Novel transposon tagging and the expression of potentially lethal constructs in *Arabidopsis thaliana*.

Thesis submitted in accordance with the requirements of

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THE UNIVERSITY *of* LIVERPOOL

1.8 Project aims

The aims of the project are therefore:

1. to use ethanol inducible transposase expression, to control and optimise the timing of transposition, for transposon tagging in *Arabidopsis thaliana*.

2. to create constitutive and ethanol inducible, gain-of-function mutations, linked to a transposon tag.

3. to use the ethanol inducible *alc* switch for the expression of potentially lethal constructs and the recovery of potentially lethal phenotypes.

Chapter 2

Materials and Methods

2.1 Microbiology

2.1.1 *E. coli* Strain

All transformations and plasmid recoveries were performed using *E. coli* strain XL1-Blue:

SupE44hsdR17recA1endA1gyrA46thirelA1lac$^-$
F'[*proAB*$^+$ *lacI*^{q}*lacZ*ΔM15Tn*10*(*tet*^{r})]

2.1.2 Growth of *E. coli*

Liquid cultures were grown in LB/TB at 37°C in a shaking incubator (250 rpm). LA plates were incubated overnight at 37°C. LA and LB media was kindly prepared by Ruth Gallagher and Liz Ward. All other media was made as described in Sambrook *et al.*, (1989).

2.1.3 Electroporation Competent *E. coli* cells

300 ml LB was inoculated using a 5 ml LB overnight culture. Cells were grown to mid log phase (OD_{550} = 0.6) before harvesting by spinning (3500 rpm 15 min) in a Sorvall® RC 5B centrifuge. Cells were washed 3-4 times in cold sterile water before re-suspending in 1/1000 volume 10% glycerol and freezing in 20-40µl aliquotes in liquid nitrogen for storage at –80°C.

2.1.4 Electroporation Transformation procedure

Electrocompetent cells were thawed on ice for 30 min. DNA was dialysed for 30 min against water using Millipore® 13 mm nitrocellulose 0.025 µm filter discs. The dialysed DNA was added to the cells and the mixture placed in a pre-cooled electroporation cuvette. The mixture was electroporated using a 2mm cuvette (field strength 2.5 kV, capacitance 25 µF, resistance 200 ohms). Cells were suspended in 1 ml TB/LB and incubated at 37°C for 30 min/1 hr before plating on selective LA and incubating at 37°C overnight.

2.1.5 Heat-Shock Competent *E. coli* cells

Competent cells for transformation by heat-shock were prepared using a protocol modified from Hanahan (1983). 5 ml overnight cultures were used to inoculate 100 ml LB. This was placed in a shaking incubator at 37°C (200 rpm) and grown to mid log phase (OD_{550} = 0.6). Cells were pelleted by spinning (10 min 3000 rpm) in a Jouan MR 1822 centrifuge at 4°C. The pellets were resuspended in 7.5 ml TFB buffer 1 and placed on ice for 10 min. Cells were again pelleted by spinning (10 min 3000 rpm). The pellet was resuspended in 4 ml TFB buffer 2 before aliquoting in 200 µl lots and freezing in liquid nitrogen for storage at –80°C.

TFB Buffer 1:
100 mM RbCl
45 mM $MnCl_2$
35 mM K-Acetate
10 mM $CaCl_2$
0.5 mM LiCl
15% Glycerol
pH 5.8 with 0.2 M Acetic acid

Sterilise by filtration

TFB Buffer 2:
10 mM MOPS
75 mM $CaCl_2$
10 mM RbCl
15% Glycerol

pH 7.0 with 0.1 M NaOH

Sterilise by filtration

2.1.6 Heat Shock Transformation procedure

Heat shock competent cells were thawed on ice before addition of DNA. The mixture was left on ice for 30 min before placing at 42°C for 90 seconds. Cells were placed back on ice for two minutes before adding 1 ml LB/TB and incubating at 37°C for 45 mins–1 hr. Cells were plated out on selective LA and incubated at 37°C overnight.

2.1.7 *Agrobacterium tumefaciens* strain

GV2260 C58C1Rif^R (pGV2260) (Deblaere *et al.*, 1985)

2.1.8 Growth of *A. tumefaciens*

Liquid cultures were grown in 2x YT (Sambrook *et al.*, 1989) at 28°C in a shaking incubator (200 rpm). LA plates were grown for 2-5 days at 28°C.

2.1.9 Electroporation competent *A. tumefaciens* cells

Electrocompetent *A. tumefaciens* were generated by a modified version of a protocol supplied by Fran Robson (personal communication). Cultures were grown in 2x YT at 28°C for 24-36 hours in a shaking incubator (200 rpm) until the OD_{600} was approximately 0.6. Cells were cooled on ice for 15-20 mins before spinning (10 min 3500 rpm) at 4°C in a Jouan MR 1822 centrifuge. The cells were washed in 1, 0.5, and 0.02 volumes of cold 10% glycerol spinning each time as above, before finally re-suspending in 0.01 volume 10% glycerol and freezing in liquid nitrogen in 40 µl aliquotes for storage at –80°C.

