Durham University

Durham E-Theses

The effects of foreign genes in transgenic Thale cress (Arabidopsis thaliana

Evans, Katherine Mary

How to cite:

Evans, Katherine Mary (1991) *The effects of foreign genes in transgenic Thale cress (Arabidopsis thaliana*, Durham theses, Durham University. Available at Durham E-Theses Online: http://etheses.dur.ac.uk/6156/

Use policy

The full-text may be used and/or reproduced, and given to third parties in any format or medium, without prior permission or charge, for personal research or study, educational, or not-for-profit purposes provided that:

- a full bibliographic reference is made to the original source
- a link is made to the metadata record in Durham E-Theses
- the full-text is not changed in any way

The full-text must not be sold in any format or medium without the formal permission of the copyright holders.

Please consult the full Durham E-Theses policy for further details.

Academic Support Office, The Palatine Centre, Durham University, Stockton Road, Durham, DH1 3LE
e-mail: e-theses.admin@durham.ac.uk Tel: +44 0191 334 6107
http://etheses.dur.ac.uk

The Effects of Foreign Genes in Transgenic Thale Cress (*Arabidopsis thaliana*).

The copyright of this thesis rests with the author.
No quotation from it should be published without
his prior written consent and information derived
from it should be acknowledged.

A thesis submitted by Katherine Mary Evans BSc (Hons)
in accordance with the requirements for the degree of
Doctor of Philosophy in the University of Durham.

Department of Biological Sciences, December 1991.

18 AUG 1992

Declaration

I declare that no part of this thesis has previously been submitted for a degree of this or any other university. Also that, unless otherwise indicated, the work presented in this thesis is entirely my own.

The copyright of this thesis rests entirely with the author. No quotation from it should be published without her prior written consent and information derived from it should be acknowledged.

Acknowledgements

I would like to thank the Department of Biological Sciences, Durham University, for the use of their laboratory facilities and Dr John Gatehouse for his supervision over the last three years. I would also like to thank Dr Nigel Robinson for his advice on the $PsMT_A$ project.

I am very grateful to Jackie Spence and John Davies for all their help with the microscopy work and to Paul Sidney and Dave Hutchinson for their photographic work. Also, thank you to Dave Bown for his advice on molecular biology and to Dr Clare Brough for our 'inspirational' tissue culture discussions.

I would especially like to thank Dr Margaret Gibbs for initially, pointing me in the right direction and latterly, for keeping me sane, and Julia Bryden for letting me mess up her lab, make up her solutions and do her washing up (how will you ever replace me?).

This work was funded by a Science and Engineering Research Council Grant.

Abstract

Agrobacterium tumefaciens-mediated transformation was used to introduce foreign genes into *Arabidopsis thaliana* (Thale Cress). Initially a simple marker gene construct (pJIT73) was used to set up the transformation system. Once established, three further constructs were introduced to test all aspects of the system: a gene; a promoter-reporter fusion; and a transposable element.

$PsMT_A$ (isolated from *Pisum sativum* [Evans *et al.*, 1990a]) encodes a polypeptide with strong homology to class I metal-binding proteins or metallothioneins. In order to determine its function *in planta*, $PsMT_A$ was introduced into *Arabidopsis thaliana* under the control of the 35S CaMV promoter. PCR analysis was used to confirm the presence of the engineered gene and its expression. F1 transgenic seedlings, grown on both copper-supplemented and control medium, accumulated copper at a higher concentration than control seedlings. This data suggests that the $PsMT_A$ gene encodes a copper chelating protein.

The structure of specialized plant cell walls varies with their function. Hydroxyproline-rich glycoproteins (HRGPs) or extensins are frequently present in the cell walls of strengthened cell types. A rape extensin gene promoter (*ext*A [Evans *et al.*, 1990b]) fused to a GUS reporter was introduced into *Arabidopsis*, using *Agrobacterium tumefaciens*-mediated transformation. *ext*A expression in the stems of transgenic plants was localized to the phloem and the epidermal tissue (using GUS histochemical staining and an anti-GUS immuno-gold assay).

Transposition of the *Tam*3 element (from *Antirrhinum majus*) has been studied in tobacco (Martin *et al.*, 1989). *Tam*3 was introduced into *Arabidopsis thaliana* in a modified version of pJIT73. Transgenic plants were identified by GUS histochemical staining, immunogold labelling and PCR analysis, and the presence of the *Tam*3 element was determined by genomic blotting. However, no evidence of a *Tam*3 transposition event was conclusive.

Abbreviations

Ala : alanine

Amp : ampicillin

AS : acetosyringone

BAP : 6-benzylaminopurine

B_5 : Gamborgs B_5 medium

BSA : bovine serum albumin

CAT : chloramphenicol acetyl transferase

cDNA : complementary DNA

CIM : callus-inducing medium

$cpm\mu g^{-1}$: counts per minute per microgramme

CTAB : cetyltrimethylammonium bromide

Cys : cysteine

2,4-D : 2,4-dichlorophenoxyacetic acid

dATP : deoxyadenosine 5'-triphosphate

dCTP : deoxycytosine 5'-triphosphate

dGTP : deoxyguanosine 5'-triphosphate

DNA : deoxyribonucleic acid

DNase : deoxyribonuclease

dTTP : deoxythymidine 5'-triphosphate

EDTA : ethylenediaminetetraacetic acid

EtBr : ethidium bromide

Glu : glutamate

Gly : glycine

GM : germination medium

GSH : glutathione

GUS : beta-glucuronidase

His : histidine

HPLC : high pressure liquid chromatography

HRGP : hygroxyproline-rich glycoprotein

IAA : indole-3-acetic acid

2ip : N^6-(2-isopentenyl)adenine

kDa : kiloDalton

kbp : kilobase pair

Kin : kinetin

λ : bacteriophage lambda

M : molar

mRNA : messenger RNA

MS : Murashige + Skoog medium

MT : metallothionein

NAA : 1-naphthaleneacetic acid

NMR : nuclear magnetic resonance

nos : nopaline synthase

NPT-II : neomycin phosphotransferase II

ORF : open reading frame

PCR : polymerase chain reaction

PEG : polyethylene glycol

Pro : proline

psi : pounds per square inch (pressure)

PVP : polyvinyl-polypyrrolidine

RFLP : restriction fragment length polymorphism

RNA : ribonucleic acid

RNase : ribonuclease

rpm : revolutions per minute

RUBP : ribulose bisphosphate

SDS : sodium dodecyl sulphate

Ser : serine

SIM : shoot-inducing medium

SOD : superoxide dismutase

T-DNA : transfer DNA

TE : tris-EDTA

TESPA : 3-aminopropyltriethoxy silane

Tris : tris(hydroxymethyl)methylamine

Tyr : tyrosine

u : unit of enzyme activity

UV : ultra-violet

vir : virulence region

X-gal : 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

X-Gluc : 5-bromo-4-chloro-3-indolyl glucuronide

CONTENTS

	Page
Title	1
Declaration	2
Acknowledgements	3
Abstract	4
Abbreviations	5
Contents	7
List of Figures	13
List of Plates	14
1. **Introduction**	15
1.1 ***Arabidopsis thaliana***	16
1.1.1 The "botanical *Drosophila*"	16
1.1.2 Mutagenesis of *Arabidopsis*	18
1.1.3 Cloned genes from *Arabidopsis*	21
1.1.4 *Arabidopsis* ecotypes	23
1.2 ***Agrobacterium tumefaciens***	24
1.2.1 Transfer DNA	25
1.2.2 The Virulence Region	26
1.2.3 The Chromosomal Virulence Region	27
1.2.4 The transfer and integration of T-DNA	28
1.2.5 *Agrobacterium* vectors	29
1.2.6 *Agrobacterium* transformation of *Arabidopsis*	30
1.2.7 T-DNA insertional mutagenesis in *Arabidopsis thaliana*	32

1.3 **Metallothionein** 34
1.3.1 Metal ions in metabolism 37
1.3.2 Metal-binding polypeptides and proteins in higher plants 39
1.3.3 *$PsMT_A$* 41
1.3.4 Metal-binding proteins in *Arabidopsis* 42
1.3.5 Engineered expression of mammalian MT in plants 42

1.4 **Transposable Elements** 44
1.4.1 Transposon tagging 45
1.4.2 *Tam3* 46
1.4.3 *Tam3* transposition 48
1.4.4 Factors affecting *Tam3* transposition 49

1.5 **Extensin** 50
1.5.1 Cell wall proteins in *Arabidopsis* 52
1.5.2 *extA* 53

1.6 **Aims** 54

2. **Materials and Methods** 55

2.1 **Materials** 56

2.1.1 Glass and plasticware 56
2.1.2 Chemical and biological reagents 56
2.1.3 *Arabidopsis thaliana* 58
2.1.4 *Agrobacterium tumefaciens* 58
2.1.5 Constructs 58
2.1.6 Primers 58

2.2 **Methods** 59

2.2.1 **Bacterial manipulations** 59

2.2.1.1 Bacterial culture media 59
2.2.1.2 Bacterial glycerol preparation 59

2.2.1.3 Bacterial transformation 60
2.2.1.4 Bacterial transformation (maximum efficiency cells) 60
2.2.1.5 Triparental mating 61

2.2.2 **DNA extraction** 62

2.2.2.1 Plasmid miniprep 62
2.2.2.2 Plasmid miniprep for sequencing 62
2.2.2.3 *Agrobacterium* plasmid miniprep 63
2.2.2.4 *Arabidopsis* DNA preparation 64
2.2.2.5 Glassware siliconization 65
2.2.2.6 Rapid plant genomic DNA extraction for PCR analysis 65

2.2.3 **DNA manipulations** 66

2.2.3.1 RNase digestion 66
2.2.3.2 Phenol/chloroform extraction 66
2.2.3.3 Silica fines extraction 67
2.2.3.4 DNA restriction digestion 68
2.2.3.5 Plate gel preparation 68
2.2.3.6 Mini-gel preparation 69
2.2.3.7 Electroelution 69
2.2.3.8 DNA visualization and photography 70
2.2.3.9 Estimation of DNA content using the spectrophotometer 70
2.2.3.10 Southern blotting 70
2.2.3.11 Colony hybridization 71
2.2.3.12 Filter prehybridization and hybridization 72
2.2.3.13 ^{32}P probe production 72

2.2.4 **DNA cloning** 73

2.2.4.1 T_4 DNA polymerase blunt-ending 73
2.2.4.2 Blunt-end cloning 74
2.2.4.3 Dephosphorylation of DNA 74
2.2.4.4 Shotgun cloning 75
2.2.4.5 Polymerase chain reaction 75
2.2.4.6 Oligonucleotide synthesis 76
2.2.4.7 Rapid mRNA extraction and cDNA synthesis 76

2.2.5 ***Arabidopsis* transformation and tissue culture** 77

2.2.5.1 Sterile techniques 77
2.2.5.2 Culture of axenic plants 78
2.2.5.3 *Agrobacterium* preparation for transformation 78
2.2.5.4 Acetosyringone induction of *Agrobacterium* 78
2.2.5.5 Root transformation 79
2.2.5.6 Kanamycin kill levels for root tissue 80
2.2.5.7 Metal toxicity levels of germination 80

2.2.6 **Histochemical GUS assay procedure** 80

2.2.7 **Tissue sectioning** 81

2.2.7.1 Fixation 81
2.2.7.2 Dehydration 81
2.2.7.3 Embedding in wax 81
2.2.7.4 Tissue sectioning and mounting 82

2.2.8 **Immuno-labelling** 82

2.2.8.1 Immuno-gold labelling 82
2.2.8.2 Silver enhancing 83

2.2.9 **Metal analysis by flame spectrophotometry** 83

3. **Results** 84

3.1 **Establishing a transformation method for *Arabidopsis*** 85

3.1.1 Kanamycin kill levels of *Arabidopsis* 85
3.1.2 *Agrobacterium tumefaciens* C58/3 85
3.1.3 pJIT73 86
3.1.4 Production of *Agrobacterium tumefaciens* pJIT73 88
3.1.5 C58/3 pJIT73 transformation of *Arabidopsis thaliana* 90

Analysis of putative transformants 93
3.1.6 DNA analysis of putative transformants 93

3.1.7 Histochemical GUS assay 95
3.1.8 Immuno-gold anti-GUS labelling 95
3.1.9 Conclusion 95

3.2 Transformation of *Arabidopsis* with the putative plant metallothionein gene *$PsMT_A$* (from *Pisum sativum*) 98

3.2.1 PCR amplification of *$PsMT_A$* 98
3.2.2 Cloning *$PsMT_A$* into pUC18 100
3.2.3 Cloning *$PsMT_A$* into pROKII 102
3.2.4 Production of the *Agrobacterium tumefaciens* *$PsMT_A$* strains 104
3.2.5 C58/3 *$PsMT_A$* transformation of *Arabidopsis thaliana* 107

Analysis of putative transformants 108
3.2.6 Detection of *$PsMT_A$* by PCR 108
3.2.7 Detection of *$PsMT_A$* mRNA expression 110
3.2.8 Quantification of mRNA by competitive PCR 110

Analysis of F_1 generation 115
3.2.9 Single leaf *$PsMT_A$* PCR 115
3.2.10 Detection of *$PsMT_A$* mRNA expression 117
3.2.11 Seed germination on zinc-containing medium 118
3.2.12 Seed germination on cadmium-containing medium 118
3.2.13 Seed germination on copper-containing medium 119
3.2.13 Metal analysis of *Arabidopsis thaliana* 120

3.3 Transformation of *Arabidopsis* with the *Antirrhinum* transposable element *Tam3* 127

3.3.1 Cloning *Tam3* into the pJIT73 vector 127
3.3.2 Production of the *Agrobacterium tumefaciens* *Tam3* strain 129
3.3.3 C58/3 *Tam3* transformation of *Arabidopsis thaliana* 132

Analysis of putative transformants 131
3.3.4 Single leaf GUS PCR analysis 131
3.3.5 Histochemical GUS assay 131
3.3.6 Immuno-gold anti-GUS labelling 134

3.3.7 Description of transformants and progeny 134
3.3.8 *Tam3* genomic blot 134

3.4 Transformation of *Arabidopsis* with a promoter-GUS fusion containing the promoter from the rape extensin gene *extA* 138

3.4.1 pAS44/45 138
3.4.2 Production of *Agrobacterium tumefaciens* pAS44/45 strains 140
3.4.3 C58/3 pAS44/45 transformation of *Arabidopsis thaliana* 142

Analysis of putative transformants 142
3.4.4 DNA analysis 142
3.4.5 Histochemical GUS expression 144
3.4.6 Immuno-gold anti-GUS labelling 144

4. Discussion 147

4.1 Summary of results 148

4.2 **Metallothionein** 149
4.2.1 Metallothionein / zinc interactions 149
4.2.2 Metallothionein / iron interactions 150
4.2.3 Metallothionein / copper interactions 152
4.2.4 Expression of $PsMT_A$ in *Arabidopsis* 154

4.3 ***Tam3*** 158
4.3.1 Dual element transposon tagging systems 160

4.4 **Extensin** 163
4.4.1 Analysis of *extA* plants 166

4.5 **Conclusion** 168

5. **References** 170

List of Figures

1. pJIT73 T-DNA 87

2. The sequence of $PsMT_A$ 99

3. p+$PsMT_A$ and p-$PsMT_A$ T-DNAs 103

4. p+$PsMT_A$ and p-$PsMT_A$ *Hin*dIII restriction sites 105

5. The comparative sequences of $PsMT_A$ and $PsMT_B$ 113

6. Copper concentrations of control and putative transgenic $PsMT_A$ Arabidopsis (copper-supplemented and non-copper-supplemented medium) 121

7. Iron and zinc concentrations of control and putative transgenic $PsMT_A$ *Arabidopsis* (copper-supplemented and non-copper-supplemented medium) 124

8. Comparison of copper, zinc and iron contents of control and putative transgenic seedlings (non-copper supplemented growth) 126

9. p*Tam*3 T-DNA 128

10. pAS44 and pAS45 T-DNAs 139

List of Plates

1.	Autoradiograph of C58/3 pJIT73 triparental mating	89
2.	Regeneration sequence of root explants	91
3.	Autoradiograph of PCR-amplified GUS from putative transgenic *Arabidopsis*/pJIT73	94
4.	Stem sections of transgenic *Arabidopsis*/pJIT73 Histochemical GUS expression	96
5.	Stem section of transgenic *Arabidopsis*/pJIT73 Immunogold GUS assay	97
6.	Autoradiograph of PCR-amplified $PsMT_A$ from pλS2	101
7+8.	Autoradiographs of C58/3 p$PsMT_A$ triparental mating	106
9.	Autoradiograph of PCR-amplified $PsMT_A$ from putative transgenic single leaf DNA	109
10.	Autoradiograph of PCR-amplified $PsMT_A$ mRNA expression	111
11.	Autoradiograph of competitively PCR-amplified $PsMT_A$/$PsMT_B$	114
12.	Autoradiograph of PCR-amplified $PsMT_A$ DNA and cDNA from F1 generation putative transgenic *Arabidopsis*	116
13.	Autoradiograph of C58/3 p*Tam*3 triparental mating	130
14.	Autoradiograph of PCR-amplified GUS from putative transgenic *Arabidopsis*/p*Tam*3	132
15.	Stem sections of transgenic *Arabidopsis*/p*Tam*3 Histochemical GUS assay	133
16.	Stem section of transgenic *Arabidopsis*/p*Tam*3 Immunogold GUS assay	135
17.	Autoradiograph of *Tam*3 genomic blot of *ASP*718-restricted putative transgenic *Arabidopsis*/p*Tam*3	137
18.	Autoradiograph of C58/3 pAS44/45 triparental mating	141
19.	Autoradiograph of PCR-amplified GUS from putative transgenic *Arabidopsis*/pAS44/45	143
20	Stem section of transgenic *Arabidopsis*/pAS44 Immunogold GUS assay	145

1. INTRODUCTION

1.1 *Arabidopsis thaliana*

Arabidopsis thaliana (Thale cress) is a small weed belonging to the *Cruciferae*. It is related to several important crops, such as cabbage, broccoli and horseradish, but is itself of no economic value and is typically found as a colonizing weed on cleared or waste ground. Although the exact geographic origin of *Arabidopsis* is unknown, it now grows well in many regions around the world, ranging from the tropics to northern Scandinavia (Meyerowitz, 1987).

The mature plant consists of a basal rosette of leaves, with a main stem and several secondary stems, each topped by an inflorescence. When grown under ideal conditions, the plant can reach 30 to 40cm high before flowering. The flowers are typical of the mustard family, consisting of four sepals alternating with four white petals, combining to produce a flower approximately 3mm long and 1mm across. The flowers normally self-fertilize and develop into fruits, known as siliques, each containing 30-60 small seeds. On average, a plant produces around 5×10^3 to 10^4 of the tiny seeds, which each weigh approximately 20µg.

1.1.1 The "botanical *Drosophila*".

Laibach, in 1943, first noted the features of *Arabidopsis* that make it suitable for laboratory work, and have led to it being referred to as the "botanical *Drosophila*" (Whyte, 1946). As the mature plant is so small and compact, hundreds can be grown in a relatively small space, either in sterile media or in soil. When grown in continuous illumination, it requires only 5 to 6 weeks to progress from seed to seed, therefore screening of seedlings is rapid and

inexpensive.

The use of *Arabidopsis* as a laboratory plant has grown with the advances in molecular biology. Assays of *Arabidopsis* DNA content indicate a haploid nuclear genome size of approximately $7x10^7$bp, which is organized into only 5 chromosomes. The contrast with other plants used in molecular genetic work is striking: tobacco has a haploid genome of $1.6x10^9$bp; the pea haploid genome is $4.5x10^9$bp; and the wheat haploid genome is $5.9x10^9$bp. Analysis of *Arabidopsis* DNA reassociation kinetics and cross-hybridization of genomic clones indicates that the nuclear genome has a very low content of repeat sequences, and any that are present are usually well dispersed (Meyerowitz, 1989; Somerville *et al.*, 1985).

It hardly seems surprising that so many research programmes have been set up using *Arabidopsis*, its size, lifestyle and genome size making it the ideal model plant system for isolation of genes which have been identified by mutated phenotype, but for which the conventional isolation method via cDNA is not applicable, or is very difficult. The molecular isolation of such genes by chromosome walking has been facilitated by the availability of restriction fragment length polymorphism maps (Nam *et al.*, 1989) and the construction of yeast artificial chromosomes containing large inserts of *Arabidopsis* DNA (Ward and Jen, 1990; Grill and Somerville, 1991). However, an alternative approach to gene isolation is possible using insertional mutagenesis. The use of endogenous and foreign transposable elements has been examined but their feasibility remains to be established (Schmidt and Willmitzer, 1989). *Agrobacterium* T-DNA insertional mutagenesis has, however, already been successfully used to isolate *Arabidopsis* genes (Koncz *et al.*, 1990; Feldmann *et al.*, 1989).

1.1.2 Mutagenesis of *Arabidopsis*

The initial genetic studies on *Arabidopsis*, and the vast majority of the genetic information reported, involved the use of various mutagens to isolate visible, biochemical and physiological mutants. Seeds are usually soaked in solutions of potent mutagens, such as ethyl methane sulphonic acid ester (EMS), or exposed to X-rays to induce mutations. Each (M1) seed contains only a few cells that give rise to the reproductive structures of the mature plant. A mutation in one of these cells will be replicated in its progeny, and will thus be present in heterozygous form in a sector of the mature plant. When the flowers that arise from this mutant sector self-fertilize, M2 seeds are produced. One-quarter of them are homozygous for the newly induced mutation, and two-thirds of the remaining seeds are heterozygous. Mutagenesis of a large number of seeds can be done in small volumes owing to the small size of the *Arabidopsis* seeds, and the small size of the mature plant allows many thousands of potential mutants to be screened.

Morphological variants comprise the largest class of characterized mutants. Mutations affecting virtually all parts of the plant are known (Meyerowitz and Pruitt, 1984): leaf shape (Rédei and Hirono, 1964); leaf hairs (Koornneef *et al.*, 1982); disposition and size of stems (Rédei, 1962; Koornneef *et al.*, 1983). Many mutations in the flower, and the genes controlling its development, have been characterized (Komaki *et al.*, 1988), the better known being the *agamous* mutants (Bowman *et al.*, 1991; Yanofsky *et al.*, 1990) and the *apetala* mutants (Irish and Sussex, 1990). A number of male-sterile mutants have also been characterized and are now being cloned (Wilson *et al.*, 1991; Chaudhury *et al.*, 1991).

The mutations most easily found are those that result in an embryo-lethal phenotype, as siliques mature with about one-quarter of the seeds aborted. The high frequency of embryo-lethal phenotypes produced indicates the large number of genes involved in embryogenesis; one group of researchers at Oklahoma State University have already isolated and characterized over 90 recessive mutants of *Arabidopsis* defective in embryo development (Patton *et al.*, 1991).

Another class of mutants characterized are those which affect the ability of the plant to synthesize or respond to growth regulators. It is hoped that the information derived from these mutants will contribute greatly to the understanding of the complex biosynthesis pathways and the mode of action of the growth regulators. Mutants have been isolated that affect, for example, the synthesis of gibberellins and auxins (Koornneef *et al.*, 1985); that fail to respond to ethylene (Bleecker *et al.*, 1988); and that are resistant to auxin (Estelle and Somerville, 1987).

Mutants have been characterized in the nitrogen metabolism pathway (eg, nitrate reductase [Crawford *et al.*, 1988]) and in several of the amino acid biosynthetic pathways (Klee *et al.*, 1987; Haughn and Somerville, 1986). Photosynthesis research has also progressed with the production of plants deficient in enzyme activities. One of the mutants isolated proved to be due to a mutation in a previously unsuspected chloroplast enzyme, RUBP carboxylase/oxygenase activase (Somerville and Ogren, 1982). Aspects of phytochrome activity have also been characterized using *Arabidopsis* mutants (Chory *et al.*, 1989; Parks *et al.*, 1989). Other isolated mutations include ones affecting lipid biosynthesis (Browse *et al.*, 1987; Browse, 1991), starch biosynthesis (Lin *et al.*, 1988), nucleotide synthesis (Moffatt and Somerville, 1988), and phototropism and gravitropism (Trisno and

Burke, 1987; Khurana and Poff, 1988).

Once characterized, these mutant genes need to be cloned. Many of these genes have unknown products so cloning them is very difficult. One strategy is to map the genes to a position on the RFLP map, and then to use the nearest polymorphic clone as a starting point for a chromosomal walk to the gene. As cloned *Arabidopsis* genes can be reintegrated into the genome and function normally, an assay for DNA regions cloned by chromosomal walking is available. The yeast artificial chromosome library (Ward and Jen, 1990) should greatly facilitate the cloning of genes through chromosome walking, and a physical map of the *Arabidopsis* genome is also being developed (Goodman *et al.*, 1989) to simplify the task of cloning genes identified by mutation. It should be possible, using these resources, to clone any gene identified by mutation in *Arabidopsis* and to examine the relationship between mutant phenotype and gene function in higher plants.

However, *Arabidopsis* has several shortcomings when it comes to mapping genes compared to other model systems. It has an absence of well-characterized deletions, duplications, inversions, and temperature-sensitive mutants which are present, and aid mapping, in other systems, eg., *Drosophila* and *Caenorhabditis elegans*. *Arabidopsis* also lacks the accessory (B) chromosomes that have facilitated linkage detection and the analysis of dosage compensation in maize (Coe *et al.*, 1988). Trisomic and telotrisomic lines have been isolated from *Arabidopsis* but have been used primarily to localize centromeres and to identify linkage groups. Complementation tests are also of limited use due to the considerable amount of time (and patience) required to make pairwise crosses between large groups of plants. It is because of this difficulty to assign a large number of mutants to map positions

that only a fraction of the mutant genes characterized so far have actually been mapped or cloned.

1.1.3 Cloned genes from *Arabidopsis*

Several *Arabidopsis* genes have now been cloned and characterized, and their structure and low copy number has led to various conclusions about the organization of the *Arabidopsis* genome. *Arabidopsis* gene families generally appear to have fewer genes than homologous families in other plant species. The genes also appear to be more compact than their homologues in other species, with fewer and smaller introns. It seems unlikely that this difference in gene structure and the number of homologues is the only factor that causes the 20-fold difference in magnitude of genome sizes but it obviously contributes to the small genome.

One of the first *Arabidopsis* genes cloned and sequenced was that coding for alcohol dehydrogenase (Chang and Meyerowitz, 1986), obtained by reduced-stringency cross-hybridization with the maize *Adh1* gene. Further analysis (Dolferus and Jacobs, 1984) has shown that, in contrast to other flowering plants, *Arabidopsis* has only one such gene. DNA sequence analysis reveals a gene interrupted by 6 introns which are identical in position to 6 of the 9 introns present in the other plant *Adh* genes. Although it encodes a protein of 379 amino acids like the other plant genes, the *Arabidopsis Adh* gene requires approximately 3-fold less DNA than the other genes.

A second class of cloned *Arabidopsis* genes are the chlorophyll a/b (*cab*) light harvesting protein genes of photosystem II (Leutwiler *et al.*, 1986). Three *cab* genes were identified by cross-hybridization

to a *cab* probe from *Lemna* (an aqueous monocot). Other species examined have shown many more homologues: *Petunia* contains at least 16 *cab* genes; 8 in pea; 7 in wheat and 10-12 in *Lemna*. All 3 *Arabidopsis* genes are coded within the same 6.5kb DNA region and all code for identical mature proteins. No introns are evident. More recently new *cab* genes have been isolated, a PSI *cab* gene and also a new smaller *cab*-like gene, called *cab4* (Zhang *et al.*, 1991). The multigene family encoding the small subunit polypeptides of ribulose-1,5-bisphosphate carboxylase/oxygenase has also been isolated and characterized (Krebbers *et al.*, 1988).

Other cloned genes include 12S-globulin seed storage protein genes (Meyerowitz, 1987), the 5-enolpyruvylshikimate-3-phosphate synthase gene (Klee *et al.*, 1987) and the α- and β-tubulin gene families (Ludwig *et al.*, 1987; Marks *et al.*, 1987b). Many other genes have been cloned using the cross-hybridization technique and are reviewed by Meyerowitz (1987). More recent additions to the list, include two auxin-inducible genes, isolated using soybean cDNA probes, (Conner *et al.*, 1990), the calmodulin clones (from the calcium mediated signal transduction pathway) isolated using barley calmodulin cDNA probes (Ling *et al.*, 1991) and the *Arabidopsis* gene encoding the 10kDa polypeptide of PSII (Gil-Gómez *et al.*, 1991).

With the development of PCR cloning techniques, several more *Arabidopsis* gene families have been isolated. Plazinski *et al.* (1991) have isolated a family of *Arabidopsis* myosin genes using PCR. At least 10 *Arabidopsis* actin genes have also been revealed (Meagher, 1991). *Arabidopsis* lends itself well to this technique, with its lack of repetitive DNA to interfere with the PCR primers and the small amount of DNA necessary for the reaction. One of the problematic aspects with *Arabidopsis* is the difficulty in extracting large amounts of DNA with

the small size of the genome and the small amount of tissue available, therefore with limiting DNA, the PCR technique is more likely to succeed than the cross-hybridization technique.

1.1.4 *Arabidopsis* ecotypes

The use of *Arabidopsis* in the laboratory is now so widespread that an *Arabidopsis* information service has been established which regularly publishes journals. A collection has also been published listing all the ecotypes with their individual characteristics and various strengths for laboratory work. The most commonly used ecotypes in molecular biology are the Columbia and Landsberg *erecta* plants. The homozygous recessive mutation *erecta* was added to the Landsberg strain to produce a more compact plant, with greater suitability for large-scale laboratory work. The ecotype Columbia was also derived from the Landsberg strain by Rédei (Pruitt and Meyerowitz, 1986).

Arabidopsis collection number C24 (the ecotype used in this project) is often known as Columbia; however C24 plants, when mature, are glabrous (have no trich omes) whereas the true Columbia ecotype has pubescent (hairy) stems and leaves.

1.2 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens was found to be the causative agent of crown gall tumours as long ago as 1907 (Smith and Townsend). This gram-negative soil phytopathogen infects at least 150 plant genera, producing tumours on most dicotyledonous and several monocotyledonous species.

The tumour production was found to be due to the presence of a large (140-235kb) plasmid, the tumour inducing (Ti) plasmid (Zaenen *et al.*, 1974). Bacteria that have been cured of their Ti plasmid are no longer able to induce tumours but when conjugated with a virulent strain, the tumour inducing capacity is restored.

In addition to tumourigenesis, the Ti plasmid encodes a number of other functions, including the production of phytohormones and one of a variety of unusual amino acid derivatives (Braun and Wood, 1976), specific to the bacteria responsible for inciting the tumour, collectively known as opines (for example, octopine and nopaline). The ability to synthesize and catabolize the various opines may be used to classify the Ti plasmids.

During infection, a short portion of the Ti plasmid (about 20kb), known as the transfer (T) DNA, is transferred from the plasmid to the plant cell. Analysis of crown gall tumour lines (Chilton *et al.*, 1980), has shown that T-DNA integrates into the nucleus of the susceptible plant, resulting in the permanent transformation described above.

The transfer process is partly controlled by the products of a region of the Ti-plasmid, known as the virulence (*vir*) region. Also involved in the transfer process, is a region of chromosomal DNA, known as the chromosomal virulence region (*chv*).

1.2.1 Transfer DNA

For the excision and transfer of the T-DNA to be precise, there must be sequences which define its borders, that are recognized by the *Agrobacterium*. Genetic analysis has shown that the T-DNA is bounded by two essentially identical 25bp direct repeats (Simpson *et al.*, 1982), the right and left borders. While deletion of the left border has no significant effect on pathogenicity, deletion of the right border will totally abolish it (Shaw *et al.*, 1984). The orientation of the right border has also to be conserved, when reversed the efficiency of transfer is greatly attenuated. This suggests that T-DNA transfer is from right to left and that the direction is determined by the border sequences. Slight differences in sequence, added to the effects of the surrounding sequences (for example, the *overdrive* enhancer [Van Haaren *et al.*, 1987]) enhance right and attenuate left border activity, resulting in polar DNA transfer (Jen and Chilton, 1986).

T-DNA sequence analysis has shown that although part of a prokaryotic bacterium, its genes have eukaryotic-like promoters, lack introns and contain a polyadenylation signal towards their 3' ends. By inducing site-directed mutations and analysing transcripts, several loci within the T-DNA have been identified. The opine synthase and secretion genes have been located (De Greve *et al.*, 1982; Bevan *et al.*, 1983; Messens *et al.*, 1985) as has the gene conferring the *Agrobacteriums* oncogenicity towards the plant host (Hooykaas *et al.*, 1988).

The *tmr*, *tms* and *tml* loci (involved in auxin and cytokinin biosynthesis) have also been identified from T-DNA (Reams *et al.*, 1983) which explains why plant cells transformed with Ti T-DNA become independent of these growth regulators and form tumourous growths

(Binns and Thomashow, 1988).

1.2.2 The Virulence Region

The *vir* region is a 35kbp non T-DNA linked region which provides most of the *trans*-acting functions for T-DNA transfer. Expression of *vir* is tightly regulated, therefore it is the activation of this region which initiates the transfer process. *Vir* activation has been linked to certain phenolic exudates, such as acetosyringone (AS) and its α-hydroxy derivative, which are produced by actively metabolizing wounded plant cells (Stachel *et al.*, 1985; Stachel *et al.*, 1986a). Other groups have identified lignin precursors such as coniferyl alcohol and sinapinic acid (Hooykaas, 1989) and even some flavonoids which may act as *vir* inducers.

Extensive analysis of the *Vir*-region of a nopaline C58 strain has shown that it is organized into at least six separate complementation groups that are either essential for (*vir* A, B, D and G) or that enhance the efficiency of (*vir* C and E) plant cell transformation.

The *vir*A and *vir*G proteins appear to function as a chemoreceptor, sensing the presence of the wound exudates (Leroux *et al.*, 1987) and transmitting the information to the inside of the bacterium. *vir*E encodes DNA binding proteins (Christie *et al.*, 1988) which cover single-stranded T-DNA molecules, protecting them from cellular nucleases and facilitating the transfer of the T-strands through bacterial membranes (Citovsky *et al.*, 1989). The functions of the *vir*B products are unknown but it has been suggested that their role may be in directing T-DNA transfer through the bacterial membrane

possibly by the formation of a pore (Engstrom *et al.*, 1987).

Mutant analysis suggests that VirD1 and VirD2 act as a site-specific endonuclease that recognizes and cleaves the lower strand of the T-DNA border sequences at the 3rd and 1st nucleotide positions allowing T-DNA release by strand-displacement DNA synthesis (Albright *et al.*, 1987). The *vir*D operon also encodes a DNA-relaxing enzyme, similar to a topoisomerase (Ghai and Das, 1989).

The virulence-related-proteins (VRPs) are also induced in *Agrobacterium* grown in the presence of AS (Engstrom *et al.*, 1987; Zambryski *et al.*, 1989), but their roles, as yet, remain to be determined.

1.2.3 The Chromosomal Virulence Region

The chromosomal virulence genes are expressed constitutively, which implies perhaps some additional roles in other bacterial-plant interactions. So far, the *chv*-region has been shown to contain four different loci which are involved in attachment of *Agrobacterium* to plant cells.

*chv*A is thought to encode a transport system which excretes the cyclic β-1-2-glucan synthesized by *chv*B (and affected by the *pscA* locus [Marks *et al.*, 1987a]) into the periplasm (Cangelosi *et al.*, 1989). The *cel* locus synthesizes the cellulose fibrils which cause the bacteria to cluster when attaching to the plant cells, thus increasing their infectivity (Matthysse, 1983).

The *att* locus, is not as well defined but affects cell surface proteins involved in bacterial attachment (Matthysse, 1987). The *ros* gene product specifically represses the *vir*C and *vir*D operons in the

vir region of the Ti plasmid, possibly by competition for binding sites (Cooley *et al.*, 1991).

1.2.4 The transfer and integration of T-DNA

The function of the T-DNA borders can be more clearly shown by the generation of a free transferable T-DNA copy, following the activation of *vir* expression. Using the two border nopaline Ti plasmid as an example (Stachel *et al.*, 1986b), a single-stranded molecule (the T-strand) is produced at approximately one copy per *vir*-induced bacterium. The T-strand corresponds to the lower strand of the T-DNA region such that its 5' and 3' ends map to the right and left T-DNA borders, respectively. Double-stranded linear molecules of T-DNA and circular T-DNA are also found in transformed plant cells (Veluthambi *et al.*, 1987; Yamamoto *et al.*, 1987). It is not known whether the T-DNA is integrated into the plant genome via a single- or double-stranded DNA intermediate. The conversion of the T-strand to a double-stranded form in bacteria or in plants has been shown to be possible (Bakkeren *et al.*, 1989; Grimsley *et al.*, 1987).

T-DNA integration also relies on the response of the wounded plant cells. Studies suggest that the plant cells must be dividing and synthesizing host DNA for the integration to be successful (Gheyson *et al.*, 1987).

Genetic mapping indicates that T-DNA insertion is randomn (Wallroth *et al.*, 1986), however the high frequency of successful gene fusions observed when inserting promoterless reporter genes indicates that T-DNAs are preferentially integrated into potentially transcribed loci (Koncz *et al.*, 1989b). Further results (Matsumoto *et al.*, 1990)

indicate that sequence homology between the incoming T-DNA and the plant DNA has an important function in integration. Rearrangements of target plant DNA, including deletions, inversions and duplications, occur during transformation, suggesting that T-DNA integration may rely on endogenous recombination. A model for T-DNA integration involving illegitimate recombination has been described by Mayerhofer *et al.* (1991).

1.2.5 *Agrobacterium* vectors

The Ti-plasmid is a highly efficient natural vector for the transfer of DNA to plant cells when combined with a virulent strain of *Agrobacterium*. Although the mechanism of DNA transfer has only begun to be understood, we can take advantage of this system to modify the plasmid for application to the genetic engineering of plants.

Several basic requirements have to be fulfilled when modifying the plasmid for use as a genetic engineering vector. As it is only the DNA between the right and left borders of the T-DNA that is transferred, these sequences defining the T-DNA must be determined and must remain intact if an accurate transfer is to occur.

Secondly, if the transformed plant cells are to be able to differentiate in a normal manner, rather than proliferate as tumours, the T-DNA coding for the oncogenic properties must be deleted.

Thirdly, once the oncogenic properties are deleted, a marker must be introduced to simplify differentiation between transformed and untransformed cells. Selectable markers and reporter genes include antibiotic resistances and expression systems such as β-glucuronidase and luciferase.

Finally, in order to make the engineered plasmid as versatile as possible, a multiple cloning site within the T-DNA makes further manipulations much more straight forward.

Two transfer systems have now been developed, the '*cis*' and '*trans*' vector systems. The '*cis*' system is based on a reconstructed Ti-plasmid, where the required DNA can be inserted into the T-region along with a drug resistance marker (Matzke and Chilton, 1981). The Ti-plasmids own virulence genes are used in the transfer, hence the '*cis*'.

