Mannheim, Germany) Table 2.2 illustrates the composition of probe labelling mixture.

Table 2.3 Composition of Probe Synthesis Mixture

Chemical	Amount
Sterile distilled water	34.00µl
10XPCR buffer with magnesium chloride 15mM	5.0 µl
Forward primer 20nm	2.0 µl
Reverse primer 20nm	2.0 µl
Taq DNA polymerase	1.0 µl
PCR DIG probe synthesis mixture (dATP, dCTP, dGTP 2 mM each dTTP1.3mMDIG-11dUTP alkali-labile pH7.0 nucleotide mixture)	5.0 µl

Probe synthesis was carried out using PCR in the Techne-Genius thermocycler using the programme described in Section 2.2.4.3. Probe concentration was calculated by electrophoresis of 5 µl of probe on a 2% agarose gel with 0.5TBE buffer run at 50 Vcm^{-2} for 2 h against 2 µl of 100bp ladder (New England Biolabs). Probe concentration was calculated to be 20ng ml^{-1}.

2.2.4.6.2 Restriction Digest

Genomic DNA extracted as described in Section 2.2.4.2 was restricted in 2 reactions per plant. In the first reaction the gene cassette was restricted at both ends using the restriction enzyme *Hin*dIII producing a fragment of 10kb, indicating full integration of the gene. In the second restriction, the DNA was restricted with the enzyme *Sac*I, which cut the DNA just inside the *nahG* gene, thereby enabling copy number to be determined.

Table 2.4 Composition of restriction digest mixtures

Chemical	Amount
*Hin*dIII	1 µl
10X reaction buffer	2 µl
Genomic DNA solution	17 µl
Sac I	1 µl
10X buffer	2 µl
Genomic DNA mixture	17 µl

Restriction mixture was incubated at 37°C for 24 h. Following restriction the fragments of DNA were separated using agarose gel electrophoresis on a 400ml 0.8% agarose gel dissolved in 0.5M tris-Borate-EDTA (TBE) run at 12 volts for 24 h in a buffer solution of 0.5TBE. The gel was stained using ethidium bromide 0.5mgl^{-1} and visualised under uv light to confirm the restriction and separation of fragments.

2.2.4.6.3 Transfer to membrane

The gel was submerged in 250mM HCL for 10 min at room temperature and then rinsed with water. The gel was then submerged in denaturation solution (0.5M NaOH, 1.5M NaCl) for 15 min at room temperature with shaking. The gel was rinsed and then submerged in neutralisation solution (0.5M Tris-HCL pH7.5, 3M NaCl) for 15 min with shaking at room temperature and then rinsed. The denaturation and neutralisation procedures were repeated. The membrane prepared for Southern transfer using the apparatus (Appendix1).

Buffer 20X SSC (750mM NaCl, 75mM sodium citrate pH 7.0) was used to transfer the DNA from the gel to the membrane. The position of the lanes was marked with pencil on the wrong side of the membrane and the top right hand corner was cut of so that the

membrane orientation could be confirmed on the X-ray film. The edges of the filter paper were sealed with Nescofilm to ensure that buffer passed through the gel and membrane. The apparatus was incubated overnight. After blotting the gel was again visualised to confirm that the DNA fragments had been transferred to the membrane. The DNA was crosslinked to the membrane using a Stratalinker for 2 min.

2.2.4.6.4 DIG labelled prehybridisation and hybridisation

Prehybridisation, hybridisation, posthybridisation washes and blocking were carried out with the membrane inside a 200ml hybridisation tube rotating slowly to ensure all the membrane was covered in a hybridisation oven. Twenty ml of DIG easy Hyb solution (Roche) was added to the hybridisation tube containing the membrane and incubated at 37^{0}C for 2 h. Prehybridisation solution was discarded and replaced with 20ml of hybridisation solution (DIG easy Hyb containing 20ngml^{-1} DIG labelled probe). The tube was incubated overnight at 37^{0}C.

The membrane was washed twice 5 min per wash in 2 X SSC (made by diluting 20 X SSC buffer) containing 0.1% SDS buffer and then washed twice 15 min per wash in 0.5 X SSC buffer containing 0.1%SDS at 37^{0}C. The membrane was then equilibrated in washing buffer (10mM maleic acid , 15 mM sodium chloride pH7.5 with 0.03% Tween 20) for 1 min. The washing buffer was discarded from the tube. The membrane was then agitated in 20 ml of blocking solution (10mM maleic acid, 15mM sodium chloride, pH 7.5 containing 1%v/v blocking solution (Roche) at 37^{0}C for 60 min.

2.2.4.6.5 DIG labelled visualisation

The antibody solution was prepared by diluting the Anti-Digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche) 1:20,000. Therefore, 1.5µl of AntiDigoxigenin-AP was added to 30ml of blocking solution. The membrane was agitated for 30 min in the antibody solution at 37^{0}C. The antibody solution was discarded and the membrane washed twice (15 min per wash) in washing buffer (10mMmaleic acid, 15mM sodium chloride, 0.03% Tween 20, pH7.5). The membrane was equilibrated in detection buffer (10mM Tris-HCl, 10mM NaCl pH 9.5) for 2 min. The chemiluminescent substrate CDP-star (Roche) was diluted 1:100 in detection buffer. The membrane was removed from the tube and placed immediately into a plastic bag (ensuring that the membrane remained wet.). Diluted CDP star was pipetted onto the

membrane (0.5ml per 100cm^2 of membrane). The bag was immediately sealed and visualised. Standard X-ray film (Amersham) was used with a 15-45 min exposure.

2.2.4.6.6 Prehybridisation and hybridisation using P^{32}

Pre-hybridisation, hybridisation and post-hybridisation washes were carried out with the membrane inside a 200ml hybridisation tube rotating slowly to ensure all the membrane was covered. Twenty ml of prehybridisation solution was added to each membrane. Prehybridisation solution (10.2 ml of sterile distilled water, 5ml of 20 X SSPC [sodium chloride 175.3 gl^{-1}, sodium dihydrogen orthophosphate 27.6gl^{-1}, sodium EDTA 7.4gl^{-1}, pH 7.4], 2ml of 50X Denhardts solution, 2 ml of 10% SDS and 0.8ml of salmon sperm 5mgml^{-1}), the final ingredient denatured by boiling for 5 min and snap cooled on ice was added to the membrane inside a 200ml hybridisation tube. This was then incubated in a hybridisation oven at 38^{0}C for 2 h. Prehybridisation solution was discarded and replaced with 10ml of hybridisation solution. Hybridisation solution contained 4µl of linearised probe denatured by boiling for 5 min then snap cooled on ice, 4.5µl of nuclease free water. This was added to the random prime labelling kit and labelled by the addition of 5µl of P^{32} UTP. The probe was denatured by incubation for 10 min at 100^{0}C and added to 10ml of prehybridisation solution. Hybridisation solution was incubated with the membrane overnight at 65^{0}C.

The membrane was washed for 10 min at 65^{0}C in washing buffer 1 (5ml of 0.5% SSC, 10ml 0.5% SDS. 180ml sterile distilled water). Washing buffer 1 was discarded and the membrane washed for 10 min in washing buffer 2 (1ml of 0.1%SSC, 10ml of 0.5% SDS, 189ml sterile distilled water) at 65^{0}C. Washing buffer 2 was discarded.

The wet membrane was immediately removed from the hybridisation tube, sealed and visualised. Standard X-ray film (Amersham) was used with a 1hour to 1week exposure. In membranes with high radioactivity a phosphoimager was used to expose the membranes.

2.2.4.7 Growth analysis

Four pots from each line were assessed. The height of each plant was measured from the top of the soil to the tip of the flag leaf using a tape measure. Stem number was counted, leaf length, leaf width and leaf thickness was measured on a sample of leaves from each pot. These measurements were carried out three times with one-month intervals between the growth analyses. Photosynthetic efficiency was measured using an SIRUS-1 (PP Systems, Hitchen, UK.) which measured stomatal conductance and

the amount of carbon dioxide consumed $cm^{-2}min^{-1}$. The carbon dioxide reference was set at 340ppm and the relative humidity reference set at 10 +/- 1 millibars. The leaf area measured was set to $1cm^2$ to account for the narrow leaves that rice possesses.

2.2.4.7.1 Chlorophyll analysis

Chlorophyll was analysed in two ways. Chlorophyll was analysed on living leaves using a Minolta SPAD 502 chlorophyll meter (Minolta Co. ltd. Japan) which when clamped onto a leaf gives a reading of total chlorophyll content.

Ten fresh leaf discs (1cm diameter, fresh weight 60mg +/- 20mg) were removed from each leaf tested. Leaf samples were stored in 1.5ml Eppendorf tubes in liquid nitrogen. Samples were ground whilst still frozen. Ground leaf samples were then suspended in 1m of 80% propanone and vortexed for 30 seconds. Samples were centrifuged for 2 min at 10,000rpm and the supernatant removed. Each sample of 1ml was aliquoted into a 1.5 ml plastic cuvette. Quantities of Chlorophyll a and b were measured using a spectrometer (Perkin Elmer uv/vis Lamda Bio) taking readings at 470nm, 647nm and 663nm. The spectrometer was blanked using a cuvette containing 1ml of 80% propanone. The results were fed into a spreadsheet and analysed using the following equations.

Chlorophyll A: 12.25 X A663 – 2.79 X A647

Chlorophyll B : 21.5 X A647 – 5.1 X A663

Total Chlorophyll: 7.15 X A663 + 18.71 X A647

Carotenoids : 1000 X A 470 –1.82 Chlorophyll A – 85.02 Chlorophyll B/198

2.2.4.7.2 Protein quantification

Protein quantification was carried out with the same samples using Bradford's reagent (Bio-RAD) measuring absorbance at 595nm in order to establish that any difference in enzyme activity was not merely a result of aberrant concentrations of protein in samples. Soluble proteins were extracted by grinding 1g of frozen leaf tissue with liquid nitrogen. Frozen ground plant material was added to 5ml of phosphate buffer (potassium dihydrogen orthophosphate 0.03M and dipotassium hydrogen orthophosphate 0.03M in a 1:1 ratio, pH 7.0). Aliquots of plant leaf extracts 10µl were added to 990µl of a 20% aqueous solution (v/v) Bradford's reagent. Absorbance was measured in a spectrometer (Perkin Elmer) at room temperature. Protein standards consisting of 5, 10, 20, 30, 40, 50 $mgml^{-1}$ BSA were calibrated.

2.2.4.7.3 Protein visualisation using SDS-PAGE gel

Samples were prepared by grinding 10mg ± 1mg of frozen leaf tissue resuspended in 17µl of extraction buffer (50mM Tris pH 8.5, 10mM EDTA pH8.5, 1mM calcium chloride, 1mM PMSF, 1mM DTT heated at 95°C then pulse centrifuged and snap cooled on ice). Samples were centrifuged for 10 min at maximum in a microfuge, 15µl of supernatant were removed and added to 15µl of loading dye (0.125M Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mecaptoethanol, 0.02% bromophemnol blue). Positions of bands were assessed using 5µl of molecular rainbow markers (Promega).

The main gel 12% (3ml water, 4ml of 30% acrylamide 1.9ml of 2 M Tris pH 8.8, 100µl of 10% SDS, 100µl of 10% ammonium persulphate, 4µl TEMED catalyst) was prepared and left to set for 40min in the fume hood. The main gel was overlaid with 300µl of water to maintain anaerobic conditions. The water was removed from the main gel and 5ml of stacking gel added (3.4ml water, 0.83ml 30% acrylamide, 0.63ml 1M Tris pH 6.8, 50µl 10% SDS, 50µl of 10% ammonium persulphate, 5µl TEMED). A comb was inserted and the stacking gel left to set for 40 min. The gel was loaded with 30µl per well of protein samples and the gel was run at 120 Vcm^{-2} for 4-5 h.

The gel was removed from the electrophoresis apparatus and stained with Coomassie Blue (0.25% Coomassie Blue in 10% acetic acid, 30% methanol) overnight. The gel was destained in 10% acetic acid and 30% methanol for 4-5 h. The gel was photographed using a transilluminator.

2.2.4.7.4 Pollen viability

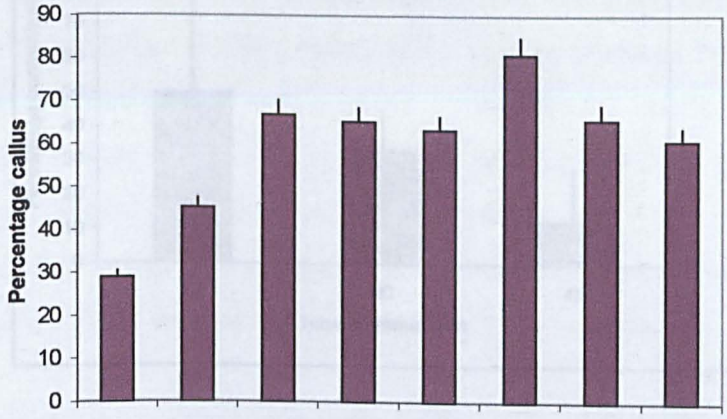
Pollen was viable only in the morning (L .Kenyanin pers. com). Therefore pollen samples were collected between 8 and 9 am, incubated for 1 h in 2% (v/v) FDA solution (fluorescien diacetate $3mgml^{-1}$ in propanone). Viable pollen was visualised by fluorescence microscopy. Seeds of transgenic plants were germinated on sterile damp filter paper in 9cm Petri dishes at 12 seeds per Petri dish and incubated at 28°C in the dark for 5 d. Following germination, viable seedlings were placed in 175ml jars each containing 40ml of MS medium with $50mgl^{-1}$ hygromycin B (Duchefa) and $30mgl^{-1}$ sucrose semi-solidified with 0.4% agarose. Leaf tissue from plants regenerated following seed viability experiments was assessed for presence of the gene using PCR (Section 2.2.4.3) and for gene transcription RT-PCR (Section 2.2.4.4).

2.3 RESULTS

2.3.1 Tissue Culture Results

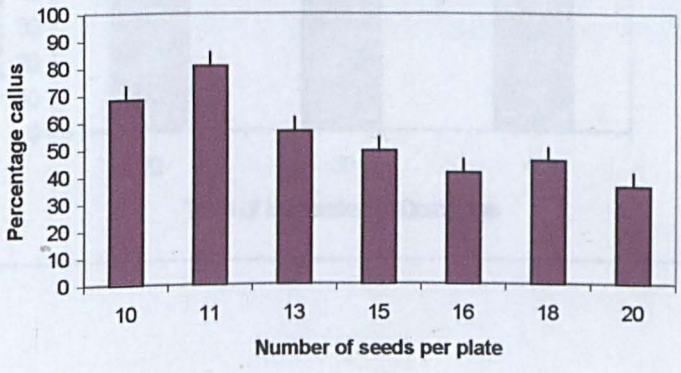
The percentage of calli induced was plotted against the number of days between subcultures in order to determine whether there was any relationship between time between subcultures and the percentage of callus (Figure 2.1). The maximum percentage callus was obtained with 18-24 days after subculture. However the relationship was statistically insignificant. In all graphs, error bars indicate the standard error.

Figure 2.1
Percentage of seeds forming callus for different intervals of subculture



Experiments were set up to determine whether increasing the number of seeds on the Petri dish would decrease the percentage callus formation (Figure 2.2), $X^2 = 5.5$, which was 5% significant with 4 degrees of freedom. The percentage callus formation decreased when the density of seeds exceeded 11per 9cm diameter Petri dish.

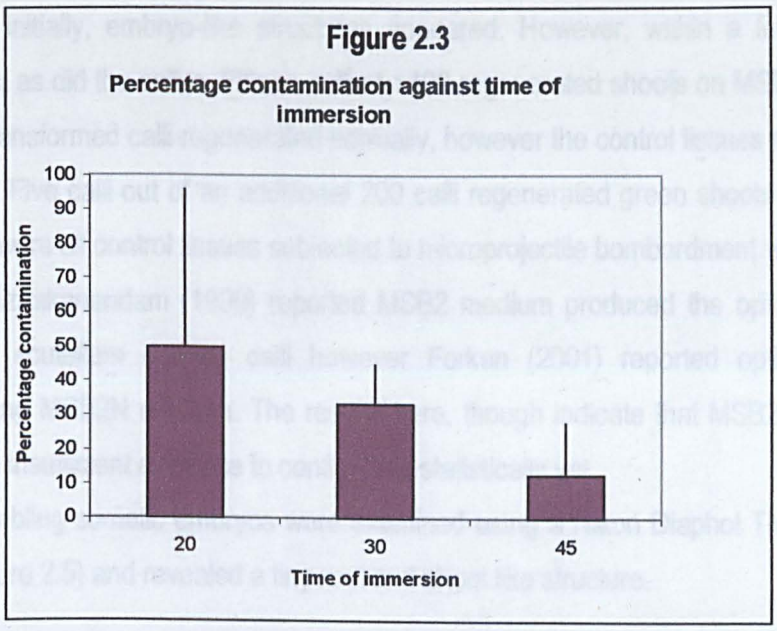
Figure 2.2
Percentage of seeds forming callus against number of seeds per plate



2.3.1.1 Percentage Contamination

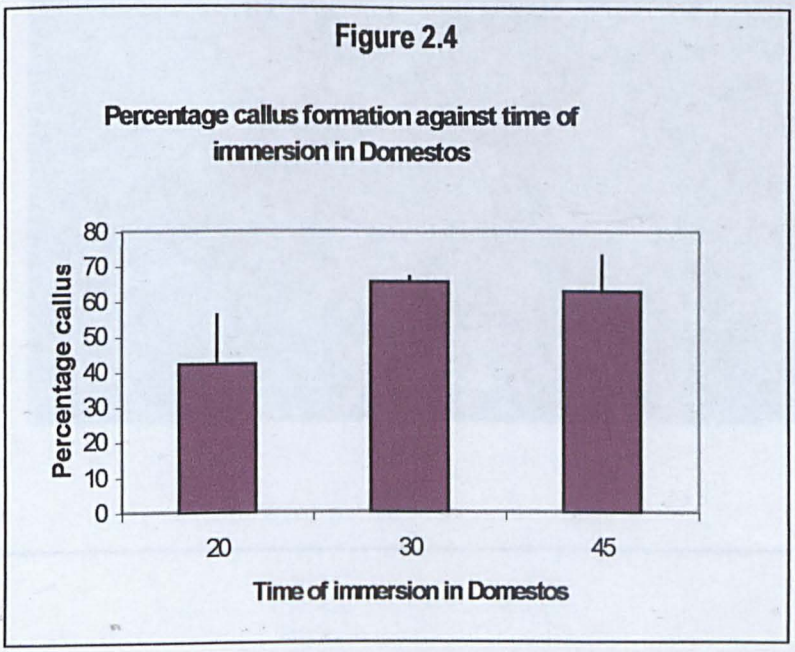
Percentage contamination was plotted against the time of immersion in 50% (v/v) "Domestos" in order to determine whether there was any reduction in contamination with longer immersion in "Domestos" (Figure 2.3). Forty-five min immersion in "Domestos" reduced percentage contamination. However, the variability within the samples resulted in the data not being statistically significant.

Figure 2.3



The percentage callus formation was plotted against time of immersion in "Domestos" to determine whether increasing the time of immersion reduced the percentage callus formation (Figure 2.4).

Figure 2.4



Extending the time of immersion in 50% (v/v) "Domestos" to 45 min did not have any detrimental effect on the percentage callus formation. However, the results were not found to be statistically significant, due to the variability within each sample.

2.3.1.2 Regeneration media

Shoots were not regenerated on MSKN medium from either putatively transformed material or control tissues. Initially, embryo-like structures appeared. However, within a few d, these became necrotic, as did the callus. Fifteen calli of >400 regenerated shoots on MSB2 medium. The putatively transformed calli regenerated normally, however the control tissues regenerated chlorotic shoots. Five calli out of an additional 200 calli regenerated green shoots on MSB2N medium. These were all control tissues subjected to microprojectile bombardment with gold not carrying DNA. Azhakanandam (1999) reported MSB2 medium produced the optimum shoot regeneration of scutellum derived calli however Forkan (2001) reported optimum plant regeneration using MSB2N medium. The results here, though indicate that MSB2 is optimal, however there is insufficient evidence to confirm this statistically yet.

Structures resembling somatic embryos were examined using a Nikon Diaphot TMD inverted microscope (Figure 2.5) and revealed a tiny root and shoot like structure.

Figure 2.5
Putative somatic embryos

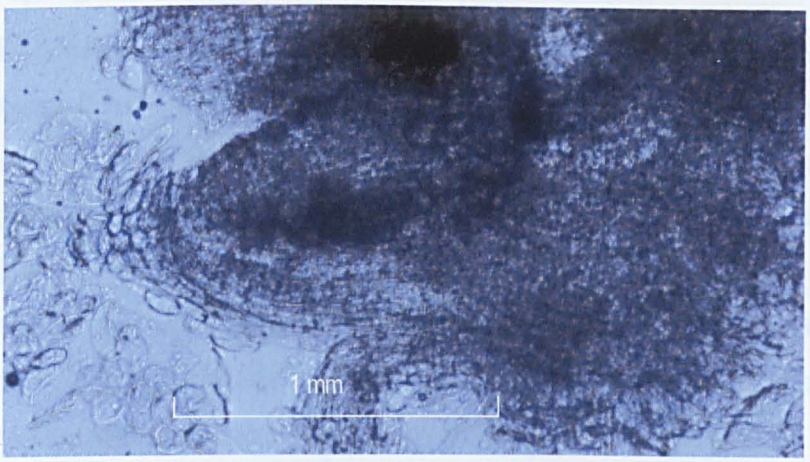
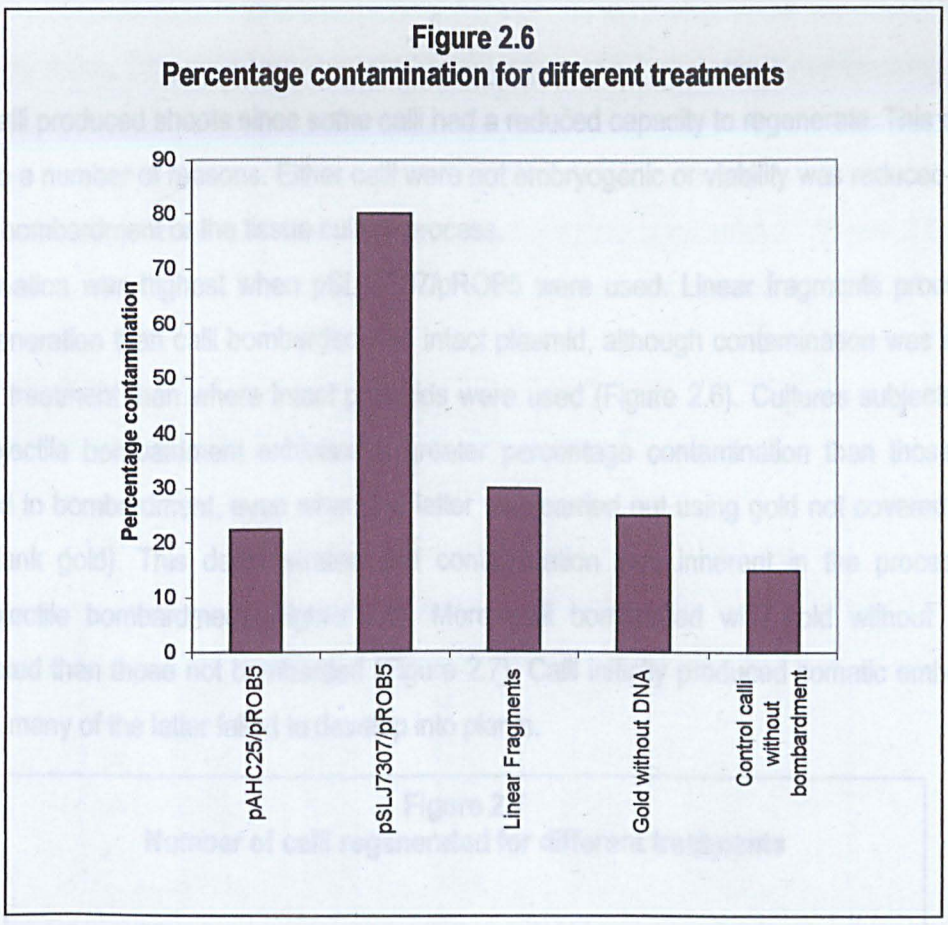


Figure 2.6
Percentage contamination for different treatments



2.3.2 Microprojectile bombardment

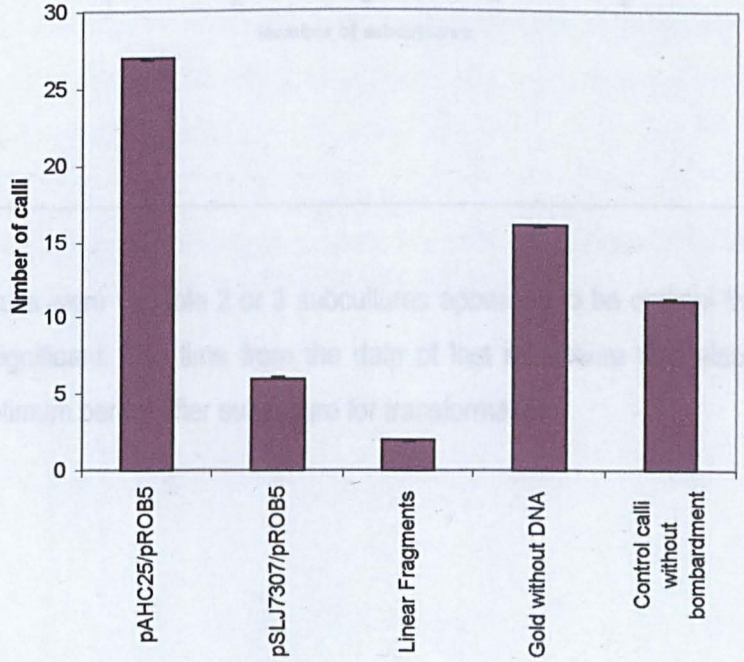
Calli derived from mature embryo scutella were subcultured for varying times and subjected to microprojectile bombardment with pROB5 with either pAHC25 or pSLJ7307 or with gold particles not carrying any DNA. The length of time in culture was assessed to determine if increasing the time in culture reduced shoot regeneration. Tissues subjected to microprojectile bombardment, were cultured on LS2.5 medium with 0.4% (w/v) agarose containing hygromycin at 50mgl^{-1}. After 28d and two subcultures they were transferred to regeneration medium. The percentage contamination was greater in tissues subject to bombardment (Figure 2.6).

However, there was regeneration in both putatively transformed tissues and those bombarded with gold particles not containing DNA, chi squared value $X^2 = 12.6$ with 4 degrees of freedom significant at the 5% level (Figure 2.7).

Not all calli produced shoots since some calli had a reduced capacity to regenerate. This could be due to a number of reasons. Either calli were not embryogenic or viability was reduced as a result of bombardment or the tissue culture process.

Contamination was highest when pSLJ7307/pROB5 were used. Linear fragments produced less regeneration than calli bombarded with intact plasmid, although contamination was lower with this treatment than where intact plasmids were used (Figure 2.6). Cultures subjected to microprojectile bombardment exhibited a greater percentage contamination than those not subjected to bombardment, even when the latter was carried out using gold not covered with DNA (blank gold). This demonstrated that contamination was inherent in the process of microprojectile bombardment (Figure 2.6). More calli bombarded with gold without DNA regenerated than those not bombarded (Figure 2.7). Calli initially produced somatic embryos, although many of the latter failed to develop into plants.

Figure 2.7
Number of calli regenerated for different treatments

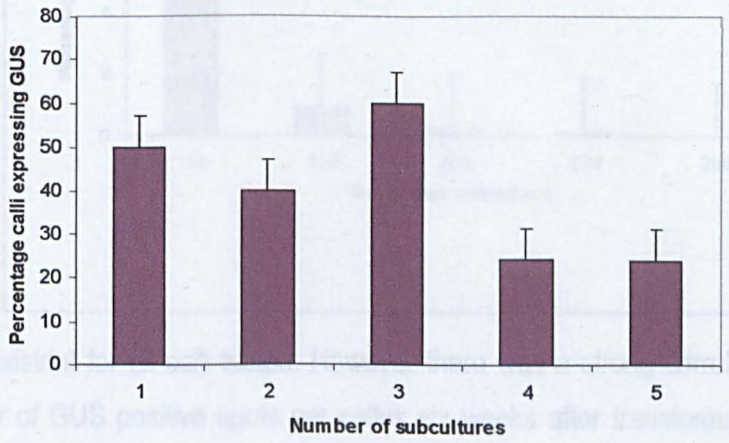


2.3.3 GUS expression of bombarded calli

GUS expression was assessed in terms of numbers of calli with indigo spots and the numbers of spots per callus. Transient gene expression was assessed 6d after transformation; long-term stability was assessed 42d after transformation

There was no clear correlation between the number of subcultures and the number of GUS positive calli that were recorded for GUS expression 42d after bombardment (Figure 2.8). Chi squared was not significant. Therefore, the null hypothesis was not rejected.

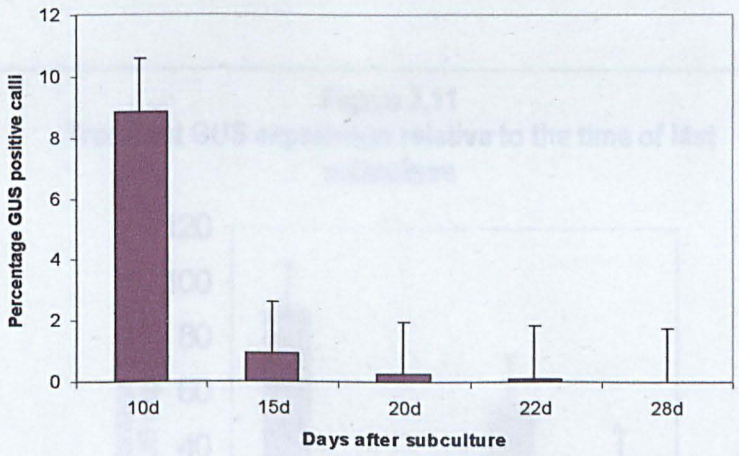
Figure 2.8
Percentage calli expressing GUS after different numbers of subcultures



Although the results were variable 2 or 3 subcultures appeared to be optimal the chi-squared value was not significant. The time from the date of last subculture was also measured to determine the optimum period after subculture for transformation.

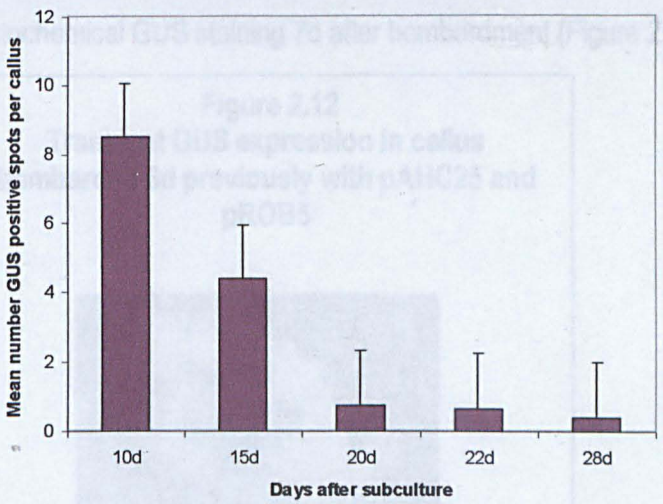
Calli bombarded when more than 15d had elapsed from the date of the last subculture exhibited virtually no GUS positive spots Chi-squared = 8.27 with 3 degrees of freedom significant at the 5% level; Figure 2.9.

Figure 2.9
Percentage GUS expression for different intervals between last subculture and bombardment



This was consistent for all calli tested. However there was a strong correlation between the mean number of GUS positive spots per callus six weeks after transformation and the time between the last subculture and bombardment Chi-squared was 9.43 significant at the 5% level with 5 degrees of freedom (Figure2.10).

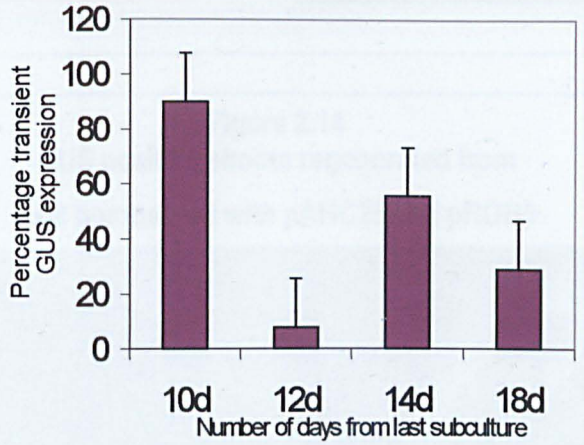
Figure 2.10
Mean number of GUS positive spots per callus for different times of transformation after subculture



Transient GUS expression was measured in order to determine whether the length of time in culture reduced the percentage transformation. The percentage transient GUS expression showed no correlation with the number of subcultures.

Percentage transient GUS expression produced no consistent correlation between the time from the last subculture. However, since bombardment was generally undertaken from 10d to 14d after the last subculture, the time may not have been variable enough to illustrate any trend (Figure 2.11).

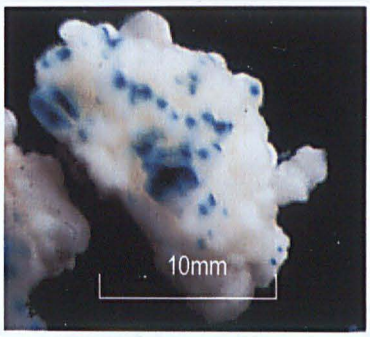
Figure 2.11
Transient GUS expression relative to the time of last subculture



For transient GUS expression, the mean number of GUS positive spots per calli had no apparent correlation with the time between the last subculture and bombardment.

Calli bombarded when more than 15d had elapsed after the last subculture exhibited virtually no GUS-positive spots. This was consistent for all calli tested. Transient GUS expression was assessed using histochemical GUS staining 7d after bombardment (Figure 2.12).

Figure 2.12
Transient GUS expression in callus bombarded 6d previously with pAHC25 and pROB5



GUS expression was assessed again six weeks after bombardment (Figure 2.13). Later shoots from GUS positive plants were assessed (Figure 2.14).

Figure 2.13

GUS expression in callus 42d after bombardment with pAHC25 and pROB5

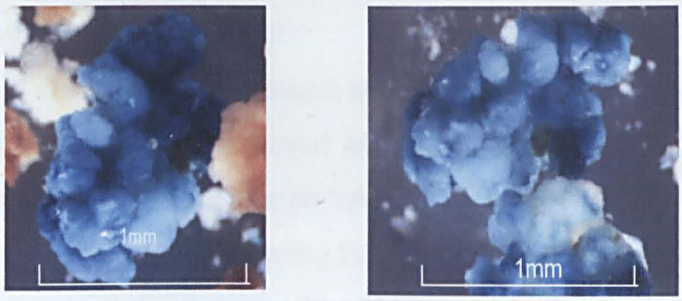
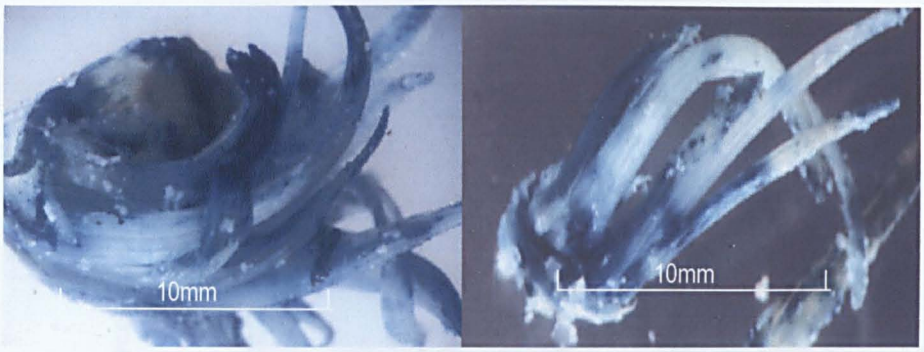


Figure 2.14
GUS positive shoots regenerated from
calli bombarded with pAHC25 and pROB5



Control calli and shoots regenerated from these tissues did not exhibited GUS expression. Rupture disk pressure had very little effect on the GUS expression in bombarded callus.

2.3.4 plating densities of callus

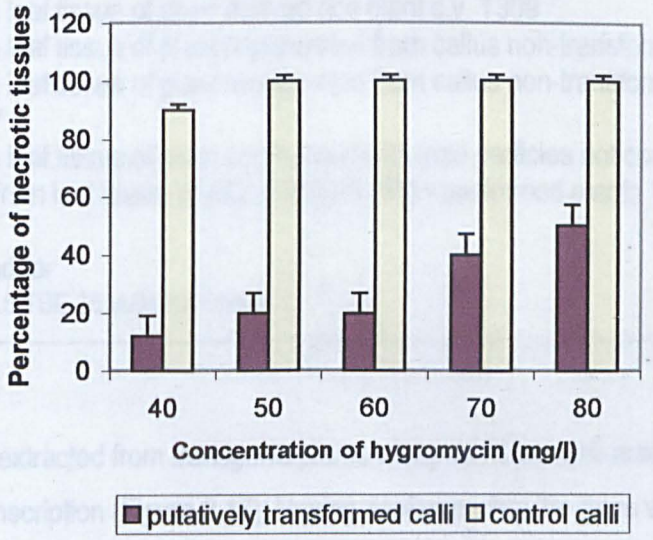
The optimum number of calli per 9cm Petri dish following bombardment was 10-12 since calli plated at greater densities became brown and flaccid 7d after transfer to shoot regeneration medium.

2.3.5 Concentrations of hygromycin for selection of putative transformants

After 28d on medium containing hygromycin 50mgl^{-1}, calli not subjected to microprojectile bombardment, had not increased significantly in size. Putatively transformed calli grew on medium when the concentration of hygromycin was 60mgl^{-1} but grew slowly with concentrations of hygromycin above 60mgl^{-1}.

Control tissues failed to survive with hygromycin above 40mgl^{-1}. Such calli were soft and flaccid when handled with forceps and were scored as non-viable (Figure 2.15). Interestingly the putatively transformed calli survived better on medium with hygromycin at 50-60mgl^{-1} than on medium with hygromycin at 40mgl^{-1} suggesting that concentration of hygromycin in the medium assisted growth of transformed calli. Above 60mgl^{-1} even putatively transformed calli exhibited increased necrosis.

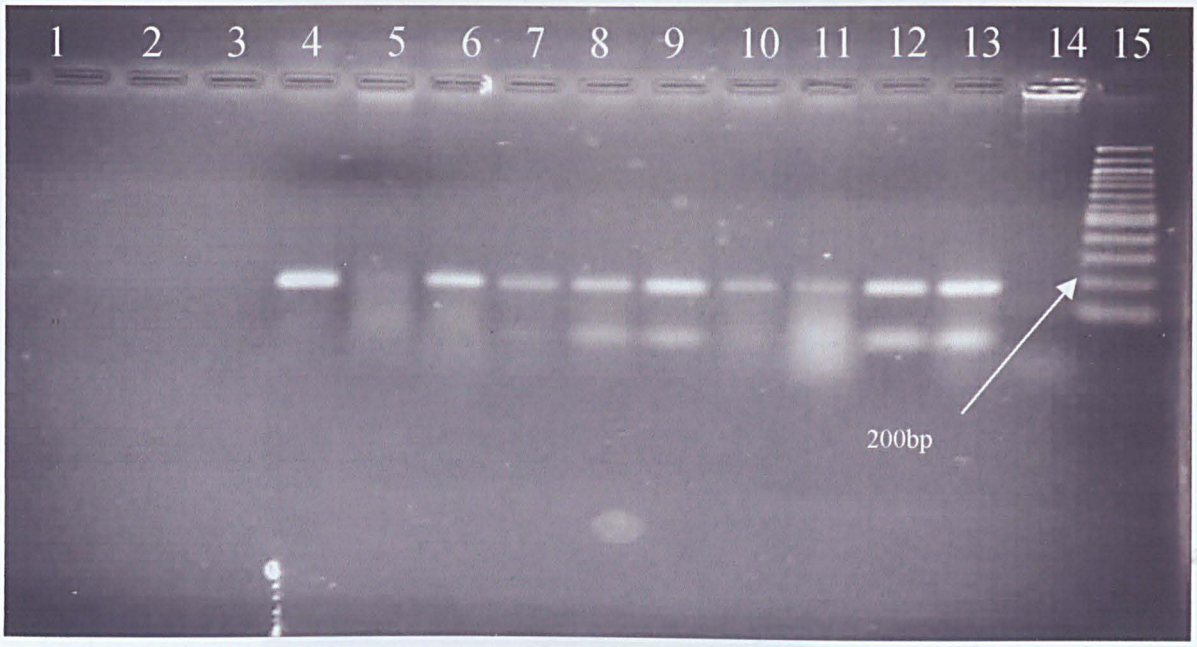
Figure 2.15
Concentration of hygromycin and percentage necrosis of control and putatively transformed calli



2.3.6 Polymerase Chain Reaction

PCR of DNA extracted from putatively transformed calli, confirmed that the calli contained the same amplified fragments as plasmid DNA and that the callus has been transformed. Similar fragments were not amplified from non-transformed calli. PCR of DNA extracted from rice leaves confirmed the transgenic status of the plants (Figure 2.16).

Figure 2.16
PCR using *nahG* primers of genomic DNA extracted from non-transformed plants and plants transformed with pSLJ7307/pROB5

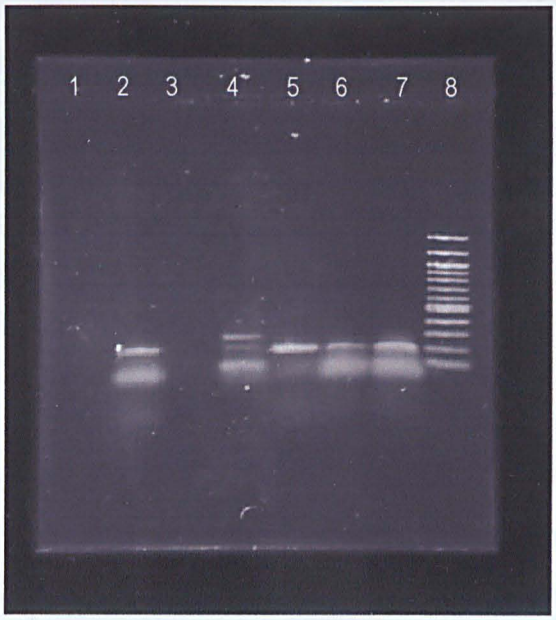


1= DNA from leaf tissue of seed derived rice plant c.v. T309
2= DNA from leaf tissue of plant regenerated from callus non-transformed 12
3= DNA from leaf tissue of plant regenerated from callus non-transformed 13
4= pSLJ7307
5= DNA from leaf tissue of plant bombarded with gold particles not coated in DNA
6-13 = DNA from leaf tissue of pSLJ7307/pROB5 transformed plants 1, 4, 7, 10, 11, 13, 14, 15
14 = pROB5
15= 100bp ladder
2% agarose 0.5 TBE 75 volts for 1 hour

2.3.7.RT-PCR

RT-PCR of RNA extracted from transgenic plants using *nahG* primers resulted in confirmation of *nahG* DNA transcription (Figure 2.17). Having confirmed that the gene was present in the regenerated plants it was necessary to assess the presence and activity of the protein salicylate hydroxylase. The presence of the protein was determined using 2D-SDS PAGE gel separation. Enzyme activity was assessed by enzyme assays.

Figure 2.17
RT-PCR of RNA extracted from non-transformed plants and plants transformed with pSLJ7307/pROB5



1= RNA reverse transcriptase not added
2 = RNA from pSLJ7307/pROB5 transformed plant 4
product size = 200 bp
3 = RNA from a non-transformed plant T309
4 = MSB2 positive control
5-7 = RNA from pSLJ7307/pROB5 transformed plants 7,10,14
8 = 100bp ladder

2.3.8 Bioinformatics

Searching for homology between the DNA sequence for the *nahG* gene and homologues in plants did not produce any homology with any plant sequence available. The protein sequence was then used to search for homology with any protein in rice. The resulting search produced no homology to any protein found in rice. However, there was significant homology to proteins found in *Arabidopsis thaliana,* (table 3).

Table 2.5

Distribution of Blast Hits on the Query Sequence

Sequences producing significant alignments:	(bits) Value
gb|AAG17703.1|AF281655_1 (AF281655) zeaxanthin epoxidase [*Arabidopsis thaliana*]	64 4e-09
dbj|BAB08942.1| (AB026640)zeaxanthin epoxidase precursor [*Arabidopsis thaliana*]	64 4e-09
gb|AAF82390.1|AF134577_1 (AF134577)zeaxanthin epoxidase [*Arabidopsis thaliana*]	63 5e-09
gb|AAD15449.1| (AC006068)putative monooxygenase [*Arabidopsis thaliana*]	63 5e-09
dbj|BAB11935.1| (AB030296) AtABA1[*Arabidopsis thaliana*]	63 7e-09
sp|P93236|ABA2_*Lycopersicon esculentum* ZEAXANTHIN EPOXIDASE PRECURSOR gi|7489	63 8e-09

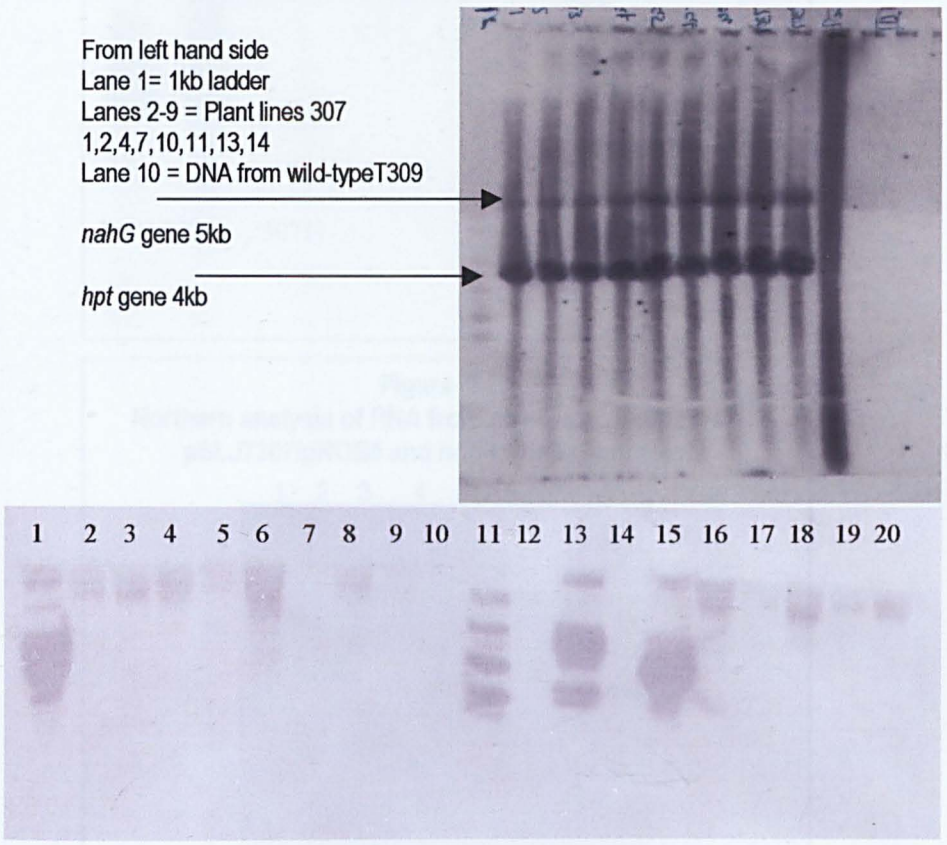
2.3.9 Plant Regeneration

In total approximately 3600 calli were subjected to microprojectile bombardment, of which approximately 1800 were lost to contamination. Ten putative transgenic lines were produced from the remainder. Further microprojectile bombardment experiments, resulted in the production of 4 more putative transgenic plants tested due to time constraints. Half of the explants for each line were transferred into compost whilst half remained in culture. Three lines of plants subjected to microprojectile bombardment with uncoated gold were regenerated, with an additional line still in tissue culture. Four lines of plants not subjected to bombardment were produced from calli at the same time as those subjected to bombardment. Plants were also produced directly from seed of Taipei 309, Pusa Basmati 1 and Martelli . Three lines of plants possessing the *gus* gene from pAHC25 and the *hpt* gene from pROB5 derived from earlier experiments (approximately 960 calli bombarded) were also maintained and half of the plants were transferred to the glasshouse. The efficiency of transformation for pSLJ7307 and pROB5 was 0.28% and for pAHC25 and pROB5 was 0.31%.

2.3.10 Southern analysis

The analysis of DNA restricted with *Hind III* and *sac I* in transgenic plants was carried out a number of times. After enhancement using paintshop pro 6 the Southern analysis can be seen (Figure 2.18)

Figure 2.18
Southern analysis of DNA from plant lines transformed with pSLJ7307/pROB5
***Hind* III restriction, illustrating presence of both genes above**
***Sac* 1 restriction illustrating copy number below**



From left SacI digest illustrating copy number
Lanes 1-9 = pSLJ7307/pROB5 transformed plants 1,2,4,7,10, 11, 13,14, 15
Lane 10 = T309 seed derived non-transformed plant
Lane 11 = 1kb ladder
Lane 12 = Uncoated gold plant 6
Lanes 13-20 = pSLJ7307/pROB5 transformed plants 16, 17, 18, 20, 21, 22, 25, 26

Some lines failed to produce evidence on copy number, in this experiment but produced positive results in subsequent experiments (Figure 2.19). However, all transgenic lines produced positive RT-PCR results and therefore the foreign gene had been transcribed. Verification of transcription was sought using northern analysis (Figure 2.20)

Figure 2.19
Southern analysis of pSLJ7307 lines with *Sac* 1 restriction illustrating copy number

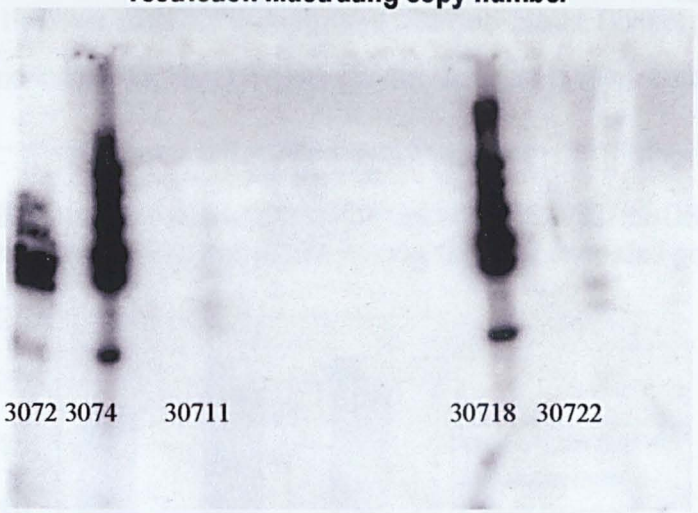
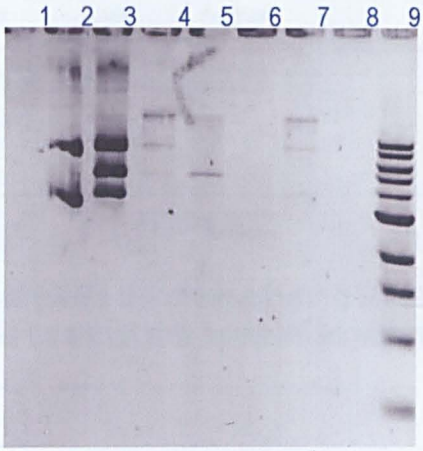


Figure 2.20
Northern analysis of RNA from plants transformed with pSLJ7307/pROB5 and non-transformed plants

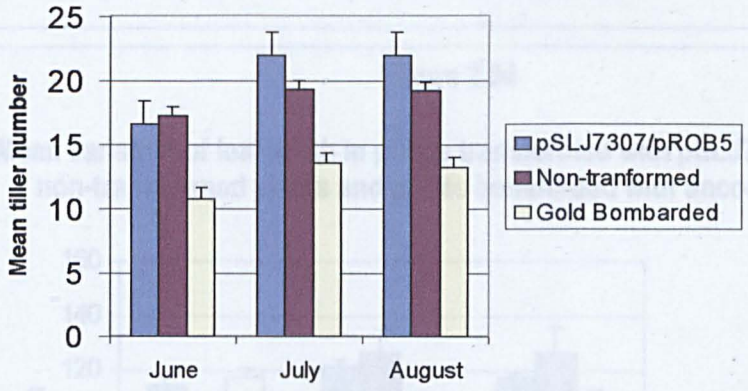


Key from left
Lane 1 = RNA from non-transformed plant
Lanes 2-8 = RNA from transformed plants 1,2,4,7,10,11.13
Lane 9 = 100 bp ladder

2.3.11 GROWTH ANALYSIS

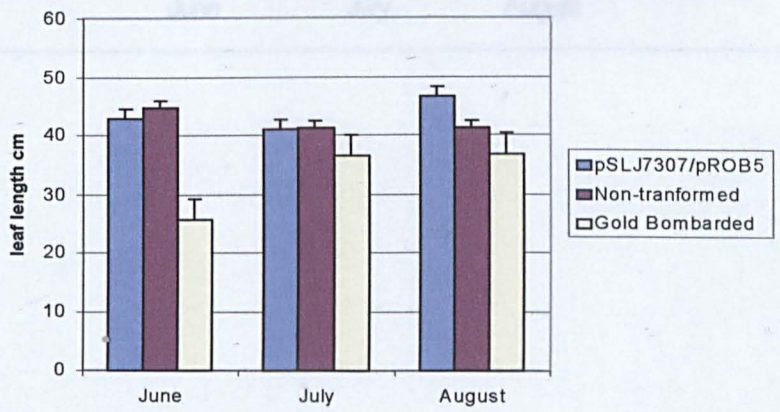
Transgenic, control and plants bombarded with uncoated gold varied significantly in aspects of their growth. Initial observations were that plants derived from calli subjected to microprojectile bombardment with uncoated gold were shorter with fewer tillers (Figure 2.31). Leaves of these plants were thinner, shorter and narrower (Figures 2.32-2.34). ANOVA carried out on these results were significant for all measurements at the 0.001% level with 25 degrees of freedom. These plants were also chlorotic. Two of the pSLJ7307/pROB5 transgenic plants exhibited these effects. However, other plants resembled wild-type plants. Control plants were taller with more tillers and longer, thicker and wider leaves. Variation in tiller number is given in Figure 2.21.

Figure 2.21
Mean tiller number for plants transformed with pSLJ7307/pROB5 non-transformed plants and plants bombarded with uncoated gold



Variation in leaf length can be seen in figure 2.22.

Figure 2.22
Mean leaf length of plants transformed with pSLJ7307/pROB5 non-transformed plants and plants bombarded with uncoated gold



Variation in plant height leaf width and leaf thickness (Figures 2.23-2.25).