2.1.10 Electroporation transformation procedure for *A. tumefaciens*

Competent cells were thawed on ice. DNA was dialysed for 30 min against water using Millipore® 13 mm nitrocellulose 0.025 µm filter discs. The dialysed DNA was mixed with the cells and the mixture placed into a pre-cooled 2 mm electroporation cuvette. Electroporation was carried out with a field strength 12.5 kV/cm, capacitance 25 µF, resistance 200 ohms and pulse length of around 9 secs. 1 ml YT was added and the cells were incubated at 28°C for 3 hrs. Cells were plated on selective LA and incubated at 28°C for 3 days.

2.1.11 Antibiotic selection of transformed colonies

Antibiotics were made up and used as described in the following table. Water soluble antibiotics were filter sterilised and all were stored at –20°C. Antibiotics were added to LA after cooling to around 50°C.

Antibiotic	Stock	Solvent	Working conc.
Ampicillin	50 mg/ml	H_2O	50 mg/l
Kanamycin	50 mg/ml	H_2O	50 mg/l
Rifampicin	100 mg/ml	70% methanol	100 mg/l
Tetracycline	10 mg/ml	70% ethanol	10 mg/l

2.1.12 Blue/White selection
Blue / white selection was carried out as described in Sambrook *et al*., (1989).

2.1.13 Colony Screening
Colony screens were produced using NEN® Colony/Plaque Screen™ hybridisation transfer membranes. Colony growth and transfer was performed as described by the manufacturer.

2.1.14 Radio-labelling DNA probes using random oligonucleotide primers
Radio-labelled probes were generated using the Random Primed DNA Labeling Kit produced by Roche. Probes were made using the standard assay as described in manufacturers instructions.

2.1.15 Diagnosis of *Agrobacterium* transformants by PCR
0.1 ml of cell suspension was pelletted by spinning (8000 rpm 5 min) in a benchtop centrifuge. The pellet was re-suspended in 20 µl of water and boiled for 10 min. Cellular debris was pelletted by spinning at 12,000 rpm for 5 min and 2 µl of supernatant was taken and used as a template for PCR.

2.2 Molecular Biology

2.2.1 Plasmid DNA mini/midi preps
Plasmid DNA was recovered using Qiagen® spin plasmid mini/plasmid midi kits. All plasmid preps were done according to manufacturers instructions.

2.2.2 Digestion of plasmid DNA
Restriction digestion was performed using enzymes from a variety of manufacturers and was carried out according to manufacturers instructions for the enzyme. Buffers were used as supplied where possible but some double digests required use of alternate buffer. In these instances enzyme activity information was used from Stratagene and Life Sciences International catalogues to optimise the buffer concentrations.

2.2.3 Partial digestion of plasmid DNA
Partial digestion was carried out by producing a set of serial dilutions of enzyme relative to the DNA concentration. In general this involved dilutions of 0.5-10 units of enzyme for 1-2 µg of plasmid. Digests were usually carried out at 37°C for 30 min. This was optimised in each case by repeated attempts with results checked by agarose gel electrophoresis.

2.2.4 Dephosphorylation of DNA using shrimp alkaline phosphatase

Shrimp alkaline phosphatase was supplied by Amersham Life Sciences and used as described in the manufacturers instructions. Reactions were carried out for 30 mins at 37°C and the enzyme was heat inactivated when required by heating at 65°C for 20 min.

2.2.5 Generation of blunt ends using Klenow enzyme

Klenow enzyme was supplied by Roche was used to generate blunt ends after restriction digestion. 3' overhangs were removed by the addition of enzyme and buffer as recommended by the manufacturer. For end-filing 5' overhangs dNTP's were added at 5 mM total concentration. Blunt ending reactions were carried out at room temperature for 30 min-1 hr.

2.2.6 Separation of fragments by agarose gel electrophoresis

Electrophoresis was carried out as described in Sambrook *et al.*, (1989). Both the running buffer and gel contained a final concentration of 1x TAE. Ethidium bromide was added to the gel before pouring at a final concentration of approximately 0.1 mg/l.

2.2.7 Molecular weight ladder

1 kb DNA Ladder (GibcoBRL) was used as a molecular weight standard during gel electrophoresis. The fragment sizes in base pairs are as follows:
12,216, 11,189, 10,180, 9,162, 8,144, 7,126, 6,108, 5,090, 4,072, 3,054, 2,036, 1,636, 1,018, (517, 506), 396, 344, 298, (220, 201, 154, 134, 75)

2.2.8 Extraction of fragments from agarose gel

Gel extraction was done using Qiagen® Qiaquick gel extraction kit as described in the manufacturers instructions. Fragments were generally eluted in pre-warmed elution buffer at approximately 50°C.