The binary or '*trans*' system uses a mini-Ti-plasmid that can replicate in *E.coli* therefore facilitating cloning techniques. The mini-Ti contains a full-length T-DNA bounded by the border sequences, into which the required DNA is added. A second plasmid, the *vir* plasmid, containing the entire virulence region, is also added to the *Agrobacterium* host and provides the transfer functions for the T-DNA in '*trans*' (Rogers *et al.*, 1987a and b).

1.2.6 *Agrobacterium* transformation of *Arabidopsis*

The suitability of *Arabidopsis thaliana* as a laboratory plant is further increased by its susceptibility to infection by *Agrobacterium tumefaciens*.

Initial attempts at transformation followed the methods developed for Petunia and Tobacco (Horsch *et al.*, 1985), using leaf disc explants of *Arabidopsis* (Columbia) cocultivated with an A208 binary *Agrobacterium* strain (Lloyd *et al.*, 1986). Transformation efficiency was later found to be increased by the presence of acetosyringone (Sheikholeslam and Weeks, 1987).

Schmidt and Willmitzer (1988) developed leaf and cotyledon transformation methods with improved efficiency. Their results showed that the *Arabidopsis* ecotype and the *Agrobacterium* strain have a far more drastic influence on the transformation efficiency than the type of explant or its age. Their results indicated that *Arabidopsis* C24 had a higher transformation rate than either Landsberg *erecta* or Wassilewskija ecotypes with the C58 *Agrobacterium* strains. The binary C58 strains were equally efficient as the *cis* C58 strains with C24 but were less efficient with the other *Arabidopsis* strains.

The system featured in this project was developed by Valvekens *et al.* (1988). The procedure is essentially the same as the leaf disc transformation but instead of using leaf tissue, root explants were cocultivated with *Agrobacterium tumefaciens*. Regeneration of the root explants is reputed to be considerably faster and more efficient than that of the leaf tissue. *Arabidopsis* ecotypes C24 and Landsberg *erecta* were found to be equally efficient when transformed with the binary C58 *Agrobacterium* strain, however, ecotype Columbia tended to be slower and less efficient at shoot formation. Further work examining the rate of spontaneous mutagenesis during root regeneration (Valvekens and Van Montagu, 1990) showed that the rate is low enough to have no severe implications when applying the root transformation method for regular use.

Other methods have been used to try to improve the efficiency of the system. Feldmann and Marks (1987) have published a non-tissue culture method in which germinating seeds of Wassilewskija *Arabidopsis* are transformed by cocultivating with a C58 *cis Agrobacterium* strain. This technique has proved successful, especially in T-DNA mutagenesis studies, but the transformation and screening of thousands of seeds is necessary in order to identify any transformants.

Arabidopsis thaliana also provides a useful expression system. The susceptibility to *Agrobacterium* transformation, and the speed and quantity of the production of first and second generation transgenic plants allows the analysis of expression of engineered genes.

1.2.7 T-DNA insertional mutagenesis in *Arabidopsis thaliana*

As well as being used to add engineered genes to the *Arabidopsis* genome, *Agrobacterium*-mediated transformation also allows new genes within the genome to be identified. Two different T-DNA insertion mutagenesis techniques have already been used to isolate genes in *Arabidopsis*.

One approach has been to use promoterless constructs to eliminate the somaclonal variants induced in culture and to focus on transformants in which T-DNA insertion into active genes has occurred (André *et al.*, 1986). Koncz *et al.* (1989a and b) further developed the technique using a promoterless kanamycin resistance reporter gene (kanamycin resistance) linked to the right end of the T-DNA, transformed into plants along with a plasmid replicon and a selectable hygromycin-resistance gene. Transcriptional and translational reporter gene fusions were identified by screening for kanamycin resistance, and the gene fusions were rescued from the plants by transformation of the T-DNA-linked plasmid and flanking plant DNA into *E.coli*. Several tissue-specific promoters were initially identified and the technique has since been used to isolate the nuclear gene, *ch*-42, encoding a novel chloroplast protein (Koncz *et al.*, 1990).

The second approach has involved screening plants for mutations produced after seed transformation with *A.tumefaciens* (Feldmann and

Marks, 1987). This technique depends on a large scale transformation programme and the screening of thousands of offspring to find mutations that co-segregate with a selectable marker gene. Approximately 19% of these random T-DNA inserts are reported to generate phenotypes (compared to only 1% reported for the previous method), resulting in a wide range of putatively tagged mutants (Feldmann *et al.*, 1989; 1990). Isolated genes include those required for trichome formation (Marks and Feldmann, 1989), floral development (Yanofsky *et al.*, 1990) and embryogenesis (Errampalli *et al.*, 1991).

1.3 Metallothionein

Certain metal ions, such as zinc, copper and iron, are essential components in a variety of enzymic reactions, but when present in higher concentrations, can be highly toxic. Other ions, such as cadmium and mercury, are not known to have any biological function and have only toxic effects. A variety of mechanisms of metal tolerance have been described in both prokaryotic and eukaryotic systems. Some mechanisms are metal-specific, while others can facilitate detoxification of a range of different metal ions.

Metallothioneins (MTs) bind to metal ions in the cytoplasm. They are believed to 'buffer' the level of free metal ions within cells and, as such, may serve to detoxify excess metal. The first MT was isolated from equine renal cortex as a cadmium-binding protein (Margoshes and Vallee, 1957), but MTs and MT genes have since been isolated from a wide range of vertebrates, invertebrates, fungi and cyanobacteria (Karin and Richards, 1982; Maroni *et al.*, 1986; Munger *et al.*, 1987; Robinson *et al.*, 1990).

Analysis revealed MTs to be low molecular weight, single chain proteins with high numbers of metal-binding cysteine residues, typically arranged in Cys-X-Cys amino acid repeat motifs (where X is an amino acid other than cysteine) which bind the metal ions with thiolate bonds. Analysis of the secondary structure by both X-ray crystallography and NMR spectroscopy revealed two metal-binding domains in animal MTs. Metal ions (such as cadmium) are bound in clusters of four and three ions formed by bridging and terminal cysteine thiolate ligands. The structure is folded into two domains; the N-terminal (β) enfolds the three metal cluster, the remaining four metal ions are bound within the C-terminal (α) domain (Furey *et al.*,

1987). Mammalian MT is stabilized by a total of 42 intramolecular Cys-metal-Cys cross-links, 24 of which are in the α-domain and only 18 in the β-domain, resulting in a lower conformational stability and collective affinity for the metal in the latter. The Cys side chains of the β-domain are therefore more accessible to alkylating agents and have a greater tendency to lose metal (Bernhard *et al.*, 1986). This is also reflected by the sequence in which the clusters are built up when metal ions are added to the apoprotein. The exchange of metal ions is thought to be considerably faster from the β-domain, so it is this one that is thought to be more likely to be involved in the transfer of metal ions to apometalloenzymes, if in fact MTs also function as a metal ion reservoir which directly donate metal ions to metal-requiring apoenzymes (Hamer, 1986). Alternatively, MT could serve as a 'metal-reservoir' but the metals could be released when required via protein degradation.

MTs isolated from most vertebrates and some fungi (for example, *Neurospora* [Lerch and Beltramini, 1983] and *Agaricus* [Munger and Lerch, 1985]) show extensive sequence homology to equine renal cortex MT, these have been designated class I MTs.

Class II MTs are found in prokaryotes such as *Synechococcus* sp. and in some yeasts, eg, *Saccharomyces cerevisiae*, (Olafson *et al.*, 1988; Hamer, 1986) and although they have no direct sequence relationships with class I, they also contain metal-binding cysteine residues, typically in Cys-X-Cys motifs, and they are synthesized following exposure to elevated metals.

Class I MTs display extensive polymorphism. Mammalian tissues usually contain two or more distinct isoforms, grouped into two major fractions, MT-1 and MT-2, differing by a single negative charge at neutral pH. In many cases, there are also subforms within these

fractions, separable by HPLC and specified as MT-1a, MT-1b etc. The various isoforms exhibit small differences in their binding affinity for metal ions, but otherwise appear to have similar biochemical properties. Some of the multiplicity of MTs has been confirmed by gene cloning experiments showing that animal MTs are encoded by a multi-gene family (Hamer, 1986).

Synthesis of MTs is induced, in response to elevated concentrations of certain trace metal ions, at the transcriptional level. This is mediated through interactions between upstream regulatory DNA sequences and *trans*-activating cellular factors (Karin *et al.*, 1984; Carter *et al.*, 1984). The *ACE1* or *CUP2* locus from yeast constitutively expresses a *trans*-activating factor, which in the apoprotein form, cannot bind to DNA. In the presence of the copper or silver ion, the amino-terminal domain undergoes a conformational switch into a folded, protease-resistant form that specifically recognizes the *CUP1* upstream activator sequence (Culotta *et al.*, 1989).

Different isoforms of human MT have shown differential transcription responses: the hMT-1_A promoter is responsive only to cadmium, whereas the hMT-2_A promoter is responsive to zinc and glucocorticoids as well as cadmium (Richards *et al.*, 1984). MT expression can also be altered by changes in gene structure, such as amplification and methylation, and by cellular differentiation and development (Compere and Palmiter, 1981).

The function of MT, even in animals, is still a subject of debate. The presence of the multi-gene family and the differential responses of the various isoforms of MTs imply that there may be diversity in the function of the different genes. A role in metal metabolism and/or detoxification is strongly suggested by its

induction and binding to metals. Other functions suggested for MT include control of the intracellular redox potential, activated oxygen detoxification or roles in sulphur metabolism, but at present, no experimental evidence is available (Hamer, 1986).

The possibility that MT is involved in transfer of metal ions to apometalloenzymes has led to several studies of MT metal exchange reactions. Mammalian zinc-MT can reactivate various zinc-dependent enzymes including carbonic anhydrase, aldolase, thermolysin and alkaline phosphatase *in vitro* with approximately the same rates as inorganic zinc salts (Udom and Brady, 1980).

MT may have a metalloregulatory function in cellular repair processes, growth and differentiation. In view of the known effects of zinc on DNA and RNA polymerases, and its serving as a structural modulator of the zinc finger domains in several DNA-binding proteins, it has been hypothesized that Zn-MT plays a part in the storage, transmission and the expression of genetic information (Kägi and Schäffer, 1988; Zeng *et al.*, 1991). Furthermore, it has recently been demonstrated that MT-accumulates within the nucleus of certain animal cells at S-phase (Tsujikawa *et al.*, 1991).

1.3.1 Metal ions in metabolism

The uptake of phytotoxic amounts of metals can result in inhibition of several enzymes and an increase in activity of others. Enzyme inhibition is predominantly by binding of the metal to sulphydryl groups involved in the catalytic activity or the structural integrity of enzymes. Inactivation can also occur through the deficiency of an essential metal in metalloproteins or metal-protein

complexes, which may be combined with substitution of a toxic metal for the deficient element. The induction of some enzymes is considered to play a significant role in the metal-induced stress metabolism (Van Assche and Clijsters, 1990).

The participation of metal ions in metabolic processes is widespread. Iron proteins are often involved in electron transfer, for example, ferredoxins and cytochromes. Copper-containing proteins are present in various forms, some acting in one-electron transfer processes (for example, plastocyanin), some as peroxide-producing oxidases (for example, galactose oxidase and amine oxidase) and some as two-electron acceptors in oxidation processes (for example, tyrosinase, an o-phenol monooxygenase) (reviewed in Clijsters and Van Assche, 1985).

Superoxide dismutase (SOD) catalyses the conversion of the superoxide radical (O_2^-) to hydrogen peroxide and oxygen (Salin, 1987). Iron-SOD has been isolated from *Euglena* (Lengfelder and Elstner, 1979) and *Brassica* (Salin and Bridges, 1980). Copper-zinc-SOD was demonstrated in animals, fungi and higher plants (Asada *et al.*, 1977) and manganese-SOD in the mitochondria of higher plants (Palma *et al.*, 1986).

Zinc has widespread functions within the organism, acting almost like a vitamin. Zinc-containing carbonic anhydrase promotes hydrolysis and hydration reactions involving carbonyl groups, for example, the hydration of acetaldehyde or the hydrolysis of a toluenesulphonic acid sulphone (Sandmann and Böger, 1983). Alcohol dehydrogenases are also dependent on zinc, as are alkaline phosphatases, phospholipases, carboxy- and amino-peptidases and RNA and DNA polymerases. Zinc deficiency also affects the synthesis of tryptophane and IAA, and ribosome stability (Clarkson and Hanson, 1980). Over recent years,

zinc has been shown to be a necessary co-factor to DNA-binding proteins including the highly publicized 'zinc-finger' proteins. These regulatory proteins are thought to recognize and bind to a specific DNA sequence via a zinc-finger structure. The zinc-binding domains are characterized by Cys-Cys...His-His or Cys-Cys...Cys-Cys sequence units with a constant spacing of 12 or 13 amino acids between the inner ligands. The zinc is bound between the pairs of Cys and His residues causing the protein to fold up into a multi-fingered structure. The number of fingers present and the spacing between them can be varied, thus a variation in the strength of the interaction and a high level of specificity in recognition can be achieved. In addition to the Zn-finger proteins, there are also a number of other Zn-requiring DNA-binding proteins which form other tertiary structures (for review, Harrison, 1991).

1.3.2 Metal binding polypeptides and proteins in higher plants

Class III MTs (cadystin; phytochelatin; γglutamyl metal-binding peptide; phyto-metallothionein and poly[γglutamyl-cysteinyl]glycine or $[\gamma EC]_nG$) have been proposed to bind and detoxify certain metal ions within higher plants. Unlike the genetically encoded classes I and II, class III MTs are synthesized from glutathione and/or γ-glutamyl cysteine and consist of repeating γglutamylcysteinyl units with a single C-terminal glycine (or β-alanine) residue ($[\gamma$Glu-Cys$]_n$Gly, where n = 2 - 11).

Metal ions are aggregated in a cluster containing several (γGlu-Cys)$_n$Gly molecules. Complexes are extremely heterogeneous because of multiple peptide components and the many possible combinations that

can arise from these mixtures.

Phytochelatins were originally described from the yeast *Schizosaccharomyces pombe* (Murasugi *et al.*, 1981) where they were found to be inducible by cadmium. The ability to synthesize these polypeptides has since been shown to be conserved through the most advanced higher plants (for example, the Orchidales) to the more primitive red, green and brown algae (Steffens, 1990).

Phytochelatin synthesis is induced by a number of different metals, with cadmium as the strongest inducer and zinc as one of the weakest. In *Euglena gracilis*, exposure to zinc did not induce synthesis of Zn-$(\gamma EC)_nG$, the majority of the zinc present was found in a low molecular weight pool (Shaw *et al.*, 1989). Data suggests that $(\gamma EC)_nG$ is only likely to weakly associate with zinc, if at all, *in vivo* (Reese and Wagner 1987a and b).

The similarity of phytochelatins to glutathione (GSH: γ-Glu-Cys-Gly) indicates that their biosynthesis shares a common enzymology. Grill *et al.* (1989) identified the heavy metal activated phytochelatin synthase as the enzyme catalysing the stepwise addition of γ-glutamylcysteine from GSH to phytochelatin oligomers.

Although it is evident that phytochelatins play a central role in the detoxification of excess metals and are also involved in metal ion homoeostasis, recent evidence suggests that they may not enjoy an exclusive role as the heavy metal-binding polypeptides of plants.

The E_c protein, isolated from wheatgerm, is known to bind zinc (Lane *et al.*, 1987), possibly acting as a zinc storage protein. Its abundant Cys-X-Cys motifs and its ability to bind zinc have led to it being designated a class II MT. The presence of either class I or II MT in vegetative plant tissue has not yet been confirmed, although the isolation of a metal-binding low molecular weight protein from maize

seedlings, probably analogous to animal MT, has been described (Leblová and Špirhanzlová, 1987).

1.3.3 *$PsMT_A$*

The *$PsMT_A$* gene, isolated from *Pisum sativum*, has an open reading frame encoding a protein with sequence homology to class I MTs (Evans *et al.*, 1990a). The coding region consists of two MT-like domains, in the first of which 14 out of 26 amino acids are identical to *Neurospora crassa* class I MT. *$PsMT_A$* transcripts are highly abundant in roots even when not exposed to high concentrations of trace metals.

Two further genes *$PsMT_B$* and *$PsMT_C$* have since been isolated, along with homologous genes from alfalfa and *Phaseolus vulgaris* (Bryden and Tommey, personal communication). Other related genes, all of which encode the two domain MT-like proteins have been detected in *Mimulus guttatus* (de Miranda *et al.*, 1990)), soybean (Chino *et al.*, unpublished), maize (de Framond, 1991) and barley (Okumura *et al.*, 1991).

The regulation and function of these genes is still under investigation. In order to confirm that the genes are in fact MT genes, characterization of their putative products is necessary as well as the determination of their action within the plant.

1.3.4 Metal-binding proteins in *Arabidopsis*

Although, no homologues have been detected using cross-hybridization to a *PsMT*$_A$ probe or that are amplified using *PsMT*$_A$ PCR primers, an MT-like gene has recently been isolated from *Arabidopsis* by Chino *et al.* (unpublished). With the range of species that have now been reported to encode an MT-like protein, it would appear highly probable that most, if not all, plant species have at least one MT gene.

The interactions of heavy metals with *Arabidopsis* has also been studied using heavy metal sensitive mutagenized seeds. Mutants have been identified which display cadmium and mercury sensitivity, but are only slightly sensitive to copper and zinc. This suggests that these are mutants in a specific detoxification mechanism. So far, the mutation has been mapped to chromosome 5, and a chromosome walk is now being undertaken to isolate the gene involved (Cobbett *et al.*, 1991).

1.3.5 Engineered expression of mammalian MT in plants

Biotechnologists have, for several years, been attempting to engineer MT into plants in order to either produce metal tolerant plants or, on a more fundamental scale, to elucidate more details of MT function and action. As far as crop production is concerned, MT may be useful for decreasing chronic, low-level cadmium exposure in humans, as the bulk of cadmium intake in man is derived from agricultural crops.

A cDNA clone of Chinese hamster MT-II was introduced by a systemic infection into turnip (*Brassica campestris*) using a

recombinant cauliflower mosaic virus (Lefebvre *et al.*, 1987). Infected tissue was found to contain MT at a level of 0.5% of the soluble leaf protein, had four times the cadmium-binding capacity of control leaves and conferred tolerance to 10mM cadmium.

Maiti *et al.*, (1988, 1989 and 1991) improved the system by transforming tobacco (*Nicotiana tabacum*) with mouse MT using *Agrobacterium tumefaciens*, resulting in seed-transmissable MT expression. Their results corroborate those of Lefebvre *et al.* (1987) in suggesting that chelation of excess free cadmium was the mechanism of tolerance. Cadmium tolerance in very young seedlings was found to be affected by levels of MT 5 to 10 times lower (0.06% to 0.1% soluble leaf protein) than those obtained by Lefebvre. Their results also suggest that MT may affect the steady state levels of cadmium by altering the rate of uptake or discharge of cadmium in leaves. Alternatively, MT may reduce the translocation of cadmium from roots to upper parts of the plants, again resulting in an overall decrease in cadmium in the leaves.

1.4 Transposable elements

A transposable genetic element (or transposon) is a piece of DNA that can move independently from place to place within a genome. Transposable elements were first identified in maize plants by Barbara McClintock (1963). Similar elements have since been found in many other organisms (Bingham *et al.*, 1981; Moerman *et al.*, 1986).

Until the late 1970s, the genome was thought to be completely stable, with each gene occupying a fixed position on a particular chromosome. Transposition was discovered from studies of unstable pigment mutations. In most cases, a mutation produces an irreversible inactivation of a gene, with a back mutation (or reversion) almost never occurring. However in a few cases, the frequency of reversion is so high that the organism is produced with patches of mutant and revertant tissue and can therefore have a variegated appearance. This variegation has since been shown to arise from the insertion of a transposable element into the gene, blocking its expression, followed by its transposition away and the restoration of function.

Transposable elements fall into two classes. One class can promote their own transposition and are, therefore, autonomous. The second class of elements are unable to transpose without the presence of an autonomous element in the same cell, acting in *trans*.

Elements of both classes have been characterized from maize, the most well-known being the *Ac/Ds* and the *Spm* (*En*) systems. *Ac* (Activator) and *Spm* (Suppressor-mutator) are both autonomous elements, but only *Ac* can transactivate *Ds* (Dissociation). The other well-characterized transposable element family is the autonomous *Tam* family from *Antirrhinum majus*.

1.4.1 Transposon tagging

Transposable elements have no obvious phenotype unless they insert into a gene (or the controlling regions surrounding a gene) that has a visible phenotype. The movement of transposons has been studied using genes that encode, for example, the biosynthesis of anthocyanin pigments of maize (McClintock, 1963) and flower pigmentation in *Antirrhinum majus* (Bonas *et al.*, 1984). By using the process in reverse, however, with a transposon-specific hybridization probe, a disrupted gene (whose products are often unknown) can be isolated. This technique has been used to isolate a variety of genes, initially in *Drosophila melangaster* (Bingham *et al.*, 1981), then further developed for use in plants (Federoff *et al.*, 1984; Martin *et al.*, 1985).

Transposon-tagging has been limited by the small number of elements that have been fully characterized and the limited number of species that contain these particular elements. This problem has since been overcome by the introduction of characterized transposons into the novel plant host via *Agrobacterium*-mediated transformation. The *Spm* element has been introduced and shown to transpose within both the *Nicotiana tabacum* and the *Solanum tuberosum* genomes (Masson and Federoff, 1989; Frey *et al.*, 1989). In addition to both these genomes, the *Ac* element has also been shown to transpose in *Lycopersicon esculentum* (Yoder *et al.*, 1988), *Glycine max* (Zhou and Atherly, 1990), *Oryza sativa* (Izawa *et al.*, 1991), *Daucus carota* and *Arabidopsis thaliana* (Van Sluys *et al.*, 1987). *Tam*3 has also been shown to transpose in tobacco (Martin *et al.*, 1989); however, the frequency of transposition appears to be less than that of *Ac*.

Complications may arise with this technique because the

transposon may insert into a great many sites in the genome, causing difficulties in the isolation of the gene of interest. A pronounced difference in dosage effect has been shown with *Ac* in maize and tobacco. In maize, the increase in number of active *Ac* elements results in a decrease of transposition, while in tobacco, more copies of *Ac* cause more transposition (Hehl and Baker, 1990).

1.4.2 *Tam3*

In *Antirrhinum majus*, transposon activity has been most intensively studied in the genes responsible for anthocyanin biosynthesis, as mutations have an easily recognizable phenotype. The *Pallida* (*pal*) locus encodes the enzyme dihydroflavanol-4-reductase from the anthocyanin pathway, which when blocked, results in ivory rather than red flowers. A highly mutable *pal*-recurrens (rec) line was isolated where the flowers have randomnly occurring red sectors indicating the presence of a transposable element. This line was crossed to a homozygous *nivea* (chalcone synthase) line in order to produce unstable mutations in this previously cloned gene.

The resultant *niv*^{rec} plant produced palely pigmented flowers with randomnly occurring red sectors. Restriction digest analysis and hybridization with a radiolabelled portion of the chalcone synthase gene led to the identification of the *Tam3* (*T*ransposon *a*ntirrhinum *m*ajus 3) element (Sommer *et al.*, 1985).

Structural analysis has shown the *Tam3* element to be 3629bp long, containing an open reading frame of 2.48kb and no intron (Sommer *et al.*, 1988). In common with many transposons, *Tam3* possesses 12bp terminal inverted repeats and generates 8bp duplications upon

integration.

The *Tam* elements fall into two different transposon families. *Tam*1, *Tam*2 and *Tam*4 have homologous termini and produce 3bp target duplications (Luo *et al.*, 1991). 12 out of 13bp of their termini are homologous to the termini of both the *Spm* and *En* elements of *Zea mays* (Upadhyaya *et al.*, 1985); they are also homologous to the terminal 5bp of the *Tgm*1 element of *Glycine max* (Vodkin *et al.*, 1983). The *Tam*1 element includes two genes (*tnp*1 and 2); the *tnp*2 transcript contains an ORF that shares 45% homology with part of the *tnp*D gene of *En/Spm* and 48% homology with an ORF of the *Tgm* element (Nacken *et al.*, 1991). All of these elements also produce 3bp target duplication, suggesting that they are members of the same family (Bonas *et al.*, 1984).

*Tam*3 appears to belong to the second transposon family, along with the maize *Ac/Ds* element system and the *P* and *hobo* elements from *Drosophila* (Kaufman *et al.*, 1989; Streck *et al.*, 1986). They all generate an 8bp duplication of target sequences on integration (although there is evidence that *Tam*3 can also produce 5bp duplications [Coen *et al.*, 1986]). *Tam*3 and *Ac* produce similar sizes of transcript (approximately 3.1kb and 3.3kb respectively), both with unusually long 5' untranslated regions, 500 nucleotides (*Tam*3) and 600-700 nucleotides (*Ac*). The proteins encoded by the elements are very similar (749 amino acids for *Tam*3 and 807 amino acids for *Ac*), with regions of fairly high homology (50-60%), probably indicating similar function.

1.4.3 *Tam3* transposition

Various mechanisms have been proposed for the transposition of transposable elements, usually based on either homologous recombination between copies of the elements within the genome (Martin *et al.*, 1988) or chromosomal breaks made at the ends of different element copies (Roiha *et al.*, 1988). Both of these systems, however, require a copy of the transposable element at each breakpoint of the rearrangement.

Chromosomal rearrangements around *Tam3* breakpoint sites in the *pal*-42 *Antirrhinum majus* line cannot be easily explained by either of these basic mechanisms. The *pal*-42 inversion has been explained as an aberrant example of a proposed transposition model where the ends of the element are cleaved sequentially rather than simultaneously (Robbins *et al.*, 1989). For this model to be correct, the donor and recipient transposon sites must become associated, and therefore transposition should occur to those sites most likely to become associated with the donor site. Association between DNA sites depends on the spatial arrangement of chromatin in the nucleus, but it would seem likely to include sites predominantly near to each other on the same chromosome. Further work (Hudson *et al.*, 1990) has indicated a strong preference for *Tam3* transposition to recipient sites that are tightly linked to the donor.

Similarly, two thirds of *Ac* transpositions from the *P* locus in maize are to linked positions and almost half are within 5 map units of *P* (Greenblatt, 1984). This, together with their similar termini and excision site footprints, implies *Tam3* and *Ac* transpose by a similar mechanism. Novick and Peterson (1981) have shown that the maize element *En* (*Spm*) also transposes preferentially to linked sites,

suggesting that this element also transposes by a similar mechanism.

1.4.4 Factors affecting *Tam3* transposition

The effect of temperature on unstable lines with the *Tam3* element in different loci was examined. In each case, the frequency of excision was found to be approximately 1000 times greater at 15°C than at 25°C (Carpenter *et al.*, 1987).

The effect of temperature on most unstable mutations in plants is much less than that described for *Tam3*: typical values range from a 1.4-fold change in the *v* locus in *Nicotiana* species hybrids (Sand, 1957) to a 5 to 13-fold change in pg^{m} of *Zea mays* (Peterson, 1958). Elements with a higher degree of temperature sensitivity have been described in *Saccharomyces cerevisiae* where the *Ty* element shows a 100-fold increase in transposition rate when grown at 15-20°C than at 30°C (Paquin and Williamson, 1986) and in *E.coli* where the Tn*3* element shows a 20-fold increase at 23°C than at 37°C (Kretschmer and Cohen, 1979). The exceptionally high rate of *Tam3* excision at 15°C compared to 25°C may reflect altered levels of activity of transposase or repressors of transposition. Alternatively, transposition may be favoured by the slower cell division cycle at 15°C.

Tam3 is also controlled by an unlinked gene *Stabiliser* (*St*), which reduces the excision rate. *St* appears to interact specifically with *Tam3* and does not affect the excision rate of *Tam1* (Harrison and Carpenter, 1973). It has been suggested that *St* and *Tam3* could act as a two component system like *Ac/Ds* or *En/I* in maize (Carpenter *et al.*, 1987) but it perhaps seems more likely that *St* acts as a repressor element and is probably also temperature sensitive.

1.5 Extensin

The plant cell wall is a complex entity which largely determines the function of the cell. Walls of distinct cell types are comprised of a characteristic combination and spatial organization of cellulose, hemicelluloses, pectic compounds, lignin, suberin, proteins and water. Specialized cell walls vary from rigid sclerenchyma to gas impermeable photosynthetic bundle sheath cells.

The best characterized and most abundant proteins in dicotyledonous plant cell walls are the hydroxyproline-rich glycoproteins (HRGPs), the 'extensins'. The extensins are characterized by a pentapeptide Ser-Pro-Pro-Pro-Pro repeat motif, that is post-translationally modified by the action of prolyl hydroxylase to yield a hydroxyproline-rich polypeptide. After hydroxylation is complete, a further modification occurs: arabinosyl transferase activity in the Golgi results in the specific linkages between arabinose residues and the hydroxyproline. After secretion into the cell wall, the extensins are slowly insolubilized by cross linking, typically with covalent isodityrosine bonds (Biggs and Fry, 1990), resulting in cell wall strengthening.

Analysis of sugar beet extensin (Li *et al.*, 1990) has revealed the presence of an insertion sequence splitting the tetrahydroxyproline block. Using this extensin sequence as a probe, homologues in both soybean and carrot cDNA sequences, previously disregarded, have been identified.

The role most associated with extensin is that of a cell wall strengthener. Electron micrographs have shown that extensin is present as thin rod-like structures with average lengths of 80-84nm (Stafstrom and Staehelin, 1986). Its rod-like structure provides the cell with

mechanical strength and rigidity.

Examination of wounded and infected tissue has shown there to be a ten times increase in the amount of extensin produced (Esquerré-Tugayé and Lamport, 1979), suggesting a probable role in defence. There are various suggested reasons for this: to produce a more dense cell wall, preventing infection spread and tissue desiccation around a wound; to provide nucleation sites for lignin deposition resulting in a tougher wall (Whitmore, 1978) and also to immobilize pathogens within the wall as the positive charge of the HRGP results in the agglutination of invading bacteria (van Holst and Varner, 1984).

Studies of tomato HRGPs show the presence of more than one type of cell wall HRGP in a given tissue, indicating the probable existence of a multigene family. Variations in the number of copies of the repeat motif and the size of the DNA insert between them or within the tetrahydroxyproline blocks would vary the number and rigidity of the 'rods' and, therefore, the strength of the extensin. With such variation found between the extensin sequences, the presence of the multigene family seems highly probable.

Two extensin clones, isolated from tomato, exhibit different responses to wounding. Although both class I and II extensin mRNAs are present in unwounded stems, class I mRNA accumulates markedly in response to wounding, while class II extensin mRNA does not. Showalter *et al.* (1991) speculated that both classes of extensin represent structural components of the cell wall but that an additional role in wound healing and defence is possible for the class I extensin clone. Such a role is particularly attractive, given the abundancy of Tyr-X-Tyr units, compared to class II extensin, and therefore the enhanced potential for the formation of intermolecular isodityrosine cross-links, in class I extensin. These cross-links would allow for the

erection of a highly dense and impenetrable cell wall barrier, serving to keep pathogens at bay as well as to prevent excessive evaporative water loss at a wound site.

It is possible that, like the hydroxyproline-containing collagens (the animal cell structural proteins), the extensins are 'tailored to their tissue'. Studies of collagen have shown that of the 10 to 11 collagen types, 4 are exclusive to cartilage tissue (Piez, 1987). In order to assign a function to each member of the proposed extensin multigene family, it is necessary to know in what type of cell it is present and its cellular location. As the extensin genes are isolated and characterized, it is possible to determine their functions and expression patterns with genetic engineering techniques.

1.5.1 Cell wall proteins in *Arabidopsis*

Very little has been reported about the cell wall structure of *Arabidopsis*. After an extensive literature search, the only details found describe the isolation of five cDNA clones encoding glycine-rich proteins (de Oliveira *et al.*, 1990). Glycine-rich proteins (GRPs) are another class of plant cell wall proteins, containing $(Gly-X)_n$ repeats. They have been isolated from a number of species for example, *Oryza sativa* (Lei and Wu, 1991) and *Phaseolus vulgaris* L. (Keller *et al.*, 1989), and do not appear to replace or exclude the presence of HRGPs. From the data published, there seems nothing to suggest that *Arabidopsis* cell walls are any different to any other dicotyledonous plant cell wall.

1.5.2 *extA*

The *extA* gene was one of several extensin genes isolated from a *Brassica napus* L. (oilseed rape) genomic library (Evans *et al.*, 1990b). It contains the characteristic Ser-Pro_4 repeat motif which is markedly more regularly repeated than in carrot extensin. The highly repetitive nature of the polypeptide produces the usual extensin alternating hydrophilic/hydrophobic hydrophilicity profile.

Northern blots and RNase protection assays showed the gene is expressed at least 400 times higher in root tissue than in other organs. Previous extensin studies have linked its expression with sclerenchyma cell formation; however no sclerenchyma have been found in the growing tip of rape roots where the *extA* expression is most abundant.

In order to elucidate the expression patterns of this particular extensin gene, promoter-reporter gene fusion constructs were made to introduce via transformation back into rape and into other species. The construction of the fusion is described fully in Shirsat *et al.* (1991).

1.6 Aims

The initial aim of this project was to establish a workable *Agrobacterium tumefaciens* transformation procedure of *Arabidopsis thaliana*. In order to test all aspects of the transformation system, three constructs were introduced: a gene; a promoter-reporter fusion; and a transposable element.

The introduction of the putative metallothionein gene, $PsMT_A$, into the *Arabidopsis* genome, under the control of a strong constitutive promoter, was to be attempted to try to elucidate its function within the plant.

Insights into the function of the cloned extensin *ext*A gene were to be obtained by localizing its expression. After introducing an *ext*A promoter/ GUS reporter fusion into *Arabidopsis*, using the *Agrobacterium* transformation method, its pattern of expression was to be determined by microscopy.

Finally, attempts to introduce the transposable element *Tam*3 into the *Arabidopsis* genome were to be followed by observing the effects of its tranposition and 'transposon tagging' any interesting mutant genes produced.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Glass and plasticware

All glassware used in DNA manipulations were siliconized and autoclaved. Plasticware and Pipetman tips were autoclaved. Pipetman tips used in tissue culture work were individually foil wrapped and autoclaved.

2.1.2 Chemicals and biological reagents

All reagents, unless listed separately below, were obtained from BDH Chemicals Ltd, Poole, Dorset and were of analytical grade or the best available.

BSA, DTT, EtBr, TESPA, RNase A, Pronase P, Proteinase K, 2,4-D, 2ip, thiamine, pyridoxin, nicotinic acid, lysozyme, acetosyringone, ampicillin, kanamycin monosulphate and spectinomycin were all obtained from Sigma Chemical Co., Poole, Dorset. 5-bromo-4-chloro-3-indoyl-β-D-glucuronide, PVP, sorbitol and Na-laurylsarcosine were also obtained from Sigma. Augmentin was supplied by Beechams Pharmaceuticals, Brentford, Middlesex.

3MM paper and filter paper were from Whatman Ltd, Maidstone, Kent; Nitrocellulose filters were from Anderman and Co. Ltd, Kingston-upon-Thames.

MS medium and Gamborgs Basal salts (without sucrose, kinetin or 2,4-D) were obtained from Flow Laboratories, Rickmansworth, Herts. Agar was obtained from Difco, Detroit, Michigan, U.S.A. Agarose was from Gibco BRL Ltd, Paisley. Petri dishes (9cm) and Chloros were supplied by A & J Beveridge, Newcastle. Sterile Acrodisc filter units (0.2μm) were from Gelman Sciences, Ann Arbor MI48106, U.S.A. 1.25cm Micropore tape was obtained from Boots Ltd, Beeston, Nottingham.

Sephadex G-50 and Ficoll-400 were from Pharmacia Fine Chemicals, Uppsala, Sweden. NaCl was from May and Baker Ltd, Dagenham. Yeast extract, bacto-tryptone and beef extract were obtained from Oxoid Ltd, Basingstoke, Hants.

$\alpha[^{32}P]$-dCTP and the IntenSE M Silver Enhancement kit were from Amersham International Plc., Amersham, Oxon. Restriction endonucleases, buffer solutions, DNA modifying enzymes, cDNA synthesis kits and dNTPs were from Boehringer Mannheim GmbH., Mannheim, Germany and Northumberland Biologicals Ltd, Cramlington. *Taq* polymerase and buffer were from Promega, 2800 Woods Hollow Road, Madison, WI 53711-5399 U.S.A.

DH5α and DH5αMAX frozen competent cells were from Gibco-BRL Ltd, Paisley, Scotland.

Goat anti-rabbit IgG gold conjugate was from Bioclin Immunogold Reagents. Paraplast plus tissue embedding medium was from Monoject Scientific Inc., Athy, Co. Kildare, Ireland; Histo-clear and Ecoscint A were from National Diagnostics, Manville, New Jersey, U.S.A.

2.1.3 *Arabidopsis thaliana*

The *Arabidopsis* strain used was C24, from the *Arabidopsis thaliana* collection (from Dr C. Dean, John Innes Institute, Norwich.)

2.1.4 *Agrobacterium tumefaciens*

The non-oncogenic C58/3 *Agrobacterium tumefaciens* strain (Dr P. Mullineaux, John Innes Institute, Norwich, [Dale *et al.*, 1989]) was used in all the transformations. 100mg/l spectinomycin and 80mg/l nalidixic acid selection was used throughout.

2.1.5 Constructs

pJIT73 was developed by Dr P.Mullineaux, John Innes, Norwich and was provided by Dr M. Gibbs, Fisons Horticulture.

Tam3 was obtained as a *Kpn*I fragment cloned into pUC18 from Dr A. Gierl, Max-Planck-Institut für Züchtungsforschung, D-5000 Köln 30.

pAS44 and pAS45 were constructed by and obtained from Dr A. Shirsat, University of Bangor.

$PsMT_A$ was obtained from Dr I.M.Evans, University of Durham, cloned into pUC18.

$PsMT_B$ was isolated and cloned by Miss J. Bryden (University of Durham).

pROKII was constructed by Dr M. Bevan and Dr T. Kavanagh, IPSR, Norwich and was provided by Dr C. Brough, University of Durham / A.G.C., Cambridge.

2.1.6 Primers

GUS gene PCR primers 216 and 217 were provided by Dr R.R.D.Croy; 283 and 284 ($PsMT_B$) were from Miss J. Bryden (University of Durham).

2.2 METHODS

2.2.1 Bacterial Manipulations

2.2.1.1 Bacterial culture media

E.coli cultures were grown in the following media:-

YT: 8g bacto-tryptone, 5g yeast extract, 5g NaCl - L^{-1}

LB: 10g bacto-tryptone, 5g yeast extract, 5g NaCl, 1g glucose - L^{-1} (Miller, 1972).