Figure 2.23

Mean variation in plant height in plants transformed with pSLJ7307/pROB5, non-transformed plants and plants bombarded with uncoated gold

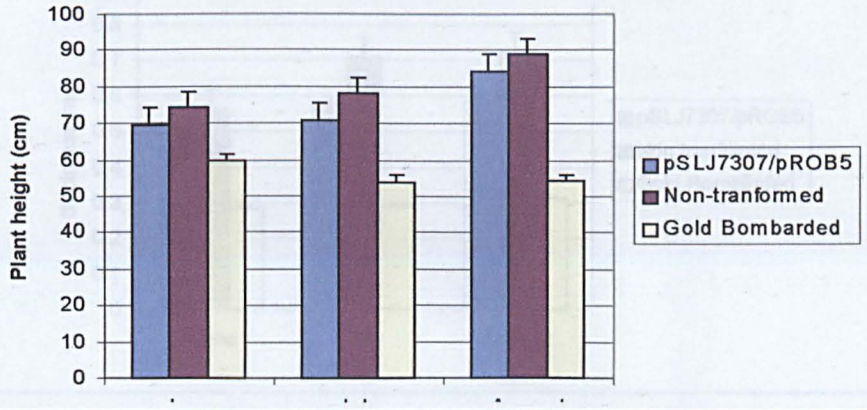


Figure 2.24

Mean variation of leaf width in plants transformed with pSLJ7307/pROB5, non-transformed plants and plants bombarded with uncoated gold

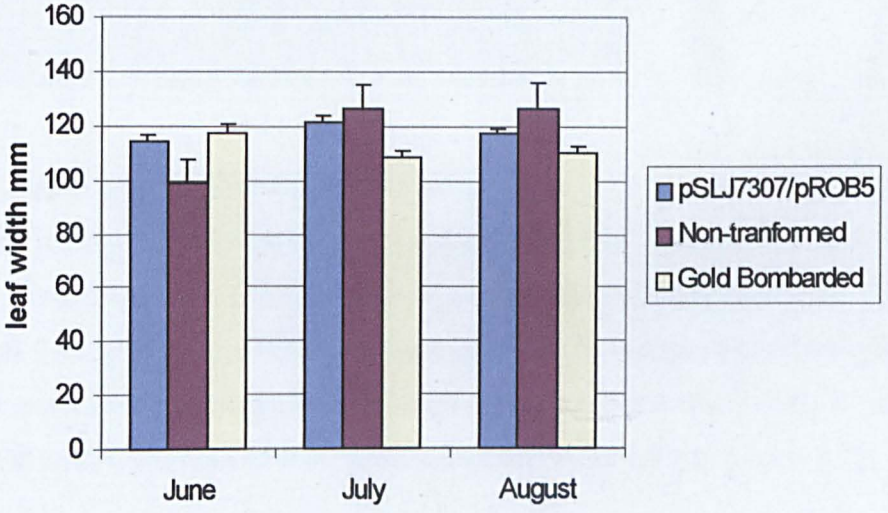
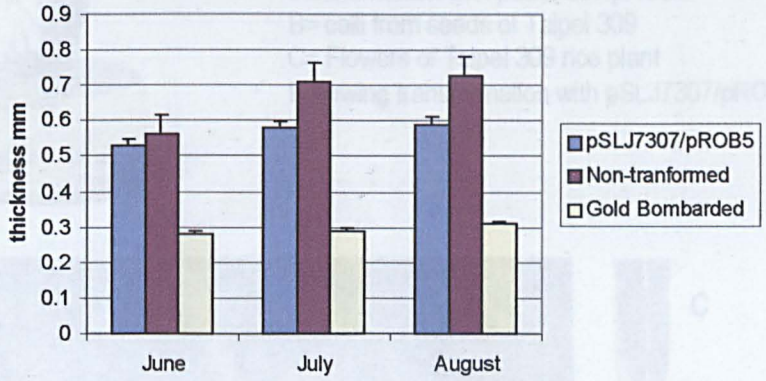


Figure 2.25

Mean variation in leaf thickness in plants transformed with pSLJ7307/pROB5, non-transformed plants and plants bombarded with uncoated gold

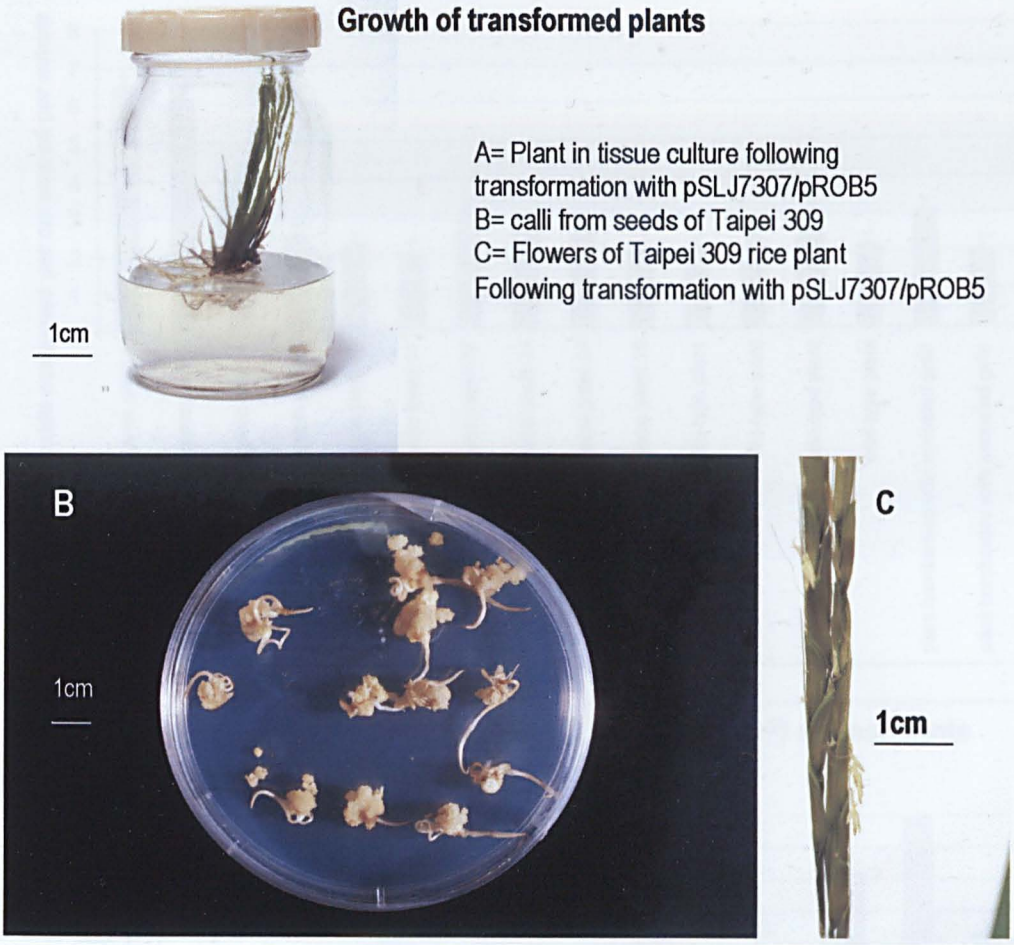


The regenerants can be seen in Figure2.26.

Figure 2.26

Growth of transformed plants

A= Plant in tissue culture following transformation with pSLJ7307/pROB5
B= calli from seeds of Taipei 309
C= Flowers of Taipei 309 rice plant
Following transformation with pSLJ7307/pROB5



3.3.12 ANALYSIS OF PHOTOSYNTHESIS

The rate of photosynthesis was lower in plants bombarded with uncoated gold than in transgenic plants (Figure 2.27). However, the effect was not statistically significant. There was considerable variation between the lines in every treatment. Plants transformed with pSLJ7307 numbers 1 and 2 were particularly high. Stomatal conductance was much lower in transgenic plants than in either control plants or in plants treated with uncoated gold (Figure 2.28).

Figure 2.27 Mean carbon dioxide consumed per plant cm^{-2} min^{-1}

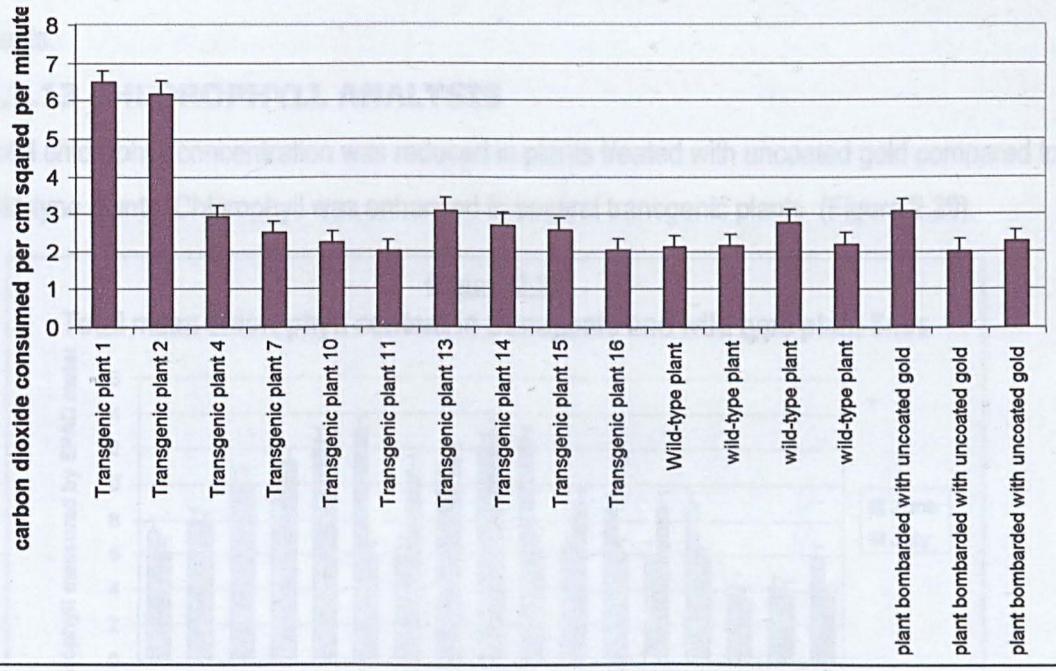
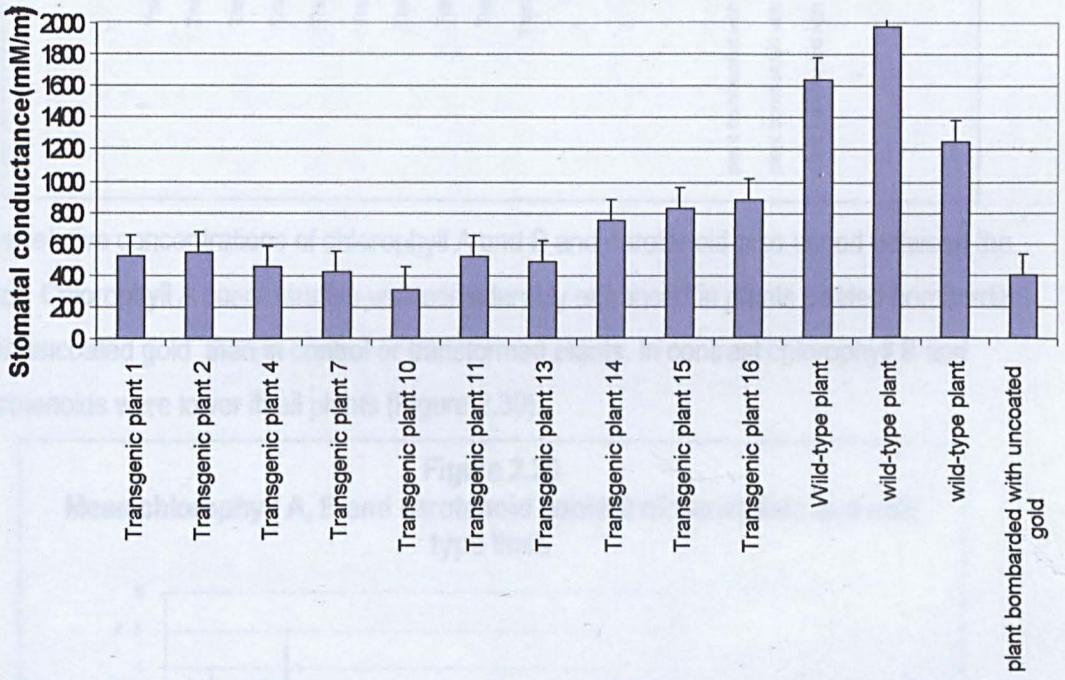


Figure 2.28 Mean stomatal conductance (mM metre^{-2}) against plants



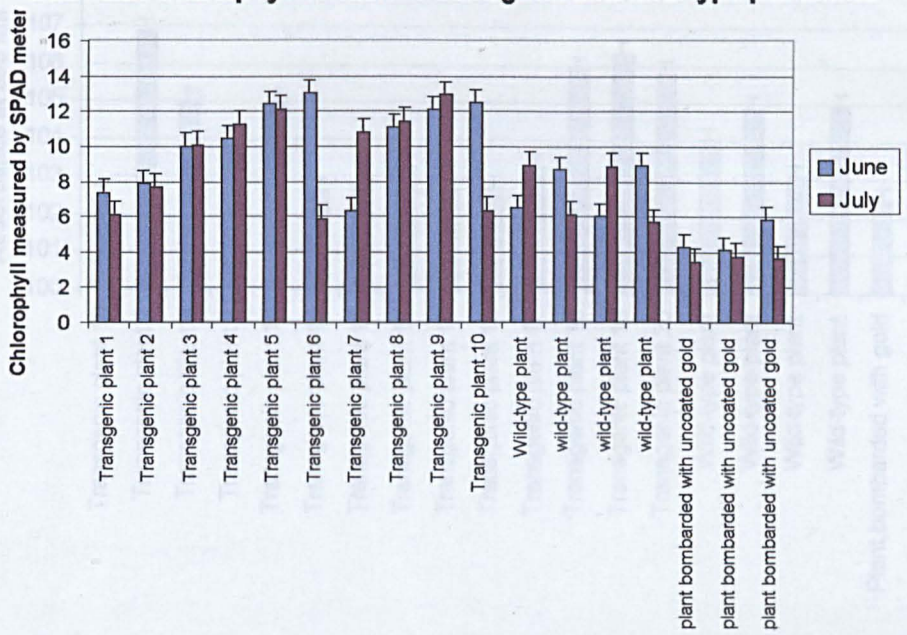
Although carbon dioxide consumed was consistent between lines of transgenic plants with the exception of plants 1 and 2, stomatal conductance was reduced across most lines of transgenic plant lines transformed with pSLJ7307/pROB5 and plants treated with uncoated gold compared with wild-type plants. This could result in enhanced drought tolerance in plants transformed with pSLJ7307 and pROB5, especially as plant growth did not appear to be reduced in

transgenic plants compared to wild-type plants. Plants treated with uncoated gold did show considerably reduced stomatal conductance. However, growth was also reduced in these plants.

2.3.13 CHLOROPHYLL ANALYSIS

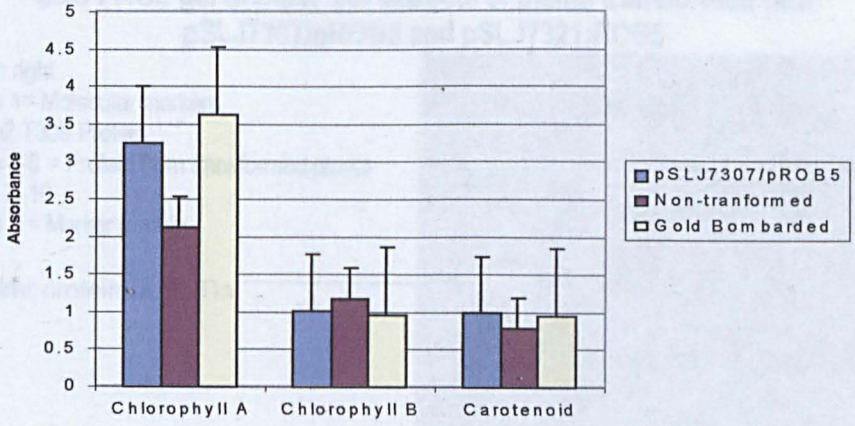
Total chlorophyll concentration was reduced in plants treated with uncoated gold compared to wild-type plants. Chlorophyll was enhanced in several transgenic plants (Figure 2.29).

Figure 2.29
Total mean chlorophyll content in transgenic and wild type plant lines



The relative concentrations of chlorophyll A and B and carotenoid also varied between the lines. Chlorophyll A concentration was considerably enhanced in plants treated bombarded with uncoated gold than in control or transformed plants. In contrast chlorophyll B and carotenoids were lower in all plants (Figure 2.30).

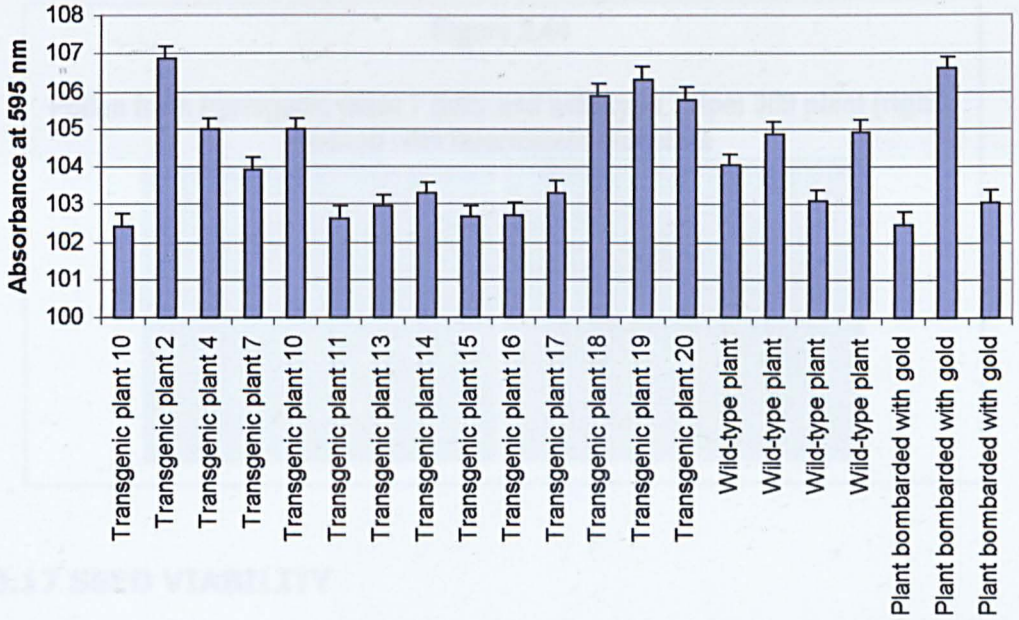
Figure 2.30
Mean chlorophyll A, B and carotenoid content of transgenic and wild type lines



2.3.14 PROTEIN QUANTIFICATION

Protein concentrations varied by 5 percent between plants, but were relatively consistent throughout (Figure 2.31).

Figure 2.31
Mean absorbance at 595 nm for leaf extracts of plants transformed with pSLJ7307/pROB5 demonstrating protein content



2.3.15 PROTEIN GEL

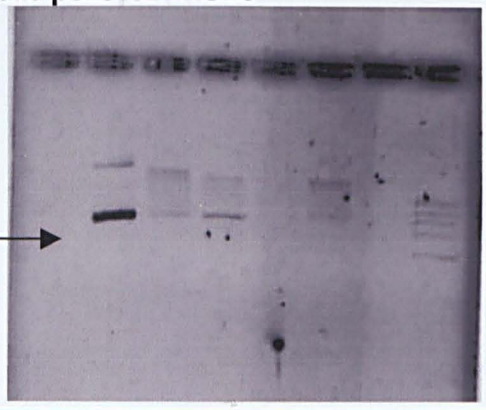
Proteins visualised using SDS-PAGE gels produced a band of just over 40kDA, consistent with the expected size of the protein (Figure 2.43).

Figure 2.323

SDS-PAGE gel of plant leaf extracts of plants transformed with pSLJ7307/pROB5 and pSLJ7321:ROB5

From right
Lane 1= Molecular markers
Lane2 T309 Protein
Lane 3-6 = Protein from transformed plants 1, 4, 7, 10
Lane 7 = Marker protein

Marker protein at 40kDa

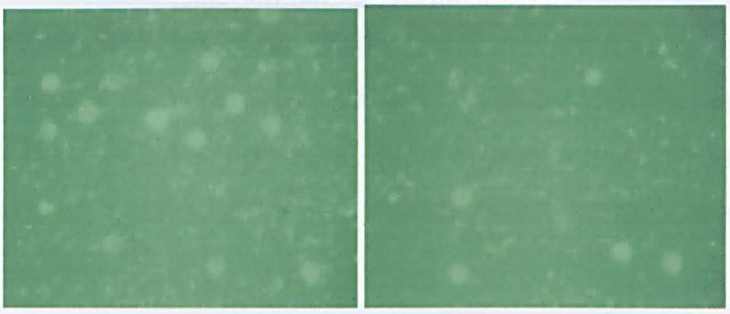


2.3.16 POLLEN VIABILITY

Few of the transgenic lines flowered (transgenic plants 1, 7, 10 11,13 and 15) and in those lines that produced seeds, small panicles and few seeds were produced. Many plants did not flower and remained vegetative (transgenic plants 2, 4, 14, 16). However, those plants that did produce pollen produced viable pollen (Figure 2.44). It was not possible to germinate this pollen *ex vivo* and therefore the viability may have been lower than indicated by the staining.

Figure 2.44

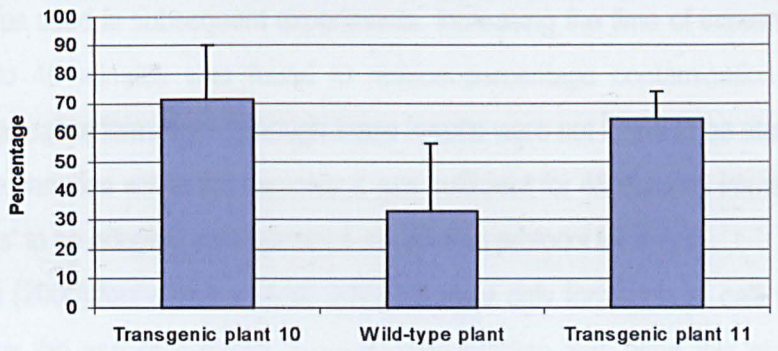
Pollen from transgenic plant 7 (left) and wild-type Taipei 309 plant (right), stained with fluorescein diacetate



2.3.17 SEED VIABILITY

Many transgenic plants were not fertile and therefore few seeds were produced. Of those seeds that were produced, initially only from transgenic plants 10 and 11, seeds germinated on damp filter paper at 25^{0}C in the light after 3 days with a mean germination percentage of 60% (Figure 2.45). Subsequently, increased growth-room humidity led to more transgenic plants (1,7,13 and 15) and control plants producing seeds. Plants from calli bombarded with uncoated gold did not flower.

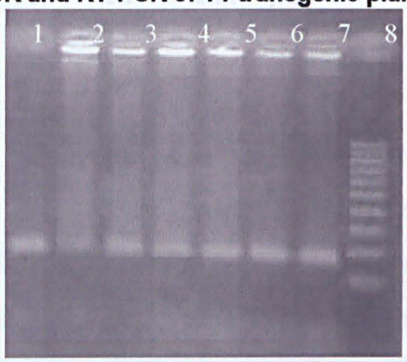
Figure 2.45
Mean percentage germination of progeny from lines pSLJ730710, pSLJ730711 and T309



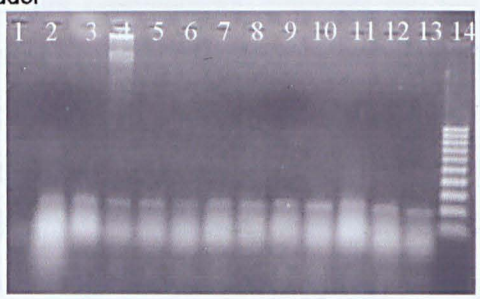
2.3.18 ASSESSMENT OF PROGENY

PCR of DNA extracted from T1 generation seedlings confirmed that they were positive for the gene (Figure 2.46). RT-PCR demonstrated that the gene was transcribed (Figure 2.46). Salicylate hydroxylase assays confirmed that the enzyme activity was enhanced in transgenic T1 plants (Chapter 4).

Figure 2.46
PCR and RT-PCR of T1 transgenic plants



From left
Lanes 1-8 = PCR of DNA with *nahG* primers of T1transgenic plants 1,7, 10, 11, 13 15
Lane 9 = 100bp ladder



From left
Lanes 1-3 and 5-14= RT-PCR of RNA from T1 transgenic plants1, 7, 10, 11, 13 15
Lane 4 = DNA from plasmid pSLJ7307 with *nahG* primers
Lane 15= 100bp ladder

2.4 DISCUSSION

2.4.1 Tissue Culture

Seed density of 11 per 9cm Petri dish was found to produce optimal callus formation and this density was used in subsequent experiments. Increasing the time of immersion in 'Domestos' solution to 45 minutes was found to reduce percentage contamination without reducing percentage callus formation. Although these results were not found to be statistically significant due to the variation within the samples it was sufficient for 45 minutes immersion in 50% (v/v) 'Domestos' to be adopted as a standard sterilisation protocol for seeds.

Al Forkan (2000) found that somatic embryos were only formed from calli when water stress induced by the use of a higher agarose concentration was used this stage was therefore incorporated into tissue culture. All cultivars demonstrated higher plant regeneration frequencies on MSB2N medium, containing BAP and NAA (Al Forkan, 2000). Azhakanandam (1999) reported MSB2 medium induced optimal shoot regeneration of scutellum-derived calli. However, Al Forkan (2000) reported optimal plant regeneration using MSB2 medium containin NAA medium. Both MSB2 and MSB2N induced plant regeneration with no statistical difference between them. MSB2N was used in the present experiments as this promoted root and shoot regeneration.

2.4.2 Bombardment

Contamination was increased when calli were subjected to microprojectile bombardment. Contamination was greatest when intact pAHC25 and pROB5 were used. Although contamination was lower following bombardment with linear fragments of pAHC25 and pROB5, transformation efficiency was reduced. The probability that the constructs fragment during the preparation of microprojectiles or during the bombardment process is proportional to the size of the plasmid. This can result in a higher percentage of fragments of DNA integrating into the plant genome, leading to a lower transformation efficiency and disruption of the metabolism and/or morphology of transformed plants (Southgate *et al.*, 1995; Marchant and Southgate, 1996). As a result, linear fragments of plasmids pAHC25 and pROB5 were used for bombardment and the transformation efficiency compared with that using intact plasmids. It was therefore unexpected that bombardment of linear fragments would have lower transformation efficiency that bombardment of intact plasmids. Several explanations have been considered. The concentration of linear fragments of DNA extracted from agarose gels following restriction could have been lower than quantification of the DNA on the gel, due owing

to losses during extraction of the linear fragments. Alternatively plasmids of 10kb (pAHC25) and 5kb (pROB5) respectively were perhaps too small to demonstrate an appreciable difference in transformation efficiency. Bobardment of linear fragments of pSLJ7307 at 20kb, and pROB5 again produced lower transformation efficiency than co-bombardment of intact pSLJ7307 and pROB5. Even when pROB5 was ligated to pSLJ7307 making a plasmid of 25kb, transformation efficiency did not decline.

Fewer calli were regenerated from control calli not subjected to microprojectile bombardment indicating that the tissue culture process rather than microprojectile bombardment inhibited plant regeneration from calli.

Optimal transformation was achieved using calli between 9 and 14 days after subculture, both transgene expression and the percentage of calli exhibiting stable transgene activity were strongly correlated with the time between the last subculture and transformation. This agrees with the results of Chen *et al.* (1998). The use of actively dividing cells is essential for transformation (Hiei, *et al.*, 1984). A time interval between the last subculture and transformation of 14 days or less ensures that calli have an increased probability of being actively dividing. The density of calli following microprojectile bombardment was found to be optimal at 10-12 calli per 9cm petri dish. Chen *et al.* (1998) also reported that reduction in transformation efficiency was not observed even after 5 subculture cycles on semi-solidified medium or after 4 to 8 subculture cycles in liquid medium prior to gene transfer. In these experiments it was found that transformation efficiency of calli was not reduced even following 5 subcultures. Transformation efficiency was found to be increased by subjecting calli to an osmotic pre-treatment prior to transformation. Zhang *et al.* (1996) found that subjecting embryogenic tissues to an osmotic treatment pre- and post-transformation increased the efficiency of both stable and transient transformation in a number of rice cultivars. Calli cultured on a medium containing 0.6-0.8 M total carbohydrate significantly increased transformation efficiency (Chen *et al.*, 1998). Zhang *et al.* (1996) found that osmotic treatment of cells for 4 hours prior to transformation and 16-20 hours post-transformation enhanced the transformation efficiency. Concentrations of hygromycin in excess of $50mgl^{-1}$ were produced 100% necrotic control tissues following two subcultures. The percentage of necrotic transformed calli increased by a mean of 20% when hygromycin concentrations in excess of 60 mgl^{-1} were used. Hygromycin concentration of 50 mgl^{-1} was therefore found to be optimal for the selection of transgenic calli whilst minimising the percentage of necrotic transgenic calli resulting from the toxic effects of the antibiotic. Zhang *et al.* (1996) tested various hygromycin concentrations on indica rice transformed with a plasmid carrying genes encoding hygromycin

phosphotransferase (*hpt*) and β-glucuronidase (*gus*) driven by the cauliflower mosaic virus CaMV 35S promoter. A hygromycin concentration of 40-50 mgl^{-1} was found to be optimal for the recovery of transgenic plants (Chen *et al.*, 1998).

2.4.3 Plant growth analysis

Initial observations that blank gold plants were stunted and chlorotic were confirmed by analysis. Two of the transgenic lines also produced stunted and chlorotic plants. Microprojectile bombardment provided little control over the site of integration of foreign DNA or the copy number. The aberrant morphology of some transgenic plant lines regenerated following microprojectile bombardment could be due to the lack of control over the site of integration, which can be seen in these stunted and chlorotic lines. Plants regenerated from calli following bombardment of uncoated gold demonstrated morphological changes being stunted and chlorotic with thinner shorter leaves and fewer tillers. These plants had not been subjected to bombardment with transgenes and therefore neither the positional effects of transgene integration, nor the copy number were responsible. The morphological abnormalities recorded must have resulted from somaclonal variation or from bombardment with uncoated gold. Plants transformed with pSLJ7307 lines did not exhibit these effects, so it is unlikely that morphological changes arose from the bombardment process. Unfortunately, calli subjected to a vacuum without bombardment also failed to regenerate, and therefore, it is not possible to state whether subjecting calli to a vacuum caused these morphological abnormalities. Interestingly the stomatal conductance in transgenic plants and in plants derived from calli bombarded with uncoated gold appeared lower this did not result in a substantial difference in photosynthetic efficiency for transgenic plants, but plants derived from calli bombarded with uncoated gold exhibited reduced growth. Lower stomatal conductance in transgenic plants implied that transgenic plants could have an increased drought resistance without loss of photosynthetic efficiency.

CHAPTER 3

AGROBACTERIUM-MEDIATED TRANSFORMATION OF RICE

3.1 INTRODUCTION

Genetic transformation of many dicotyledonous plants is routine using *Agrobacterium tumefaciens*. However, genetic transformation of monocotyledons has developed more slowly as these plants are not natural hosts for *A. tumefaciens*. Direct DNA uptake procedures, including microprojectile bombardment, microinjection and DNA uptake into protoplasts, resulted in multiple copies of genes integrated into the genome in addition to gene fragmentation, rearrangement and a high frequency of sterile plants (Finnegan and McElroy, 1994). It appears from the literature that several factors are of critical importance in the *Agrobacterium*-mediated transformation of rice, which may explain the initial difficulty experienced in *Agrobacterium*-mediated rice transformation.

3.1.1 History of Rice transformation

Hiei *et al.* (1994) were the first workers to produce unequivocal evidence for the production of hygromycin resistant rice plants that also exhibited GUS activity following *Agrobacterium*-mediated transformation. This approach usually results in discreet unrearranged segments of DNA being inserted into the recipient genome at a low copy number (Hiei *et al.*,1994; Dong *et al.*, 1996). The transformation frequency reported was similar to that obtained by the methods used routinely for the transformation of dicotyledonous species (20-30%) by *Agrobacterium* and was higher than for other direct DNA transfer methods such as microprojectile bombardment and protoplast transformation (Azhakanandam, 1999). One to 20% of transgenic rice plants were fertile (Hiei *et al.*, 1997). One of the main advantages of *Agrobacterium*-mediated gene delivery was that relatively large fragments of DNA with defined ends of more or less predictable size were integrated into the plant genome.

Several factors influence the efficiency of *Agrobacterium*-mediated transformation of rice, such as the target explant material, exposure of the bacterial cells to the phenolic wound signal molecule acetosyringone to activate *vir* gene expression, use of super virulent bacterial strains and the development of super binary vectors combined with appropriate selectable markers. All these parameters increased the transformation efficiency for this species (Azhakanandam, 1999; Chan *et al.*, 1993; Hiei *et al.*, 1994).

Efficient DNA delivery into recipient cells is essential for plant transformation with the choice of tissue for transformation being crucial. The use of actively dividing cells, such as those in embryogenic scutellum-derived calli, has been reported to be preferable (Hiei *et al.*, 1994).

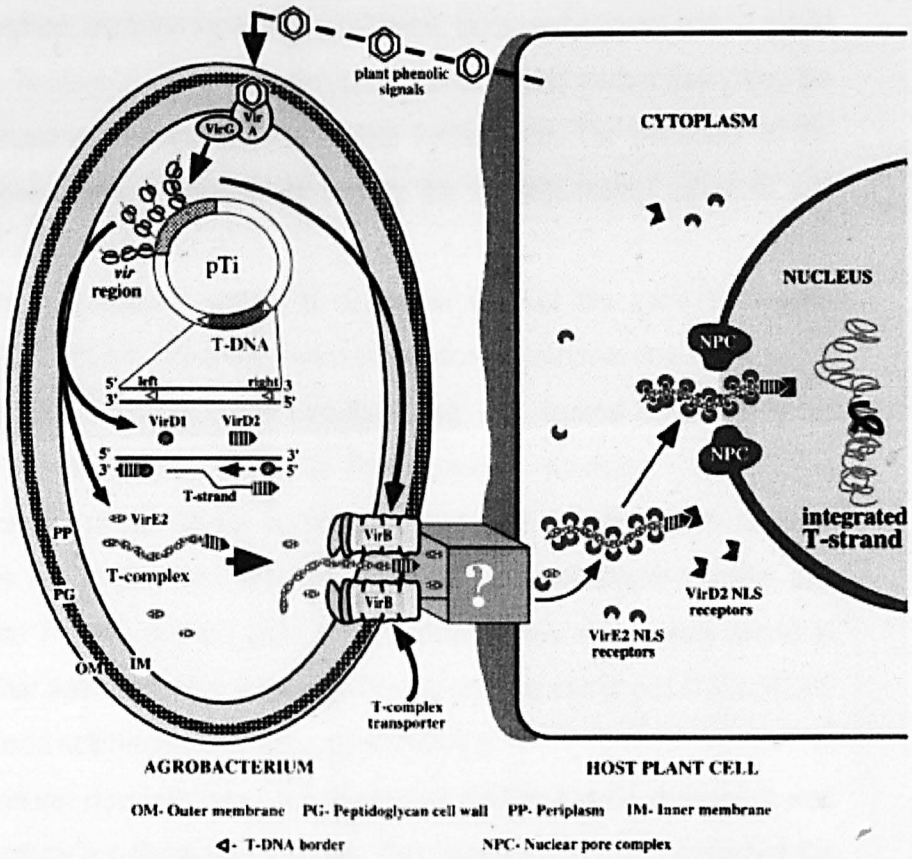
3.1.2 Constructs for rice transformation

Agrobacterium tumefaciens strain LBA4404 has been used with success for gene transfer in rice (Hoekema *et al.*, 1983; Hiei *et al.*, 1994; Azhakanandam, 1999; Al-Forkan 2000). The use of LBA4404 with a super binary vector (such as pTOK233) was most effective for rice transformation (Azhakanandam, 1999; Al-Forkan, 2000). The use *Agrobacterium* strains with extra copies of the virulence genes increased the transformation efficiency, enabling transformation of rice cv.s not previously susceptible to *Agrobacterium* transformation (Raineri *et al.*, 1990; Gould *et al.*, 1991; Chan *et al.*, 1993; Hiei *et al.*, 1994; Azhakanandam, 1999; Al-Forkan, 2000). Upon perception of the inducing signals by the receptor *VirA*, the transcription factor *VirG* is activated by phosphorylation. Activated *virG* induces expression of other *vir* genes. The VirA and Vir G proteins, a 2 component regulatory system, mediate control of gene expression.

VirA protein detects the small phenolic compounds released by wounded plants, resulting in autophosphorylation. VirA phosphrylation of VirG protein leads to activation of gene transcription (Zupan and Zambryski, 1995). Generation of a single-stranded copy of the T-DNA, composed of nucleoprotein associated with at least 2 types of virulence protein, then follows. VirD2 and VirE2 proteins are encoded by Ti plasmid in the bacteria (Yusibov *et al.*,1994) and are the only two proteins essential for the transfer of the T-DNA (Tinland, 1996). These two proteins recognise and nick the 25bp border sequence, which results in attachment of the VirD2 protein to a specified nucleotide on the 5' end of the lower strand of T-DNA. After nicking, VirD2 remains tightly bound to the 5' end of the single stranded (ss) T-DNA transferred to the plant cell (Zupan and Zambryski, 1997), through a VirB-VirD4 mediated bacterial pore enabling the export of the VirD2-ss-T-DNA (Figure 3.1). The single stranded DNA passes through a VirB-VirD channel chaperoned by the VirE1 protein (Dumas *et al.*, 2001). VirE1 protein blocks the binding properties of VirE2 to single stranded ss-T-DNA and facilitates the passage of VirE2 across the bacterial membrane. VirE2 protein facilitates pore formation in the plant cell membrane, binds to ss-T-DNA in the plant cytoplasm to form the VirD2/VirE2 complex attached to the ss-T-DNA (Figure 3.1). This facilitates the passage of the

structure through the nuclear pore complex. VirD2 docks the ss-T-DNA complex to the nuclear pore and VirE2 ensures that the VirD2-ss-T-DNA complex is less than 10nm in diameter for transport into the nucleus. Once inside the cytoplasm, VirE2 protein binds to ss-T-DNA and thus coats the ss-T-DNA protecting it against nucleases in the plant genome (Figure 3.1; Zupan and Zambryski, 1997). Extra copies of *VirG* (Liu *et al.*, 1992 Hansen *et al.*, 1994) or *VirG* containing segments have been reported to increase the efficiency of *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994). Therefore, strain LBA4404, has been supplemented with a constitutive *VirG* mutant gene *VirGN54D* in a *SacI/Hind* III fragment of 1250 base pairs on a compatible plasmid BBR1MCS (Kovach *et al.*, 1994). This has been used in conjunction with a separate binary construct containing an antibiotic gene and gene of interest on a different plasmid, and has been reported to increase transformation efficiency of *Catharanthus roseus* in a 'ternary' transformation system (Van der Fits *et al.*, 2000). The chloramphenicol resistance determinant on pBBR1MCS was not compatible with LBA4404. Therefore, a pBBR1MCS-5 derivative was constructed with a gentamycin resistance gene (Kovach *et al.*, 1995). Transformation efficiency of this system was enhanced when 100µM acetosyringone was used, in addition to a low pH (6.3) and a monosaccharide (10-50mM glucose; Scheeren-Groot *et al.*, 1994). Cloning the *gus*A intron and *bar* genes from pPh1223INT, as *Eco*RI/XbaI and *Eco*RI fragments into pMOGB22 created the binary vector pMOGB22 (Van der Fits *et al.*, 2000).

Figure 3.1
Virulence genes in *Agrobacterium* transformation



(Zupan *et al.*, 2000)

3.1.3 Aims and objectives of the investigation involving *Agrobacterium*-mediated transformation of rice

These experiments aimed to transform the Japonica cv. Taipei 309 with the *nahG* gene using *Agrobacterium*-mediated transformation to modify the production of the enzyme salicylate hydroxylase, encoded for by the *nahG* gene. Increased enzyme activity would thus modify the production of the rice volatiles, veratrole and methyl salicylate, the breakdown products of the product of salicylate hydroxylase. The behaviour of the brown planthopper, which is attracted to rice by the volatiles methyl salicylate and veratrole may then be modified.

Additionally these experiments aimed to determine whether the extra *VirG* gene (*VirG54D*) on pBBR1MCS-5 increased the transformation efficiency of rice.

Whilst it is possible to transform rice simultaneously with several constructs using microprojectile bombardment using co-bombardment (Christou, 1997), the simultaneous transfer of two binary vectors into rice using *Agrobacterium*-mediated gene delivery is not routine and would necessitate the simultaneous transfer and integration of two T-DNAs into the genome. Therefore, these experiments aimed to determine whether both the binary vector pMOGB22 and the vector pSLJ7321::ROB5 could be transferred at the same time using pBBR1MCS-5.

In order to transform rice calli using *Agrobacterium*-mediated gene delivery, it was necessary to produce a suitable binary vector. The plasmid SLJ72321 possessed the selectable marker *npt* II which encoded for the neomycin phosphotransferase gene that conferred resistance to kanamycin sulphate. Kanamycin is widely used with dicotyledons, but is unsuitable and ineffective in monocotyledons (Potrykus *et al.*, 1985; Peng *et al.*, 1992) and, particularly, in rice (Datta, 1999). Consequently, it was necessary to produce a construct for transformation that possessed both the gene of interest namely *nahG,* and a suitable selectable marker. Shimamoto *et al.* (1989), Azhakanandam (1999) and Al-Forkan (2000) used the *hpt* gene as a selectable marker and produced fertile transgenic plants following selection on hygromycin. Therefore, it was decided to restrict pSLJ7321 at the *Hind* III site and to ligate the *hpt* gene restricted as a *Hind* III segment from pROB5 into pSLJ7321, thus making the vector pSLJ7321::ROB5.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of the construct for rice transformation

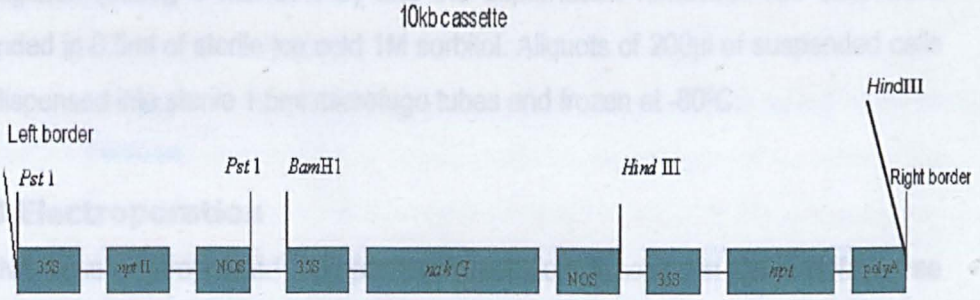
The *hpt* gene from pROB5 (map in Appendix), was restricted using *Hind* III (New England Biolabs, Beverley, Maryland, USA) in the following reaction mixture, 5µl of 10X reaction buffer, 1µl of *Hind* III restriction enzyme, 1µl of BSA (New England Biolabs), 43µl of aqueous solution containing 2µg of pROB5. The reaction mixture and plasmid were incubated at 37^{0}C overnight. Restriction was confirmed by agarose gel electrophoresis of 5µl of the sample.

The plasmid SLJ7321 (map in Appendix) was restricted at the *Hind* III site in a reaction mixture consisting of 5µl of 10X reaction buffer, 1µl of *Hind* III restriction enzyme, 1µl of BSA, 40µl of DNA solution containing 2µg of pSLH7321 and 3µl of sterile deionised water. The reaction mixture and plasmid were incubated at 37^{0}C overnight. Restriction was confirmed by agarose gel electrophoresis of 5µl of the sample. The plasmid was dephosphorylated to prevent self-ligation of the vector. The following components were added to the digested DNA mixture in a sterile microfuge tube, which was incubated at 37^{0}C for 60 min; 10µl of 10X reaction buffer (Promega, Chilworth Research Centre, Southampton, UK), 2µl of calf intestinal alkaline phosphatase (CIAP) and 38µl of nuclease free water. The reaction was terminated with addition of 2.0µl of 0.5M EDTA to the reaction mixture and heating to 65^{0}C for 20 min. The dephosphorylated DNA was extracted using phenol followed by ethanol precipitation.

The ligation of the *hpt* fragment derived from pROB5 into pSLJ7321 was carried out in a sterile 1.5 ml microfuge tube, using 200ng of vector DNA (pSLJ7321), 100ng of insert DNA (the *hpt* gene), 6µl of 10X T4DNA ligase buffer (Promega) and 1µl of T4DNA ligase (Promega). This was incubated at room temperature overnight. Ligation was confirmed by agarose gel electrophoresis of a sample of the reaction mixture. A diagram of the ligated cassette can be seen in Figure 3.2. This construct was transformed into competent cells of *E. coli* using the calcium chloride method (Sambrook *et al.*, 1989). Transgenic bacteria were selected with the antibiotics ampicillin 50mgl^{-1} (selects for the *hpt* fragment) and tetracyclin 5mgl^{-1} (selects for pSLJ7321), followed by extraction using a Midi-prep kit (Sigma-Aldrich, Gillingham, UK). Orientation of the *hpt* gene was confirmed with restriction digests of the ligated

plasmids and the presence of both genes in the ligated plasmid was confirmed with PCR (Chapter 2).

Figure 3.2
pSLJ7321::ROB5 construct



3.2.2 Preparation of competent cells of *Agrobacterium tumefaciens*

Colonies of *A. tumefaciens* were taken from storage (-80^{0}C) and grown on 9 cm Petri dishes each containing 20ml per dish of semi-solid AB medium (dipotassion hydrogen orthophosphate 6.0gl^{-1}, sodium dihydrogen orthophosphate 2.0gl^{-1}, NH_4Cl 2.0gl^{-1}, $MgSO_4.7H_20$ 0.60gl^{-1}, KCl 0.30gl^{-1}, $CaCl_2$ 0.02gl^{-1}, $FeSO_4.7H_20$ 5mgl^{-1}, glucose 10gl^{-1}, pH7.0, Bacto agar 15gl^{-1}).

Three sterile flasks each containing 5ml of liquid AB medium were each inoculated with a loop of one of the 3 constructs used; LBA4404 or LBA4404 containing the plasmid BBR1MCS-5 *virG54D,* or LBA4404 containing the plasmids BBR1MCS-5 and MOGB22. These were incubated overnight at 27^{0}C ± 1^{0}C with shaking at 250rpm. Each culture was maintained separately. An aliquot (1ml) of each culture was used to inoculate one of 3 sterile flasks, each containing 50ml of liquid AB broth. These were incubated overnight at 27^{0}C ± 1^{0}C with shaking at 250rpm. An aliquot of each bacterial culture was used to inoculate several flasks of 500ml. These were incubated at 27^{0}C ± 1^{0}C, shaking at 120rpm until the cells reached a density of 5 X 10^{7}cells ml^{-1}. The cells were decanted into 500ml centrifuge bottles and pelleted by centrifugation at 3000g for 10 min at 4^{0}C. The supernatant was discarded and the centrifuge tubes with the cell

pellets placed on ice. Ice cold 10% glycerol was added to each tube (50ml per tube). The tubes were vortexed to resuspend the cells and 10% (v/v) glycerol added to increase the volume to 500ml. The cells were pelleted by centrifugation at 3000g for 10 min at 4°C. The supernatant was discarded. The cells were again resuspended in 50ml of 10% (v/v) ice cold glycerol, vortexed and centrifuged 3000g for 10 min at 4°C. The supernatant was discarded and the cells suspended in 5ml of sterile ice cold 10% (v/v) glycerol and transferred to a sterile 50ml polypropylene tube. Cells were pelleted by centrifugation (3000g 5 min at 4°C) and the supernatant removed. The cells were suspended in 0.5ml of sterile ice cold 1M sorbitol. Aliquots of 200µl of suspended cells were dispensed into sterile 1.5ml microfuge tubes and frozen at -80°C.

3.2.3 Electroporation

The DNA to be electroporated (2µg per electroporation dissolved in 5µl of RNase free water) was pippetted into sterile microfuge tubes. These were stored on ice.

Sterile SOC medium [20gl^{-1} Bacto-tryptone, 5gl^{-1} Bacto-yeast extract, sodium chloride 0.5gl^{-1}, 10ml of 250mM potassium chloride (pH 7.0), 20mM glucose added after autoclaving and 5ml of 2M magnesium sulphate sterilised by filtration and added after autoclaving)] was dipensed into 1ml aliquots per electroporation in sterile 1ml microfuge tubes and stored on ice.

The electrocompetent cells of *A. tumefaciens* were thawed and stored on ice. Cuvettes, (1mm diameter, Biorad, Hemel Hempsted, UK.) were also stored on ice. For each electroporation 20µl of electrocompetent cells were added to 5µl of DNA solution together with 40µl of sterile ice-cold nuclease-free water. The mixture was pippetted into the cuvette, which was tapped to ensure the contents were mixed.

The micropulser was set to 1.1KV and 192Ω. For each electroporation, the cuvette was inserted into the slide chamber and the latter was pushed into the chamber of the electroporator (Biorad) until the cuvette was seated between the contacts in the base of the chamber. The machine was set to pulse and the parameters were recorded. Each cuvette was immediately removed from the chamber and 1ml of sterile ice cold SOC medium was added. The medium was pippetted up and down a few times to ensure mixing, before being transferred to a sterile 1.5ml microfuge tube. The cells were incubated at 30°C for 3h with shaking at 250rpm. The cells were then centrifuged 10 sec and 800µl of supernatant removed from each tube. One hundred µl aliquots of cells from each electroporation were each plated onto 9cm diameter Petri dishes containing

semi-solidified AM medium [dipotassion hydrogen orthophosphate 6.0gl^{-1}, sodium dihydrogen orthophosphate 2.0gl^{-1}, NH_4Cl 2.0gl^{-1}, $MgSO_4$ .7H $_2$0 0.60gl^{-1}, KCl 0.30gl^{-1}, $CaCl_2$ 0.02gl^{-1}, $FeSO_4$. 7H $_2$0 5mgl^{-1}, glucose 10gl^{-1} (pH7.0) Bacto agar 15gl^{-1}] containing the appropriate selective antibiotics and were incubated for 48 h at 30^{0}C. The treatment structure, antibiotics and controls, can be seen in Table 3.1. LBA4404 containing pSLJ7321::ROB5 was selected using rifampicin 20mgl^{-1}.

LBA4404 containing pBBR1MCS-5 and pSLJ7321::ROB5 was selected using rifampicin 20mgl^{-1} and gentamicin 20mgl^{-1}. LBA4404 containing pBBR1MCS-5, pSLJ7321::ROB5 and pMOGB22 selected on rifampicin 20mgl^{-1}, kanamycin 20mgl^{-1} gentamicin 20mgl^{-1}. The treatment structure ensured that the efficacy of the antibiotics was tested. Cultures were subjected to the electroporation process without the addition of a plasmid and then cells were cultured to confirm that the cells were viable following electroporation.

Table 3.1

Treatment structure for Electroporation

(Bold characters are the treatments. The others were positive or negative controls)

Treatment	**Antibiotics 20mgl^{-1}**
LBA4404	rifampicin , gentamicin (negative control)
LBA4404 pSLJ7321:: ROB5	**rifampicin , kanamycin**
LBA4404 pBBR1MCS-5 pSLJ7321:: ROB5	**rifampicin, kanamycin , gentamicin**
LBA4404 pBBR1MCS-5 pMOGB22 pSLJ7321: ROB5	**rifampicin, hygromycin, kanamycin, gentamicin**
LBA4404 pBBR1MCS-5 pMOGB22	rifampicin, kanamycin, gentamicin (confirmation of viability of bacteria)
LBA4404 pBBR1MCS-5 pMOGB22	rifampicin, hygromycin, kanamycin, gentamicin (negative control)
LBA4404	rifampicin (confirmation of viability of bacteria)
LBA4404 pBBR1MCS-5 virG54D	rifampicin, kanamycin, gentamicin (negative control)
LBA4404 pBBR1MCS-5 virG54D	rifampicin, gentamicin (confirmation of viability of bacteria)

3.2.4 Colony Hybridisation

Colonies produced following electroporation were assessed by colony hybridisation, only those plates where separate colonies were visible being suitable. Positively charged nylon membrane was cut to the size of the Petri dishes and placed on top of the colonies. The membranes were marked at the edge with a needle for future orientation. Membranes were removed from the plates using forceps and placed onto clingfilm. Denaturing solution (0.5M sodium hydroxide, 1M sodium chloride) was pipetted onto the clingfilm in 0.5ml aliquots. The membranes were placed onto the denaturing solution, colony side uppermost, and incubated for 5 min. Membranes were transferred to 0.5ml aliquots of neutralisation solution (1M Tris pH 7.4, 1M sodium chloride) colony side up and incubated for 5 min. The neutralisation step was repeated and the DNA was cross-linked under a UV light (Stratalinker) exposed for 2 min. The membranes were rinsed in 2X SSC (750mM NaCl, 75mM sodium citrate pH 7.0) 0.1% (w/v) SDS for 2 min. The membranes were laid colony side down onto paper towel and blotted with another paper towel to remove lysed bacterial protein without smearing the colonies. The membranes were hybridised using the same protocol as Southern hybridisation (Chapter 2). Colonies identified as positive from hybridisation were tested using PCR (Chapter 2) to confirm the presence of the gene.

All bacterial strains were maintained in 20% (v:v) glycerol at -70^{0}C for long term storage. These glycerol stocks were prepared by adding 750µl of an overnight liquid culture of bacteria to 750µl of sterile LB: glycerol mixture. The latter was prepared by mixing 60ml of liquid LB containing the appropriated antibiotics to 40ml of sterile glycerol. Stocks were transferred to 2ml cryopreservation vials and frozen in liquid nitrogen prior to storage at -70^{0}C. Growth of bacteria on semi-solid medium was initiated by approximately 100µl of frozen culture. Samples were spread onto 20ml aliquots of semi-solid LB (0.8% w:v agar containing the appropriate antibiotics. Bacteria were grown at 26±1^{0}C for 2-3 d on semi-solid LB medium before use.

3.2.5 *Agrobacterium*-mediated rice transformation

Agrobacterium strain LBA4404 containing the plasmids detailed in Table 3.1 were cultured with the appropriate antibiotics. Liquid cultures were initiated by transferring a loopful of bacteria from a 3-d-old bacterial colony on semi-solid medium to 20ml aliquots of liquid LB containing the appropriate antibiotics in 100ml Erlenmeyer flasks. Cultures were incubated on a horizontal shaker (180 rpm, 28±1^{0}C, dark) for 16-17h.

Overnight cultures were grown to an optical density of 1.0-1.5 before diluting [1:1, 1:20, 1:50 (v:v)] 1ml of bacterial suspension with liquid LS2.5 medium in 9cm Petri dishes.

3.2.5.1 Preparation of and inoculation of explants

Embryogenic calli (31 d old) were removed from pre-cultivation medium and immersed in the bacterial suspensions for 15-20 min in sterile 9cm Petri dishes each containing 20ml of bacterial culture. Calli were blotted with sterile filter paper to remove excess bacteria. Inoculated calli were co-cultivated with *Agrobacterium* on LS2.5 medium with the addition of 100µm acetosyringone (pH6.2) in the dark at 27±1°C for 3 d.

After co-cultivation, calli were transferred to LS2.5 with the addition of 250mgl^{-1} cefotaxime for 5-7 d, before transfer to LS2.5 medium with the addition of hygromycin 50mgl^{-1} for 14 d. Histochemical GUS analysis was carried out using the procedure of Jefferson *et al.* (1987) on a selection of calli (4 per experiment) 6d after co-cultivation and at 42 d after co-cultivation. *Agrobacterium* transformation was repeated 3 times.

Tissues were sub-cultured twice onto selection medium for a further 14 d and then transferred to MSB2 medium with 50mgl^{-1} hygromycin and 250mgl^{-1} cefotaxime 1% (w/v) agarose. Cultures were incubated in the dark at 27±1°C until somatic embryogenesis. The number of calli producing somatic embryos was noted for each plate and Chi-squared analysis performed.

3.2.5.2 Plant regeneration from inoculated tissues

Calli producing somatic embryos were transferred to MSB2 medium with 50mgl^{-1} hygromycin and 250mgl^{-1} cefotaxime 1% (w/v) but with the agarose content reduced to 0.4% (w:v), and incubated with a 16 h photoperiod (47µE m^{-2} sec^{-1}, Cool White Florescent tubes) at 26±1°C. The shoot regeneration frequency was recorded after 25 d as the percentage of scutellum-derived calli producing 1 or more shoots. Shoots (2cm in height) were detached from individual calli and transferred to 40ml aliquots of shoot multiplication medium [MSB2 medium with the addition of 50gl^{-1} sucrose, 0.5mgl^{-1} NAA and 250mgl^{-1} cefotaxime, semi-solidified using 0.25% (w:v) Phytagel (Sigma-Aldrich, Gillingham, UK)] in 175 ml capacity screw capped glass jars for 20-25 d. Any unrooted plants were transferred to MS medium with sucrose 30gl^{-1} and NAA 1.5mgl^{-1} semi-solidified with Phytagel (0.25% w:v) pH6.8 for 7-10 d. Regenerated plants were maintained in this medium. Fifty percent of each line of rooted, regenerated transformed

and non-transformed somatic embryo-derived plants were transferred to the glasshouse.

3.2.5.3 Transfer of regenerated plants from culture

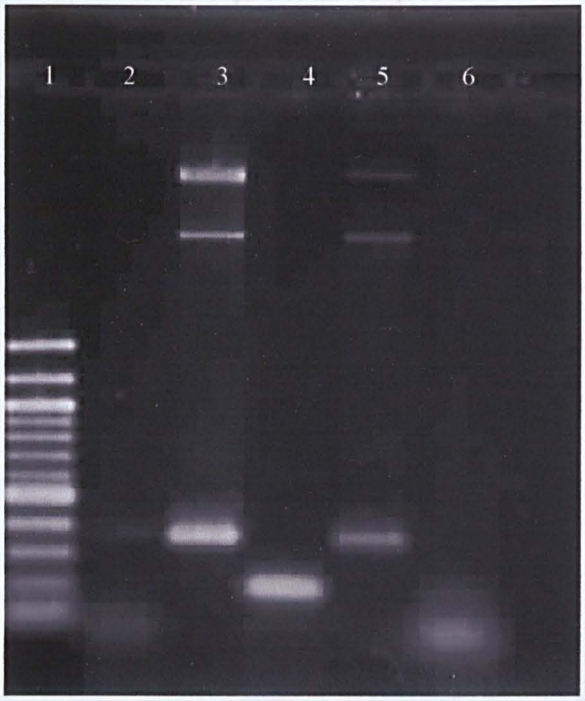
Regenerated plants each approx 5-7 cm in height were removed from MSB2N or MS1.5N and their roots washed thoroughly with tap water. Plants were transferred to 7.5cm diameter plastic pots each containing a mixture 6:6:6:1 by volume of Perlite, Vermiculite, Levington M3 compost and John Innes No.3 compost and well watered. Plants and pots were covered with plastic bags and transferred to a controlled environment room with a temperature of 27±1^{0}C, and a 16h photoperiod (180µE m^{-2} sec^{-1}, Cool White florescent tubes). Seed-derived plants were also grown alongside those derived from calli. After 14 d the corners of each bag were removed and after a further 14 d the bags were removed. Leaf samples were collected for enzyme analysis. Transfer to glasshouse, PCR, RT-PCR Southern analysis and growth analysis were carried out following the methods described in Chapter 2.

3.3 RESULTS

3.3.1 Preparation of the construct pSLJ7321::ROB5

Restriction digest of the ligated construct pSLJ7321::ROB5 showed the presence of both fragments. This was confirmed by PCR (Figure 3.3).