2.2.9 In gel reactions

In gel reactions were carried out after electrophoresis using Nuseive® GTG® agarose (FMC BioProducts). Reactions were carried out using protocols from the manufacturers instructions (FMC BioProducts Sourcebook).

2.2.10 TA Cloning

TA cloning was carried out using the Promega pGEM®-T Easy Vector System 1 and reactions were carried out according to the manufacturers instructions.

2.2.11 Ligation of fragments using T4 DNA Ligase

T4 DNA ligase was used as described in the manufacturers instructions. Ligations were typically carried out at either 37°C, 23°C or 8°C over time periods of 1 hr-7 days. For ligations lasting longer than this more ligase was added to the reactions after around 7 days.

2.2.12 PCR

PCR was carried out using *Taq* polymerase as described in the manufacturers instructions. Proofreading PCR was carried out using the EXPAND™ high fidelity PCR system from Roche, again according to manufacturers instructions. Primer annealing temperatures were estimated by using the formula 2(A+T) + 4(G+C) –5 and optimised by trial and error and by use of a gradient block.

2.2.13 DNA Sequencing

Sequence analysis was carried out using both Lycor and ABI sequencers by Angela Rosin.

2.3 Plant Growth, transformation and analysis

2.3.1 *Arabidopsis* cultivar

The *Arabidopsis thaliana* ecotype Columbia was used throughout the project.

2.3.2 Plant growth room conditions

Plants were grown under banks of fluorescent strip lights containing a 1:1 mixture of Phillips Aquarelle and Fluotone bulbs. A cycle of 16 hours light / 8 hours dark was used for experimental growth and 24 hr light was used for selection using harvest and for growing plants for transformation. The maximum temperature of the rooms during hours of light was 22°C.

2.3.3 Soil mixture

Plants were grown in a mixture (3:3:1 ratio) of John Innes No.3, Levington MH, and coarse pearlite. This was treated with Intercept insecticide (Levington Horticulture Ltd.) at 0.02 g/l compost before or during seed vernalisation.

2.3.4 Stratification of seed

Seed was sown onto wet soil and placed at 8°C in the dark, covered with a propagator lid for 3-7 days before placing in the growth room. Propagator lids were left on for 4-6 days to keep the humidity high and encourage germination. After this time the vents were opened for 1-2 days before removal of lids.

2.3.5 *Arabidopsis* transformation by floral dip

Transformation was performed using a protocol modified from (Clough and Bent, 1998; modified from Bechtold *et al*., 1993). Approximately 5-10 plants per pot were grown for 3-4 weeks on soil under growth room conditions. Primary bolts were removed to stimulate secondary bolting. Plants were first transformed around 4-5 days later when secondary bolts were 4-10 cm in length. 300 ml cultures of *A. tumefaciens* were grown for 24-36 hrs at 28°C (200 rpm) in YT containing tetracycline (10 μg/ml), kanamycin (50 μg/ml), and rifampicin (100 μg/ml). Cells were pelleted by spinning (3500 rpm 10 min) in a Sorvall® RC 5B centrifuge. The pellet was resuspended in 5% sucrose, 0.1% MES, (pH 5.7) before addition of 0.05% Silwet L-77. Whole plants were dipped in the *Agrobacterium* suspension for between 5-45 seconds before watering and placing back in the growth room covered by propagator lids to retain humidity. Dipping was repeated 2-3 times at 5-7 day intervals. Plants were grown to maturity as normal and once the initial siliques began to dry, watering was stopped and plants were removed from their pots for drying and seed collection. Seed was harvested from dried plants, left to further dry for at least 7-10 days and sown for selection of putative transformants.

2.3.6 Collection of seed

Plants were grown until the florescences had all begun to form siliques. This was either done in groups or individually, separated by using Aracon tubes (supplied by Lehle Seeds). Once the earliest siliques began to dry, plants were up-rooted and laid on their side on Whatman® 3MM paper to aid drying. Seed was collected once all the siliques had dried. Shattering was aided by hand and debris was removed by sieving. Seed was stored initially in plastic tubes and later in paper bags.

2.3.7 Selection of transformants using Harvest™

Plants were sprayed after around 10 days growth whilst still at the two leaf stage. The glufosinate-ammonium herbicide Harvest™ (Aventis CropScience) was used at a working concentration of 75 g/l. Spraying was done by hand and plants were sprayed until run-off. Plants were grown under growth room conditions and re-sprayed up to two times after 6-9 day intervals. Healthy green plants were transplanted and grown without further selection to maturity for either experimentation or seed collection. Harvest™ selection was carried out under 24 hour light conditions.