Agrobacteria were cultured on YEB medium:-

1g yeast extract, 5g beef extract, 5g bacto-tryptone, 5g sucrose, 2mM $MgSO_4.7H_2O$ - L^{-1} pH 7.2 (Vervliet *et al.*, 1975).

All media were used at pH 7.2 and, when necessary, were solidified with 1.5% bactoagar. Sterilization was by autoclaving at 121°C, 15 p.s.i. for 20 minutes. Media was cooled to below 60°C before any necessary antibiotics were added.

2.2.1.2 Bacterial glycerol preparation

A sample of bacteria was streaked out, using a sterile loop, onto YT (*E.coli*) or YEB (*A.tumefaciens*) plates containing the appropriate antibiotic selection. The bacteria were left to grow at 37°C (*E.coli*) or 28°C (*A.tumefaciens*) until single colonies could be seen clearly and any contamination, if present, was obvious. Using a sterile loop, a single colony was transferred thickly onto a new plate with the same selection and grown up until the plate was covered.

The bacteria were then scraped off the plate with the sterile loop and suspended in 1ml YT or YEB (with no selection added). Once adequately suspended, 1ml sterile 80% glycerol was added and mixed. The sample was then stored at -80°C ready for use.

2.2.1.3 Bacterial transformation

A 50µl aliquot of *E. coli* DH5α cells was thawed slowly on ice before 1-3µl (maximum 0.01µg) DNA was added and dispersed carefully throughout the cells. The contents were mixed by tapping the tube and were then incubated on ice for 30 minutes. The cells were then heat-shocked at 37°C for 2 minutes followed by 2 minutes on ice.

200µl L.B. was added and the cells were grown on a shaker at 37°C for 1 hour. 50µl and 200µl cells were spread onto L.B. plates, with 100µg/ml ampicillin, 50µg/ml X-gal (in dimethylformamide) and further appropriate antibiotics as necessary, and were incubated at 37°C overnight.

2.2.1.4 Bacterial transformation (maximum efficiency cells)

A 20µl aliquot of *E.coli* DH5αMAX cells was treated as above. 1-3µl (maximum 0.01µg) DNA was added and dispersed through the cells, which were then treated as above.

80µl S.O.C. broth (Sambrook *et al.*, 1989) was added to the cells prior to the hour incubation. 10µl and 90µl aliquots were plated out, as above.

2.2.1.5 Triparental mating

In order to transfer a plasmid from a donor *E. coli* strain into a recipient *Agrobacterium*, a mobilizing strain must be used, for example, *E.coli* pRK2013. The antibiotic resistances of all of the strains were confirmed prior to the mating, by the growth or lack of growth on media containing each of the various antibiotics individually.

A 5ml culture of each strain was grown overnight with appropriate selection at either 37°C (*E.coli*) or 28°C (*Agrobacterium*). The cells were harvested by centrifugation for 10 minutes at 900g and washed by resuspension in 5ml sterile 10mM $MgSO_4$. Using 1ml of each suspension, a 1:1:1 mix was made. 1ml of the mix was plated onto non-selection L.Agar. Plates were also prepared with 1ml *E.coli* pRK2013; 1ml *Agrobacterium* recipient strain; 1ml *E.coli* donor strain containing the plasmid; and, as a sterility check, 1ml 10mM $MgSO_4$. The suspensions were rolled rather than spread around the plates and were then grown, overnight (not inverted), at 28°C (the 2 *E.coli* strains were grown at 37°C).

The growth was scraped and washed off the plates with 3ml sterile 10mM $MgSO_4$. A serial dilution of each of the strains was plated onto YEB with full selection for the *Agrobacterium* containing the plasmid and grown for 24 - 48 hours.

Single colonies from the 1:1:1 mix plates were picked off and grown (with selection) on a reference plate and in 5ml cultures ready for miniprepping.

2.2.2 DNA Extraction Techniques

2.2.2.1 Plasmid miniprep (Birnboim and Doly, 1979)

Single bacterial cultures were inoculated into 5ml YT medium containing the appropriate antibiotics and also transferred to a reference plate. These were incubated overnight at 37°C. 1.5ml of the culture was transferred to an eppendorf tube and centrifuged for 1 minute to pellet the bacteria. The pellet was resuspended by pipetting in 100µl ice-cold solution I (10mM EDTA; 25mM Tris HCl pH 8; 50mM glucose; 4mg/ml lysozyme added just before use). The tubes were incubated at room temperature for 5 minutes.

200µl of freshly prepared, ice-cold solution II (0.2N NaOH; 1% SDS) was added and mixed by inverting rapidly several times before leaving on ice for 5 minutes.

Then 150µl ice-cold potassium acetate (60ml 5M potassium acetate; 11.5ml glacial acetic acid and 28.5ml water) was added, mixed by inversion for 10 seconds and stored on ice for 5 minutes. The RNA was pelleted by centrifugation for 5 minutes at 4°C. The DNA was then phenol/chloroform extracted and ethanol precipitated.

2.2.2.2 Plasmid miniprep for sequencing

This method (Mierendorf and Pfeffer, 1987) is slightly modified from the alkaline lysis method above. It produces DNA that is clean enough for sequencing.

The cells are grown and prepared as above, then resuspended in solution I but no lysozyme is added. The rest of the miniprep is

identical to the alkaline lysis method until the final centrifugation. The supernatant was transferred to a fresh tube and the centrifugation was repeated. The supernatant was then RNase treated (see section 2.2.3.1), before an equal volume of phenol/chloroform was added and mixed for 30 seconds. After centrifugation, the aqueous layer was precipitated with 2.5 volumes of ethanol at -70°C for 5 minutes.

The DNA was pelleted by centrifugation and then rinsed with prechilled 70% ethanol, before being dried under vacuum. The pellet was dissolved in 16µl sterile deionized water and was mixed with 4µl 4M sodium chloride and then 20µl 13% PEG (MW 8000). The PEG precipitation was incubated at 0°C for 20 minutes, then centrifuged for 10 minutes and the pellet rinsed and dried as before. The DNA was dissolved at an appropriate concentration for sequencing on the ABI 373A DNA Sequencer.

2.2.2.3 *Agrobacterium* plasmid miniprep

Following Dhaese *et al.* (1979), single colonies from the triparental mating were inoculated onto a reference plate and into 5ml YEB with selection and grown overnight at 28°C. A 1.5ml aliquot of the culture was pelleted by centrifugation for 30 seconds at 4200rpm. The pellet was resuspended by vortexing in 380µl pronase buffer: 50mM Tris pH 8; 20mM EDTA; 0.8% Na-lauryl sarcinate. 20µl 20mg/ml pronase P (previously self-digested by incubation at 42°C for 2 hours) was added and the mixture was incubated at 37°C for 1 hour until colourless.

The lysate was sheared carefully by passing it, several times, through first a 1.1mm diameter needle followed by a 0.6mm diameter needle until the viscous liquid became fluid. The DNA was

deproteinized with 2 phenol extractions (500µl phenol, 400µl sterile distilled water) then 4 chloroform extractions.

The DNA was ethanol precipitated (with added 0.3M sodium acetate) then pelleted immediately. The pellet was resuspended in TE and the ethanol precipitation repeated. The pellet was washed twice in 80% ethanol and dried before finally resuspending ready for restricting.

2.2.2.4 *Arabidopsis* DNA preparation (Bancroft, personal communication)

2 to 3g of frozen tissue was ground to a fine powder in a liquid nitrogen-cooled pestle and mortar. 12.5ml extraction buffer (140mM sorbitol; 220mM Tris pH 8; 22mM EDTA; 800mM NaCl; 1% Na - lauryl sarcosine; 0.8% CTAB; pH 8 and autoclaved) was added and transferred to a 30ml siliconized Corex tube. The tissue was incubated at 65°C for 20 minutes, with occasional vigorous shaking. 5ml chloroform was added and shaken at room temperature for 20 minutes. The phases were separated by centrifugation (5000rpm for 10 minutes).

8.5ml isopropanol was mixed with the aqueous phase and left on ice for 10 minutes. The precipitate was collected by centrifugation (10000rpm for 10 minutes), then dissolved in 2ml TE (10mM Tris; 1mM EDTA; pH 8 and autoclaved) by gentle pipetting. The RNA was precipitated by a 20 minute incubation on ice after the addition of 2ml 4M lithium acetate and pelleted by centrifugation (10000rpm for 10 minutes).

The DNA was ethanol precipitated and collected by centrifugation (8000rpm for 15 minutes). The tube was then drained and inverted for

10 minutes before resuspending the pellet ready for cleaning (phenol/chloroform extraction or silica fines).

2.2.2.5 Glassware siliconization

The glassware was washed and rinsed in distilled water and dried well. After degreasing with chloroform and leaving it to evaporate, the glassware was rinsed with siliconizing fluid (any excess was poured off). It was then left to evaporate for ½ hour before washing thoroughly with distilled water. Once the glassware was dry, it was autoclaved and was then ready for use in all DNA experiments.

2.2.2.6 Rapid plant genomic DNA extraction for PCR analysis

The tissue samples for analysis were macerated in a sterile eppendorf for 15 seconds at room temperature with no buffer (Edwards *et al.*, 1991). 400µl extraction buffer (200mM Tris HCl pH 7.5; 250mM NaCl; 25mM EDTA; 0.5% SDS) was added and the sample was vortexed for 5 seconds.

The extracts were centrifuged at 13 000rpm for 1 minute, 300µl of the supernatant was removed and mixed with an equal volume of isopropanol. After incubation at room temperature for 2 minutes, the extract was centrifuged at 13 000rpm for 5 minutes. The pellet was vacuum dried before being resuspended in 100µl TE.

10 or 2.5µl of this solution was used as the template for a 35 cycle polymerase chain reaction.

2.2.3 DNA Manipulations

2.2.3.1 RNase digestion

The crude DNA pellet was resuspended in TE pH 8.0, with DNase-free RNase (RNase was dissolved in water [10mg/ml] and boiled for 5 minutes) added to a final concentration of 50-100μg/ml and incubated for ½-1 hour at 37°C. The solution was then extracted with either phenol/chloroform or silica fines and ethanol precipitated.

2.2.3.2 Phenol/chloroform extraction

To the crude DNA solution, an equal volume of phenol was added, vortexed and then centrifuged for 2 minutes. The supernatant was removed and the phenol layer back-extracted (to ensure maximum DNA recovery) by the addition of an equal volume TE pH 7.5, followed by vortexing and centrifugation. This second supernatant was removed and added to the first.

An equal volume of phenol-chloroform (1:1) was added, then vortexed and centrifuged. To the supernatant, an equal volume of chloroform (with 4% v/v isoamylalcohol to aid phase separation) was added before vortexing and centrifuging. The supernatant was removed and the last step repeated.

A tenth volume 3M sodium acetate pH 5.2 was added, followed by 1000μl 100% ethanol at -20°C, and the tubes left at -20°C for 1 hour. After centrifugation at 4°C for 15 minutes, the supernatant was removed. The pellet was then washed in 70% ethanol (-20°C), repelleted

and the ethanol removed. The pellet was then vacuum dried and resuspended in an appropriate volume of TE pH 7.5.

2.2.3.3 Silica fines extraction

This method was developed (and the silica fines provided) by Dr N.J.Robinson, Durham University, based on the method of Golden *et al.* (1987).

The crude DNA pellet was resuspended in 0.35ml sterile distilled water then mixed with 0.7ml sodium iodide solution (90.8g NaI; 1.5g Na_2SO_3; 100ml distilled water; filter sterilized; 0.5g Na_2SO_3 added to saturation and stored in the dark at 4°C). After shaking the fines, 10µl was added to the DNA and mixed well.

A 10 minute incubation at room temperature bound the DNA to the fines, which were then pelleted in a microcentrifuge for 15 seconds. The supernatant was aspirated off and the fines resuspended in 1ml 70% ethanol (70% ethanol; 30% TE buffer). The fines were pelleted again and as much supernatant removed as possible. The fines were resuspended in 50µl TE buffer and incubated at 37°C for 10 minutes to elute the DNA. The fines were pelleted again and the DNA solution was ready for use.

2.2.3.4 DNA restriction digestion

DNA restrictions were set up in sterile eppendorfs, each made up to a final volume of 10μl for every 1μg DNA used. The amount of restriction enzyme used also varied and was based on 1 unit of enzyme digesting 1μg DNA in 1 hour at 37°C.

Using the correct dilution of the appropriate enzyme buffer, the reaction tubes were set up containing the DNA sample, the restriction enzyme and, in most cases, some RNase to ensure the sample is clean. These were then incubated at 37°C for 1-2 hours before the loading buffer was added ready for running on the gel.

2.2.3.5 Plate gel preparation

Perspex formers (150mm x 185mm) were sealed onto ethanol-cleaned plates with silicone grease and the well-former positioned at the correct height. 200ml Alec's gel buffer (from a 10x stock of 96.8g Tris and 7.4g EDTA in 2l, pH7.7 with glacial acetic acid) was solidified with 0.8% agarose by heating in the microwave until dissolved. Once the gel had cooled, 1mg/l ethidium bromide was added before pouring.

The gel (without the formers) was placed onto the platform of the horizontal submarine electrophoresis tank, with sufficient Alec's gel buffer (containing 1mg/l ethidium bromide) added to cover the gel by 2-5mm. Having loaded the samples with the loading buffer (Fast orange: 0.1% Orange G, 20% Ficoll 400, 0.1M EDTA pH 8, autoclaved) into the wells (making sure to have added a marker), the gel was run with a maximum voltage of around 100-120V.

2.2.3.6 Mini-gel preparation

50ml TBE (108g/l Tris, 55g/l Boric acid, 9.3g/l EDTA, pH 8.3) was heated with 0.7% agarose and when cool, 1mg/l ethidium bromide was added. This was poured into a cleaned mini-gel apparatus (Cambridge Uniscience). 50ml TBE with 1mg/l ethidium bromide was used as the buffer.

2.2.3.7 Electroelution

The DNA fragment required was cut out of the gel using an ethanol-sterilized scalpel. The dialysis tubing was prepared by boiling for 10 minutes in 1mM EDTA then washing thoroughly with sterile distilled water. The gel was placed into the tubing along with 500μl TBE (90mM Tris; 90mM Boric Acid; 2.5mM EDTA; pH 8.3) making sure that the end is sealed securely. After all the air bubbles had been removed, the second end was sealed and the tubing was placed transversely into a minigel tank containing sufficient TBE to cover it.

A current of 60mA was run across the tubing for 20 minutes, followed by a 20 second burst of reversed polarity to release the DNA from the side of the tubing. Having checked the DNA had in fact eluted using UV light, the buffer (containing the DNA) was removed from the tubing which was rinsed with more buffer to remove any traces of DNA left. The DNA was then cleaned using either phenol/chloroform or silica fines, ready for ethanol precipitation.

2.2.3.8 DNA visualization and photography

DNA, stained with ethidium bromide, was visualized on a U.V. transilluminator, wavelength 300nm. When required, photographs were taken using a Polaroid Land camera with 3000 ASA Type 667 Polaroid film. The exposure was F11 for 3 seconds, with an orange filter.

2.2.3.9 Estimation of DNA content using the spectrophotometer

Using the Pye-Unicam SP8-150 Spectrophotometer, the absorbance of the DNA samples was measured at 260nm. The samples were prepared in 1ml glass cuvettes, 1µl original sample was made up to 1ml with water.

1mg/ml DNA has an absorbance of 20 at a wavelength of 260nm. Using this fact and taking note of the scale used, the concentration of DNA in the samples can be calculated from the printout.

The absorbance of the DNA samples was also measured with the Philips PU8700 series UV/Visible Spectrophotometer.

2.2.3.10 Southern blotting

The method used was based on that of Southern (1975). Prior to blotting, the gel was soaked (with agitation) in various solutions to denature and neutralize the DNA: 30 minutes in 0.25M HCl (if the fragments of interest are larger than 15kbp); 2 x 15 minutes and 1 x 30 minutes in 1.5M NaCl and 0.5M NaOH; and 3 x 30 minutes in 1.5M NaCl, 0.5M Tris HCl (pH 7.2) and 1mM Na_2EDTA.

The gel blotting apparatus was a platform covered with 3 layers of 3MM filter paper soaked in 20xSSC (3M NaCl; 0.3M $CH_3COO.Na$; pH 7.0 with 10N NaOH) including a filter paper wick immersed in a tank of 20xSSC. The gel was smoothed down on top of the filter paper and was surrounded with cling film to ensure that the 20xSSC passed only through the gel. A nitrocellulose filter, soaked first in distilled water then in 20xSSC for 20 minutes, was smoothed down on top of the gel, making sure that no air bubbles were present. This was topped with 3 layers of dry 3MM filter paper, 3 layers of absorbent nappy pads and a 500g weight.

After blotting overnight, the apparatus was dismantled carefully making sure that the edge and lanes of the gel were marked onto the filter (in ink) before the filter was removed and dried in a vacuum oven.

2.2.3.11 Colony hybridization

Replica L.Agar plates, with appropriate selection, were overlaid with gridded nitrocellulose filters as described by Sambrook *et al.* (1989). Colonies produced from transformations were streaked, within the grids, on replica plates and grown overnight at 37°C.

One filter of each pair was sealed and stored at 4°C while the other was prepared for probing. This was done by sandwiching the filter between dampened blotting paper and immersing it in various solutions (drying inbetween each): 3 minutes in 10% SDS; 5 minutes in 1.5M NaCl and 0.5M NaOH; 5 minutes in 1.5M NaCl, 0.5M Tris HCl (pH 7.2) and 1mM Na_2EDTA; and finally 5 minutes in 2xSSC. The filter was air dried for 30 minutes then baked at 80°C for 1 hour.

2.2.3.12 Filter prehybridization and hybridization

Prehybridization of the filter was carried out following Sambrook *et al.* (1989) in a sealed plastic bag containing 100ml (per filter) prehybridization solution: 5xSSC; 5xDenhardt's [0.1% Ficoll; 0.1% PVP; 0.1% BSA]; and 100-200μg/ml boiled herring sperm DNA. The filter was prehybridized for at least an hour at 65°C in a shaking waterbath.

The bag was drained before 50ml (per filter) hybridization solution was added: 5xSSC; 1xDenhardt's; 100μg/ml boiled herring sperm DNA. The radiolabelled probe was boiled for 5 minutes before it was added to the filter, the bag was resealed and incubated at 65°C, in a shaking waterbath, overnight.

The probe was retained and stored frozen for 1-2 months. The filter was washed in dilutions of SSC: 30 minutes in 2xSSC; 2 x 15 minutes in 1xSSC; and 2 x 15 minutes in 0.1xSSC, all warmed to 65°C before use. The filter was checked inbetween the washes for bands of radioactivity which could be distinguished above the background level. The filter was then exposed, with markers, to flashed X-ray film with an intensifying screen overnight, or longer if necessary, before the film was developed.

2.2.3.13 ^{32}P probe production

^{32}P probes were produced by nick translation following Rigby *et al.* (1977). The DNA fragment to be radiolabelled was isolated as a band on an agarose gel, then excised and eluted from the gel. 0.1-0.25μg DNA was dissolved in 26μl sterile distilled water and boiled

for 5 minutes.

10μl OLB (Sambrook *et al.*, 1989) was mixed into the sample followed by 2μl RNase-free BSA (10mg/ml). 10μl ^{32}PdCTP (100μCi) was added and the reaction was initiated with 2μl (2u) Klenow enzyme. The reaction was incubated overnight in a lead pot at room temperature.

The probe was purified from the unincorporated label by chromatography on Sephadex G-50 column (buffer: 150mM NaCl; 10mM EDTA; 50mM Tris; 0.1% SDS; pH 7.5). 500μl aliquots were collected from the column and the radioactivity was measured by scintillation. 1μl from each 500μl aliquot was added to 5ml scintillation fluid (Ecoscint A). The scintillation was measured on a Packard Tri-Carb Prias scintillation counter.

2.2.4 DNA Cloning Techniques

2.2.4.1 T_4 DNA Polymerase blunt-ending

The DNA to be blunt-ended was dissolved in 15μl sterile deionized water and mixed with 2μl 10x Polymerase buffer (0.33M Tris acetate pH 7.9; 0.66M potassium acetate; 0.10M magnesium acetate; 5.00mM DTT; 1mg/ml BSA). 2μl dNTP mix, containing 0.125mM of each of the 4 dNTPs was added followed by 1μl (2.5 u) T_4 DNA Polymerase.

The mixture was incubated at 37°C for 5 minutes, then the reaction was stopped by incubation at 65°C for 5 minutes. The blunt-ended DNA was diluted with 200μl TE, then cleaned with a phenol/chloroform extraction.

2.2.4.2 Blunt-end cloning

The vector, into which a blunt-ended insert is to be cloned, must first be restricted with a blunt cutting restriction enzyme, usually *Hinc*II.

The blunt-ended insert, from above, was diluted with TE and mixed with 300µl phenol, then left on ice until the vector restriction was ready. The correct concentration of restricted vector was added and mixed with the phenol and insert. A ratio of 3:1 insert:vector of molar sizes was used, with ≥0.1 pmol insert. The phenol/chloroform extraction was completed, then the DNA was precipitated with 2.5 volumes of a 1:10 mixture of sodium acetate:ethanol. The pellet was resuspended in 8µl water and mixed with 1µl 10x ligase buffer and 1µl ligase. The ligation was incubated overnight at 15°C.

2.2.4.3 Dephosphorylation of DNA

Prior to the addition of the insert DNA to the vector, during cloning, the 5' restricted vector ends were treated with alkaline phosphatase to reduce self-religation. Sufficient $ZnCl_2$ was added, post-restriction, to the vector to produce a 1mM solution, into which 0.5-1.0u alkaline phosphatase was mixed. The solution was incubated at 37°C for 30 minutes, then at 65°C for 10 minutes. After phenol/chloroform extraction and ethanol precipitation, the DNA was resuspended ready for cloning.

2.2.4.4 Shotgun cloning

The vector and insert DNA, at the correct molar ratio, were restricted together, usually with a restriction enzyme that gives a large overhang. The restriction was checked by running half of it on a mini-gel. The remainder was cleaned up with phenol/chloroform, then ethanol precipitated. The pellet was resuspended in 8μl water and mixed with 1μl 10x ligation buffer and 1μl ligase. The reaction was incubated overnight at 15°C.

2.2.4.5 Polymerase chain reaction (PCR)

The reactions were carried out following Saiki *et al.* (1988) in a volume of 100μl and, at all times, care was taken to avoid contamination with other DNA by the use of positive displacement pipettes. Sterile deionized water was pipetted into an eppendorf tube, followed by 10μl *Taq* reaction buffer, 16μl dNTP mix (1.25mM solution of each of the 4 dNTPs), 5μl of each 20mM forward and reverse primer solutions, 1μl (4.5u) *Taq* Polymerase and finally 15μl template DNA. The contents were mixed by tapping the tube and then overlaid with 100μl mineral oil, ensuring all air bubbles were removed. The tubes were UV-irradiated for approximately 5 minutes to reduce cross-contamination (Sarkar and Sommer, 1990). The tube was then placed into a thermal cycler (Pharmacia LKB Gene ATAQ Controller) and the 3 stage denaturation, annealing and extension reaction begun.

For each reaction, a negative control was set up, which was identical except it contained no template DNA. This enabled any contamination to be identified.

The temperatures used in the reactions were calculated to suit the individual primers, using their melting temperatures (Sambrook *et al.*, 1989); obviously, the annealing temperature has to be below the melting temperature.

Once the amplification cycles were completed, an aliquot of the reaction mixture was run on an agarose plate gel, with the control and a λ size marker, to determine the results.

2.2.4.6 Oligonucleotide synthesis

Oligonucleotide primers were synthesized by Mr J. Gilroy (Durham University) on the ABI 381A DNA Synthesizer, using the standard synthesis programme. The primers were dissolved in sterile distilled water and stored at -80°C.

2.2.4.7 Rapid mRNA extraction and cDNA synthesis

Following Gilliland *et al.* (1990), mRNA can be extracted and analysed from as few as 10 cells.

mRNA handling precautions, sterile equipment and diethyl-pyrocarbonate treatments were used throughout the extraction and reverse transcriptase reaction, following Sambrook *et al.* (1989). A small amount of leaf tissue was chopped up into a sterile eppendorf and 20µl buffer (0.5% NP-40; 10mM Tris pH 8.0; 10mM NaCl; 3mM $MgCl_2$) was added. After a 5 minute incubation on ice, the nuclei and debris were removed by centrifuging at 13 000rpm for 2 minutes.

10µl of the mRNA supernatant was used in the reverse

transcription reaction. 20 to 50pmol oligo(dT) primer was added to the mRNA together with 500µM of each dNTP, 1 x *Taq* polymerase PCR buffer, 1mM DTT, 2 units RNase inhibitor, 5 units Reverse transcriptase and made up to 20µl with diethylpyrocarbonate-treated water. The mixture was incubated for 1 hour at 37°C.

10µl of this reaction mixture was used as the template in a 35 cycle PCR.

2.2.5 *Arabidopsis* Transformation and Tissue Culture

2.2.5.1 Sterile techniques

All tissue culture procedures were carried out in a laminar flow cabinet, which was sprayed liberally with 70% ethanol before use. All the equipment used was sprayed with 70% ethanol before being placed in the cabinet and either autoclaved or flamed with 80% ethanol before use. All media used in the experiments were autoclaved for 20 minutes at 15 p.s.i. (121°C) and cooled to 60°C before the addition of any necessary antibiotics.

All the sterile procedures were completed quickly and with as little contact as possible, to avoid contamination. It is important to remember that the rear of the cabinet is the most sterile area and whenever any sterile object is uncovered, other equipment should be moved around it rather than over it.

2.2.5.2 Culture of axenic plants

The seeds were sterilized in 20% sodium hypochlorite (Chloros) for 20 minutes, with frequent shaking. After 3 rinses with sterile water, the seeds were spaced out onto sterile GM (MS salts; 1% sucrose; 1.0mg/l thiamine; 0.5mg/l pyridoxine; 0.5mg/l nicotinic acid; pH 5.7; solidified with 0.8% agar) in a petri dish. The dishes were then sealed with Micropore and placed in the growth room for about 3 to 4 weeks before the tissue was ready for use.

2.2.5.3 *Agrobacterium* preparation for transformations

5ml YEB, with the appropriate selection, was inoculated with a loop of *Agrobacterium* glycerol. This was incubated on a shaker at 28°C overnight. The culture was then transferred to 25ml YEB with selection in a 100ml conical flask and incubated overnight.

For use in a transformation, the *Agrobacterium* was pelleted using the benchtop centrifuge at 4200rpm for 10 minutes at room temperature. The pellet was then washed 3 times in sterile 2mM $MgSO_4$, resuspending and centrifuging each time. After the final pelleting, the *Agrobacterium* was resuspended in the appropriate transformation medium.

2.2.5.4 Acetosyringone induction of *Agrobacterium*

After growing overnight in 25ml YEB, the *Agrobacterium* was pelleted and resuspended in 25ml induction medium (MS salts , 3%

sucrose, 12.5mM Na_2HPO_4 [Sheikholeslam and Weeks, 1987]) with antibiotic selection and 1.5mM acetosyringone. The flask was then incubated at 28°C overnight and washed with sterile 2mM $MgSO_4$, as described above.

2.2.5.5 Root transformation

Root tissue transformation was attempted following Valvekens *et al.* (1988). Intact root systems were excised from sterile plants and incubated on CIM (Gamborgs B_5 salts; 2% glucose; 0.5mg/l 2,4-D; pH5.7; 0.8% agar) for 3 days. The roots were then cut into 5mm explants, placed in liquid medium with an added overnight *Agrobacterium* culture and then mixed gently for about 2 minutes. After blotting on sterile filter paper, the root explants were plated out in clumps on CIM and incubated for 48 hours in the Growth Room.

The explants were then rinsed, blotted and plated onto SIM (Gamborgs B_5 salts; 2% glucose; 5mg/l 2ip; pH5.7; 0.8% agar) with 200mg/l augmentin and 50mg/l kanamycin monosulphate selection. The plates were sealed with 1.25cm Micropore, to reduce the condensation, and kept under constant illumination. The explants were transferred onto fresh medium every fortnight. Any shoots produced were transferred onto GM (see 2.2.5.2) in tall Beatson bottles with petri dish lids and sealed with Micropore. In order to increase regeneration efficiency, only 2 to 4 shoots were put in each Beatson.

Note: in order to facilitate the infections and washes, small autoclaved plastic baskets with 100µm mesh bases were used. The explants were put into the sieves ready for infection or washing. This

allowed the explants to be removed easily from the mesh rather than "fishing" them out of the liquid medium. The time needed for infection and washing can therefore be judged more accurately.

2.2.5.6 Kanamycin kill levels for root tissue

Sterile *Arabidopsis* root tissue was excised and plated onto CIM then transferred onto SIM plates after 3 days. The media of both sets of plates was supplemented with kanamycin concentrations of 0, 10, 20, 30, 40, 50, 60mg/l. The tissue was examined daily until the kill level could be determined.

2.2.5.7 Metal toxicity levels of germination

Sterilized *Arabidopsis* seeds were plated onto GM (see Methods 2.2.5.2) containing increasing concentrations of heavy metals. The plates were examined regularly and the level of germination and growth was compared to the unsupplemented control plates.

2.2.6 Histochemical GUS assay procedure

Tissue was chopped up finely and incubated overnight at 37°C with 10μl 0.1M sodium phosphate buffer pH 7.0 (30.5 units 0.2M Na_2HPO_4 and 19.5 units 0.2M NaH_2PO_4)and 10μl 2mM X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide dissolved in phosphate buffer), following Jefferson (1987). The tissue was then dehydrated with 100% ethanol (fresh ethanol was added when necessary). Any positive, expressing tissue shows a blue colouration, seen more clearly using a light microscope.

2.2.7 Tissue Sectioning

2.2.7.1 Fixation

5mm lengths of X-Gluc stained *Arabidopsis* tissue were placed into a freshly prepared 3% paraformaldehyde and 1.25% gluteraldehyde solution in 0.05M phosphate buffer pH 7.0. The samples were fixed for at least 4 hours at room temperature, with gentle agitation, on a 45° rotating platform at 2 r.p.m.

2.2.7.2 Dehydration

The tissue samples were dehydrated at room temperature, with gentle agitation, in increasing concentrations of ethanol. The tissue was immersed for 30 minutes in each of the following concentrations of ethanol: 2.5%, 25%, 50%, 75%, 95% and 100%.

2.2.7.3 Embedding in wax

The tissue was infiltrated overnight at room temperature in a 1:1 mixture of 100% ethanol : Histo-clear with gentle agitation. The mixture was replaced with 100% Histo-clear which was changed twice daily over 24 hours. The tissue was infiltrated overnight in a 1:1 mixture of Histo-clear : wax (Paraplast plus) at 56°C and then 100% wax at 56°C for 36 hours, replacing the wax twice daily. The tissue was then embedded in fresh wax.

2.2.7.4 Tissue sectioning and mounting

5 to 25μm sections were cut from the wax blocks using a Leitz 1512 microtone. The sections were floated in a beaker of water, heated to 45°C, until they unrolled. Sections were picked up onto TESPA-coated microscope slides and dried overnight. (Slides were TESPA coated by immersing in a 2% TESPA in acetone solution for 10 - 15 seconds, rinsed twice in 100% acetone then in distilled water and dried in a dust-free environment.)

The slides were immersed in Histo-clear to dissolve the wax, rinsed with 100% ethanol and then distilled water. After drying and cleaning, coverslips were added with D.P.X.mountant.

Sections were examined and photographed using a Nikon Optiphot microscope.

2.2.8 Immuno-labelling

2.2.8.1 Immuno-gold labelling

5 to 25μm sections were cut from the wax blocks and processed (as in 2.2.7), up to and including the dewaxing with Histo-clear. The slides were washed in distilled water then in PBS (0.1M phosphate-buffered saline). The sections were then incubated (still on the slides) in 5% BSA in PBS for 30 minutes at room temperature to block the non-specific sites. After rinsing in PBS, the sections were incubated in a 1:50 concentration of specific antibody (provided by Dr A.Shirsat, University of Bangor) in 5% BSA in PBS for 4 hours at room temperature. The sections were washed again in PBS, 3 x 15 minutes, then were incubated in 1nM goat anti-rabbit IgG (gold label) in 5% BSA

in PBS, for 2 hours at room temperature. After washing a further 3 x 15 minutes in PBS, the sections were rinsed in distilled water and then milli Q water, ready for silver enhancing.

2.2.8.2 Silver enhancing

Silver enhancing was carried out using the IntenSE M Silver Enhancement kit from Amersham. Equal volumes of enhancer and initiator solutions were mixed and added onto the sections, which were incubated until a brown signal was visible. At which point, the sections were rinsed well in distilled water, allowed to air dry and then mounted in D.P.X.mountant.

2.2.9 Metal analysis by flame spectrophotometry

Individual putative transgenic and control plantlets were analysed for copper content after 3 to 4 weeks on control or 100μM $CuSO_4$-containing medium. Each plantlet was washed thoroughly in de-ionized distilled water to remove any traces of medium. After blotting dry on Whatmann filter paper, the plantlets were weighed (in as little time as possible to avoid dehydration). The plantlets were frozen in liquid nitrogen and ground in eppendorfs. 1ml 70% nitric acid was added to each plantlet and incubated overnight at room temperature. The debris was centrifuged out of the solution, and the supernatant removed.

A Perkin Elmer Atomic Absorption Spectrophotometer was used to measure the metal content of these supernatants, and the concentration was calculated by comparison with standard curves.

3. RESULTS

3.1 Establishing a Transformation Method for *Arabidopsis thaliana*.

3.1.1 Kanamycin kill levels of *Arabidopsis*

From the concentrations of kanamycin tested (Methods 2.2.5.6), 50mg/l was chosen for subsequent selections. Growth of the control tissue started to decline when the kanamycin concentration increased over 30mg/l but ceased totally at 50mg/l. These results are in agreement with Valvekens *et al.* (1988).

3.1.2 *Agrobacterium tumefaciens* C58/3

This strain was developed by Dr P. Mullineaux (John Innes Institute, Norwich) as a binary vector system for plant transformations. The T-DNA from the pTi C58 was deleted and replaced by a spectinomycin / streptomycin resistance gene. C58/3 was isolated as a spectinomycin / streptomycin^{R} nalidixic acid^{R} kanamycin^{S} colony (Dale *et al.*, 1989).

3.1.3 pJIT73

pJIT73 is a 17.5kb chimaeric construct, developed by Dr P. Mullineaux, based on pBIN19 (Bevan, 1984). It contains a broad host range origin of replication (enabling it to replicate in both *E.coli* and *Agrobacterium*) and selectable bacterial antibiotic marker genes. It also contains a T-DNA region, bounded by right and left border repeats, recognizable by the *Agrobacterium* and can therefore be used as the donor plasmid in an *Agrobacterium* binary vector system.

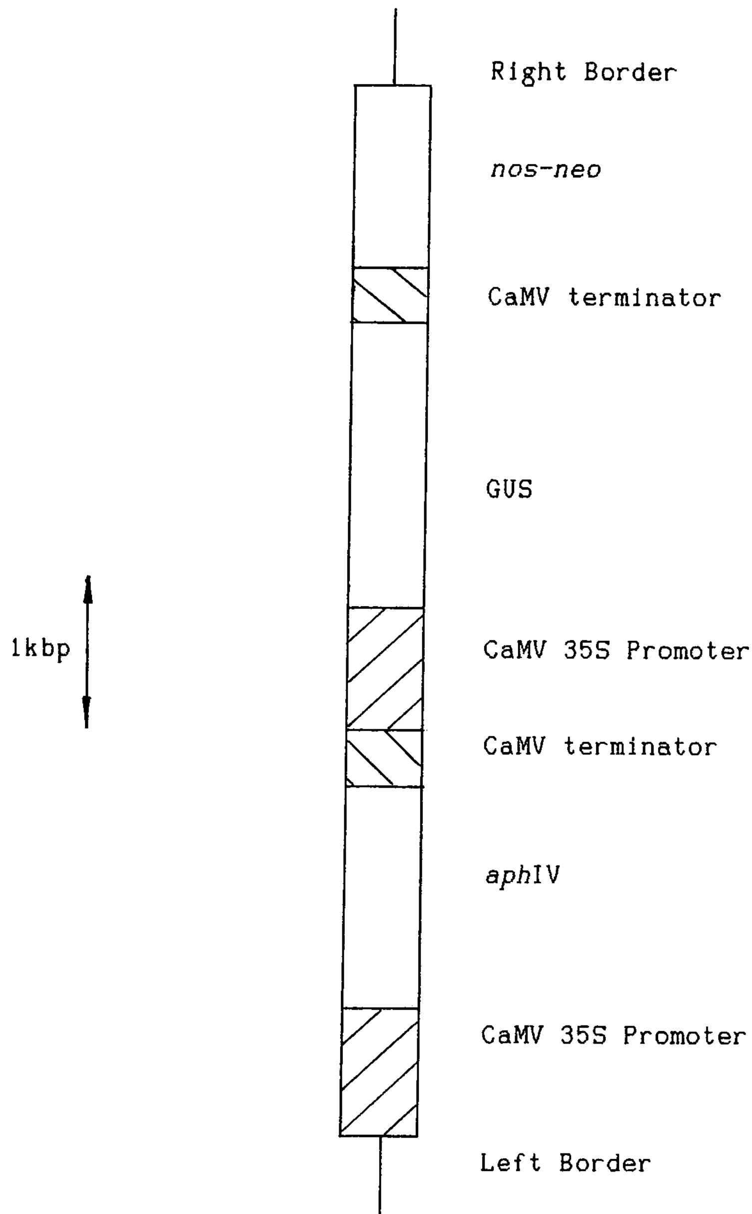
Figure 1 shows the components of the T-DNA, the hygromycin (*aph*IV) and neomycin (*nos-neo*) phosphotransferases for selection and the GUS reporter gene. The GUS gene encodes the easily detectable β-Glucuronidase activity and, as no endogenous GUS activity could be detected in *Arabidopsis thaliana* (see Results 3.1.7), it was an ideal scorable reporter gene.

The GUS and *aph*IV genes are both constitutively expressed, under the control of 35S CaMV promoters. Although levels of expression of genes promoted by 35S CaMV have been shown to vary in transgenic plants (Sanders *et al.*, 1987) (possibly due to T-DNA positional effects not the CaMV promoter), it has been shown to produce expression levels more than 30 times higher than the commonly used nopaline synthase promoter.

This combination of selectable markers and the easily scorable reporter gene made pJIT73 an obvious choice to use whilst establishing the transformation procedure.

Figure 1

pJIT73 T-DNA



Structure of the pJIT73 construct (P.M.Mullineaux), containing the *nos-neo* (neomycin phosphotransferase) kanamycin resistance gene, the *aph*IV hygromycin resistance gene and the GUS marker gene.