Figure 3. 3
PCR of pSLJ7321::ROB5 for *nahG* and *hpt* genes



From Left
Lane 1 = 100 bp ladder
Lane 2 = primers only
Lane 3 = pSLJ7321::ROB5 *hpt* primers
Lane 4 = pSLJ7321::ROB5 *nahG* primers
Lane 5 = pROB5 *hpt* primers
Lane 6 = no primers

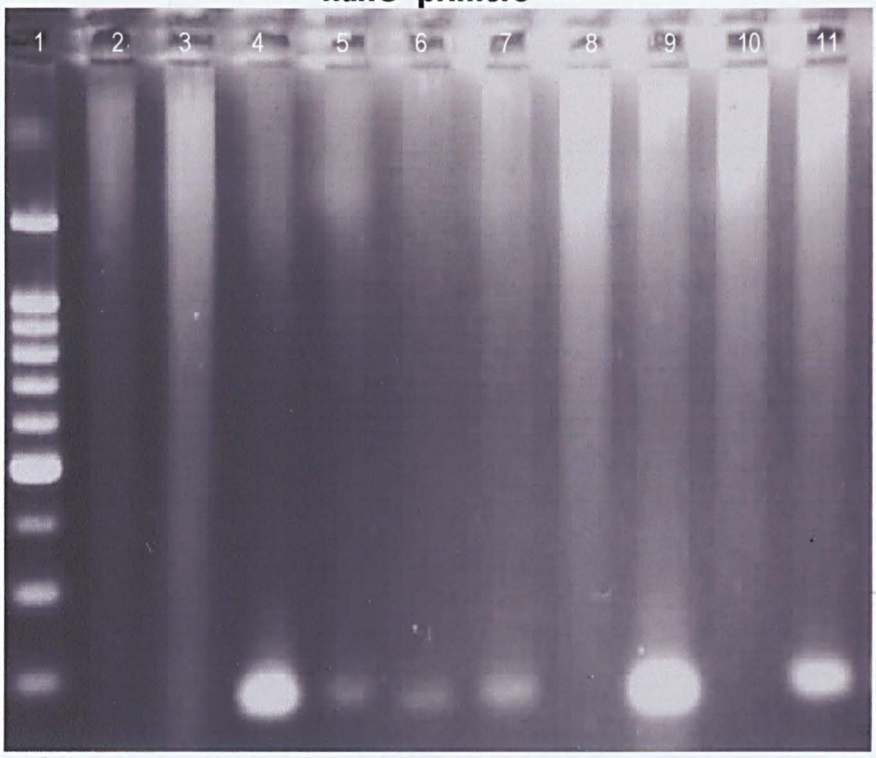
3.3.2 Electroporation of pSLJ7321::ROB5 into *Agrobacterium*

Agrobacterium tumefaciens strains LBA4404, LBA4404 containing pBBR1MCS and LBA4404 containing pBBR1MCS with pMOGB22 proved difficult to grow. Reducing the concentration of the antibiotics to 20mgl^{-1} resulted in improved growth. Increasing stringency of preparation improved the electroporation efficiency. However, colonies were produced only following electroporation after several months. Colony hybridisation

identified several colonies as possibly transgenic. PCR was used to confirm the transgenic status of the colonies Figure 3.4.

Figure 3.4

PCR of DNA extracted from *A. tumefaciens* electroporated with pSLJ7321:: ROB5
***nahG* primers**



From left

Lane 1= 100bp ladder
Lane 2 = No DNA
Lane 3 = No Primers
Lane 4 = LBA4404 with pSLJ7321::ROB5
Lanes 5-7 = LBA4404 pBBR1MCS pSLJ7321::ROB5
Lane 9 = LBA4404 pBBR1MCS, pMOGB22, pSLJ7321::ROB5
Lane 11 = pSLJ7321::ROB5

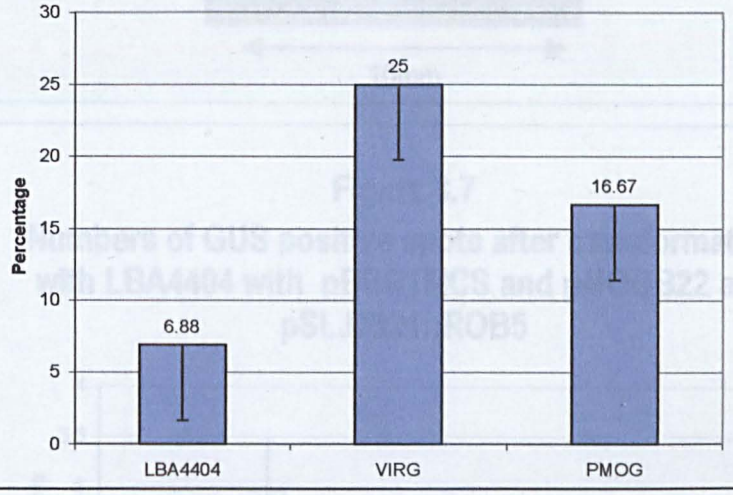
3.3.3 Production of somatic embryos from calli putatively transformed by *Agrobacterium*

Calli transformed using pBBR1MCS, in addition to pSLJ7321::ROB5 produced most somatic embryos 2 months after co-cultivation with *A. tumefaciens*. Calli transformed

using the binary vector pMOGB22 in addition to pBBR1MCS and pSLJ7321::ROB5 produced fewer somatic embryos. However, calli co-cultivated with LBA4404 and pSLJ7321::ROB5 produced the least number of somatic embryos (Figure 3.5).

There was wide variation in the number of somatic embryos produced following inoculation of calli with LBA4404 carrying pSLJ7321::ROB5 and pBBR1MCS and in calli inoculated with LbA4404 carrying pBBR1MCS, SIJ7321:ROB5 and pMOGB22. Standard deviations for each treatment were 6.88, 26.71 and 29.3, respectively. Very few plates were contaminated during the experiment compared to the number that were contaminated using microprojectile bombardment to transform rice callus.

Figure 3.5
Percentage of transformation following *Agrobacterium* co-cultivation



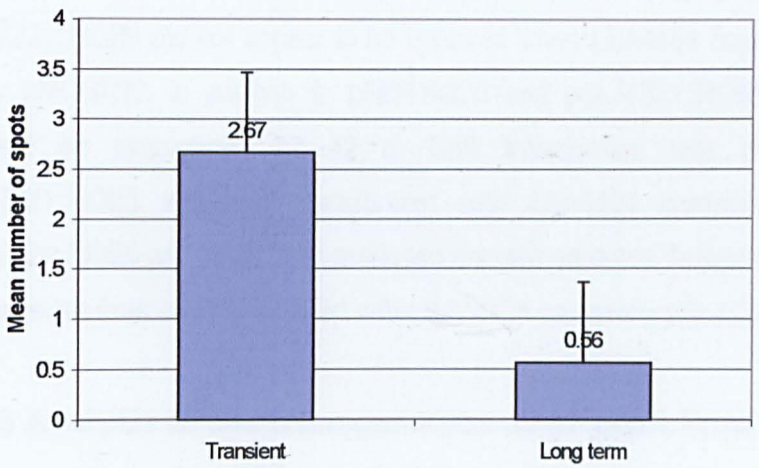
3.3.4 Histochemical GUS Assessment

Histochemical GUS analysis (Figure 3.6) was carried out 6d and 42d after co-cultivation with pMOGB22. The percentage of transient GUS positive calli was significantly greater than those exhibiting long term GUS expression. The numbers of transient GUS positive spots were also higher than those tested after 42 d (Figures 3.7 and 3.8). Error bars represent the standard error for each treatment and demonstrate the inconsistency between calli.

The distribution of transient GUS positive spots on calli can be seen in Figure 3.6.

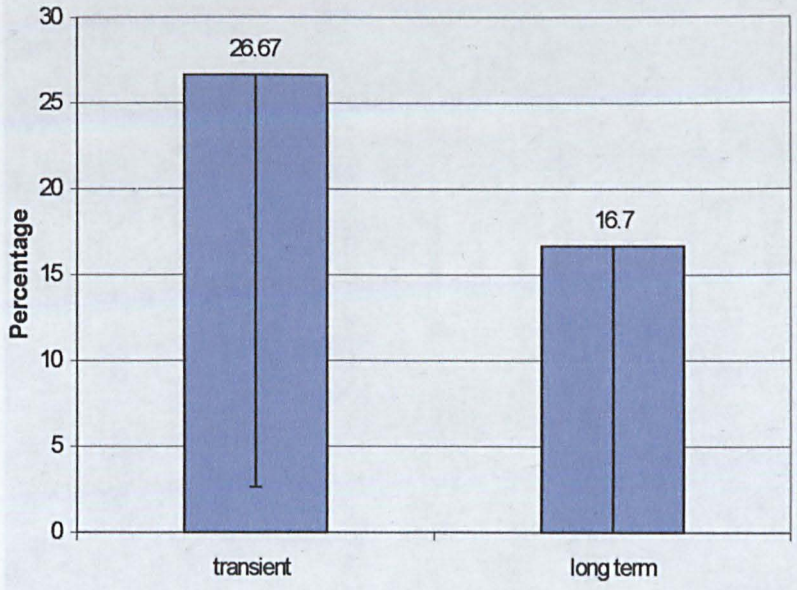
Figure 3.6
Distribution of GUS positive spots on callus 6 d after co-cultivation with LBA4404 pBBR1MCS pSLJ7321::ROB5 and pMOGB22

Figure 3.7
Numbers of GUS positive spots after transformation with LBA4404 with pBBR1MCS and pMOGB22 and pSLJ7321::ROB5



Chi-squared = 3.9 with 1 degree of freedom, significant at the 5% level.

Figure 3.8
Percentage of GUS positive calli after transformation with LBA4404 with pBBR1MCS and pMOGB22 and pSLJ7321::ROB5



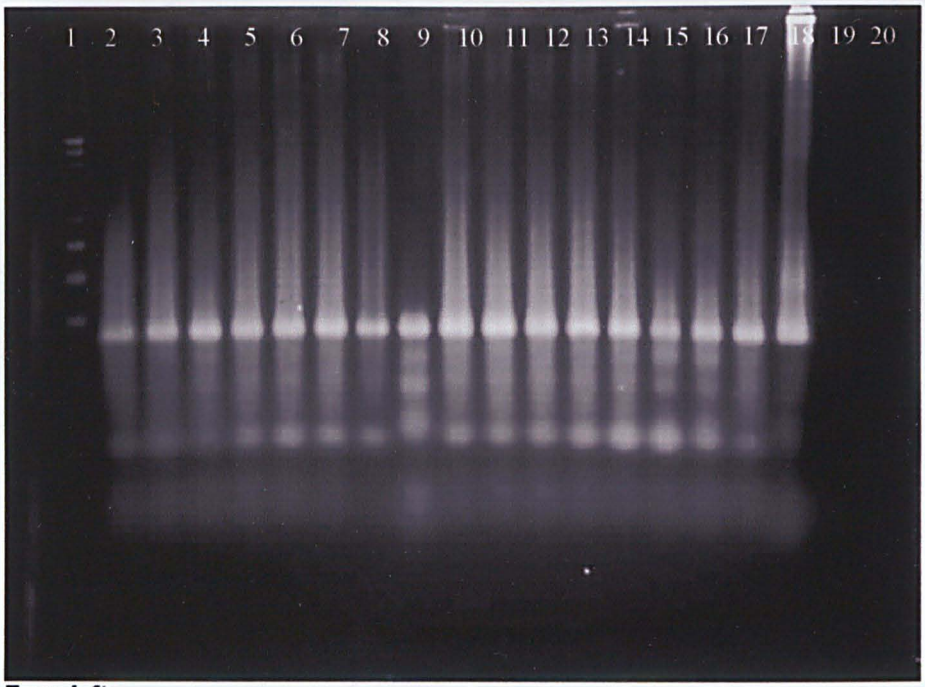
Chi-squared = 4.5 with 1 degree of freedom, significant at 5% level.

Organogenesis of calli co-cultivated with LBA4404 containing pBBR1MCS and pSLJ7321::ROB5 did not appear to be impaired when LBA4404 contained the binary vector pMOGB22, in addition to pBBR1MCS and pSLJ7321::ROB5. All calli were selected on hygromycin for 42 d. Calli transformed with pBBR1MCS and pSLJ7321::ROB5 and calli transformed with LBA4404 containing pBBR1MCS, pSLJ7321::ROB5 and pMOGB22 produced somatic embryos, however embryos failed to regenerate from calli transformed with LBA24404 containing pSLJ7321::ROB5.

3.3.5 Analysis of the transgene status of plant lines

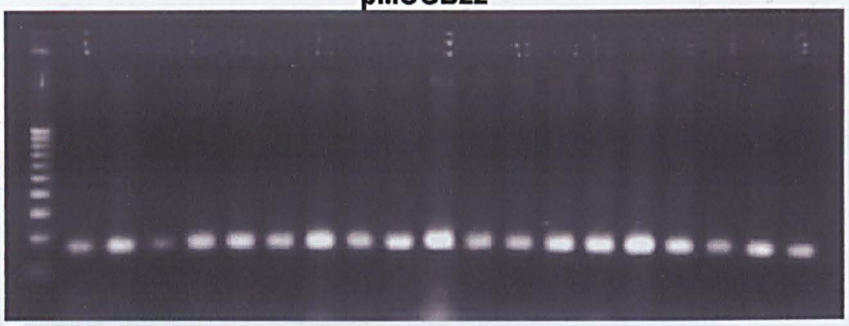
Leaf tissue was used for PCR and RT-PCR to confirm that both the *nahG* and the *hpt* genes were inserted and transcribed (Figures 3.9, 3.10). Southern analyses confirmed copy number and integration (Figures 3.11, 3.12) and enzyme assays indicated that enzyme activity was enhanced (Chapter 4). Histochemical GUS assays were carried out on only a small sample of tissues, as this assay was destructive.

Figure 3.9
PCR of DNA from leaf tissue of plants transformed with LBA4404, pBBR1MCS, pSLJ7321::ROB5 and (lanes 2 and 3) pMOGB22



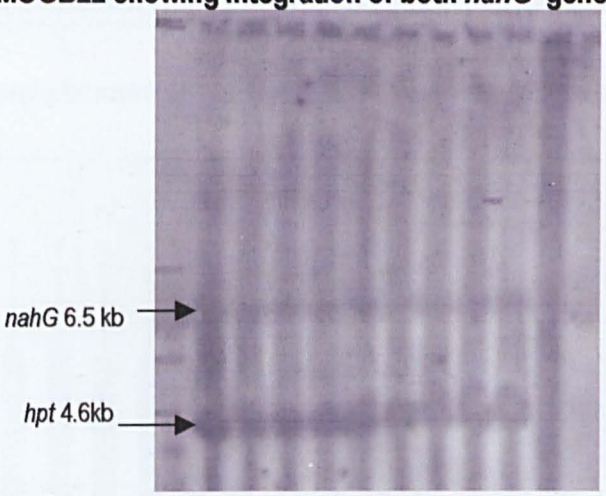
From left
Lane 1=100bp Ladder
Lanes 2-3 =LBA4404, pBBR1MCS pSLJ7321::ROB5 and pMOGB22
Lanes 4-17 =LBA4404 pBBR1MCS pSLJ7321::ROB5
Lane 18 = pSLJ7321::ROB5 positive control
Lanes 19-20= No DNA and no Primers negative controls
2% (w/v) agarose gel in 0.5 TBE buffer, 75V, 2 h. PCR used *nahG* primers

Figure 3.10
RT-PCR of RNA extracted from leaf tissues of plants transformed with LBA4404, pBBR1MCS, pSLJ7321::ROB5 and in lanes 2-4 pMOGB22



From Left
Lane 1 = 100bp ladder
Lanes 2-4 = LBA4404, pBBR1MCS, pSLJ7321::ROB5 pMOGB22
Lanes 4-18 = LBA4404, pBBR1MCS, pSLJ7321::ROB5
Lane 19 = pSLJ7321::ROB5
2% (w/v) agarose gel in 0.5 TBE, 75V, 2 h. PCR used *nahG* primers
Negative controls used on bottom half of gel

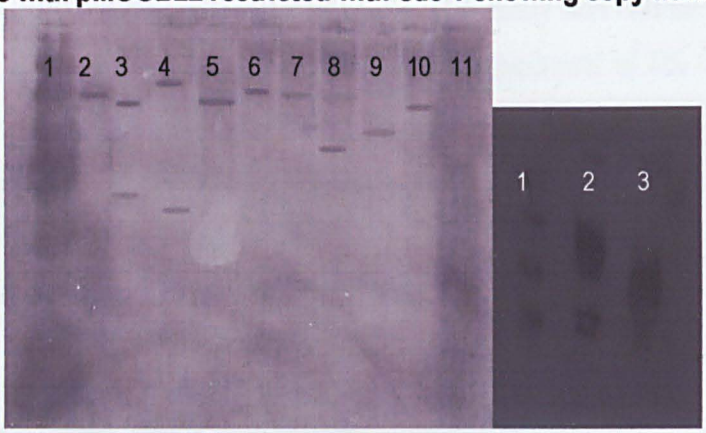
Figure 3.11
Southern analysis of DNA from leaf tissue transformed with LBA4404, pBBR1MCS, pSLJ7321::ROB5 and in lane 11 with pMOGB22 showing integration of both *nahG* gene and *hpt* gene



From left
Lane 1= 1kb ladder
Lanes 2-10= plants transformed with pSLJ7321:ROB5, PBBR1MCS (numbers 1, 2, 4, 6, 7, 10, 12, 15, 17) probed with *nahG* and *hpt* probes
Lane 11 = plant transformed pSLJ7321:ROB5, pBBR1MCS, pMOGB22
Lane 12 = T309 DNA (control plant)

Figure 3.12

Southern analysis of DNA from leaf tissue transformed with LBA4404, pBBR1MCS, pSLJ7321::ROB5 and in right hand side picture with pMOGB22 restricted with *Sac* 1 showing copy number



Left membrane from left

Lane 1 = 1Kb ladder

Lanes 2-10= plants transformed with pSLJ7321:ROB5, pBBR1MCS (numbers 1, 2, 4, 6, 7, 10, 12, 15, 17) probed with *nahG* probe

Lane 11 = T309 (control plant)

Right membrane from left

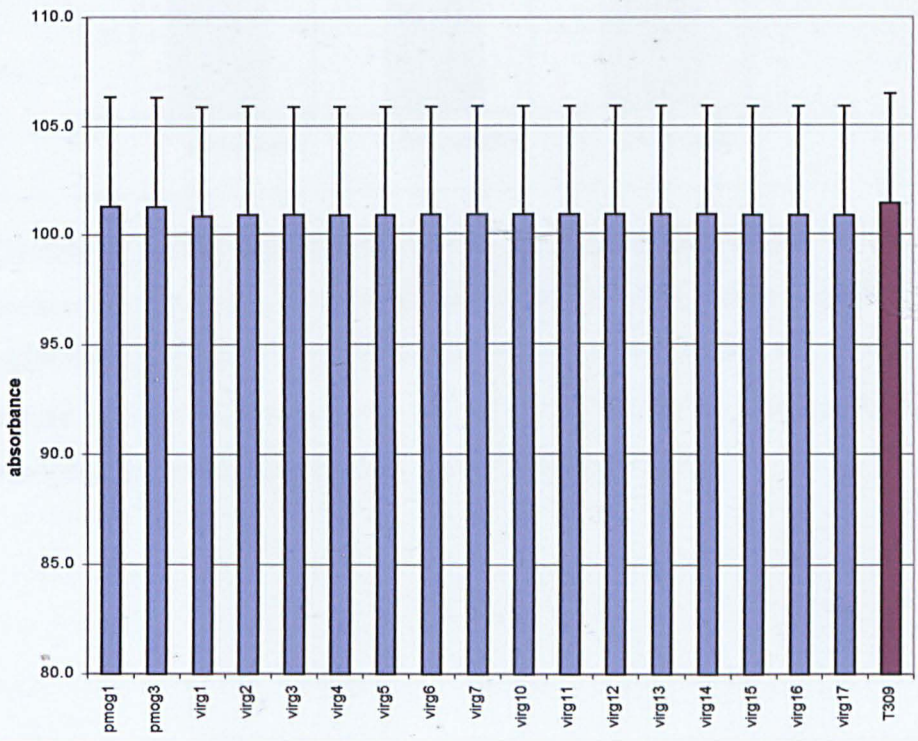
Lane 1= ladder I Kb

Lanes 2-3= plants 1& 3 transformed pSLJ7321:ROB5, pBBR1MCS, pMOGB22 probed with *nahG* probe

3.3.6 Protein analysis of plant lines

Figure 3.13

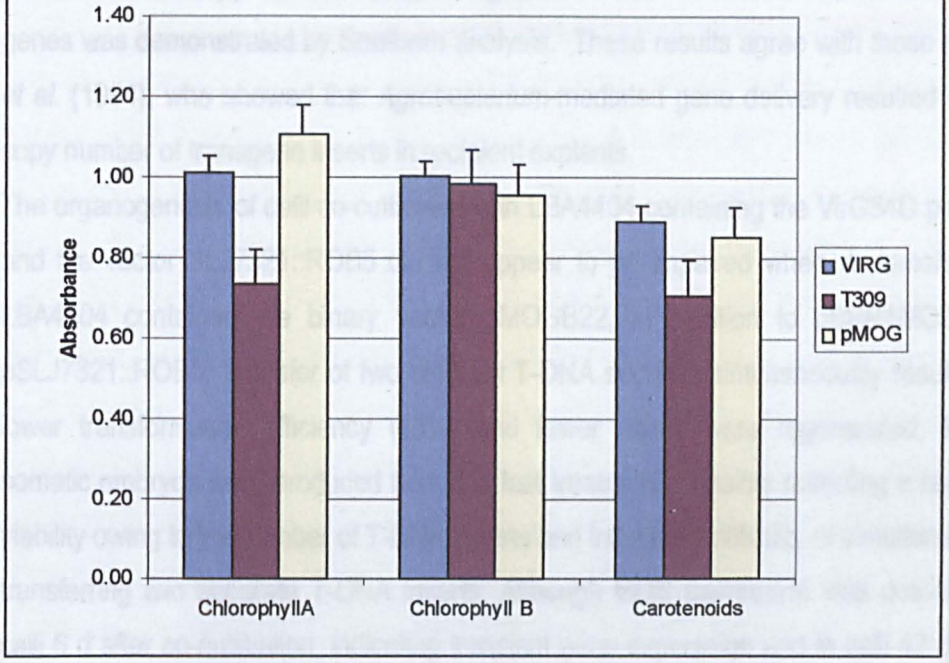
Mean protein content of leaf tissue from *Agrobacterium*-transformed plants



Protein content was consistent in different plants. Each leaf sample had 3 replicates taken each time the assay was carried out. The assay was carried out on 5 separate occasions (Figure 3.13). Samples varied by a maximum of 5% between samples. Enhanced salicylate hydroxylase activity could not be attributed to differences in protein content within plant samples.

3.3.7 Chlorophyll analysis of plant lines

Figure 3.14
Mean chlorophyll content of leaf tissue from *Agrobacterium*-transformed plants



Chlorophyll content was carried out for 3 replicates per sample on 5 separate occasions. Chlorophyll A content was lower in plant tissue transformed with pBBR1MCS compared to wild type T309 (Figure 3.14), but it was enhanced in leaf tissues of plants transformed with pMOGB22. Carotenoid content was enhanced in transgenic leaf tissues compared to tissues of wild-type T309.

3.4 DISCUSSION

De fits *et al.* (2000) recorded an increase in transformation efficiency with the use of pBBR1MCS carrying the extra copy of the *virG* gene in the transformation of *Catharanthus roseus*. The present results demonstrate a similar increase in transformation efficiency using rice. Azhakanandam (1999) recorded increased transformation efficiency using the superbinary vector pTOK233 with the Japonica cv. Taipei 309. Al-Forkan (2000) demonstrated similar increases in transformation efficiency following the use of the superbinary vector pTOK233 with Indica rice cv.s. In the present experiments, similar increases in transformation efficiency resulted from using pBBR1MCS carrying the *VirG54D* gene. Regenerated transgenic plants possessed low copy numbers (1-3). Integration of both selectable marker and *nahG* genes was demonstrated by Southern analysis. These results agree with those of Hiei *et al.* (1994), who showed that *Agrobacterium*-mediated gene delivery resulted in low copy number of transgene inserts in recipient explants.

The organogenesis of calli co-cultivated with LBA4404 containing the VirG54D plasmid and the vector SLJ7321::ROB5 did not appear to be impaired when *A. tumefaciens* LBA4404 contained the binary vector pMOGB22, in addition to pBBR1MCS and pSLJ7321::ROB5. Transfer of two different T-DNA sections simultaneously resulted in lower transformation efficiency (16%) and fewer plants were regenerated. Fewer somatic embryos were produced from this last treatment, possibly reflecting a reduced viability owing to the number of T-DNA inserts and increased difficulty of simultaneously transferring two separate T-DNA inserts. Although GUS expression was detected in calli 6 d after co-cultivation, indicating transient gene expression and in calli 42 d after transformation, GUS activity was not recorded in plants regenerated following co-cultivation with LBA4404 containing pBBR1MCS, pSLJ7321::ROB5 and pMOGB22. Therefore, stable integration of both genes was not demonstrated. However, if such a protocol could be perfected it may be possible to transfer a selectable marker on a separate plasmid to the gene of interest and thereby allow for segregation in subsequent generations to produce marker-free plants. The transformation frequency obtained was similar (25%) to those obtained by the methods used routinely for the transformation of dicotyledonous species (20-30%) (De Fits *et al.*, 2000) and was higher than for other direct DNA transfer methods, such as microprojectile

bombardment and polyethylene glycol transformation of protoplasts (Repellin, 2001). Combinations of antibiotics resulting from selectable markers on constructs pBBR1MCS, pMOGB22 and on pSLJ7321::ROB5 impaired the growth of bacterial in liquid medium and, as a result, concentrations of antibiotics were reduced to $20mgl^{-1}$. The use of three or more antibiotics significantly increased necrosis of calli. Reduction of the concentration of antibiotics did not result in increasing numbers of escapes. However, a high proportion of calli died whilst undergoing selection and this could have contributed to the lower transformation efficiency found with the simultaneous transfer of LBA4404 containing pBBR1MCS and pMOGB22 and pSLJ7321::ROB5. Stable integration of the *gus* gene in regenerated rice plants could not be established using histochemical GUS analysis of leaf tissue although the plants exhibited positive RT-PCR for the *nahG* gene and demonstrated increased salicylate hydroxylase activity.

Microprojectile bombardment has several disadvantages, including little control over both the position of the DNA inserted in the genome and the number of copies of the gene inserted (Repellin *et al.*, 2001). Transgene copy number also has an effect on co-expression of introduced genes. In general, increased copy number results in reduced co-expression (Christou, 1990). In the present experiments, there was no correlation between the copy number, as detected by Southern analysis, and the expression of the introduced genes. Both *Agrobacterium*-mediated transformation and microprojectile bombardment resulted in enhanced salicylate hydroxylase activity (Chapter 4), consistent for wounded plant tissue and old leaf tissue (6 months). Enhanced salicylate hydroxylase activity correlated with reduced salicylic acid concentration (Chapter 4) and enhanced catalase and ascorbate peroxidase activity. Plants regenerated following *Agrobacterium*-mediated transformation displayed no morphological abnormalities. Protein concentration and chlorophyll content were not affected in these plant lines (Figures 3.14 and 3.13). However, due to time constraints, detailed growth analyses were not carried on these plants.

CHAPTER 4 ENZYME ASSAYS

4.1 INTRODUCTION

Reactive oxygen species are toxic compounds produced by plants as part of their metabolic processes. In order to protect plant cells from the cytotoxic effects of these compounds, plants possess antioxidant enzymes. These enzymes include superoxide dismutase (SOD), catalases and peroxidases. In addition, plants contain low-molecular-mass antioxidants such as ascorbate (ASC), reduced glutathione (GSH) and α-tocopherol. The literature indicates that salicylate hydroxylase by interfering with the plant's ability to accumulate salicylic acid may interfere with the activity of these enzymes. These experiments aimed to establish whether salicylate hydroxylase activity was enhanced in transgenic plant lines, whether salicylic acid accumulation was inhibited as a result, and how this affected other antioxidant enzymes and reactive oxygen species.

4.1.1 Reactive oxygen species

Free radicals are defined as atoms or compounds, which contain an unpaired electron. They are generally considered as intermediates, reacting with non-radicals to produce a new radical. Oxygen radicals and oxygen-derived free radical species are collectively termed reactive oxygen species (ROS). Reactive oxygen species can be generated by two different mechanisms; by absorption of sufficient energy to reverse the spin on one of its two unpaired electrons, or by monovalent reduction. The Singlet State, in which the two electrons have opposite spins, is achieved by the absorption of sufficient energy. The production of reactive oxygen species by monovalent reduction (dismutation) involves the stepwise reduction of O_2 to $O^{\bullet}{}_2^-$ and subsequent reduction of $O^{\bullet}{}_2^-$ to H_2O_2. Several enzymes such as xanthine, urate and D-amino acid oxidases can also generate H_2O_2. Superoxide can inactivate some metal containing enzymes such as ferrodoxin-linked nitrate reductase, catalases and peroxidases (Asada and Takahshi, 1987), as well as haem moieties and iron-sulphur containing groups, causing damage to amino acids and loss of protein and enzyme function (Halliwell and Gutteridge, 1999). Superoxide can reversibly react with H^+ to form the more reactive hydroperoxyl radical ($HO^{\bullet}{}_2$) that, due to its uncharged nature, can cross lipid membranes more readily (Halliwell and Gutteridge, 1999) and can also oxidise NADH directly. However, the ratio of $O^{\bullet}{}_2^-$ to $HO^{\bullet}{}_2$ in most physiological systems is between

100/1 and 1000/1 (pH 6.8 to pH 7.8, respectively). The cooperative interaction of H_2O_2 and $O^{\bullet}{}_2^-$ in the metal-catalysed Fenton reaction (Halliwell and Gutteridge, 1992) (Figure 4.1) causes the production of OH^-.

$Fe^{2+} + H_2O_2 \longrightarrow$ intermediate complexes $\longrightarrow Fe^{3+} + OH^{\bullet} + OH^-$

$Fe^{3+} + O^{\bullet}{}_2^- \longrightarrow Fe^{2+} + O_2$

The net sum of these reactions is known as the Haber-Weiss reaction

$H_2O_2 + O^{\bullet}{}_2^- \longrightarrow O_2 + OH^{\bullet} + OH^-$

A common source of hydrogen peroxide is the dismutation of $O^{\bullet}{}_2^-$ by superoxide dismutase. Super oxide dismutate (SOD) is, therefore, both a scavenger and a source of reactive oxygen species.

Additionally, OH^- can be generated via UV-induced homolytic fission of the O-O bond in H_2O_2, ethanol metabolism, peroxynitrous acid decomposition, reactions between hypoclorous acid and O_2^-

$HOCl + O^{\bullet}{}_2^- \longrightarrow O_2 + Cl^- + OH^{\bullet}$

or HOCL with iron chelates such as ferrocyanide

$HOCL + Fe(CN)_6^{4-} \longrightarrow OH^{\bullet} + Cl^- + Fe(CN)_6^{3-}$.

The hydroxyl radical is extremely reactive, so much so that it cannot be scavanged enzymatically. Antioxidant systems in plants therefore rely on enzymatic scavenging of $O^{\bullet}{}_2^-$ and H_2O_2. The concentration of $OH^{\bullet}$ *in vivo* is virtually zero due to its high reactivity. However, $OH^{\bullet}$ radicals initiate self-propagating reactions and the formations of longer lived free radicals whilst directly causing peroxidation of lipid membranes, mutations and breaks in DNA strands and destruction of proteins (Slooten *et al.*, 1998). Peroxidised membranes become rigid and lose their integrity (Asada and Takahashi, 1987). Failure to remove reactive oxygen species leads to the destruction of cellular components and is termed oxidative stress. Oxidative stress is manifested by peroxidation of lipid membranes, protein and nucleic acid degradation. If unchecked, this leads to cellular degradation and cell death. Wounding of plant tissue leads to release of increased reactive oxygen species which are associated with oxidative damage of lipids, proteins and nucleic acids initiation of signal cascades for the production of jasmonic acid and the hypersensitive response.

4.1.1.1 Peroxidation of lipid membranes

Lipid peroxidation is initiated by the abstration of an H^+ from the methylvinyl group on a fatty acid ($OH^\bullet + LH \longrightarrow L^\bullet + H_20$). The remaining carbon centred radical forms a resonance structure sharing an unpaired electron among the adjacent carbons. The reaction results in the formation of a peroxyl radical through triplet oxygen ($L^\bullet + O_2 \longrightarrow LOO^\bullet$). The peroxy radical then removes an H^+ from a second fatty acid, forming a lipid hydroperoxide and another carbon centred free radical ($LOO^\bullet LH \longrightarrow L^\bullet + LOOH$). The reaction is self-propagating. The lipid hydroperoxides will participate in the Fenton reaction (Figure 4.1), leading to the formation of reactive alkaloids ($LOOH + Fe^{2+} \longrightarrow OH^- + Fe(III) + LO^\bullet$). In the presence of Fe therefore, the chain reactions leading to lipid peroxidation and free radical propagation are both self-propagating and amplified. These reactions terminate when the carbon centred radicals and peroxy radicals cross-link to form stable conjugated non-radical products resulting in accumulation of high molecular weight cross-linked fatty acids and phospholipids.

$L^\bullet + L^\bullet \longrightarrow L\text{-}L$;

$L^\bullet + LOO^\bullet \longrightarrow LOOL$;

$LOO^\bullet + LOO^\bullet \longrightarrow LOOL + O_2$.

In plants, an alternative mechanism has been suggested for fatty acid degradation (Barclay and McKersie, 1994). Superoxide has been shown to produce free fatty acids via the de-esterification of the phospholipid bi-layer (Senaratna *et al.*, 1985). Superoxide interacts with esters via nucleophilic additions resulting in the formation of a peroxyl radical ($LC(O)OO^\bullet$). Following H^+ removal, the resulting hydroperoxide decomposes into a free fatty acid (Figure 4.1) ($LCOO^A$). The predominance of this mechanism for lipid peroxidation in some plant tissues may be due in part to the presence of lipid-soluble membrane antioxidants such as phenols, flavanoids and quinines (Barclay and McKersie, 1994).

4.1.1.2 Protein degradation

ROS degrade protein function by fragmentation of peptide chains, site-specific amino acid modifications, alteration of the electric charge, aggregation and increasing susceptibility to proteolysis (Halliwell and Gutteridge, 1992). While neither hydrogen peroxide nor $O_2^{\bullet -}$ alone are capable of causing DNA strand breaks under

physiological conditions *in vitro*, $OH^{\bullet}$ produced through the metal catalysed Fenton reaction *in vitro*, would account for characteristic DNA damage. In the bacteria *Escerichia coli*, it has been demonstrated that a Fenton active metal bound to DNA is reduced by NADPH or $O^{\bullet}{}_2^-$. A subsequent reaction of the $O^{\bullet}{}_2^-$ would cause the formation of the $OH^{\bullet}$ radical and the oxidation of adjacent sugar or base moieties leading to breakage of DNA chains (McKersie, 1996). Hydroxyl radicals can also induce DNA-protein cross-links these can result in cell damage should replication or transcription precede repair.

4.1.1.3 Production of reactive oxygen species

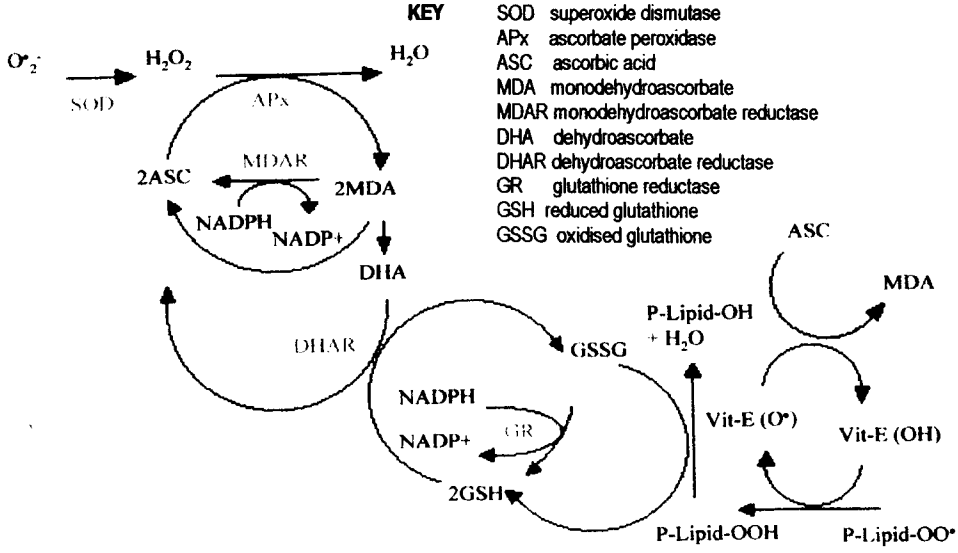
ROS are produced under physiological conditions through many processes. The most prevalent are the Mehler reaction and photorespiration (Noctor *et al.*, 2000). Where the supply of electrons by photosystem 1 (PSI) is in excess and the supply of NADP is limiting, the reducing side of PSI contributes to the monovalent reduction of O_2 to $O^{\bullet}{}_2^-$ and the subsequent production of H_2O_2 by dismutation or reduction. Uptake of O_2 is increased by the stromal iron-sulphur protein ferredoxin leading to production of $O^{\bullet}{}_2^-$ by reduction of O_2 as ferredoxin is reduced by PSI. The chlorophyll pigments also produce $^1O_2$, a highly reactive radical (Foyer, 2002). The decay of chlorophyll from its excited state to its ground state results in either the transfer of electrons, radiative decay (fluorescence) or the conversion of chlorophyll to a triplet state. Triplet chlorophyll ($^3CHl^*$) interacts with ground-state triplet O_2 producing $^1O_2$. Saturation of thermal dissipation leads to the accumulation of singlet excited chlorophyll and, consequently, $^3CHl^*$ accumulation, photoinhibition and the generation of $^1O_2$. Conditions that prevent utilisation of electrons by the electron transport chain enhance $^3CHl^*$ production (Foyer, 2002). Photosystem II oxidation facilitates the transfer of electrons from water to the PSII reaction centre, releasing ground-state triplet O_2. Electrons from the reaction centre may contribute to ROS production in the form of H_2O_2 and $O^{\bullet}{}_2^-$ (McKersie, 1996). During photorespiration, rubisco catalyses the addition of oxygen to RuBP, forming phosphoglycolate and subsequently phospoglycerate. Photorespiration acts as a sink for the energy produced during photosynthesis and any down-regulation of photorespiration would lead to a rise in the formation of ROS. While photorespiration does not directly lead to the formation of ROS in the chloroplast, subsequent oxidation of glycollate in the peroxisome generates high levels of H_2O_2 (Foyer *et al.*, 1994). Generation of reactive oxygen species has been shown to occur at the flavin

mononucleotide group (FMN) within complex I of the mitochondrial electron transport chain (Liu *et al.*, 2002).

Cytochrome P_{450} catalyses the addition of oxygen into an organic substrate by mixed function oxygenases ($RH + NADPH + H^+ + O_2 \longrightarrow ROH + NADP^+ + H_3O$) and, in so doing, produces activated oxygen. The complex P_{450}-RHOO produced during these reactions can decompose to form P_{450}-RH releasing O_2^- (McKersie, 1996). Subsequent addition of an electron to the P_{450}-RHOO complex followed by decomposition can also generate H_2O_2 (Halliwell and Gutteridge, 1992). An oxoiron porphyrin radical ($Fe(IV)O^{\bullet}$) is produced following protonation of the Fe(III)-P_{450}- substrate-O_2^{2-} complex. Later, oxygen addition generates a carbon radical and iron-bound hydroxyl radical (Halliwell and Gutteridge, 1992). Peroxisomes contain many of the cellular enzymes that generate H_2O_2, such as xanthine oxidase, glycolate oxidase, urate oxidase and flavoprotein dehydrogenases (Halliwell and Gutteridge, 1999). Flavoproteins generate $O^{\bullet}{}_2^-$ (McKersie, 1996).

Production of ROS is enhanced by environmental factors such as extremes of temperature, nitrogen deficiency, pollution, high light intensity, drought conditions and pathogen attack, that interrupt energy transfer from light to carbon dioxide, resulting in excessive irradiation of the chloroplast (Foyer, 2002). If oxidative damage to the photosynthetic apparatus occurs then this is ultimately deleterious to plant growth and productivity. Oxidative stress tolerance in agricultural crops would therefore be of great economic benefit, leading to increased crop yields during growth under fluctuating environmental conditions.

Figure 4.1
Production and detoxification of reactive oxygen species



4.1.2 Antioxidants

To protect plant cells from the cytotoxic effects of ROS, plants possess antioxidant enzymes such as, superoxide dismutase (SOD), catalases and peroxidases. Plants contain low-molecular-mass antioxidants such as ascorbate (ASC) reduced glutathione (GSH) and α- tocopherol.

SOD is an enzyme complex that catalyses the dismutation of $O^{\bullet}{}_2^-$ to hydrogen peroxide at diffusion controlled rates $O^{\bullet}{}_2^- + O^{\bullet}{}_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$. SODs are classified as Fe-, Mn- or Cu/Zn SODs based on their metal cofactor. The phylogenetic and tissue distribution of SODs has been reviewed extensively (Van Camp *et al.*, 1994). Chloroplasts contain mainly Cu/ZnSOD. Mitochondria contain MnSOD whose reaction rates decrease at alkaline pH. Similarly, while FeSODs are located in the cytosol, like MnSODs they exhibit decreased reaction rates concomitant with increases in alkaline pH.

Hydrogen peroxide produced in peroxisomes and mitochondria are further detoxified. Hydrogen peroxide is reduced to water and oxygen, a process catalysed by ascorbate peroxidases. These heme containing enzymes use the large pool (up to 10mM) of ascorbate (Perl-Treves and Perl, 2002) present in the chloroplast and oxidise it to monodehydroascorbate (MDA) according to the following reaction.

$$2 \text{ ascorbate} + H_2O_2 \xrightarrow{APX} 2MDA + 2\,H_2O$$

Although glutathione is present in plastids its utilisation for direct reduction of H_2O_2 is not an important process in plants (Perl-Treves and Perl, 2002).

In the plastids, MDA may give rise to dehydroxyascorbate (DHA) which must be reduced to regenerate the ascorbate pool, either non-enzymatically by ferrodoxin (Figure 4.1):

$$MDA + Fd_{red} \longrightarrow \text{ascorbate} + Fd_{ox},$$

by reducing MDA to ascorbate using monodehydroxyascorbate reductase (MDAR) in the stroma using NADPH:

$$2MDA + NADPH \xrightarrow{MDAR} 2\text{ascorbate} + NADP^+$$

or by reducing DHA to ascorbate by dehydroxyascorbate redutase (DHAR) with NADPH dependent glutathione (GSH) as the reducing substrate:

$$DHA + 2GSH \xrightarrow{DHAR} \text{ascorbate} + GSSH.$$

Scavenging occurs at the thylakoid surface near PSI, minimising the risk of escape and reaction of ROS with each other. Scavenging also operates in the stroma to protect the sensitive enzymes of the Calvin cycle. An ascorbate peroxidase based cycle for removal may also operate in the plant cytoplasm (Dalton *et al.*, 1987).

Glutathione reductase (GR), a flavoprotein that is found mostly in chloroplasts completes the 'Asada-Halliwell pathway' by regenerating the glutathione pool with NADPH as electron donor (Foyer and Halliwell, 1976):

$$GSSG + NADPH \xrightarrow{GR} 2GSH + NADP^{+}.$$

GR is also found in mitochondria and the cytosol. Overproduction of GR in transgenic tobacco plants transformed with Pea and *E. coli* GR, led to increased ozone tolerance (Creissen, 1996).

In plants, salicylic acid is derived from the phenyl propanoid pathway that converts phenyl alanine to *trans*-cinnamic acid using the enzyme phenylalanineammonia lyase (PAL). Cinnamic acid then follows a β-oxidation pathway to benzoic acid, which can be stored conjugated to a sugar molecule or converted to salicylic acid (Leon and Raskin, 1995). Salicylic acid is present in the cytoplasm in much higher concentrations in rice leaves than in tobacco (Silvermann *et al.*, 1995). Salicylic acid has been measured by gas chromatography (Muljone, 1998) and HPLC (Yalpani *et al.*,1992; Coudray, 1995). The colour change produced by salicylic acid with iron III chloride was demonstrated by Klämbt (1962). However, there is an additional pathway by which salicylic acid may be derived which derives from chorismate via a two-step process. Plants transformed with the gene that converts chorismate to salicylic acid showed a 500-1000 fold increase in salicylic acid (Verbenne *et* al., 2000) and constitutive expression of pathogenesis related proteins.

4.1.3 Additional proteins and enzymes

Thioredoxin is a small ubiquitous protein that plays a redox-regulatory role in plants, that has been suggested to protect organisms by scavenging reactive oxygen as well as regenerating oxidised proteins (Takemoto *et al.*, 1998). Transferrin, a plasma Fe carrier protein, ferritin an intracellular Fe-storing complex and ceryloplasm, a Cu-binding glycoprotein, have all been suggested to have antioxidant functions *in vivo* (Perl-Treves and Perl, 2002). Additional cellular processes that are activated after severe oxidative damage may also play a part in repairing oxidative stress such as phopholipases and proteases that degrade and repair biological macromolecules, whose activities are

induced by oxidative stress. Mechanisms that enable a cell to regain its homeostasis could also be regarded as secondary defence enzymes such as glutathione-S-transferases (GST). These enzymes catalyse the conjugation of a *y*-glutamylcysteinyl glycine (GSH) molecule to a variety of chemical compounds such as the products of lipid peroxidation or oxidative DNA degradation, which are then secreted to the apoplast or vacuole through glutathione pumps. Interestingly, GSTs are induced amongst others by ROS, ozone, wounding, ethylene, heavy metals and pathogen attack. They may also function as peroxidases and scavenging radicals (Perl-Treves and Perl, 2002). Ascorbate is an important antioxidant not only for its role as APX substrate that scavenges hydrogen peroxide in the chloroplast stroma, but also for its additional role on the thylakoid surface, protecting or regenerating oxidised carotenes and tocopherols. Tocopherol, a phenolic lipid soluble antioxidant present in plants, is very important as a chain terminator of free radical reactions that cause lipid peroxidation. Carotenoids, lipid soluble molecules, protect plants from oxidative damage. In the photosynthetic apparatus, β-carotene quenches both excited triplet-state chlorophyll and singlet oxygen, preventing them from initiating lipid peroxidation.

4.1.4 Salicylate hydroxylase

The *nahG* gene from *Pseudomonas putida* encodes for the enzyme salicylate hydroxylase. Plants expressing this gene are, therefore, unable to accumulate salicylic acid and, consequently, are unable to express systemic acquired resistance. Catalase and ascorbate peroxidase catalyse the breakdown of hydrogen peroxide, which is an initiator of the jasmonic acid pathway and an early signal in the hypersensitive response. It has been reported that in some rice plants catalase activity remains unaffected by salicylic acid concentration, whilst in other rice tissues catalase activity is reduced by increased concentrations of salicylic acid. In these experiments, the concentrations of antioxidant enzymes were assessed in plants unable to accumulate salicylic acid and in wild-type plants. Shoots of rice seedling have been demonstrated to exhibit high concentrations of salicylic acid (Silverman *et al.*, 1995), while rice root tissue and cell suspensions have much lower concentrations of salicylic acid (Chen *et al.*, 1997).

The isolation and characterisation of salicylate hydroxylase from the soil bacterium *Pseudomonas putida* was first carried out by Yamamoto *et al.* (1965). The enzyme catalyses the formation of catechol from salicylate and reduced pyridine nucleotide in

the presence of flavin adenine dinucleotide as a specific cofactor. The mechanism of the salicylate hydroxylase reaction was elicited (Katagiri *et al.*, 1966). The equation was found to be:

FAD-salicylate $\longrightarrow$ $FADH_2$-salicylate + NAD + O_2 $\longrightarrow$ FAD +catechol+ CO_2 +H_2O.

The rate of salicylate-dependent NADH oxidation was strictly proportional to that of catechol formation from salicylic acid. It was later found that salicylate hydroxylase forms an enzyme-substrate complex with salicylate. (Takemori *et al.*, 1969). The nature of chemically and phytochemically reduced salicylate hydroxylase was investigated and the intermediate was found to be transitory (White-Stevens and Kamin, 1972). A red flavoprotein radical was detected in the absence of substrate (Takemori *et al.*,1969). Binary and ternary complexes between salicylate hydroxylase and components such as FAD substrate and NADH, were studied to determine dissociation constant and stoichiometric ratio of each component (Suzuki *et al.*, 1969). The protein was isolated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the amino acid structure determined. A range of behaviour intermediate between the substrate (salicylate) and pseudosubstrate (benzoate) modes are hydroxylated and some of the oxygen utilised diverted to hydrogen peroxide (White-Stevens and Kamin, 1972).

4.1.5 Salicylic acid and catalase

It has been shown that salicylic acid forms a compound with catalase (Ryals *et al.*, 1994). The production of hydrogen peroxide, the resulting hypersensitive response, lipid peroxidation and production of jasmonic acid from the octadecanoid pathway, would all be affected by the inhibitory effect of salicylic acid. It might be expected that plants unable to accumulate salicylic acid would have no inhibition of catalase or ascorbate peroxidase activity and, therefore, lower levels of hydrogen peroxide which may alter the reactive oxygen species and their concentrations, released upon plant wounding. Hydrogen peroxide and catalase have been quantified in lettuce and in cotton leaf tissue (Garratt *et al.*, 2001).

It has been demonstrated that catalase in rice shoots is insensitive to salicylic acid concentrations (Higo and Higo, 1996), whilst catalase in rice roots is susceptible to salicylic acid, which correlates to tissue specific expression of two catalase genes (*cat A* and *cat B*). *Cat A* was found to express at high levels in rice seedling shoots and be insensitive to salicylic acid concentration (Higo and Higo, 1996), whilst *cat B* was found

to express in rice roots and cell suspensions and be sensitive to salicylic acid concentrations. It has been reported that ascorbate peroxidase activity is inhibited by salicylic acid (Durner and Klessig, 1996).

4.1.6 Aims of the experiments reported in this chapter

- These experiments aimed to establish whether leaf tissue from transgenic plant lines resulted in an increase in salicylate hydroxylase activity.
- If increased salicylate hydroxylase activity was established, these experiments aimed to establish if this correlated with a decrease in the substrate salicylic acid.
- Further experiments aimed to determine if altered salicylate hydroxylase activity and concomitant reduced salicylic acid concentrations had enhanced or inhibited the activity of ascorbate proxidase, catalase and ascorbate oxidase and whether this altered the concentration of hydrogen peroxide in the leaf tissues of transgenic plant lines. Following detection of such a change, assaying the activity of SOD indicated whether this was due to reduced inhibition from decreased salicylic acid accumulation or to enhanced production of hydrogen peroxide from increased SOD activity.
- Assaying extracts from damaged and old (6 months after transplanting) leaves produced data on how consistent these results were and whether there was any effect on the burst of reactive oxygen species that was released following wounding.
- Finally, assaying ascorbic acid provided indications on the state of the ascorbate pool in these plant lines.

4.2 MATERIALS AND METHODS

Samples of leaf tissue 1g ± 0.1g fresh weight from all transgenic and wild-type lines were collected and immediately frozen in liquid nitrogen. Three separate samples were taken from each line and the experiment repeated 3 times. Samples were ground whilst still frozen. Samples used for catalase assays or salicylate hydroxylase were resuspended in 5ml of ice cold phosphate buffer (potassium dihydrogen orthophosphate 0.03M and dipotassium hydrogen orthophosphate 0.03M in a 1:1 v/v ratio, pH 7.0). Protein quantification was carried out with the same samples using 20% (v/v) Bradford reagent (Bio-RAD, Hemel Hempsted UK). Each cuvette contained 990µl of 20% (v/v) Bradford reagent (BIO-RAD) and 10µl of cell extract. Absorbance was measured at 595nm. Protein concentrations were determined and these were standardised in order to establish that any difference in enzyme activity was not merely a result of aberrant concentrations of protein in samples (Section 2.4.7.2).

4.2.1 Salicylate hydroxylase assays

The method used was based on that described by Yamamoto *et al.* (1965). Each 3ml capacity quartz cuvette contained 400µl of reaction mixture (200µl of 200nm salicylic acid, 100µl of 100nm NADH, 100µl of 60nm Tris-HCl, pH 8.0) together with 600µl of cell extract. The decrease in absorbance at 340nm was measured over 5 min at room temperature using a λ-Bio UV spectrophotometer (Perkin-Elmer, Beaconsfield, UK). The decrease in absorbance of standard enzyme concentrations (1, 2, 4 and 8 units) of salicylate hydroxylase (Sigma-Aldrich) was measured to establish empirical curves.

4.2.2 Salicylic acid

Plant samples were purified using a Sephadex G50 column (Sigma-Aldrich). To each 1.5ml polystyrene cuvette (Scientific Laboratory Supplies) was added 900µl of 0.01M iron III chloride and 100µl of cell extract. Absorbance was measured at 550nm using a λ-Bio UV spectrophotometer and salicylic acid standards (10nM, 1µM, 10µM, 100µM, and 0.5mM). Three replicates were taken for each plant line tested on 5 separate occasions.

4.2.3 Catalase assays

Samples were poured through Miracloth (Calbio) to remove leaf debris and kept on ice. One hundred µl of cell extract were added to 1.9ml of the reaction mixture (25ml of phosphate buffer pH7.0, 33µl of 30 volume hydrogen peroxide) in a quartz cuvette (3ml capacity) thermostatically controlled to 25^{0}C. After mixing, the decrease in absorbance at 240nm (AB_{240nm}) with ΔAB_{240} constant, was measured, once the samples had equilibrated to room temperature, over a 2 min period using a λ-Bio UV spectrophotometer. Samples of catalase of known concentration (10, 20, 30, 40, 50 and 80 U) were also tested in order to establish the decrease in absorbance empirically. Cell extracts (100µl) were purified using a Sephadex G50 column and decrease in absorbance was measured.

4.2.4 Hydrogen peroxide assays

Samples of leaf tissue (500mg ± 10mg) were collected and immediately frozen in liquid nitrogen. Tissues were ground whilst still frozen and homogenised tissues were added to 1ml of ice cold extraction buffer (50mM N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid] (HEPES) buffer pH7.5, 1mM ethylenediamine-tetraacetic acid (EDTA), 45mM magnesium chloride and 0.2% v/v Triton X-100). The homogenates were microfuged (10,000Xg, 10min) at 4^{0}C. One ml aliquots of the supernatants were transferred to clean microfuge tubes and maintained on ice. Samples were stored at –80^{0}C until they were analysed. Four hundred µl of cell supernatant were added to 400µl of chloroform:methanol (2:1 v:v), vortexed and centrifuged (10,000g, 3 min). The upper aqueous phase was removed. Chloroform methanol extraction was repeated 5 times. Fifty µl of aqueous upper phase was aliquoted into a 3ml optical methylacrylate (flourimetric) cuvette with 2.96ml of reaction buffer (2.9ml HEPES pH 7.5, 30µl 50mm homovanillic acid and 30µl of 4µM peroxidase). Samples were incubated for 10 min at 25^{0}C. Standards consisted of 0.5, 1, 5 and 10nm hydrogen peroxide in the reaction mixture. The reaction was quantified using a flourimeter (Perkin-Elmer 3000) with excitation at 315nm and emission at 425nm.

4.2.5 Asorbate peroxidase assays

Ascorbate peroxidase activity was determined spectrophotometrically using the method described by Durner and Klessig (1995). Oxidation of ascorbic acid was followed by the

decrease in absorbance at 290nm. The reaction rates measured were linear for 3 min and were corrected for the auto-oxidation of ascorbic acid. Each 3.5ml quartz cuvette contained 1ml of phosphate buffer (potassium dihydrogen orthophosphate 0.03M and dipotassium hydrogen orthophosphate 0.03M in a 1:1 ratio, pH 7.0), 750µl of cell extract (1g +/- 10mg of ground plant leaf tissue in 1ml of phosphate buffer) , 200µl of ascorbic acid (1.0mM), and 20µl of hydrogen peroxide (3% v/v). Decrease in absorbance was measured at 290nm using a λ-Bio UV spectrophotometer every 20 seconds for 5 min. Each plant line had 3 replicates measured on 5 separate occasions. Decrease in absorbance of cell extract spiked with 100 µl of 1mM salicylic acid was measured to determine the extent of ascorbate peroxidase inhibition with increased salicylic acid concentration. Cell extract was passed through a Sephadex G50 column and the decrease in absorbance measured as above.

4.2.6 Ascorbate oxidase assays

Frozen plant leaf samples of 100mg+/-10mg were ground in liquid nitrogen. One ml of sodium phosphate buffer (0.2m sodium dihydrogen orthophosphate and 0.2M disodium hydrogen phosphate, pH 6.5, containing EDTA 0.5mM) was added to each sample and vortexed. Ascorbate oxidase is membrane bound and so samples were not filtered. To each sample, 10ml of sodium phosphate buffer (0.2m sodium dihydrogen orthophosphate and 0.2M disodium hydrogen phosphate, pH 6.5) was added. Each 3.5ml quartz cuvette contained 950 µl of sodium phosphate buffer, 950 µl cell extract and 100 µl of 100µM ascorbic acid. Samples were incubated at room temperature for 2 min, then decrease in absorbance was measured at 265nm using a λ-Bio UV spectrophotometer every 20 sec for 5 min. Standards consisted of 1, 2, 3, 4, 5, 10, 20, 40, 50 units of asorbate oxidase (Promega). Cell extracts (1ml) were spiked with the addition of 100µl of 1mM salicylic acid and the decrease in absorbance measured, in order to determine whether increased salicylic acid concentration decreased ascorbate oxidase activity.

4.2.7 Ascorbic acid assays

Plant leaf samples (200±10mg) were frozen in liquid nitrogen and ground whilst frozen. Samples were transferred into 1.5ml Eppendorf tubes and 1ml of 6% v/v aqueous solution of trichloroacetic acid (TCA) added. Samples were centrifuged at 12,000rpm in a microfuge at 4^{0}C for 2 min and the supernatants removed. Standard concentrations of

10nM, 20nM, 30nM 40nM and 50nM ascorbate were prepared in 6% TCA. A 20µl aliquot of either sample or standard was pipetted into each well of a microtitre plate 20µl of sodium phosphate buffer 0.2M (disodium hydrogen orthophosphate 0.4M and sodium dihydrogen orthophosphate 0.4M, pH 7.4) per well were added, 10µl of DTT solution (16mg of dithiothreitol in 10ml sodium phosphate buffer giving a 10mM solution) was added. The microtitre plate, were incubated for 15 min at 42^{0}C. Aliquots of 10µl per well of 0.5% N-ethylmaleimide aqueous solution at 42^{0}C were added. Absorbances were read in a plate reader at 550nm

Aliquots of 20µl of sodium phosphate buffer were pipetted into each well together with 20µl aliquots of either samples or standards, and 80µl per well aliquots of colour reagent (16mg of dithiothreitol in 10ml sodium phosphate buffer). The plates were incubated for 45 min at 42^{0}C and read in a plate reader at 550nm. Six replicates per line were tested twice.