2.3.8 Recipe for Lehle tissue culture media

1 M KNO_3	5	ml/l	
1 M KH_2PO_4	2.5	ml/l	
1 M $MgSO_4$	2	ml/l	
1 M $Ca(NO_3)_2$	2	ml/l	
Sequestrene 1:8:1	2.5	ml/l	(2.5g $FeSO_4$.7(H20) in 400 ml H_2O, add 3.36 g NaEDTA. Bring to the boil then stir for 30 min whilst cooling. Make up to 450ml.)
25x Micronutrient mix	1	ml/l	70 mM H_3BO_3 14 mM $MnCl_2$ 0.5 mM $CuSO_4$ 1 mM $ZnSO_4$ 0.2 mM $NaMoO_4$ 10 mM NaCl 0.01 mM $CoCl_2$
Agarose	8	g/l	

2.3.9 Recipe for GM tissue culture media

MS Basal Salt Micronutrient	1 x
MS Basal Salt Macronutrient	1 x
B5 Vitamins	1 x
Sucrose	20 g/l
Bacto Agar	8 g/l

MS media and B5 vitamins were obtained as 10x concentrate from SIGMA Cell Culture™.

2.3.10 Tissue culture antibiotic selection

Streptomycin was made up as 200 mg/ml stock in water, sterilised by filtration, and used at 200 mg/l. Streptomycin selection plates contained 10 g/l glucose. Hygromycin was used at 35 mg/l diluted from liquid stock.

2.3.11 Sterilisation of seed for tissue culture

Seed was sterilised for 5 min in 70% ethanol, washed with sterile water, and soaked for 20 min in 10% (v/v) household bleach before washing 5-6 times in sterile water.

2.3.12 Vernalisation of seed for growth on tissue culture plates

Vernalisation of seed on tissue culture plates was carried out using a protocol supplied by Dr Mike Salter (personal communication). Seed was sterilised and sown on plates before placing in the dark at 6-8°C for 4 days. Plates were then exposed to light at room temperature for 4 hours, left in the dark at room temperature overnight and placed in growth room conditions.

2.3.13 Histochemical GUS Staining of whole plants / leaves

Histochemical GUS staining was performed using a protocol modified from Jefferson *et al*. (1987). Cuticles were removed by dipping briefly in chloroform for 4-6 seconds. The tissue was left to dry briefly before immersing in GUS staining buffer. Vacuum infiltration was used to increase the uptake of buffer. A vacuum was applied until the staining buffer started to boil then the pressure was allowed to increase before re-applying the vacuum 2-3 times. Tissue was left immersed in staining buffer, at 37°C overnight. Visualisation of staining was enhanced upon removal of chlorophyll by boiling for 5 min in 70% ethanol.

GUS staining Buffer:

50 mM	$NaPO_4$
1 mM	EDTA
1 mM	X-gluc

2.3.14 Quick plant DNA extraction for diagnostic PCR

The protocol for quick plant DNA extraction was supplied by John Doonan (via Mike Bevan, personal communication). Plant material was ground in Rapid Extraction Buffer (REB) before phenol:chloroform extraction. Nucleic acid was precipitated by the addition of 1/10 volume 3 M NaAc pH 5.2 and two volumes of EtOH. After precipitation by spinning (12,000 rpm 15 mins) in a microcentrifuge, the pellets were re-suspended in 0.2 ml water and approximately 1 µl was used as a template for PCR.

Rapid Extraction Buffer:

50 mM	Tris
25 mM	EDTA
250 mM	NaCl
0.5%	SDS

2.3.15 Whole plant RNA extraction

RNA extraction was performed using a modified protocol supplied by Dr KP Croft (personal communication). Plant tissue was ground in liquid nitrogen using a pestle and mortar. The frozen powder was placed in a 30 ml centrifuge tube and 10 ml 2x NETS (80°C) added followed immediately by 10 ml phenol (80°C). The mixture was vortexed thoroughly before releasing the pressure and placing on ice for 15-30 mins. Samples were spun at 4000 g for 10 min and the supernatant was removed and re-extracted using 5 ml room temperature phenol. The aqueous layer was recovered and nucleic acid was precipitated by the addition of 1/10 volume 3 M NaAc pH 5.2, and 2 volumes EtOH (–20°C). Samples were left at –20°C overnight before precipitation by centrifugation at 12,000 g for 15 mins. RNA was resuspended in DEPC treated H_2O.

2x NETS:
200 mM NaCl
2 mM EDTA
20 mM Tris
pH 7.5
1% (w/v) SDS

2.3.16 Northern analysis of whole plant RNA

Northern analysis was carried out using formaldehyde denaturing gels as described in Sambrook *et al*., (1989). RNA was transferred onto NEN Genescreen Plus® membrane as described in Sambrook *et al*., (1989) and the manufacturers instructions. The RNA was fixed to the membrane by UV-crosslinking and baking the membrane at 80°C for 45 mins. Radiolabeled probes were made by using the Random Primed DNA Labelling Kit (Roche) and hybridised following the membrane manufacturers instructions.