3.1.4 Production of *Agrobacterium tumefaciens* pJIT73

The procedure for the introduction of a broad host range plasmid into *Agrobacterium* by a triparental mating is described in Methods 2.2.1.5. The antibiotic resistance phenotypes of the conjugative (pRK2013) and donor (pJIT73) *E.coli* strains were confirmed: both grew on $50\mu gml^{-1}$ kanamycin but failed to grow on $100\mu gml^{-1}$ spectinomycin or $80\mu gml^{-1}$ nalidixic acid. The reverse situation occurred when the *Agrobacterium* strain C58/3 was tested.

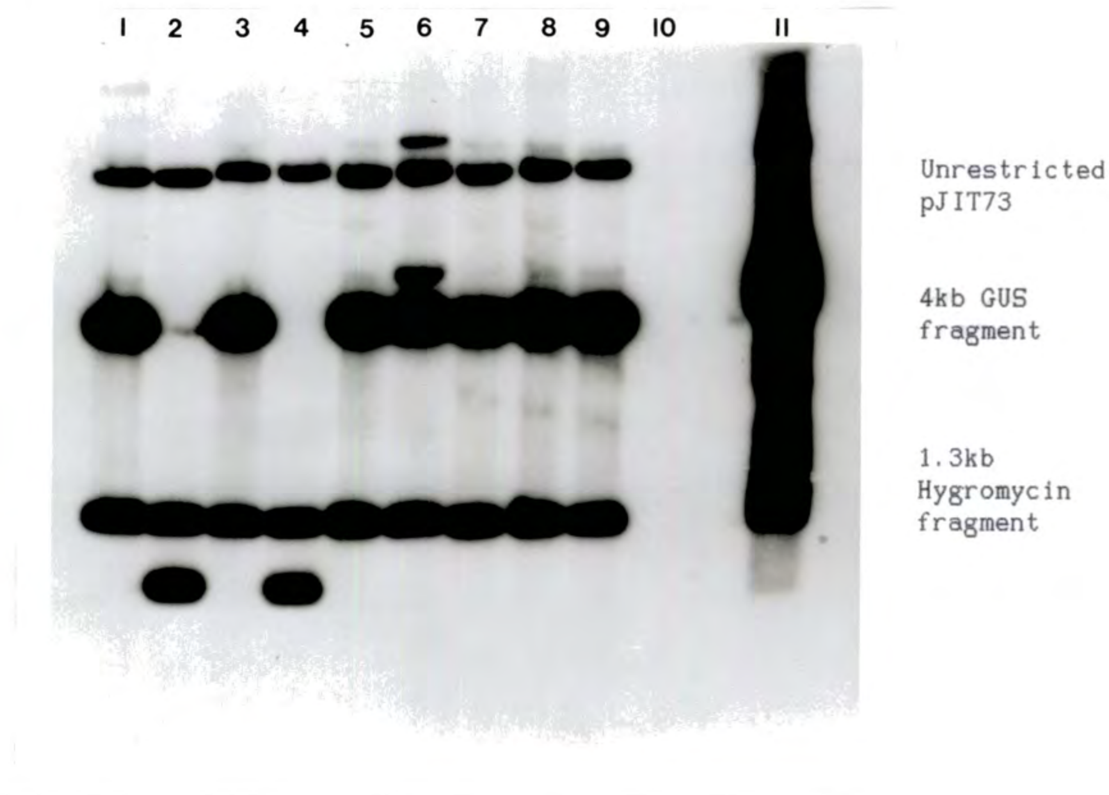
After the triparental mating, 9 single colonies were picked off the selection plates and grown up overnight for minipreping. DNA was extracted (Methods 2.2.2.3) and restricted with *Hin*dIII. The restriction was then run on a 0.8% agarose gel together with *Hin*dIII restricted DNA from the *Agrobacterium* recipient strain as a negative control, *Hin*dIII restricted DNA from the *E.coli* pJIT73 donor strain as a positive control and a λ size marker. The gel was Southern blotted (Methods 2.2.3.10) and the DNA was baked onto the nitrocellulose filter.

The 4kb GUS fragment and the 1.3kb Hygromycin fragment from *Hin*dIII-restricted pJIT73 were labelled by nick translation (Methods 2.2.3.13) to a specific activity of 8.5×10^7 counts min^{-1} μg^{-1} and were used to probe the filter (Methods 2.2.3.12). After washing to a stringency of 0.1 x SSC for 30 minutes at 65°C, the filter was exposed overnight.

The results are shown in Plate 1. Colony 1 (track 1) was chosen for subsequent plant transformations as the hybridizing bands present correspond to the 4.0kb GUS fragment and the 1.3kb Hygromycin fragment (as do tracks 3, 5, 7, 8 and 9).

Plate 1

Autoradiograph of C58/3 pJIT73 Triparental Mating



1-9. Putative C58/3 pJIT73 *Hin*dIII-restricted samples

10. C58/3 *Hin*dIII-restricted negative control

11. *E.Coli* pJIT73 *Hin*dIII-restricted positive control

3.1.5 C58/3 pJIT73 transformation of *Arabidopsis thaliana*

Transformation of *Arabidopsis* root tissue was carried out, following Methods 2.2.5.5, with acetosyringone-induced (Methods 2.2.5.4) *A. tumefaciens* C58/3 pJIT73. The regeneration sequence of transformed root explants, compared to control root explants on 50mg/l kanamycin selection, is shown in Plates 2a-d; shoots were large enough for excision within 3 to 4 weeks.

Putative transgenic shoots which came through the 50mg/l kanamycin selection were excised and planted into large Beatson jars containing germination medium (GM). Tissue samples and seeds were collected from each plant.

Regeneration Sequence of Root Explants

Plate 2a

Transformed root explants, after 7 days on SIM (+selection).

Plate 2b

Transformed root explants, after 14 days on SIM (+selection).

Plate 2c

Transformed root explants, after 21 days on SIM (+selection)

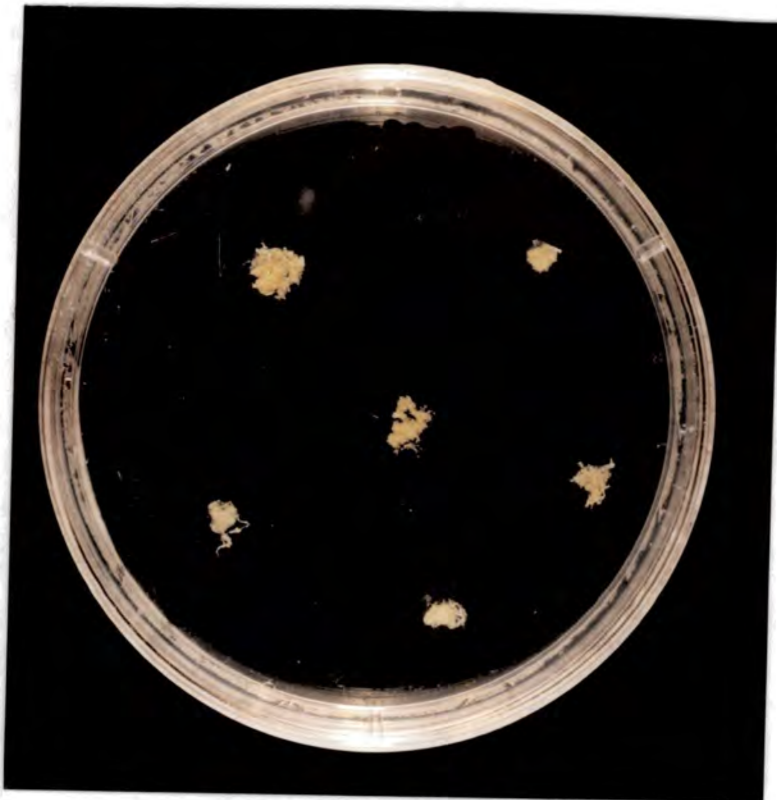


Plate 2d

Control root explants, after 21 days on SIM (+selection)

3.1.6 DNA analysis of putative transformants

DNA was extracted from single putative transgenic *Arabidopsis* leaves and single control *Arabidopsis* leaves, following Methods 2.2.2.6. A 35 cycle PCR (polymerase chain reaction) amplification, using primers specific for the GUS gene (216 and 217), was used to confirm the presence of the introduced gene in transformants. Each PCR cycle consisted of 45s at 94°C denaturation, 45s at 45°C annealing and 2 min. at 72°C extension (with a 5 minute final extension in the 35th cycle). A 50µl reaction was set up for each sample (Methods 2.2.4.5) using 5µl DNA extract in each reaction, with pJIT73 as a positive control. All the reactions were UV-irradiated for 7 minutes before being placed in the thermal cycler.

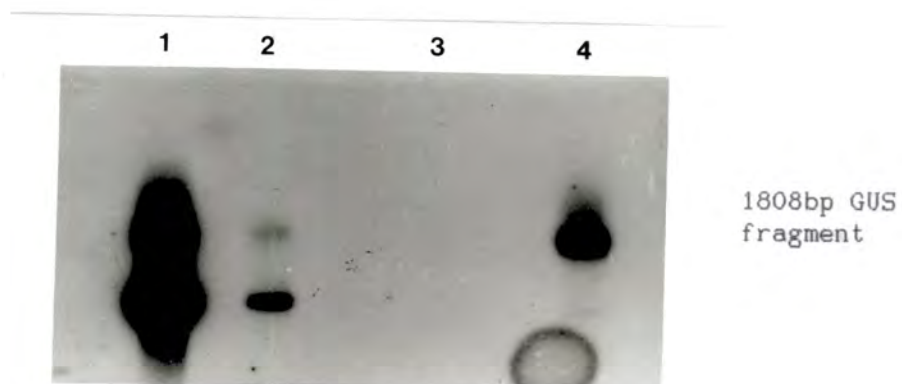
20µl of each reaction was run on a 0.8% agarose gel, together with a λ size marker, which was then Southern blotted (Methods 2.2.3.10). The 4kb *Hin*dIII GUS fragment of pJIT73 was ^{32}P labelled by nick translation (Methods 2.2.3.13) to a specific activity of 2.6 x 10^{9} counts min^{-1} μg^{-1} and was hybridized to the filter, overnight, at 65°C. The filters were washed to a stringency of 1 x SSC for 30 minutes at 65°C before being exposed for two hours to X-ray film (Methods 2.2.3.12).

The results are shown in Plate 3. The amplified product is predicted to be 1808bp in length. A band of the correct size, hybridizing to the GUS probe, is present in the transgenic plant tested, but not in the control.

Plate 3

Autoradiograph of PCR-amplified GUS

from Putative Transgenic ***Arabidopsis*** pJIT73



1. pJIT73 GUS PCR positive control
2. GUS PCR of putative transgenic *Arabidopsis* pJIT73
3. PCR negative control
4. GUS PCR of putative transgenic *Arabidopsis* pJIT73

3.1.7 Histochemical GUS assay

Putative transformed tissue, together with control tissue, was incubated in X-Gluc (Methods 2.2.6). The transformed tissue was clearly blue in colour when compared to the green control tissue. Stained tissue was fixed, dehydrated and embedded in wax (Methods 2.2.7), then sectioned and photographed (Plates 4a and b). The transformants show an all over blue colouration, indicating the non-tissue specific expression pattern of the 35S CaMV promoter. The control tissue showed no blue colouration at all.

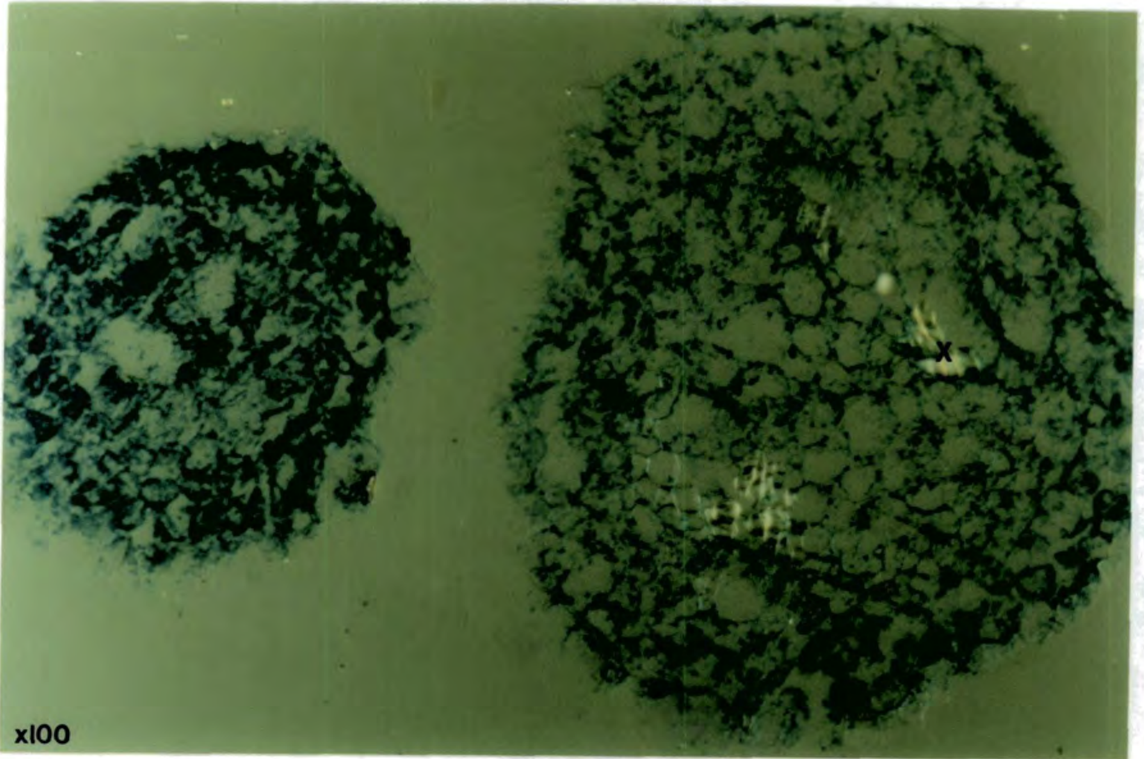
3.1.8 Immuno-gold anti-GUS labelling

Sections of tissue, prepared as above, were incubated with the anti-GUS antibody, then immuno-gold labelled and silver enhanced (Methods 2.2.8). After mounting in D.P.X. mountant, the sections were photographed under epi-polarized light (Plate 5). The silver particles that enhance the immuno-gold label were clearly seen all over the transgenic section whereas no label was present on the control sections.

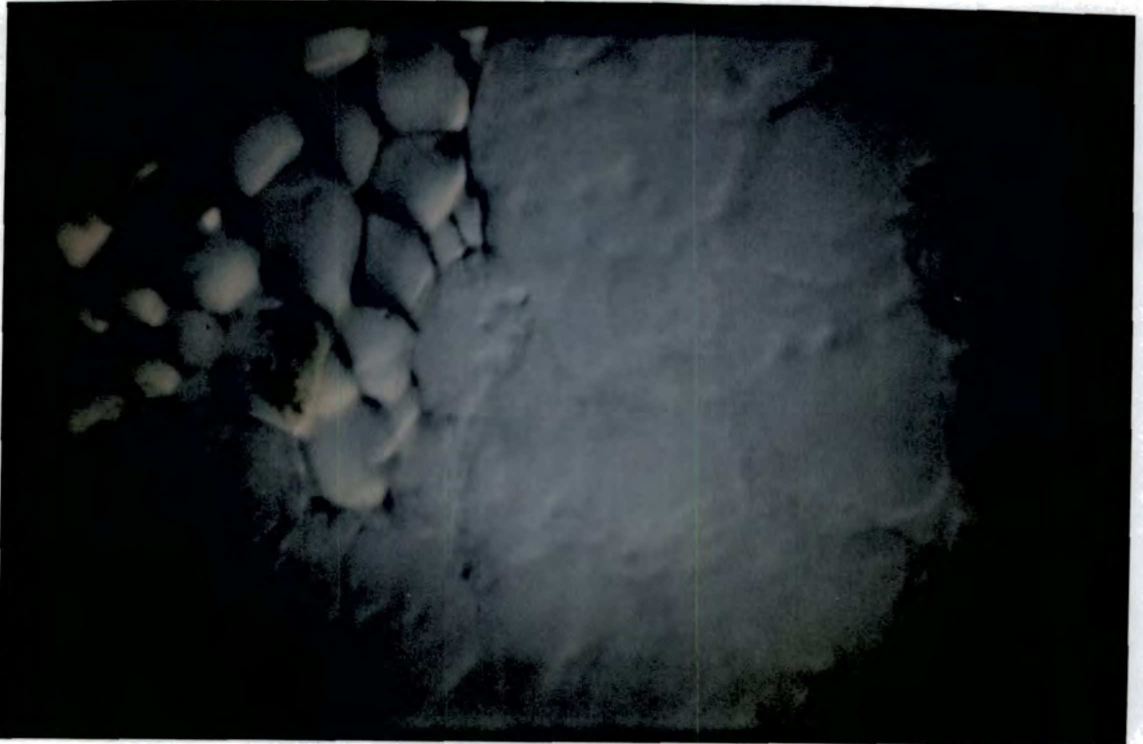
3.1.9 Conclusion

It was concluded from this data that the root transformation system described was sufficiently fast and efficient for use in future experiments. The GUS marker gene has also proved to be useful in detecting transformants. The histochemical assay is quick and easy, and with the availability of the GUS PCR primers, the presence of the gene at the DNA level is readily detected.

Plate 4 (a and b)
Stem Sections of Transgenic *Arabidopsis* / pJIT73
Histochemical GUS Expression



4a : Stem sections of GUS histochemically stained transgenic *Arabidopsis* / pJIT73, showing a strong blue colouration throughout the sections (except in some of the more highly refractive xylem cells [X]), which indicates a high level of non-tissue-specific 35S CaMV-GUS expression.



4b : Section of a GUS histochemically stained control *Arabidopsis* stem, showing the lack of blue colouration, and therefore lack of GUS expression, when compared to Plate 4a.

Plate 5

Stem Section of Transgenic *Arabidopsis* / pJIT73

Immunogold GUS Assay

Stem section of *Arabidopsis* / pJIT73 after immunogold anti-GUS labelling and silver enhancing, showing the accumulation of silver particles over the entire section. This correlates to the GUS expression pattern shown in Plate 4a of non-tissue-specific expression which is the expected expression pattern of the 35S CaMV promoter.

3.2 Transformation of *Arabidopsis* with the Putative Plant Metallothionein Gene *$PsMT_A$* (from *Pisum sativum*).

3.2.1 PCR amplification of *$PsMT_A$*

The *$PsMT_A$* coding region was contained in a cloned segment of pea genomic DNA, pλS2 (3.1kb insert in pUC18). The cloning strategy employed to obtain the coding sequence in a form suitable for incorporation in *A. tumefaciens* expression vectors was to amplify the *$PsMT_A$* coding region using the polymerase chain reaction (PCR), at the same time adding a specific restriction site to both ends. A two stage cloning would then follow, first into pUC18, then into the pROKII vector ready for the *A. tumefaciens* triparental mating.

Figure 2 shows the $PsMT_A$ sequence, and the position and sequence of both the forward (270) and reverse (271) primers.

The PCR forward primer (270) was designed to prime from base -44 (T) and was a 15bp perfect match. A *Kpn*I (and *ASP*718) restriction site was added, together with two extra bases at the 5' end to ensure that the *Kpn*I site would be amplified intact, as one or two bases are often lost from the ends of the PCR product during amplification.

The reverse PCR primer (271) was designed to prime from base 883 (G) and was also a 15bp perfect match. A *Kpn*I restriction site and two extra bases were added to the 5' end as on the forward primer.

*Kpn*I restriction sites were chosen for the primer construction because the *$PsMT_A$* coding region contains no internal sites and the pROKII vector has a usefully positioned *Kpn*I cloning site. Also the *Kpn*I isoschizomer, *ASP*718, produces a larger overhang than *Kpn*I and was therefore used in the *$PsMT_A$* cloning.

Figure 2

The Sequence of *$PsMT_A$*

```
5'..GAAGATAACAGCAACCAAGCAATTAATATCAATTGTTGTTTGCAAAAAATCTTAGGTTCTGAAAAT
     ATGGTACCAGCAACCAAGCAAT (Primer 270)

ATG TCT GGA TGT GGT TGT GGA AGC AGT TGC AAC TGT GGT GAT AGC TGC AAGTAA
Met Ser Gly Cys Gly Cys Gly Ser Ser Cys Asn Cys Gly Asp Ser Cys Ly
                                Domain 1

GGATCCACCACCTTAATTCTTTGTTGTTTTTCTGTATAATTTTTTCATTACAATTATTTGTATGTCTATT

TTTAATCATATAGATGATTCTTTGGAGATTTTTTTAAATAATTTGTTTAGTTTTATCGCATCGAATAATA

TATGATCTGAGCATGAGAAAAATAAATTTAATATAGACGGATTGTTTTTTATAAATGAATTAGGCTGAAT

CTAAATTCTAAGACTATGAATATGGTTCATAATTCTATGTTAAATCATTTTGTGTAGTGAAATTGGGCAA

TTTTATGTGTAAACGCATAATTTTGAGGTTTAAAATAAGGATCGTGCTGTCGCGATAGTTTAAGTGTCGA

TTGTAGTCGCGTAAAGGCTTTTCTGATTTCGGTTGGTTTAAGTGTTGATTGCAATCGTGTAAAGAGTTTT

CTAATTTCGGTTGGTTTAGGTGCGATTGCAGTCGGGTAAAGATTTTCGTGATTGTCGTCGTTGCGGTGTG

AATTAATCACAATTTCTTCTTTATCATAAAAACGTTGAATAACATATCGATATCGATTTGAAAACCTTTT

TCGTGTAACGGTCTTTCGAAAACCTTTATTTTGACAACCAAGTTTATAATTGATTTGTTTTGCTTGACAG

A  TGC AAC AAG AGG  TCT AGT GGA TTG AGC TAC TCC GAA ATG  GAA  ACC  ACC
s  Cys Asn Lys Arg  Ser Ser Gly Leu Ser Tyr Ser Glu Met  Glu  Thr  Thr

GAA ACC GTG ATT  CTT GGC GTC GGT CCG  GCG AAG ATC CAG TTT GAA GGT  GCT
Glu Thr Val Ile  Leu Gly Val Gly Pro  Ala Lys Ile Gln Phe Glu Gly  Ala

GAA ATG AGT GCT GCT TCT GAG GAT GGT GGC TGC AAG TGT GGT GAT AAC TGC
Glu Met Ser Ala Ala Ser Glu Asp Gly Gly Cys Lys Cys Gly Asp Asn Cys
                                                  Domain 2

ACT TGT GAC CCT TGC AAC TGC AAA TGAAGTGTAACATATAAAAGCTTGAAGCAGAGATATTG
Thr Cys Asp Pro Cys Asn Cys Lys                          CTTCGTCTCTATAAC

AAACCATTATGTTT.......3'
CATGGTA (Primer 271)
```

The sequence of *$PsMT_A$* and the predicted amino acid sequence of its product (Evans *et al.*, 1990a). Primers 270 and 271 both contain a *Kpn*I / *ASP*718 restriction site (GGTAC/C) at the 5' ends. A PCR using these primers on $PsMT_A$ should produce a 969bp product from chromosomal DNA and a 335bp product from cDNA.

A 10 cycle PCR was used with a 1½ minute denaturation step at 94°C, a 1 minute annealing step at 50°C (well below the primer melting temperatures of 64°C and 62°C) and a 2 minute extension step at 72°C. A 5 minute extension step was added to finish the 11th cycle (Methods 2.2.4.5).

20µl of the PCR product was run on a 1% agarose gel along with a PCR negative control (no template DNA was added to the reaction) and a λ size marker. The gel was blotted and probed with the 3.1kb *$PsMT_A$* *Eco*RI/*Sal*I fragment of pλS2, nick translation labelled to a specific activity of 1.35 x 10^9 cpmµg^{-1}. The filter was washed to a stringency of 0.1 x SSC for 30 minutes at 65°C and exposed overnight (Methods 2.2.3.10/12/13).

The results are shown in Plate 6. The strongly hybridizing band is 969bp and therefore corresponds to *$PsMT_A$*, (the smaller band corresponds to the primers). The remainder of the PCR was run on a 1% agarose gel and the 969bp band was electroeluted (Methods 2.2.3.7).

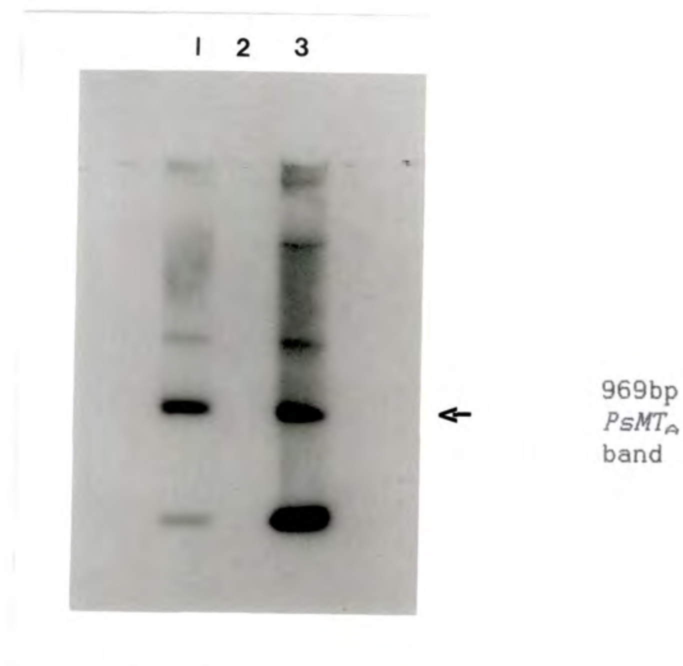
3.2.2 Cloning *$PsMT_A$* into pUC18

Attempts to clone the *$PsMT_A$* coding region into pUC18 using the *Kpn*I sites were unsuccessful. Similar problems have arisen in other groups with the lack of recognition by restriction enzymes to sites towards the ends of PCR amplified products.

Blunt-end cloning was the obvious next strategy to attempt. The *$PsMT_A$* was blunt-end cloned into *Hinc*II-restricted pUC18 (Methods 2.2.4.1/2). The ligation was transformed into *E.coli* DH5αMAX competent cells which were plated onto Xgal/Amp plates (Methods 2.2.1.4). 3 white colonies were selected, grown overnight and minipreped. The DNA

Plate 6

Autoradiograph of PCR-amplified *PsMT*$_A$ from pλS2



1. PCR of pλS2 using *PsMT*$_A$ primers 270 and 271

2. PCR negative control

3. PCR of pλS2 using *PsMT*$_A$ primers 270 and 271

was *ASP*718 (*Kpn*I isoschizomer)-restricted and run on a gel with a λ size marker. 2 of the 3 colonies showed a 969bp insert. DNA from both of these colonies was prepared for sequencing (Methods 2.2.2.2) and sequenced by J. Bryden using the ABI 373A DNA sequencer. The results confirmed the sequence to be that of $PsMT_A$.

3.2.3 Cloning $PsMT_A$ into pROKII

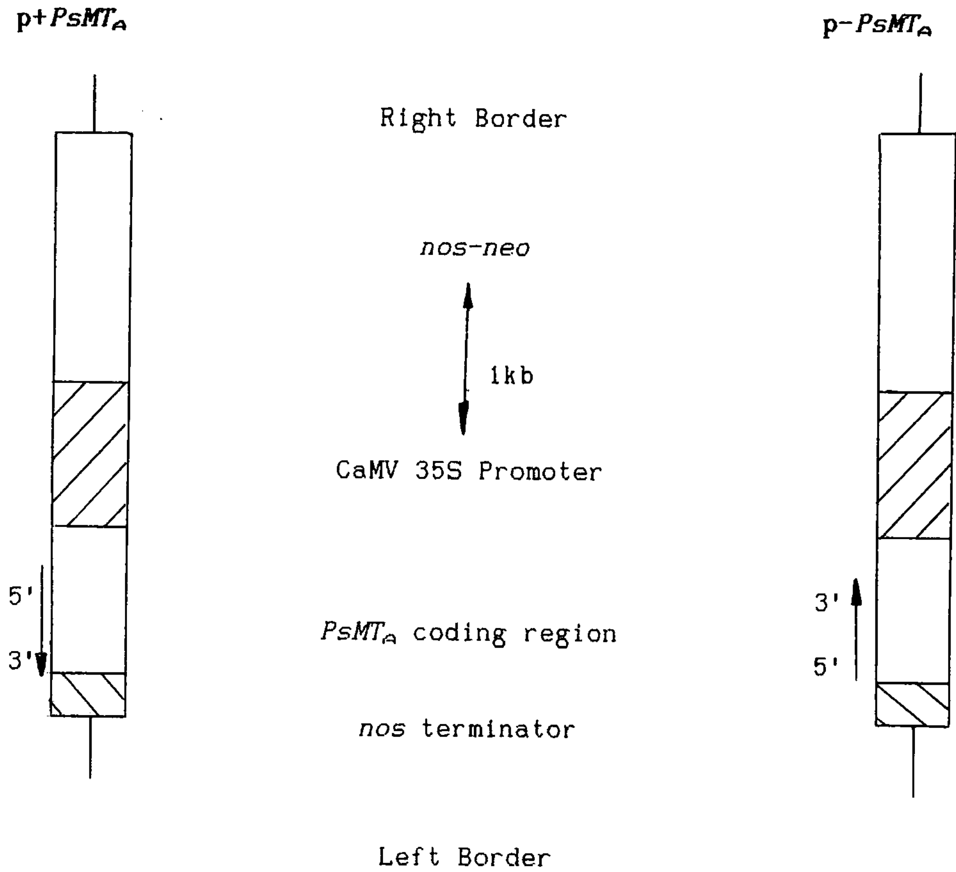
Figure 3 shows the pBIN19 based pROKII constructs with the $PsMT_A$ coding region in between the 35S CaMV constitutive promoter and the *nos* terminator, and the *nos-neo* kanamycin resistance gene.

pROKII was restricted with *ASP*718 to produce larger overhangs than *Kpn*I (therefore improving cloning efficiency), and alkaline phosphatase treated (Methods 2.2.4.3) to reduce religation. The *ASP*718 $PsMT_A$ fragment was electroeluted from a gel and ligated in a 1:1 molar ratio with the *ASP*718 restricted pROKII. 1μl of the ligation was transformed into *E.coli* DH5α competent cells which were then plated onto $50\mu gml^{-1}$ kanamycin YT (Methods 2.2.1.3). Approximately 60 colonies grew overnight.

The colonies were picked off onto 2 replica plates for a colony screen, a $PsMT_A$ positive control was added, and one filter was probed with the $PsMT_A$ probe (as results 3.2.1). After washing to a stringency of 0.1 x SSC for 10 minutes at 65°C, and overnight exposure, 25 colonies were shown to contain $PsMT_A$.

6 of these colonies were picked off and grown overnight to miniprep (Methods 2.2.2.1). The DNA was restricted with *ASP*718 and run on a 0.8% agarose gel, with a λ size marker to reconfirm the presence of the 969bp $PsMT_A$ band. All 6 colonies were positive, each showing a

Figure 3



Structure of the p+$PsMT_A$ and p-$PsMT_A$ constructs, containing the *nos-neo* kanamycin resistance gene and either the positive or negative orientation of $PsMT_A$. Details of the construction are in Results 3.2.3.

band which, when compared to the λ size marker, appeared to be 969bp long.

The orientation of the *PsMT*$_A$ insert was confirmed by restricting the DNA with *Hin*dIII. The only *Hin*dIII sites in the construct are shown in Figure 4. When restricted, the positive orientation clone should contain the large pBIN19 band and the 1744bp 35S CaMV-*PsMT*$_A$ band whereas the negative orientation clone should contain the pBIN19 band and the 825bp 35S CaMV band. Of the 6 clones tested, 2 were in the positive orientation and 4 were in the negative orientation.

3.2.4 Production of the *Agrobacterium tumefaciens PsMT*$_A$ strains

The antibiotic resistance phenotypes of the *E.coli PsMT*$_A$ positive and negative strains were confirmed by their growth on 50μgml^{-1} kanamycin and failure to grow on 100μgml^{-1} spectinomycin and 80μgml^{-1} nalidixic acid.

After the triparental matings, 5 colonies from each of the positive and negative orientation strains were picked off the selection plates and grown overnight for minipreping (Methods 2.2.2.3). The DNA was extracted and half of it restricted with *ASP*718, the remainder with *Hin*dIII. A 0.8% agarose gel was run for each set of restrictions, with positive and negative strains together with a C58/3 negative control, *E.coli* positive controls and a λ size marker on each. The gels were Southern blotted and the filters probed with the *PsMT*$_A$ probe (as 3.2.1). After washing to a stringency of 0.1 x SSC for 10 minutes at 65°C, the filters were exposed overnight (Methods 2.2.3.10/12/13). The results are shown in Plates 7 and 8.

Figure 4

p+/-*PsMT*$_A$ *Hin*dIII Restriction Sites

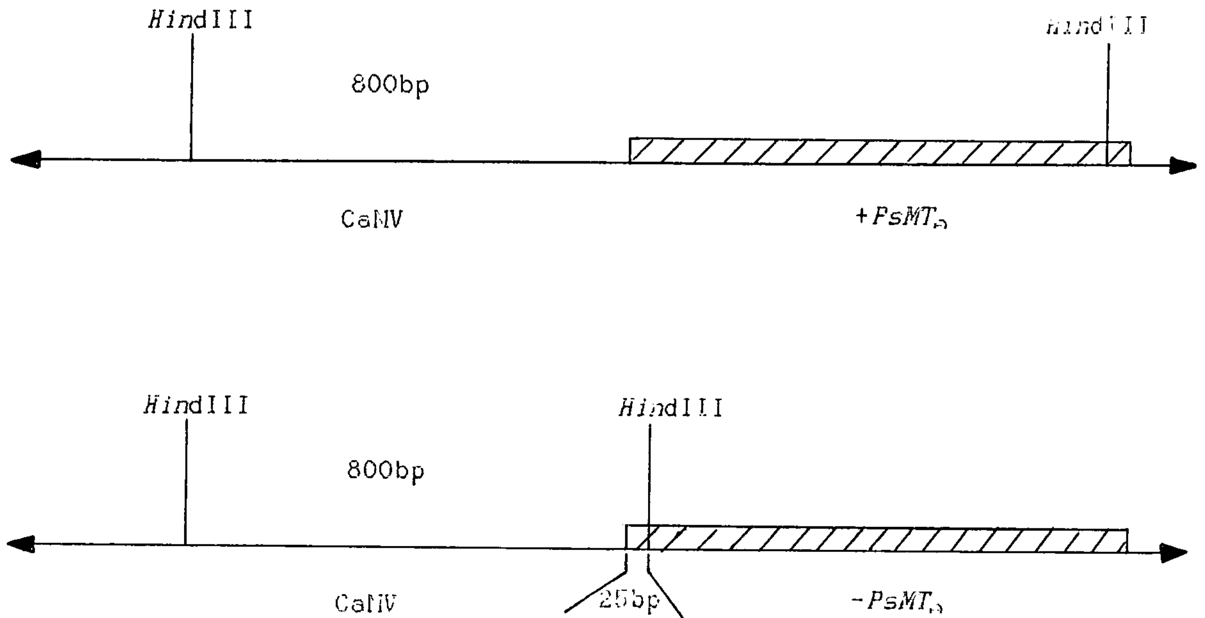
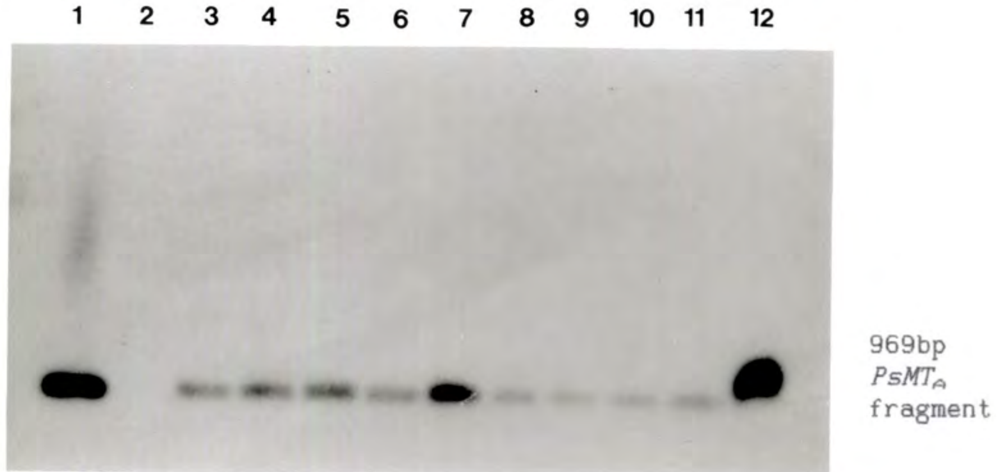


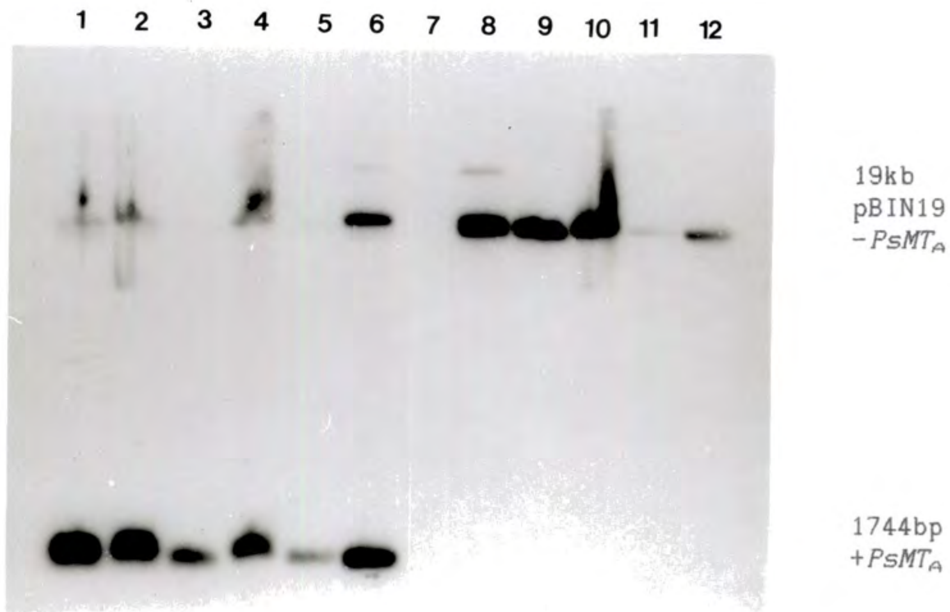
Figure 4 shows the *Hin*dIII restriction sites used to determine the orientation of the 969bp *PsMT*$_A$ insert within the T-DNA. When restricted, the positive orientation clone should result in the large pBIN19 band and the 1744bp 35S CaMV-*PsMT*$_A$; the negative orientation clone should result in the pBIN19 band and the 825bp 35S CaMV band.