4.2.8 Superoxide dismutase

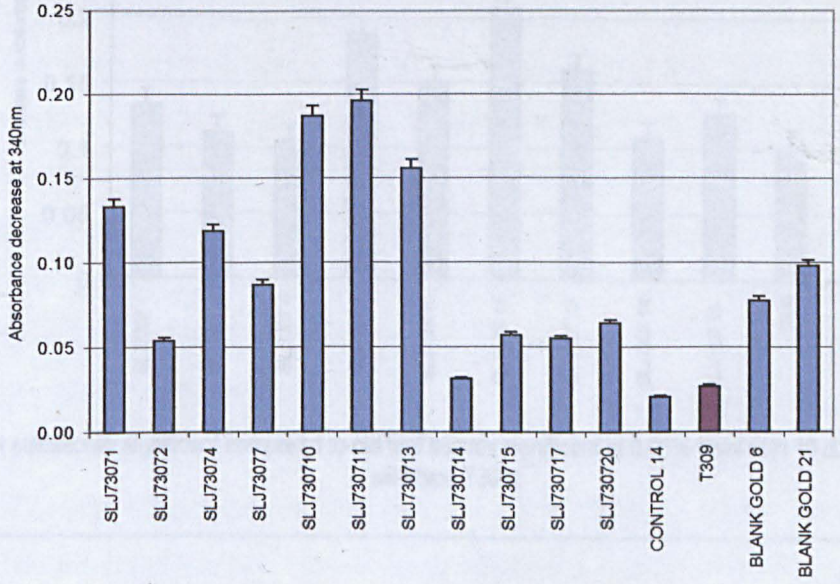
Total superoxide activity was measured by determining the amount of enzyme required to produce a 50% inhibition of cytochrome C reduction (Halliwell and Gutteridge, 1999). In a 3ml quartz cuvette, 2.9ml of solution A [10ml xanthine solution (0.76mg xanthine in 10ml 0.001m sodium hydroxide) solution mixed with 100ml of cytochrome C solution (24.8mg cytochrome C in 100ml of 50mM potassium phosphate buffer, pH 7.8)] were mixed with 50µl of cell extract supernatant. The reaction was initiated by the addition of 50µl of solution B [xanthine oxidase (Grade 1, Sigma) in sodium EDTA buffer (50mM pH7.8 containing 0.1mM EDTA) to give an activity of 10Uml^{-1}] kept on ice until use. Inhibition of the reduction of cytochrome C by O_2 was monitored at 550nm (25^{0}C) using a λ-Bio UV spectrophotometer. Standards containing 1, 3, 6, 12, 23, 47, 94 and 188 U of SOD (where 1 U was defined as the amount of enzyme that inhibited the rate of reduction of cytochrome C by 50%) were used to construct a standard curve.

4.3 RESULTS

4.3.1 Salicylic hydroxylase

Levels of salicylate hydroxylase were variable from plant to plant. However, it was clearly demonstrated that salicylate hydroxylase activity was enhanced in transgenic plants compared to wild-type Taipei 309 (designated T309 in all figures) and control plants that had been subjected to the tissue culture process without transformation (designated C in all Figures). Plants from calli subjected to bombardment with uncoated gold (designated BG in all Figures) were sometimes anomalous with exceptionally low or high readings. Comparison of salicylate hydroxylase activity measured by decrease in absorbance over time between tissues extracted from plants co-transformed by microprojectile bombardment with pSLJ7307 and pROB5, control plants subjected to tissue culture, blank gold plants and wild type Taipei 309 can be seen in Figure 4.2. Microprojectile transformed plants are designated in every graph by the plasmid followed by a number. Tissues extracted from old plant leaf tissue (leaf tissue collected 6 months after transplantation) produced similar results to young plant tissue (collected 1 month after transplantation) (Figure 4.3), as did wounded tissues (Figure 4.4) and samples purified by passing the extract through a Sephadex G50 column (Figure 4.5). This purification reduced the values measured, though the pattern of reduction of absorbances between plants remained similar. In all figures, bars represent the standard error.

Figure 4.2
Mean decrease in absorbance for leaf tissues extracted from plants transformed with pSLJ7307/pROB5 tested for salicylate hydroxylase activity



(Significant at 0.01% level with 10 degrees of freedom)

Figure 4.3
Mean decrease in absorbance for old leaf tissue from plants transformed with pSLJ7307/pROB5 and wild-type plants tested for salicylate hydroxylase activity

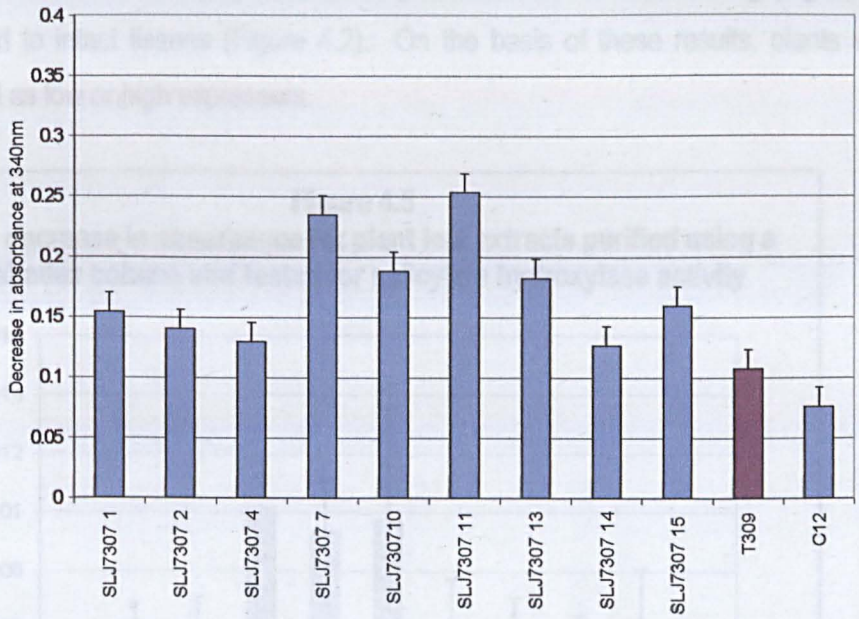
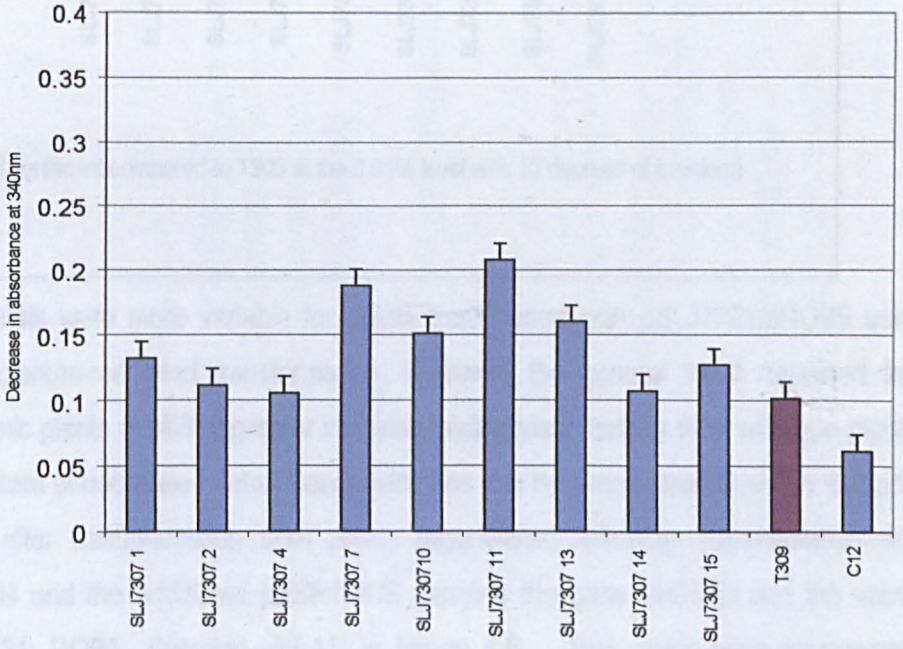


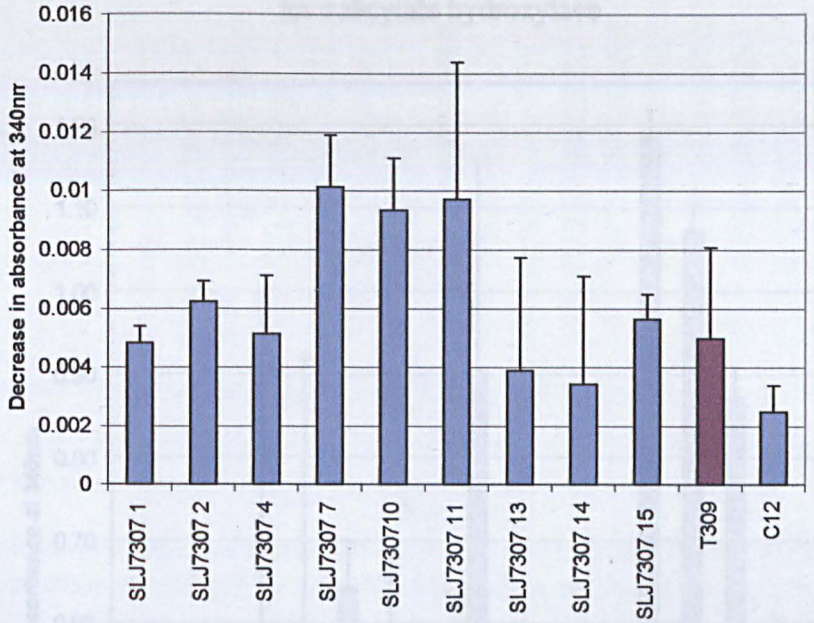
Figure 4.4
Mean decrease in absorbance for wounded plant tissue transformed with SLJ7307/pROB5 and tested for salicylate hydroxylase activity



(not statistically significant compared to old leaf tissues significant at 0.05% level with 10 d.f against wild-type T309)

Decrease in absorbance was highest in old tissues (Figure 4.3) and lowest in tissues purified using Sephadex (Figure 4.5). There was no significant difference between the decrease in absorbance for plant leaf tissue collected 4 h after wounding (Figure 4.4) compared to intact tissues (Figure 4.2). On the basis of these results, plants were classified as low or high expressors.

Figure 4.5
Mean decrease in absorbance for plant leaf extracts purified using a Sephadex column and tested for salicylate hydroxylase activity

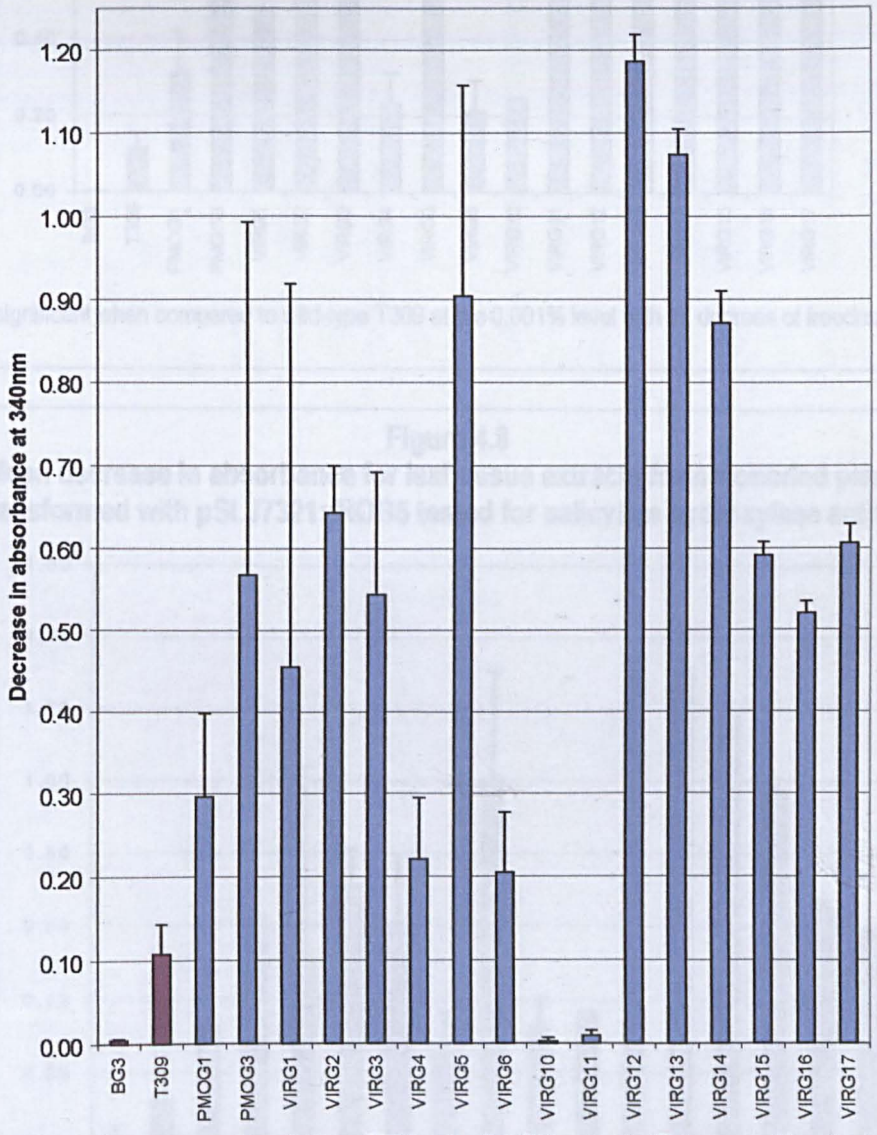


(significant compared to T309 at the 0.01% level with 10 degrees of freedom)

The results were more variable for plants transformed with pSLJ7321/pROB5 using *Agrobacterium*-mediated transformation. However, the general trend remained that transgenic plants exhibited greater salicylate hydroxylase activity than wild-type plants. The pattern of decrease in absorbance with time can be seen in leaf tissues collected 1 month after transplantation from plants regenerated following transformation with LBA4404 and the additional pBBR1MCS carrying the gene *virG54D* and the vector pSLJ7321: ROB5 (labelled vir1-17) in Figure 4.6. Two plants were regenerated following transformation with pBBR1MCS, pSLJ7321::ROB5 and the binary vector pMOGB22 carrying the *gus* and *bar* genes (labelled PMOG 1 & 3). Similar patterns can be seen across the plants from old leaf tissue collected 6 months after transplantation

(Figure 4.7) and from wounded tissue (Figure 4.8) following purification using a Sephadex G50 column, where the change in absorbances were reduced. However, the pattern remained similar (Figure 4.9). Error bars represent the standard error of the mean; the statistical significance resulting from an analysis of variance and the degrees of freedom are included.

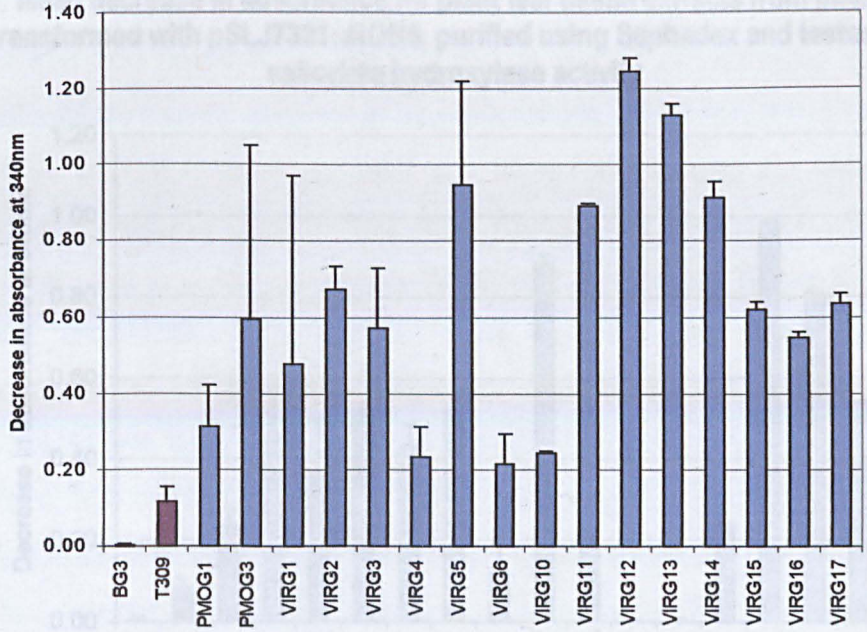
Figure 4.6
Mean decrease in absorbance for leaf tissue extracts collected from plants transformed with *A. tumefaciens* containing pSLJ7321:: ROB5 and tested for salicylate hydroxylase



(significant when compared to wild-type T309 at the 0.01% level with 17 degrees of freedom)

Figure 4.7

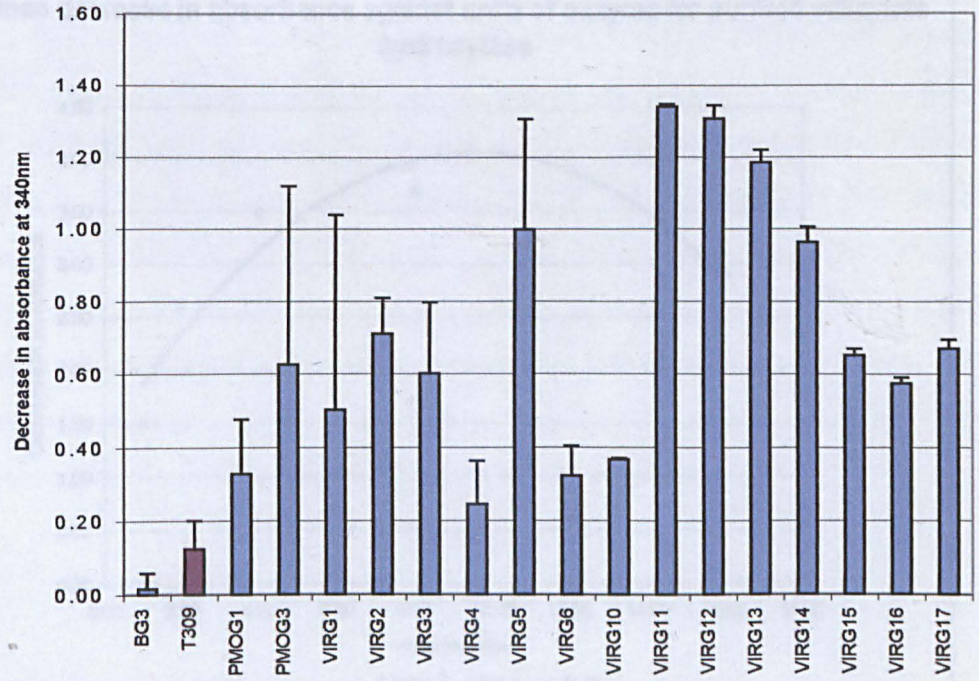
Mean decrease in absorbance for extracts of old leaf tissue (6 months after transplantation) from plants, transformed with pSLJ76321:: ROB5 and tested for salicylate hydroxylase



(significant when compared to wild-type T309 at the 0.001% level with 17 degrees of freedom)

Figure 4.8

Mean decrease in absorbance for leaf tissue extracts from wounded plants transformed with pSLJ7321::ROB5 tested for salicylate hydroxylase activity



(not statistically significant compared to old tissues but statistically significant at the 0.001% level with 17 degrees of freedom compared to wild type plants)

Figure 4.9

Mean decrease in absorbance for plant leaf tissue extracts from plants transformed with pSLJ7321::ROB5, purified using Sephadex and tested for salicylate hydroxylase activity

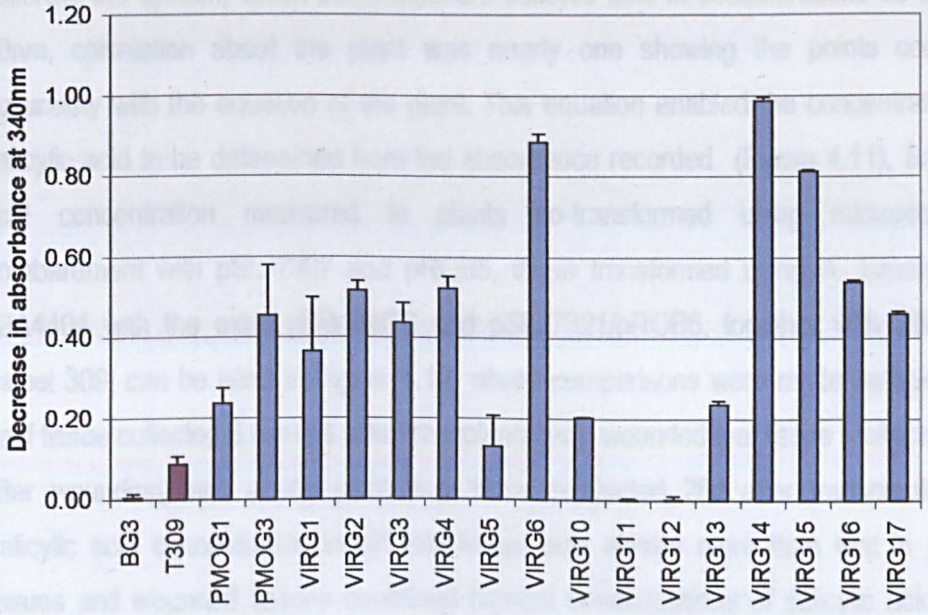
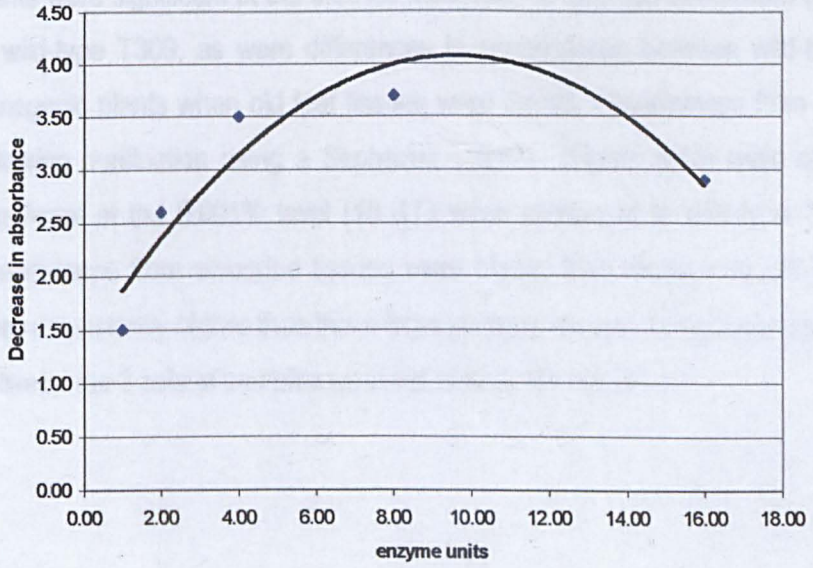


Figure 4.10

Mean decrease in absorbance against units of enzyme for purified salicylate hydroxylase



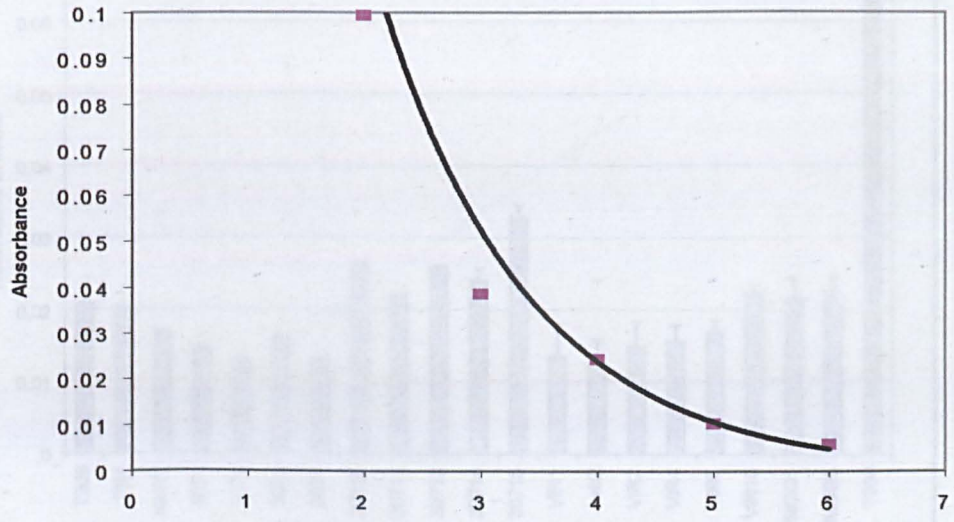
Where the equation of the plant is $y = -0.0298x^2 + 0.5726x + 1.3235$

and the correlation about the plant is $R^2 = 0.8619$

4.3.2 Salicylic acid

Salicylic acid is the substrate for the enzyme salicylate hydroxylase and, consequently, an increase in salicylate hydroxylase would be expected to correlate to a decrease in salicylic acid concentration. Salicylic acid of various concentrations was used to calibrate the system, which could measure salicylic acid in concentrations as low as 10nm, correlation about the plant was nearly one showing the points correlate accurately with the equation of the plant. This equation enabled the concentration of salicylic acid to be determined from the absorbance recorded (Figure 4.11). Salicylic acid concentration measured in plants co-transformed using microprojectile bombardment with pSLJ7307 and pROB5, those transformed using *A. tumefaciens* LBA4404 with the extra pBBr1MCS and pSLJ7321/pROB5, together with wild-type Taipei 309, can be seen in Figure 4.12, where comparisons were made between old (leaf tissue collected 6 months after transplantation), wounded leaf tissue (collected 4 h after wounding) and young plant leaf tissue (collected 28d after transplantation). Salicylic acid concentration in old leaf tissue was always more than that in young tissues and wounded tissues contained highest concentrations of salicylic acid. The error bars representing the standard deviations indicated that the range of values was uniform. Differences in absorbances of extracts between plants transformed with pSLJ7307/pROB5, pSLJ7321:: ROB5 and wild-type T309 were statistically significant at the 0.001% level with 22 degrees of freedom. Absorbances of samples from wounded plants were significant at the 0.001% level with 18 degrees of freedom (d.f.) compared to wild-type T309, as were differences in absorbances between wild-type T309 and transgenic plants when old leaf tissues were tested. Absorbances from plant samples following purification using a Sephadex column (Figure 4.13) were also statistically significant at the 0.001% level (18 d.f.) when compared to wild-type T309. Although absorbances from wounded tissues were higher than those from old tissues, which were consistently higher than those from younger tissues, comparisons of absorbances between the 3 sets of samples were not statistically significant.

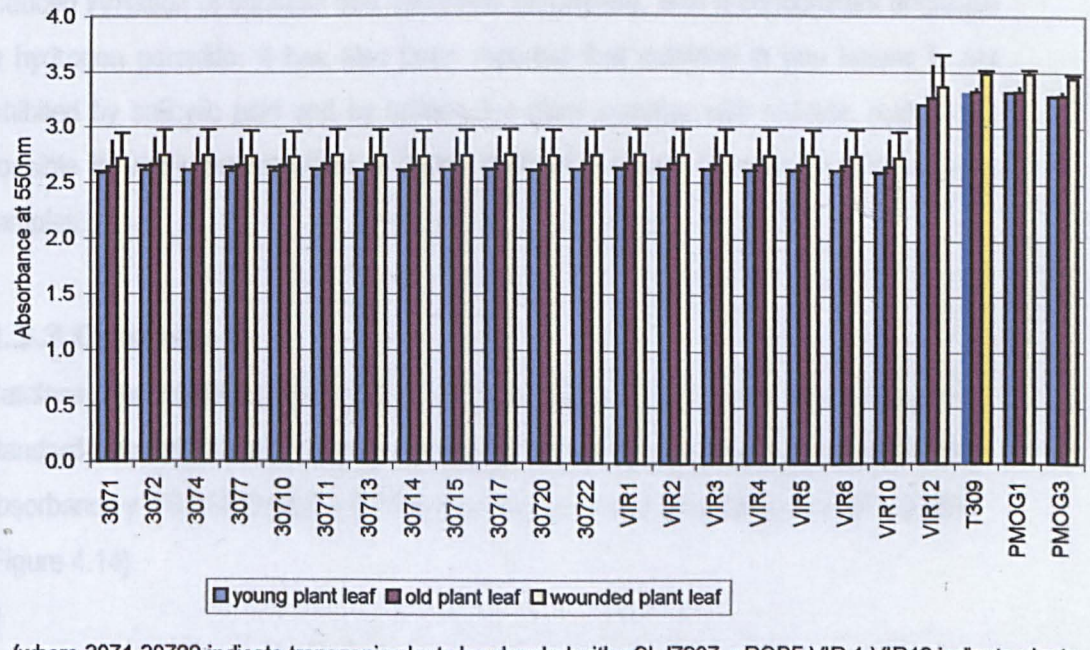
Figure 4.11
Absorbance against concentration for standards of salicylic acid



2 100 µ M
3 10 µ M
4 1 µ M
5 100 n M
6 10 n M

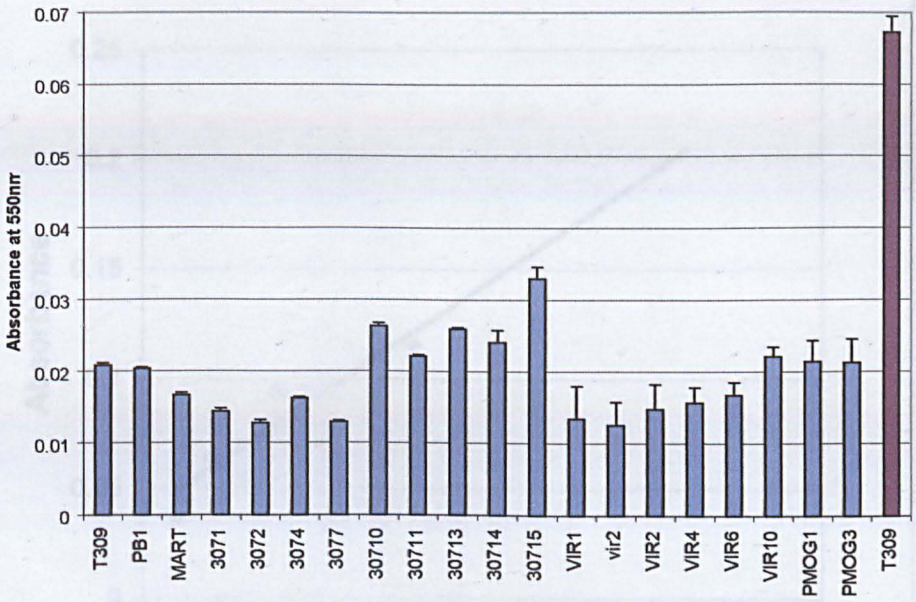
$y = 0.5642e^{-0.7909x}$ Is the equation of the plant
$R^2 = 0.9772$ Is the correlation about the line

Figure 4.12
Mean absorbance of extracts from leaf tissue of plants transformed with pSLJ7307 and pROB5 or pSLJ7321::ROB5 and wild-type T309 plants tested for salicylic acid concentration



(where 3071-30722 indicate transgenic plants bombarded with pSLJ7307, pROB5 VIR 1-VIR12 indicate plants transformed pSLJ7321::ROB5 and pBBR1MCS and pMOG indicates plants transformed with pMOGB22)

Figure 4.13
Mean absorbances of extracts from plant leaf samples, purified using Sephadex, tested for salicylic acid concentration



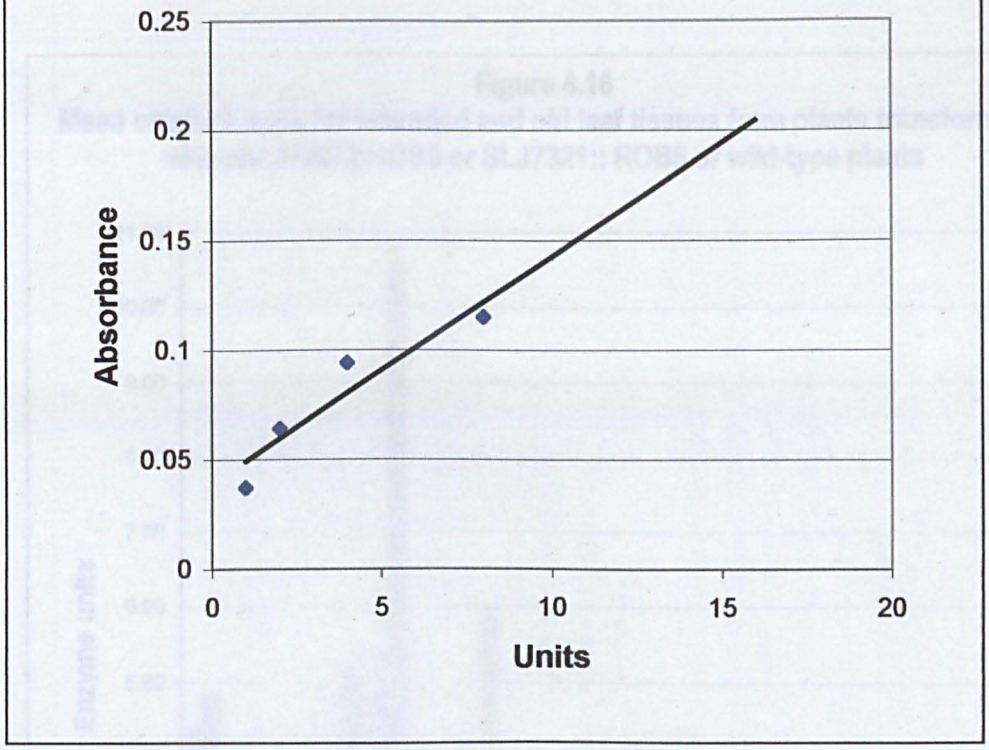
(where 3071-30722 indicate transgenic plants bombarded with pSLJ7307, pROB5 VIR 1-VIR12 indicate plants transformed pSLJ7321::ROB5 and pBBR1MCS and pMOG indicates plants transformed with pMOGB22)

Salicylic acid has been reported to inhibit activity of the enzymes catalase and ascorbate peroxidase (Durner and Klessig, 1995; Chen *et al.,* 1993), which degrade hydrogen peroxide. Therefore, it would be expected that in plants over-expressing salicylate hydroxylase with a concomitant decrease in salicylic acid, there would be reduced inhibition of catalase and ascorbate peroxidase, with a concomitant decrease in hydrogen peroxide. It has also been reported that catalase in rice leaves is not inhibited by salicylic acid and by spiking the plant samples with salicylic acid it was possible to verify the inhibition or insensitivity of catalase to salicylic acid in these samples.

4.3.3 Catalase

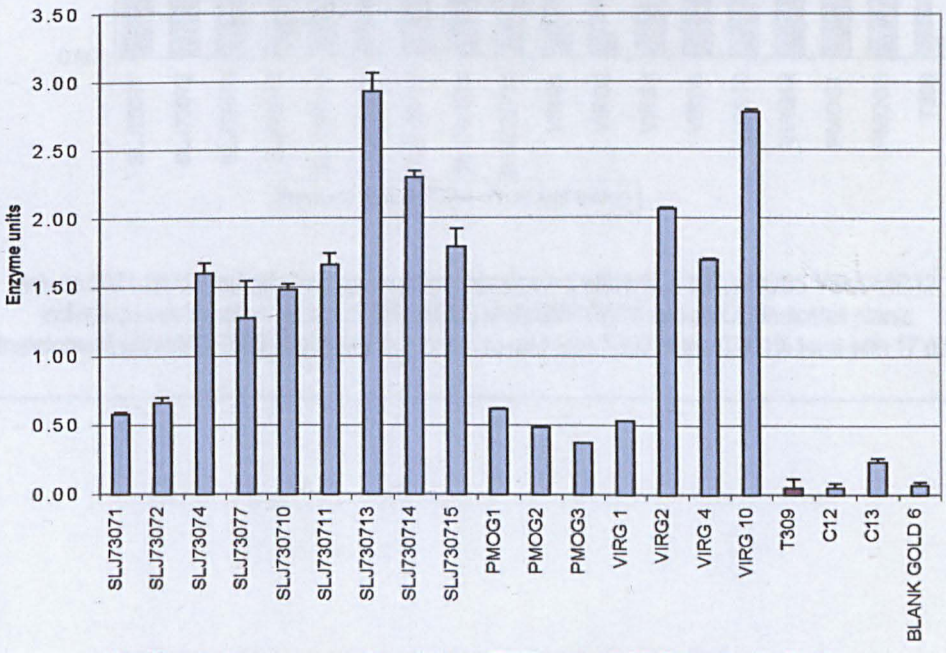
Catalase concentrations were established by setting up a calibration curve based on standard concentrations of catalase, where the equation of the plant demonstrated that absorbance y = 0.0104(units) + 0.0386 and the correlation about the plant $R^2 = 0.8852$ (Figure 4.14).

Figure 4.14
Decrease in absorbance against catalase units



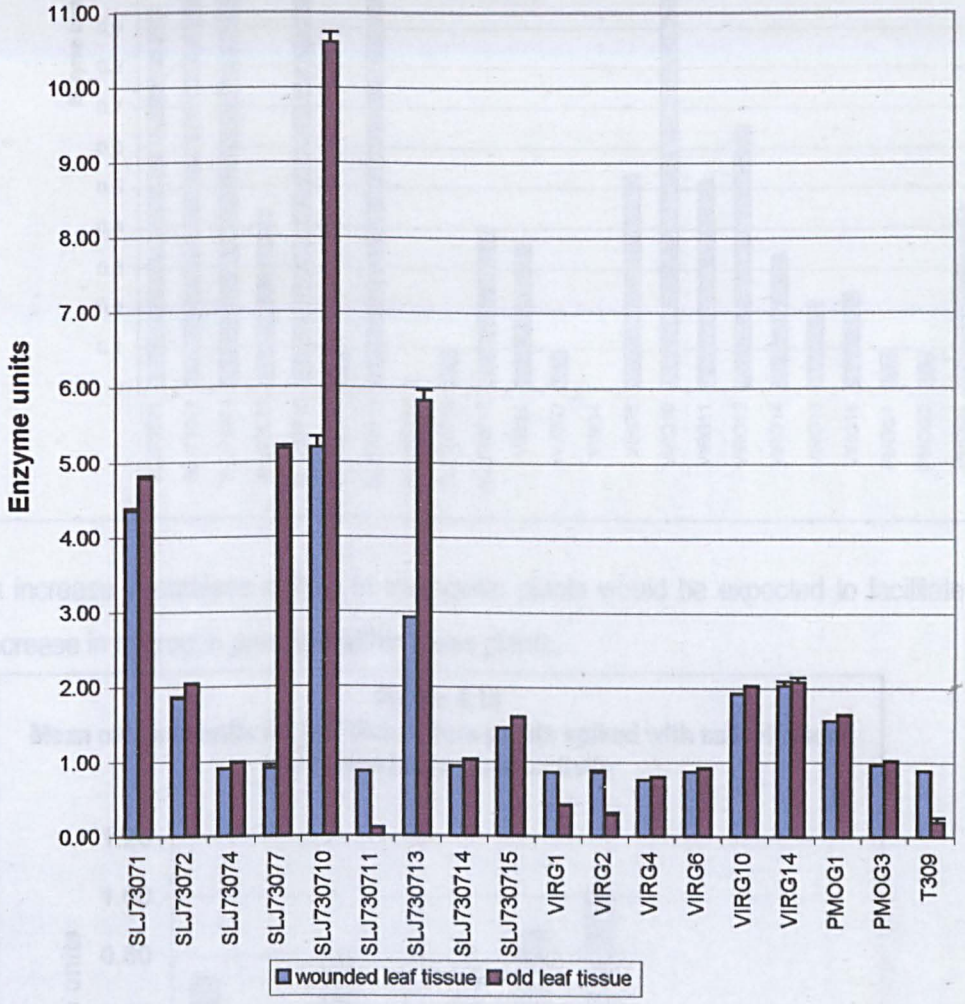
The enzyme concentration for the plant plants was calculated and displayed (Figure 4.15).

Figure 4.15
Mean units of catalase for leaf tissues of plants transformed with pSLJ7307/pROB5, pSLJ7321::ROB5 and wild-type plants



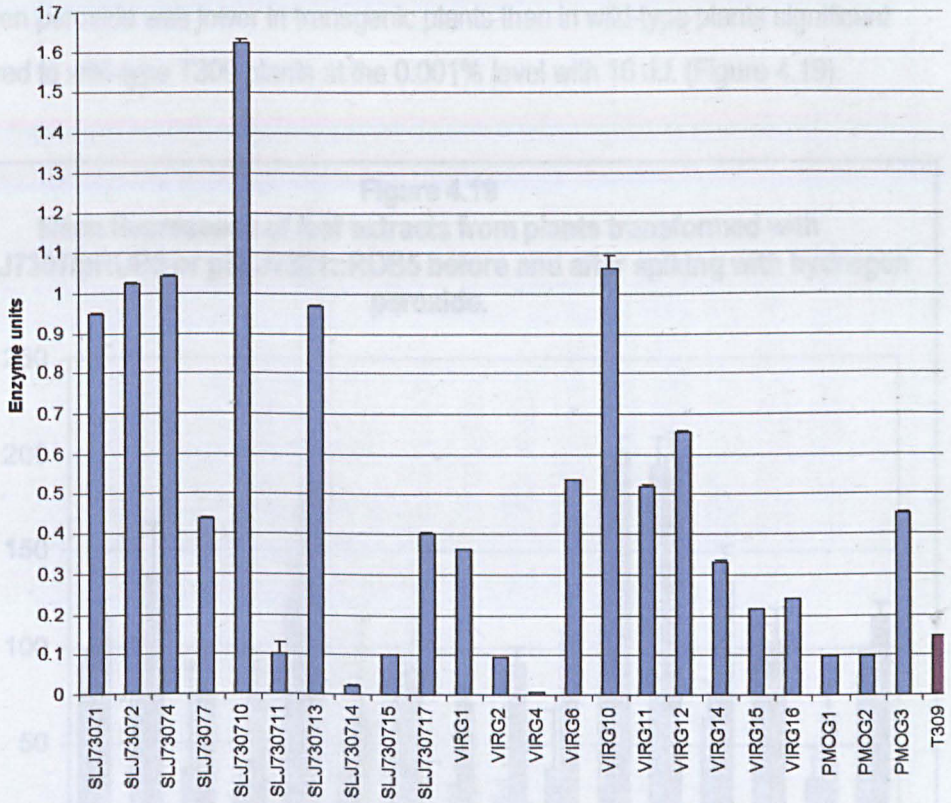
Significant when compared to wild-type T309 at the 0.001% level with 23 d.f.
(where 3071-30722 indicate transgenic plants bombarded with pSLJ7307, pROB5 VIR 1-VIR12 indicate plants transformed pSLJ7321::ROB5 and pBBR1MCS and pMOG indicates plants transformed with pMOGB22)

Figure 4.16
Mean catalase units for wounded and old leaf tissues from plants transformed with pSLJ7307/pROB5 or SLJ7321:: ROB5 or wild-type plants



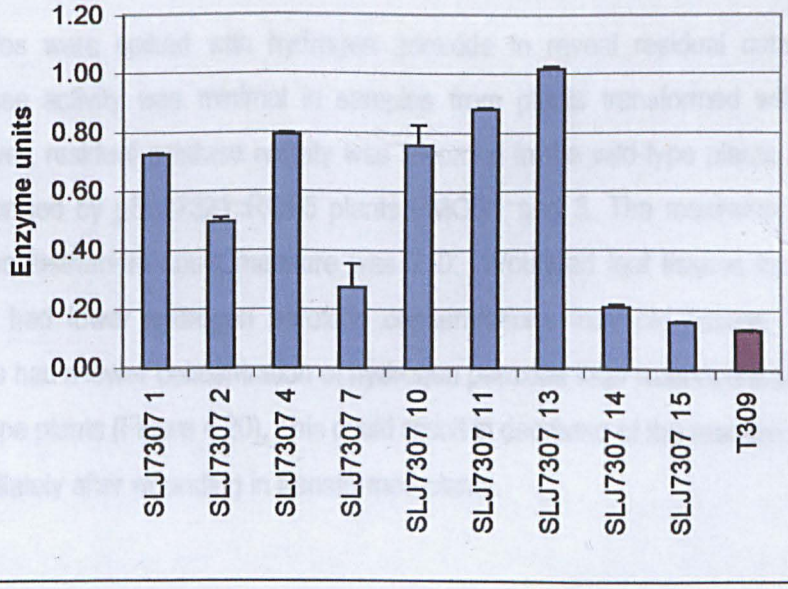
(where 3071-30722 indicate transgenic plants bombarded with pSLJ7307, pROB5 VIR 1-VIR12 indicate plants transformed pSLJ7321::ROB5 and pBBR1MCS and pMOG indicates plants transformed with pMOGB22)Significant compared to wild-type T309 at the 0.001% level with 17 d.f.

Figure 4.17
Mean catalase units for plant extracts purified with sephadex and tested for catalase activity



An increase in catalase activity in transgenic plants would be expected to facilitate a decrease in hydrogen peroxide within these plants.

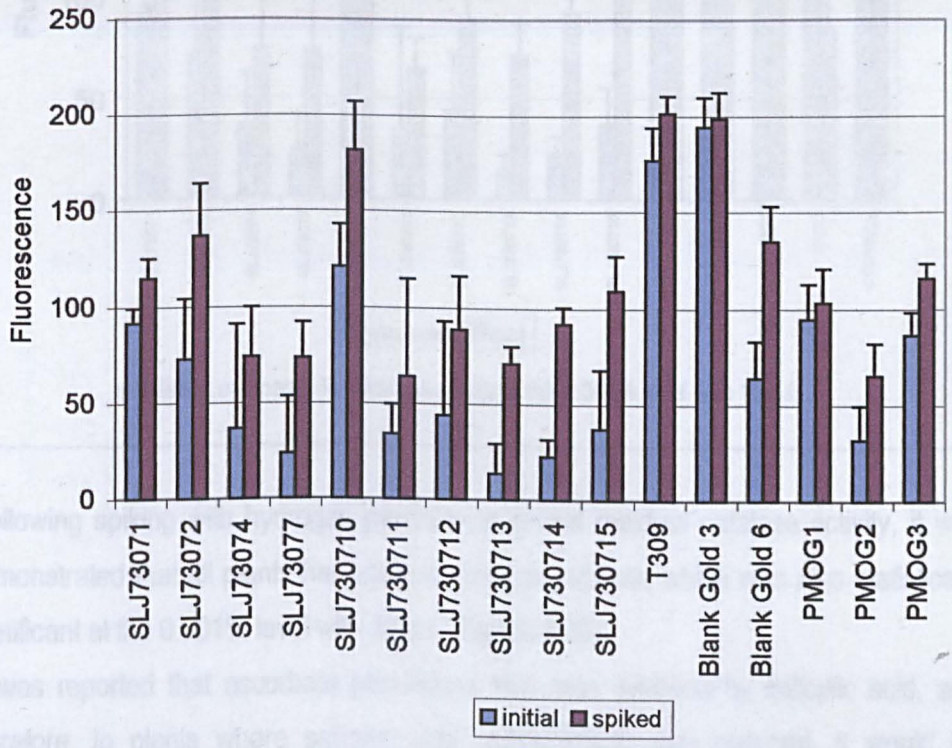
Figure 4.18
Mean catalase units for leaf tissue from plants spiked with salicylic acid and tested for catalase activity



4.3.4 Hydrogen peroxide

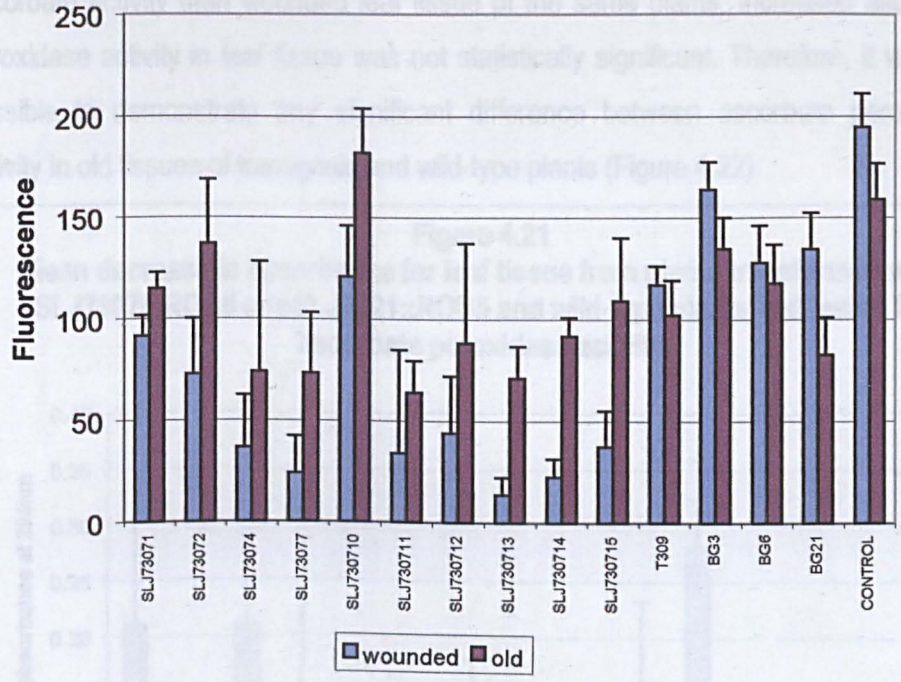
Hydrogen peroxide was lower in transgenic plants than in wild-type plants significant compared to wild-type T309 plants at the 0.001% level with 16 d.f. (Figure 4.19).

Figure 4.19
Mean fluoresence of leaf extracts from plants transformed with pSLJ7307/pROB5 or pSLJ7321::ROB5 before and after spiking with hydrogen peroxide.



Samples were spiked with hydrogen peroxide to reveal residual catalase activity. Catalase activity was minimal in samples from plants transformed with pSLJ7307. However, residual catalase activity was recorded in the wild-type plants and in plants transformed by pSLJ7321::ROB5 plants PMOG1 and 3. The maximum fluorescence that the instrument could measure was 210. Wounded leaf tissues from transgenic plants had lower hydrogen peroxide concentrations than old tissues, whereas old tissues had a lower concentration of hydrogen peroxide than wounded leaf tissues from wild-type plants (Figure 4.20). This could result in decrease of the reactive oxygen burst immediately after wounding in transformed plants.

Figure 4.20
Mean fluorescence from unspiked, freshly wounded and old plants transformed with pSLJ7307/ pROB5 or pSLJ7321:: ROB and wild-type plants



significant compared to wild-type plants at the 0.001% level with 15 d.f.

Following spiking with hydrogen peroxide to reveal residual catalase activity, it was demonstrated that all plants had minimal catalase activity, which was also statistically significant at the 0.001% level with 15 d.f. (Figure 4.20).

It was reported that ascorbate peroxidase was also inhibited by salicylic acid, and therefore, in plants where salicylic acid concentration was reduced, it would be expected that ascorbate peroxidase activity would be enhanced. To discern whether antioxidant activity was enhanced and pathways upregulated or whether increasing salicylate hydroxylase with concomitant decrease in salicylic acid removed inhibitors of the activity of catalase and ascorbate peroxidase, other antioxidant activities were measured. These antioxidants comprised superoxide dismutase, that catalyses the generation of hydrogen peroxide, ascorbate oxidase, a cell wall bound peroxidase and ascorbic acid.

4.3.5 Ascorbate peroxidase

Ascorbate peroxidase activity was enhanced in transgenic plants compared to wild-type plants significant at the 0.05% level with 14 d.f. Wounded tissues exhibited higher peroxide activity than old leaf tissue, statistically significant at the 0.01% level with 14 d.f.

(Figure 4.22), although in three transgenic plants old leaf tissues had a greater ascorbate activity than wounded leaf tissue of the same plants. Increased ascorbate peroxidase activity in leaf tissue was not statistically significant. Therefore, it was not possible to demonstrate any significant difference between ascorbate peroxidase activity in old tissues of transgenic and wild-type plants (Figure 4.22).

Figure 4.21
Mean decrease in absorbance for leaf tissue from plants transformed with SLJ7307/pROB5 or pSLJ7321::ROB5 and wild-type plants and tested for ascorbate peroxidase activity

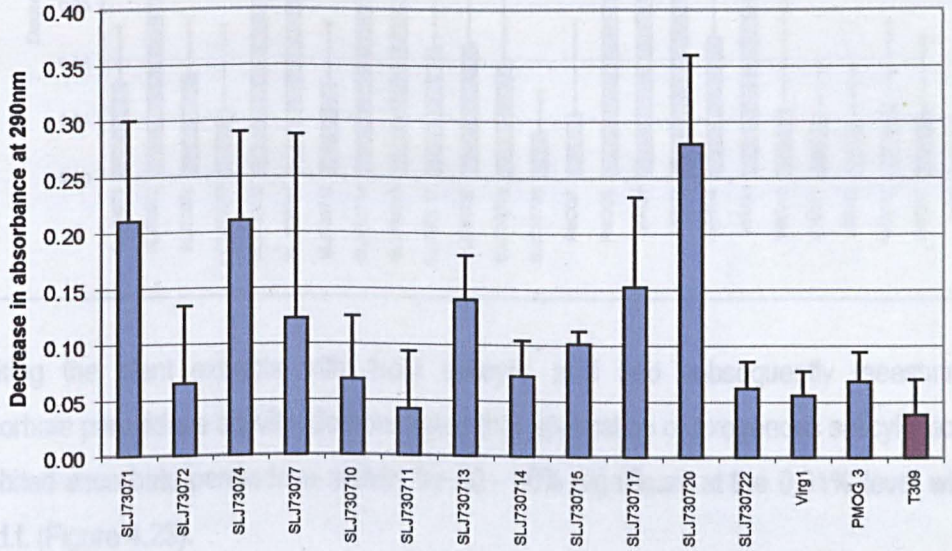


Figure 4.22
Mean decrease in absorbance for old leaf tissue and wounded tissues from plants transformed with pSLJ7307/pROB5 or pSLJ7321:: ROB5 and wild-type plants and tested for ascorbate peroxidase activity

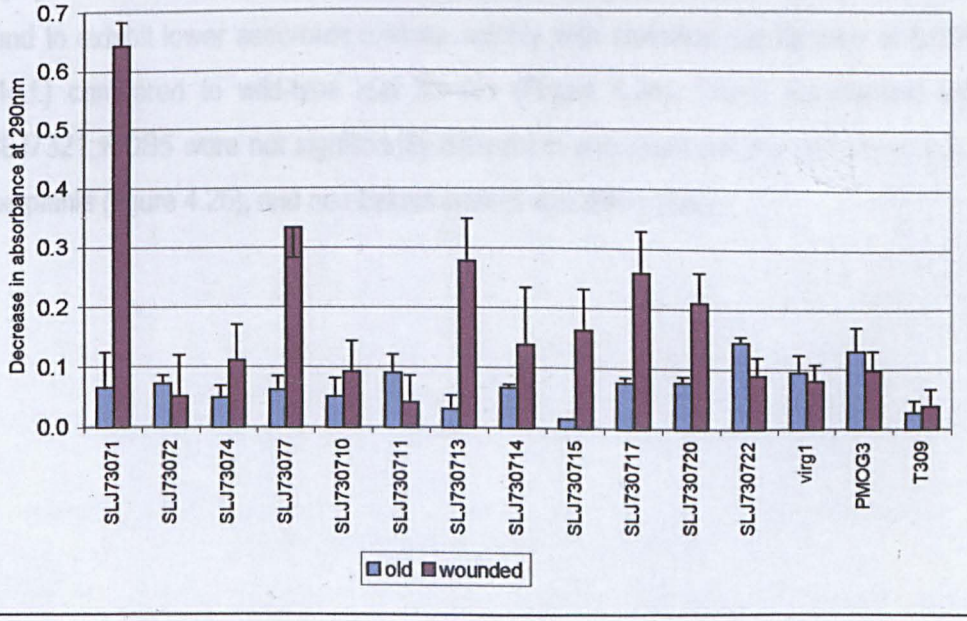
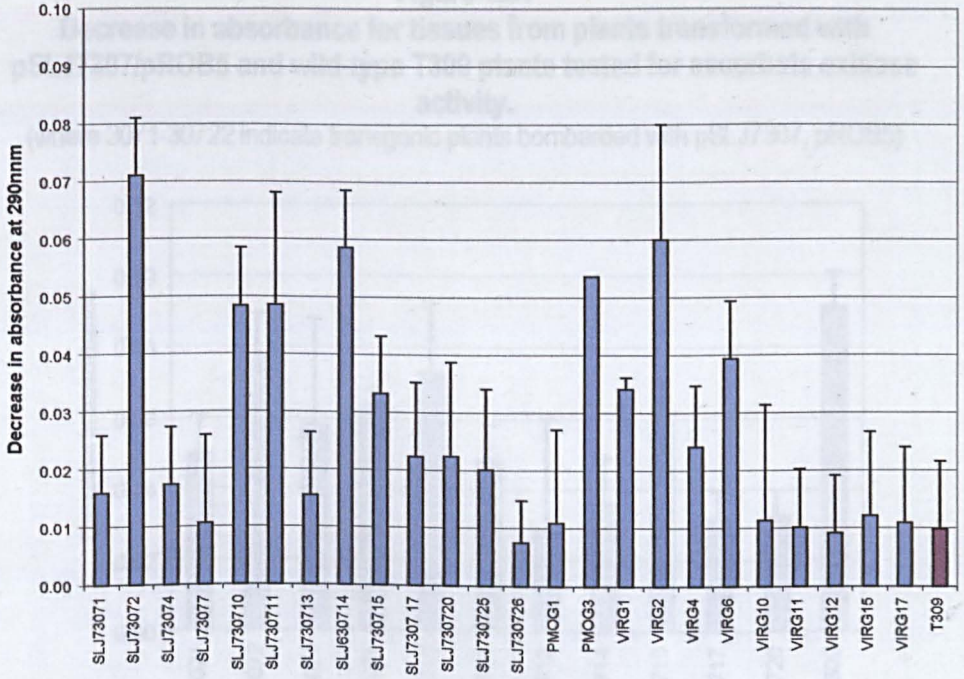


Figure 4.23
Mean decrease in absorbance for extracts of plants spiked with salicylic acid



Spiking the plant extracts with 1mM salicylic acid and subsequently measuring ascorbate peroxidase activity, demonstrated that application of exogenous salicylic acid inhibited ascorbate peroxidase activity by 50 - 90% significant at the 0.01% level with 14 d.f. (Figure 4.23).