2.3.17 Visualisation of Northern analysis

Northern blots were visualised using a Molecular Dynamics Storm 860 Machine after exposure to Molecular Dynamics Storage Phosphor Screens. The results were analysed using Imagequant software.

2.3.18 Crossing plants

Plants were grown on soil for crossing. The plants were grown until primary bolts appeared and these were often removed to induce secondary bolting. Once the plants had developed 4-6 bolts and the first flowers had begun to open the plants were selected for crossing.

Initially 2-3 bolts were used per plant. The auxiliary buds were removed from these bolts but the leaves were left intact. Flowers that had already opened were removed from the cluster of buds at the apex of the bolt, along with any very young flower buds. Buds were chosen that were swollen but still contained immature stamens with greenish-yellow anthers. The sepals were opened using forceps and the anthers removed. Pollen was taken from the donor plant and placed on the end of the stigma. The bolts were marked with tape and the flowers were allowed to mature and form siliques. Once the siliques were mature the bolts were removed and allowed to dry for seed collection.

Chapter 3

Novel transposon tagging using the *alc* system

3.1 Introduction

Many different transposon tagging systems based on the *Ac*/*Ds* system from maize have previously been described (for review see Osborne and Baker, 1995; Sundareson, 1996; Maes *et al*., 1999). Novel genes have been identified by insertional mutagenesis, and novel expression patterns by promoter/enhancer trapping using elements carrying reporter constructs. Wilson *et al*. (1996) described a transposon tagging system designed to generate dominant gain-of-function mutations. The *Ds* transposon contained an outward facing *35S* promoter.

Outward expression after insertion of a transposon into or close to a gene can produce either sense, or antisense transcripts dependent upon the orientation and insertion site of the transposon. Increased or ectopic expression creates dominant or semi-dominant phenotypes. Use of an inducible promoter to express outward from a transposon would potentially create inducible dominant mutations. This could lead to the recovery of mutants whose phenotype would normally result in lethality. Insertional mutants would also be created by direct disruption of transcription.

Balcells *et al*. (1994) described a system where inducible transposition was controlled by expression of *Ac* transposase from a soybean heat shock promoter.

Transposase expression was induced in transformed plants after heat shock. Transposition was limited in this system to embryonic cells, partly due to the specificity of the heat shock promoter. The inducibility of the promoter allowed lower levels of transposase expression than using a constitutive *35S* promoter. This can result in increased re-insertion frequency. The use of an inducible promoter in this way also removes the need to cross with transposase expressing lines and allows reversion to be studied by re-mobilisation of the transposon. A promoter, which does not show the specificity for expression in embryonic cells, would further improve this system.

The aim of this set of experiments was to generate novel systems for transposon tagging in *Arabidopsis*. Both of the systems described utilise the *Ac/Ds* transposon system originally described by Barbara McClintock (McClintock, 1951) and the *alc* inducible promoter switch described by Caddick *et al*. (1998) and Salter *et al*. (1998). The transposable elements in both systems carry outward facing promoters. Both of the systems use inducible promoters to control expression of *Ac* transposase. This allows control of the timing of transposase expression and therefore optimisation of transposition.

The first system described uses the *alcA* promoter to control transposase expression. The transposable element contains a constitutive *35S* outfacing promoter. Any aberrant expression pattern created by expression outward from the site of insertion in this system would be constitutive.

The second system uses the *Gmhsp* 17.3-B soybean heat shock promoter to control transposase expression. The transposable element contains an *alcA* outfacing

promoter. Any aberrant expression pattern created by expression outward from the site of insertion in this system would be inducible. Plants showing mutant phenotypes created by this expression can potentially be identified and rescued for further study.

Building the two systems involved generating transposable elements and transposase constructs in suitable cloning vectors. Markers were required for both primary selection and to identify successful transposition events. The transposable elements contain a bar selectable marker which contains the *pat* gene from *S. hygroscopicus* (De Block *et al*., 1987) for selection with the herbicide glufosinate. This is used for both selection of primary transformants and removal of plants containing unsuccessful re-insertion events after transposition. The streptomycin phosphotransferase fusion developed by Jonathan Jones (Jones *et al*., 1989) was used to identify excision events. The transposon is cloned into the untranslated leader sequence between a *35S* constitutive promoter and a mutated version of the streptomycin phosphotransferase (SPT) gene from *E. coli* Tn*5*. Transposition results in the subsequent expression of SPT and is scored by resistance to streptomycin.