Plates 7 and 8

Autoradiographs of C58/3 p*PsMT*$_A$ Triparental Mating



1. *E.coli* p*PsMT*$_A$ *ASP*718 restricted positive control
2. C58/3 *ASP*718 restricted negative control
3-7. Putative C58/3 p+*PsMT*$_A$ *ASP*718 restricted samples
8-12. Putative C58/3 p-*PsMT*$_A$ *ASP*718 restricted samples



1-5. Putative C58/3 p+*PsMT*$_A$ *Hin*dIII restricted samples
6. *E.coli* p+*PsMT*$_A$ *Hin*dIII restricted positive control
7. C58/3 *Hin*dIII restricted negative control
8. *E. coli* p-*PsMT*$_A$ *Hin*dIII restricted negative control
9-12. Putative C58/3 p-*PsMT*$_A$ *Hin*dIII restricted samples

Track 3, Plate 7, (track 1, Plate 8) was selected for the *A. tumefaciens* C58/3 *PsMT$_A$* positive strain as it shows the expected 969bp band when *ASP*718-restricted, and the expected 1744bp band when *Hin*dIII-restricted which hybridize to the *PsMT$_A$* probe. Track 9 (Plates 7 and 8) was selected for the *A. tumefaciens* C58/3 *PsMT$_A$* negative strain as it shows the expected 969bp band when *ASP*718-restricted, and the expected 19kb band when *Hin*dIII-restricted, which hybridize to the *PsMT$_A$* probe. The restriction sites and band size explanations are shown in Figure 4.

3.2.5 C58/3 *PsMT$_A$* transformation of *Arabidopsis thaliana*

The *Arabidopsis* root explants were transformed as in Methods 2.2.5.5, using acetosyringone-induced *Agrobacteria*. The explants were plated out onto shoot-inducing medium (SIM) containing 200mg/l augmentin and 50mg/l kanamycin. The shoots were excised after 5 weeks and transferred onto germination medium (GM). The siliques were allowed to develop and the seeds collected.

Analysis of putative transformants

3.2.6 Detection of *$PsMT_A$* by PCR

DNA was extracted from single putative transgenic p+*$PsMT_A$* and p-*$PsMT_A$* *Arabidopsis* leaves, single control *Arabidopsis* leaves and a comparable sized piece of a *Pisum sativum* leaf, following Methods 2.2.2.6. A 35 cycle PCR of 45 seconds at 94°C, 45 seconds at 55°C and 2 minutes at 72°C, with a 5 minute extension time added to the 35th cycle, was used. 50μl PCRs were set up with primers 270 and 271 (see 3.2.1) and 10 or 2.5μl of the *Pisum sativum*, control or transgenic leaf extracts. A pλS2 positive control and PCR negative controls were also set up. The reactions were all UV-irradiated for 7 minutes before being placed into the thermal cycler.

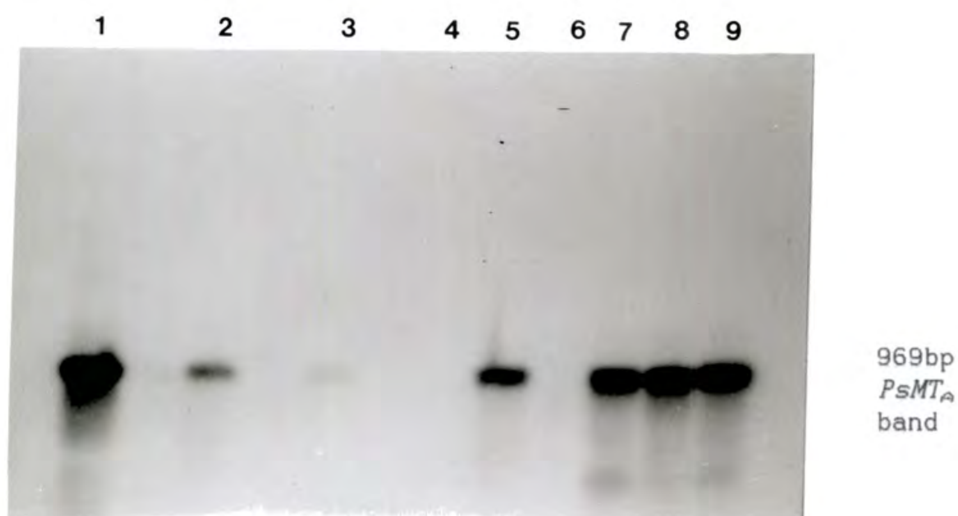
The products were run on a 1.5% agarose gel along with a λ size marker. The gel was Southern blotted and the filters probed with the *$PsMT_A$* probe, as 3.2.1. After washing to a stringency of 0.1 x SSC for 30 minutes at 65°C, the filter was exposed overnight (Methods 2.2.3.10/12/13).

The results are shown in Plate 9, and show 969bp bands that are strongly hybridizing to the *$PsMT_A$* probe in the pλS2 and *Pisum sativum* positive controls as well as the putative transgenic *Arabidopsis* p+ and p-*$PsMT_A$* strains. A faint background band can be seen in the PCR negative control but both of the *Arabidopsis* negative controls show no trace of *$PsMT_A$* hybridization.

Plate 9

Autoradiograph of PCR-amplified $PsMT_A$

from Putative Transgenic Single Leaf DNA



1. pλS2 $PsMT_A$ PCR positive control

2. *Pisum sativum* $PsMT_A$ PCR positive control

3. PCR negative control

4. Control *Arabidopsis* $PsMT_A$ PCR negative control

5. Putative transgenic *Arabidopsis/p-*$PsMT_A$ PCR

6. Control *Arabidopsis* $PsMT_A$ PCR negative control

7-9. Putative transgenic *Arabidopsis*/p+$PsMT_A$ PCR

3.2.7 Detection of *PsMT$_A$* mRNA expression

mRNA was extracted, following Methods 2.2.4.7, from control and transgenic p-*PsMT$_A$* and p+*PsMT$_A$* tissue. Using reverse transcriptase, cDNA was made from each sample.

10μl of each cDNA solution was used in a 50μl PCR using primers 270 and 271 and the 35 cycle PCR described in 3.2.6. Positive and negative PCR controls were set up as before. 20μl of each reaction was run on a 1.5% agarose gel together with a λ size marker. The gel was Southern blotted and the filter probed with the *PsMT$_A$* probe as 3.2.1. After washing to a stringency of 0.1 x SSC for 30 minutes at 65°C, the filter was exposed overnight (Methods 2.2.3.10/12/13).

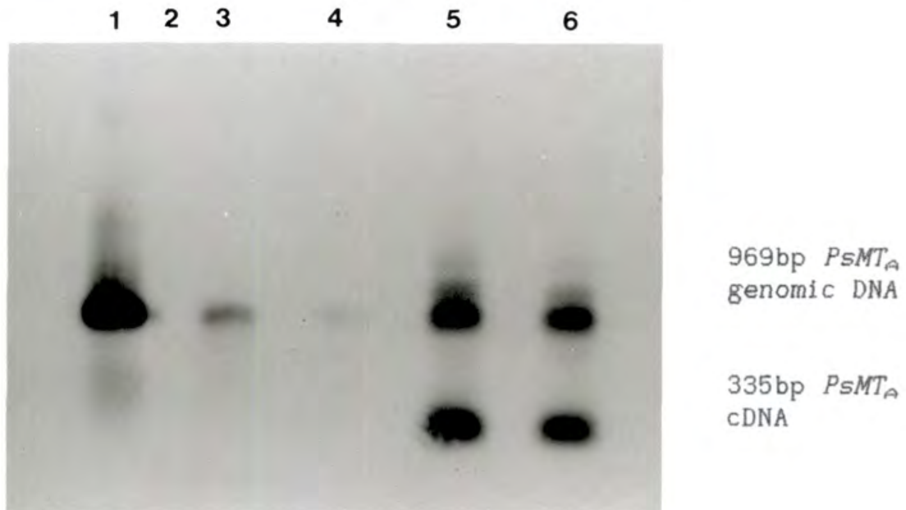
The results are shown in Plate 10. The 969bp band is the PCR product of contaminating genomic DNA or unprocessed mRNA and corresponds to *PsMT$_A$* complete with its intron. The second smaller band (335bp) corresponds to *PsMT$_A$* with no intron and is therefore the product of the mRNA/cDNA PCR. The p-*PsMT$_A$* clone shows no 335bp band, as expected.

3.2.8 Quantification of mRNA by competitive PCR

Following Gilliland *et al.* (1990), the quantity of template DNA present in a PCR can be estimated by comparing the amount of product with the amount of product of a competitive template of known concentration. As long as the template concentration is the only limiting factor, the concentration of the products should be directly proportional to the concentration of the templates.

Plate 10

Autoradiograph of PCR-amplified *PsMT$_A$* mRNA Expression



1. pλS2 *PsMT$_A$* PCR positive control
2. PCR negative control
3. Putative transgenic *Arabidopsis*/p-*PsMT$_A$* genomic and cDNA PCR
4. Control *Arabidopsis* genomic and cDNA PCR

5+6. Putative transgenic *Arabidopsis*/p+*PsMT$_A$* genomic and cDNA PCR

The sequence of $PsMT_B$ is shown in Figure 5, along with the two primers (283 and 284) designed to prime both $PsMT_B$ and $PsMT_A$. $PsMT_B$ was an ideal competitive template because as well as having an identical priming site to $PsMT_A$, its PCR product (405bp) is a different size to both the $PsMT_A$ chromosomal DNA PCR product (875bp) and the cDNA PCR product (241bp).

The $PsMT_B$ competitive template was prepared by minipreping the pUC18-$PsMT_B$ plasmid from *E.coli* (Methods 2.2.2.1) and restricting with *Eco*R1 and *Hin*d111. The $PsMT_B$ fragment was electroeluted (Methods 2.2.3.7), phenol/chloroform extracted and ethanol precipitated. After resuspension, the DNA concentration was determined and dilutions of 1.00pg/µl, 0.50pg/µl, 0.10pg/µl and 0.05pg/µl were produced.

mRNA was extracted and cDNA synthesized from a 2mm square portion of leaf tissue from two different plants, following Gilliland *et al.* (1990) (Methods 2.2.4.7), 5µl of the 20µl cDNA preparation was mixed with 5µl of each of the $PsMT_B$ dilutions for the PCR templates. A 35 cycle PCR (45 seconds at 94°C, 45 seconds at 45°C and 2 minutes at 72°C with an extra 3 minutes added to the final extension) was carried out with the primers 283 and 284 (see Figure 5).

25µl of each reaction was run on a 1.2% agarose gel, along with a positive and negative control and a λ size marker. The gel was Southern blotted and probed with the $PsMT_A$ probe (as before). After washing to a stringency of 0.1 x SSC at 65°C for 30 minutes, the filter was exposed overnight (Methods 2.2.3.10/12/13).

The results are shown in Plate 11. The larger band corresponds to the 405bp $PsMT_B$ PCR product and the smaller one corresponds to the 241bp $PsMT_A$ cDNA PCR product. From these results, a 2mm square of plant 5 shows the presence of approximately 2.0pg $PsMT_A$ cDNA and of plant 6 shows approximately 14.0pg $PsMT_A$ cDNA when corrected.

Figure 5

The Comparative Sequences of $PsMT_A$ and $PsMT_B$

$PsMT_A$5'..GAAGATAACAGCAACCAAGCAATTAATATCAATTGTTGTTTGCAAAAAATCTTAG

GTTCTGAAAATATGTCTGGATGTGGTTGTGGAAGCAGTTGCAACTGTGGTGATAGCTGCAAGTAAGGATC
$PsMT_B$ GTCTGGATGTGGTTGTGGAAGCAGTTGCAACTGTGGTGATAGCTGCAAGTAAGGATC
GCGAATTCGTCTGGATGTGGTTGTGG (Primer 284)

CACCACCTTAATTCTTTGTTGTTTTTCTGTATAATTTTTTCATTACAATTATTTGTATGTCTATTTTTAA
CACCACCTTAACTCTTCCTTACATTAAAATCTATATAATTATTTTTTTCTATATGGTTATTAATCTGT--

TCATATAGATGATTCTTTGGAGATTTTTTTAAATAATTTGTTTAGTTTTATCGCATCGAATAATATATGA

TCTGAGCATGAGAAAAATAAATTTAATATAGACGGATTGTTTTTTATAAATGAATTAGGCTGAATCTAAA

TTCTAAGACTATGAATATGGTTCATAATTCTATGTTAAATCATTTTGTGTAGTGAAATTGGGCAATTTTA

TGTGTAAACGCATAATTTTGAGGTTTAAAATAAGGATCGTGCTGTCGCGATAGTTTAAGTGTCGATTGTA

GTCGCGTAAAGGCTTTTCTGATTTCGGTTGGTTTAAGTGTTGATTGCAATCGTGTAAAGAGTTTTCTAAT

TTCGGTTGGTTTAGGTGCGATTGCAGTCGGGTAAAGATTTTCGTGATTGTCGTCGTTGCGGTGTGAATTA

ATCACAATTTCTTCTTTATCATAAAAACGTTGAATAACATATCGATATCGATTTGAAAACCTTTTTCGTG
TGGTATTTATTTTTTTTCTTAA

TAACGGTCTTTCGAAAACCTTTATTTTGACAACCAAGTTTATAATTGATTTGTTTTGCTTGACAGATGCA
GAATCAATCTTGCAAAAAATTTGTTAATATTTTATTGGATTATTTTGATTTTTATTGCTGTGCAGATGCA

ACAAGAGGTCTAGTGGATTGAGCTACTCCGAAATGGAAACCACCGAAACCGTGATTCTTGGCGTCGGTCC
ACAAGAGGTCTAGTGGATTGAGCTACTCCGAAATGGAAACCACAGAAACCGTGATTCTTGGCGTCGGTCC

GGCGAAGATCCAGTTTGAAGGTGCTGAAATGAGTGCTGCTTCTGAGGATGGTGGCTGCAAGTGTGGTGAT
GGCGAAGATCCAGTTTGATGGTGCTGAAATGAGTGTTGCAGCTGAGGATGGTGGCTGCAAGTGTGGTGAT

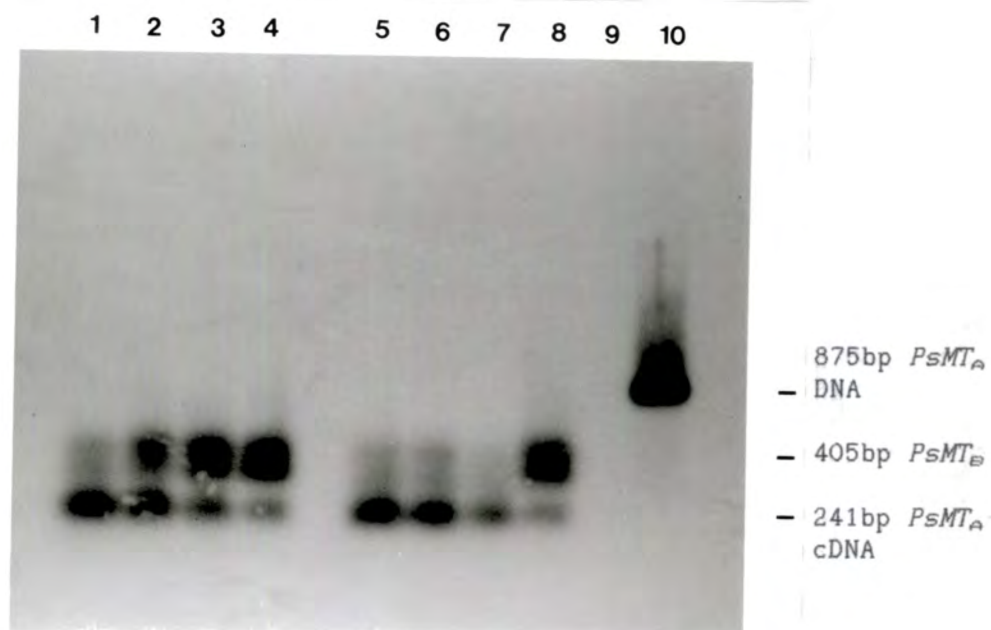
AACTGCACTTGTGACCCTTGCAACTGCAAATGAAGTGTAACATATAAAAGCTTGAAGCAGAGATATTGAA
AGCTGCACTTGTGACCCTTGCAACTGCAAA
GTGACCCTTGCAACTGCAAAGTCGACGGCC (Primer 283)

ACCATTATGTTT.......3'

The comparative sequences of $PsMT_A$ and $PsMT_B$ (Bryden, personal communication). A PCR with primers 283 and 284 from $PsMT_B$ should produce a 405bp product. If $PsMT_A$ is the template, a 875bp product from the chromosomal DNA and a 241bp product from the cDNA would be expected.

Plate 11

Autoradiograph of competitively PCR-amplified $PsMT_A$ / $PsMT_B$



Putative transgenic *Arabidopsis* $PsMT_A$ PCR (Plant 5) with:-

1. 0.25pg $PsMT_B$
2. 0.5pg $PsMT_B$
3. 2.5pg $PsMT_B$
4. 5.0pg $PsMT_B$

Putative transgenic *Arabidopsis* $PsMT_A$ PCR (Plant 6) with:-

5. 0.25pg $PsMT_B$
6. 0.5pg $PsMT_B$
7. 2.5pg $PsMT_B$
8. 5.0pg $PsMT_B$

9. PCR negative control
10. pλS2 $PsMT_A$ PCR positive control

Analysis of the F1 generation

3.2.9 Single leaf *PsMT*$_A$ PCR

DNA was extracted from single F1 putative *PsMT*$_A$ leaves and single control *Arabidopsis* leaves, following Methods 2.2.2.6. A 35 cycle PCR with 45 seconds at 94°C denaturation, 45 seconds at 55°C annealing and 2 minutes at 72°C extension with a final 5 minute extension in the 35th cycle, was carried out on each sample with primers 270 and 271. A PCR positive control (pλS2) and negative control were also set up (Methods 2.2.4.5).

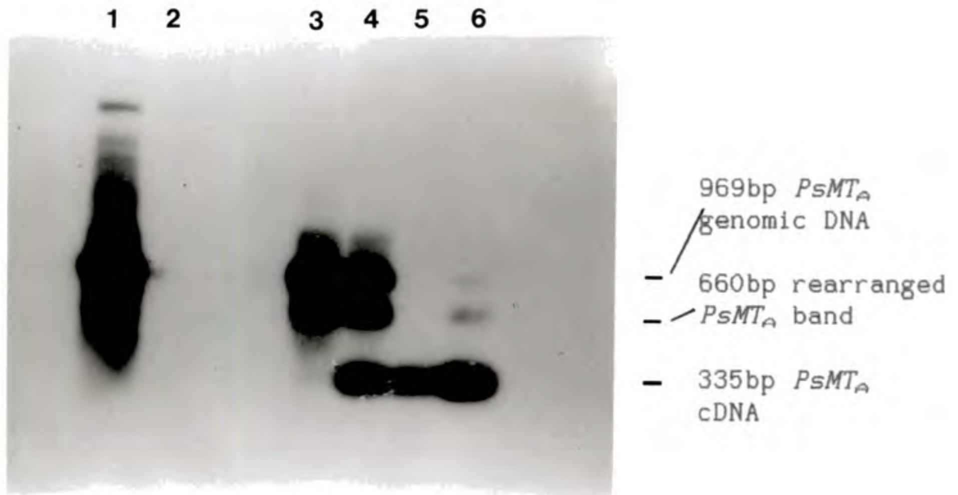
20µl of each reaction was run on a 1% agarose gel, together with a λ size marker, and was then Southern blotted. The filter was hybridized overnight at 65°C to a *PsMT*$_A$ probe (as 3.2.1), then washed to a stringency of 0.1 x SSC at 65°C for 30 minutes, before being exposed to X-ray film (Methods 2.2.3.10/12/13).

The results are shown in Plate 12, along with those of 3.2.10. Track 3 shows a single leaf PCR result, where the expected 969bp *PsMT*$_A$ is present, as well as a 660bp band which also hybridizes to the *PsMT*$_A$ probe. This could be the result of a rearrangement (deletion) of the added gene.

Plate 12

Autoradiograph of PCR-amplified *PsMT$_A$* DNA and cDNA

from F1 Generation Putative Transgenic *Arabidopsis*



1. pλS2 *PsMT$_A$* PCR positive control.

2. PCR negative control.

3. *PsMT$_A$* PCR of putative transgenic *Arabidopsis* single leaf DNA.

4-6. *PsMT$_A$* PCR of putative transgenic *Arabidopsis* cDNA

3.2.10 Detection of *$PsMT_A$* mRNA expression

mRNA was extracted, following Methods 2.2.4.7, from putative transgenic F1 leaf tissue. Using reverse transcriptase, cDNA was made from each sample.

10µl of each cDNA solution was used in a 50µl PCR using primers 270 and 271 and the 35 cycle PCR described in Results 3.2.6. Positive and negative PCR controls were set up as usual. 20µl of each reaction was run on a 1.5% agarose gel together with a λ size marker. The gel was Southern blotted and the filter probed with the *$PsMT_A$* probe as before. After washing to a stringency of 0.1 x SSC for 30 minutes at 65°C, the filter was exposed overnight (Methods 2.2.3.10/12/13).

The results are shown in Plate 12. The 969bp band is the PCR product of contaminating genomic DNA or unprocessed mRNA and corresponds to *$PsMT_A$* complete with its intron. The 335bp corresponds to *$PsMT_A$* with no intron and is therefore the product of the mRNA/cDNA PCR. The intermediate band (660bp) could be explained by the presence of several copies of the coding sequence, one of which having undergone a rearrangement or recombination.

3.2.11 Seed germination on zinc-containing medium

Control *Arabidopsis* seeds were planted onto medium supplemented with concentrations of $ZnCl_2$ of 0, 10μM, 100μM, 1mM, 5mM or 10mM (Methods 2.2.5.7). After 2 weeks growth, the seedlings on the 10 and 100μM $ZnCl_2$ appeared healthy and normal when compared to seedlings on control medium. Seedlings on 1mM $ZnCl_2$ were slightly stunted when compared to the others but still appeared healthy. Both 5mM and 10mM $ZnCl_2$ reduced germination, resulting in a few stunted seedlings, with slightly more growth on 5mM than 10mM $ZnCl_2$.

F1 seeds from a $PsMT_A$-expressing transgenic *Arabidopsis* were planted onto 1mM and 5mM $ZnCl_2$ germination medium. After 2 weeks, the germination levels were very low and the resulting seedlings were stunted and totally bleached.

3.2.12 Seed germination on cadmium-containing medium

Control *Arabidopsis* seeds were planted onto medium supplemented with concentrations of $CdCl_2$ of 0, 0.1μM, 1μM, 10μM, 50μM, 100μM, 500μM or 1mM (Methods 2.2.5.7). After 2 weeks, the seedlings on Cadmium concentrations less than 500μM were all growing well and appeared healthy. Seedlings on 500μM $CdCl_2$ were slightly smaller but still appeared healthy when compared to the 1mM CdCl seedlings. Although these seeds germinated, the seedlings were stunted and unhealthy.

F1 seeds from a $PsMT_A$-expressing transgenic *Arabidopsis* were planted onto 500μM $CdCl_2$ germination medium. After 2 weeks, the germination levels were low and the resulting seedlings looked much less healthy than the comparable control plants.

3.2.13 Seed germination on copper-containing medium

Control *Arabidopsis* seeds were planted onto medium supplemented with concentrations of $CuSO_4$ of 0, 25µM, 50µM, 75µM, 100µM, 125µM and 150µM (Methods 2.2.5.7). After 2 weeks, the gradation in seedling size was obvious, with the 25µM seedlings only slightly smaller than the controls and the 150µM seedlings extremely stunted, not having progressed very far from the initial germination.

F1 seeds from *PsMT*$_A$-expressing transgenic and control *Arabidopsis* were planted onto 100µM $CuSO_4$ and control germination medium. After 3 weeks, although the germination levels were low, very little difference could be detected in the size of the control and putative transgenic seedlings on 100µ $CuSO_4$, although the putative transgenics that were planted onto control medium looked less healthy (being generally smaller and more chlorotic) than the control seedlings.

3.2.14 Metal analysis of *Arabidopsis*

Control and putative transgenic *Arabidopsis* were grown as described in Methods 2.2.5.7. The seeds used were from 4 different transgenic parents (F,D,G and C) all of which had shown *$PsMT_A$* mRNA expression. Metal was extracted (Methods 2.2.9) and the copper, iron and zinc content were determined using Atomic Absorption Spectrophotometry.

Figures 6a-g show the copper contents of control and F1 selfed progeny of transgenic *$PsMT_A$* *Arabidopsis* grown on control and copper-supplemented media. The control seedlings (Figures 6a and 6c) showed very little variation in their copper content, although, as expected, the control plants on the copper-supplemented medium accumulated more copper than those on the unsupplemented medium. As *$PsMT_A$* segregation was expected within the putative transgenic seed populations, a variation in the copper accumulation was also expected. The putative transgenic (F) seedlings planted onto non-supplemented medium (Figure 6b) showed the same lower basal level of copper accumulation as the corresponding controls, however, 15 out of 20 seedlings show a higher level of accumulation, possibly due to a high concentration of the metal-binding *$PsMT_A$* product, with 3 of these accumulating 6-8 times more copper than the controls. The putative transgenic seedlings planted on copper-supplemented medium show a similar type of variation of copper accumulation (Figures 6d-g). The G and F populations show only 1 out of 24 and 4 out of 24 seedlings that exhibit a copper concentration higher than the controls, however, the D and C populations contain several more high accumulators (14 out of 33 and 9 out of 10 seedlings), suggesting that the *$PsMT_A$* product does in fact accumulate high concentrations of copper and that the variation shown

Copper concentrations
Control and Putative Transgenic Seedlings
(no copper supplement)

Figure 6a: Control

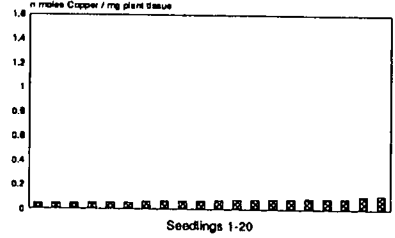
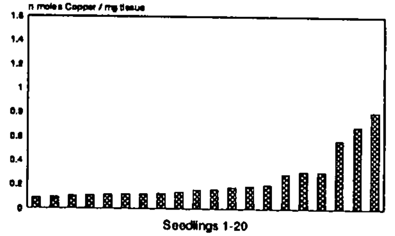


Figure 6b: Putative Transgenic (F)



Copper Concentrations of Copper Supplemented Seedlings

Figure 6c: Control

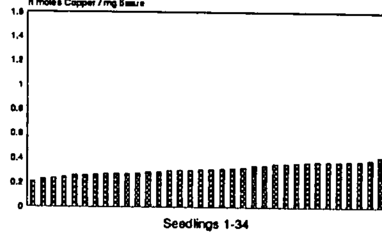


Figure 6d: Putative Transgenic (F)

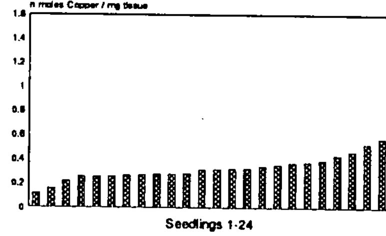


Figure 6e: Putative Transgenic (D)

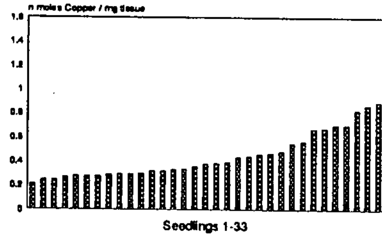


Figure 6f: Putative Transgenic (G)

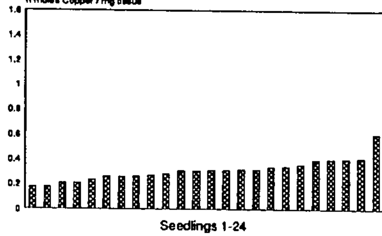
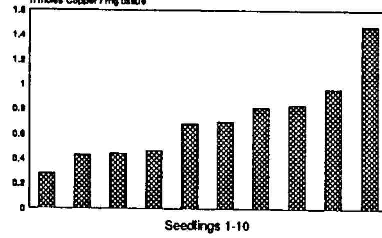


Figure 6g: Putative Transgenic (C)



is the result of normal segregation and variation within the seed populations.

One possible explanation for the variation in copper concentration could be that it was the result of a variation in efficiency of the metal extraction technique. If this was true, the iron and zinc concentrations of the seedlings would positively correlate with the copper concentration. Figures 7a-g show the change in iron and zinc concentrations with the increasing copper concentration of control and putative transgenic *PsMT$_A$* *Arabidopsis* on control and copper supplemented media. As can be seen from the data there is no correlation between the iron or zinc concentrations and the copper concentration, thus suggesting that the variation in copper concentrations was not the result of the metal-extraction technique. Furthermore, if it was due to variation in extraction, the non-transgenics would be anticipated to show the same distribution of accumulation as the transgenic seedlings. One further interesting fact arises from this data; the control and putative transgenic seedlings planted on the unsupplemented medium accumulate more iron and zinc than the seedlings planted on copper-supplemented medium. The difference is statistically significant when analysed with the t test. This perhaps suggests that there is some antagonism between the uptake or storage of copper, and iron and zinc.

Figures 8a-f show the variation in concentrations of iron and zinc of control and putative transgenic *PsMT$_A$* *Arabidopsis*, compared to those of copper. The iron and zinc concentrations both show a similar pattern of distribution to the copper, however in both cases, the difference between the controls and putative transgenic seedlings is negligible when compared to the difference between the copper seedlings.

Iron and Zinc Concentrations of Seedlings on no Copper Supplement

Figure 7a: Control

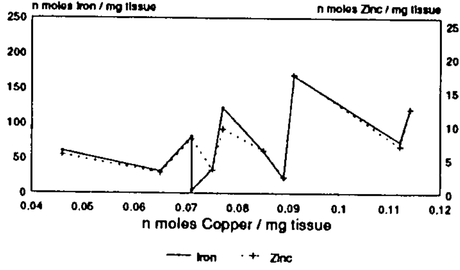
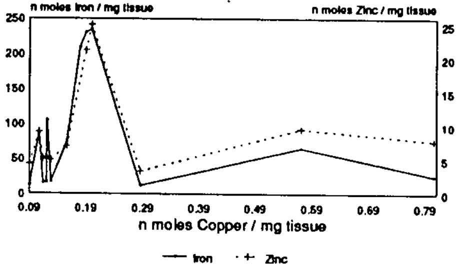


Figure 7b: Putative Transgenic (F)



Iron and Zinc Concentrations of Copper Supplemented Seedlings

Figure 7c: Control

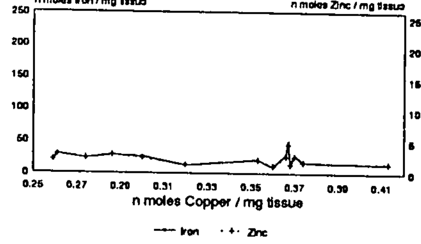


Figure 7d: Putative Transgenic (F)

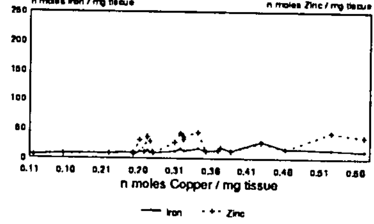


Figure 7e: Putative Transgenic (D)

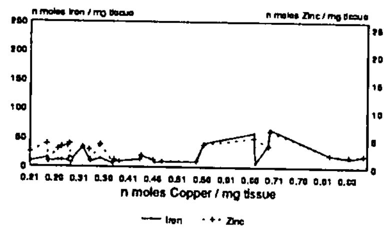


Figure 7f: Putative Transgenic (G)

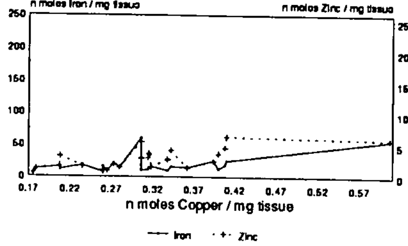


Figure 7g: Putative Transgenic (C)

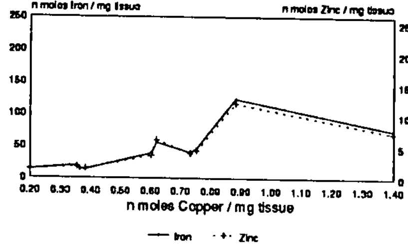


Figure 8

Comparison of Copper, Zinc and Iron Contents

of Control and Putative Transgenic Seedlings

(non-Copper Supplemented Growth)

Figure 8a: Copper

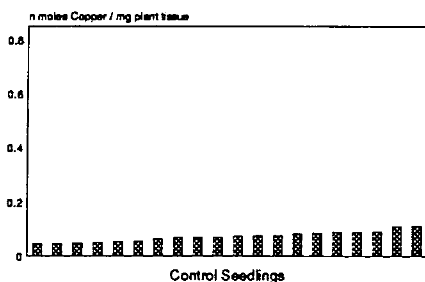


Figure 8b: Copper

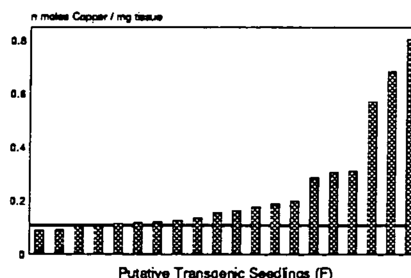


Figure 8c: Zinc

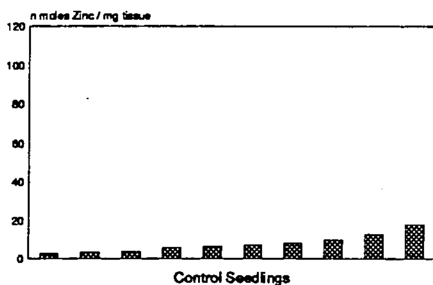


Figure 8d: Zinc

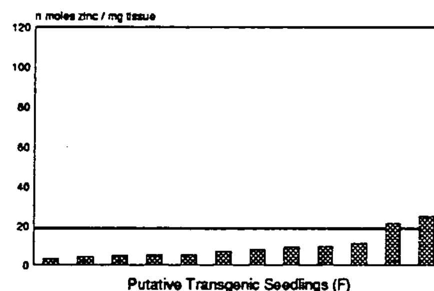


Figure 8e: Iron

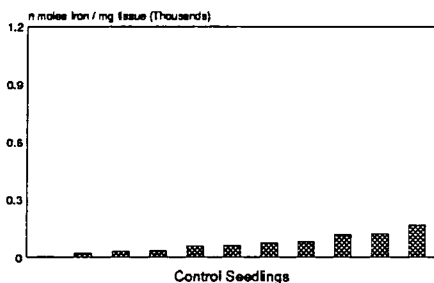
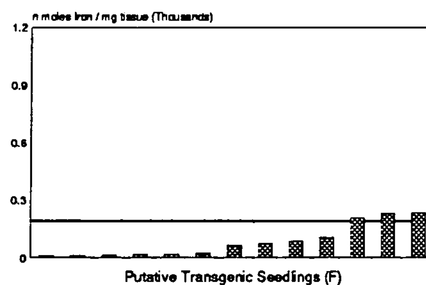


Figure 8f: Iron



The maximum metal ion content of the control seedlings is indicated across the corresponding putative transgenic values, clearly showing the seedlings exceeding the control value and by how much they exceed it. By adjusting the scales to be proportionally equal, the difference within the values of copper content is much greater than within the zinc or iron values in the transgenic seedlings.

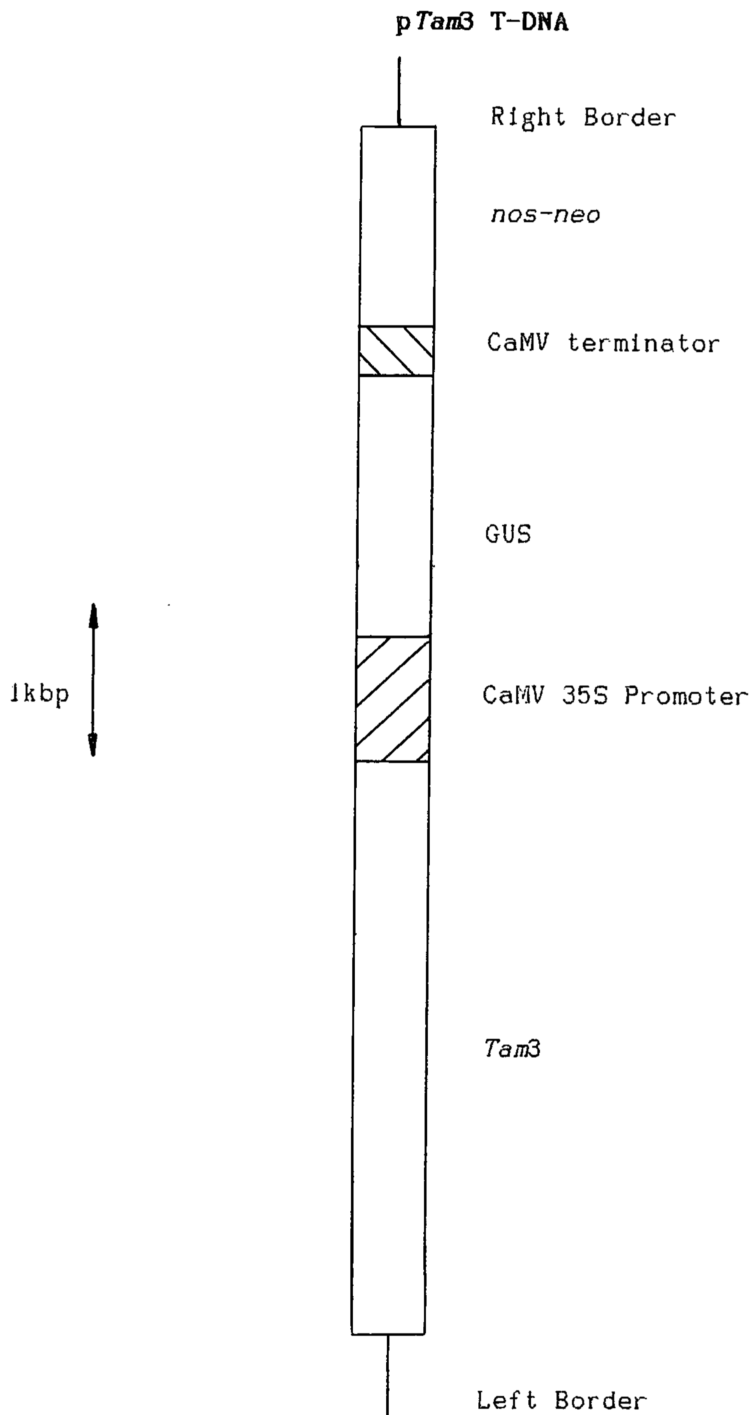
3.3 Transformation of *Arabidopsis* with the *Antirrhinum* Transposable Element *Tam3*

3.3.1 Cloning *Tam3* into the pJIT73 vector

The *Tam*3 element was obtained as a 3.7kb *Kpn*I fragment, containing the element with 150bp flanking sequences, cloned into pUC18. The plasmid was transformed into *E.coli* DH5α competent cells (Methods 2.2.1.3). 6 single colonies were isolated and grown overnight. The DNA was minipreped (Methods 2.2.2.1) and then restricted with *Kpn*I. When run on a 0.6% agarose gel, along with a λ size marker, all 6 tracks confirmed the presence of the 3.7kb *Tam*3 fragment.