4.3.6 Ascorbate oxidase

Plant leaf tissues tested for ascorbate oxidase were not filtered, as this enzyme is largely cell wall bound. Leaf tissue from plants transformed with pSLJ7307/pROB5 were found to exhibit lower ascorbate oxidase activity with statistical significance of 0.05% (11d.f.) compared to wild-type leaf tissues (Figure 4.24). Plants transformed with pSLJ7321::ROB5 were not significantly different in ascorbate oxidase activity to wild - type plants (figure 4.25), and no obvious pattern was discernable.

Figure 4.24

Decrease in absorbance for tissues from plants transformed with pSLJ7307/pROB5 and wild-type T309 plants tested for ascorbate oxidase activity.

(where 3071-30722 indicate transgenic plants bombarded with pSLJ7307, pROB5)

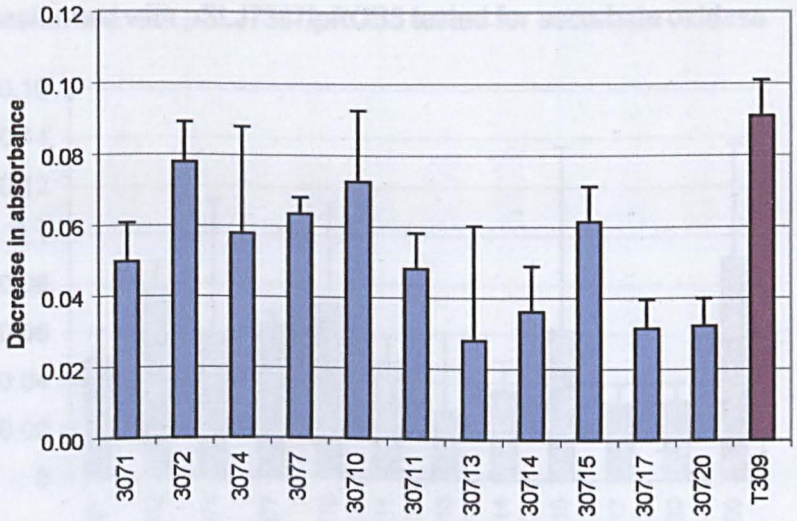
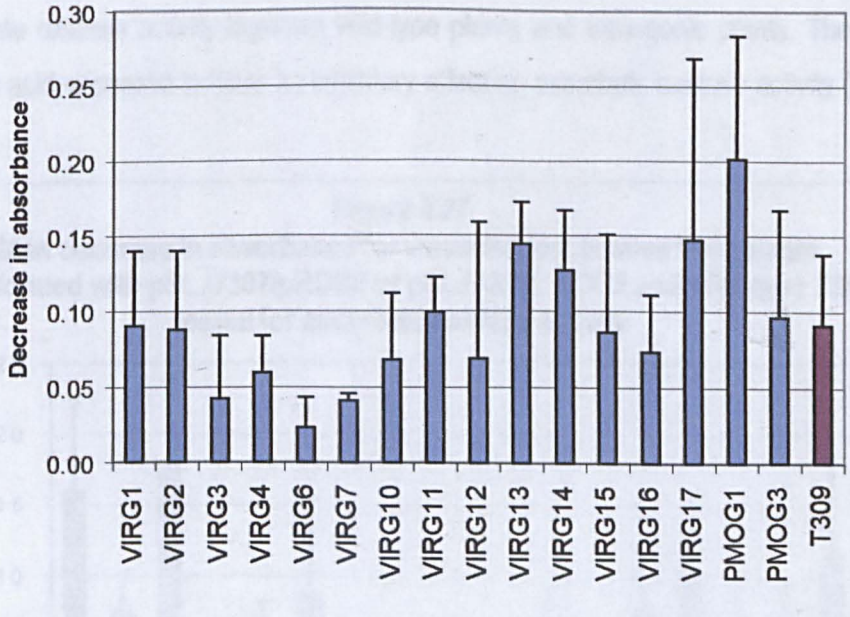


Figure 4.25

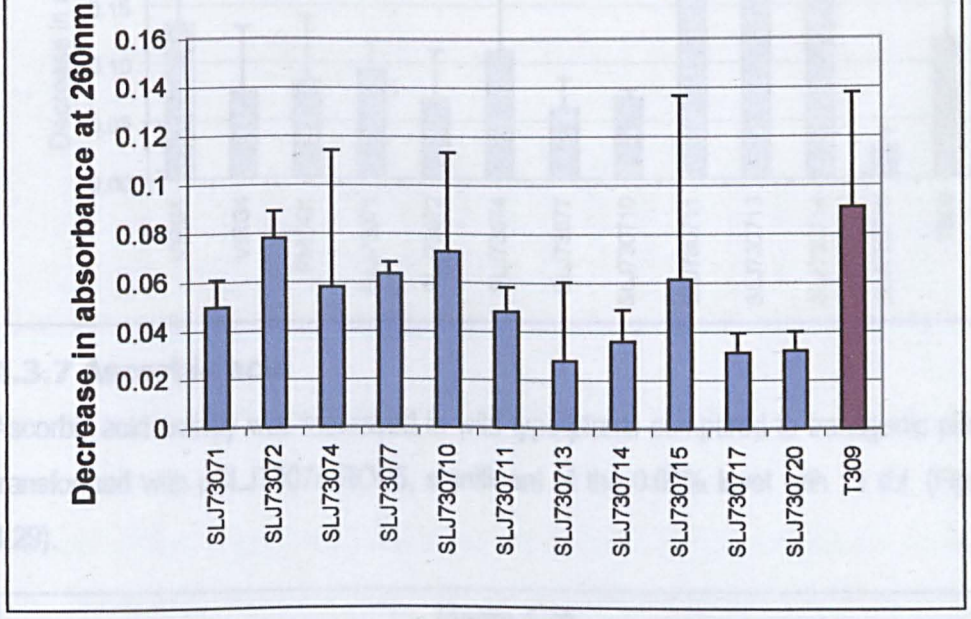
Mean decrease in absorbance for tissue from plants transformed with pSLJ7321:: ROB5 and wild-type T309 plants, tested for ascorbate oxidase activity



(where VIR 1-VIR12 indicate plants transformed pSLJ7321::ROB5 and pBBR1MCS and pMOG indicates plants transformed with pMOGB22)

Old leaf tissue from transgenic pSLJ7307/pROB5 plants produced no statistically significant difference between absorbance of wild-type plants and transgenic plants (Figure 4.26).

Figure 4.26
Mean decrease in absorbance for old leaf tissues from plants transformed with pSLJ7307/pROB5 tested for ascorbate oxidase



Similarly, no statistically significant difference was found between wounded tissues of wild-type plants and transgenic plants tested for ascorbate oxidase (Figure 4.27). Plant tissues spiked with salicylic acid exhibited no statistically significant difference in ascorbate oxidase activity between wild-type plants and transgenic plants. Therefore, salicylic acid appeared to have no inhibitory effect on ascorbate oxidase activity (Figure 4.28).

Figure 4.27
Mean decrease in absorbance for wounded leaf tissues from plants transformed with pSLJ7307/pROB5 or pSLJ7321:: ROB5 and wild–type T309 tested for ascorbate oxidase activity

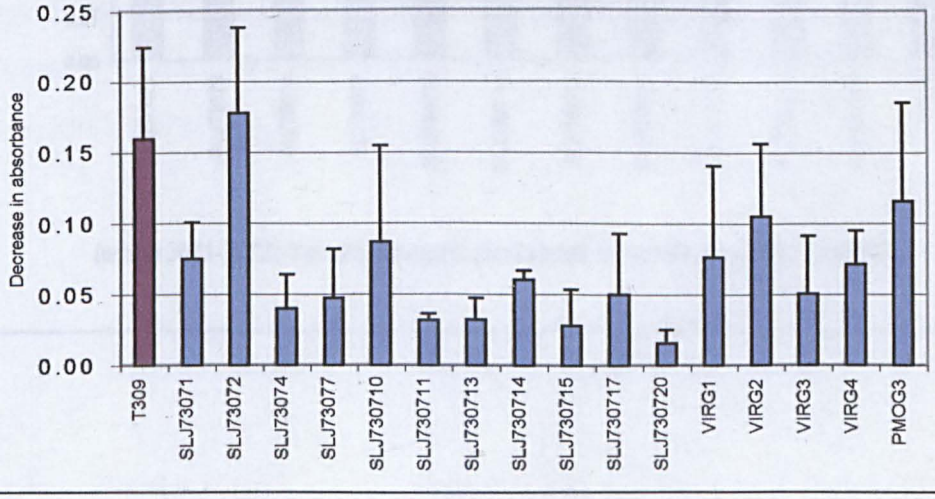
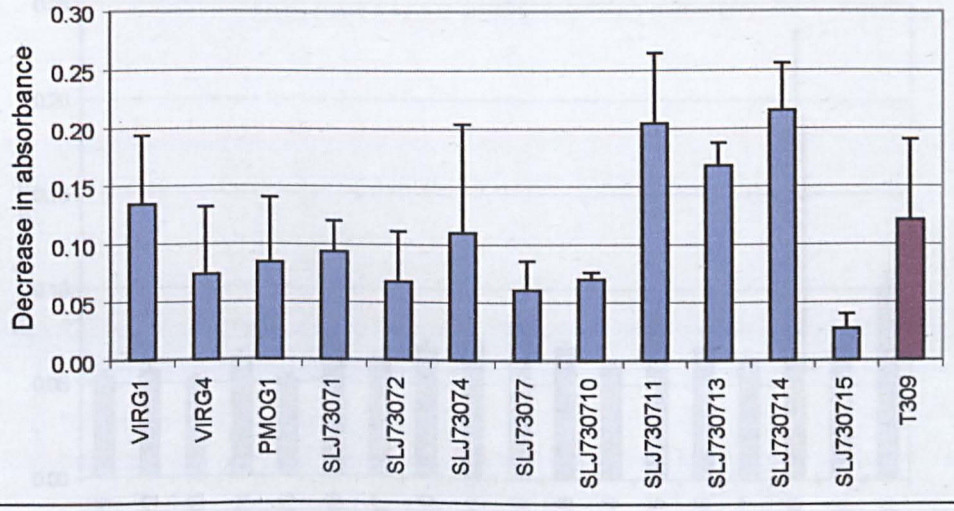


Figure 4.28

Mean decrease in absorbance for leaf tissue spiked with salicylic acid from plants transformed with pSLJ7307/pROB5 or pSLJ7321:: ROB5 or wild-type plants

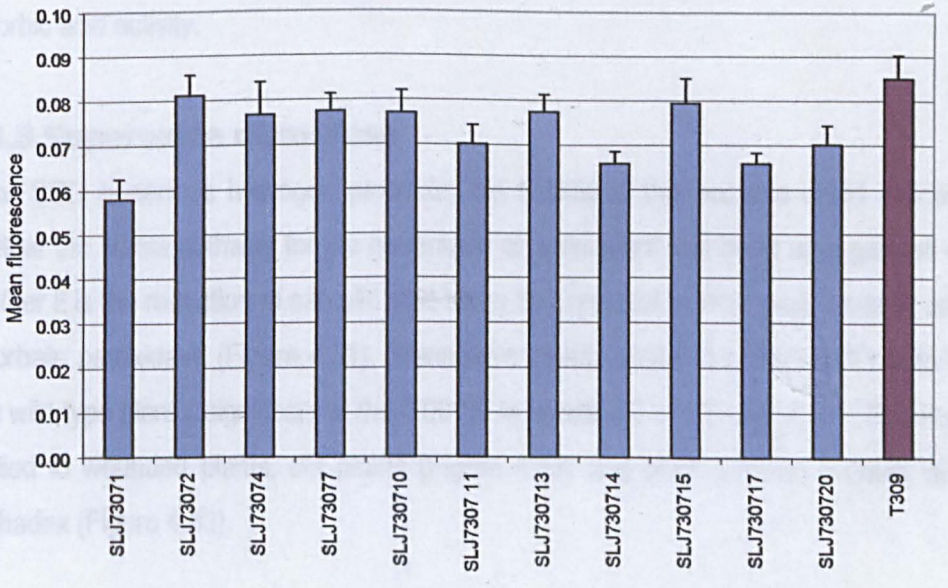


4.3.7 Ascorbic acid

Ascorbic acid activity was increased in wild-type plants compared to transgenic plants transformed with pSLJ7307/pROB5, significant at the 0.01% level with 11 d.f. (Figure 4.29).

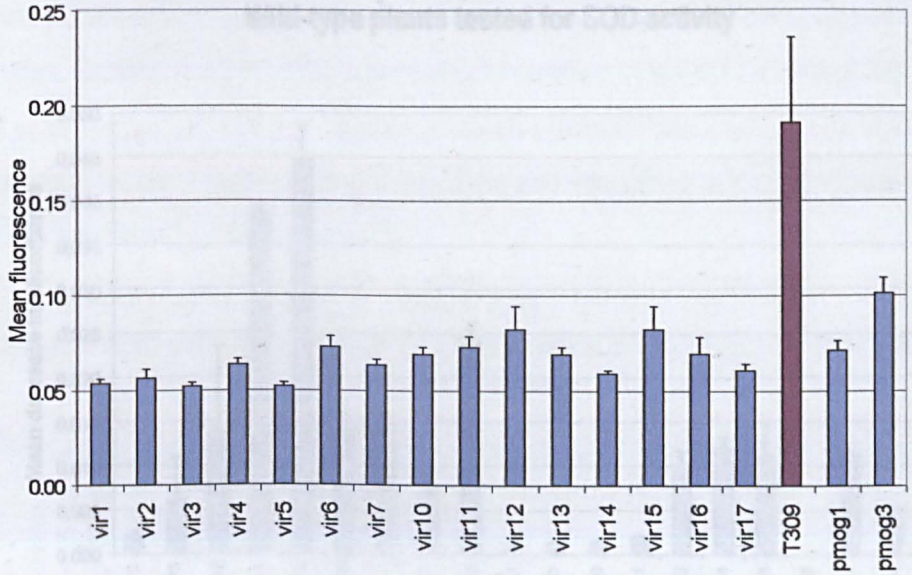
Figure 4.29

Mean fluorescence of leaf sample extracts from plants transformed with SLJ7307/pROB5 and wild-type plants assessed for ascorbic acid activity.



(where 3071-30722 indicate transgenic plants bombarded with pSLJ7307, pROB5)

Figure 4.30
Mean fluorescence for leaf samples from plants transformed with pSLJ7321:: ROB5 tested for ascorbic acid activity



Plant leaf extracts from plants transformed with pSLJ7321::ROB5 exhibited consistently lower fluorescence than those from wild-type plants significant at the 0.001% level with 17 d.f. when tested for ascorbic acid activity (Figure 4.30). Wounded plant leaf samples, old plant leaf samples and plant tissues spiked with salicylic acid, were not tested for ascorbic acid activity.

4.3.8 Superoxide dismutase

Since SOD generates hydrogen peroxide, the activity of this enzyme could indicate whether the whole pathway for the generation of antioxidant has been up-regulated or whether it is the reduction of salicylic acid alone that resulted in increased catalase and ascorbate peroxidase (Figure 4.31). Transgenic plants exhibited greater SOD activity than wild-type plants, significant at the 0.001% level with 22 d.f. (Figure 4.31). This also applied to wounded plants, old plants (Figure 4.32) and plant samples purified with Sephadex (Figure 4.33).

Figure 4.31
Mean decrease in absorbance for plant leaf samples from transgenic and Wild-type plants tested for SOD activity

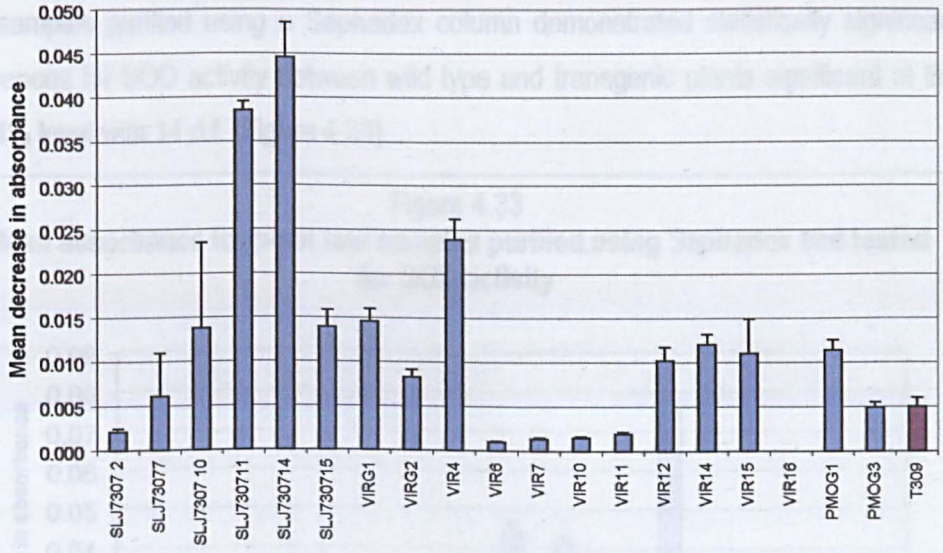
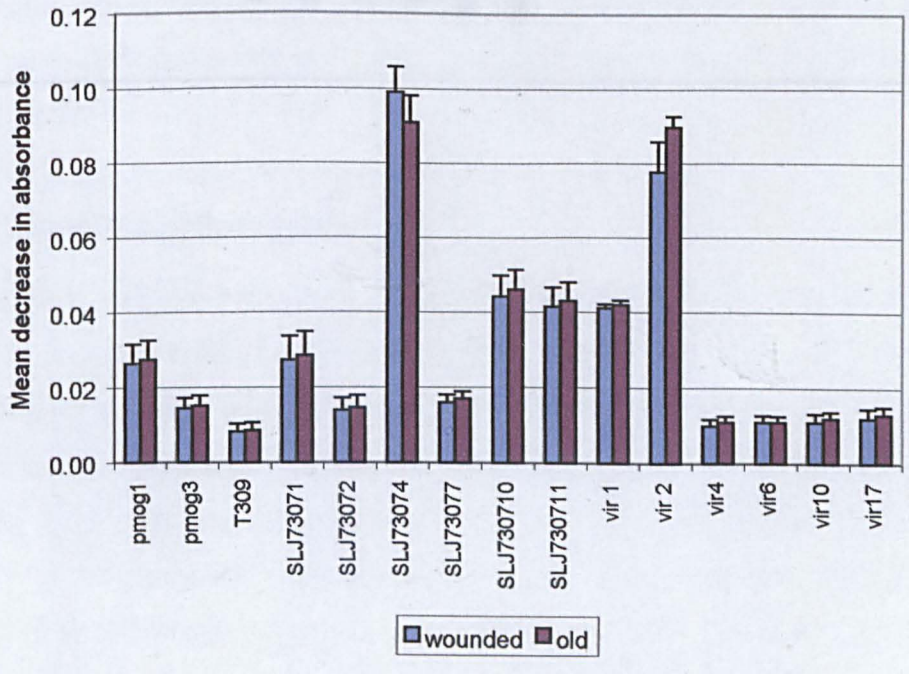
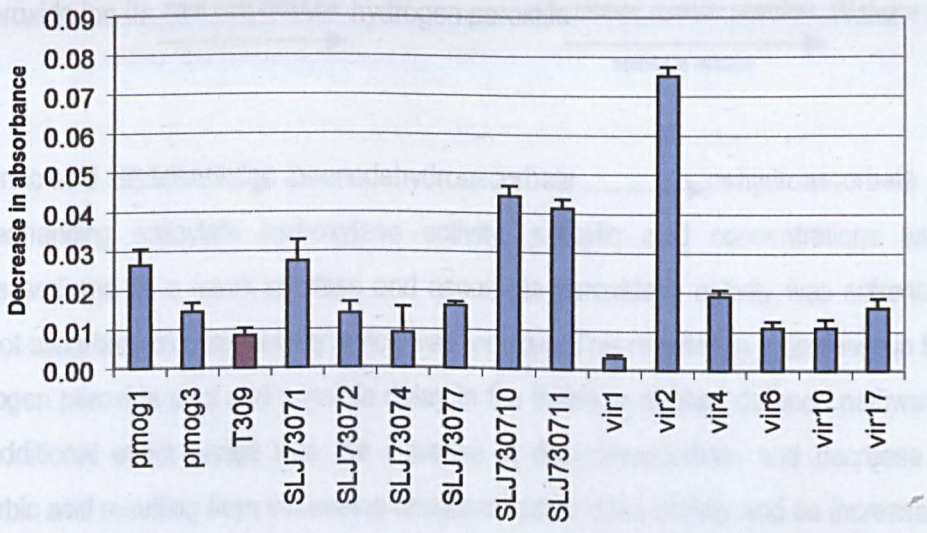


Figure 4.32
Mean absorbance of wounded and old plant tissues from transgenic and wild-type plants tested for SOD activity



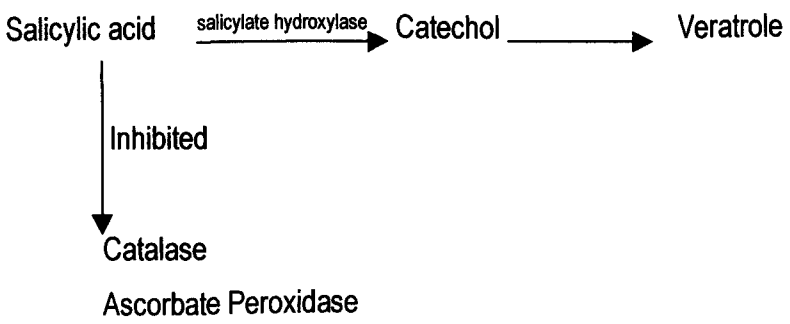
Differences in SOD activity between wounded and old tissues were not statistically significant. However, both wounded and old plant tissues demonstrated statistically significant differences between the absorbance of wild-type and of transgenic plant leaf samples, significant at the 0.001% level with 14 degrees of freedom (Figure 4.32). Plant leaf samples purified using a Sephadex column demonstrated statistically significant differences for SOD activity between wild-type and transgenic plants significant at the 0.001% level with 14 d.f. (Figure 4.33).

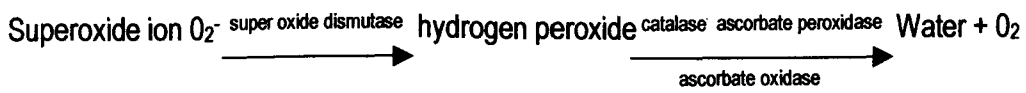
Figure 4.33
Mean absorbance for plant leaf samples purified using Sephadex and tested for SOD activity



4.4 DISCUSSION

The basic assumptions that were tested can be summarised in the following equations





By enhancing salicylate hydroxylase activity, salicylic acid concentrations were decreased and as a result catalase and ascorbate peroxidase activity was enhanced but not ascorbate oxidase activity which was reduced. This resulted in a decrease in the hydrogen peroxide pool and possible delay in the initiation of plant defence pathways. An additional effect tested was the increase in dehydroascorbate and decrease in ascorbic acid resulting from increasing ascorbate peroxidase activity and an increase in the superoxide dismutase activity in SLJ7307 plants, but a decrease in SOD activity in SLJ7321 plants.

4.4.1 Salicylate hydroxylase

The increase in salicylate hydroxylase activity in transgenic pSLJ7307/pROB5 plants was up to twice that in wild-type plants and although statistically significant, these results were not as definite as had beenhypothesised. Salicylate hydroxylase catalyses the stoichiometric formation of catechol from salicylic acid and reduced pyridine nucleotide, in the presence of flavin adenine dinucleotide (FAD) as a specific cofactor (Katagiri *et al.*, 1966). The enzyme forms an enzyme substrate complex with salicylate, the FAD moiety of the complex being reduced with NADH. Formation of catechol was strictly proportional to the rate of salicylate dependant NADH oxidation. Therefore, the experiment measured the rate of NADH oxidation. Oxidation of NADH is not specific to

salicylate hydroxylase and, consequently, it was important to verify that concentrations of the substrate salicylic acid were reduced in the plants over-expressing salicylate hydroxylase activity. Salicylate hydroxylase activity in the samples could have been limited by feedback inhibition from the catechol, or through insufficient FAD in the reaction mixture. Both salicylic acid and NADH were added to the samples. In plants transformed with pSLJ7321::ROB5, the activity of salicylate hydroxylase was up to 10 times greater than that found in wild-type plants, although for these plant samples, the enzyme activity was less consistent for samples between plants and from the same plant.

Older plant tissues exhibited enhanced salicylate hydroxylase activity compared to younger leaf tissues, while wounded leaf tissue demonstrated highest enzyme activity. However, the difference between enzyme activity in older leaf tissue and that in wounded leaf tissue was not statistically significant. Wounded plants produce ROS such as hydrogen peroxide, superoxide and the hydroxyl radical as a result of the disruption of cell walls and membranes. These compounds initiate signal cascades leading to the production of jasmonic acid. It is possible that salicylate hydroxylase is up regulated as a result of these signal cascades. Alternatively, salicylate hydroxylase may initiate plant signal cascades, leading to plant defence responses or the production of methyl salicylate. Salicylic acid is toxic in concentrations greater than 1mM. Therefore, excess salicylic acid in intact cells is conjugated to a glucose molecule and stored as β-O-D-glucosyl salicylic acid (Chen *et al.*, 1993). Disruption of the cell wall during wounding could therefore lead directly to an increase in salicylic acid concentration in the cytoplasm, necessitating increased salicylate hydroxylase activity.

4.4.2 Salicylic acid

Salicylic acid was reduced in the pSLJ7307/pROB5 transformed plants compared to wild-type plants. However, there was no significant difference in salicylic acid content between PMOG plants and wild-type plants. Most plants transformed with pBBR1MCS *virG*54D plants had reduced salicylic acid content compared to wild-type plants with the exception of plant VirG12. Old leaf tissue demonstrated increased salicylic acid concentration compared to young leaf tissue. Some plants appeared to be exceptions showing both high levels of salicylate hydroxylase and high levels of salicylic acid. However, salicylic acid was reduced in most plants in which salicylate hydroxylase activity was enhanced. This could have implications for the defence response of these

plants as salicylic acid has been demonstrated to be necessary in such plant defence responses. It has been reported that salicylic acid concentrations are much higher in rice leaf tissue than in many other plants (Silverman *et al.*, 1995). It is possible that salicylate hydroxylase is stored in older leaf tissues, which could be part of enzyme changes leading to senescence. Many of the signals that encode for plant defence genes and stress response genes are also triggered in senescent leaf tissues (Buchanan-Wollaston *et al.*, 2003).

Wounded leaf tissue had the highest salicylic acid content in all plants. Increase in salicylic acid content in wounded leaf tissue would support the theory that wounding releases salicylic acid that was stored as the inactivated compound β-0-D-glucosyl salicylic acid in the vesicles. Tissue extracts purified with Sephadex demonstrated the highest difference in salicylic acid content between transgenic and wild-type plants. There is an additional pathway by which salicylic acid is produced via chorismate by a two-step process (Verbenne *et al.*, 2000). Plants transformed with the gene that converts chorismate to salicylic acid showed a 500-1000 fold increase in salicylic acid (Verbenne *et* al., 2000) and constitutive expression of pathogenesis-related proteins. Consequently, salicylic acid may be manufactured as a result of at least two separate pathways and it is therefore unlikely that all salicylic acid in leaf tissue would be eliminated by enhanced salicylate hydroxylase activity. Additionally, it has been reported that salicylic acid is present in much greater concentrations in the leaves of rice plants than in tobacco (Silvermann *et al.*, 1995).

Salicylic acid has been shown to inhibit the activity of a number of enzymes such as catalase (Chen *et al.*, 1993) and ascorbate peroxidase (Durner and Klessig, 1995), possibly due to the ability of salicylic acid to chelate iron.

Salicylic acid binds to and inhibits catalase, leading to an increase in hydrogen peroxide and production of ROS. Hydrogen peroxide has been shown to have a direct action against pathogens and act as intermediaries in wound signal cascades (Mauch-Mani and Metréaux 1998). In addition, high concentrations of hydrogen peroxide have been shown to lead to an increase in salicylic acid production (Leon 1995). There is therefore more than one mode of action for salicylic acid. Not only does salicylic acid bind to a receptor and lead to the initiation of systemic acquired resistance, but infected leaves producing increased amounts of salicylic acid inhibit the activity of catalase and ascorbate peroxidase. This leads to an increase in the concentration of hydrogen peroxide that prolongs the oxidative burst that initiates signal cascades for plant wound

response and local defence responses. The increased hydrogen peroxide stimulates the production of salicylic acid (Mauch-Mari and Slusarenko, 1996). Some plant defence genes in *Arabidopsis thaliana* can be initiated by a salicylic acid independent pathway (Iris *et al.*, 1996). When the pathway for the production of salicylic acid is blocked, either by design as in *nahG* transformed plants or by mutations such as the knock-out inhibitor plants *nim* and *ndr*, the defence of *A. thaliana* plants is compromised (Mauch-Mari and Slusarenko, 1996). If the pathway is stimulated by the application of exogenous compounds, such as 2,6-INA or by mutation such as in the over-expressing plants *cpr, lst, A. thaliana* host defence is strengthened. A 25kD soluble salicylic acid binding protein identified in tobacco leaves was found to be an isozyme of catalase (Du and Klessig, 1997).

4.4.3 Hydrogen peroxide and catalase

Hydrogen peroxide was lower in transgenic plants than in wild-type plants, corresponding with the increase in peroxide degrading enzymes, ascorbate peroxidase, ascorbate oxidase and catalase, in transgenic plants. In wild-type plant extracts, wounded tissues showed maximal hydrogen peroxide content. However, in transgenic plants, old leaf tissues exhibited greater hydrogen peroxide content than wounded tissues. A transient increase in hydrogen peroxide is consequent on wounding as part of the ROS production resulting from the disruption of cell membranes (Leon *et al.*, 2001). Therefore, it is possible that the transient burst of ROS was not detected in these assays. Alternatively, the increase in peroxide degrading enzyme activity in transgenic plants could lead to hydrogen peroxide from the reactive oxygen burst following wounding being 'mopped up'. Another alternative is that the increase in hydrogen peroxide operates only within redox limits within the cell and there is a summation effect. Hydrogen peroxide production as a consequence of stress response, wounding or defence gene initiation, would be limited depending on the redox state of the cell. Prolonged increase in hydrogen peroxide in the cell could affect the redox balance of the cell and was shown to activate the expression of PR genes (Durner and Klessig, 1995). Wound signalling, resulting from production of hydrogen peroxide from SOD was maximal several minutes after wounding (Felton,1999). Hydrogen peroxide concentrations appear to be enhanced in older plant tissues possibly accumulating as part of the senescence process.

Salicylic acid has been shown to inhibit wound-activated jasmonic acid-mediated responses (Peña-Cortez *et al.*, 1995; Doares *et al.*, 1995). Activation of jasmonic acid-mediated wound response is initiated from by-products of cell wall degrading enzymes of plant pathogens (Héruart *et al.*, 1993). Hydrogen peroxide is a well-characterised stress response to both biotic and abiotic stresses involved in the hypersensitive response, chilling tolerance and cross-tolerance to a variety of stresses (Perl-Treves and Perl, 2002). Catalase can utilise hydrogen peroxide for the oxidation of various organic substrates. Peturbation in the glutathione metabolism of plants has been shown to induce the genes for phenyl alanine ammonia lyase and cytosolic superoxide dismutase (Perl-Treves and Perl, 2002).

Catalase activity was enhanced in transgenic plants compared to wild-type plants. Spiking the plant samples with salicylic acid reduced catalase activity by a mean of 60%. Salicylic acid did therefore inhibit catalase activity. Older leaf tissues demonstrated enhanced catalase activity compared to wounded leaf tissues, showing an accumulation of catalase in older tissues. Possibly, the breakdown of catalase was inhibited in transgenic plants especially as highest activity was recorded in plants transformed biolistically with pSLJ7307/pROB5 where multiple or fragmentary inserts could destroy the transcription of a number of genes. The function of the inhibition of both catalase and ascorbate by salicylic acid may be to prevent the cell from depleting its reserves of NAD(P)H. Whilst catalase degrades hydrogen peroxide without consuming reducing equivalents [NAD(P)H], the regeneration of oxidised ascorbic acid requires NAD(P)H, since both monodehydroascorbate reductase and glutathione reductase use NAD(P)H as an electron donor (Nocton and Foyer, 1998).

Whereas NAD(P)H is not limiting in plastids under light (Heineke *et al.*, 1991), prolonged increase of hydrogen peroxide concentrations due to complete or even partial inhibition of catalase activity by salicylic acid, together with a functional ascorbate-glutathione pathway, could deplete NAD(P)H. Therefore, co-ordinated inhibition of catalase and ascorbate peroxidase not only enhances hydrogen peroxide concentrations but may also prevent the cell from the consumption of reducing equivalents, that may be required for the production of anti-microbial compounds and proteins necessary for the establishment of resistance to pathogen attack.

4.4.4 Ascorbate peroxidase

Transgenic plant samples demonstrated significantly greater ascorbate peroxidase activity than those from wild-type plants. This activity was highest in wounded plant tissues. There was no significant difference between ascorbate peroxidase in old and young leaf tissue. Spiking the samples with 1mM salicylic acid reduced enzyme activity by 50 - 90%, demonstrating that salicylic acid did inhibit ascorbate peroxidase in these samples. Ascorbate peroxidase uses 2 molecules of ascorbic acid to reduce hydrogen peroxide to water with concomitant generation of 2 molecules of monodehydroascorbate MDHA, which require NAD(P)H to regenerate ascorbic acid. Ascorbate peroxidase fulfils a similar role to catalase in detoxifying hydrogen peroxide. Seven different ascorbate peroxidases have been distinguished in plants. The various isoforms are different in several molecular and enzymatic properties, such as molecular weight, electron donor specificity, lability in the absence of ascorbate, pH optimum, and ascorbate and hydrogen peroxide affinity (Jespersen *et al.*, 1997). In general, chloroplastic isoforms are very specific for ascorbate as an electron donor, whereas the cytosolic ascorbate peroxidase can also oxidise pyrogallol (Koshiba, 1993). In maize paclobutrazol-mediated chilling tolerance correlates with an induction of ascorbate peroxidase and superoxide dismutase activities in leaves (Pinhero *et al.*, 1997). When seedlings and callus tissue were treated with methyl jasmonate the activities of SOD, catalase and ascorbate peroxidase increased and remained higher in the resistant cultivar. High light stress induced cytosolic ascorbate peroxidase gene expression following increases in hydrogen peroxide concentration (Foyer, 2003). If ascorbate is not rapidly regenerated by ferrodoxin in the chloroplast membrane or by monodehydroascorbate reductase in the stroma, it dissociates to ascorbic acid and dehydroascorbate DHA. This is reduced to ascorbic acid by the action of the enzyme dehydroascorbate reductase using reduced glutathione as an electron donor. Oxidized glutathione is then recycled by NADPH-consuming glutathione reductase.

This GST ascorbic acid cycle fulfils roles in the adjustment of cellular redox potentials and regulation of gene expression. Exploitation of antioxidants could be used to manipulate cellular redox states. Promoters sensitive to reduced or oxidised forms of glutathione or ascorbate, may indicate redox sensing as central to cellular chemistry of ascorbate.

4.4.5 Ascorbate oxidase

Leaf sample extracts from plants transformed with pSLJ7307/pROB5 exhibited lower ascorbate oxidase activity than extracts from wild-type plants. Most leaf extracts from plant plants transformed with pSLJ7321::ROB5 (the PMOG and VIRG plants) also exhibited lower ascorbate oxidase activity. This pattern was also observed in old plant leaf tissue, although there was no significant difference between activity in old and young tissues. Wounded leaf tissues produced the same expression pattern as the other treatments though ascorbate oxidase was higher than in other treatments. Ascorbate oxidase, which is largely cell wall bound, may be activated by plant wounding and the destruction of plant cell walls. Ascorbate oxidase catalyses the reduction of ascorbic acid to dehydroascorbate via the free radical monodehydroascorbate, with the concomitant reduction of a molecule of oxygen to water was found to be involved in plant cell wall expansion and growth, and was active in the apex of growing tissues (Davey *et al.*, 2002).

In leaf tissues spiked with salicylic acid, the pattern was slightly different with several pSLJ7307/pROB5 derived plants exhibiting higher ascorbate oxidase activity than wild-type plants and some plants exhibiting inhibited ascorbate oxidase activity. No pattern was observed that correlated application of exogenous salicylic acid with any inhibition or over-expression of ascorbate oxidase. Ascorbate oxidase activity was found to be relevant at certain stages of growth, fruit development, ripening and senescence. This correlated to a decrease in ascorbic acid (Nocton and Foyer, 1998). Plants over-expressing ascorbate oxidase were shown to be more susceptible to ozone. High concentrations of ascorbate oxidase mRNA and enhanced enzyme activity correlate with slow cycling of cells of the quiescent centre. Concentrations of ascorbate oxidase and glutathione in the symplast were not affected by ascorbate oxidase over-expression. However, the redox state was altered with ascorbate reduced and reduced glutathione increased (Sanmartin *et al.*, 2003). Ascorbate oxidase has been assigned various biochemical functions. However, its role in the redox balance of the ascorbate pool is largely unknown (Nocton and Foyer, 1998).

4.4.6 Ascorbic acid

Ascorbic acid was significantly lower in transgenic than in wild-type plant samples, this was particularly noticeable in plants transformed with pSLJ7321::ROB5 (the VirG and PMOG plants). Dehydroascorbate accounted for approximately 50% of the ascorbic

acid measured in wild-type plants but approximately 70% of ascorbic acid measured in transgenic plants. Though both ascorbic acid and ascorbate oxidase expression was reduced in transgenic plants, the level of dehydroascorbate was higher in transgenic plants than in wild-type samples. There is support for these findings in the literature (Nocton and Foyer, 1998). It has been reported that ascorbate oxidase over-expression in tobacco leaves did not result in change in total ascorbic acid and dehydroascorbate content. These findings were consistent with a lack of correlation between ascorbate oxidase activity and total ascorbic acid and dehydroascorbate content across a range of developmental stages in leaves. This resulted in the oxidation of the ascorbic acid pool in the apoplast and caused a shift of approximately 5% in the redox state of ascorbic acid and glutathione in the symplast. The ascorbate pool was more oxidised and the glutathione pool more reduced. An observed decrease in the ascorbic acid redox state in the symplast of transgenic plants suggests that the rate of return of dehydroascorbate from the apoplast exceeded the capacity for the regeneration of ascorbic acid, which may represent a shift in the antioxidant metabolism to counter the enhanced loss of ascorbic acid through the decomposition of dehydroascorbate (Nocton and Foyer, 1998).

Ozone tolerance was correlated to ascorbic acid content of the leaf apoplast. Regulation of apoplast ascorbic acid pool is achieved through the balance between facilitated shuttling of ascorbic acid and dehydroascorbate (Nocton and Foyer, 1998).

4.4.7 Superoxide dismutase

In leaf samples from plants transformed with pSLJ7307/pROB5 plants superoxide dismutase activity was enhanced whereas in pSLJ7321::ROB5 plants it was suppressed compared to wild-type plant samples. The major SOD enzymes are located in the chloroplasts (Cu/ZnSOD and FeSOD in some species), cytoplasm (Cu/ZnSOD), and mitochondria (MnSOD). CuZnSOD and FeSOD are sensitive to hydrogen peroxide (Mano, 2002). The dismutation is catalysed by the metal ion at the active site SOD also exists in peroxisomes and glyoxisomes (Perl-Treves and Perl, 2002). Superoxide dismutates the superoxide ion to hydrogen peroxide and oxygen using water. Overexpression has been implicated in enhanced stress tolerance depending on factors such as the nature of the stress imposed the isozyme over-expressed the leaf age, growth conditions and strength of over-expression (Allen, 1995). Increases in SOD were associated with recovery from high light and low temperature conditions (Karpinski

et al., 1993). This implies that SOD has an important role in the low temperature induced oxidative stress response. Cold and dark stored and then illuminated tomato leaves were shown to have significantly lower activity of CuZnSOD (Michalski and Kaniuga, 1981). Generally, all reports show that transgenic plants with moderately enhanced activities of MnSOD or Cu/ZnSOD have a higher tolerance to oxidative stress (Kaprinski *et al.*, 2002). Environmental stresses that generate reactive oxygen species such as UV light, ozone, air pollutants, low temperatures, salt stress, drought, heat shock, and pathogen attack, have been shown to induce plant SOD activities (Van Camp *et al.*, 1994).

4.4.8 Conclusion

Enzyme/substrate	Result in transgenic lines Compared to wild type Plants
Salicylate hydroxylase	Increased activity
Salicylic acid	Lower concentration
Catalase	Increased activity
Ascorbate peroxidase	Increased activity
Ascorbate oxidase	Decreased activity
Hydrogen peroxide	Lower concentration
Superoxide dismutase	Enhanced activity in SLJ7307 plants Decreased activity in SLJ7321 plants
Ascorbic acid	Lower concentration
Monodehydroascorbate	
Dehydroascorbate	Higher concentration

In conclusion, it is possible that the stress tolerance of the transgenic plants has been enhanced with elevated SOD activity, catalase activity, and ascorbate peroxidase activities. The detoxification of superoxide was enhanced in these transgenic plants and the hydrogen peroxide produced subsequently detoxified. Ascorbic acid and ascorbate oxidase activity in transgenic plants was reduced. However the proportion of dehydroascorbate was increased, indicating that dehydroascorbate was accumulated more rapidly than regeneration to ascorbic acid. Hydrogen peroxide, produced as a consequence of the wounding response, was rapidly detoxified, thus resulting in a

reduced burst of reactive oxygen species on wounding and possibly affecting signal cascades initiated by it's concentration. Reduction in salicylic acid not only reduced inhibition of catalase and ascorbate peroxidase activities, but could also prevent the induction of systemic acquired resistance consequent on pathogen attack.

CHAPTER 5. PLANT DEFENCE RESPONSES

5.1 INTRODUCTION

5.1.1 Systemic acquired resistance (SAR)

Plant defence responses are initiated following pathogen attack. These responses are not restricted to tissues in close proximity to the site of attack, but extend to distal tissues which become more resistant to a second challenge by the same or another pathogen (Ryals *et al.*, 1996). This response, termed systemic acquired resistance (SAR), has been studied for a number of years and occurs in many species such as *Arabidopsis thaliana* (Unkes *et al.*, 1992; Unkes *et al.*, 1993)), tomato (Doares *et al.*, 1995), cucumber (Métraux *et al.*, 1990) and tobacco (Mur *et al.*, 1997). Early transcriptional changes in and around lesions develop following pathogen recognition. These include the localised induction of genes encoding enzymes in the phenylpropanoid synthesis pathway. The lower leaves develop necrotic lesions at the same time as the rest of the plant becomes noticeably resistant to a variety of bacterial, fungal and viral pathogens. At the same time that resistance appears systemically in the plant, expression of at least nine sets of genes is induced (Ward *et al.*, 1994). A number of these genes encode pathogenesis related (PR) proteins. The products of these proteins are thought to be involved with establishing SAR (Ward *et al.*, 1994).

5.1.2 Involvement of salicylic acid in systemic acquired resistance

Considerable evidence supports the involvement of salicylic acid (SA) 2-hydroxybenzoic acid in the induction of SAR (Yalpani *et al.*, 1991). Salicylic acid binds to a catalase isoform in tobacco leaves inhibiting its activity (Chen *et al.*, 1993). Grafting experiments performed using transgenic rootstocks that were unable to accumulate SA were used to test whether SA is the mobile system in SAR. Transgenic rootstocks, although unable to accumulate SA, were capable of delivering a signal that rendered the non-transformed scions resistant to pathogen infection (Vernooij *et al.*, 1994). Therefore the translocated signal is not SA, although SA is required in the distant tissues to induce SAR. Alternatively, SA was required in minute amounts to initiate SAR and low concentrations of SA remaining were sufficient to initiate SAR. Ryals (1996) argued that one promoter used by Vernooij (1994) was free to

enter the phloem and be dispersed, conferring SAR. Salicylic acid has been shown to accumulate in the phloem of inoculated plants and in tissues that are about to become resistant (Malamy *et al.,* 1990; Métraux *et al.,* 1990). SAR can be induced by exogenous application of SA (Ward *et al.*, 1994). Systemically protected plants often contain prominent proteins that are synthesized in response to primary inoculation. PR proteins are extracellular acidic polypeptides, some of which have chitinase or β-1-3, glucanase activity to degrade fungal cell walls. Many of the genes that encode for these proteins have been defined (Ward *et al.*, 1994) In the laboratory, SAR can be induced by primary inoculation of plants with a pathogen or non-pathogenic bacteria, viruses or fungi (Ward *et al.*, 1991). Acquired resistance can also be triggered by natural or synthetic compounds (Kessmann *et al.*, 1994). For example, 2,6-dicloroisonicotinc acid (2,6-INA) has been demonstrated to induce acquired disease resistance in rice leading to accumulation of PR proteins 1-9, enhanced lipoxygenase activity and enhanced levels of jasmonic acid (Schweizer *et al.*, 1997).

Confirmation of the involvement of SA was obtained using plants that had been transformed by the *nahG* gene. The bacterium *Pseudomonas putida* contains an enzyme salicylate hydroxylase that converts salicylic acid to catechol, which forms part of the napthalene degradative pathway. The gene for this enzyme, *nahG,* was engineered for expression in plants and has been transformed into a number of species (tomato, tobacco and *Arabidopsis*). Transformed plants exhibited markedly reduced salicylic acid accumulation around the lesion forming at the point of infection. These plants failed to exhibit both local and systemic PR protein accumulation. Plants possessing this gene could not accumulate SA and were unable to express SAR in response to fungal, bacterial or viral pathogens clearly indicating the importance of SA in SAR (Bi *et al.,* 1995; Brading *et al.,*2000; Felton *et al.,* 1999; Mur *et al.*, 1997). Catechol has no resistance inducing effect and does not trigger SAR expression (Felton *et al.,* 1999; Ryals *et al.*, 1994; Sticker *et al.*, 1997). Schweizer *et al.* (1998) found that post-wounding rice plants induced an accumulation of PR proteins and subsequent enhanced resistance to the rice blast fungus *Magnaporthe grisea.*

5.1.3 Biochemical pathways leading to the production of salicylic acid

There is much evidence to support the hypothesis that benzoic acid generated by the phenyl propanoid pathway is the immediate precursor of SA (Leon *et al.*, 1995). Following the work of Vernooij *et al.* (1994), it is possible that systemic benzoic acid dispersal could contribute to SAR. Benzoic acid is not a substrate for *nahG* (Strange, 1994). However, reducing phenylpropanoid biosynthesis in transgenic tobacco has been shown to compromise SAR, whilst increasing phenylpropanoid biosynthesis enhances SAR (Felton *et al.*, 1999). The ability of SA homologues to compete with SA by binding or inhibiting catalase activity, correlates to their ability to induce SAR. Catalase activity reduces levels of hydrogen peroxide. Therefore, a reduction in catalase activity will result in increased hydrogen peroxide concentrations. Hydrogen peroxide induces accumulation of PR proteins associated with SAR. This model implies that hydrogen peroxide acts downstream of SA (Bi *et al.*, 1995). Hydrogen peroxide and a catalase inhibitor were found to be weak inducers of PR gene induction in tobacco and unable to induce PR-1 in plants expressing *nahG* (Chen *et al.*, 1993).

Classically, the increased resistance associated with SAR has been linked to the action of PR proteins (Brading *et al.*,2000; Felton *et al.*, 1999; Mur *et al.*, 1997). The function of some PR proteins is unknown whilst some have been found to be β-glucanases and chitinases. Thus, given that β-glucans and chitins are molecular constituents of fungal cell walls, such enzymes have an obvious anti-microbial role. It is less clear how such proteins could be effective against bacterial or viral pathogens (Mur *et al.*, 1996).

Catechol has no resistance-inducing effect and does not trigger SAR expression (Felton *et al.*, 1999; Ryals *et al.*, 1994; Sticker *et al.*, 1997). Schweizer *et al.* (1998) found that post-wounding rice plants induced an accumulation of PR proteins and subsequent enhanced resistance to the rice blast fungus, *Magnaporthe grisea*. Transcription of PR genes in rice plants unable to accumulate SA will determine whether SA acid is essential for the initiation of PR gene expression post wounding or after induction of SAR using 2,6-INA.

5.1.4 Hypersensitive response

An incompatibility interaction often results in rapid cell death in the plant, known as a hypersensitive response, limiting the spread of the infection. It is unclear whether the hypersensitive response causes restriction of the disease lesion or whether it is a downstream effect of resistance.

An initial result of the hypersensitive response appears to be the production of active oxygen species (Anderson *et al.*, 1991). These would be expected to cause lipid peroxidation resulting in membrane disfunction. A second mechanism by which membranes may be attacked is through the operation of the enzymes lipolytic acid hydrolase and lipoxygenase. Lipolytic acid releases fatty acids from membrane lipids and these are subject to oxidation by lipoxygenase. Lipoxygenase itself is also reported to generate singlet oxygen (Croft *et al.*, 1990). Ohta *et al.* (1991) showed lipoxygenase activity increased in rice following infection by the *Magnporthe grisea*. The increases were greater in plants inoculated with an incompatible race of the fungus compared to a compatible one. Recently, it has been found that strategies have been evolved by plants to counteract a variety of biotic/abiotic stresses, including induction of genes encoding PR proteins, in particular PR gene family.

The hypersensitive response is characterised by the production of reactive oxygen species (ROS). An oxidative burst characterises the generation and release of ROS and cross-linking of plant cell wall proteins. Superoxide generated by NADPH oxidase mediated reduction of Fe^{3+} to Fe^{2+}, dismutes to form H_2O_2, which subsequently reacts with Fe^{2+} via the Fenton reaction, forming $OH^{\bullet}$, or with Fe^{3+} to form $O^{\bullet}{}_2^-$. The hypersensitive response production of $O^{\bullet}{}_2^-$ has been linked to flavoproteins. Generation of H_2O_2, by pH dependent cell-wall peroxidase has been deemed responsible for some, if not all the H_2O_2, which is produced during the hypersensitive response (Bolwell and Wojtaszek, 1997). It has been demonstrated that some pathogenic fungi secrete oxalic acid as part of the infection process, this oxalic acid catalyses the conversion of oxalate to CO_2 and H_2O_2 (HOOC-COOH + O_2 $\longrightarrow$ $2CO_2 + H_2O_2$). Plant amine oxidases have been demonstrated to have peroxidase activity and generate H_2O_2 (Bolwell and Wojtaszek, 1997). Hydrogen peroxide participates in a number of responses, such as phytoalexin biosynthesis, lignification, cross-

linking of cell wall glycoprotein and transcription of defence proteins. After the primary burst around the innoculation site, secondary 'micro-bursts' occur that are required for systemic resistance. Reactive oxygen appears to participate in plant-pest interactions. Several genes whose expression is upregulated after nematode infection have been isolated from potatoes (Niebel *et al.*, 1995). Catalase is a substrate for hydrogen peroxide. One gene isolated from potato encoded a catalase isoform *Cat2St,* whose mRNA levels increased throughout the infected root. Soybeans attacked by the insect, *Helicoverpa zea,* demonstrated a shift in their oxidative status, resulting in increases in lipid peroxidation and hydroxyl radical formation (Bi and Fenton, 1995). The activity of several enzymes, including lipoxygenase, peroxidases, ascorbate oxidase and NADH oxidase has been shown to increase following insect attack (Hildegrand *et al.,* 1986). In some cases, the pathogen elicits a systemic response in the plant and the resistance of distant tissues to subsequent infection by a yet unknown mobile signal.

5.1.5 Wounding

Responses activated by wounding lead to healing of damaged tissue and the activation of defence mechanisms to prevent further damage. From a few minutes to several hours after wounding a process occurs of perception, generation, release and transduction of specific signals for subsequent activation of wound-related defence genes. These genes encode proteins which repair damaged plant tissue and produce substances inhibiting growth of predator insects by lowering digestibility of plant tissue or toxin production. The proteins also activate and participate in wound signalling pathways and adjust plant metabolism to imposed nutritional demands. Damaged tissues undergo severe disordering of cell structures associated with de-compartmentalisation and release of stored materials, together with a drastic loss of water.

5.1.6 Initial wounding response

Earliest events detected upon wounding in leaves include ion fluxes across plasma membranes, followed by changes in cytoplasmic calcium concentration and changes in protein phosphorylation. Several minutes after wounding there is transient production of ROS including the super oxide anion in damaged tissues. Hydrogen peroxide is produced

both locally and systemically. Hydrogen peroxide may be generated in systemin related responses. For example, non-wounded plants treated with systemin-generated hydrogen peroxide both locally and systemically (Orozco-Cardenas and Ryan, 1999). Structurally diverse molecules play a regulatory role in wound signalling. These molecules include oligopeptides such as systemin (Pearck *et al.*, 1991), oligosaccharides produced from damaged cell walls (Bishop *et al.*, 1981) and signal molecules such as jasmonates (Farmer and Ryan, 1990) and ethylene (O'Donnell *et al.*, 1996). Frequently, the induction of a wound response requires simultaneous action of different signals and regulators. Often the quantitative and quantitative participation of any putative signal in the activation of a wound response depends also on the plant species. Wounding of leaves by chewing insects induces the synthesis of defence proteins in both wounded leaves and distal unwounded leaves. Several chemical signals regulate this response, including oligosaccharides derived from polygalacturonic acid, abscisic acid, auxins and products of the octadecanoid pathway including linolenic acid and jasmonic acid (JA). Acetyl SA and SA are signals for initiation of SAR towards pathogens. These signals inhibit the wound induced, JA induced, systemin induced and oligosaccharide elicited accumulation of protease inhibitors by blocking the synthesis of JA. The induction of low molecular weight cysteine-rich basic proteins by pathogens appears to occur via a SA independent pathway, requiring functional components of the JA and ethylene response pathways. Wounding drastically alters a plant's pattern of gene expression, both locally and systemically. Physical wounding results in loss of compartmentation, exposing the host cell wall to endogenous polygalacturonases. Polysaccharides may be released by invading pathogens or in the saliva of some insects, such as aphids. The oligosaccharides liberated can initiate membrane depolarization, affecting ion flux, in particular calcium ions (Bowles, 1992) and are endogenous elicitors of gene expression. However, their mobility is restricted due to their size. Evidence supports the implication of calcium and calcium binding proteins in the regulation of wound responses. It is well known that wound signal molecules promote rapid membrane depolarisation with concomitant proton influx (Thain *et al.*, 1995; Moyen and Johannes, 1996). In tomatoes, elevation of intracellular levels of calcium (Moyen *et al.*, 1998) and changes in pattern of protein phosphorylation are part of the response to wounding (Schaller and Oaking, 1999). Membrane depolarization is accompanied by potassium efflux

and a decrease in cytoplasmic pH (Thain *et al.*, 1990). Systemin is an 18 amino acid peptide generated from a larger protein precursor Prosystemin (McCurl and Ryan, 1992). There is considerable evidence that it is a primary long distance transmittable signal in plants (Pearce *et al.*, 1991, Ryan, 2000) The function of systemin or a related polypeptide has been demonstrated in solanaceous species (Constabel *et al.*, 1998). Activation of wound responses led to transcription activation of two families of proteinase inhibitor genes (*pin*) in transgenic tomato plants over-expressing the prosystemin gene (McCurl *et al.*, 1994), indicated an essential role for systemin in local and systemic wound activated responses. Wound induced *pin* expression was suppressed by antisense prosystemin. A putative systemin receptor has been identified in tomato (Scheer and Ryan 1999). Jasmonic acid (JA) receptors have not been identified even though JA is known to be one of the major inducers of gene activation in response to wounding. For full activation of the jasmonate-dependent wound signalling pathway in tobacco a rapidly wound-induced protein kinase (WIPK), a mitogen activated protein (MAP) kinase homologue has to be active (Seo *et al.*, 1995, 1999). Reversible protein phosphorylation regulates both JA dependent and independent wound signalling in *Arabidopsis*, protein phosphatase also regulates wound induced *pin* activation in potato and tomato. Protein kinase inhibitors block WIP PIN-kinase, whereas protein phosphatase inhibitors activate WIP PIN-kinase in tomato (Dammann *et al.*, 1997, Rojo *et al.*,1998).

5.1.7 Secondary signals post-wounding

The production, perception of primary signal activation of ion channels and reversible protein phosphorylation occur within a few minutes of wounding. These events lead to the generation of a second wave of wound-regulated signals to propagate defence response and to activate defence function. These secondary substances are primarily oxylipins, widespread key regulators of wound-activated gene expression. JA is derived from α-linolenic acid, the most abundant fatty acid in membrane lipids in leaves. In the first hours post-wounding, unsterified fatty acids are released from lipids by the action of wound-inducible phospholipases activated by calcium and modulated by reversible phosphorylation (Conconi *et al.*, 1996; Lee *et al.*, 1997). It is assumed that phospholipase activity releases linolenic acid from complex lipids to provide the unsterified fatty acid

substrate for lipoxygenases, which introduce molecular oxygen initiating the octodecanoid pathway (Vick, 1993). Wound responses mediated through the action of JA require the production of unsterified fatty acids as substrates for the octadecanoid pathway. As the enzymes involved in the JA pathway may combine to catalyse reactions with linolenic and hexadecatrienic acids, they are likely to yield a family of related products, the jasmonates (Grundlack and Zenk, 1998). The availability of JA insensitive mutants of *Arabidopsis* (Feys *et al.*, 1994; Berger *et al.*, 1996; Titarenko *et al.*,1997) enabled elucidation of the role of JA in wound activated responses. Cloning of the *COI1* gene that confers JA insensitivity and inactivation of JA-mediated wound signalling has revealed the protein to be an F-box protein (Xie *et al.*, 1998). This may function by recruiting regulators of wound defence for modification and subsequent degradation. However, it is still necessary to determine how this kind of modification regulates subsequent JA dependent gene expression, which could be used to limit JA production with signal transduction pathways that control JA-mediated plant development and defence responses to environmental stresses.