Coupland *et al*. (1988) found that only 238 bp of sequence at the 5' end and 209 bp at the 3' end of *Ac* are required for efficient transposition. It was decided to generate minimal transposon constructs containing only these ends of the *Ac* sequence. These were generated from a transposon template by PCR. The other parts of the system were generated from a series of plasmids as PCR or restriction fragments and cloning strategies are described fully in this chapter.

The pGreen / pSoup transformation system (Hellens *et al*., 2000) was used to

transform whole plants by a modified floral dip protocol (Clough and Bent, 1997). Figures 3.1 and 3.10 contain schematic diagrams summarising the cloning strategies used for the generation of each set of constructs.

Figure 3.1 Cloning strategy for the generation of system 1

Schematic diagram illustrating the cloning strategy described in the following chapter. The enzymes used for the generation of fragments and cloning are shown. Each step is labeled to correspond to the appropriate description in Chapter 3.2. Promoter elements shown in inverse type indicate right to left promotion of transcription. Many of the plasmids are described in further detail later in the chapter.

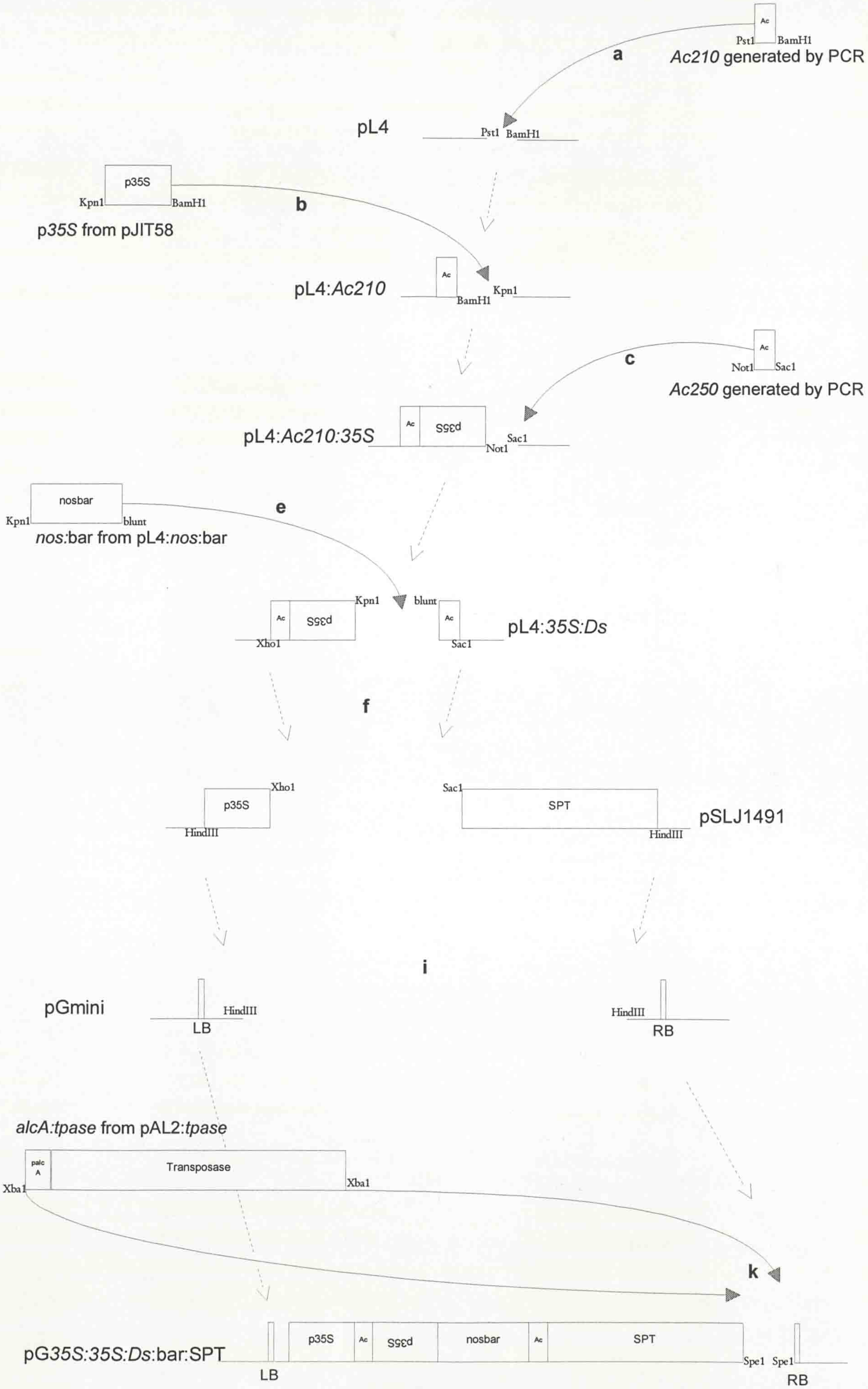
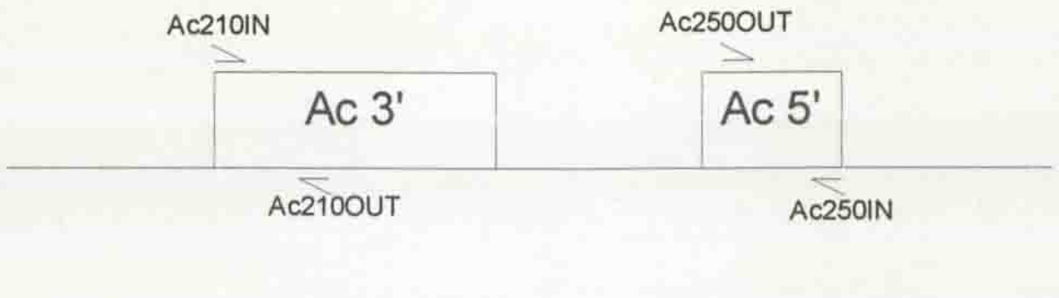