Shotgun cloning was attempted in order to replace the *Kpn*I Hygromycin fragment of pJIT73 with the *Kpn*I *Tam3* fragment (Methods 2.2.4.4), see Figures 9 and 1. A 1:1 molar ratio of pJIT73 : *Tam*3 DNA were mixed together and restricted with *ASP7*18 (which recognizes the same sites as *Kpn*I but produces a 5 bp overhang rather than a 3 bp overhang). Half of the restriction mixture was run on a 0.6% minigel, with a λ size marker, to determine the efficiency of the restriction, the remainder was phenol/chloroform extracted and ethanol precipitated. The pellet was resuspended and ligated in a concentrated ligation. The ligation was then transformed into *E.coli* DH5α competent cells which were plated out onto YT plates with ampicillin / kanamycin selection (Methods 2.2.1.3). Colonies that had grown overnight were picked off onto 2 replica nitrocellulose filters for a colony screen (Methods 2.2.3.11).

Figure 9



Structure of the p*Tam*3 construct, containing the *nos-neo* kanamycin resistance gene and the GUS marker gene (with the 35S CaMV promoter), as well as the *Tam*3 transposable element. Details of the construction are in Results 3.3.1.

A probe was made by nick translation of the *ASP718* *Tam3* fragment to a specific activity of 9.2 x 10^8 $cpm\mu g^{-1}$ and hybridized to one of the filters. After washing to a stringency of 0.1 x SSC for 10 minutes at 65°C, the filter was exposed (Methods 2.2.3.12/13). 2 colonies showed up as positives, one of which was selected and a glycerol prepared ready for the triparental mating.

3.3.2 Production of the *Agrobacterium tumefaciens* *Tam3* strain

The *E.coli* p*Tam3* antibiotic resistance phenotype was confirmed by its growth on $50\mu gml^{-1}$ kanamycin and failure to grow on $100\mu gml^{-1}$ spectinomycin and $80\mu gml^{-1}$ nalidixic acid.

After the triparental mating (Methods 2.2.1.5), 9 colonies were picked off the selection plates and grown up overnight for minipreping (Methods 2.2.2.3). The DNA was extracted and restricted with *ASP718*. The restriction was run down a 0.8% agarose gel together with *ASP718*-restricted *A.tumefaciens* recipient DNA as a negative control, *ASP718* restricted *E.coli* p*Tam3* DNA as a positive control and a λ size marker. The gel was Southern blotted and the filter probed with the *Tam3* probe from Results 3.3.1. The filter was washed to a stringency of 0.1 x SSC for 10 minutes at 65°C and exposed overnight (Methods 2.2.3.10/12/13). The results are shown in Plate 13. The track 7 strain was selected for the C58/3 p*Tam3* strain, as the expected 3.7kb band is present, strongly hybridizing to the *Tam3* probe.

Plate 13

Autoradiograph of C58/3 p*Tam*3 Triparental Mating

1. *E. coli* p*Tam*3 *ASP*718 restricted positive control

2. C58/3 *ASP*718 restricted negative control

3-11. Putative C58/3 p*Tam*3 *ASP*718 restricted samples

3.3.3 C58/3 p*Tam3* transformation of *Arabidopsis thaliana*

Root explants of *Arabidopsis* were transformed (Methods 2.2.5.5) with acetosyringone-induced *A. tumefaciens* C58/3 p*Tam3*. Shoots which came through the 50mg/l kanamycin selection (see Plates 2a-d) were excised and planted into no selection germination medium (GM). Tissue samples and seeds were collected when appropriate.

Analysis of putative transformants

3.3.4 Single leaf GUS PCR analysis

The presence of the GUS gene was confirmed using the PCR technique with DNA extracted from single putative transgenic *Arabidopsis* leaves as described in Results 3.1.6. The results are shown in Plate 14. The GUS probe hybridizes to the 1808bp band which corresponds to the band expected from the GUS primers 216 and 217.

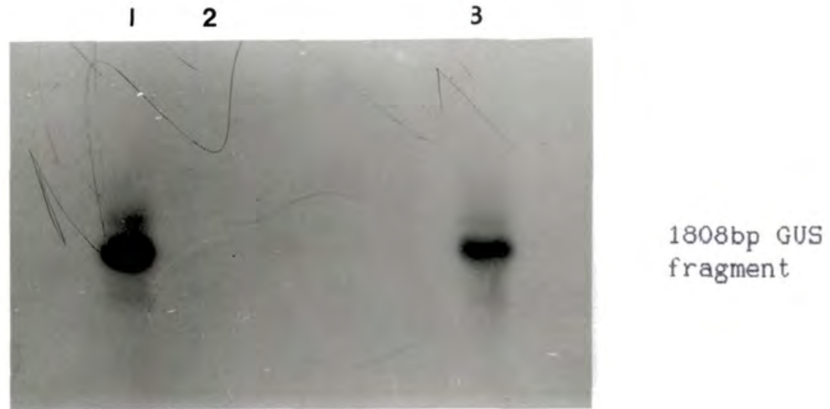
3.3.5 Histochemical GUS assay

Tissue samples were excised from putative transgenic *Arabidopsis* and control *Arabidopsis* and incubated at 37°C in an X-Gluc solution (Methods 2.2.6). Transgenic tissue was strongly expressing, producing a blue colouration within a couple of hours, when compared to the green controls. The tissue was fixed, dehydrated and embedded in wax (Methods 2.2.7). The tissue was then sectioned and photographed (see Plates 15a and b). The blue colouration was non-tissue specific as seen in 3.1.7, being under the control of the 35S CaMV promoter.

Plate 14

Autoradiograph of PCR-amplified GUS from Putative Transgenic

Arabidopsis/p*Tam*3

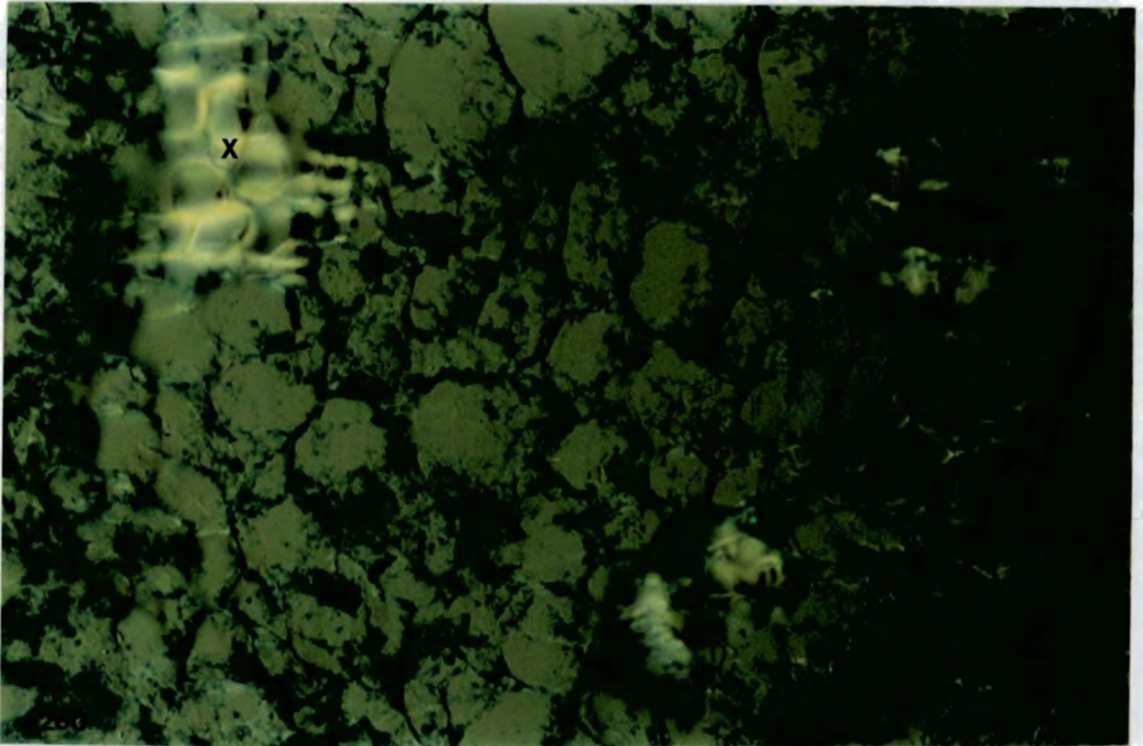


1. pJIT73 GUS PCR positive control

2. PCR negative control

3. GUS PCR of putative transgenic *Arabidopsis* p*Tam*3

Plate 15 (a and b)
Stem Sections of Transgenic *Arabidopsis* / p*Tam3*
Histochemical GUS Assay



15a : Stem section of GUS histochemically stained transgenic *Arabidopsis* / p*Tam3*, showing a strong blue colouration throughout the section (except in some of the more highly refractive xylem cells [X]), which indicates a high level of non-tissue-specific 35S CaMV-GUS expression.

15b : Section of a control *Arabidopsis* stem after GUS histochemical staining showing the lack of blue colouration, and therefore the lack of GUS expression, when compared to Plate 15a.

3.3.6 Immuno-gold anti-GUS labelling

Sections of stem tissue were treated as above, then incubated with anti-GUS antibody, immuno-gold labelled and silver enhanced (Methods 2.2.8). After mounting in D.P.X. mountant, the sections were photographed under epi-polarized light (see Plate 16). The silver particles enhancing the immuno-gold assay were present in a non-tissue specific pattern, as in 3.1.8.

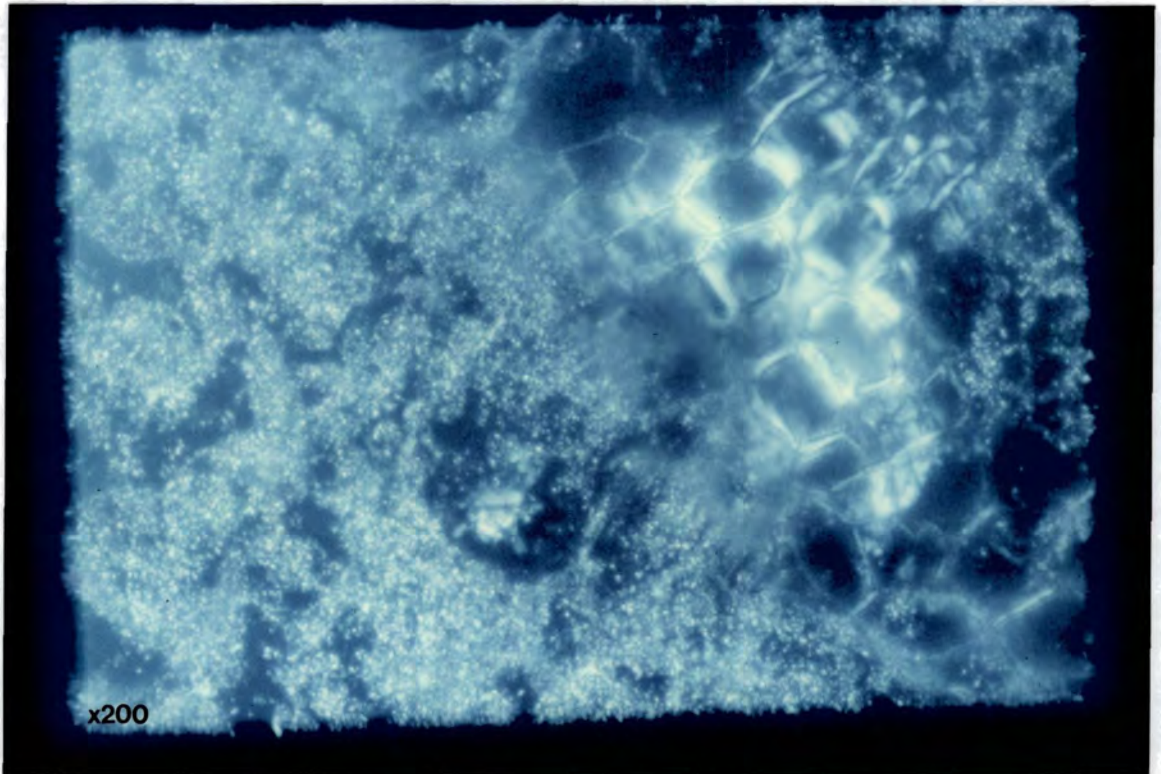
3.3.7 Description of transformants and progeny

F1 putative transgenic seeds were sterilized and planted onto germination medium (Methods 2.2.5.2) with added 50mg/l kanamycin selection. After 3 to 4 weeks, the seedlings which had survived through the selection were transferred to soil and grown on to maturity. The plants were examined at all stages for any visible mutation that could indicate a transposition event. Of the 43 plants examined, no phenotypic mutations were obvious. This could indicate a lack of transposition events within the plants or alternatively transposition could have occurred but *Tam3* was not blocking the expression of a gene that would result in a phenotypic change.

3.3.8 *Tam3* genomic blot

DNA was extracted from F1 putative transgenic *Arabidopsis* tissue and control *Arabidopsis* tissue, following Methods 2.2.2.4. The DNA was restricted with *ASF*718 and was run, together with the 3.7kb *Tam3*

Plate 16
Stem Section of Transgenic *Arabidopsis* / p*Tam3*
Immunogold GUS Assay



Stem section of transgenic *Arabidopsis* / p*Tam3* after immunogold anti-GUS labelling and silver enhancing, showing the accumulation of silver particles over the entire section. This correlates to the GUS expression pattern shown in Plate 15a of non-tissue-specific expression which is the expected expression pattern of the 35S CaMV promoter.

fragment as a positive control, on a 0.8% agarose gel together with a λ size marker. The gel was Southern blotted (Methods 2.2.3.10) and the filter was vacuum dried.

The *ASP*718 *Tam*3 fragment previously cloned into pUC18 was ^{32}P labelled by nick translation (Methods 2.2.3.13) to a specific activity of 4.3 x 10^{9} cpmμg^{-1}. The probe was hybridized to the filter (after prehybridization) overnight, at 65°C. After washing to a stringency of 1 x SSC for 30 minutes at 65°C, the filter was exposed for 10 days to X-ray film (Methods 2.2.3.10/12).

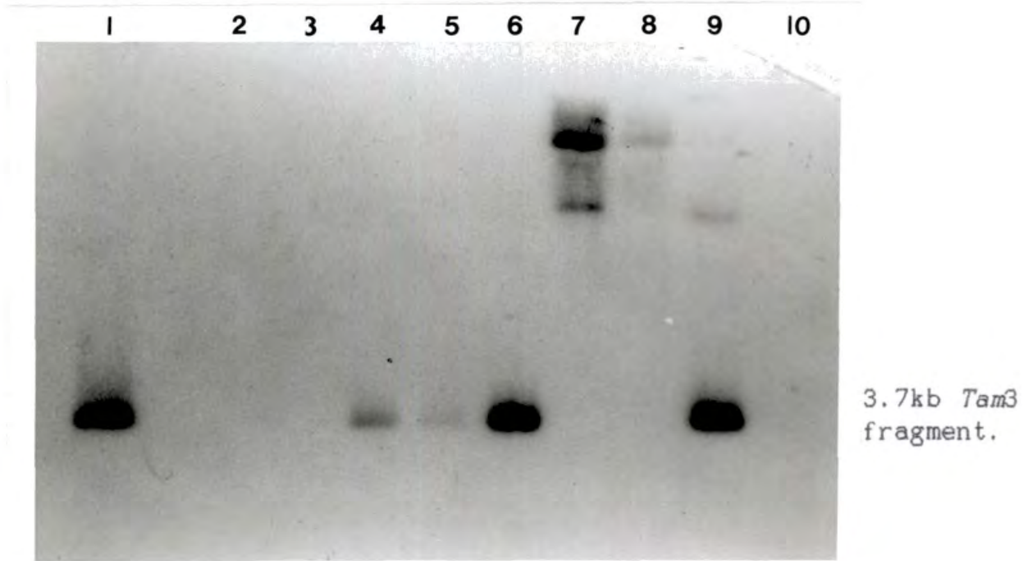
The results are shown in Plate 17. The transgenic lines are clearly hybridizing to the *Tam*3 probe, showing a 3.7kb band. If the *Tam*3 element had transposed, the 3.7kb band would no longer be present unless more than one copy of the *Tam*3-T-DNA was present initially. Theoretically, if transposition had occurred, the probe would hybridize to the *Tam*3 element elsewhere in the genome, presumably a larger *ASP*718 band, but it would also hybridize to the 150bp flanking regions remaining from the original 3.7kb *ASP*718 fragment (the empty donor site). Due to the small size of the residual flanking sequences, it is possible that the hybridized band would fail to be detected. The larger hybridizing bands seen in tracks 7, 8 and 9 are most likely to be the result of partially restricted genomic DNA rather than a transposition.

In order to determine whether or not a transposition event had occurred, further restriction analysis would need to be undertaken. By using an enzyme that would restrict within *Tam*3, transposition would result in a different pattern of bands that would hybridize to a *Tam*3 probe or to a probe specific to the empty donor site.

Plate 17

Autoradiograph of *Tam3* Genomic Blot of *ASP*718-restricted

Putative Transgenic *Arabidopsis*/p*Tam3*



1. *ASP*718 *Tam3* fragment (positive control).

2-3. *ASP*718-restricted control *Arabidopsis* DNA.

4-10. *ASP*718-restricted putative transgenic *Arabidopsis*/p*Tam3* DNA.

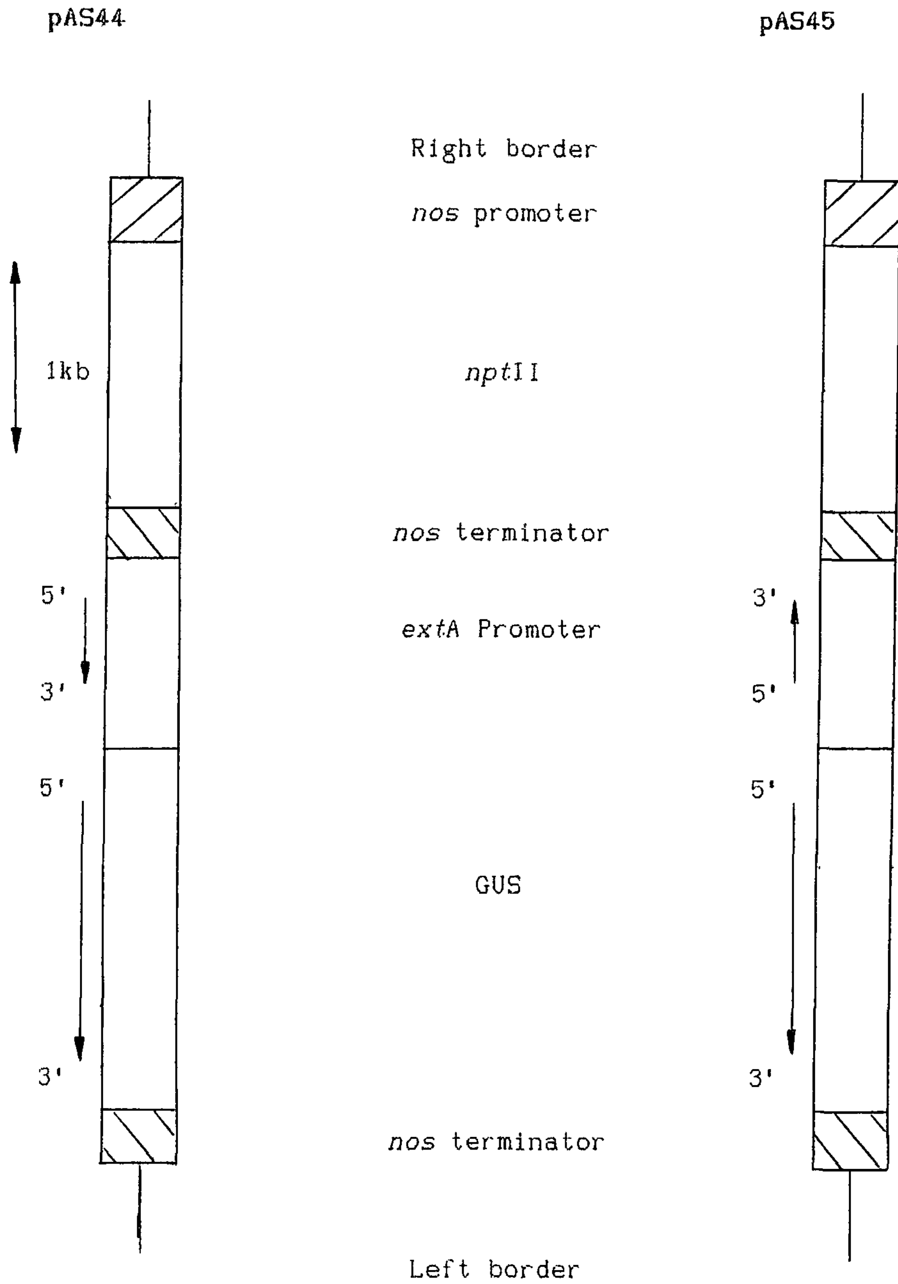
3.4 Transformation of *Arabidopsis* with a Promoter-GUS Fusion containing the Promoter from the Rape Extensin Gene *extA*

3.4.1 pAS44/45

pAS44 and pAS45 were constructed and donated by Dr A.Shirsat (University of Bangor). pAS44 is a pBIN19 based vector containing a *ext*A 1.0kb promoter-GUS coding sequence translational fusion. Three in-frame ATG initiator codons were present, which would result in an 11, 7 or 0 amino acid N-terminal extension to the GUS enzyme (having only a minimal effect on its activity). A neomycin phosphotransferase II gene is also contained within the T-DNA right and left borders (see Figure 10). pAS45 is identical except that the *ext*A promoter is in reverse orientation, therefore acting as a negative control by its failure to promote GUS expression (Shirsat *et al.*, 1991).

Reporter gene fusion systems were developed to simplify the analysis of gene activity by fusing specific promoter sequences to readily assayable reporter genes. The easiest to quantify and most sensitive reporter gene system developed so far uses the β-glucuronidase (GUS) gene (Jefferson, 1987; Jefferson *et al.*, 1987), measurable quantitively by fluorimetry and qualitively by histochemistry. Even though the enzyme (isolated from *E.coli*) is absent in most dicotyledonous plants tested to date, it has no obvious ill effects when highly expressed in transformed plants. Expression can be localized to a single cell using the histochemical assay with the X-Gluc substrate.

Figure 10



Structure of the pAS44 and pAS45 T-DNA constructs, containing the *npt*II kanamycin resistance gene under the control of the nopaline synthase promoter (*nos*) and the GUS marker gene under the control of either the positive (44) orientation or negative (45) orientation of the *extA* promoter. Details of the construction are in Results 3.4.1.

3.4.2 Production of *Agrobacterium tumefaciens* pAS44/45 strains

E.Coli pAS44/45 antibiotic resistance phenotypes were confirmed by their growth on 50$\mu g ml^{-1}$ kanamycin and failure to grow on 100$\mu g ml^{-1}$ spectinomycin and 80$\mu g ml^{-1}$ nalidixic acid.

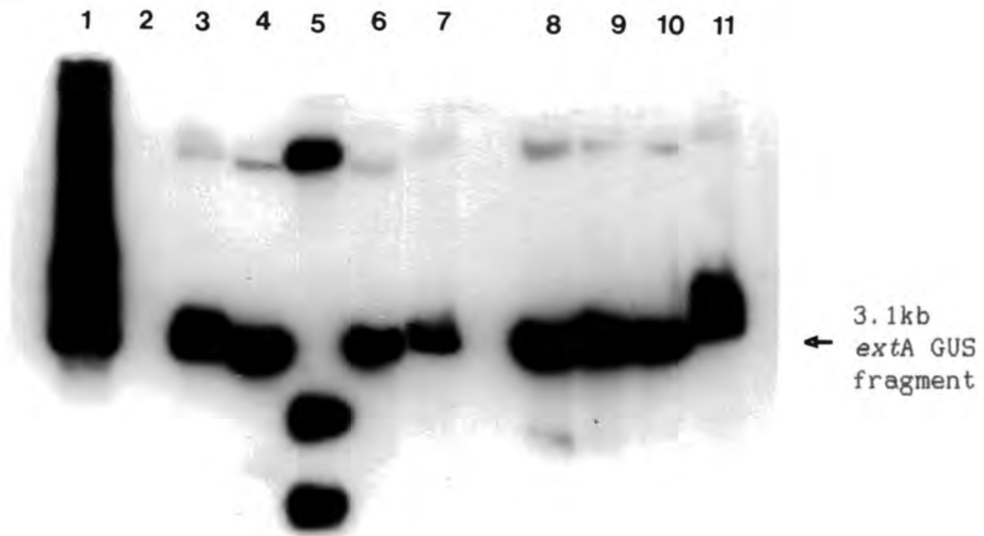
After the triparental mating (Methods 2.2.1.5), 10 single colonies were picked off the selection plates and grown up overnight for minipreping. The DNA was extracted (Methods 2.2.2.3) and restricted with *Bam*HI and *Eco*RI. The restriction was then run on a 0.8% agarose gel together with *Bam*HI/*Eco*RI *Agrobacterium tumefaciens* recipient DNA as a negative control, *Bam*HI/*Eco*RI restricted *E.coli* pAS44 DNA as a positive control and a λ size marker.

After Southern blotting, the filters were probed with a 4.5 x 10^{9} cpmμg^{-1} GUS probe made from nick translating the 4kb *Hin*dIII GUS fragment of pJIT73. The filters were washed to a stringency of 2 x 0.1 SSC for 15 minutes at 65°C and exposed overnight (Methods 2.2.3.10/12/13).

The results are shown in Plate 18. Track 7 was selected for the C58/3 pAS44 strain and track 8 was selected for the C58/3 pAS45 strain, both showing the expected 3.1kb *Bam*H1 / *Eco*R1 *ext*A / GUS band hybridizing to the GUS probe.

Plate 18

Autoradiograph of C58/3 pAS44/45 Triparental Mating



1. *E.coli* pAS44 *Bam*H1/*Eco*R1 restricted positive control

2. C58/3 *Bam*H1/*Eco*R1 restricted negative control

3-7. Putative C58/3 pAS44 *Bam*H1/*Eco*R1 restricted samples

8-12. Putative C58/3 pAS45 *Bam*H1/*Eco*R1 restricted samples

3.4.3 C58/3 pAS44/45 transformation of *Arabidopsis thaliana*

Arabidopsis root explants were transformed (Methods 2.2.5.5) using acetosyringone-induced *A. tumefaciens* C58/3 pAS44/45 (Methods 2.2.5.4). Shoots which came through the 50mg/l kanamycin selection were excised and planted into no selection germination medium. Once rooted, the shoots were transplanted into soil and the seeds saved.

Analysis of putative transformants

3.4.4 DNA analysis

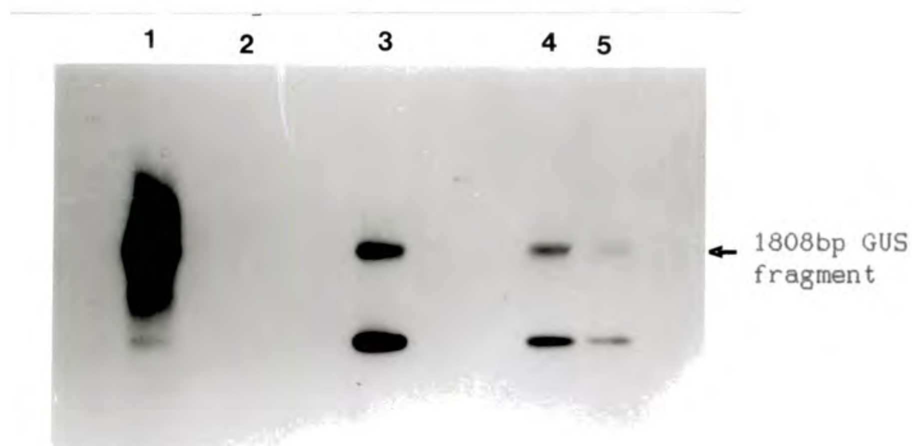
The presence of the GUS gene in putative transgenic tissue was confirmed using the GUS PCR technique (Results 3.1.6) on DNA extracted from single putative transgenic leaves and single control *Arabidopsis* leaves, following Methods 2.2.2.6. The filter was hybridized to a GUS probe as in 3.4.2.

The results are shown in Plate 19. The GUS primers were expected to produce a 1808bp product, a band of this size can be seen in the transgenic strains but not in the control. This 1808bp band hybridizes to the GUS probe.

Plate 19

Autoradiograph of PCR-amplified GUS from

Putative Transgenic *Arabidopsis* pAS44/45



1. pJIT73 GUS PCR positive control

2. PCR negative control

3. GUS PCR of putative transgenic *Arabidopsis* pAS44

4-5. GUS PCR of putative transgenic *Arabidopsis* pAS45

3.4.5 Histochemical GUS expression

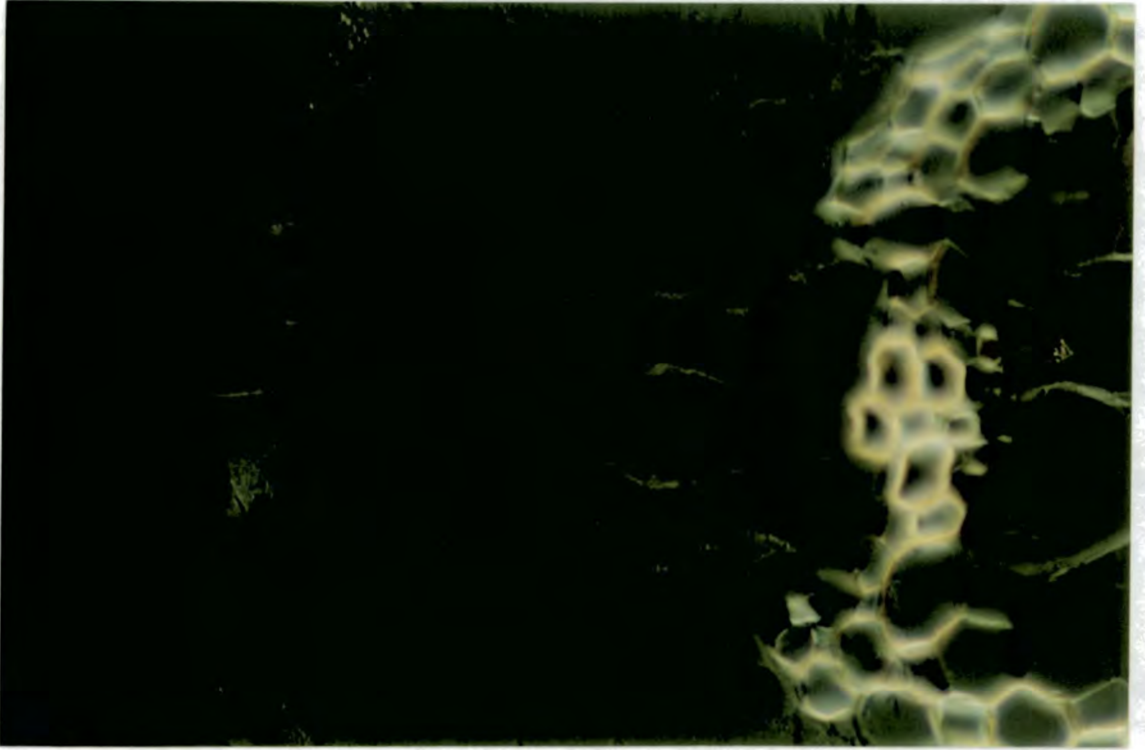
Tissue samples from the putative transformants and control plants were excised and incubated in X-Gluc (Methods 2.2.6). The samples were embedded in wax and sectioned (Methods 2.2.7). Both the control stem samples and the pAS45 putative transgenic stem samples showed no blue colouration. Sections of pAS44 transgenic stem tissue showed discrete blue staining in the phloem and around the epidermis which was unfortunately too faint to photograph (blue being a colour particularly difficult to distinguish when using the microscope), see Plate 20a. No blue colouration could be seen in the control or either of the transgenic root tissues, however the sections were very poor.

3.4.6 Immuno-gold anti-GUS labelling

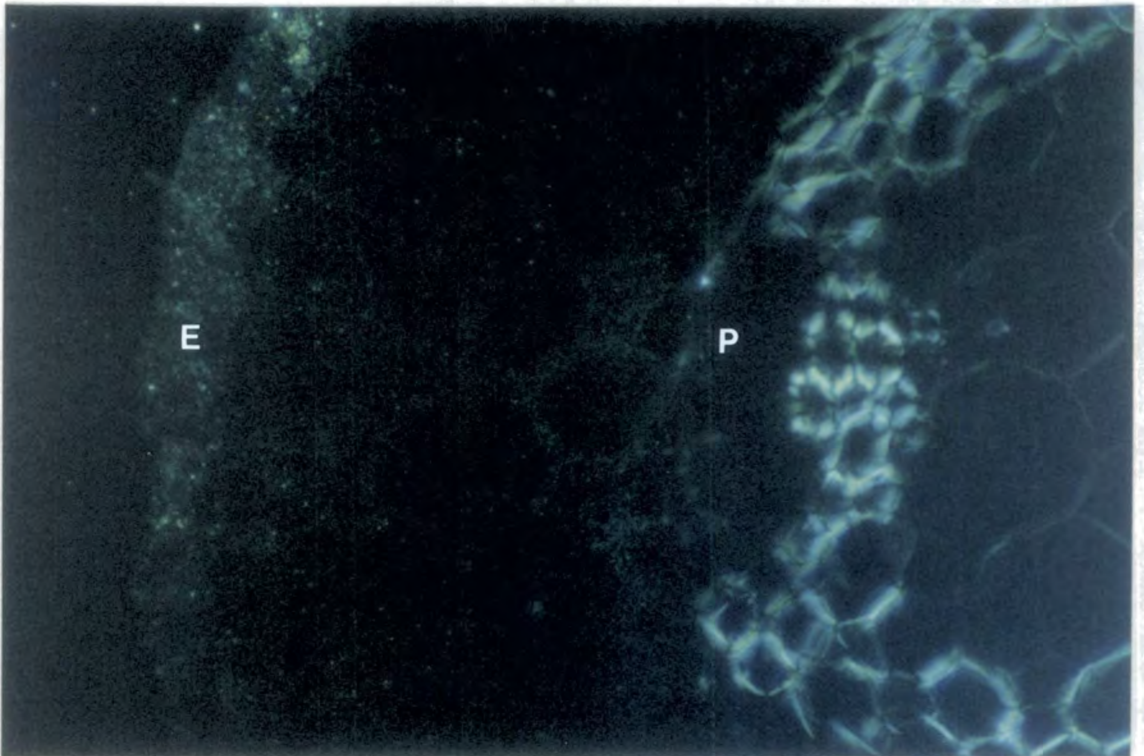
Tissue samples from the putative transformants and control plants were excised and incubated in X-Gluc (Methods 2.2.6.2). The samples were embedded in wax and sectioned (Methods 2.2.7). The sections were then anti-GUS immuno-gold labelled and silver enhanced (Methods 2.2.8). The sections were then photographed under epi-polarized light (see Plates 20b and c). The silver particles that enhance the immuno-gold label can be seen in the phloem and around the epidermal layer of the pAS44 transformants stem tissue. No silver particles could be seen on the pAS45 transformant sections, as expected, or on the control sections.

Due to the particularly small size of the transgenic roots, it proved extremely difficult to process and to cut usable sections. Consequently, no conclusive data was obtained to confirm the presence or absence of root *extA* / GUS expression.

Plate 20 (a and b)
Stem Section of Transgenic *Arabidopsis* / pAS44
Immunogold GUS Assay



20a : Stem section from transgenic *Arabidopsis* / pAS44 under a normal light source, after histochemical GUS staining, with blue colouration too faint to photograph. Photographs of control *Arabidopsis* sections appeared identical to 20a (see Plate 4b).



20b : Stem section of transgenic *Arabidopsis* / pAS44 after immunogold anti-GUS labelling and silver enhancing, showing the accumulation of silver particles in the epidermal layer (E) and the phloem (P).

Plate 20c

20c : Stem section of transgenic *Arabidopsis* / pAS44 after immunogold anti-GUS labelling and silver enhancing, under higher magnification than in 20b, confirming the specific phloem (P) expression.

4. DISCUSSION

4.1 Summary of results

The initial aim of the project, to establish a workable *Agrobacterium*-mediated transformation method for *Arabidopsis thaliana*, was achieved. It was used to introduce three diverse constructs, encoding either a gene, a promoter-reporter fusion or a transposable element, to confirm that the transformation method could successfully be used for the full range of possible gene constructs.

The putative metallothionein gene, $PsMT_A$, was introduced into *Arabidopsis thaliana* under the control of the constitutive 35S CaMV promoter, in order to determine its function. Transformants were shown to contain and express the $PsMT_A$ gene using PCR. Metal analysis revealed that F1 transgenic seedlings accumulated copper to a higher concentration than control seedlings thus suggesting that the $PsMT_A$ gene encodes a copper-chelating protein.

The promoter from the rape *extA* extensin gene was fused to a GUS reporter gene and transformed successfully into *Arabidopsis thaliana*. Using stem sections with an anti-GUS immuno-gold assay, *extA* expression was localized to the phloem and the epidermal tissue, suggesting a probable role in cell wall strengthening.

The transposable element *Tam3* (with the GUS marker gene) was introduced into the *Arabidopsis thaliana* genome. Its presence was confirmed by Southern blotting, GUS PCR and histochemical staining. Unfortunately, no evidence of a transposition event was found.

4.2 Metallothionein

The metal-binding properties of metallothionein (MT) suggest a probable role in metal metabolism and detoxification. A more encompassing role was suggested whereby MT functions homoeostatically as a reservoir, able to sequester and/or donate its metal intracellularly to metal-requiring metalloproteins.

4.2.1 Metallothionein / zinc interactions

Exogenously administered zinc has been shown to be recovered as zinc-thionein (Kägi and Schaffer, 1988) forming a possible reservoir of available but not 'free' zinc. The second step, the reactivation of zinc-requiring apo-enzymes, has also been demonstrated with, for example, alcohol dehydrogenase, aldolase, thermolysin, alkaline phosphatase and carbonic anhydrase (Udom and Brady, 1980), at approximately the same rate as inorganic zinc salts. Similar results have been obtained for phytochelatins (class III MTs), which were shown to reactivate diamino oxidase and carbonic anhydrase apoenzymes as effectively as free metal ions (Thumann *et al.*, 1991).