5.1.8 Cross talk between SAR and wound signalling pathways

Pathogen infection is greatly facilitated in damaged leaves. It is likely, therefore, that wounding triggers defence responses, which activate specific developmental programmes, in order to prepare the plant to resist further pathogen attack. Transgenic tobacco plants over-expressing a MAP-kinase WIPK gene accumulated SA in response to wounding, a phenomenon that led to enhanced pathogen resistance (Sano *et al.*, 1994; Seo *et al.*, 1995). Interactions between pathogen defence and wounding are generally negative. There appears to be an inverse relationship between pathogen resistance and insect herbivory (Felton *et al.*, 1999). Inhibition of wound activated JA-mediated responses by SA or pathogens has been reported extensively (Pena-Cortes *et al.*, 1993; Doares *et al.*, 1995; Preston *et al.*, 1999). However, activation of JA mediated wound responses by the products of cell wall-degrading enzymes of *Erwinia carotovora* has been reported (Norman *et al.*,1999). Simultaneous treatment of tobacco with SA and JA or JA and ethylene led to over-induction of PR1 (pathogenesis related protein). Compared to PR1 produced in seedlings when treated with one compound only (Xu *et al.*, 1994), *Arabidopsis* mutants impaired in JA sensitivity are less resistant to pathogens. Therefore It has been concluded

that JA perception may be necessary for activating defences against certain pathogens. It has been suggested that integration of wound and pathogen activated responses at the level of MAP-kinase catalysed phosphorylation may be widely functional in plants (Zhang and Klessig, 1998). These kinases are closely related to the previous MAP-kinases induced by wounding (WIPK) and SA (SIPK) in tobacco (Seo *et al.*, 1995; Zhang and Klessig, 1997, 1998). It has been proposed that SA has a dual function constraining constitutive expression of disease and insect resistance mechanisms and reciprocally switching selective action from one to the other. Salicylic acid has been shown to inhibit synthesis of proteinase inhibitors induced by systemin and jasmonic acid in tomato leaves (Doares *et al.*, 1995).

5.1.9 Aims and objectives

These experiment aimed to demonstrate whether transgenic plants with reduced ability to accumulate salicylic acid were compromised in their ability to initiate systemic acquired resistance.

Systemic acquired resistance was measured by transcription of pathogenesis related proteins. In order to demonstrate wound-induced PR gene transcription in transgenic rice plants unable to accumulate SA following wounding and activation of SAR, PR genes were chosen that had been documented in rice after both wounding and following treatment with 2,6-INA (Schweizer *et al.*, 1998).

pr1 has been described as a antifungal protein of 14-17 kD, *pr5* has been described as an antifungal thaumatin like protein similar to alpha amylase/trypsin inhibitors and pbz has been described as a peroxidase like protein with homology to a probenazole inducible protein (http://abstracts.aspb.org/pb2003/public/P60/0479.html).

5.2 MATERIAL AND METHODS

5.2.1 Preparation of *Oryza sativa* cv.Taipei 309 plants

Seeds of Japonica rice cv. Taipei 309 were cultured to produce scutellum-derived calli. The latter were subjected to microprojectile bombardment at 1350 psi (Section 2.2.1 and Section 2.2.2)

5.2.2 Treatment of plants with 2,6-dichloroisonocotinic acid (2,6-INA)

Eight weeks after transfer to the growth room, rice plants (both wild-type and transgenic) were watered with 2,6-INA formulated as a 25% wettable powder applied as a soil drench. Plant pots were watered with 100ml per plant of suspension containing 2,6-INA at concentrations of 10 and 100 ppm (0.1 - 1mgl^{-1}). Samples of leaf tissue (1g ± 0.1g) were removed at 0, 6, 12, 24, 48 and 60 h after 2,6-INA treatment from both treated and untreated plants.

5.2.3 Stress treatment of plants

Leaves were wounded by squeezing with blunt-ended ribbed forceps every 3 to 5mm. Samples of leaf tissue (1g ± 0.1g) were removed at 0, 6, 12, 24, 48 and 60 h after stress treatment from both the wounded leaf and the leaf adjacent to the wounded leaf. Initial experiments on DNA isolated from leaves of control and transgenic rice plants using the method of Dellaporta *et al.* (1983) followed by PCR confirmed the presence of pathogenesis-related genes *pir1*, a homologue of the pathogen induced wheat gene (*wir1*) (Bull *et al.*,1992) *pr5* (Rimmann and Dudler, 1993), *pr9* (Reimmann *et al.*, 1993) and *pr10* (Midoh and Iwata, 1996). Reaction parameters consisted of 35 cycles (94^{0}C 1 min 60^{0}C for 30 sec 72^{0}C 1 min) and one section of (72^{0}C for 3 min followed by 4^{0}C for 5 min). PCR was carried out in a Techne Genius instrument.

5.2.4 RNA Isolation

Total RNA was isolated from samples (100mg) of leaf tissue using an RNA mini kit (Promega) and following instructions in the RNeasy mini handbook 06/2001 available on-

line at http://www.promega.com. In order to eliminate the possibility of DNA present following elution of the RNA, RNase–Free DNase was used (Promega). To each microfuge tube containing 50µl of RNA solution 6 µl of 10X buffer was added and 2 µl of RNase-Free DNase. The mixture was incubated at 37^{0}C for 1 h. One µl of DNase stop solution was added to the mixture, which was then incubated at 65^{0}C for 10 min to inactivate the DNase. This mixture was used for RT-PCR.

5.2.5 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out using the ABgene ReverseIT 1st strand synthesis kit using anchored oligonucleotides dTs for the synthesis of the first strand and MSB2, the positive control supplied with the kit, to confirm that the reaction was successful. Ten µl of the RNA mixture was used in each reaction to which was added 2 µl of anchored oligo dTs. This was heated at 70^{0}C for 5 min and then cooled to 5^{0}C. The first strand was synthesised by the addition of first strand synthesis buffer, 5mM dNTP mix reverse transcriptase (25 units per µl) and RNase inhibitor in a 1 : 2 : 1 : 1 (v/v) ratio. This mixture was incubated at 42^{0}C for 1 h after which the RT was inactivated by incubation at 75^{0}C for 10 min. PCR was then immediately carried out using 5 µl of this reaction mixture as the DNA template and reducing the amount of sterile water correspondingly. The primers used were derived from genes *pir1* (Bull *et al.*, 1992), *pr5* (Reimmann and Dudler, 1993) and *pbz*, a homologue of *pr9* (Midoh and Iwata, 1996), using the PRIMER 3 programme.
Thirty-five cycles of PCR confirmed the transcription of *pir1, pr5* and *pbz*.

Primers from *pir1* embl/M94959/TAW1R1PR Wheat WIR1

Forward primer: acgtgcacaactagctttcg

Reverse primer: gacgttagggtcgagagcac

Primers from *pr5* embl/X68197/OSTHLP/O.sativa for thaumatin like protein

Forward primer: gcagccaggacttctacgac

Reverse primer: tgatgcattatgggcagaag

Primers from *pbz1* embl/D38170/OSP1PPBZ1

Forward primer: ccggagaaggagaaggacat

Reverse primer: gactcaaacgccacgagaat

All product sizes were 200bp.

5.2.6 Semi-Quantitative RT-PCR

All PCR products were 200 bp long. Once the background transcription of RNA was determined, semi-quantitative RT-PCR was carried out to establish differences in expression. In order to determine the background transcription of the defence genes used, samples were removed from the PCR thermocycler after 0, 5, 7, 10, 14, 16, 18, 21, 23, 25, 28, 30 and 35 cycles of PCR repeated 3 times to confirm accuracy of the result. Semi-quatitative RT-PCR was based on the premise that enhanced transcription would result in the product being visible after fewer cycles of PCR than background expression. Semi-quantitative RT-PCR of RNA extracted from distal and proximal tissues collected at timed intervals after treatment of high expressers, low expressers and wild-type plants. Plants treated with 2,6-INA, wounding, or wounding followed by treatment with 2,6-INA, were used to established whether pathogenesis related (PR) genes were induced in these plants.

5.3 RESULTS

5.3.1 Enzyme and substrate levels

Following somatic embryogenesis and regeneration, plants producing mRNA for the transgene, as confirmed using RT-PCR, were assayed for enhanced salicylate hydroxylase activity and reduced SA concentration (Figures 5.1, 5.2). In all Figures, error bars equal the standard error of the mean. Plants were also assayed for enhanced methyl salicylate and veratrole production using gas chromatography and mass spectrometry (Chapter 6). Plants were classified on the basis of being high expressors of the transgene depending on the salicylate activity measured (Figure 5.1). Plants SLJ7307 7, 11 and 13 were designated as high, whilst SLJ7307 2, 14 and 15 were designated as low.

Figure 5.1
Mean salicylate hydroxylase activity for tissues from transgenic and wild-type plants

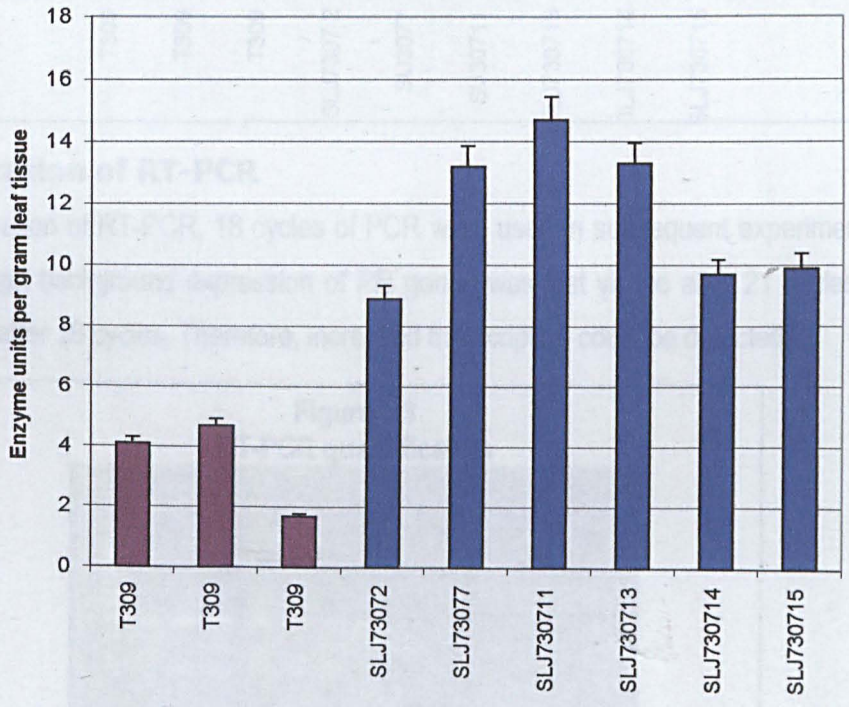
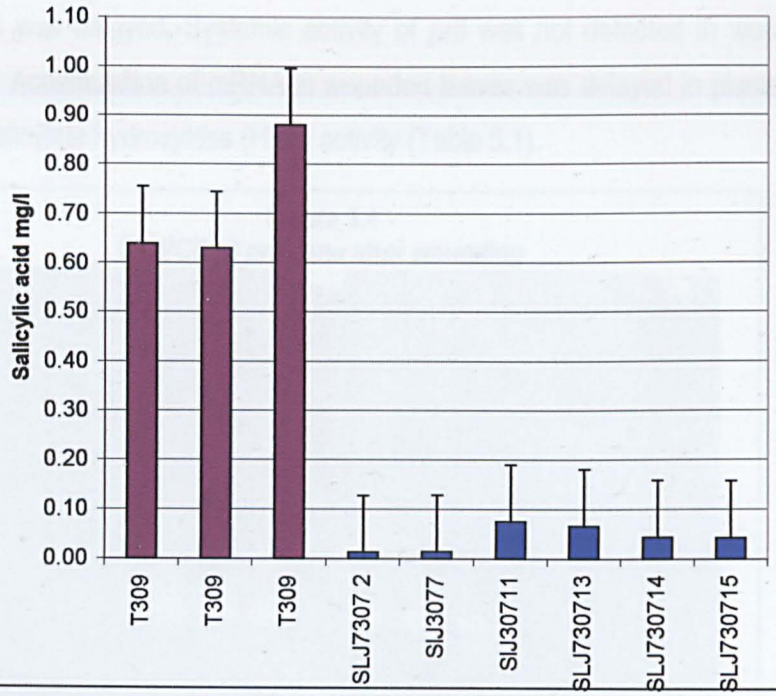


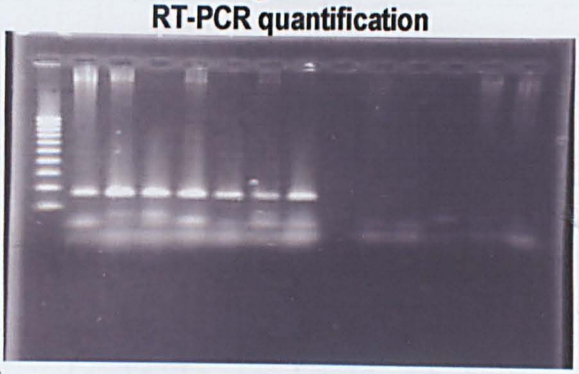
Figure 5.2
Concentration of salicylic acid in 1g samples of leaf tissues from transgenic and wild-type plants



5.3.2 Calibration of RT-PCR

Following calibration of RT-PCR, 18 cycles of PCR were used in subsequent experiments because although background expression of PR genes was just visible after 21 cycles it was not visible after 18 cycles. Therefore, increased transcription could be detected.

Figure 5.3
RT-PCR quantification

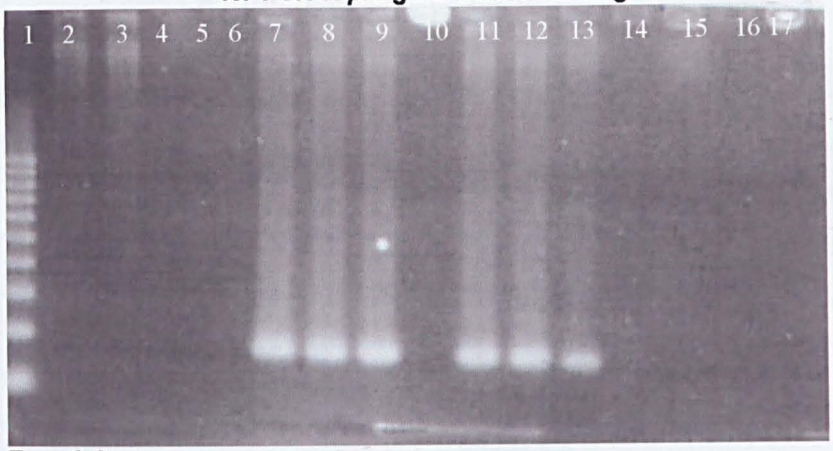


From left:
lane 1 = 100 bp ladder
lane 2 = 25 cycles PCR
lane 3 =28 cycles PCR
lane 4 = 30 cycles PCR
lane 5 = 35 cycles PCR
lane 6 = 23 cycles PCR
lane 7 = 21 cycles PCR
lane 8 = 23 cycles PCR (in error)
lanes 9-15 = 0, 5, 7, 10, 14, 16, 18 cycles PCR

5.3.3 Accumulation of pathogenesis-related genes after wounding

Wounding of leaves led to accumulation of mRNA for PR genes (*pr1, pr5, pbz)* in all plants (Figure 5.4). However, in tissues distant to the site of wounding, accumulation of mRNA for these genes was delayed. Systemic activity of *pr5* was not detected in wounded plants (Table 5.1). Accumulation of mRNA in wounded leaves was delayed in plants expressing enhanced salicylate hydroxylase (High) activity (Table 5.1).

Figure 5.4
RT-PCR of *pr5* gene after wounding



From left
Lane 1 = 100bp ladder
Lanes 2, 3& 4 = *pr5* 12 hours after wounding in high low and control plants
Lanes 5, 6 & 7 = *pr5* 24 hours after wounding in high low and control plants
Lanes 8, 9 & 10 = *pr5* in control low and high plants 48h after wounding
Lanes 11, 12 & 13 = *pr5* in control low and high plants 60 h after wounding
Lanes 14, 15 & 16 = *pr5* in control low and high plants in leaves adjacent to wounded leaf
Lane 17 = No reverse transcriptase

Table 5.1
PR gene transcription in leaves of wounded rice plants

Time after wounding (h)	6	6	6	12	12	12	24	24	24	48	48	48	60	60	60
Control (C) Low (L) High (H) expressor	C	L	H	C	L	H	C	L	H	C	L	H	C	L	H
pr1 local	+	-	-	-	-	-	+	-	-	+	+	-	+	+	+
pr1 systemic	-	-	-	-	-	-	-	-	-		-	-	+	-	-
pr5 local	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+
pr5 systemic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pbz local	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
pbz systemic	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+

5.3.4 Accumulation of PR gene transcription following induction of SAR

Treatment of plants with 2,6-INA (10mgl^{-1} and 100mgl^{-1}) led to accumulation of mRNA for all PR genes 24 h after treatment in all plants (Figure 5.5) at both concentrations of 2,6-INA (Table 5.2). There were no differences observed between low and high expressing plants

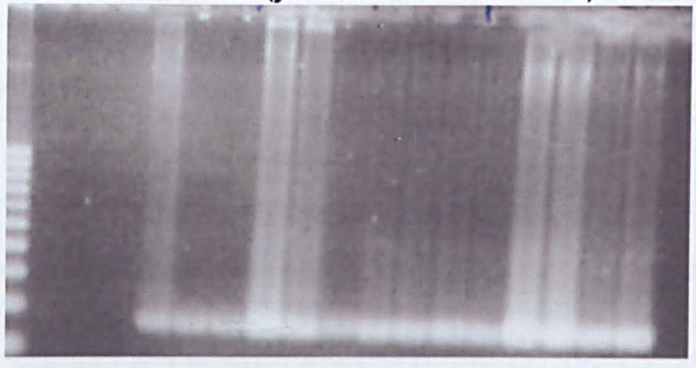
Table 5.2

PR gene transcription following treatment with 2,6-Dichloroisonicotinic acid

Time after wounding (h)	6	6	6	12	12	12	24	24	24	48	48	48	60	60	60
Control (C), Low (L), High (H)	C	L	H	C	L	H	C	L	H	C	L	H	C	L	H
pr1	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+
pr5	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+
pbz	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+

Figure 5.5

RT-PCR of defence genes after treatment with 2,6-INA

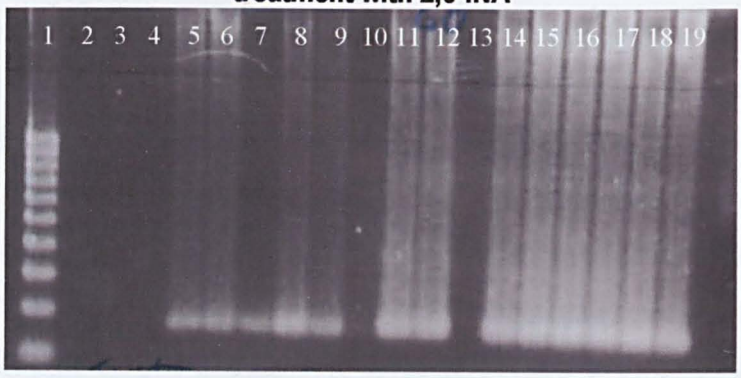


From left
Lane 1 = 100bp ladder
Lanes 2-4 = control low and high plants 12 h after treatment with 2,6-INA *pr1*
Lanes 5-7 = control low and high plants 24 h after treatment with 2,6-INA *pr1*
Lanes 8-10 = control low and high plants 48 h after treatment with 2,6-INA *pr1*
Lanes 11-13 = control low and high plants 60 h after treatment with 2,6-INA *pr1*
Lanes 14-18 = control high and low plants 24 and 48 h after treatment with 2,6-INA *pr5*

5.3.5 Transcription of PR genes after wounding and subsequent induction of SAR

Treatment of plants with 2,6-INA following wounding led to accumulation of *pr1* and *pbz* mRNA 12 h after treatment in both systemic and local tissues of control plants and accumulation of *pr5* locally and systemically 24 h after treatment in T309 control plants. Expression of PR genes was delayed in tissues of plants over-expressing SAH, although a faint band was visible 12 h after wounding (Figure 5.6, lane 7). Local and systemic expression of all three genes continued until 60 h after treatment with 2,6-INA.

Figure 5.6
RT-PCR of plant defence genes after wounding of leaves and subsequent treatment with 2,6-INA



From left
Lane 1 = 100bp ladder, lanes 2-4 *pr1* 6 h after treatment with 2,6-INA, lanes 5-7 *pr1* 12 h after treatment with 2,6-INA, lanes 8-10 *pr1* 24h after treatment with 2,6-INA, lanes 11-13 *pr1* 48 h after treatment with 2,6-INA, lanes 14-16 *pr1* 60h after treatment with 2,6-INA

Table 5.3
PR gene transcription in leaves of rice plants wounded and subsequently treated with 2,6-INA

Time after wounding (h)	6	6	6	12	12	12	24	24	24	48	48	48	60	60	60
Control (C) Low (L) High (H) expressor	C	L	H	C	L	H	C	L	H	C	L	H	C	L	H
PR1 local	-	-	-	+	+	+	+	+	-	+	+	-	+	+	+
PR1 systemic	-	-	-	-	-	-	+	+	-	+	+	-	+	+	+
PR5 local	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
PR5 systemic	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+
PBZ local	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
PBZ systemic	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+

Rice plants transformed with the *nahG* gene over-expressed SAH compared to wild-type plants and accumulated less SA than wild-type plants. Transcription of PR genes post-wounding was delayed compared to wild-type plants and this delay correlated with over-expression of SAH and concomitant decrease in SA concentrations. Local transcription of genes was found to be optimal 48 h after wounding whilst systemic gene transcription only occurred in leaf tissues of plant lines highly over-expressing SAH 60 h after wounding. Gene transcription was visible 24 h after wounding for genes *pr1*, *pbz* and for *pr5*. There was no delay detected in *pbz* gene transcription after wounding and therefore control, low and high expressing plants produced an identical local response after wounding. Systemic expression of *pr5* was not detected in control, low or high expressing plants. Transcription of PR genes was induced following treatment with 2,6-dichloroisonicotinic acid (2,6-INA) a synthetic inducer of systemic acquired resistance (SAR). Pathogenesis related gene transcription in control T309 plants developed 12 h after application of 2,6-INA compared with 24 h post-wounding. All plant lines demonstrated gene transcription 24 h after treatment with 2,6-INA even high expressing lines. Pathogenesis related proteins were not detected on SDS-PAGE gels and therefore it was not possible to demonstrate that salicylic acid is necessary for the induction of systemic acquired resistance.

5.4 DISCUSSION

5.4.1 Systemic acquired resistance

Much of the literature asserts that an inability to accumulate SA results in inhibition of SAR (Ryals *et al.*, 1996; Mur *et al.*, 1997; Felton *et al.*, 1999), which is evidenced by production of PR proteins. However, some recent literature has asserted that some defence pathways are SA independent and, therefore, SAR can be initiated without SA (Brading *et al.*, 2000). In these experiments, although transgenic plants were demonstrated to possess low concentrations of SA, the transcription of defence genes *pr1*, *pr5* and *pbz* was demonstrated following wounding and treatment with 2,6-INA. There are several explanations for this. It could be that these genes only require a small amount of SA to initiate transcription. An alternative explanation is that these genes are initiated independently of SA. A further possible explanation is that as gene transcription was delayed in plants over-expressing SAH, the low concentration of SA may have delayed induction of transcription of *pr1*, *pr5* and *pbz*. There were differences in the delay in transcription between the 3 genes following wounding, but not following treatment with 2,6-INA, suggesting that the low concentration of SA affected transcription of the 3 genes differently and possibly the genes had different sensitivities to the concentration of SA. There was no difference in gene transcription following treatment with 10ppm 2,6-INA or 100 ppm, so it is possible that rice is very sensitive to 2,6-INA and a lower concentration would need to be used to illustrate any differences in gene transcription. Accumulation of mRNA for pathogenesis related genes *pr5* and *pbz* was detected 12 h after treatment with 2,6-INA by Schweizer *et al.* (1997), but in the present experiments it was detected after 24 h for *pr5* and *pbz* and 12 h for *pr1*. This could indicate that over-expression of SAH could lead to delay in transcription of *pr5* and *pbz*.. Pathogenesis-related proteins activated as a result of plant infection do not always coincide with those activated by application 2,6-dichloroisonicotinic acid, nor does PR gene activation coincide with levels of SA in the plants (Corné and Pieterse, 1999). It would be interesting to evaluate the induction of SAR with a rice pathogen, but facilities were not available to test this option. SAR is a complex phenomenon and although there are indications that these *nahG* expressing plants had altered expression of SAR, a small experiment can only produce indications. A more

accurate picture of enhanced PR gene expression could be obtained with the use of a quantitative PCR machine.

5.4.2 Pathogenesis related gene transcription post-wounding

Salicylic acid has been shown to inhibit wound-activated jasmonic acid mediated responses (Peña-Cortez *et al.*, 1995; Doares *et al.*, 1995). Activation of jasmonic acid-mediated wound response is initiated from by-products of cell wall degradation and enzymes produced by plant pathogens (Héruart *et al.*, 1993). In plants unable to accumulate SA enhanced wound response would be expected. In these experiments PR gene transcription was delayed following wounding in transgenic plants. Therefore, this indicates that PR genes used in these experiments appear to be more responsive to SA concentration than to plant wounding. Transcription of PR genes *pr1, pr2, pr3* and *pbz* was detected in rice leaves 12 hours after wounding by Schweizer *et* al. (1998). However, only *pr1* mRNA exhibited this pattern in the experiments reported here, PR gene transcription was delayed in plants with enhanced SAH expression.

Salicylic acid is toxic in concentrations higher than 1mM. Therefore, excess salicylic acid in intact cells is conjugated to a glucose molecule and stored as *β-0-D*-glucosyl salicylic acid (Chen *et al.*, 1993). Disruption of the cell wall during wounding therefore releases SA previously stored in an inactive form leading to a burst of SA and concomitant PR gene transcription. This could explain the delay in PR gene transcription in transgenic plants following wounding. Not only does SA bind to a receptor and lead to the initiation of SAR, but infected leaves produce increased amounts of SA. SA inhibits the activity of catalase and ascorbate peroxidase leading to an increase in the concentration of hydrogen peroxide. This prolongs the oxidative burst initiating signal cascades for plant wound response and local defence response. Hydrogen peroxide has been shown to have a direct action against pathogens and act as an intermediary in wound signal cascades (Mauch-Mani and Metréaux 1998). Increased hydrogen peroxide stimulates the production of SA (Mauch-Mari and Slusarenko, 1996).

5.4.3 Gene transcription following wounding then application of 2, 6-INA

An inverse relationship exists between resistance to pathogen attack as demonstrated by the onset of SAR mediated by SA and the initiation of wound response, mediated by hydrogen peroxide and catalase (Felton *et al.*, 1999). It has been widely reported that the SAR pathway and the octadecanoid pathway leading to the production of JA are antagonistic (Mur *et al.*, 1997; Felton *et al.*, 1999). However, at the level of SA there appears to be some interaction. Wounding releases ROS, which include hydrogen peroxide and this has been shown to induce the JA pathway and in excess to lead to the production of SA (Leon 1995) as well as inducing transcription of PR genes (Brading *et al.*, 2000). Schweizer *et al.* (1998) demonstrated that post-wounding, rice plants accumulated PR proteins, which resulted in enhanced resistance of rice to the rice blast fungus. In the present experiments there were indications of cross talk between gene induction post-wounding and post-induction of SAR. PR gene transcription was detected 24h after treatment with 2,6-INA in transgenic plants, whereas in plants treated with 26-INA after wounding they were detected 12 h post-treatment. There appears to be cross talk between the signals for plant defence and those for wound initiation. Concentrations of SA have been reported as being particularly high in rice plants (Silverman *et al.*, 1995), in addition SA concentrations of 50-200μM have been shown to be toxic, enhancing initial oxidative burst and cell death (Shirasu *et al.*, 1997).

Although PR gene transcription was delayed in plants expressing enhanced salicylate hydroxylase activity, it was detected both locally and systemically in these plants following wounding and treatment with 2,6-INA. It is therefore probable that SA is unlikely to be a signal for systemic acquired resistance in rice or that SAR in rice plants operates independently of SA. Finally, it is important to note that these experiments are only an indication of relative gene transcription and empirical measurements of transcription are only possible using a quantitative PCR machine.

CHAPTER 6 PLANT VOLATILE ANALYSIS

6.1 INTRODUCTION

6.1.1 Volatiles released from plants

Plants and animals naturally produce behaviour volatile semiochemicals. Such chemicals are known to play a part in host plant location for food by adults and larvae of many insects. Examples include isothiocyanate volatiles released from cruciferous species that attract specialist insects, such as *Delia brassica* (Free and William, 1978) and volatiles of oilseed rape volatiles that attract cabbage seed weevil (*Ceutorhynchus assimilis*) (Evans and Williams, 1992). Plant odours are a complex mixture of compounds, only a proportion of which are typically used by an insect to locate its host (Visser, 1986). Many plants are only suitable for insects at a particular stage of their development. Therefore, volatile cues from plants may involve the production of different volatiles compounds or different concentrations of compounds (Masri, 1995). Some plant volatiles are specific, whilst others are common, to many green leaf plants. Green leaf volatiles, formed by oxidative degradation of leaf lipids, are continuously released into the air due to plant ageing and injury (Visser, 1979). Green 'odour' is derived from 8 volatile compounds (Hartanaka, 1993) made of 6-carbon aldehydes and alcohols, including the aldehyde *E*-2-hexanal and the alcohol *Z*-3-hexanol. These compounds are synthesized in green leaves from α-linolenic acid and linoleic acids via their respective hydroperoxides. Individual green leaf odour compounds may have a specific function in attracting specialist insects. For example, hexanal has been shown to attract *Drosophila melanogaster* (McAlpine *et al.*, 1984). Other plant volatiles have synergistic effects where components of a plant odour blend. Hexanal released with another plant volatile, *(E)*- asarone, demonstrated increased attraction for the carrot fly (*Psila rosea*) (Guerin *et al.*, 1983).

6.1.2 Insects' response to plant volatiles

There has been little commercial use of volatile plant-produced semiochemicals to control insects. However, volatile plant attractants when incorporated into *Cucurbita sp.* stimulated feeding of the insect pest, *Diabrotica sp.* (Metcalf and Metcalf, 1992). Terpenoid compounds released in higher concentrations from young apples were more attractive to codling moth (*Cydia pomonella*) females, than volatiles produced from more mature fruit with lower concentrations of terpenoids (Bengtsson *et al.*, 2001).

Some insect species can discriminate between the different mixture of volatiles produced by plants subject to larval feeding compared to undamaged plants during oviposition (Jonsson and Anderson, 1999).

6.1.3 Volatiles produced by rice plants

Obata *et al.* (1983) used steam distillation to collect volatile chemicals released from rice plants of the cv. Nipponbare. These chemicals included 27 volatile substances, 14 esters, 7 carbonyl compounds, 5 alcohols and triallyl isothiocyanate. Methyl and ethyl esters of palmitate, oleate, linoleate and linolenate represented major constituents. Hernendez *et al.* (1989) used air entrainment and thermal desorption to identify 28 volatiles from the rice cvs. Mars and PI 346883. Hexanal, *(E)*-2-hexanal, *(Z),(Z)*-heptadienal and *(E), (E)* 2,4-heptadienal comprised over 50% of the total volatiles produced. Ramachandran *et al.* (1990) listed 1-heptanol, *(Z)*-2-hepten-1-ol, 2-heptanane and linalool, together with a number of hydrocarbons, monoterpenes, sesquiterpenes and aromatic compounds including methyl salicylate and methyl benzoate in the headspace collected from rice plants. Masri (1995) identified β-ionone, 2-nonanone and 4-oxoisophorone, as well as methyl benzoate, methyl salicylate and veratrole, as volatile compounds released from rice leaves of the cv. Balilla. Linalool, 2, nonanone, methyl benzoate, methyl salicylate and veratrole were found to be present in volatile mixtures collected from intact rice plants, mechanically damaged leaves and planthopper infested rice leaves. The concentration of volatiles was greatest from planthopper infested rice leaves, intact rice plants produced the lowest concentration of volatiles (Masri, 1995).

6.1.4 Response of *Nilaparvata lugens* to rice volatiles

Obata *et al.* (1986) found that the volatiles released from rice plants played a significant part in attracting 3 species of planthopper (*Nilaparvata lugens, Sogatella furchifera, Laodelphax striatellus*) to rice plants. Annectomised planthoppers were not attracted, suggesting receptors in antennae responded to plant volatiles. This was confirmed by Masri (1995). Volatiles from rice extracted as steam distillates from plants, susceptible or resistant to *N. lugens* were used to examine the attraction, settling and feeding behaviour of *N. lugens* (Saxena and Okech, 1985). Gas chromatography (GC) revealed more than 30 peaks within the different rice cvs. Taichung Native 1 (TN1) and the resistant cvs. PTB 33, ASD7, ARC 6650, Rathu, Heenati and Mungo. Most peaks were common to all cvs. However, unique peaks were present in some cvs.. More female

planthoppers settled and fed on tillers of the susceptible rice cv. TN1 sprayed with its own extract than TN1 sprayed with the extract of either cvs. ARC6650 or PTB 33. Sixteen compounds of headspace volatiles from rice seedlings were collected using Tenax (Omega Specialist instruments Madison USA.) and analysed with GC and GC with mass spectrometry (GC-MS). Undamaged plants emitted lower concentrations of volatiles than mechanically damaged plants and those infested by *N. lugens* for 1-2 days. Plants infested by *N. lugens* for 4-5 days emitted much higher concentrations of volatiles and these infested plants were highly attractive to *N. lugens* females. Plants infested with *Spodoptera litura* released higher levels of volatiles than all other treatments. However, rice plants damaged by *S. litura* had a clearly repellant effect on *N. lugens* females (Xu *et al.*, 2002). Both qualitative and quantitative differences in volatiles have been demonstrated between rice plants susceptible and resistant to the white-backed planthopper *Sogatella furchifera* (Khan and Saxena, 1988; Liu *et al.*, 1989). This suggests that attractants and repellents in susceptible or resistant rice varieties may have a role to play in host plant selection. Gas chromatography, coupled with insect antennograms identified veratrole as the volatile producing the greatest insect response; methyl benzoate, 4, oxoisophorone and methyl salicylate also gave appreciable results (Masri, 1995).

6.1.5 Aims and objectives of the work presented in this chapter

Masri (1995) demonstrated that veratrole and methyl salicylate were highly attractive rice leaf volatiles for *N. lugens.* Plants were transformed with pSLJ7307 or with pSLJ7321::ROB5, containing *nahG* that encodes salicylate hydroxylase. This enzyme catalyses the breakdown of salicylic acid and leads eventually to the production of veratrole and possibly methyl salicylate. Therefore, in high expressing transgenic plants, it would be expected that the concentrations of the volatiles veratrole and/or methyl salicylate would be enhanced. These experiments aimed to test that hypothesis. Mechanically damaged plants have been shown to release more volatiles than intact plants. Collection of volatiles from mechanically damaged and intact palnts and their analysis by GC and GC-MS was used to evaluate this possibility.

6.1.6 Collection and measurement of plant volatiles

Extraction, distillation and air entrainment have been used to collect and to identify volatiles released from plants. Rearrangement or decomposition of many labile

compounds often occurs when they are subjected to heat. Collection of chemicals in a natural environment is difficult as plants are sensitive to movement, such as wind and mechanical stress. Changes in the growth rate of plants and the amounts of volatiles such as ethylene, that are released, increases with increasing stress (Kimmerer and Kozlowski, 1982).

Identification of volatiles from plants involves the separation and characterisation of compounds which are frequently present only in minute amounts (Blight, 1990). Masri (1995) used dynamic headspace air entrainment techniques to collect volatiles from vessels containing plants and extracted the volatiles obtained by eluting the adsorbent (Poropak Q) with purified solvent (hexanol). This technique provided mixtures of rice volatiles in the ratios in which they were present in the air surrounding the plants. Volatiles were also collected from blank vessels, vessels containing planthopper-damaged plants and vessels containing mechanically damaged, but uninfested, plants. Extracts of volatiles collected were separated on a 50m X 0.32mm i.d. methyl silicone bonded phase fused silica capillary column (HP-1) in a Hewlett Packard 5880A gas chromatograph equipped with split/splitless injector and flame ionisation detector. The carrier gas was hydrogen. The oven temperature was maintained at 40^{0}C for 5 min then programmed to rise 5^{0}C min^{-1} to 150^{0}C increasing at 10^{0}C min^{-1} to 250^{0}C.

6.1.7 Kovat's indices

In 1958, E.S. Kovats devised a system to report retention times relative to one another. The system he developed compared a compound of interest's retention time relative to the n-alkane series. This meant that values were only dependent on the stationary phase and to a lesser extent on column temperature. Column length, diameter, inlet pressure and flow rate were not important in determining Kovat's indices. In addition, it did not matter whether a packed or capillary column was used, since when a homologous series of hydrocarbons was chromatographed, the intramolecular forces are relatively constant. This meant that the Kovat's index for a particular substance would be relatively constant whatever GC machine was used to separate the sample. This facilitated comparison of substances separated on different GC machines. The separation of n-alkanes is controlled primarily by differences in vapour pressure. A linear relationship was observed when the log of the retention time was plotted against Kovat's index; for n-alkanes 1 is equal to 100 multiplied by the number of carbon atoms they possess i.e. hexane = 600, decane = 1,000

6.2 MATERIAL AND METHODS

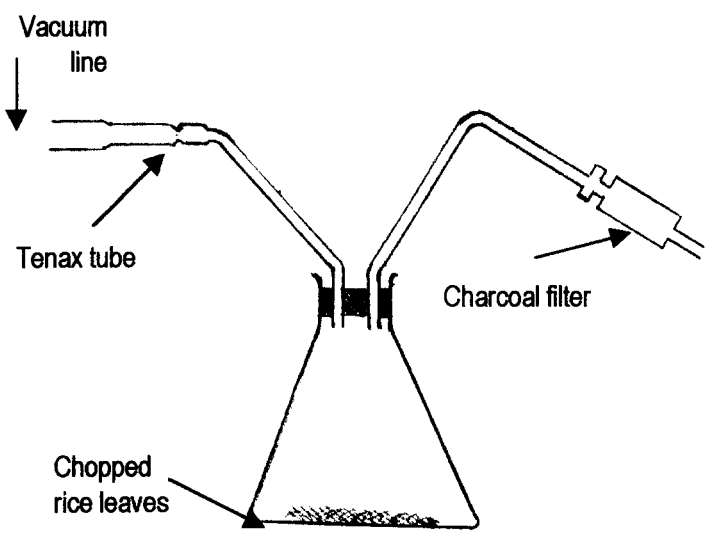
6.2.1 Preparation of Taipei 309 plants

Japonica rice seeds (*Oryza sativa* cv Taipei 309) were cultured to produce scutellum-derived calli. The latter were subjected to microprojectile bombardment at 1350 psi with pSLJ7307 containing the *nahG* gene encoding salicylate hydroxylase (Section 2.2.1 and Section 2.2.2).

6.2.2 Initial volatile collection

Plant leaf samples 1.0g± 0.1g were collected from plants 2 months after transfer from tissue culture. Leaf material was chopped finely in a liquidiser and the volatiles collected by drawing air into the liquidiser and out through a tube into which was inserted a Tenax tube containing 50mg of Tenax TA (Omega Specialist instruments Madison USA.) for 5 min. The liquidiser did not produce uniformly macerated tissue and, therefore, in subsequent experiments, plant leaf samples of 1.0g ± 0.1g were chopped into lengths of 0.25cm and immediately placed into a 100ml Erlenmeyer flask (Figure 6.1). The vacuum line was connected to a rubber tube, which fitted into the neck of the flask. A charcoal filter was connected to another tube that connected into the bung in the top of the flask. Air was drawn from the laboratory through the charcoal filter and into the flask containing the chopped rice leaf material. The vacuum line ensured that air passed over the chopped rice leaf material and through the Tenax tube for 15 min. Following collection, Tenax tubes were sealed to prevent contamination.

Figure 6.1
Volatile collection from macerated plants



Air entered the charcoal filter, passed over the rice leaves and through the Tenax tube before being drawn into the vacuum line

6.2.3 Initial measurement of volatiles

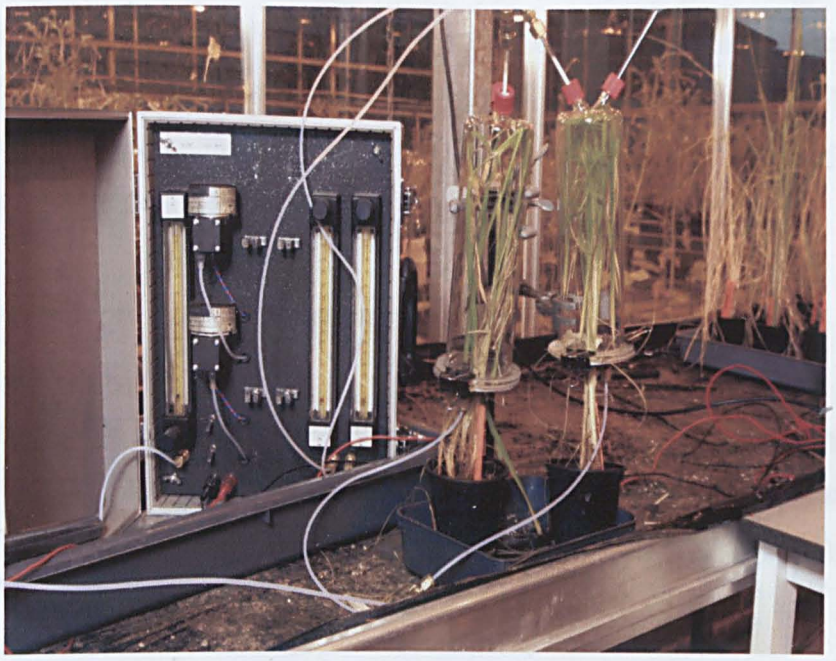
Tenax tubes were sealed and the rice plant samples discarded. Each Tenax Tube was inserted into an Agilent 6890 (model 122-5522) GC column, (Agilent Technologies Cheadle, Stockport Cheshire UK.) (DB-5ms, 0.25mm X 25m X 0.25µm). The carrier gas was helium and the instrument was programmed with the same temperature profile reported by Masri (1995), specifically 40^{0}C for 5 min rising 5^{0}C min^{-1} to 150^{0}C, followed by 10^{0}C min^{-1} to 250^{0}C. Three samples were taken for each plant analysed; 2 control plants and 8 transgenic plants were analysed.

The peak for 1,2-dimethoxybenzene (veratrole) was analysed from the MS library supplied with the GC machine and the profiles for the transgenic and control plants compared.

6.2.4 Collection of volatiles over a four day period

Volatiles were collected from intact plants by encasing the plants in a vessel with an opening for the stem (Figure 6.2). Air was pumped from the glasshouse through a charcoal filter into the base of the vessel, out of the vessel at the top through a Tenax tube and out through the pump. The apparatus was set up for 4d for each sample. Plants were each sampled twice using intact plants. Leaves were wounded by squeezing each leaf with blunt ended ribbed forceps every 3-5mm. Following wounding, volatiles were collected from plants as described above. Wounded plant lines were sampled twice.

Figure 6.2
Volatile collection from intact plants



6.2.5 Gas chromatography of volatiles collected in Tenax tubes

Samples were analysed using a 6890 Hewlet Packhard GC (GMI Line 50 Jackson Avenue, Minnesota, USA) with splitless injector on a 25m capillary column of 0.25mm diameter and a film thickness of 0.25µm with a maximum temperature of 350^{0}C. Initial flow was 0.5ml min^{-1} and average velocity was 29 cm min^{-1}. Helium was the carrier gas. The GC machine was programmed for 40^{0}C for 5 min, rising to 150^{0}C at 5^{0}C min^{-1} followed by at 10^{0}Cmin^{-1} to 250^{0}C.

6.2.6 Concentrations of volatiles

Dilutions of veratrole (100ng μl^{-1}, 50ng μl^{-1} and 10ng μl^{-1}) and methyl salicylate were made in hexane. Samples (each 1µl) were injected into a Tenax tube and run on the GC machine. This dilution series was then used to establish the relationship between peak area and concentrations of veratrole and methyl salicylate, enabling the calculation of a concentration curve. Concentrations of veratrole and methyl salicylate in leaf samples of transgenic and wild-type lines were calculated from this data.

6.2.7 Co-injection

In order to confirm that the peaks ascribed to veratrole and methyl salicylate from the GC traces of volatiles collected from plants, samples of veratrole µl of 50ngμl^{-1} and methyl salicylate 1µl of 50ngμl^{-1} were injected into a Tenax tube containing plant leaf volatiles. The increase in peak area could then be ascribed to the spiking of the Tenax tube with veratrole and methyl salicylate. Mass spectrometry was also used to identify veratrole and methyl salicylate in volatiles collected from plant leaf samples.

6.2.8 Retention times of veratrole and methyl salicylate and Kovat's indices

A solution of alkanes of 9 - 25 carbon atoms in hexane was injected 1µl of a 50ngμl^{-1} solution into a Tenax tube and run in the GC machine. The retention times were used to calculate the Kovat's Indices (KI) for the retention time of the peaks of interest using the equation of Figure 6.3.

Figure 6.3
Calculation of Kovat's index

$$1 = 100 \times A + 100 \times \left[\frac{\log_{10} RTB - \log_{10} RTC}{\log_{10} RTD - \log_{10} RTC} \right]$$

A = number of carbon atoms in alkane peak immediately before peak of interest
RTB = retention time of peak of interest
RTC = retention time of alkane immediately before peak of interest
RTD = retention time of alkane peak immediately after peak of interest

6.3 RESULTS

6.3.1 Initial results from Gas Chromatography

Transgenic plants released greater amounts of volatiles than wild-type plants (Tables 6.1, 6.2), although the amounts of volatiles varied from plant to plant (Tables 6.1, 6.2). The amount of veratrole detected was greater in most transgenic plants than in wild-type plants (Figure 6.4). In some wild-type plants, veratrole could not be detected in the volatiles collected. The peak at which veratrole was released was not always pure as a squat peak was observed over a period of time rather than a sharp peak at a particular instant (Figure 6.4). However, the same peaks were present in both the reference sample of veratrole (Figure 6.5) and the peak eluted confirming the identity of the peak (Figure 6.4). The amounts of veratrole found were minute. However *N. lugens* were found to emit a 2mV antennogram response to 10μl of extract from mechanically damaged rice plants. Veratrole, at concentrations as low as 1μg, produced an 80% response when expressed as a percentage relative to the hexanal standard (Masri, 1995). When tissue was wounded with a sharp needle to mimic tissue damage by insects, the amounts of volatiles released were so small they could not be identified using the GC machine with any degree of certainty. Macerating leaf tissues in a blender with air entrainment system attached produced greater quantities of volatiles. However, the tissues were fibrous and damage was therefore not uniform and not quantitative. Consequently tissues of uniform weight (1.0g) were therefore chopped into 0.25cm lengths to generate the results (Table 6.1). Retention times (RT) varied between samples and a slight variation in Kovat's index (KI) calculated from RT was correlated with these variations.

Table 6.1

Retention times (RT) and quantities of veratrole released from 1g of leaf tissue chopped into 0.25cm lengths, trapped onto Tenax and measured by gas chromatography

Plant	Peak Area	RT (min)	KI	concentration ($ng\ g^{-1}$)
SLJ7307 2	200	13.6	1103	19.34
SLJ7307 4	200	13.6	1104	19.3
SLJ7307 7	125	13.6	1104	13.6
SLJ7307 10	100	13.6	1103	11.7
SLJ7307 11	130	13.7	1103	14
SLJ7307 13	145	13.6	1104	15.1
SLJ7307 14	150	13.7	1104	15.5
SLJ7307 15	200	13.5	1102	19.3
Taipei 309	12	13.6	1103	4.9
Taipei 309	11	13.6	1102	4.9

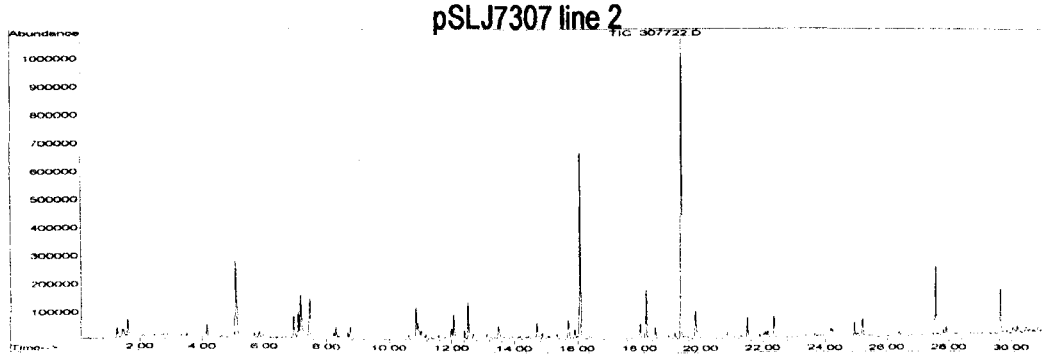
Table 6.2

Retention times (RT) and quantities of methyl salicylate released from 1g of leaf tissue chopped into 0.25cm lengths, trapped onto Tenax and measured by gas chromatography

Plant	Peak Area	RT (min)	KI	Concentration (ng g^{-1})
SLJ7307 2	700	16.9	1178	57.6
SLJ7307 4	600	16.2	1178	49.9
SLJ7307 7	300	16.2	1177	27
SLJ7307 10	180	16.2	1178	17.8
SLJ7307 11	200	16.2	1180	19.3
SLJ7307 13	300	16.2	1177	27
SLJ7307 14	400	16.1	1177	34.7
SLJ7307 15	150	16.2	1178	15.5
Taipei 309	30	16.2	1178	6.3
Taipei 309	57	16.2	1178	8.4

Figure 6.4
Chromatograms showing peak height against time for SLJ7307 plant 2 and wild-type plant 13

pSLJ7307 line 2



Control line 13

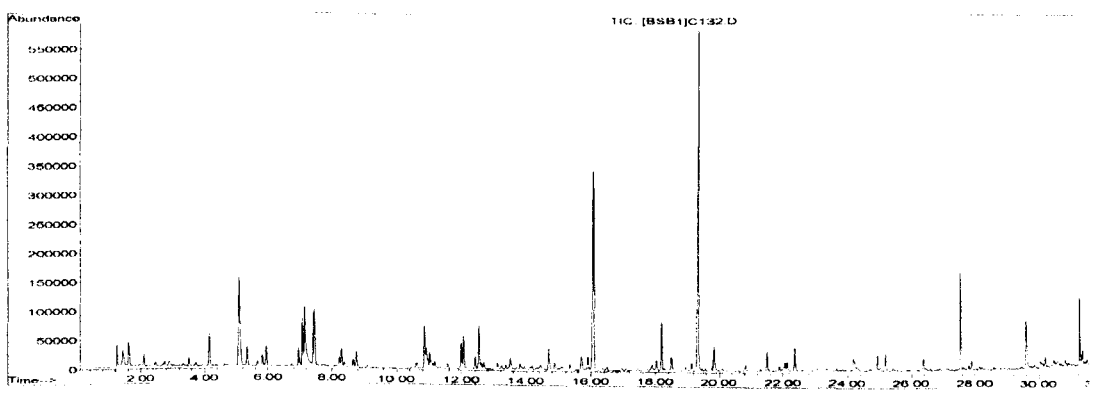
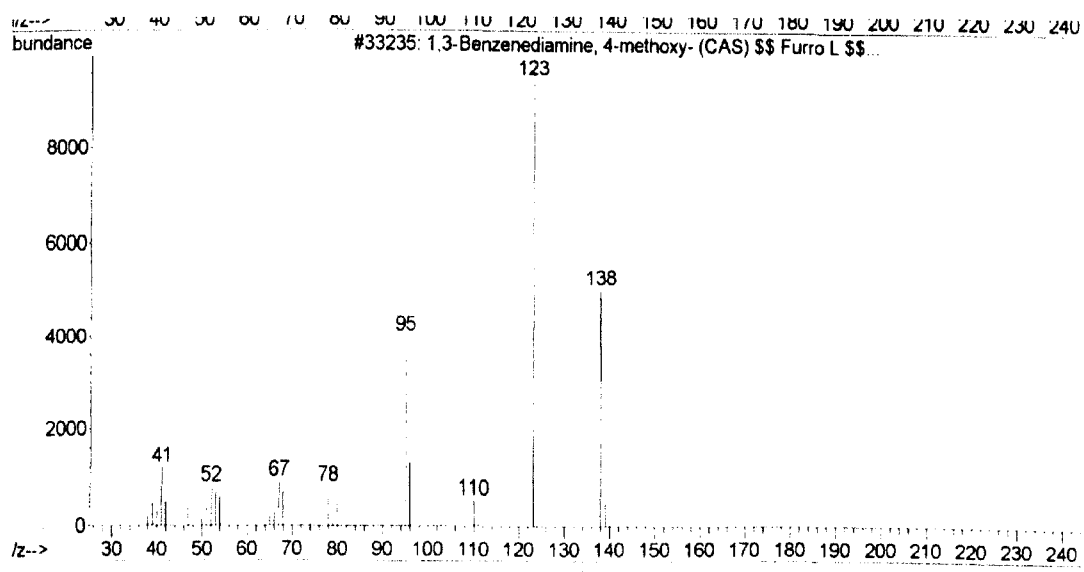
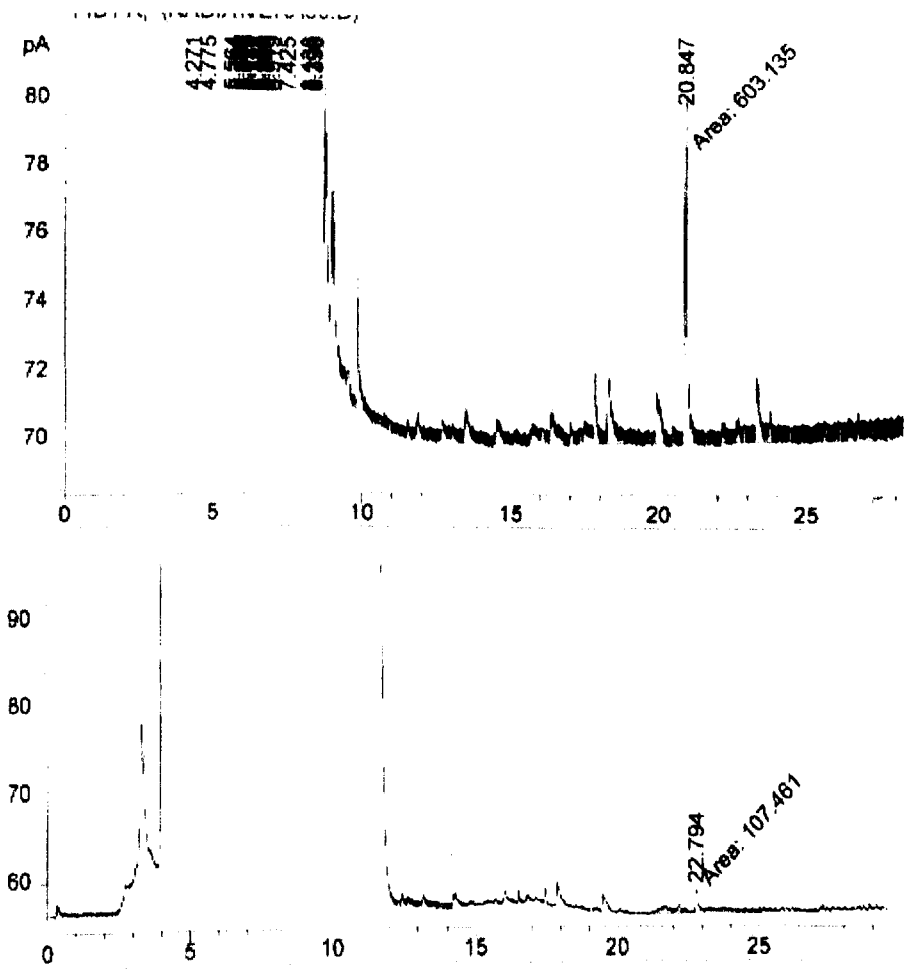


Figure 6.5
Mass spectrometer of 1,2-dimethoxy benzene (veratrole) showing the ionisation pattern



transgenic rice plants released greater amounts of volatiles from their leaves than control (wild-type) plants. Injection of veratrole and methyl salicylate confirmed the retention times of veratrole as 20.85 min and the retention time for methyl salicylate as 22.84 min (Figure 6.6). Gas chromatographs vary in the retention times of identical chemicals. Therefore, it was necessary to determine the retention time of methyl salicylate and veratrole from standard concentrations. The KI for samples run using this method was calculated independently from those following collection of volatiles for 4 d.

Figure 6.6
Gas chromatogram of 50ng of veratrole (top) and 10ng of methyl salicylate (below) illustrating peak height against retention time min



Different concentrations of veratrole and methyl salicylate injected into a Tenax tube and run on the GC resulted in calibration curves for veratrole and methyl salicylate (Figures 6.7, 6.8). Volatiles separated from plant volatiles could therefore be quantified for the amount of veratrole and methyl salicylate they contained. Concentration was more difficult to determine, since whole plants were sampled and although the plants were of the same age, plant masses were not identical.