Ac
Pst1
BamH1
a
Ac210 generated by PCR
pL4
Pst1
BamH1
p35S
Kpn1
BamH1
b
p35S from pJIT58
Ac
pL4:Ac210
BamH1
Kpn1
c
Ac
Not1
Sac1
Ac250 generated by PCR
pL4:Ac210:35S
Ac
p35S
Not1
Sac1
nosbar
Kpn1
blunt
e
nos:bar from pL4:nos:bar
Ac
p35S
Xho1
Kpn1
blunt
Ac
Sac1
pL4:35S:Ds
f
p35S
Xho1
HindIII
Sac1
SPT
HindIII
pSLJ1491
i
pGmini
HindIII
LB
HindIII
RB
alcA:tpase from pAL2:tpase
palc A
Transposase
Xba1
Xba1
k
pG35S:35S:Ds:bar:SPT
LB
p35S
Ac
p35S
nosbar
Ac
SPT
Spe1
Spe1
RB

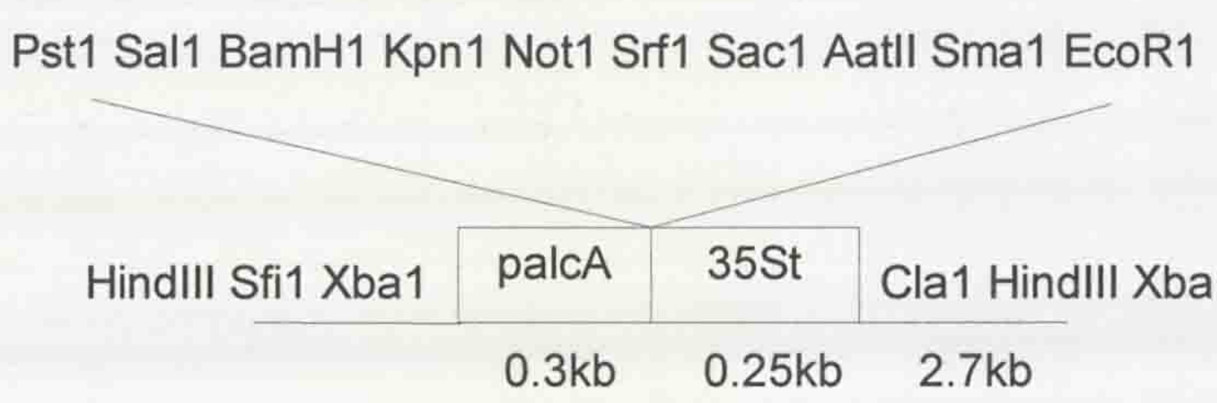
Figure 3.2 Plasmids used in cloning strategies

a.