Since the discovery of zinc-finger proteins and other zinc-requiring DNA-binding proteins, a more fundamental level of control has been hypothesized for MT. Many regulatory proteins are now known to require zinc to form their structural binding domains, for example, Xfin (Lee *et al.*, 1989), GAL4 (Pan and Coleman, 1990) and the glucocorticoid receptor (Luisi *et al.*, 1991). It has been proposed that by regulating the amount of zinc available to these regulatory proteins and transcription factors, MT could be involved in gene

control (Zeng *et al.*, 1991).

Metal analysis revealed that 2 putative *PsMT_A*-transgenic F1 seedlings showed a slightly higher zinc concentration than the controls (Figures 8c and d), however the difference is not statistically significant. This data, although limited, suggests that *PsMT_A* is not primarily involved in zinc metabolism.

4.2.2 Metallothionein / iron interactions

One recent theory of the function of the proteins encoded by these newly isolated plant 'MT-like' genes is that they are involved in iron efficiency (Okumura *et al.*, 1991). Iron efficiency systems in bacteria use exuded chelating molecules (siderophores) to bind the sparingly soluble inorganic iron, solubilizing it and making it available for uptake. A similar system is the major iron accumulation strategy in plants belonging to the Gramineae (and to a lesser extent in other higher plants), where the chelating of the iron is thought to be by phytosiderophores (hydroxy- and amino-substituted imino-carboxylic acids), such as mugineic or avenic acid (Römheld, 1987). The onset of iron deficiency strongly stimulates the release of phytosiderophores into the rhizosphere where they chelate any available iron and are then taken back into the roots as the Fe^{3+}-phytosiderophore chelate molecule. Other iron accumulation strategies that rely more on the ability to reduce Fe^{3+} (present in the medium) to Fe^{2+} (the form used by the cell) are used by most other higher plants, and to a lesser extent by the Gramineae. These include the extrusion of reducing substances (in addition to phytosiderophores) which reduce the Fe^{3+} as well as chelating it and increasing the

concentration of available iron; the extrusion of H^+, which decreases the pH of the rhizosphere thus enhancing the reduction of Fe^{3+}; and by iron reductases on the root surfaces which reduce Fe^{3+} chelates to Fe^{2+} (Römheld, 1987).

Comparisons of the iron concentration of control and putative *PsMT$_A$*-transgenic F1 seedlings revealed only 3 putative transgenics with an iron content higher than that of the controls (Figures 8e and f). These differences were minimal and proved to be statistically insignificant, suggesting that *PsMT$_A$* is not primarily involved in iron metabolism.

Okumura *et al.* (1991) isolated the MT-like *ids-1* gene from barley which encodes an mRNA that increases in concentration during iron deficiency. When the growth regimes of the other plant species from which MT-like genes have been isolated were re-examined, they all appear to be deficient in iron. This could explain the apparent high levels of basal expression seen in the species and the absence of induction in response to elevated concentrations of metal ions such as copper and zinc. Okumura *et al.* (1991) have further proposed that *ids-1*, and other MT-like genes in plants, may have a role in iron-efficiency, possibly analogous to the bacterial *fur* genes. The MT-like genes could encode proteins which bind iron and are involved in regulating the expression of genes encoding enzymes which are responsible for the synthesis of the phytosiderophores. However, there is an alternative explanation for the observed induction of these genes in response to low iron which is consistent with their proposed role as MT-like copper-chelating molecules, possibly analogous to the *CUP1* gene system of yeast.

4.2.3 Metallothionein / copper interactions

Analysis of the copper concentration of control and putative *PsMT$_A$*-transgenic F1 seedlings revealed an obvious statistically significant difference (Figures 8a and b), with the transgenic seedlings accumulating up to 8 times more copper than the controls.

Determination of the metal binding properties of the *PsMT$_A$* protein was necessary in order to confirm it as a metallothionein. The *PsMT$_A$* coding sequence has been cloned via a pGEX vector into *E.coli*, where it was expressed as a fusion product with glutathione-S-transferase (GST) (Tommey *et al.*, 1991). The protein was cleaved and partially sequenced to confirm its identity. From the data obtained, the sequence suggests a structure most suited to copper binding. This correlates with the analysis of the putative *PsMT$_A$*-transgenic seedlings, descibed above.

Estimations of the pH of half dissociation of the metal from the protein is a criterion commonly used to distinguish MT from non-MT metal-binding proteins. The half dissociation values of zinc, cadmium and copper from equine MT were determined to be 4.50, 3.00 and 1.80 respectively, and of cadmium and copper from *Schizosaccharomyces pombe* phytochelatin, 4.00 or 5.40 (depending upon whether or not the complexes contained additional inorganic sulphide) and 1.30. The pH of half dissociation of the *PsMT$_A$* fusion product, purified from *E.coli* grown in metal ion supplemented media of zinc, cadmium and copper were estimated to be 5.25, 3.95 and 1.45 respectively (Tommey *et al.*, 1991). A comparison of these values to equine MT and phytochelatin suggest that *PsMT$_A$* also has high affinities for certain metal ions, particularly copper, supporting the idea that it is a plant MT. Final confirmation that this protein is a plant MT requires the

characterization of its metal-binding properties *in planta*.

The copper accumulation properties of the *E.coli* expressing the *PsMT*$_A$-GST fusion product were compared to those of the *E.coli* control expressing only GST. The *PsMT*$_A$-GST strain accumulated a much higher concentration of copper than the controls (W.P.Lindsay *et al.* unpublished).

The involvement of MT in copper homoeostasis in yeast has been well documented by Hamer (1986). The yeast copper-MT gene (*CUP1*) transcript was shown to increase steadily to a plateau with increasing copper concentrations, the MT acting as a copper store to prevent the toxic effects of the excess ions. Copper, like iron, is reduced (from Cu^{2+} to Cu^{+}) when absorbed into the cell. Further work on yeast, has led to the identification of a cell surface copper reductase (Hamer, 1991 in press) from a mutant deficient in copper accumulation. The mutant was complemented and the gene identified. Its sequence was found to be identical to the yeast iron reductase gene (*FRE1*) indicating that its reductase activity is not specific to iron, as was previously thought, but instead also reduces copper ions. *FRE1* is expressed in copper- and/or iron-deficient conditions enabling the reduction and therefore the uptake of these essential ions, but it is switched off with sufficiency. *FRE1* was found to be less sensitive to iron than to copper, therefore under iron-deficient conditions when the *FRE1* gene is highly expressed, the cells will be accumulating the necessary iron but will also be accumulating copper at a much higher, potentially toxic, rate. This excess copper would stimulate the expression of the *CUP1* copper-MT which binds the copper therefore detoxifying the cell. No reports of this theory having been examined have been published.

If the iron reductase of plants is also a copper reductase, it

could explain the gradual increase of the *PsMT*$_A$ transcripts during germination. As the seedlings grew (in increasingly iron deficient conditions) the iron/copper reductase would be activated, co-accumulating excess copper as well as the necessary iron. The transcription of a copper chelator may therefore be increased to bind the free copper.

Some recent unpublished data (Kochian *et al.* in press) has recorded variation in the copper accumulation of pea plants grown with or without an iron supplement. Plants grown in iron-sufficient conditions were shown to accumulate only 8.1μg copper per gramme of fresh weight tissue compared to plants grown in iron-deficient conditions which accumulated 104.1μg/g.

Experiments are underway in order to try and determine whether the iron deficiency theory could be correct. Pea plants, grown in iron-sufficient conditions will be analysed for *PsMT*$_A$ expression (previously thought to be constitutively expressed in the mature seedlings). If the iron-deficiency theory is correct, it should be possible to switch on expression of the *PsMT*$_A$ gene by transferring the plants to iron-deficient conditions. Furthermore, *PsMT*$_A$ expression may be re-activated under iron-sufficient conditions in the presence of excess copper.

4.2.4 Expression of *PsMT*$_A$ in *Arabidopsis*

In addition to responding to the presence of metal ions, animal MT genes show changes in the level of expression through embryogenesis into maturity (Kägi and Schaffer, 1988). As the organism matures, the concentration of MT present generally decreases. In plants, very

little is known about the regulation of expression, or function, of MT-like proteins.

The class II MT (E_c), isolated from wheat, (Hanley-Bowdoin and Lane, 1983) is abundant in the embryonic tissue (wheatgerm) but its abundance declines after germination. Studies of the expression pattern of the *PsMT* genes revealed a lack of *PsMT* transcripts in RNA isolated from embryonic radicle but an increase in abundance in the roots after germination, apparently the opposite of the class II MT results in wheat (Robinson *et al.*, in press). However, *PsMT* transcripts of a slightly smaller size were detected in embryonic cotyledons.

The presence of $PsMT_A$ in *Arabidopsis* was confirmed by PCR analysis of genomic DNA with $PsMT_A$ primers (Results 3.2.6). mRNA expression was also confirmed using poly A-primed cDNA synthesis and $PsMT_A$ PCR primers (Results 3.2.7). F1 seedlings were similarly analysed and showed the presence of a rearrangement, probably a deletion, in one copy of the $PsMT_A$ coding region. A previously undetected 660bp band, as well as the 969bp $PsMT_A$ coding region, hybridize to a $PsMT_A$ probe (Results 3.2.9/10). The $PsMT_A$ / $PsMT_B$ competitive PCR data suggests some variation in the level of expression of $PsMT_A$ mRNA between individual transformants. However, the initial mRNA extraction technique used was too variable for the data to be used as anything other than an indication (Results 3.2.8).

With the isolation of an *Arabidopsis* MT-like gene (Chino *et al.* unpublished), it was important to verify from the sequence whether or not the PCR primers (270 and 271) used to identify transformants would also prime the amplification of the native gene. From the data available, these primers are not compatible with the *Arabidopsis* MT-like gene and would therefore only amplify the introduced $PsMT_A$ gene.

The lack of amplification of the *Arabidopsis* MT-like gene(s) with primers 270 and 271 is demonstrated in Plate 9.

The *PsMT*$_A$ metal-binding data obtained from the *E.coli*-expressed protein, suggested that the *Arabidopsis* metal-binding studies should concentrate initially on copper. The subsequent copper analysis of putative transgenic *Arabidopsis* grown on control or copper-supplemented media corroborated the *E.coli* data. Although a wide range of values of copper content were obtained from the putative *PsMT*$_A$ transgenics, a clear over-accumulation of copper in several individuals could be seen when compared to the controls. It is possible that if a plant was expressing a particularly high level of *PsMT*$_A$ protein, it would be unlikely to survive beyond germination, due to the lack of available essential metal ions. No correlation was seen between the high copper levels and either the iron or zinc levels; however it was noticeable that both the control and putative transgenic seedlings accumulated more iron and zinc when planted on the non-copper-supplemented medium. More extensive analyses must be carried out, however, before any conclusions can be drawn about the possible antagonistic effect between the metals.

With hindsight, these metal-binding results would have been more complete if a tissue sample from each plant had been removed, prior to the metal extraction, for Western blot analysis of *PsMT*$_A$ expression, using the recently made *PsMT*$_A$ antibody. This would have enabled the high copper accumulation to be linked specifically to those seedlings that were expressing the highest levels of *PsMT*$_A$.

The experiments could have perhaps also been improved by using control *Arabidopsis* that had regenerated through a tissue culture cycle (the p-*PsMT*$_A$ *Arabidopsis* transformants would have been ideal), and would therefore remove the possibility of higher variation within

the populations being due to the influence of the somaclonal variation induced by prolonged tissue culture. However, the root transformation system used was developed for its speed of regeneration and therefore reduces the time spent in tissue culture. Results have shown that the actual rate of spontaneous mutation is low enough to have no severe implications on experimental work (Valvekens and Van Montagu, 1990).

Further potentially useful data could be obtained by doing the same analyses but with seedlings germinated on media supplemented with iron or zinc, or even cadmium to compare the $PsMT_A$ proteins binding ability *in planta* with that of the *E.coli*-expressed protein.

In conclusion, the copper-binding data from both the *E.coli* and the *Arabidopsis*-expressed $PsMT_A$ appears to support the suggestion that the $PsMT_A$ protein binds copper *in vivo*.

4.3 *Tam3*

Transposable elements have the ability to move independently around the genome of their host plant, blocking the expression of any gene into which they insert. When the sequence of the transposable element is known, it can be used to 'tag' the gene into which it has inserted, thus potentially being a method to isolate every gene in the plant. With the completly random transposition event, *Arabidopsis* has a natural advantage over other plants, it contains very little repetitive non-coding DNA therefore there is a higher probability of transposon insertion within a gene. However, one limitation of the transposon tagging system with *Arabidopsis* and, in fact, most plant species, is the lack of native characterized transposable elements. This problem has been partially overcome by the introduction of transposable elements into foreign hosts using *Agrobacterium*-mediated transformation. Several transposable elements have been introduced into foreign hosts, for example, the maize elements *Ac* (Van Sluys *et al.*, 1987) and *Spm* (Frey *et al.*, 1989), and the *Tam*3 element from *Antirrhinum majus* (Martin *et al.*, 1989).

Although many laboratories have set out to develop a transposon tagging system for *Arabidopsis*, no published genes have yet been isolated by this method. The maize *Ac* element has been shown to transpose in *Arabidopsis* at frequencies high enough to make insertional mutagenesis a realistic possiblity in primary transformants right through to the F2 generation of seedlings (Schmidt and Willmitzer, 1989).

One of the newly developed visual markers being used in such chimaeric constructs is streptomycin phosphotransferase (SPT). When plated onto streptomycin, excision events can be detected as green

sectors on white cotyledons, with germinal excision events detected as fully green seedlings (Jones *et al.*, 1989). Besides the antibiotic resistance markers, another well-used system involves the *bar* gene conferring resistance to the herbicide Phosphinotricin (Altmann *et al.*, 1990).

The *Tam*3 transposon tagging system was originally developed to take advantage of its temperature response. Experiments in its native host, *Antirrhinum majus*, showed a significant increase in transposition frequency when the temperature was lowered to 15°C. A system where transposition could be stimulated by a simple drop in temperature and suppressed by raising the temperature, resulting in stable mutations, was very appealing.

*Tam*3 has been shown to transpose when introduced into the tobacco genome (Martin *et al.*, 1989), however it appeared as if the frequency of transposition, initially greater than 10%, dropped significantly in the progeny of the transgenic plants. The integration event of *Tam*3 in tobacco is somewhat more irregular than in *Antirrhinum*. This could be due to the less precise action of the tobacco enzymes supplementing the transposase than those of the native host enzymes. A similar imprecise insertion of *Ac* in tobacco has also been observed (Martin *et al.*, 1989).

A re-induction of transposition in transgenic tobacco plants was attempted by a temperature shift to 15°C. Although the initial analysis of transgenic plants indicated a high frequency of transposition, no evidence of further transposition could be detected, suggesting that only conditions early in transformation/regeneration may be favourable for *Tam*3 transposition. This relative stability of *Tam*3 in transgenic tobacco plants suggests that movement may be suppressed in some way by the host plant. Methylation has been

strongly correlated to the inactivation of several maize elements (Bennetzen, 1987; Schwarz and Dennis, 1986) and, indeed, when examined, *Tam3* from the transgenic tobacco was found to be rapidly and heavily methylated within the inverted repeats.

The transgenic *Arabidopsis* produced were shown to contain a *Tam3* element but the screening of *Tam3* transposition proved inconclusive and would have required extensive DNA analysis before any definite results would have been obtained. It was decided that due to the amount of analysis necessary and *Tam3* data published since the onset of this project, the project would be abandoned. Although results showing transposition could possibly have been obtained (see Results 3.3.8), a higher priority was given to the other transformation experiments undertaken. The design of the p*Tam3* construct was poorly thought out, analysis would have been quicker and more efficient if an excision marker gene, such as SPT or even GUS, had been employed. A construct in which the transposable element is blocking the expression of a second screenable marker gene would simplify the detection of excision events.

4.3.1 Dual element transposon tagging systems

One of the disadvantages of using an autonomous transposon (like *Tam3*) for gene tagging is that it gives rise to potentially unstable mutations. The two element *Ac/Ds* system has been used to develop a more controllable system: an internally deleted *Ac* element (essentially a *Ds*) and the *Ac* transposase gene under the control of inducible or pollen-specific promoters that will induce transposition in independent gametes (Balcells *et al.*, 1990). Promoters with a more

pronounced effect on the timing of transposition, for example the *Arabidopsis* ADH promoter (Chang and Meyerowitz, 1986) and the *Petunia* CHI-A2 promoter (Van Tunen *et al.*, 1989), have been selected but no definite results have yet been reported.

The non-autonomous element can also be manipulated to optimize the transposon tagging procedure. By equipping it with a genetic marker, it is possible to confirm directly that induced mutations are linked to the tag. To facilitate the recloning of tagged genes, the non-autonomous element can be equipped with bacterial cloning features (for example, an origin of replication and a bacterial resistance gene). The methotrexate resistance gene, DHFR, has been shown to fit into the *Ds* element without significantly altering the capacity to be *trans*-activated (Masterson *et al.*, 1989). Other possible markers include the GUS reporter gene which is also small enough to be cloned into the *Ds* element. Further applications have been proposed for non-autonomous elements, such as cloning in a strong promoter and using random insertion to isolate, by over-expression, the genes around it (Haring *et al.*, 1991b).

In order to try and take advantage of the temperature sensitive transposition control of *Tam3* as a transposon tag, a two element system was developed. Haring *et al.* (1991a) produced a transgenic tobacco line containing an artificial d*Tam3* element (with a deletion in the presumptive transposase coding region) and showed it could be *trans*-activated by an activator *Tam3* element (immobilized by the deletion of one inverted repeat). As in the previous transgenic lines, no enhancement of *Tam3* excision could be detected with the temperature downshift, again suggesting that the enhanced excision is not encoded by *Tam3* itself but that other host plant features are involved.

In conclusion, the relatively low excision rate and integration

frequency in transgenic tissue of *Tam3* and the failure of the temperature-sensitive transposition switch, made the system less suitable for gene tagging than previously thought. Future gene tagging work should concentrate on the *Ac* system which seems to be the most successful system characterized to date. Future *Tam3* work should be focused on trying to determine the host plant factors that are present in *Antirrhinum majus* that are involved in the temperature-sensitive transposition switch. If these factors could be determined, it would theoretically be possible to use them to control transposition resulting in a highly efficient controllable gene tagging system.

4.4 Extensin

Extensins constitute a class of cell wall hydroxyproline-rich glycoproteins (HRGPs) present in a wide variety of plants. They contain the repetitive sequence Ser-Hyp-Hyp-Hyp-Hyp and are apparently isodityrosine cross-linked into the primary wall. Although obviously involved in some way with cell wall architecture, the range of extensin functions are still under speculation. In order to elucidate their distribution and possible function within the plant, extensin expression patterns have been analysed by various groups over recent years.

Cassab and Varner (1987) demonstrated the accumulation of HRGPs during the development of soybean seeds, primarily localized in the seed coat, hilum and the vascular elements. Hydroxyproline had previously been reported as being abundant in the seed coat of several plant families (van Etten *et al.*, 1961) indicating a possible protective and mechanical function in the testa. Extensin was further localized to the sclerenchyma, a cell type highly abundant within the seed coat and present in many plant organs, enabling them to withstand various strains, such as stretching, bending, weight and pressure, without undue damage to the softer, thin-walled parenchyma cells.

Although some of the early results, based on crude HRGP extracts, may be misleading, the variation in the extensin expression pattern, between different species, is wide ranging. Extensin was found to be abundant in carrot root phloem parenchyma cells (Stafstrom and Staehelin, 1988), whereas Benhamou *et al.* (1990), found HRGPs were only minor components in tomato root tissue. A specific role in lateral root initiation was suggested for extensin by Keller and Lamb (1989), having found a distinct tobacco HRGP that is synthesized and

accumulates at such sites.

Stiefel *et al.* (1990) produced *in situ* hybridization results showing extensin expression in maize was particularly high in regions initiating vascular elements and associated sclerenchyma. Further analysis of maize has shown the major maize HRGP (PC-1) was localized in vascular bundles and the epidermis of stem tissue (Hood *et al.*, 1991). The data presented in Results 3.4.6 shows that the pattern of expression of the rape *extA* promoter when introduced into *Arabidopsis thaliana* mirrors that of PC-1 in maize, suggesting that they share a similar function.

Hydroxyproline was found in the cell walls of stems, leaves, roots, tassels and silks, with stem nodes containing the highest concentration (probably due to the large number of vascular bundles present). The silks (maize stigmatic material) also contain a surprisingly large amount of extensin, which may be functioning by giving strength to the silk but, unlike lignin, would also allow the necessary flexibility. The presence of extensin in tobacco and bean stigmas has also been shown (Wycoff *et al.*, 1990).

Various evidence has been presented to suggest that extensins are encoded by a multi-gene family and that more than one type of cell wall extensin is present in a given tissue (Smith *et al.*, 1984; 1986). Showalter *et al.* (1991) have synthesized extensin cDNAs from mRNAs isolated from wounded and unwounded tomato tissue. From the sequence data of the clones, five distinct classes were obvious, of which classes I and II exhibit extensin characteristics. Class I clones contain Ser-$(Pro)_4$-Ser-Pro-Ser-$(Pro)_4$-$(Tyr)_4$-Lys repeats and were found to accumulate markedly upon wounding, whereas class II clones contain Ser-$(Pro)_4$-Ser-Pro-Ser-$(Pro)_4$-Thr-$(Tyr)_{1-3}$-Ser repeats and showed no accumulation upon wounding. As both class I and class II

clones are present in unwounded tissue, it was assumed that they both represent structural components within the tomato cell wall. However, it was speculated that because of the abundance of Tyr-X-Tyr units (where X is usually Tyr or Val), giving class I clones an enhanced potential for the formation of intermolecular cross-links (therefore increasing their insolubility), that an additional role in wound healing and plant defence was particularly attractive. The isodityrosine cross-links could allow for the erection of a highly dense and impenetrable cell wall barrier, thus serving to keep pathogens at bay as well as to prevent excessive evaporative water loss from a wound site. While the class II extensin sequences are novel, the class I extensin sequences are homologous to those of other plant extensins, for example, the bean clones isolated by Corbin *et al.* (1987).

Studies of the race:cultivar-specific interactions of *Colletotrichum lindemuthianum* with *Phaseolus vulgaris* L cells revealed differences in the responses of three distinct HRGP mRNA species. In the incompatible interaction (a resistant host), there was an early increase in HRGP mRNA correlated with the expression of hypersensitive resistance, whereas in a compatible interaction (a susceptible host), the HRGP mRNA accumulates as a delayed response at the onset of lesion formation (Showalter *et al.*, 1985). This polymorphism may reflect subtle regulatory and structural differences related to the specific functions of the protein products in defence.

These multiple RNAs may be the result of a multi-gene family or could possibly be encoded by a single gene and arise by alternative splicing. However, it appears that there are several different extensin types, present in the same tissue, that are differentially expressed and apparently have differing functions.

4.4.1 Analysis of *extA* plants

The *extA* / GUS fusion constructs (pAS44 and 45) were designed and constructed to localize the expression of the *extA* extensin gene isolated from *Brassica napus* L. (Evans *et al.*, 1990b). GUS reporter fusion constructs have previously been used successfully in studies of the tissue-specific expression of several genes, for example, a bean cell wall glycine-rich protein (Keller *et al.*, 1989), the anaerobically induced maize *Adh1* promoter (Kyozuka *et al.*, 1991) and leaf epidermal cell expression of the pea plastocyanin, pea ferredoxin:NADP$^+$ reductase and tobacco *rbcS* genes (Dupree *et al.*, 1991).

The *extA* constructs were transformed, initially, into *Brassica napus* L. using *Agrobacterium rhizogenes* (Shirsat *et al.*, 1991). GUS expression was localized to the vascular cylinders of the transgenic hairy roots, specifically in the phloem. Expression of GUS in the pericycle cells of the emerging lateral root was not seen, suggesting that *extA* in *Brassica napus* L. has a different function to the tobacco extensin isolated by Keller and Lamb (1989). The *extA* coding sequence with its promoter was transformed into tobacco (Shirsat *et al.*, 1991), and its expression examined by northern blot analysis. *extA* expression was localized to the root tissue, but was also present to a lesser extent in the stems.

In order to confirm these expression patterns, the construct was transformed into *Arabidopsis*, a member of the same family (the *Cruciferae*) as *Brassica napus* L. The *Arabidopsis* expression pattern was similar. The expression in the stem was localized to the phloem, as in *Brassica napus* L., however, the immuno-gold anti-GUS labelling also detected *extA* expression in the epidermis. No expression was

found in the root tissue, however this could have been simply because of the small size of the roots and the difficulty this caused in their manipulation.

From both sets of data, it would appear that the *ext*A gene is highly tissue specific, mostly linked to the phloem cells. Phloem has to be able to withstand high pressures but also maintain flexibility, extensin could potentially fulfill both of these requirements, therefore its expression is only to be expected. Expression of *ext*A in epidermal cells of *Arabidopsis* was not surprising if the gene product is acting as a wall strengthener. It is possible that expression was not found in *Brassica napus* L. epidermal cells because the plants sectioned were older and the role of cell wall strengthener had been taken over by lignin. The expression pattern in *Arabidopsis* appears to be the same as that reported for the major maize HRGP (PC-1) (Hood *et al.*, 1991). It would be interesting to compare the sequences of these two extensins to check for any homology.

Future work with this transgenic line will probably concentrate on identifying the role of the *ext*A gene in the development from seed to plant, following work by Stiefel *et al.* (1990) and Ye and Varner (1991). Using the GUS fusion constructs, it should be possible to localize cell specific expression at various stages of development of the seedlings and to identify any variation within those patterns of expression. Although, at times, the small size of the *Arabidopsis* plants will be a hindrance (for example, when tissue sections are required), the large number of seeds produced and the size of the seedlings permits a possible vast screening programme of *ext*A expression, by taking seedlings at various intervals and simply incubating the whole plant in the GUS histochemical stain.

4.5 Conclusions

A workable *Agrobacterium tumefaciens*-mediated transformation system for *Arabidopsis thaliana* was established and used to successfully transform three test constructs (a gene, a promoter-reporter fusion and a transposable element) into the plant genome. Analysis of plants containing the putative metallothionein gene, $PsMT_A$ has provided further data to suggest that $PsMT_A$ functions within copper metabolism. Transgenic plants containing the rape extensin *ext*A promoter-GUS fusion enabled the determination of its phloem and epidermal tissue-specific expression.

This project illustrates some of the many applications of a workable transformation system of *Arabidopsis*. However, the main advantage of *Arabidopsis* to the molecular biologist is its small genome, and therefore its potential for gene isolation, by mutagenesis, cross-hybridization, PCR techniques and transposon tagging. Although recent data suggests that the *Tam3* gene tagging system is less efficient than previously thought, the project illustrates the ease of engineering a gene tagging system into *Arabidopsis*. The results of other transposable elements (notably *Ac*) suggest their greater potential.

There are several seldomly mentioned disadvantages in the use of *Arabidopsis* which must be considered before a project is undertaken. Its size, although a major advantage in terms of the small amount of space required for growth, can be a severe handicap when it proves necessary to extract large amounts of DNA; the lack of available tissue, together with the small genome size, can be a problem.

Although used successfully to study physiological mutations (Komaki *et al.*, 1988; Haughn and Somerville, 1988), its small size can

again cause problems. Detailed studies of morphology require much greater manual dexterity and therefore more time, when compared to, for example, tobacco. The basic phenotype of *Arabidopsis*, its simple flower morphology, lack of petal pigmentation and simple leaf shape, reduces the ease of identification of many classes of mutant, when compared to maize or tobacco.

In conclusion, the uses of *Arabidopsis* are indeed widespread and its value to the molecular biologist as a source of cloneable genes has already been proved. However, there has been a tendency over recent years to use *Arabidopsis* in research projects without considering its potential disadvantages.

5. REFERENCES

Albright, L.M., Yanofsky, M.F., Leroux, B., Nester, E.W. (1987)Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. J. Bacteriol., **169**, p1046-1055.

Altmann, T., Schmidt, R., Willmitzer, L. (1990) Induced transposition of a *Ds*-element carrying a bacterial and a plant selectable marker gene in *Arabidopsis thaliana.* Fourth International Conference on *Arabidopsis* Research, Abstracts p26.

André, D., Colau, D., Schell, J., Van Montagu, M., Hernalsteens, J.P. (1986) Gene tagging in plants by a T-DNA insertion mutagen that generates APH(3')II-plant gene fusions. M.G.G., **204**, p512-518.

Asada, K., Kanematsu, S., Uchida, K. (1977) Superoxide dismutases in photosynthetic organisms: Absence of the cuprozinc enzyme in eukaryotic algae. Arch. Biochem. Biophys., **179**, p243-256.

Bakkeren, G., Koukoliková-Nicola, Z., Hohn, B. (1989) Recovery of *Agrobacterium tumefaciens* T-DNA molecules from whole plants early after transfer. Cell, **57**, p847-857.

Balcells, L., Swinburne, J., Ingle, K., Scofield, S., Jones, J., Coupland, G. (1990) Development of a transposon tagging system using the maize *Ac* element. Fourth International Conference on *Arabidopsis* Research, Abstracts p27.

Benhamou, N., Mazau, D., Esquerré-Tugaye, M.T. (1990) Immunocytochemical localisation of hydroxyproline-rich glycoproteins in tomato root cells infected by *Fusarium oxysporum* f. sp. radicis-lycopersici: Study of a compatible interaction. Mol. Plant Path., **80**, p163-173.

Bennetzen, J.L. (1987) Covalent DNA modification and the regulation of *Mutator* element transposition in maize. M.G.G., **208**, p45-51.

Bernhard, W.R., Vašák, M., Kägi, J.H.R. (1986) Cadmium binding and metal cluster formation in metallothionein: A differential modification study. Biochem., **25**, p1975-1980.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. Nucl. Acid Res., **12**, p8711-8721.

Bevan, M., Barnes, W., Chilton, M.D. (1983) Structure and transcription of the nopaline synthase gene region of T-DNA. Nucl. Acid Res., **11**, p369-385.

Biggs, K.J., Fry, S.C. (1990) Solubilization of covalently bound extensin from capsicum cell-walls. Plant Physiology, **92**, p197-204.

Bingham, P.M., Levis, R., Rubin, G.M. (1981) Cloning of DNA sequences from the *white* locus of *D.melangaster* by a novel and general method. Cell, **25**, p693-704.

Binns, A.N., Thomashow, M.F. (1988) Cell biology of *Agrobacterium* infection and transformation of plants [Review]. Ann. Rev. Microbiol., **42**, p575-606.

Birnboim, H.C., Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant DNA. Nucl. Acid Res., **7**, p1513-1523.

Bleecker, A.B., Estelle, M.A., Somerville, C., Kende, H. (1988) A dominant mutation confers insensitivity to ethylene in *Arabidopsis thaliana*. Science, **241**, p1086-1089.

Bonas, U., Sommer, H., Saedler, H. (1984) The 17-kb *Tam1* element of *Antirrhinum majus* induces a 3-bp duplication upon integration into the chalcone synthase gene. EMBO J., **3**, p1015-1019.

Bowman, J.L., Drews, G.N., Meyerowitz, E.M. (1991) Expression of the *Arabidopsis* floral gene *AGAMOUS* is restricted to specific cell types late in flower development. Plant Cell, **3**, p749-758.

Braun, A.C., Wood, H.N. (1976) Suppression of the neoplastic state with the acquisition of specialized functions in cells, tissues and organs of crown gall teratoma of tobacco. P.N.A.S., **73**, p496-500.

Browse, J., Kunst, L., McCourt, P., Somerville, C.R. (1987) Genetic manipulation of membrane lipid composition in *Arabidopsis*. UCLA Symp. Mol. Cell. Biol., **63**, p437-447.

Browse, J. (1991) The contribution of *Arabidopsis* mutants to understanding plant lipid metabolism. Abstracts of the Robertson Symposium on '*Arabidopsis thaliana* and the molecular basis of plant biology.' 30.9.91-1.10.91. Australian National University, Canberra.

Cangelosi, G.A., Martinetti, G., Leigh, J.A., Lee, C.C., Theines, C., Nester, E.W. (1989) Role of *Agrobacterium tumefaciens* ChvA protein in export of β-1,2 glucan. J.Bacteriol., **171**, p1609-1615.

Carpenter, R., Martin, C., Coen, E.S. (1987) Comparison of genetic behaviour of the transposable element *Tam3* at two unlinked pigment loci in *Antirrhinum majus*. M.G.G., **207**, p82-89.

Carter, A.D., Felber, B.K., Walling, M.J., Jubier, M.F., Schmidt, C.J., Hamer, D.H. (1984) Duplicated heavy metal control sequences of the mouse metallothionein-I gene. P.N.A.S., **81**, p7392-7396.

Cassab, G.I., Varner, J.E. (1987) Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. J. Cell Biol., **105**, p2581-2588.

Chang, C., Meyerowitz, E.M. (1986) Molecular cloning and DNA sequence of the *Arabidopsis thaliana* alcoholdehydrogenase gene. P.N.A.S., **83**, p1408-1412.

Chaudhury, A.M., Craig, S., Blomer, K., Farrell, L., Chapple, R., Sherman, B., Dennis, L. (1991) Male-fertility mutants: male-sterile sporophytic nuclear mutants. Genetic control of male-fertility in *Arabidopsis thaliana*. Abstracts of the Robertson Symposium on '*Arabidopsis thaliana* and the molecular basis of plant biology.' 30.9.91-1.10.91. Australian National University, Canberra.

Chilton, M-D., Saiki, R.K., Yadav, N., Gordon, M.P., Quetier, F. (1980) T-DNA from *Agrobacterium* Ti plasmid is in the nuclear DNA fraction of crown gall tumour cells. P.N.A.S., **77**, p4060-4064.

Chory, J., Peto, C.A., Ashbaugh, M., Saganich, R., Pratt, L., Ausubel, F. (1989) Different roles for phytochrome in etiolated and green plants deduced from characterization of *Arabidopsis thaliana*. Plant Cell, **1**, p867-880.

Christie, P.J., Ward, J.E., Winans, S., Nester, E.W. (1988) The *Agrobacterium tumefaciens vir*E2 gene product is a single stranded DNA binding protein that associates with T-strands. J.Bacteriol., **170**, p2659-2667.

Citovsky, V., Wong, M.L., Zambryski, P. (1989) Cooperative interaction of *Agrobacterium* VirE2 protein with single-stranded DNA: Implications for the T-DNA transfer process. P.N.A.S., **86**, p1193-1197.

Clarkson, D.T., Hanson, J.B. (1980) The mineral nutrition of higher plants. Ann. Rev. Plant Phys., **31**, p239-298.

Clijsters, H., Van Assche, F. (1985) Inhibition of photosynthesis by heavy metals. Photosynthesis Res., **7**, p31-40.

Cobbett, C., Hanrahan, C., Howden, R. (1991) Heavy metal-sensitive mutants of *Arabidopsis thaliana*. Abstracts of the Australian Society of Plant Physiologists. 31st Annual General Meeting, October 1991. **P17**, Australian National University.

Coe, E.H.Jr, Neuffer, M.G., Hoisington, D.A. (1988) The genetics of corn. In Corn and Corn Improvement, 3rd edn. eds Sprague, G.F., Dudley, J.W. Am. Soc. Agron., Madison, Wisconsin, p81-258.

Coen, E.S., Carpenter, R., Martin, C. (1986) Transposable elements generate novel spatial patterns of gene expression in *Antirrhinum majus*. Cell, **47**, p285-296.

Compere, S.J., Palmiter, R.D. (1981) DNA methylation controls the inducibility of the mouse metallothionein-1 gene in lymphoid cells. Cell, **25**, p233-240.

Conner, T.W., Goekjian, V.H., LaFayette, P.R., Key, J.L. (1990) Structure and expression of two auxin-inducible genes from *Arabidopsis*. Plant Mol. Biol., **15**, p623-632.

Cooley, M.B., D'Souza, M.R., Kado, C.I. (1991) The *virC* and *virD* operons of the *Agrobacterium* Ti plasmid are regulated by the *ros* chromosomal gene: analysis of the cloned *ros* gene. J.Bacteriol., **173**, p2608-2616.

Corbin, D.R., Sauer, N., Lamb, C.J. (1987) Differential regulation of a hydroxyproline-rich glycoprotein gene family in wounded and infected plants. Mol. Cell Biol., **7**, p4337-4344.

Crawford, N.M., Smith, M., Bellissimo, D., Davis, R.W. (1988) Sequence and nitrate regulation of the *Arabidopsis thaliana* mRNA encoding nitrate reductase, a metalloflavoprotein with three functional domains. P.N.A.S., **85**, p5006-5010.

Culotta, V.C., Hsu, T., Hu, S., Fürst, P., Hamer, D. (1989) Copper and the ACE1 regulatory protein reversibly induce yeast metallothionein gene transcription in a mouse extract. P.N.A.S., **86**, p8377-8381.

Dale, P.J., Marks, M.S., Brown, M.M., Woolston, C.J., Gunn, H.V., Mullineaux, P.M., Lewis, D.M., Kemp, J.M., Chen, D.F., Gilmour, D.M., Flavell, R.B. (1989) Agroinfection of wheat: inoculation of *in vitro* grown seedlings and embryos. Plant Sci., **63**, p237-245.

De Framond, A.J. (1991) A metallothionein-like gene from maize (*Zea mays*): Cloning and characterization. FEBS Letts., **290**, p103-106.

De Greve, H., Dhaese, P., Seurwick, J., Lemmers, M., Van Montagu, M., Schell, J. (1982) Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti plasmid-encoded octopine synthase gene. J. Molec. Appl. Genet., **1**, p499-511.

De Miranda, J.R., Thomas, M.A., Thurmann, D.A., Tomsett, A.B. (1990) Metallothionein genes from the flowering plant *Mimulus guttatus*. FEBS Letts., **260**, p277-280.

De Oliveira, D.E., Seurinck, J., Inzé, D., Van Montagu, M., Botterman, J. (1990) Differential expression of five *Arabidopsis* genes encoding glycine-rich proteins. Plant Cell, **2**, p427-436.

Dhaese, P., De Greve, H., Decraemer, H., Schell, J., Van Montagu, M. (1979) Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmids of *Agrobacterium tumefaciens*. Nucl. Acid Res., **7**, p1837-1849.

Dolferus, R., Jacobs, M. (1984) Polymorphism of alcoholdehydrogenase in *Arabidopsis thaliana* (L.) Heynh.: genetical and biochemical characterization. Biochem. Genet., **22**, p817-838.

Dupree, P., Pwee, K-H., Gray, J.C. (1991) Expression of photosynthesis gene-promoter fusions in leaf epidermal cells of transgenic tobacco plants. Plant J., **1**, p115-120.

Edwards, K., Johnstone, C., Thompson, C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucl. Acid Res., **19**, p1349.

Engstrom, P., Zambryski, P., Van Montagu, M., Stachel, S.E. (1987) Characterization of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. J.Mol.Biol., **197**, p635-645.

Errampalli, D., Patton, D., Castle, L., Mickelson, Ł., Schnall, J., Feldmann, K., Meinke, D. (1991) Embryonic lethals and T-DNA insertional mutagenesis in *Arabidopsis*. Plant Cell, **3**, p149-157.