Figure 6.7
Peak area for different concentrations of methyl veratrole

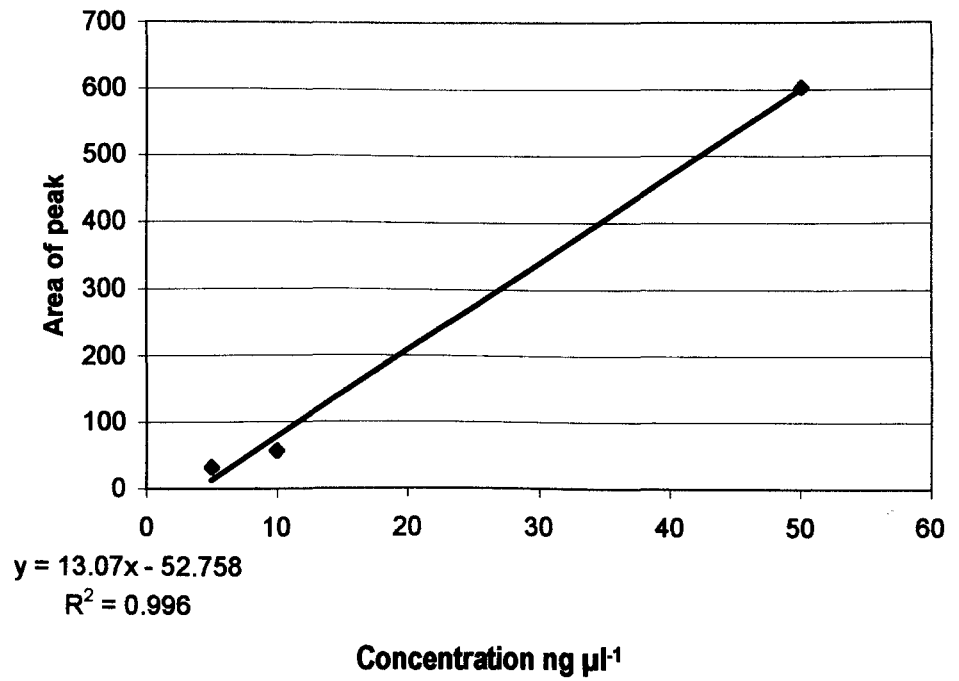
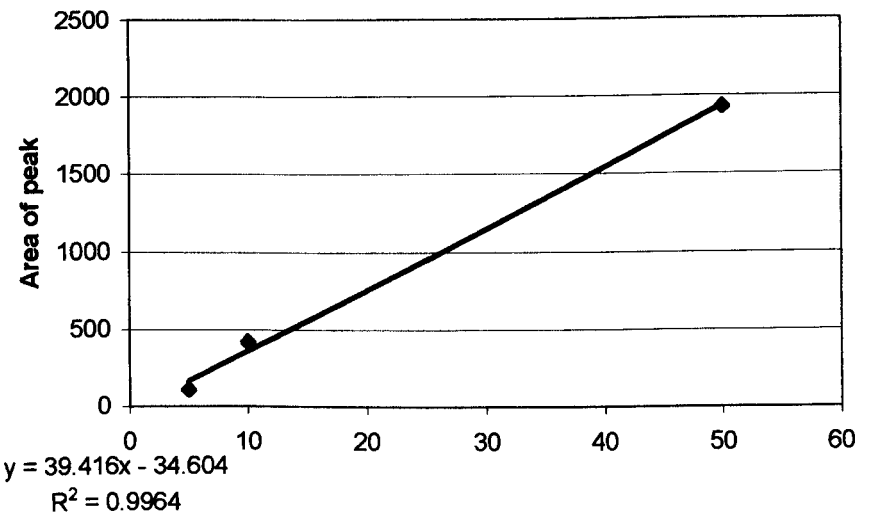
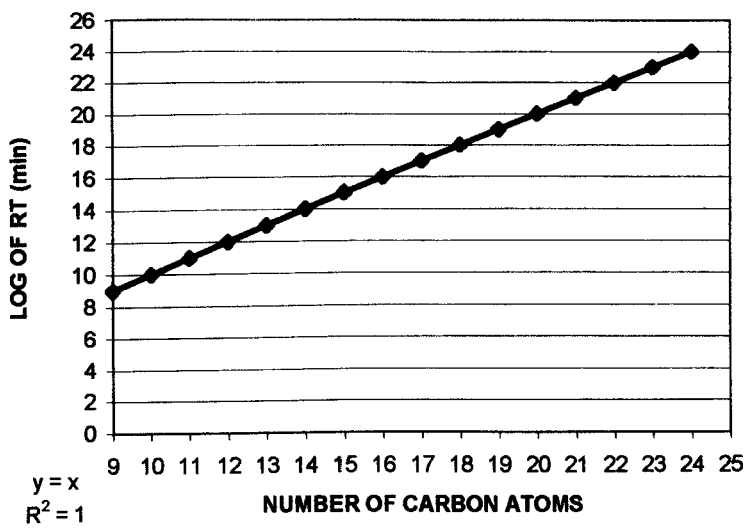


Figure 6.8
Area of peak for different concentrations of methyl salicylate



Injection of an alkane series onto the chromatography column resulted in a series of peaks for each alkane (Figure 6.9) and enabled the calculation of the Kovat's index for peaks ascribed to veratrole and methyl salicylate (Figure 6.9).

Figure 6.9
Retention time for an alkane series with 9 to 25 carbon atoms



6.3.2 Gas Chromatography of volatiles collected for four days from intact plants

The results demonstrated that veratrole and methyl salicylate were present from wild-type plants of Taipei 309, Pusa Basmati rice and Martelli. Concentrations of veratrole were enhanced in Pusa Basmati compared to Taipei 309 and reduced in Martelli. Methyl salicylate concentrations were enhanced in leaf extracts of Martelli and Pusa Basmati compared to Taipei 309 (Table 6.3). Kovat's index (KI) calculated from retention times (RT) produced some variation in the values for veratrole and methyl salicylate. Retention times varied from 20.8 - 20.9 minutes for veratrole and retention times of 22.78 – 22.89 min for methyl salicylate. These retention times produced a KI value for veratrole of 1111 – 1115 and a KI value for methyl salicylate of 1177 – 1178.

Table 6.3

Retention times and amounts of volatiles from different wild-type rice cultivars

Cultivar	RT (min)	KI	Peak Area	Amount ng per plant
Veratrole				
Taipei 309	20.8	1111	62.6	8.8
Pusa Basmati 1	20.9	1115	110	12.5
Martelli	20.8	1112	29.3	6.3
Methyl salicylate				
Taipei 309	22.9	1178	25.8	1.5
Pusa Basmati 1	22.9	1177	40.8	1.4
Martelli	22.9	1177	29.7	1.9

Damaged plants released greater quantities of volatiles from all rice varieties (Table 6.4).

Table 6.4

Retention times and amounts of volatiles from mechanically damaged plants of different wild-type rice cultivars

Line	RT (min)	KI	Peak Area	Amount ng per plant
Veratrole				
Taipei 309	20.7	1108	284	25.8
Pusa Basmati 1	20.8	1111	124	13.5
Martelli	20.8	1113	38.7	7
Methyl salicylate				
Taipei 309	22.9	1178	139.6	4.4
Pusa Basmati 1	22.9	1178	45.3	2
Martelli	22.9	1177	34.4	1.8

Transgenic plant lines regenerated following microprojectile bombardment, released higher concentrations of veratrole (Table 6.5) and methyl salicylate (Table 6.6), damaged plants from transgenic lines released higher concentrations of veratrole (Table 6.7) and methyl salicylate (Table 6.8).

Table 6.5

Retention times and amounts of veratrole released by whole plants transformed with pSLJ7307

Plant	RT (min)	KI	Peak area	Amount ng per plant
SIJ7307 2	20.8	1113	7.8	4.6
SLJ7307 7	20.8	1113	51.1	7.9
SLJ7307 11	20.4	1097	67.3	9.2
SLJ7307 14	20.8	1110	51.5	8
SLJ7307 15	20.8	1112	7.1	4.6

Table 6.6

Retention times and amounts of methyl salicylate released by whole plants transformed with pSLJ7307

Plant	RT (min)	KI	Peak area	Amount ng per plant
SLJ7307 2	22.7	1171	23.4	5.8
SLJ7307 7	22.8	1174	49.1	7.8
SLJ7307 11	22.5	1165	67.3	9.2
SLJ7307 14	22.8	1173	46.0	7.6
SLJ7307 15	22.8	1174	115.0	12.8

Table 6.7

Retention times and amounts of veratrole released by mechanically damaged plants transformed with pSLJ7307

Plant	RT (min)	KI	Peak area	Amount ng per plant
SLJ7307 2	20.8	1112	100.5	11.7
SLJ7307 7	20.8	1113	595.5	49.6
SLJ7307 11	20.7	1109	119.7	13.2
SLJ7307 14	20.7	1110	105.1	12.1
SLJ7307 15	20.8	1113	63.7	8.9

Table 6.8

Retention times and amounts of methyl salicylate released by mechanically damaged plants transformed with pSLJ7307

Plant	RT (min)	KI	Peak area	Amount ng per plant
SLJ7307 2	22.9	1176	74.5	9.7
SLJ7307 7	22.9	1175	103.2	11.9
SLJ7307 11	22.8	1173	92.9	11.2
SLJ7307 14	22.8	1174	105.2	12.1
SLJ7307 15	22.8	1175	218.9	28.4

Volatiles extracted from intact and damaged plants transformed using *Agrobacterium* produced greater amounts of veratrole (Table 6.9) and methyl salicylate (Table 6.10). In all lines tested, damaged plants released more volatiles than intact plants.

Table 6.9

Retention times and amounts of veratrole and methyl salicylate released from intact plants transformed with pSLJ7321::ROB5

Plant	RT (min)	KI	Peak area	Amount ng per plant
Veratrole				
VirG 11	20.7	1109	12.8	5
VirG 14	20.4	1100	43.3	7.4
PMOG 1	20.7	1110	1.4	4.1
Methyl salicylate				
VirG 11	22.8	1174	9.5	4.8
VirG 14	22.7	1172	6.8	4.6
PMOG 1	22.7	1172	7.5	4.6

Table 6.10

Retention times and amounts of veratrole and methyl salicylate released by mechanically damaged plants transformed with pSLJ7321::ROB5

Plant	RT (min)	KI	Peak area	Amount ng per plant
Veratrole				
VirG 11	20.7	1109	204	19.6
VirG 14	20.4	1109	129.6	14
PMOG 1	20.7	1109	69.5	9.4
Methyl salicylate				
VirG 11	22.8	1174	517.8	43.7
VirG 14	22.8	1174	54.8	8.2
PMOG 1	22.8	1174	72.5	9.6

6.4 DISCUSSION

6.4.1 Volatiles in wild-type cultivars of rice

It is recognised that green leaf volatiles, consisting of saturated and unsaturated 6 carbon alcohols and aldehydes, play a significant role in host odour recognition in phytophagous insects (Visser and Ave, 1978). Very little research has been carried out on the volatiles produced by different rice cvs. and their susceptibility to *N. lugens*. However, Liu *et al.* (1989) found that cvs. that were susceptible to white backed planthopper (*Sogatella furchifera*) produced different volatiles and different amounts of volatiles than cvs. resistant to white-backed planthopper. Volatiles also varied in air entrained leaf samples from different cvs. of rice this was correlated with more female white-backed planthoppers that settled on the susceptible varieties (Saxena and Okeck, 1985).

Masri (1995) found that of 20 volatile compounds tested, 7 produced a medium to strong response with an insect antennogram, but only 2 of these compounds, 2-hexanal and *E*-2-hexanal are classified as green leaf volatiles. Greater quantities of volatiles and a wider range of compounds were produced from the susceptible cv. Balilla than from the less susceptible cv. IR26 (Masri, 1995).

In these experiments, it has been demonstrated that the Indica rice cv. Pusa Basmati 1 produced more volatiles than either of the Japonica cvs. Taipei 309 or Martelli. This was confirmed in every sample that was collected from these different wild-type rice cvs..

It has been demonstrated that rice plant volatiles attract *N. lugens* towards rice plants (Obata *et al.*, 1986). However *N. lugens* was also attracted by the green colour (Obata *et al.*, 1981) and the high humidity (Obata *et al.*, 1983) in addition to the odour (Saxena and Pathak, 1979). It is therefore important to recognise the complexity of insect plant interactions and that volatiles are only a part of insect host-plant location strategies.

6.4.2 Differences in volatiles between wounded and intact rice plants

It might be argued that mechanically damaged plants would produce a greater quantity of volatiles, both as a result of the wounding process and as part of the octadecanoid wounding pathway. Such volatile chemicals could attract an insect pest to an already wounded plant and may even attract a predator as plant wounding is often a consequence of insect attack (Schmelz *et al* ., 2001). The chromatograms of volatiles

entrained from control vessels with intact plants of the cv. Balilla, were shown to produce small traces of few compounds, whereas the extracts of volatiles from mechanically damaged plants contained complex mixtures of volatiles (Masri, 1995). There were peaks in the chromatograms from *N. lugens* damaged Ballila that were not found in intact plants. The quantity of volatiles entrained from *N. lugens* damaged rice was 2 –3 times that of mechanically damaged rice plants which produced greater amounts of plant volatiles than intact plants (Masri, 1995). Undamaged rice seedlings produced lower concentrations of volatiles in headspace entrainment than mechanically damaged rice seedlings or seedlings infested with *N. lugens* for 1-2 days (Xu *et al.*, 2002). Undamaged, mechanically damaged and plants infested with *N. lugens* for 1 or 2 days, emitted much lower amounts of volatiles compared to plants infested with *N. lugens* for 3-5 days (Xu *et al.*, 2002). Plants infested for 3-5 days released additional chemicals to those found in other treatments; methyl salicylate and terpenoid concentrations increased dramatically. Plants infested by white-backed planthopper released much higher levels of volatiles than those in other treatments. However, these plants had a repellent effect on *N. lugens* in dual choice flight tunnel experiments (Xu *et al.*, 2002). *Nilaparvata lugens* preferred to settle and feed on the exposed resistant rice cv. Rathu Heenati than on the parafilm masked but susceptible rice cv. Taichung Native 1 (Liu *et al.*, 1994), demonstrating that the volatile profile given off by the plants played an important role in feeding choice.

6.4.3 Comparisons of volatiles from different experiments

Former studies of rice plant volatiles show little agreement in the host searching behaviour, perhaps in part because of the use of different cvs. but also due to the employment of different techniques. Obata *et al.* (1983) analysed volatiles after ether extraction followed by steam distillation on cv. Nipponbare. This method can lead to the introduction of contaminants (Blight *et al.*, 1990). Hernandez *et al.* (1989) and Ramachandran *et al.* (1990) used the preferred headspace techniques for cvs. Mars and PI346833 and IR36, respectively, but their lists of volatiles showed no overlap with those of Obata *et al.* (1983). Hernandez *et al.* (1989) used thermal desorption to flush off volatiles that may have affected some heat sensitive compounds. Ramachandran *et al.* (1990) and Obata *et al.* (1983) both report the presence of the monoterpene, linalool, and the aromatic methyl benzoate and ethyl benzoate as some of the most

potent compounds in evoking an antennogram response in *N. lugens* (Obata *et al*, 1983) compounds that were not detected in the present experiments. Volatiles that produced an antennogram response have been determined via isolation, Tenax trapping and gas chromatogaphy thermal desorption (Wang and Kays, 2002).

6.4.4 Differences between veratrole and methyl salicylate concentrations in transgenic plants compared to wild-type plants

In all experiments volatiles entrained from transgenic plants produced greater amounts of volatiles than those from wild-type plants. The range of volatiles was not significantly different. However, the amounts of veratrole and methyl salicylate were generally higher in chromatograms of volatiles entrained from transgenic plants than from wild-type plants. These results were consistent for many plant lines tested and over replicates and indicate that the oxidative decarboxylation of SA encoded for by the enzyme SAH has led to an increase in both the amount of veratrole and the amounts of methyl salicylate released from these plants. Felton (1999) stated that there was no evidence that enhancing SAH activity led to an increase in catechol. However, these experiments suggested that veratrole, the breakdown product of catechol, was enhanced in plants that over-expressed SAH. The effect of *N. lugens* on the production of rice volatiles in these transgenic lines was not assessed. Masri (1995) indicated that rice plants infested with *N. lugens* produced 2-3 times the amount of volatiles collected from intact plants, which was recently confirmed by Xu *et al.* (2002). In the present experiments, insufficient numbers of *N. lugens* were available to carry out such experiments. It would be useful and interesting to ascertain whether transgenic plants produced different volatiles and/or greater amounts of volatiles following infestation with *N. lugens*.

CHAPTER 7 INSECT BIOASSAYS

7.1 INTRODUCTION

7.1.1 Attraction of insects to rice volatiles

Behaviour-modifying chemicals produced naturally by plants and animals offer an environmentally beneficial alternative to chemical pest control (Masri, 1995). Plants release complex mixtures of volatiles important in the interaction with insects and other organisms. Behavioural studies have indicated that chemoreception plays a part in host plant selection and acceptance in the control of feeding by the rice brown planthopper, *Nilaparvata lugens* (Bernays and Chapman, 1994). Most studies reported receptor responses as generalist broad-spectrum responses to green leaf volatiles (Visser, 198 6). However, there is only limited information available regarding olfactory orientation in *N. lugens.* Saxena and Pathak (1979) showed *N. lugens* was attracted to the odour of plant extracts, as well as to the odour from green plants and high humidity. Behavioural orientation to intact rice plants was demonstrated, as well as attraction to rice extracts (Obata *et al.,* 1983). *N. lugens* was also attracted to non-host plants to which volatile host plant chemicals had been applied. Rice plant volatiles obtained as steam distillates significantly affected the behaviour of *N. lugens* (Valusamy *et al.,* 1986).

7.1.2 Attraction of *Nilaparvata lugens* to rice volatiles

Obata *et al.* (1981) reported that rice volatiles play a part in attracting hoppers onto a plant and suggested that the receptors involved are located on the antennae of the insects. The presence of the monoterpene linalool and the aromatic compound methyl benzoate as components of rice volatile mixtures was confirmed (Ramachandran *et al.,* 1990; Obata *et al.,* 1983). The distal plaque organ on the antennea barely showed any response to linalool (Youn, 2002). However, methyl benzoate and ethyl benzoate were two of the most potent compounds in evoking an insect antennogram (EAG) response in *N. lugens* (Obata *et al,* 1983). Antennectomised *N. lugens* did not respond to rice volatiles (Masri, 1995). *N. lugens* made more frequent probes on resistant rice than susceptible rice although probes were of shorter duration but stopped within 5 min. Attraction of hoppers to the rice cvs. Mudgo (gene *Bph1*), IR26 with gene *Bph1*, ASD7 carrying the resistance gene *bph2*, Norin PL10 with resistance gene *Bph3* and Babawee (*bph4*) was tested. Forty five to 87% of females survived and reproduced on ASD7, 49-98% of females reproduced on Mudgo, whilst 5-27% of females reproduced

on Norin PL10 and Babawee. It was suggested, therefore, that resistance to *Bph1* was breaking down in addition to the *bph2* gene (Tanaka and Matsumura, 2000). An ether extract of the cv. Nipponbare attracted *N. lugens* in bioassays (Obata *et al.*, 1983). In addition, acetopherone and benzaldehyde present in the ether extract of Nipponbare leaf volatiles were demonstrated to elicit a dose-dependent response on the distal plaque organ in the antennae of *N. lugens*. These compounds elicited a response of the distal plaque organ on antennae of *N. lugens* at high concentrations (10^3 µg). *N. lugens* have been shown to respond to host plant volatiles in laboratory bioassays. Antennae of both male and female planthoppers gave a response to air from above chopped rice leaves. The responses were recorded to air above very small quantities of rice plants (1g for leaf blade) (Youn, 2002). A dose-related response was obtained for 16 rice volatiles tested with EAG on *N. lugens* antennae (Youn, 2002). These compounds were hexanal, isoamylacetate, *(E)*-2-hexanalamyl acetate, acetopherone, ethyl benzoate, *(E)*-3-hexan-1-ol, methyl benzoate, 1-hexanol, *(Z)*-3-hexen-1-ol, 2-heptanol and linalool. Interestingly linalool and acetopherone had much lower responses in EAG experiments (Masri, 1995).

7.1.3 Attraction of *N. lugens* to volatiles from damaged, intact and infested rice plants

It has been suggested that herbivores would react to volatiles produced by herbivore-infested plants due to potential changes, either positive or negative, in the acceptability of the host plant (Xu *et al.*, 2002). This hypothesis was tested for the *N. lugens* in the laboratory. Sixteen components of the headspace volatiles from rice seedlings were collected with Tenax-TA and analyzed by GC and GC-MS. Significant differences in volatile emissions were observed for rice plants with different treatments. Undamaged control plants, mechanically damaged plants and plants infested by *N. lugens* for 1 or 2 d emitted much lower amounts of volatiles compared to plants infested by *N. lugens* for 3 or 5 d. The plants infested by *N. lugens* for 3 or 5 d emitted several volatiles that were not detected in undamaged control plants, mechanically damaged plants or the plants infested by *N. lugens* for 1 or 2 d. *Spodoptera litura* infested plants released much higher concentrations of volatiles than those in all other treatments. The concentration of several green leaf volatiles, methyl salicylate and terpenoids increased dramatically. In dual-choice flight tunnel experiments; adult *N. lugens* females showed no significant preference between the untreated healthy plants and mechanically damaged plants, or

the plants infested by *N. lugens* adult females. However, rice plants damaged by *S. litura* had a clearly repellent effect on *N. lugens* adult females compared to healthy undamaged plants, mechanically damaged plants or plants infested by *N. lugens* (Xu *et al.*, 2002).

7.1.4 Aims and objectives of the current work

These experiments aimed to determine whether the transgenic rice plants were more attractive to *Nilaparvata lugens* than wild-type plants using the Pettersson olfactometer and the Y-tube olfactometer.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of rice plants for olfactometry

Japonica rice seeds (*Oryza sativa* cv. Taipei 309) were cultured to produce scutellum-derived calli. These were subjected to microprojectile bombardment at 1350 psi with pSLJ7307 containing the *nahG* gene encoding SAH (Section 2.2.1 and Section 2.2.2) or transformed (Chapter 3) using *A. tumefaciens* LBA4404 with the additional pBBR1MCS and pSLJ7321:ROB5 containing the *hpt* gene coding for hygromycin phosphotransferase and pSLJ7321 containing the *nahG* gene coding for salicylate hydroxylase.

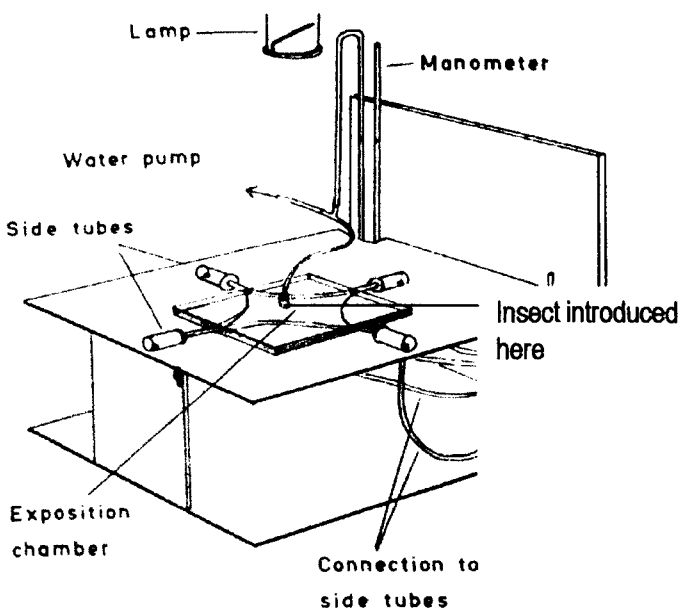
7.2.2 Preparation of plant tissues

Plant leaf samples (1g ± 0.1g) were collected from plants 2 months after transfer from tissue culture. The plant leaf tissue was used to place in either one arm of the Pettersson olfactometer or one arm of the Y-tube olfactometer. Both olfactometers were washed in detergent and rinsed with distilled water after use. A final rinse was carried out with ethanol and the equipment left to dry overnight. After each day the same procedure was adopted to ensure that the olfactometers were perfectly clean.

7.2.3 Pettersson olfactometer

The basic design of the olfactometer was similar to that used by Pettersson (1970) (Figure 7.1).

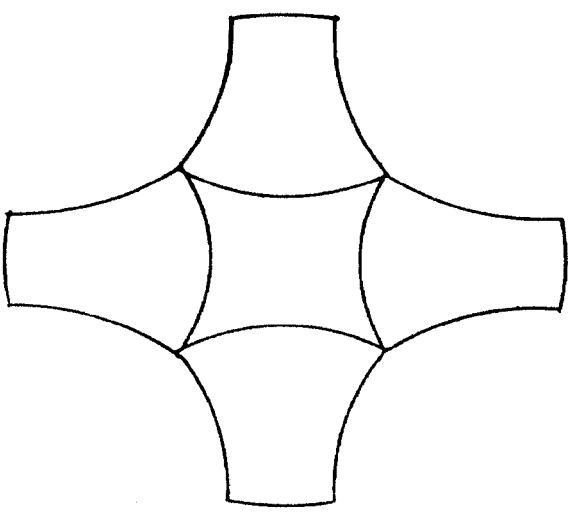
Figure 7.1
Pettersson olfactometer set up



The perspex apparatus was set up. The base was covered with a sheet of filter paper moistened with distilled water to maintain humidity. The olfactometer was housed in a controlled environment room at 27 ± 1°C, 60 ± 5% relative humidity and illuminated with fluorescent tubes (60μE m^{-2} s^{-1} Cool White Fluorescent Tubes Thorn EMI), but with no natural daylight. The air stream through the olfactometer was withdrawn by a vacuum pump (DA7C, Charles Austin Pumps Ltd, Cambridge UK) connected (once the insect had been introduced) to the apparatus by the central hole through 8mm inner diameter polythene tubing (Fisher Scientific UK). Air was drawn into the apparatus through the 4 arms 3 of which contained a 2 cm square of filter paper moistened with deionised water. The fourth contained 1g of rice leaf material. Insects were captured and retained in a plastic container (1 X 4cm) with damp filter paper to maintain humidity and kept there for up to 60 min.

In order to assess the response of *N. lugens* to volatiles released from the rice leaf material, an insect was introduced to the olfactometer through the central hole (Figure 7.1). The olfactometer was divided into zones (Figure 7.2).

Figure 7.2
Zones of the Pettersson olfactometer



Movement of the insect was monitored for 16 min. The length of time the insect spent in each zone was measured as was the number of times each zone was visited and the zone of first choice. Attraction of the insect for a particular arm was determined by the frequency each zone was visited, the length of time in that treatment and the treatment as first choice. The entrance to side tubes containing moistened filter paper or rice leaf

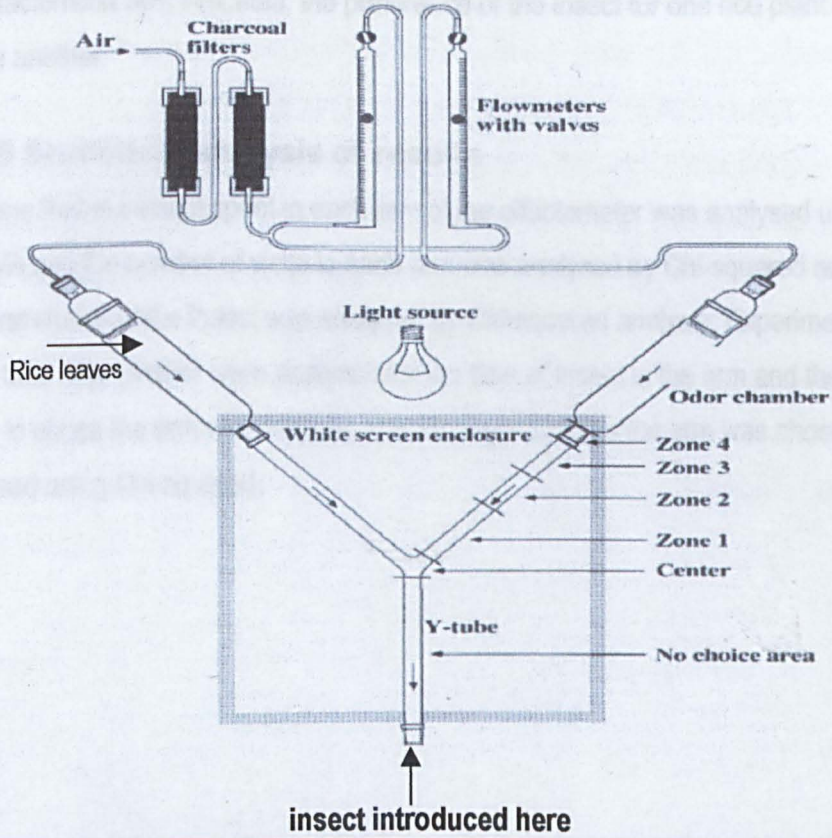
tissue was blocked using nylon mesh, so the insect could not escape. The apparatus was enclosed with white screens to prevent any visual cues presented to the insect and lit from above. Every 2 min, the apparatus was rotated by 90^0 to prevent any directional bias influencing the insect.

If the insect remained stationary for more than 5 min the experiment was considered invalid. The arm treated was varied randomly and the zones were numbered randomly 1- 4 Zone 5 was always in the centre.

7.2.4 Y-tube olfactometer

The Y-tube olfactometer offered the insect a choice of 2 pathways rather than the 4 arms of the Petterson olfactometer. The olfactometer was set up as in Figure 7.3.

Figure 7.3
Y-tube Olfactometer set up



To assess the response of *N. lugens* to the volatile chemicals released from rice leaves. Four freshly excised leaves were maintained inside a cylindrical 1x4cm PVC vial full of water. The vial was connected to one arm of the olfactometer and the same number of

vials with leaves from an alternative rice plant were placed a similar vial connected to the other arm of the olfactometer. Insects *N. lugens* were captured in a plastic container (1 X 4 cm) with damp filter paper to maintain humidity and kept there for up to 60 min. To assess the response to volatiles released from transgenic and wild-type rice plant leaves, an insect was placed in the single tube that led to the 2 arms of the olfactometer. Movement of the insect was timed for 5 min. Air from the laboratory passed through a charcoal filter to remove any extraneous odours and volatiles. It was then passed through flow meters and through the odour chambers into the arms of the olfactometer. The arms of the olfactometer and the single tube were lit and enclosed with a white screen to prevent visual cues from influencing the behaviour of the insect. The arms of the olfactometer were divided into zones (Figure 7.3). Once the insect had travelled 1cm into an arm of the olfactomter (zone 2), a positive choice for that arm was recorded. The number of times *N. lugens* travelled towards a particular rice plant, the faster the choices were made and the greater the distance travelled by the insect inside the olfactometer arm indicated, the preference of the insect for one rice plant compared to one another.

7.2.5 Statistical analysis of results

The time that the insect spent in each arm of the olfactometer was analysed using ANOVA and the number of visits to each arm was analysed by Chi-squared analysis. The first choice of the insect was analysed by Chi-squared analysis. Experiments using the Y-tube olfactometer were analysed for the time of insect in the arm and the time taken to chose the arm with ANOVA. The numbers of times the arm was chosen was analysed using Chi-squared.

7.3 RESULTS

7.3.1 Different rice cultivars in the Pettersson olfactometer

Rice cvs. Pusa basmati1 (PB1), Maratelli (Mart) and Taipei 309 (T309) were used in the olfactometer to determine which cv. was preferred by *N. lugens*. Preference was determined by the amount of time *N. lugens* spent in the treatment arm when that cv. was used, the number of visits to the treatment arm and the first choice of treatment arm.

7.3.1.1 Controls and blanks

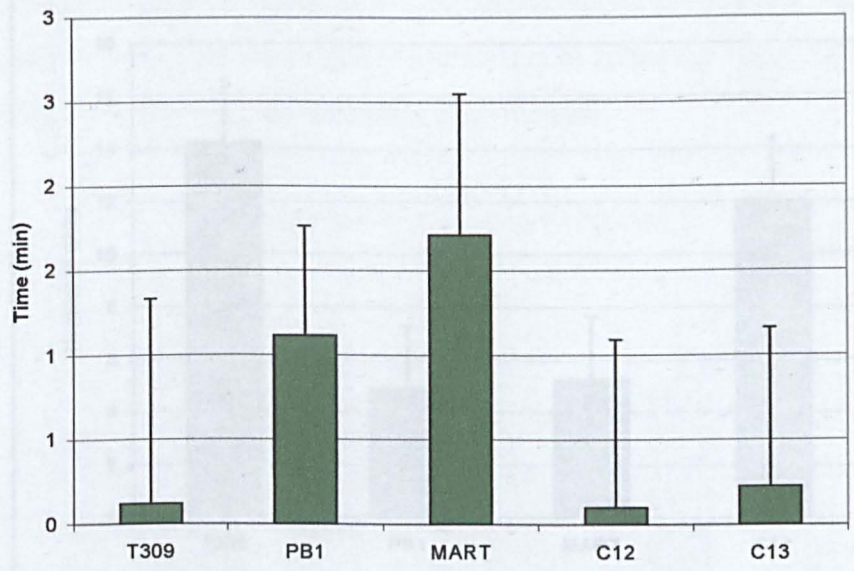
Initial experiments were used to ascertain that there was no preference for any position in the olfactometer, or preference for a particular arm when rice leaves were absent. Insects introduced into the olfactometer when damp filter paper was provided in each treatment arm either remained in the central area for the entire duration of the experiment or entered arms randomly. The olfactometer was rotated by 90 degrees every 2 min to eliminate any positional preference by the insect. Statistical analysis produced non-significant results with ANOVA, with a P value of 0.375 for duration in the treated arm and similar results for the number of visits to the treated arm. Chi squared analysis on first choice of arm was not significant. Therefore it was concluded that *N. lugens* exhibited no preference for any position within the olfactometer, or any bias towards a particular treatment arm.

Therefore, one arm of the olfactometer contained rice leaf tissue and the other 3 contained filter paper moistened with distilled water. This was repeated 8 times with 8 different insects but leaf tissue from the same rice plant. Each rice plant was tested in this manner. Subsequently 2 arms of the Pettersson olfactometer were used simultaneously.

7.3.1.2 Time spent in treatment arm

Nilaparvata lugens spent most time in the arm containing Maratelli (Figure 7.4).

Figure 7.4
Mean time (mins) spent in each treatment arm



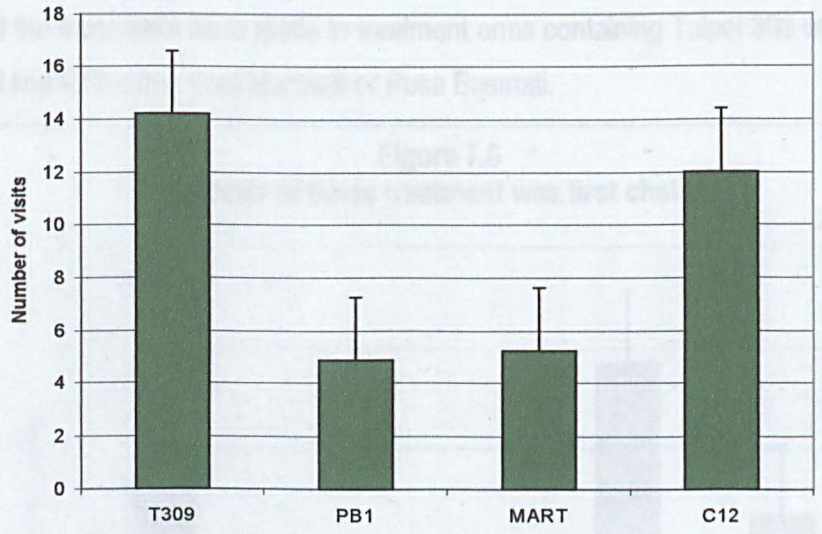
T309 = Taipei 309
PB1 = Pusa Basmati
MART = Maratelli
C12 C13 = Non transformed Taipei 309 from tissue culture

This result was variable as demonstrated by the error bars (representing the standard error). ANOVA comparing the time spent in each treatment arm from 8 replicates per treatment produced an F value of 55.9, with 3 degrees of freedom, significant at the 99.9% level. The insect clearly demonstated a preference for this cv. despite Maratelli producing fewer volatiles than either Pusa Basmati 1 or Taipei 309.

7.3.1.3 Number of visits to treatment arm

Most visits were made to the treatment arm when that arm contained leaf tissue of Taipei 309 (Figure 7.5).

Figure 7.5
Mean number of visits to arm containing each rice cv.



T309 = Taipei 309
PB1 = Pusa Basmati
MART = Maratelli
C12 = Non transformed Taipei 309 from tissue culture

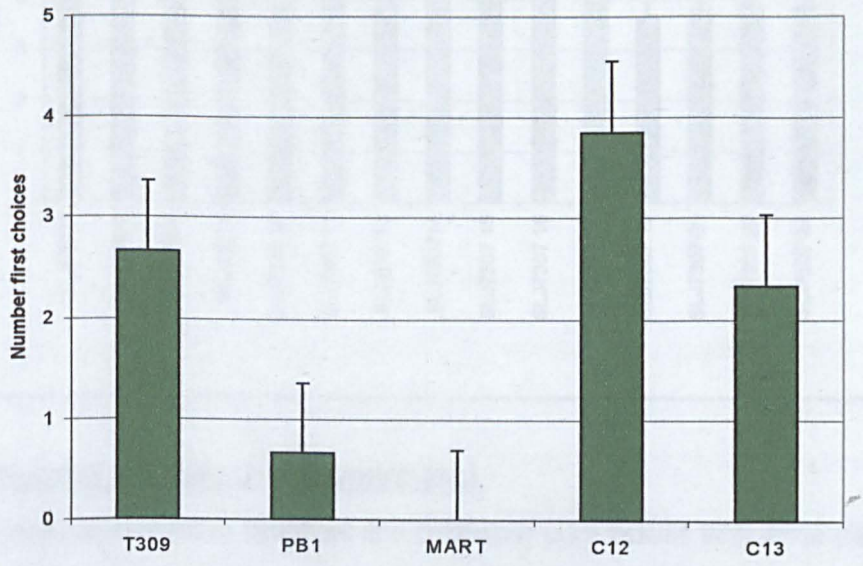
Interestingly, although most time was spent in the treatment arm containing leaves of Maratelli, most visits were made to the treatment arm containing Taipei 309. Analysis of these results produced a chi-squared value of 14.21 with 5 degrees of freedom for Taipei 309 significant at the 95% level. Analysis of the number of visits to treatment arms containing leaves of control Taipei 309 (C12) subjected to tissue culture but not transformed, produced a Chi-squared value of 12.83 with 5 d.f significant at the 95%. Level. Analysis of the number of visits made by insects to the arm containing Pusa Basmati 1 produced a Chi-squared value of 4.89 and analysis of number of visits to treatments containing leaf tissue of Maratelli was 5.253 with 12 d.f. and therefore not significant. The results were consistent as demonstrated by the uniform error bars

7.3.1.4 First choice of treatment arm

Nilaparvata lugens chose the treatment arm containing the rice cv. Taipei 309 as a first choice most often (Figure 7.6) either as wild-type T309 or as tissue cultured control

lines, C12 or C13. Results were consistent within limits as demonstrated by the similar error bars. Chi-squared analysis assumed that there would be no bias and each treatment would have similar appeal and produced a value of 120.5 with 7 degrees of freedom. This was significant at the 99.9% level. There is a clear paradox, most time was spent in treatment arm containing Maratelli, although the first choice was clearly most frequently Taipei 309 either as a tissue cultured control line C12 or a wild-type line T309 and the most visits were made to treatment arms containing Taipei 309 wild–type or control line C12 rather than Maratelli or Pusa Basmati.

Figure 7.6
Number of times treatment was first choice



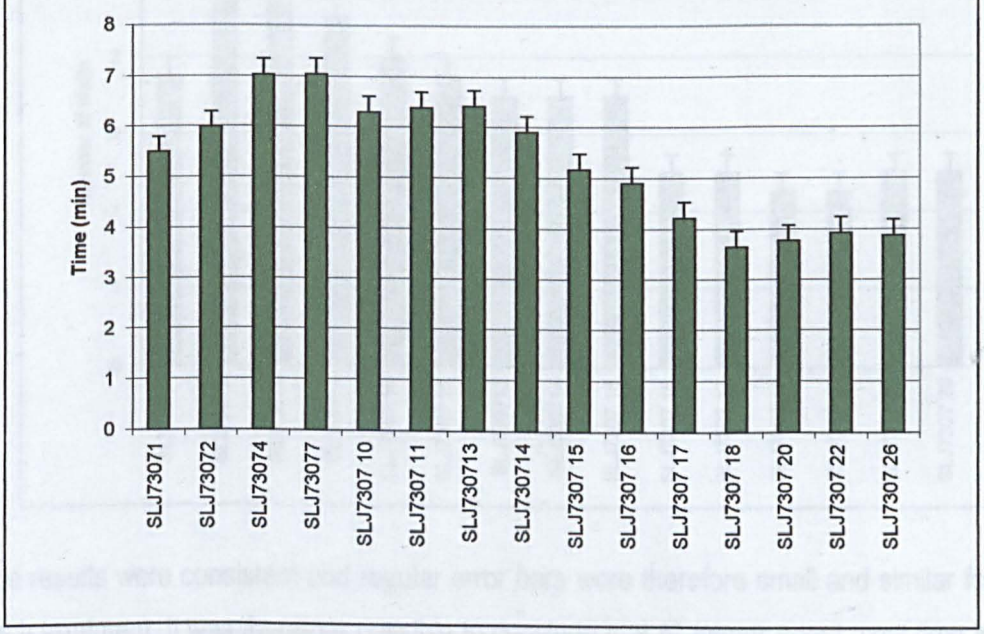
T309 = Taipei 309
PB1 = Pusa Basmati
MART = Maratelli
C12 and C 13 = Non transformed Taipei 309 from tissue culture

7.3.2 Biolistically transformed rice plants

Biolistically transformed lines containing pSLJ7307 and pROB5 were tested with leaf tissue in one arm of the olfactometer and the other 3 containing moistened filter paper results (Figure 7.6) are the mean of eight replicates for each plant tested. Each test lasted 16 minutes. Clear preference was demonstrated for the plants that highly expressed salicylate hydroxylase in terms of the time spent in the treatment arm. *N. lugens* spent most time in treatment arms containing plant tissues from high expressing lines (Figure 7.7). Transformed plants are designated by the plasmid used followed by a

number in all future graphs. ANOVA of these results produced an F value of 11.61, with 8 degrees of freedom, significant at the 99.9% level. Results were consistent and regular as demonstrated by small standard error bars.

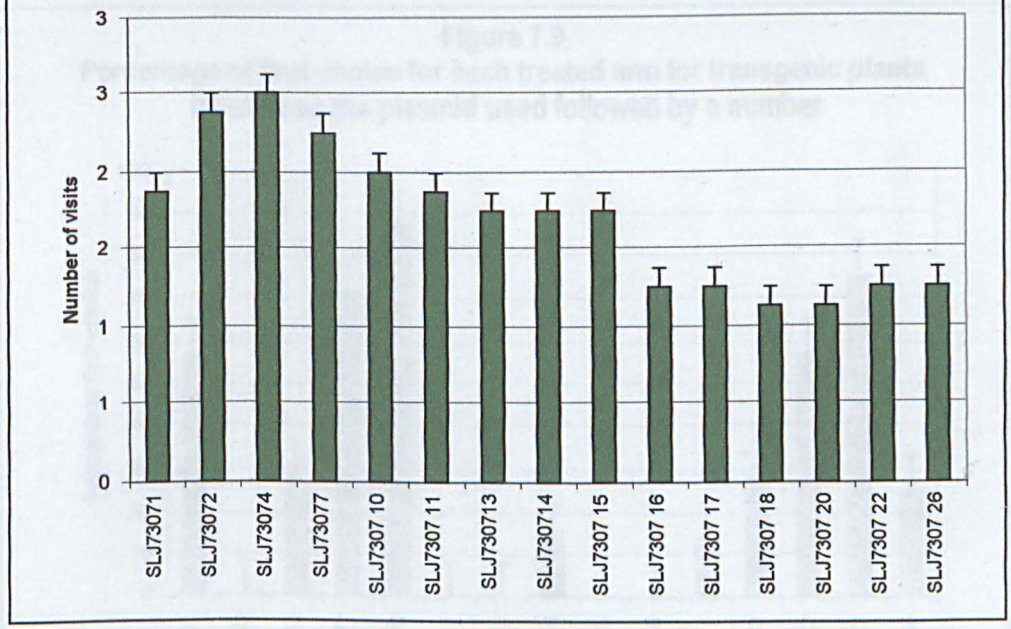
Figure 7.7
Mean time spent in treated arm for each plant



7.3.2.1 Number of visits to treatment arm

The most visits were made to treatment arm containing plant tissues from those plants that highly expressed salicylate hydroxylase (Figure 7.8). Chi-squared analysis produced a value of 50.38 with 14 degrees of freedom, significant at the 99.9% level. The number of visits clearly mirrored the most time spent in treatment arms (Figure 7.9). Individual Chi-squared analyses for each treatment were not statistically significant.

Figure 7.8
Mean number of visits for treated arm for transgenic plants labelled with the plasmid used and a number



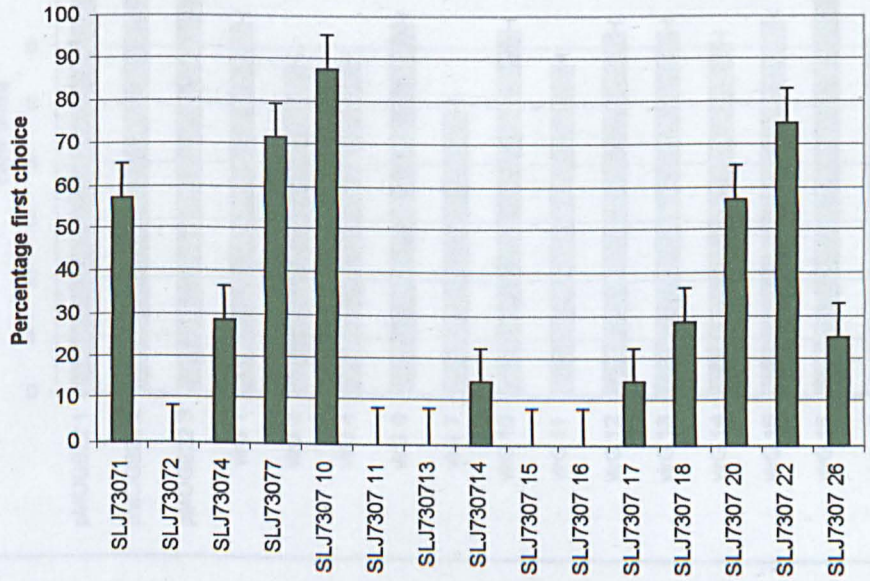
The results were consistent and regular error bars were therefore small and similar for each treatment. It was therefore possible to conclude that *N. lugens* spent most time in treatment arms containing leaf tissue from plants that over-expressed salicylate hydroxylase and visited treatment arms containing leaf tissue from those plants more frequently, demonstrating a clear preference for high expressing plants.

7.3.2.2 First choice of treatment arm

Unfortunately, the result for the first choice treatment arm (Figure 7.9) was not as consistent as the amount of time spent in each treatment arm or the number of visits. There were two groups of treatment arms that were visited most frequently as a first choice (Figure 7.9). One group contained plant tissue from the high expressing lines previously demonstrated, but the other group were treatment arms containing plant tissue from lines 18, 20, 22 and 26 that were not high over-expressors of salicylate hydroxylase. It is of course possible that whilst not over-expressing salicylate hydroxylase such lines did over-express methyl salicylate, an alternative attractive volatile for *N. lugens*. Unfortunately as these lines were only generated towards the end of the experimental period they were not available for testing for their volatile content.

The results were uniform for treatments tested and consistent and, therefore, the standard error was low. Chi-squared analysis on the results produced a value of 66.9 with 13 degrees of freedom, significant at the 99.9% level.

Figure 7.9
Percentage of first choice for each treated arm for transgenic plants labelled as the plasmid used followed by a number



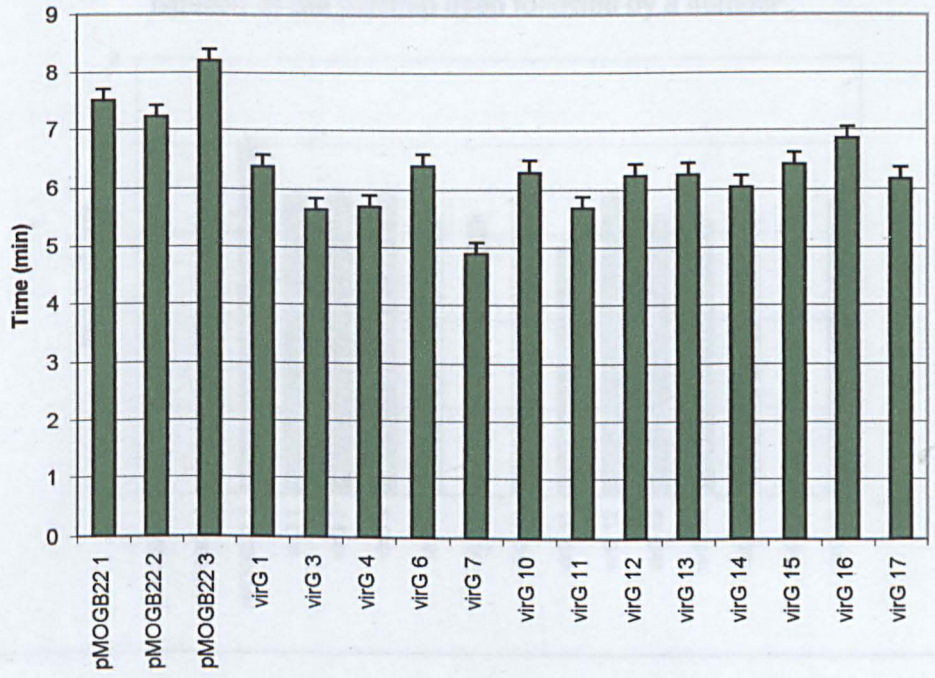
7.3.3 Response of *N. lugens* to *Agrobacterium* transformed plants

Plants transformed with *A. tumefaciens* LBA4404 with pBBR1MCS and pSLJ7321::ROB5 carrying the *nahG* gene encoding SAH were tested individually in the olfactometer with 8 replicates per plant .

7.3.3.1 Time spent in treatment arm

The time spent in each arm was consistent for each treatment with little variation. The insects spent most time in the lines transformed with LBA4404 pBBR1MCS pMOGB22 and pSLJ7321::ROB5 (Figure 7.10). These lines over-expressed SAH. VirG lines 1-10 also over-expressed SAH. However, leaf tissues from these lines did not prove so attractive to *N. lugens*. Transgenic plants are designated by the gene that they are transformed with and a number. As they are all transformed with pSLJ7321::ROB5, virG indicates those plants possessing pBBR1MCS and pMOGB22 indicates plants containing virG and pMOGB22.

Figure 7.10
Mean time spent in each treated arm for transgenic plants labelled as the plasmid used followed by a number

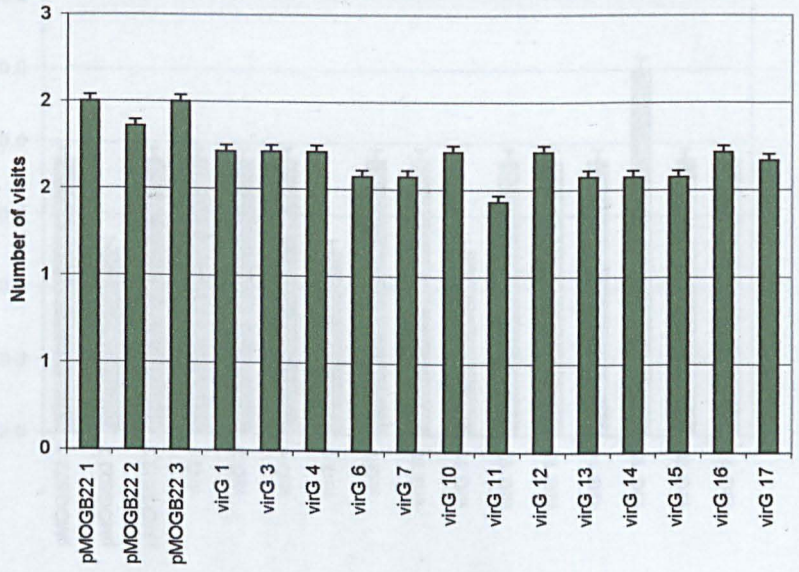


ANOVA for the time spent in each treatment arm produced an F value of 8.03 with 15 degrees of freedom, which was significant at the 99% level. Student's T- test revealed that time spent in treatment arm containing tissues from plants virG1-11 were significant at the 99.9% level, whilst pMOGB22 plants were significant at the 95% level. VirG plants 12-17 were not significant.

7.3.3.2 Number of visits to treatment arm

No overall pattern was observed in the number of visits of *N. lugens* to each treatment. It appeared that there was some preference for treatment arm containing leaf tissue from plant lines pMOGB22 (Figure 7.11). The number of visits to each arm treated was not statistically significant when analysed with Chi-squared analysis (4.61 with 15 degrees of freedom). There was no correlation between high expression of salicylate hydroxylase in a particular plant and the number of visits made by the insects to arms of the olfactometer treated with leaf tissue from that plant.

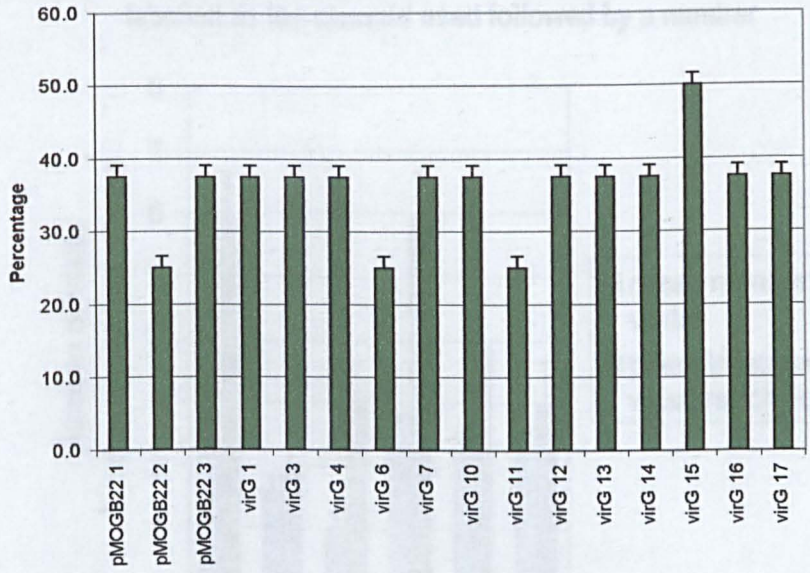
Figure 7.11
Mean number of visits to each treated arm for each transgenic plant labelled as the plasmid used followed by a number



7.3.3.3 First choice of treatment arm

There was no clear pattern as to the first choice made by the insects (Figure 7.12). Leaf tissues from plants virG 6 and 11 and pMOGB22 2 were visited as a first choice less frequently than other treatments (Figure 7.12). Leaf tissue from plant VirG15 was visited as a first choice more frequently than other plant lines. When assuming that the treatment arm would be a first choice one quarter of the time, Chi-squared analysis showed the results to be significant at the 99.9% level (Chi squared = 98 with 15 degrees of freedom).

Figure 7.12
Percentage of times treatment arm was first choice for insect



7.3.4 Two plants used simultaneously in Pettersson olfactometer.

Placing rice leaf tissue in one arm of the olfactometer established that *N. lugens* preferred the area containing rice to the control areas containing only damp filter paper. In order to obtain an indication of the comparative preferences of *N. lugens* for transformed or wild-type rice, 2 arms of the Pettersson olfactometer were used simultaneously. Both opposite and adjacent arms were used, in order to account for any bias of *N. lugens* to a positional effect.

7.3.4.1 Adjacent olfactometer arms used to assess response of N. lugens to rice leaves of 2 different plants at the same time

The number of visits to each arm and the first choice (Figure 7.13) were analysed using Chi-squared analysis (Table 7.1) Time spent each arm (Figure 7.14) was analysed using ANOVA (Table 7.1).