Esquerré-Tugayé, M.T., Lamport, D.T.A. (1979) Cell surfaces in plant-microorganism interactions. I. A structural investigation of cell wall hydroxyproline-rich glycoproteins which accumulate in fungus-infected plants. Plant Physiol., **64**, p314-319.

Estelle, M.A., Somerville, C. (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. M.G.G., **206**, p200-206.

Evans, I.M., Gatehouse, L.N., Gatehouse, J.A., Robinson, N.J., Croy, R.R.D. (1990a) A gene from pea (*Pisum sativum* L.) with homology to metallothionein genes. FEBS Letts., **262**, p29-32.

Evans, I.M., Gatehouse, L.N., Gatehouse, J.A., Yarwood, J.N., Boulter, D.B., Croy, R.R.D. (1990b) The extensin gene family in oilseed rape (*Brassica napus* L.): Characterization of sequences of representative members of the family. M.G.G., **223**, p273-287.

Federoff, N.V., Furtek, D.B., Nelson, O.E.Jr. (1984) Cloning of the *bronze* locus in maize by a simple and generalizable procedure using the transposable controlling element *Activator* (*Ac*). P.N.A.S., **81**, p3825-3829.

Feldmann, K.A., Marks, M.D.,(1987) *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: A non-tissue culture approach. M.G.G., **208**, p1-9.

Feldmann, K.A., Marks, M.D., Christianson, M.L., Quatrano, R.S. (1989) A dwarf mutant of *Arabidopsis* generated by T-DNA insertional mutagenesis. Science, **243**, p1351-1354.

Feldmann, K.A., Carlson, T.J., Coomber, S.A., Farrance, C.E., Mandel, M.A., Wierzbicki, A.M. (1990) T-DNA insertional mutagenesis in *Arabidopsis thaliana*. In: Horticultural Biotechnology, A.B.Bennett, S.D.O'Neill, eds (New York: Wiley-Liss) p109-120.

Frey, M., Tavantzis, S.M., Saedler, H. (1989) The maize *En-1/Spm* element transposes in potato. M.G.G., **217**, p172-177.

Furey, W.F., Robbins, A.H., Clancy, L.L., Winge, D.R., Wang, B.C., Stout, C.D., (1987) Crystal structure of Cd.Zn.metallothionein. Exp. Suppl., **52**, p139-148.

Ghai, J., Das, A. (1989) The *vir*D operon of *Agrobacterium tumefaciens* Ti plasmid encodes a DNA-relaxing enzyme. P.N.A.S., **86**, p3109-3113.

Gheyson, G., Van Montagu, M., Żambryski, P. (1987) Integration of *A.tumefaciens* T-DNA involves rearrangements of target plant DNA sequences. P.N.A.S., **84**, p6169-6173.

Gil-Gómez, G., Marrero, P.F., Haro, D., Ayté, J., Hegardt, F.G. (1991) Characterization of the gene encoding the 10kDa polypeptide of Photosystem II from *Arabidopsis thaliana*. Plant Mol. Biol., **17**, p517-522.

Gilliland, G., Perrin, S., Bunn, H.F. (1990) Competitive PCR for quantitation of mRNA. In: PCR Protocols: A Guide to Methods and Applications., Academic Press. p60-69.

Golden, S.S., Brusslan, J., Haselkorn, R. (1987) Genetic engineering of the cyanobacterial chromosome. In : Wu, R., Grossman, L. (eds) Methods in Enzymology, **153**. Acad. Press, London. p215-231.

Goodman, H.M., Hauge, B., den Boer, B., Giraudat, J., Nam, H.G. (1989) Isolation of developmentally important genes of *Arabidopsis*. J. Cell Biochem. Suppl., **13D**, p233.

Greenblatt, I.M. (1984) Chromosome replication pattern deduced from pericarp phenotypes resulting from the movements of the transposable element Modulator in maize. Genetics, **108**, p471-485.

Grill, E., Somerville, C. (1991) Construction and characterisation of a yeast artificial chromosome library of *Arabidopsis* which is suitable for chromosome walking. M.G.G., **226**, p484-490.

Grill, E., Löffler, S., Winnacker, E-L., Zenk, M.H. (1989) Phytochelatins, the heavy-metal binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). P.N.A.S., **86**, p6838-6842.

Grimsley, N., Hohn, T., Davies, J.W., Hohn, B. (1987) *Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants. Nature, **325**, p177-179.

Hamer, D.H. (1986) Metallothionein. Ann. Rev. Biochem., **55**, p913-951.

Hanley-Bowdoin, L., Lane, B.G. (1983) A novel protein programmed by the mRNA conserved in dry wheat embryos. Eur. J. Biochem., **135**, p9-15.

Haring, M.A., Teeuwen-de Vroomen, M.J., Nijkamp, H.J.J., Hille, J. (1991a) *Trans*-activation of an artificial d*Tam3* transposable element in transgenic tobacco plants. Plant Mol. Biol., **16**, p39-47.

Haring, M.A., Rommens, C.M.T., Nijkamp, H.J.J., Hille, J. (1991b) The use of transgenic plants to understand transposition mechanisms and to develop transposon tagging strategies. Plant Mol. Biol., **16**, p449-461.

Harrison, B.J., Carpenter, R. (1973) A comparison of the instabilities at the *nivea* and *pallida* loci in *Antirrhinum majus*. Heredity, **31**, p309-323.

Harrison, S.C. (1991) A structural taxonomy of DNA-binding domains. Nature, **353**, p715-719.

Haughn, G.W., Somerville, C. (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. M.G.G., **201**, p430-434.

Haughn, G.W., Somerville, C.R. (1988) Genetic control of morphogenesis in *Arabidopsis*. Dev. Genet., **9**, p73-89.

Hehl, R., Baker, B. (1990) Properties of the maize transposable element *Activator* in transgenic tobacco plants: a versatile interspecies genetic tool. Plant Cell, **2**, p709-721.

Hood, K.R., Baasiri, R.A., Fritz, S.E., Hood, E.E. (1991) Biochemical and tissue print analyses of hydroxyproline-rich glycoproteins in cell walls of sporophytic maize tissues. Plant Physiol., **96**, p1214-1219.

Hooykaas, P.J.J., Den Dulk-Ras, H., Schilperoort, R.A. (1988) The *Agrobacterium tumefaciens* T-DNA gene 6b is an *onc* gene. Plant Mol. Biol., **11**, p791-794.

Hooykaas, P.J.J. (1989) Transformation of plant cells via *Agrobacterium*. Plant Mol. Biol., **13**, p327-336.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T. (1985) A simple and general method for transferring genes into plants. Science, **227**, p1229-1231.

Hudson, A.D., Carpenter, R., Coen, E.S. (1990) Phenotypic effects of short-range and aberrant transposition in *Antirrhinum majus*. Plant Mol. Biol., **14**, p835-844.

Irish, V.F., Sussex, I.M. (1990) Function of the *apetala-1* gene during *Arabidopsis* floral development. Plant Cell, **2**, p741-753.

Izawa, T., Miyazaki, C., Yamamoto, M., Terada, R., Iida, S., Shimamoto, K. (1991) Introduction and transposition of the maize transposable element *Ac* in rice (*Oryza sativa* L.). submitted.

Jefferson, R.A. (1987) Assaying chimaeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep., **5**, p387-405.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., **6**, p3901-3907.

Jen, G., Chilton, M.D. (1986) The right border region of pTiT37 T-DNA is intrinsically more active than the left border region in promoting T-DNA transformation. P.N.A.S., **83**, p3895-3899.

Jones, J.D.G., Carland, F.M., Maliga, P., Dooner, H.K. (1989) Visual detection of transposition of the maize element *Activator* (*Ac*) in tobacco seedlings. Science, **244**, p204-207.

Kägi, J.H.R., Schäffer, A. (1988) Biochemistry of metallothionein. Biochem. **27**, p8509-8515.

Karin, M., Richards, R.I. (1982) Human metallothionein genes: molecular cloning and sequence analysis of the mRNA. Nucl. Acid Res., **10**, p3165-3173.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I.,Krauter, P., Westphal, H.M., Beato, M. (1984) Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-II_A gene. Nature, **308**, p513-519.

Kaufman, P.D., Doll, R.F., Rio, D.C. (1989) *Drosophila* P element transposase recognizes internal P element DNA sequences. Cell, **59**, p359-371.

Keller, B., Lamb, C.J. (1989) Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. Genes Dev., **3**, p1639-1646.

Keller, B., Schmid, J., Lamb, C.J. (1989) Vascular expression of a bean cell wall glycine-rich protein - β-glucuronidase gene fusion in transgenic tobacco. EMBO J., **8**, p1309-1314.

Khurana, J.P., Poff, K.L. (1988) Characterization of *Arabidopsis thaliana* mutants with altered phototropism. Plant Physiol. **86**, p29.

Klee, H.J., Muskopf, Y.M., Gasser, C.S. (1987) Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. M.G.G., **210**, p437-442.

Komaki, M., Okada, K., Nishino, E., Shimura, Y. (1988) Isolation and

characterization of novel mutants of *Arabidopsis thaliana* defective in flower development. Development, **104**, p195-203.

Koncz, C., Redei, G.P., Schell, J., Koncz-Kalman, Z. (1989a) Insertional mutations in *Arabidopsis* generated by T-DNA. Genetics, **122**, pS38-S38.

Koncz, C., Martini, N., Mayerhofer, R., Koncz-Kalman, Z., Korber, H., Redei, G.P. Schell, J. (1989b) High-frequency T-DNA mediated gene tagging in plants. P.N.A.S., **86**, p8467-8471.

Koncz, C., Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Reiss, B., Redei, G.P. Schell, J. (1990) Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in *Arabidopsis thaliana*. EMBO J., **9**, p1337-1346.

Koornneef, M., Dellaert, L.W.M., van der Veen, J.H. (1982) EMS- and radiation-induced mutation frequencies at individual loci in *Arabidopsis thaliana*. Mut. Res., **93**, p109-123.

Koornneef, M., van Eden, J., Hanhart, C.J., Stam, P., Braaksma, F.J., Feenstra, W.J. (1983) Linkage map of *Arabidopsis thaliana*. J.Hered., **74**, p265-272.

Koornneef, M., Cone, J.W., Karssen, C.M., Kendrick, R.E., van der Veen, J.H., Zeevaart, J.A.D. (1985) Plant hormone and photoreceptor mutants in *Arabidopsis* and tomato. In: Plant Genetics, UCLA Symp. Mol. Biol., **35**, p103-114.

Krebbers, E., Seurinck, J., Herdies, L., Cashmore, A.R., Timko, M.P. (1988) Four genes in two diverged subfamilies encode the ribulose-1,5-bisphosphate carboxylase small subunit polypeptides of *Arabidopsis thaliana*. Plant Mol. Biol., **11**, p745-759.

Kretschmer, P.J., Cohen, S.N. (1979) Effect of temperature on translocation frequency of the Tn3 element. J. Bacteriol., **139**, p515-519.

Kyozuka, J., Fujimoto, H., Izawa, T., Shimamoto, K. (1991) Anaerobic induction and tissue-specific expression of maize *Adh1* promoter in transgenic rice plants and their progeny. M.G.G., **228**, p40-48.

Laibach, R.O. (1943) *Arabidopsis thaliana* (L.) Heynh als Objekt für genetische und entwicklungsphysiologishe Ontersuchungen. Bot. Archiv., **44**, 439-455.

Lane, B.G., Kajioka, R., Kennedy, T. (1987) The wheat-germ E_c protein is a zinc-containing metallothionein. Biochem. Cell Biol., **65**, p1001-1005.

Leblová, S., Špirhanzlová, E. (1987) The intake of Cd, Cu, Pb and Zn by germinating seeds of maize; induction of metallothionein. Exp. Suppl., **52**, p710, abstract 30.

Lee, M.S., Gippert, G.P., Somon, K.V., Case, D.A., Wright, P.E. (1989) Three-dimensional solution structure of a single zinc finger DNA binding domain. Science, **245**, p635-637.

Lefebvre, D.D., Miki, B.L., Laliberté, J-F. (1987) Mammalian metallothionein functions in plants. Bio/tech., **5**, p1053-1056.

Lei, M., Wu, R. (1991) A novel glycine-rich cell wall protein gene in rice. Plant Mol. Biol., **16**, p187-198.

Lengfelder, E., Elstner, E.F. (1979) Cyanide insensitive iron superoxide dismutase in *Euglena gracilis*. Comparison of the reliabilities of different test systems for superoxide dismutases. Z. Naturforsch., **34c**, p374-380.

Lerch, K., Beltramini, M. (1983) *Neurospora* copper metallothionein: molecular structure and biological significance. Chemica Scripta, **21**, p109-115.

Leroux, B., Yanofsky, M.F., Winans, S.C., Ward, J.E., Ziegler, S.F., Nester, E.W. (1987) Characterization of the *vir*A locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. EMBO J., **6**, p849-856.

Leutwiler, L.S., Meyerowitz, E.M., Tobin, E.M. (1986) Structure and expression of three light-harvesting chlorophyll *a/b*-binding protein genes in *Arabidopsis thaliana*. Nucl. Acid Res., **14**, p4051-4076.

Li, X.B., Kieliszewski, M., Lamport, D.T.A. (1990) A Chenopod extensin lacks repetitive tetrahydroxyproline blocks. Plant Physiol., **92**, p327-333.

Lin, T.-P., Caspar, T., Somerville, C.R., Preiss, J. (1988) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADP glucose pyrophosphorylase activity. Plant Physiol., **86**, p1131-1135.

Ling, V., Perera, I., Zielinski, R.E. (1991) Primary structures of *Arabidopsis* calmodulin isoforms deduced from the sequences of cDNA clones. Plant Physiol., **96**, p1196-1202.

Lloyd, A.M., Barnason, A.R., Rogers, S.G., Byrne, M.C., Fraley, R.T., Horsch, R.B. (1986) Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. Science, **234**, p464-466.

Ludwig, S.R., Oppenheimer, D.G., Silflow, C.D., Snustad, D.P. (1987) Characterization of the alpha tubulin gene family of *Arabidopsis thaliana*. P.N.A.S., **84**, p5833-5837.

Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R., Sigler, P.B. (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature, **352**, p497-505.

Luo, D., Coen, E.S., Doyle, S., Carpenter, R. (1991) Pigmentation mutants produced by transposon mutagenesis in *Antirrhinum majus*. Plant J., **1**, p59-69.

Maiti, L.B., Hunt, A.G., Wagner, G.J. (1988) Seed transmissable expression of mammalian metallothionein in transgenic tobacco. Biochem. Biophys. Res. Commun., **150**, p640-647.

Maiti, I.B., Wagner, G.J., Yeargan, R., Hunt, A.G. (1989) Inheritance and expression of the mouse metallothionein gene in tobacco: impact on Cd tolerance and tissue Cd distribution in seedlings. Plant Phys., **91**, p1020-1024.

Maiti, I.B., Wagner, G.J., Hunt, A.G. (1991) Light inducible and tissue-specific expression of a chimaeric mouse metallothionein cDNA gene in tobacco. Plant Science, **76**, p99-107.

Margoshes, M., Vallee, B.L. (1957) A cadmium protein from equine kidney cortex. J. Am. Chem. Soc., **79**, p4813-4814.

Marks, J.R., Lynch, T.J., Karlinsey, J.E., Thomashow, M.F. (1987a) *A. tumefaciens* virulence *psc*A is related to the *Rhizobium meliloti exo*C locus. J. Bacteriol., **169**, p5835-5837.

Marks, M.D., West, J., Weeks, D.P. (1987b) The relatively large beta tubulin gene family of *Arabidopsis* contains a member with an unusual transcribed 5' noncoding sequence. Plant Mol. Biol., **10**, p91-104.

Marks, M.D., Feldmann, K.A. (1989) Trichome development in *Arabidopsis thaliana*. 1. T-DNA tagging of the *GLABROUS1* gene. Plant Cell, **1**, p1043-1050.

Maroni, G., Otto, E., Lastowski-Perry, D. (1986) Molecular and cytogenic characterization of a metallothionein gene of *Drosophila*. Genetics, **112**, p493-504.

Martin, C., Carpenter, R., Sommer, H., Saedler, H., Coen, E.S. (1985) Molecular analysis of instability in flower pigmentation of *Antirrhinum majus* following isolation of *pallida* locus by transposon tagging. EMBO J., **4**, p1625-1630.

Martin, C., MacKay, S., Carpenter, R. (1988) Large-scale chromosomal restructuring is induced by the transposable element *Tam3* at the *nivea* locus of *Antirrhinum majus*. Genetics, **119**, p171-184.

Martin, C., Prescott, A., Lister, C., MacKay, S. (1989) Activity of the transposon *Tam3* in *Antirrhinum* and tobacco: possible role of DNA methylation. EMBO J., **8**, p997-1004.

Masson, P., Federoff, N.V. (1989) Mobility of the maize Suppressor-mutator element in transgenic tobacco cells. P.N.A.S., **86**, p2219-2223.

Masterson, R.V., Furtek, D.D., Grevelding, C., Schell, J. (1989) A maize *Ds* transposable element containing a dihydrofolate reductase gene transposes in *Nicotiana tabacum* and *Arabidopsis thaliana*. M.G.G., **219**, p461-466.

Matsumoto, S., Ito, Y., Hosoi, T., Takahashi, Y., Machida, Y. (1990) Integration of *Agrobacterium* T-DNA into a tobacco chromosome: Possible involvement of DNA homology between T-DNA and plant DNA. M.G.G., **224**, p309-316.

Matthysse, A.G. (1983) Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. J. Bacteriol., **154**, p906-915.

Matthysse, A.G. (1987) Characterization of non-attaching mutants of *Agrobacterium tumefaciens*. J. Bacteriol., **169**, p313-323.

Matzke, A.J.M., Chilton, M.-D. (1981) Site-specific insertion of genes into T-DNA of the *Agrobacterium* tumour-inducing plasmid: an approach to genetic engineering of higher plant cells. J. Mol. Appl. Genet., **1**, p39-49.

Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Bakkeren, G., Crameri, A., Angelis, K., Redei, G.P., Schell, J., Hohn, B., Koncz, C. (1991) T-DNA integration: a mode of illegitimate recombination in plants. EMBO J., **10**, p697-704.

McClintock, B.(1963) Further studies of gene-control systems in maize. Carnegie Inst. Washington Year Book, **62**, p486-493.

Meagher, R.B. (1991) Historical contingency and evolution of plant actin diversity: the *Arabidopsis* actin gene family. Abstracts of the Robertson Symposium on '*Arabidopsis thaliana* and the molecular basis of plant biology.' 30.9.91-1.10.91. Australian National University, Canberra.

Messens, E., Lenaerts, A., Van Montagu, M., Hedges, R.W. (1985) Genetic basis for opine secretion from crown gall tumour cells. M.G.G., **199**, p344-348.

Meyerowitz, E.M. (1987) *Arabidopsis thaliana*. Ann. Rev. Genet., **21**, p93-111.

Meyerowitz, E.M. (1989) *Arabidopsis* a useful weed. Cell, **56**, p263-269.

Meyerowitz, E.M., Pruitt, R.E. (1984) Genetic variations of *Arabidopsis thaliana*. Pasadena: Calif. Inst. Technol. **39**.

Mierendorf, R.C., Pfeffer, D. (1987) Direct sequencing of denatured plasmid DNA. Methods in Enzymology, **152**, Guide to Molecular Cloning, p556-562.

Miller, H.J. (1972) Experiments in molecular genetics. Cold Spring Harbour, N.Y.

Moerman, D.G., Benian, G.M., Waterston, R.H. (1986) Molecular cloning of the muscle gene *unc*-22 in *Caenorhabditis elegans* by *Tc*1 transposon tagging. P.N.A.S., **83**, p2579-2583.

Moffat, B., Somerville, C.R. (1988) Positive selection for male-sterile mutants of *Arabidopsis* lacking adenine phosphoribosyl transferase activity. Plant Physiol., **86**, p1150-1154.

Munger, K., Lerch, K. (1985) Copper metallothionein from the fungus *Agaricus bisporus*: chemical and spectroscopic properties. Biochem., **24**, p6751-6756.

Munger, K., Germann, U.A., Lerch, K. (1987) The *Neurosporra crassa* metallothionein gene. J. Biol. Chem., **262**, p7363-7367.

Murasugi, A., Wada, C., Hayashi, Y. (1981) Cadmium-binding peptide induced in fission yeast, *Schizosaccharomyces pombe*. J. Biochem., **90**, p1561-1564.

Nacken, W.K.F., Piotrowiak, R., Saedler, H., Sommer, H. (1991) The transposable element *Tam1* from *Antirrhinum majus* shows structural homology to the maize transposon *En/Spm* and has no sequence specificity of insertion. M.G.G., **228**, p201-208.

Nam, H-G., Giraudot, J., den Boer, B., Moonan, F., Loos, W.D.B., Hauge, B.M., Goodman, H.M. (1989) R.F.L.P. linkage map of *Arabidopsis thaliana*. Plant Cell, **1**, p699-705.

Novick, E.M., Peterson, P.A. (1981) Transposition of the enhancer controlling element system in maize. M.G.G., **183**, p440-448.

Okumura, N., Nishizawa, N.-K., Umehara, Y., Mori, S. (1991) An iron deficiency-specific cDNA from barley roots having two homologous cysteine-rich MT domains. Plant Mol. Biol., **17**, p531-533.

Olafson, R.W., McCubbin, W.D., Kay, C.M. (1988) Primary- and secondary-structural analysis of a unique prokaryotic metallothionein from *Synechococcus* sp. cyanobacterium. Biochem., **251**, p691-699.

Palma, J.M., Sandalio, L.M., Del Rio, L.A. (1986) Manganese superoxide dismutase and higher plant chloroplasts : a reappraisal of a controverted cellular localisation. J. Plant Phys., **125**, p427-439.

Pan, T., Coleman, J.E. (1990) GAL4 transcription factor is not a "zinc finger" but forms a $Zn(II)_2Cys_6$ binuclear cluster. P.N.A.S., **87**, p2077-2081.

Paquin, C.E., Williamson, V.M. (1986) Ty insertions at two loci account for most of the spontaneous antimycin A resistance mutations during growth at 15°C of *Saccharomyces cerevisiae* strains lacking ADH1. Mol. Cell Biol., **6**, p70-79.

Parks, B.M., Shanklin, J., Koornneef, M., Kendrick, R.E., Quail, P.H. (1989) Immunochemically detectable phytochrome is present at normal levels but is photochemically nonfunctional in the *hy1* and *hy2* long hypocotyl mutants of *Arabidopsis*. Plant Mol. Biol., **12**, p425-437.

Patton, D.A., Franzmann, L.H., Meinke, D.W. (1991) Mapping genes essential for embryo development in *Arabidopsis thaliana*. M.G.G., **227**, p337-347.

Peterson, P.A. (1958) The effect of temperature on the mutation rate of a mutable locus in maize. Heredity, **44**, p121-124.

Piez, K.A. (1987) Collagen types: a review. In: Development and Diseases of Cartilage and Bone matrix, ed. K.A.Piez. New Series **46**, UCLA Symp. on Molecular and Cellular Biology. Alan R.Liss, Inc. p1-19.

Plazinski, J., Hurley, U.A., Williamson, R.E. (1991) Isolation of *Arabidopsis* myosin genes using the polymerase chain reaction. Abstracts of the Robertson Symposium on '*Arabidopsis thaliana* and the molecular basis of plant biology.' 30.9.91-1.10.91. Australian National University, Canberra.

Pruitt, R.E., Meyerowitz, E.M. (1986) Characterization of the genome of *Arabidopsis thaliana*. J. Mol. Biol., **187**, p169-183.

Reams, L.W., Gordon, M.P., Nester, E.W. (1983) Multiple mutations in the T region of the *Agrobacterium tumefaciens* tumour-inducing plasmid. P.N.A.S., **80**, p1660-1664.

Rédei, G.P. (1962) Single locus heterosis. Z. Vererbungsl., **93**, p164-170.

Rédei, G.P., Hirono, Y. (1964) Linkage studies. *Arab.* Inf. Serv., **1**, p9-10.

Reese, R.N., Wagner, G.J. (1987a) Effects of buthionine sulfoximine on Cd-binding peptide levels in suspension-cultured tobacco cells treated with Cd, Zn, or Cu. Plant Physiol., **84**, p574-577.

Reese, R.N., Wagner, G.J. (1987b) Properties of tobacco (*Nicotiana tabacum*) cadmium binding peptide(s). Biochem.J., **241**, p641-647.

Richards, R.I., Heguy, A., Karin, M. (1984) Structural and functional analysis of the human metallothionein-1_A gene: differential induction by metal ions and glucocorticoids. Cell, **37**, p263-272.

Rigby, P.W.J., Dieckman, M., Rhodes, C., Berg, P. (1977) Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol., **113**, p237-251.

Robbins, T.P., Carpenter, C., Coen, E.S. (1989) A chromosome rearrangement suggests that donor and recipient sites are associated during *Tam3* transposition in *Antirrhinum majus*. EMBO J., **8**, p5-13.

Robinson, N.J., Gupta, A., Fordham-Skelton, A.P., Croy, R.R.D., Whitton, B.A., Huckle, J.W. (1990) Prokaryotic metallothionein gene characterization and expression: chromosome crawling by ligation-mediated PCR. Proc. R. Soc. Lond., **B242**, p241-247.

Rogers, S., Horsch, R.B., Fraley, R.T. (1987a) Gene transfer in plants: production of transformed plants using Ti plasmid vectors. Methods Enz., **118**, p634-637.

Rogers, S., Klee, H., Horsch, R., Fraley, R., (1987b) Improved vectors for plant transformation: Expression cassette vectors and new selectable markers. Methods Enz., **118**, p627-633.

Roiha, H., Rubin, G.M., O'Hare, K. (1988) *P* element insertions and rearrangements at the *singed* locus of *Drosophila melangaster*. Genetics, **119**, p75-83.

Römheld, V. (1987) Different strategies for iron acquisition in higher plants. Physiol. Plant., **70**, p231-234.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, **239**, p487-491.

Salin, M.L. (1987) Toxic oxygen species and protective systems of the chloroplast. Phys. Plant., **72**, p681-689.

Salin, M.L., Bridges, S.M. (1980) Isolation and characterization of an iron-containing superoxide dismutase from a eukaryote, *Brassica campestris*. Arch. Biochem. Biophys., **201**, p369-374.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular Cloning: A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press.

Sand, S.A. (1957) Phenotypic variability and the influence of temperature on somatic instability in cultures derived between *Nicotiana langsdorffii* and *N.sanderae*. Genetics, **42**, p685-703.

Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G., Fraley, R.T. (1987) Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucl. Acid Res., **15**, p1543-1559.

Sandmann, G., Böger, P. (1983) The enzymological function of heavy metals and their role in electron transfer processes in plants. In : Encyclopedia of Plant Physiology, **15B**: Inorganic Plant Nutrition (eds A.Lauchli & R.L.Bieleski) p563-596. Springer-Verlag, Berlin.

Sarkar, G., Sommer, S.S. (1990) Shedding light on PCR contamination. Nature, **343**, p27.

Schmidt, R., Willmitzer, L. (1988) High efficiency *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* leaf and cotyledon explants. Plant Cell Rep., **7**, p583-586.

Schmidt, R., Willmitzer, L. (1989) The maize autonomous element *Activator (Ac)* shows a minimal germinal excision frequency of 0.2%-0.5% in transgenic *Arabidopsis thaliana* plants. M.G.G., **220**, p17-24.

Schwartz, D., Dennis, E. (1986) Transposase activity of the *Ac* controlling element in maize is regulated by its degree of methylation. M.G.G., **205**, p476-482.

Shaw, C.H., Watson, M.D., Carter, G.H., Shaw, C.H. (1984) The right hand copy of the nopaline Ti plasmid 25bp repeat is required for tumour formation. Nucl. Acid Res., **12**, p6031-6041.

Shaw III, C.F., Petering, D.H., Weber, D.N., Gingrich, D.J. (1989) Inorganic studies of the cadmium-binding peptides from *Euglena gracilis*. In: Winge, D., Hamer, D. (eds), Metal Ion Homeostasis, Alan R. Liss Inc., New York.

Sheikholeslam, S.N., Weeks, D.P. (1987) Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*. Plant Mol. Biol., **8**, p291-298.

Shirsat, A., Wilford, N.W., Evans, I.M., Gatehouse, L.N., Croy, R.R.D. (1991) Expression of a *Brassica napus* extensin gene in the vascular system of transgenic tobacco and rape plants. Plant Mol. Biol., **17**, p701-709.

Showalter, A.M., Bell, J.N., Cramer, C.L., Bailey, J.A., Varner, J.E., Lamb, C.J. (1985) Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection. P.N.A.S., **82**, p6551-6555.

Showalter, A.M., Zhou, J., Rumeau, D., Worst, S.G., Varner, J.E. (1991) Tomato extensin and extensin-like cDNAs: structure and expression in response to wounding. Plant Mol. Biol., **16**, p547-565.

Simpson, R., O'Hara, P., Lichtenstein, C., Montoya, A.L., Kwok, K., Gordon, M.P., Nester, E.W. (1982) The DNA from A6S/Z tumour contains scrambled Ti plasmid sequence near its junction with plant DNA. Cell, **29**, p1005-1014.

Smith, E.F., Townsend, C.O. (1907) A plant-tumour of bacterial origin. Science, **25**, p671-673.

Smith, J.J., Muldoon, E.P., Lamport, D.T.A. (1984) Isolation of extensin precursors by direct elution of intact tomato cell suspension cultures. Phytochem., **23**, p1233-1239.

Smith, J.J., Muldoon, E.P., Willard, J.J., Lamport, D.T.A. (1986) Tomato extensin precursors P1 and P2 are highly periodic structures. Phytochem., **25**, p1021-1030.

Somerville, C.R., Ogren, W.L. (1982) Isolation of photorespiration mutants in *Arabidopsis thaliana*. In: Methods in Chloroplast Molecular Biology. M.Edelman, R.B.Hallick, N.H.Chua, eds (New York: Elsevier Biomedical Press), p129-138.

Somerville, C.R., McCourt, P., Caspar, T., Estelle, M., Keith, K. (1985) *Arabidopsis thaliana* as a model system for plant genetics and molecular biology. Plant Genetics. Alan R Liss Inc. UCLA Symposium on Molecular and Cellular biology New Series, **35**, ed M Freeling. p651-660.

Sommer, H., Carpenter, R., Harrison, B.J., Saedler, H. (1985) The transposable element *Tam3* of *Antirrhinum majus* generates a novel type of sequence alterations upon excision. M.G.G., **199**, p225-231.

Sommer, H., Hehl, R., Krebbers, E., Piotrowiak, R., Lönnig, W-E., Saedler, H. (1988) Transposable elements of *Antirrhinum majus*. Plant Transposable Elements, Nelson, O. (ed) Plenum Press, N.Y. p227-235.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by electrophoresis. J. Mol. Biol., **98**, p503-517.

Stachel, S.E., Messens, E., Van Montagu, M., Zambryski, P. (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature, **318**, p624-629.

Stachel, S.E., Nester, E.W., Zambryski, P.C. (1986a) A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. P.N.A.S., **83**, p379-383.

Stachel, S.E., Timmerman, B., Zambryski, P. (1986b) Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature, **322**, p706-711.

Stafstrom, J.P., Staehelin, L.A. (1986) Cross-linking patterns in salt-extractable extensin from carrot cell walls. Plant Physiol., **81**, p234-241.

Stafstrom, J.P., Staehelin, L.A. (1988) Antibody localization of extensin in cell walls of carrot storage roots. Planta, **174**, p321-332.

Steffens, J.C. (1990) The heavy metal-binding peptides of plants. Ann. Rev. Plant Physiol., **41**, p553-575.

Stiefel, V., Ruiz-Avila, L., Raz, R., Vallés, M.P., Gómez, J., Pagés, M., Martinez-Izquerdo, J.A., Ludevid, M.D., Langdale, J.A., Nelson, T., Puigdomènech, P. (1990) Expression of a maize cell wall hydroxyproline-rich glycoprotein gene in early leaf and root vascular differentiation. Plant Cell, **2**, p785-793.

Streck, R.D., McGaffay, J.E., Beckendorf, S.K. (1986) The structure of *hobo* transposable elements and their insertion sites. EMBO J., **5**, p3615-3623.

Thumann, J., Grill, E., Winnacker, E.-L., Zenk, M.H. (1991) Reactivation of metal-requiring apoenzymes by phytochelatin-metal complexes. FEBS Letts., **284**, p66-69.

Tommey, A.M., Shi, J., Lindsay, W.P., Urwin, P.E., Robinson, N.J. (1991) Expression of the pea gene $PsMT_A$ in *E.Coli*: Metal-binding properties of the expressed protein. FEBS Letts., **292**, p48-52.

Trisno, G., Burke, P.V. (1987) Mutants of *Arabidopsis thaliana* with altered geotropism. Genetics, **116**, s2.

Tsujikawa, K., Imai, T., Kakutani, M., Kayamori, Y., Mimura, T., Otaki, N., Kimura, M., Fukuyama, R., Shimizu, N. (1991) Localization of metallothionein in nuclei of growing primary cultured adult rat hepatocytes. FEBS Letts., **283**, p239-242.

Udom, A.O., Brady, F.O. (1980) Reactivation *in vitro* of zinc-requiring apo-enzymes by rat liver zinc-thionein. Biochem.J., **187**, p329-335.

Upadhyaya, K.C., Sommer, H., Krebbers, E., Saedler, H. (1985) The paramutagenic line *niv*-44 has a 5kb insert, *Tam2*, in the chalcone synthase gene of *Antirrhinum majus*. M.G.G., **199**, p201-207.

Valvekens, D., Van Montagu, M., Van Lijsebettens, M. (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. P.N.A.S., **85**, p5536-5540.

Valvekens, D., Van Montagu, M. (1990) Spontaneous mutagenesis associated with *Arabidopsis* root regeneration. Fourth International Conference on *Arabidopsis* Research. Abstracts p46.

Van Assche, F., Clijsters, H. (1990) Effects of metals on enzyme activity in plants. Plant, Cell and Env., **13**, p195-206.

Van Etten, C.H., Miller, R.W., Earle, F.R., Wolff, I.A., Jones, Q. (1961) Hydroxyproline content of seed meals and distribution of the amino acid in the kernal, seed coat and pericarp. J.Agric. Food Chem., **9**, p433-435.

Van Haaren, M.J., Sedee, N.J.A., Schilperoort, R.A., Hooykaas, P.J.J. (1987) Overdrive is a T-region transfer enhancer which stimulates T-strand production in *A.tumefaciens*. Nucl. Acid Res., **15**, p8983-8997.

Van Holst, G.J., Varner, J.E. (1984) Reinforced polyproline II conformation in a hydroxyproline-rich cell wall glycoprotein from carrot root. Plant Physiol., **74**, p247-251.

Van Sluys, M.A., Tempé, J., Federoff, N. (1987) Studies on the introduction and mobility of the maize *Activator* element in *Arabidopsis thaliana* and *Daucus carota*. EMBO J., **6**, p3881-3889.

Van Tunen, A.J., Hartman, S.A., Mur, L.A., Mol, J.N. (1989) Regulation of chalcone flavanone isomerase (CHI) gene-expression in *Petunia hybrida*: the use of alternative promoters in corolla, anthers and pollen. Plant Mol. Biol., **12**, p539-551.

Veluthambi, K., Jayaswal, R.K., Gelvin, S.B. (1987) Virulence genes A, G and D mediate the double-stranded border cleavage of T-DNA from the *Agrobacterium* Ti plasmid. P.N.A.S., **84**, p1881-1885.

Vervliet, G., Holsters, M., Teuchy, H., Van Montagu, M., Schell, J. (1975) Characterization of different plaque-forming and defective phages in *Agrobacterium* strains. J. Gen. Virol., **26**, p33-48.

Vodkin, L.O., Rhodes, R.R., Goldberg, R.B. (1983) A cA lectin gene insertion has a structural feature of a transposable element. Cell, **34**, p1027-1031.

Wallroth, M., Gerats, A.G.M., Roger, S.G., Fraley, R.T., Horsch, R.B. (1986) Chromosomal localization of foreign genes in *Petunia hybrida*. M.G.G., **202**, p6-15.

Ward, E.R., Jen, G.C. (1990) Isolation of single-copy-sequence clones from a yeast artificial chromosome library of randomnly sheared *Arabidopsis thaliana* DNA. Plant Mol. Biol., **14**, p561-568.

Whitmore, F.W. (1978) Lignin-protein complex catalysed by peroxidase. Plant Science Lett., **13**, p241-245.

Whyte, R.O. (1946) Crop Production and Environment. (London: Faber and Faber).

Wilson, Z.A., Dawson, J., Aarts, M.G.M., Anthony, P., Briarty, L.G., Mulligan, B.J. (1991) Genetic male sterility in *Arabidopsis thaliana*. Abstracts of the Robertson Symposium on '*Arabidopsis thaliana* and the molecular basis of plant biology'. 30.9.91-1.10.91. Australian National University, Canberra.

Wycoff, K.L., Powell, P.A., Dixon, R.A., Lamb, C.J. (1990) Tissue-specific and wound responsive expression of a hydroxyproline-rich glycoprotein gene promoter in transgenic tobacco. J. Cell Biochem. Suppl., **14E**, p344.

Yamamoto, A., Iwahashi, M., Yanofsky, m.f., Nester, E.W., Takebe, I., Machida, Y. (1987) The promoter proximal region in the *vir*D locus of *Agrobacterium tumefaciens* is necessary for the plant-inducible circularization of T-DNA. M.G.G., **206**, p174-177.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. Nature, **346**, p35-39.

Ye, Z.-H., Varner, J.E. (1991) Tissue-specific expression of cell wall proteins in developing soybean tissues. Plant Cell, **3**, p23-37.

Yoder, J.I., Palys, J., Alpert, K., Lassner, M. (1988) *Ac* transposition in transgenic tomato plants. M.G.G., **213**, p291-296.

Zaenen, I., Van Laerbeke, N., Teuchy, H., Van Montagu, M., Schell, J. (1974) Supercoiled circular DNA in crown gall inducing *Agrobacterium* strains. J. Mol. Biol., **86**, p109-127.

Zambryski, P., Tempe, J., Schell., J.(1989) Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants (review). Cell, **56**, p193-201.

Zeng, J., Heuchel, R., Schaffner, W., Kägi, J.H.R. (1991) Thionein (apometallothionein) can modulate DNA binding and transcription activation by zinc finger containing factor Spl. FEBS Letts., **279**, p310-312.

Zhang, H., Hanley, S., Goodman, H.M. (1991) Isolation, characterization , and chromosomal location of a new *cab* gene from *Arabidopsis thaliana*. Plant Physiol., **96**, p1387-1388.

Zhou, J.H., Atherly, A.G. (1990) *In situ* detection of transposition of the maize controlling element (*Ac*) in transgenic soybean tissues. Plant Cell Rep., **8**, p542-545.