Figure 7.13
Number of visits of insect to each transgenic plants and first choice labelled as the plasmid used followed by a number

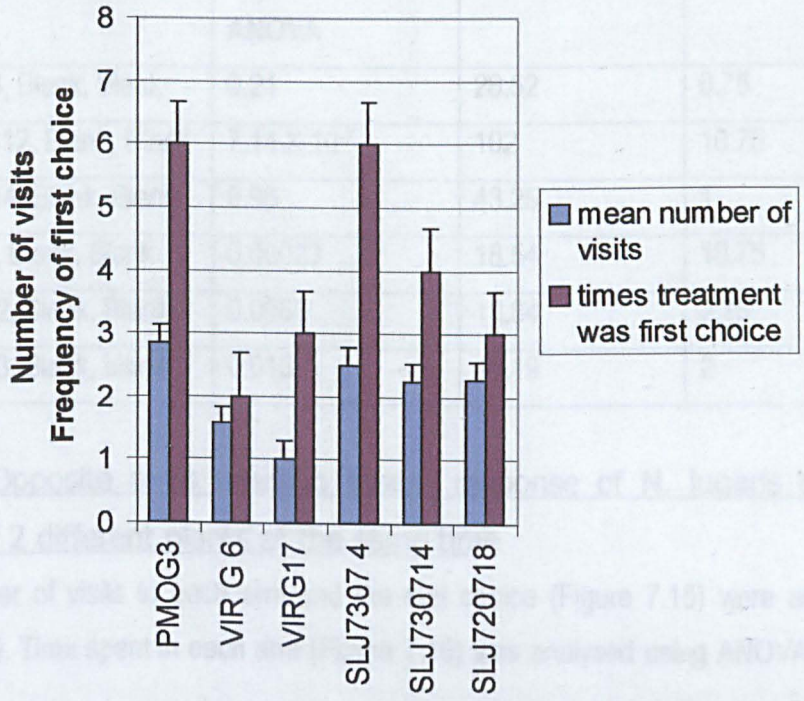


Figure 7.14
Mean time insects remained in arm containing plants from each treatment labelled as the plasmid used followed by a number

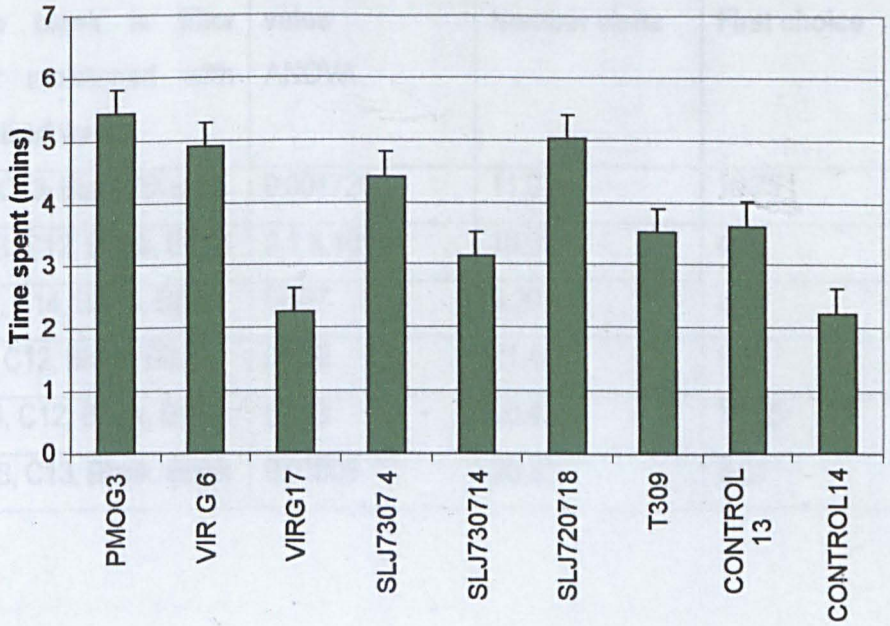


Table 7.1 Statistical results for time spent number of visits and first choice of treatment			
Treatment in each arm where blank is filter paper moistened with deionised water	**Time spent (min) in treatment Probability value ANOVA**	**Number visits Chi squared**	**First choice Chi squared**
VirG6, C13, Blank, Blank	0.21	28.52	0.75
PMOG2, C12, Blank, Blank	7.14×10^{-7}	102	10.75
VirG17, C14, Blank, Blank	0.96	43.25	1
3077, C12, Blank, Blank	0.00023	18.54	10.75
30714, C12, Blank, Blank	0.0063	18.84	2.75
30718, C13, Blank, blank	0.015	16.19	2

7.3.4.2 Opposite arms used to assess response of N. lugens to rice leaves of 2 different plants at the same time

The number of visits to each arm and the first choice (Figure 7.15) were analysed (Table 7.2). Time spent in each arm (Figure 7.16) was analysed using ANOVA (Table 7.2).

Table 7.2 Statistical results for time spent number of visits and first choice of treatment			
Treatment in each arm where blank is filter paper moistened with deionised water	**Probability value ANOVA**	**Chi squared Number visits**	**Chi squared First choice**
Vir 6, C13, Blank, Blank	0.001726	11.07	10.75
PMOG, C12, Blank, Blank	2.1×10^{-5}	16.59	4.75
Vir 17, C14, Blank, Blank	0.527	5.20	2.75
3077, C12, Blank, Blank	0.118	21.48	0.75
30714, C12, Blank, Blank	0.153	20.43	10.75
307 18, C13, Blank, Blank	0.01008	20.32	6.01

Figure 7.15
Mean number of visits of insects to each treatment and number of times that treatment was the first choice

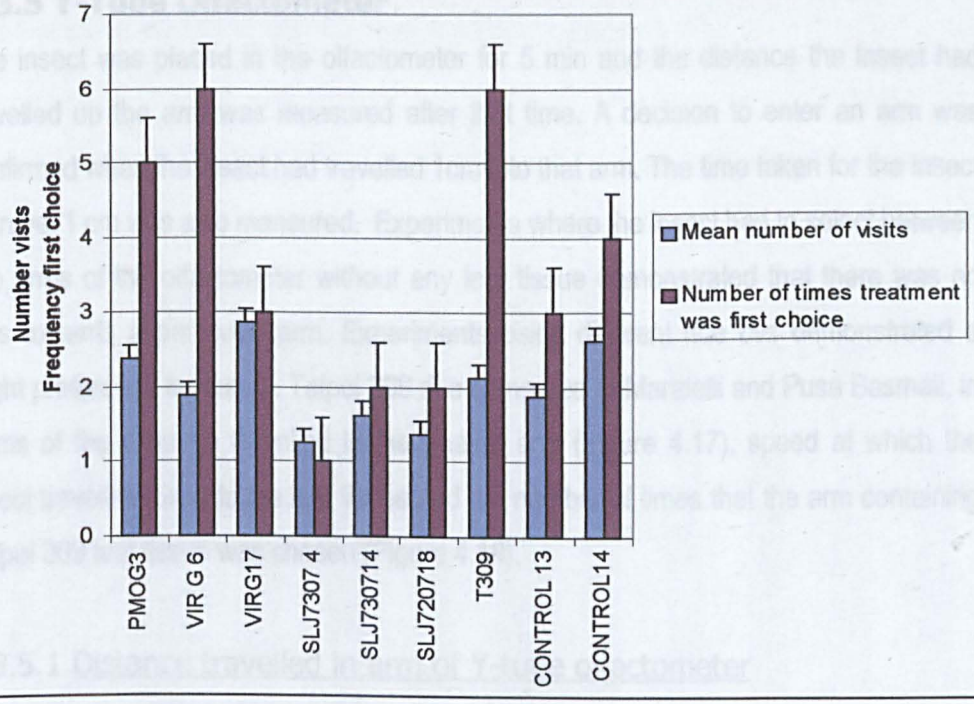
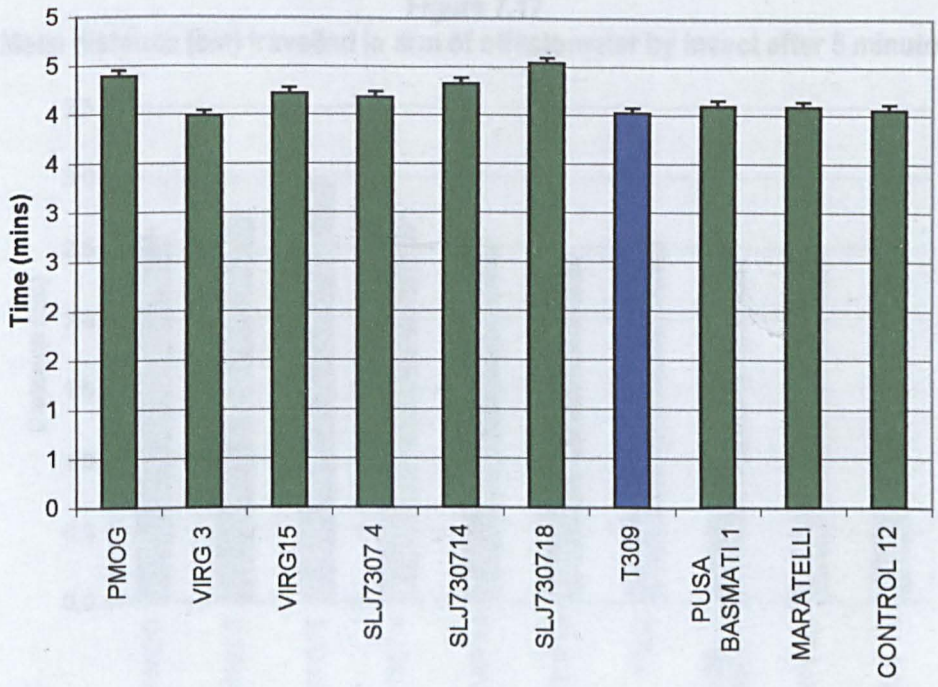


Figure 7.16
Mean time that insect remained in arm of olfactometer containing leaf tissue of transgenic and wild-type plants



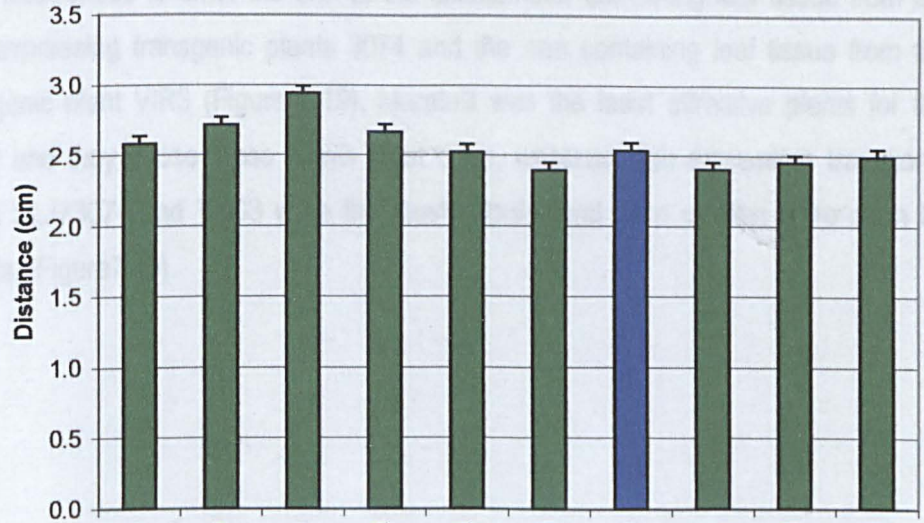
7.3.5 Y-Tube Olfactometer

The insect was placed in the olfactometer for 5 min and the distance the insect had travelled up the arm was measured after that time. A decision to enter an arm was confirmed when the insect had travelled 1cm into that arm. The time taken for the insect to travel 1 cm was also measured. Experiments where the insect had to select between two arms of the olfactometer without any leaf tissue demonstrated that there was no bias towards a particular arm. Experiments using different rice cvs demonstrated a slight preference for the cv. Taipei 309 rice compared to Maratelli and Pusa Basmati, in terms of the distance travelled in the treated arm (Figure 4.17), speed at which the insect travelled towards the leaf tissue and the number of times that the arm containing Taipei 309 leaf tissue was chosen (Figure 4.18).

7.3.5.1 Distance travelled in arm of Y-tube olfactometer

The insect travelled furthest towards leaf tissue of transgenic plant line VirG 15 (Figure 4.17). However, the difference between the distance travelled in the olfactometer treated with wild-type rice leaf tissue and leaf tissues from transgenic lines was not significant.

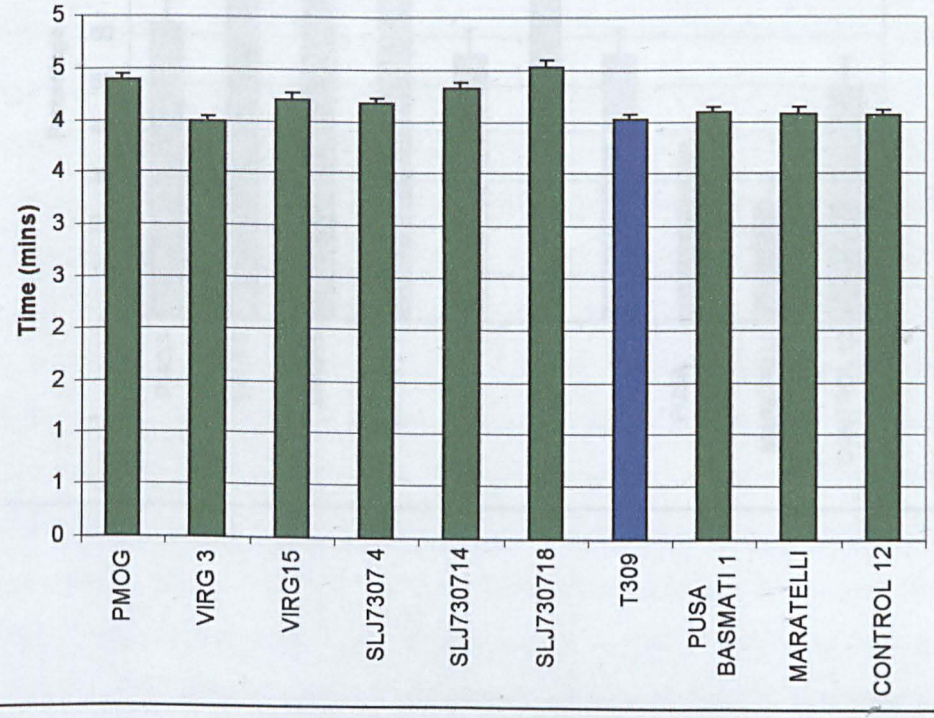
Figure 7.17
Mean distance (cm) travelled in arm of olfactometer by insect after 5 minutes



7.3.5.2 Time taken to travel 1cm

The insects travelled 1 cm into the treatment arm fastest in the arm containing Taipei 309 leaf tissue compared with the other treatments (Figure 4.18).

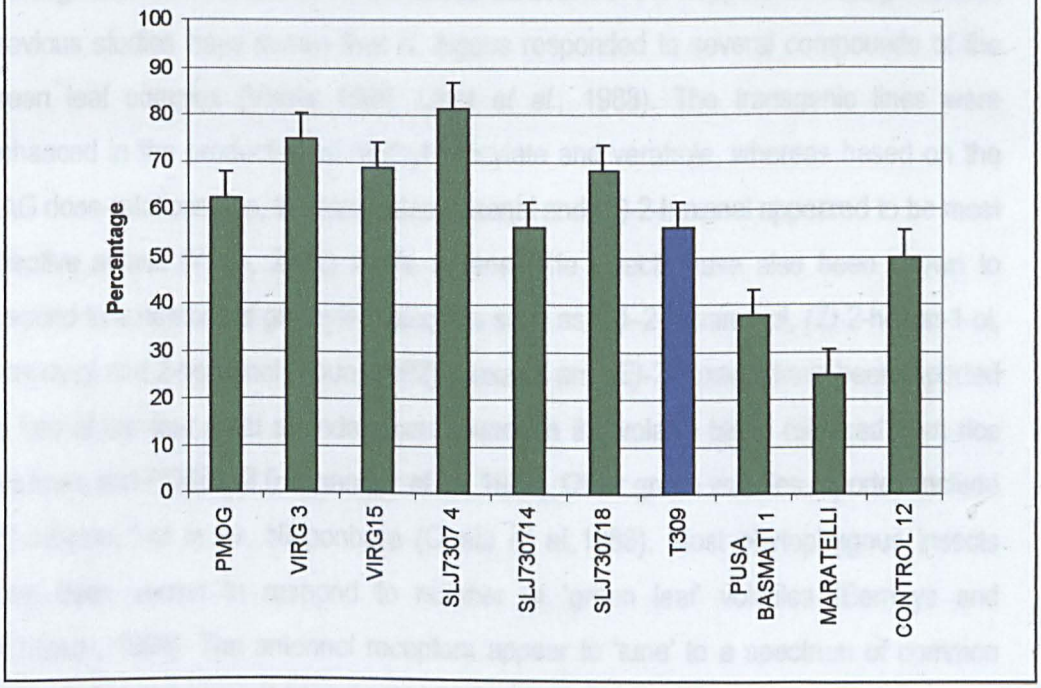
Figure 7.18
Mean time (mins) for insects to travel 1 cm along the treatment arm



7.3.5.3 Choice of plant

The insect chose to enter the arm of the olfactometer containing leaf tissue from the high expressing transgenic plants 3074 and the arm containing leaf tissue from the transgenic plant VIR3 (Figure 7.19). Maratelli was the least attractive plants for the insect and they chose these plants least often, whereas high expressing transgenic plants SLJ73074 and VirG3 were the most popular and were chosen more often by insects, (Figure7.19).

Figure 7.19
Percentage of times plants were chosen by the insect



7.4 DISCUSSION

7.4.1 Response of *Nilaparvata lugens* to volatile mixtures

Although there was evidence for increased attraction of the hoppers for transgenic rice, previous studies have shown that *N. lugens* responded to several compounds of the green leaf complex (Visser 1986, Light *et al.,* 1988). The transgenic lines were enhanced in the production of methyl salicylate and veratrole, whereas based on the EAG dose-relationships, the aldehydes hexanal and *(E)*-2-hexanal appeared to be most effective stimuli (Youn, 2002) for *N. lugens*. The insects have also been shown to respond to a number of green leaf alcohols such as *(E)*–2-hexan-1-ol, *(Z)*-2-hexan-1-ol, 1-hexanol and 2-heptanol (Youn, 2002). Hexanal and *(E)*-2-hexanal have been reported as two of the four most abundant compounds in the volatile blend released from rice cvs Mars and PI346833 (Hernandez *et al.,* 1989). Other green volatiles reported include *(Z)*-3-hexan-1-ol in cv. Nipponbare (Obata *et al.,*1983). Most phytophagous insects have been shown to respond to number of 'green leaf' volatiles (Bernays and Chapman, 1994). The antennal receptors appear to 'tune' to a spectrum of common green leaf volatiles in addition to the tuning of antennal receptors to the insect's own host plant volatiles. (Light *et al.,* 1988) and consequently an increase in two aromatic compounds though it may modify the behaviour of *N. lugens* would be unlikely to control it as the whole spectrum of green leaf volatiles have been shown to contribute to this response. Bioassays and trapping have demonstrated synergistic effects amongst components of a odour blend which enhances attraction of *N. lugens* (Masri, 1995).

7.4.2. Conclusions of present experiments

Nilaparvata lugens spent longer in areas containing transgenic rice leaf tissues. More visits were made to those areas with *Agrobacterium* –mediated transgenic plants and those areas were the first choice of *N. lugens* more frequently. In Y-tube olfactometer experiments *Nilaparvata lugens* chose the arm containing transgenic rice leaf tissue more frequently than the other arm. The insect travelled further towards the transgenic rice leaf tissue more rapidly. Initial experiments in the Pettersson olfactometer and Y-tube olfactometer confirmed that the insect exhibited a consistent, rapid, positive response towards methyl salicylate (1µl of 50nM) and veratrole (1µl of 50nM).

CHAPTER 8. GENERAL DISCUSSION

8.1 MICROPROJECTILE BOMBARDMENT

Despite the size of pSLJ7307 transformation frequency did not appear to be reduced even when the *hpt* gene from pROB5 was ligated into pSLJ7307. This is in contradiction to Marchant and Southgate (1996) who predicted that the probability that the constructs fragment during the preparation of microprojectiles for bombardment or during the bombardment itself, is proportional to the size of the plasmid. This can result in a higher percentage of fragments of DNA integrating into the plant genome, leading to a lower transformation efficiency and disruption of the metabolism and/or morphology of transformed plants. Transgene copy number also has an effect on co-expression of introduced genes (Christou, 1990). In general, increased copy number results in reduced co-expression. However, despite a copy number of 3-10 in transgenic rice plants in these experiments, there appeared no reduction in expression and all transgenic plants exhibited higher salicylate hydroxylase activity than wild-type plants.

8.2 *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF RICE

Microprojectile bombardment transformation has several disadvantages, little control over both the position of the DNA inserted in the genome and the number of copies of the gene inserted (Repellin *et al.*, 2001). *Agrobacterium*-mediated transformation results in discreet, non-rearranged segments of DNA being inserted in the recipient genome at a low copy number (Hiei *et al.*, 1994; Dong *et al.*, 1996). The transformation frequency was similar to that obtained by the methods used routinely for the transformation of dicotyledenous species (20-30%) and was higher than for other direct DNA transfer methods (Azhakanandam, 1999), 1-20% of transgenic plants recovered were normally fertile (Hiei *et al.*, 1997). Similar transformation efficiencies were recorded in the present experiments. Al-Forkan, (2000) found plant regeneration was only possible when water stress was used induced by higher agarose concentration. All cvs. demonstrated higher plant regeneration frequencies on MSB2N medium, containing both BAP and NAA. This protocol was adopted and proved highly successful.

Calli transformed with *A. tumefaciens* LBA4404 supplemented with the constitutive *vir* G mutant gene *vir*GN54D and the binary vectors pSLJ7321::ROB5 and pMOGB22,

produced transient GUS expression in co-cultivated calli but stable integration was not detected in plants. Only two lines were regenerated from this treatment. It would be interesting to verify whether stable integration of several plasmids could be verified following simultaneous *Agrobacterium*-mediated transformation. These results were entirely contradicted for experiments with cv. Maratelli where plants were regenerated only following treatment with LBA4404 (Lenin Kaniayin pers. Com.). This implies that the increased transformation efficiency is cv specific or dependent on the particular binary vector employed. Future experiments to attempt to understand the reasons for this will be useful. Certainly these present experiments have confirmed the results of De fits *et al.* (2000) who found that the use of an additional plasmid (BBR1MCS) carrying an extra *virG* gene enhanced transformation efficiency in species normally recalcitrant to *Agrobacterium*-mediated transformation. Future work to extend this enhanced transformation efficiency to other rice cvs. will be of potential agronomic importance. Rice is the most important crop in the world and, as world population increases, the necessity for increasing yields of rice may make the use of genetically modified rice essential.

8.3 ANTIOXIDANT ENZYMES

Plants expressing the *nahG* gene exhibited higher activity of the enzyme salicylic hydroxylase and lower salicylic acid. Salicylic acid has been shown to inhibit the activity of a number of enzymes such as catalase (Chen *et al.*, 1993) and ascorbate peroxidase (Durner and Klessig, 1995), possibly due to the ability of salicylic acid to chelate iron. Hydrogen peroxide has been shown to have a direct action against pathogens and acts as an intermediary in wound signal cascades (Mauch-Mani and Metréaux 1998). Hydrogen peroxide was lower in transgenic plants than in wild-type plants, corresponding with the increase in peroxide degrading enzymes, ascorbate peroxidase ascorbate oxidase and catalase. In wild-type plant extracts, wounded tissues showed maximal hydrogen peroxide content. Ascorbic acid was significantly lower in transgenic plant samples than in wild-type lines, this was particularly noticeable in plant lines transformed with pSLJ7321:: ROB5 (the VirG and PMOG lines). This ascorbic acid cycle fulfils roles in the adjustment of cellular redox potentials and regulation of gene expression. Exploitation of antioxidants could therefore be used to manipulate cellular redox states. Promoters sensitive to reduced or oxidised forms of glutathione or ascorbate, may indicate redox sensing as central to cellular chemistry of ascorbate.

Environmental stresses that generate reactive oxygen species, such as UV light, ozone, air pollutants, low temperatures, salt stress, drought, heat shock and pathogen attack have been shown to induce plant antioxidant activities (Van Camp *et al.*, 1994). It is therefore possible that the stress tolerance of the transgenic plant lines has been enhanced with elevated SOD activity, elevated catalase activity, elevated ascorbate peroxidase activity. The indications that the antioxidant status of the plants was altered have implications for the defence responses of the plants. The measurement of salicylic acid was rather crude in these experiments and it would be satisfying to verify these results by measuring salicylic acid in the transgenic plants produced in these experiments using HPLC in future experiments. Clearly, antioxidant enzyme pathways are extremely complicated and although the work here has indicated changes in antioxidant status of the plants, more experiments are needed to confirm these changes.

8.4 PLANT DEFENCE

Transcription of PR genes post-wounding was found to be delayed compared to those in wild-type plants. Transcription of genes was found to be optimal 24 h after wounding in wild-type plants, whilst maximum levels of PR proteins accumulated 48 h after wounding for transgenic plants. Transcription of PR genes was also delayed following initiation of SAR by treatment with 2,6-INA. PR gene transcription developed mor rapidly after application of 2,6-INA followed by wounding compared with wounding alone. However, transgenic plants still demonstrated delaying transcription of PR genes compared to wild-type plants. These experiments indicated demonstrated that salicylic acid is necessary for the rapid induction of SAR, but SAR develops eventually even when the ability to accumulate SA is reduced. Cross-talk between gene induction post-wounding and post-induction of SAR was confirmed with a common signal transduction. It is important to remember that these experiments are only indications of SAR and wounding responses. Future experiments with pathogens of rice could confirm whether there is any major effect. Western blots for the proteins of PR genes used in these experiments would also be of value in determining how far delayed transcription delayed protein accumulation.

8.5 INSECT STUDIES

Previous studies of rice plant volatiles have shown little agreement in the host searching behaviour. This could be due to the use of different rice varieties, but also be to the use of different extraction techniques. Responses to rice plants were lower than those recorded for individual chemicals. Perhaps this was due to an enhanced concentration in individual chemicals. Males demonstrated a consistently lower response than females (Youn, 2002).

The response of *N. lugens* to methyl salicylate was erratic from experiment to experiment and adult females exposed to 2.1 $\mu g min^{-1}$ for 20 min exhibited 70% mortality (Masri, 1995). Female hoppers demonstrated a highly significant response to rice plants cv. Balilla ($p \leq 0.001$) in linear track olfactometer experiments (Masri, 1995). There appeared to be an equal attraction of female hoppers to the resistant cv. IR62 (Masri, 1995). The resistance of a rice cv. to attack may therefore be dependent on antifeedant or physical contact stimulus rather than volatile emissions. Kimmins (1989) reported that hoppers on IR62 had a higher probing frequency and reduction in feeding. Possibly the stylet mouthparts were unable to reach the preferred site or factors inhibited sustained ingestion. In EAG experiments Masri (1995) demonstrated that the green leaf odour chemicals *(E)*-2-hexan-1-ol, *(Z)*-3-hexan-1-ol, *(Z)*-2-hexan-1-ol, *(E)*-3-hexen-1-ol, hexanal and *(E)*-2-hexanal and the aromatic compounds methyl salicylate, methyl benzoate and veratrole elicited a electrical response from the antennae of *N. lugens.* Insect bioassay and feeding tests indicated that homozygous lines containing the snowdrop lectin *gna* gene significantly inhibited *N. lugens* by decreasing hopper survival and fecundity, retarding hopper development and declining hopper feeding (Sun *et al.,* 2001). However, initial attraction towards the rice was not inhibited the inhibition of hopper attack relied on the antifeedant and toxic effects of the rice once feeding had commenced. *N. lugens* have been demonstrated to make more probes on resistant than susceptible rice cvs., but probes were of shorter duration and ceased within 5 min (Hattori, 2001). Evidence suggested that the effects of a resistant rice variety were significantly toxic to *N. lugens* adult females, causing up to 98% mortality (Liu *et al.,* 1999).

Field trials and wind tunnel experiments would be advantageous in future studies to establish the preferences of *N. lugens* for transgenic rice plants over-expressing salicylate hydroxylase, enhanced veratrole and methyl salicylate as demonstrated in

bioassays in these experiments. This would account for any abberrant behaviour of the insects in a laboratory setting.

Table 8.1

Summary of plants transformed with microprojectile bombardment using SLJ7307 as high and low expressors

Plant	SAH, APX, CAT, AO SOD, ASa	Veratrole	Methyl salicylate	Insect reaction	Classification of plant activity
SLJ73071	SAH high APX high	High	High	Enhanced	**High expressors**
SLJ73072	AO high ASa high	High	High	Enhanced	**Low expressors**
SLJ73074	APX high ASa high	High	High	Enhanced	**Low expressors**
SLJ73077	SAH high APX high ASa high	High	High	Enhanced	**High expressors**
SLJ730710	SAH high ASa high	High	High	Enhanced	**High expressors**
SLJ730711	SAH high SOD high	High	High	Enhanced	**High expressors**
SLJ730713	SAH high CAT high APX high ASa high	High	High	Enhanced	**High expressors**
SLJ730714	CAT high SOD high	High	High	Enhanced	**Low expressors**
SLJ730715	CAT high ASa high	High	High	Enhanced	**Low expressors**
SLJ730717	APX high	High	High	Less enhanced	**Low expressors**
SLJ730720	SAH Low APX high	Low	Low	Less enhanced	**Low expressors**

Table 8.2

Summary of plants transformed by *Agrobacterium*-mediated transformation with SLJ7321:ROB5 as high and low expressors

Plant	SAH, APX, CAT, AO SOD	Veratrole	Methyl salicylate	Insect reaction	Classification of plant activity
VIRG1	SAH low	Not tested	Not tested	High	**Low**
VIRG2	SAH high CAT high	Not tested	Not tested	High	**High**
VIRG3	SAH high	Not tested	Not tested	High	**High**
VIRG4	CAT high SOD high	Not tested	Not tested	High	**Low**
VIRG5	SAH high	Not tested	Not tested	High	**High**
VIRG10	CAT high	Not tested	Not tested	High	**Low**
VIRG11	SAH high	HIgh	High	High	**High**
VIRG12	SAH high SOD high	Not tested	Not tested	High	**High**
VIRG14	SAH high SOD high	High	High	High	**High**
VIRG16	SAH high SOD high	Not tested	Not tested	High	**High**
VIRG17	SAH high	Not tested	Not tested	High	**High**
PMOG1	AO high ASa high	High	High	High	**High**
PMOG3	SAH high	High	High	High	**High**

8.6 POTENTIAL FOR DEVELOPING A PEST CONTROL STRATEGY

These experiments have indicated that it is possible to enhance the production of volatile chemicals from rice by over-expression of an enzyme that leads to their production. However, the quantity of volatiles produced is only one factor in the complex interaction between the insect and host plant. The evidence of Xu *et al.* (2002) suggested that *N. lugens* responded as much to the mixture of volatiles as the amount of the volatiles released. Indeed, plants colonised by the white-backed planthopper released more volatiles than any other treatments, but had a repellent effect on *N. lugens*. It has been demonstrated that *N. lugens* responds to a number of volatile chemicals of which veratrole and methyl salicylate are highly attractive to the insect (Masri, 1995). If the amounts of these chemicals could be reduced, then the insect may well respond to other 'less attractive' volatiles. Insect attack by *N. lugens* may therefore be reduced, but it is unlikely to be completely eliminated by manipulation of the volatiles released by the host rice plants. In addition to attraction by volatiles, *N. lugens* has been shown to respond to other stimuli from the host plant visual cues and taste has been documented as generating a response from *N. lugens* (Masri, 1995). Whilst it is therefore concluded that suppression of the volatiles from rice plants using a gene silencing approach would have a limited effectiveness in reducing infestation by *N. lugens*, over-expression of the pathway has resulted in an increase in volatiles produced by rice plants. This has the possibility of creating a trap crop for the insect. A trap crop has several advantages over a genetically modified resistant crop and could be grown alongside a crop carrying conventional *BPH/bph* resistance genes to *N. lugens*. Humans would not have to eat genetically modified rice and the pollen would be unlikely to spread. Little pollen was produced from transgenic plants in these experiments and, correspondingly, very few seeds were produced. A small area of the trap crop could be used to lure the insects and then either a limited use of pesticide or biological control with the use of predators and parasitiods could be used to eliminate the pests. This approach would be unlikely to eliminate *N. lugens* entirely, but may reduce the infestation to below the level of economic impact. Additionally, the use of multiple approaches with resistance genes and trap crops with subsequent pest control would ensure that *N. lugens* has to evolve many strategies to overcome those employed by the grower.

8.7 CONCLUSIONS

This work has demonstrated that:

- Microprojectile and *Agrobacterium*-mediated transformation of rice with the *nahG* gene, derived from *Pseudomonas putida* and encoded for salicylate hydroxylase, enhanced activity of the salicylate hydroxylase enzyme. This resulted in increased veratrole production and increased insect attraction.
- Transformation of rice using an *Agrobacterium*-mediated tertiary transformation system developed by De fits *et al.*(2000) for use with *Cathranthus roseus* indicated that this system enhanced transformation efficiency in rice. Although two distinct binary T-DNAs were transferred simultaneously, regenerated plants only demonstrated the presence of the vector they were selected for. Further work with this concept could perfect the technique.
- Transgenic plants with reduced levels of salicylic acid resulted in higher activity of the enzymes catalase and ascorbate peroxidase and lower levels of the substrate hydrogen peroxide despite the increased activity of superoxide dismutase (that generates hydrogen peroxide). Ascorbate oxidase activity was unchanged in transgenic plants. However ascorbic acid converted by ascorbate peroxidase to monodehydroascorbate was demonstrated to be reduced in some transgenic plants. The reduced form of ascorbic acid dehydroascorbate was shown to be increased in these plants. It can therefore be concluded that transgenic plants produced had altered metabolism for antioxidant enzymes and reactive oxygen species.
- Salicylic acid has been implicated as essential in the signal pathway for the expression of plant defence genes to enable the initiation of systemic acquired resistance (Malamy *et al.*, 1990; Métraux *et al.*, 1990) and the octadecanoid pathway (Leon *et al.*, 2001) associated with plant wounding. Transgenic plants with reduced ability to accumulate salicylic acid demonstrated a delay in the transcription of plant defence pathways following wounding and the application of 26, INA. Such plants may therefore be compromised in their ability to respond to pathogen attack and mechanical wounding.

- Gas chromatography indicated that transgenic p[lants produced more volatiles and more of the breakdown products of salicylic acid namely veratrole and methyl salicylate.
- Screening of plant leaf material for enhanced insect attraction in bioassays confirmed that rice plant over-expressing salicylate hydroxylase resulted in increased insect attraction in terms of the number of visits and the time spent in the treatment.

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APPENDIX 1 Media/ Buffer Recipes

LB Medium

Component	Concentration (mg l^{-1})
Sodium chloride	5000
Tryptone	10000
Yeast Extract	5000
Agar (for semi-solid form)	15000
pH 7.0	

LB medium was made up in reverse osmosis water and autoclaved at 120°C for 20 minutes (after Sambrook *et al.*, 1989)

10X TBE Tris Borate EDTA Buffer for electrophoresis Recipe for 10X

108g Tris base
55g Boric acid
9.3g EDTA disodium salt
made up to 1 litre with deionised water

50X TAE Tris Acetate EDTA Buffer for electrophoresis Recipe for 50X

242g Tris base
57.1 ml of glacial acetic acid
18.6g EDTA disodium salt
Made up to a volume of 1 litre with deionised water

Bromophenol Blue Loading Buffer for DNA gle electrophoresis

50% glycerol

0.1% Bromophenol Blue

Made up in 1X TBE

Formulation of AA medium

Macronutrients, Micronutrients, Vitamins and other Supplements

Component	Concentration (mg l^{-1})
Macronutrients	
$CaCl_2$	440.0
KH_2PO_4	170.0
$MgSO_4$	370.0
KCl	2940.0
Micronutrients	
KI	0.830
H_3BO_3	6.200
$MnSO_4$	22.300
$Na_2MoO_4.2H_2O$	0.250
$ZnSO_4.7H_2O$	8.600
$CuSo_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.850
Na_2EDTA	37.250
Vitamins	
Myo-inositol	100.0
Nicotinic acid	0.5
Pyridoxine HCl	0.1
Thiamine HCl	0.5
Glycine	75.0
L-Glutamine	877.0
L-Aspartic acid	266.0
L-Arginine	228.0
Other Supplements	
2,4-dichlorophenoxyacetic acid	2.0
Gibberellic acid	0.1
Kinetin	0.2
Sucrose	30000.0
PH	5.8
Sterilisation	Filter

AA medium was made up as a single-strength solution in reverse osmosis water and filer sterilised through a 0.2 µm pore size membrane.

Formulation of Media – Macronutrients, Micronutrients, Vitamins and other Supplements

Component	Concentration (mg l^{-1})	
	MS medium	**LS2.5 medium**
Macronutrients		
KNO_3	1900.0	1900.0
NH_4NO_3	1650.0	1650.0
$CaCl_2$	440.0	440.0
KH_2PO_4	170.0	170.0
$MgSO_4$	370.0	370.0
Micronutrients		
KI	0.830	0.830
H_3BO_3	6.200	6.200
$MnSO_4$	22.300	22.300
$Na_2MoO_4.2H_2O$	0.250	0.250
$ZnSO_4.7H_2O$	8.600	8.600
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
$FeSO_4.7H_2O$	27.850	27.850
Na_2EDTA	37.250	37.250
Vitamins		
Myo-inositol	100.0	100.0
Nicotinic acid	0.5	
Pyridoxine HCl	0.5	
Thiamine HCl	0.1	0.1
Glycine	2.0	
Other Supplements		
2,4- dichlorophenoxyacetic acid	2.5	2.5
Sucrose	30000.0	30000.0
PH	5.8	5.8

MS, LS2.5 media were made up as a double-strength solution in reverse osmosis water and autoclaved as 121°C for 20 minutes. The media were prepared by mixing equal volumes of double-strength salt-based components of the respective media with 0.8% (w:v) aqueous molten agarose (40°C).

MSB2 Medium (Plant Regeneration/Multiplication Medium)

Component		Concentration (mg l^{-1})
Macronutrients	As in MS medium	As in MS medium
Micronutrients	As in MS medium	As in MS medium
Vitamins	As in MS medium	As in MS medium
Carbon source	Sucrose or maltose	50000.0
PGR's	BAP	2.0
pH 5.8		

MSB2 medium was made up as a double-strength solution in reverse osmosis water and autoclaved at 121°C for 20 minutes. Double-strength MSB2 medium was added to an equal volume of autoclaved 0.8% (w/v) aqueous molten agarose, to make semi-solid MSB2 medium.

Multiplication medium was prepared as above except that 0.8% (w:v) agarose was replaced by 0.2% (w/v) Phytagel.

MSB2N was made with MSB2 medium with the addition of NAA 0.5 mg l^{-1}

MSKN Medium (Plant Regeneration/Multiplication Medium)

Component		Concentration (mg l^{-1})
Macronutrients	As in MS medium	As in MS medium
Micronutrients	As in MS medium	As in MS medium
Vitamins	As in MS medium	As in MS medium
Carbon source	Sucrose or maltose	50000.0
PGR's	Kinetin	2.0
	NAA	0.5

MSKN medium was made up as a double-strength solution in reverse osmosis water and autoclaved at 121°C for 20 minutes. Double-strength MSKN medium was added to an equal volume of autoclaved 0.8% (w/v) aqueous molten agarose, to make semi-solid MSKN medium.

Multiplication medium was prepared as above except that 0.8% (w:v) agarose was replaced by 0.2% (w/v) Phytagel.

SOB Medium

Per litre:

To 950 ml of de-ionised H_2O, add

bacto-tryptone	20 g
bacto-yeast extract	5 g
NaCL	0.5 g

Shake until the solutes have dissolved. Add 10 ml of a 250 mM solution of KCl. (This solution is made by dissolving 1.86 g of KCl in 100 ml of de-ionised H_2O.) Adjust the pH to 7.0 with 5 N NaOH (~ 0.2 ml). Adjust the volume of the solution to 1 litre with de-ionised H_2O. Sterilise by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.

Just before use, add 5 ml of a sterile solution of 2 M $MgCl_2$. (This solution is made by dissolving 19 g of $McCl_2$ in 90 ml of de-ionised H_2O. Adjust the volume of the solution to 100 ml with de-ionised H_2O and sterilise by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.)

SOC Medium

SOC medium is identical to SOB medium, except that it contains 20mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60°C or less and then add 20 ml of a sterile I M solution of glucose. (This solution is made by dissolving 18 g of glucose in 90 ml of de-ionised H_2O. After the sugar has dissolved, adjust the volume of the solution to 100 ml with de-ionised H_2O and sterilise by filtration through a 0.22-micron filter).

APPENDIX 2 Extraction protocols colony hybridisation

Dellaporta DNA isolation method

1. Place tissue in an eppendorf and freeze in liquid nitrogen. Grind the tissue to a fine powder. Add 600 µl extraction buffer and 80 µl 10% SDS. Grind the tissue further and then vortex vigorously for 30 seconds. Add 200 µl of 5M KOAc. Vortex again for 30 seconds and then place on ice for 20 minutes.
2. Spin at 10000g for 15 minutes. Remove supernatent to a fresh eppendorf. If there is any carry over of bits of tissue etc. then spin again briefly in a microfuge and again remove the supernatent to a fresh eppendorf.
3. Add 0.7 volumes of isopropanol, mix thoroughly and place at -20°C for at least 30 minutes. Spin at 10000g for 20 minutes.
4. Remove and discard the aqueous phase leaving the pelleted material. Wash the pellet with cold 70% ethanol and allow the pellet to air dry.
5. Resuspend the pellet in 100 µl of TE buffer. Add 1 µl of RNase (2mg/ml). Mix and incubate at 37°C for 30 minutes.
6. Spin the sample in a microfuge for 1 minute at 13000 rpm to pellet any particulate matter. Remove the aqueous phase to a fresh eppendorf. Add 0.7 volumes of isopropanol and 0.1 volume of 3M NzOAc (pH 5.8). Mix well and place on ice for 10-20 minutes. Spin at 10000 g for 20 minutes. Wash the pellet with cold 70% ethanol and allow the pellet to air dry.
7. Resuspend the DNA in an appropriate volume of TNE buffer (usually between 5 and 20 µl is suitable).

Cetyltrimethylammonium bromide (CTAB) DNA Isolation Method

1. Heat CTAB extraction solution (2% (w/v) CTAb, 100mM Tris-Cl pH 8.0, 20mM EDTA pH 8.0, 1.4M NaCl) to 65^{0}C
2. Add β-mecaptoethanol to CTAB extraction solution to give a final concentration of 2%

All further steps need to be carried out in a fume hood once β-mecaptoethanol has been added. Wear gloves!

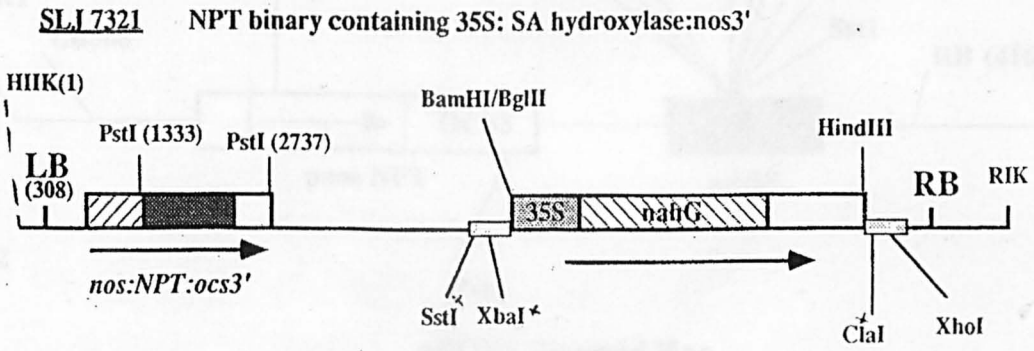
3. Grind plant material using a pestle and mortar using liquid nitrogen transfer to a solvent resistant centrifuge tube
4. Add warm CTAB extraction solution (containing β-mecaptoethanol) to pulverized plant tissue mix add 1% polyvinylpyrrolidine (PVP) to absorb phenolic compounds and incubate for 60 mins.
5. Extract the homogenate with an equal volume of 24:1 chloroform:isoamyl alcohol
6. Mix well and centrifuge 5min at 7500g (8000rpm in a JA-20)
8. Recover the top aqueous phase
9. Add 1/10 vol CTAB/NaCl solution (100mM EDTA pH 8.0 100mM Tris-Cl pH 8.0, 5M NaCl) solution to the recovered aqueous phase and mix well by inversion
10. Add an equal volume of 24:1 chloroform isoamyl alcohol mix well and centrifuge 5 min at 7500g
11. Add exactly 1 vol of CTAB precipitation solution (1% (w/v) CTAB, 50mM tris-Cl pH 8.0, 10mM EDTA pH 8.0) Mix well by inversion if precipitate is visible proceed otherwise incubate for 30min at 65^{0}C
12. Centrifuge for 5 min at 500g (2000rpm in JA-20) at 4^{0}C
13. If there is no pellet then add more precipitation solution incubate overnight at 37^{0}C centrifuge at 500g for 5 mins at 4^{0}C
14. Remove supernatant and resuspend pellet in high salt TE buffer (10mM Tris-Cl pH 8.0, 0.1mM EDTA pH 8.0, 1M NaCl)
15. Precipitate DNA using 3M sodium acetate and 2 vol ice cold ethanol
16. chill at -20^{0}C for 60min centrifuge and collect the DNA pellet

Colony Hybridization method

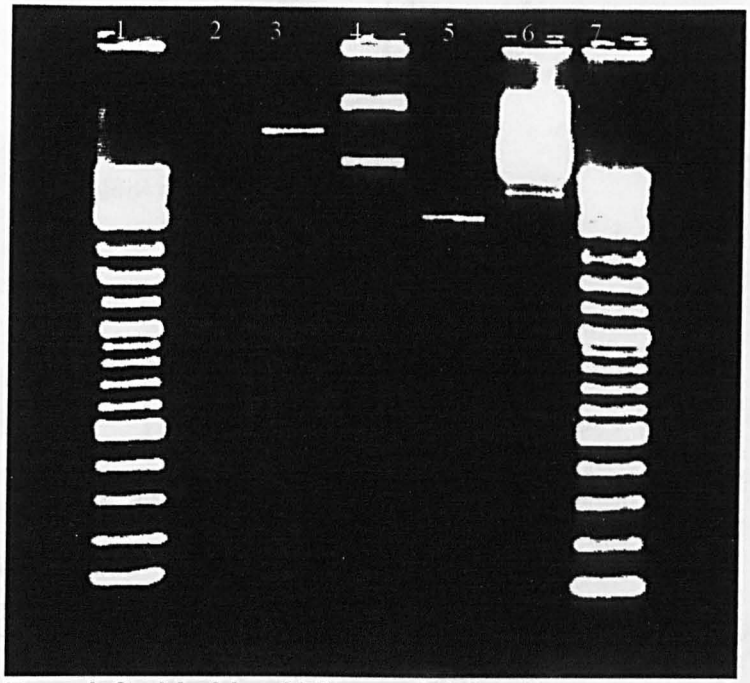
1. Prepare serial 10-fold dilutions of bacteria and spread 100 µL onto LB/Amp plates. Incubate at 37°C overnight. Also plate out positive and negative control bacteria.
2. Select plates that have an optimal density of bacteria (i.e. 2mm spacing between colonies). Put selected plates at 40°C for one hour.
3. Lay 83mm circular nylon membrane on surface of agar plate until it becomes thoroughly wetted. Poke 3 asymmetrical holes through the filters and that agar at the edge of the dish with a large needle for future orientation.
4. Place Saran wrap on bench top. Spot out 0.5 mL puddles denaturing solution (x1) and neutralising solution (x2) for each filter at 6" intervals on Saran wrap.
5. Using forceps, carefully peel nylon membrane from agar surface and place it colony side up onto a puddle of denaturing solution. The colonies should stick to the membrane and not to the plate. Incubate for 5 minutes. Blot it briefly on a paper towel (colony side up) and then transfer it to the first puddle of neutralising solution for 5 minutes. Repeat this with a second puddle of neutralising solution.
6. Cross link the DNA under a UV light (Stratalinker).
7. Rinse the filters in 2X SSC + 0.1% SDS on a rotator.
8. Lay the filer colony side down onto a paper towel. Lay a second paper towel on top and gently press on the filter to blot away most of the lysed bacterial protein. Be careful not to smear the colonies. They can be kept moist or dry until hybridisation.
9. Regrow colonies on the plates by returning them to 37°C for 4 hours and then store them at 40°C.
10. Hybridise the filters and wash them as usual. Monitor the washes with a Geiger counter to be sure that most of the counts have been washed off. If an oligo is used as a probe then the hybridisation should be done in the refrigerator without formamide and the washes should be done at low stringency (1x SSC) at room temperature.
11. Dry the filters and lay them out on an old piece of film. Place florescent markers at the corners of the film. Wrap the film in Saran wrap to hold the filters in place. Expose to film at -70°C for 4 hours to overnight.
12. Develop and dry the autorad. Line up the autorad with the filters such that the fluorescent markers line up precisely. Dot the locations of the filter pinholes on the autorad with a Sharpie. Place the autoraad on a light box. Place the bacterial dishes on the autorad lining up the agar pinholes directly over the ink marks on the film. Pick colonies that correspond to the autorad spots.

pSLJ7321

A BglII + HindIII cassette containing the 35S: SA hydroxylase:nos3' gene was inserted into the binary SLJ 44024 cut with BamHI and HindIII to create SLJ7321



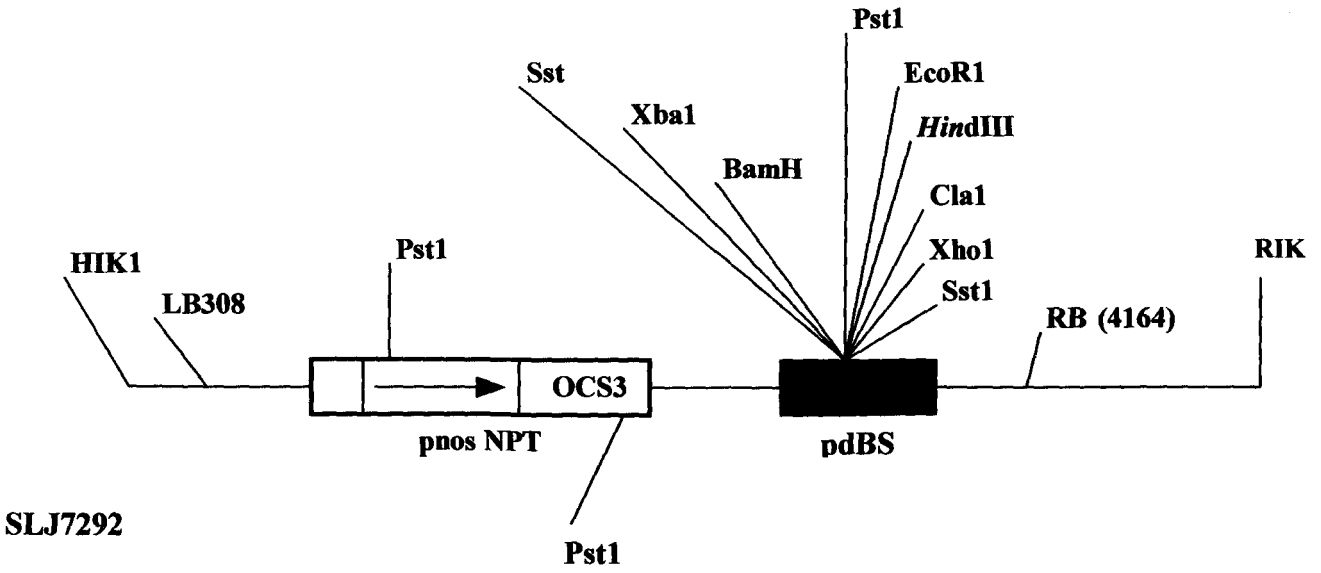
pSLJ7307 did not come with a plasmid map. However it is listed as salicylic acid subclone in pdBS from SLJ7292 at Hind III site. Restriction digest of pSLJ7307 confirmed presence of multiple cloning sites.



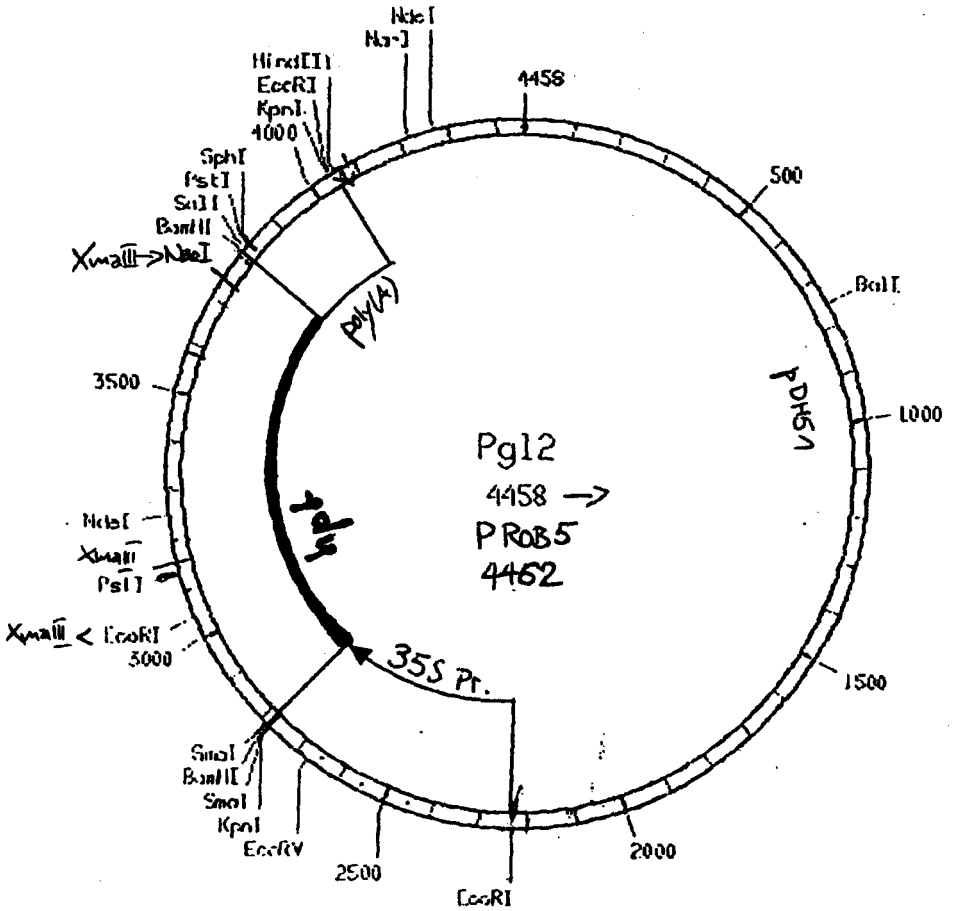
From left 1.ladder 100bp and 1kb

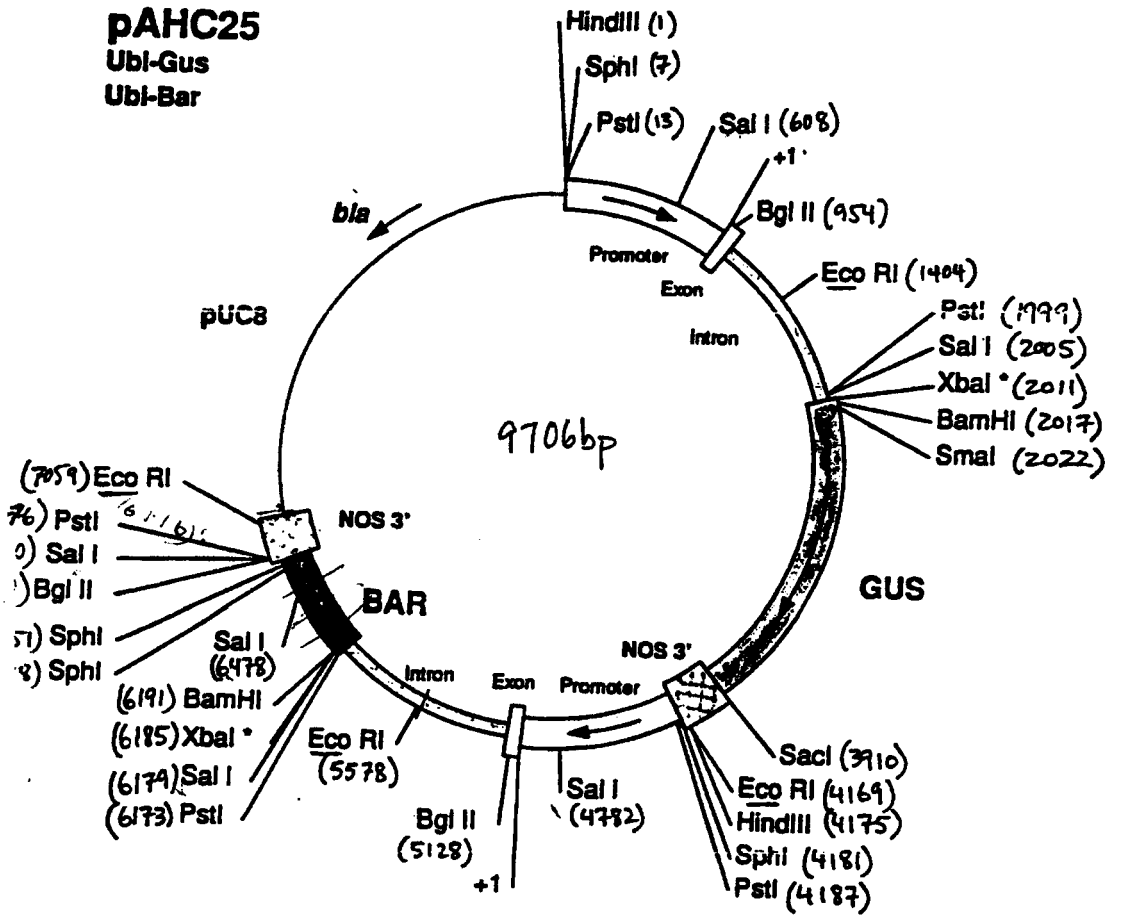
2.SLJ7307, 3.SIJ7307 HindIII restriction, 4.SLJ7321 BamH1 HindIII restriction, 5.SLJ7307 Sac1 restriction, 6.SLJ7307 HindIII Sac1 restriction

SLJ7292



pROB5 Plasmid Map





Primer Sequences

Gene	Accession Number	Product size (bp)	Annealing Temp (°C)	Forward primer 5' to 3'	Reverse Primer 5' to 3'
hpt	EMBL AB003142	374	60	ctg acc tat tgc atc tcc cg	gta ttg acc gat tcc ttg cc
nahG	EMBL AB X83926.	196	65	ctc act ttt ccg gtg agg aa	aga gag ttg gtg gtc ggg atg
pir1 Wheat *WIR1*	embl/M94959/ TAW1R1PR	198	60	acg tgc aca act agc tttc g	gac gtt agg gtc gag agc ac
pr5 thaumatin like protein	embl/X68197/ OSTHLP	196	60	gca gcc agg act tct acg ac	tga tgc att atg ggc aga ag
pbz1	embl/D38170/ OSP1PPBZ1	200	60	ccg gag aag gag aag ga cat	gac tca aac gcc acg aga at