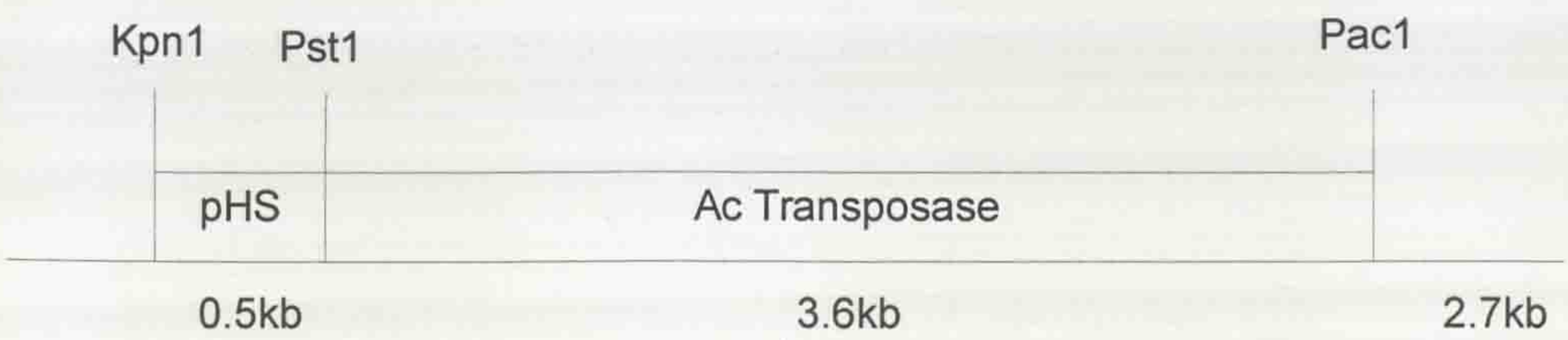
Schematic diagram of *Ds* transposon in pJJ43690. This was used as a template for PCR generation of *Ac* transposon ends in both strategies (Ac210/Ac250). The plasmid was kindly supplied by David Stevenson (John Innes Centre, Norwich).

b.



Schematic diagram of pL4 containing an *alcA* promoter (palcA), polycloning site, and a *CaMV35S* terminator (35St). The polycloning site was used as the basis of strategy 1. The plasmid is also the origin of pL4:bar (Figure 3.3b) and pAL2 (Figure 4.1).

c.



Schematic diagram of both the *Gmhsp* 17.3-B heat shock promoter (pHS) and *Ac* transposase in pACT6. The restriction sites used in the cloning strategies are indicated. The plasmid was kindly supplied by David Stevenson (John Innes Centre, Norwich).

3.2 System 1: *alcA* inducible transposition creating constitutively expressing insertional mutations

3.2a Generation of pL4::*Ac210*

Primers were designed to amplify the 5' end of the *Ds* transposon (Ac210in/Ac210out Appendix A). The ends of the primers contained *Pst*1 and *Bam*H1 restriction sites respectively. The 210 bp fragment was generated by proofreading PCR from pJJ43690 (Figure 3.2), digested with *Bam*H1/*Pst*1 and purified by gel extraction. pL4 (Figure 3.2) was linearised by digestion with *Bam*H1/*Pst*1. Linear plasmid was recovered by gel extraction and ligated with the PCR product for 24 hrs at 8°C. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pL4::*Ac210* by restriction analysis.

3.2b Generation of pL4:*Ac210::35S*

pL4:*Ac210* was linearised using *Kpn*1/*Bam*H1. The *35S* promoter was released as a *Kpn*1/*Bam*H1 fragment from plasmid pJIT58 (Guerineau *et al*., 1990). Both pL4:*Ac210* and the *35S* fragment were purified by gel extraction and ligated for 1 hour at 37°C. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pL4:*Ac210::35S* by restriction analysis.

3.2c Generation of pL4:*Ac210:35S::Ac250* (pL4::*35s:Ds*)

Primers were designed to amplify the 3' end of the *Ds* transposon (Ac250in/Ac250out Appendix A). The ends of the primers contained *Sac*1 and *Not*1

Abstract

The *alc* system is an ethanol responsive gene switch developed for the control of transgene expression in higher plants. The system originates from *Aspergillus nidulans* where it controls a pathway for sensing and utilising ethanol as a carbon source. The AlcR transcription factor regulates expression of the alcohol dehydrogenase 1 (*alcA*) gene via a number of AlcR specific binding sites situated in the *alcA* promoter. The system has been developed and characterised for use in tobacco, tomato, *Arabidopsis thaliana* and a number of crop species. The AlcR transcription factor is constitutively expressed and allows transgene expression downstream of an *alcA* promoter upon induction with ethanol.

Previously the system has been demonstrated to be functional in *Arabidopsis* where it exhibits the favourable characteristics of rapid switching, high levels of inducible expression and low levels of basal expression. The project described in this thesis involves using the *alc* system to analyse and improve the potential of generating, identifying, and recovering phenotypic mutants using both forward and reverse genetic approaches in *Arabidopsis*.

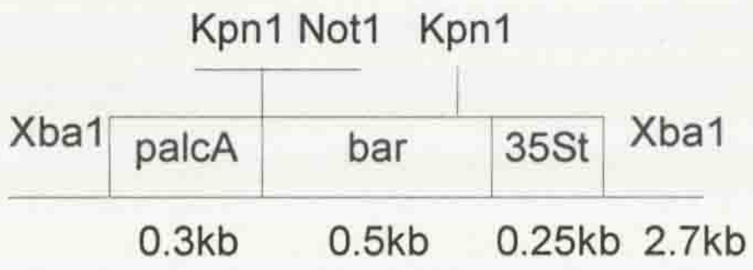
Transposon tagging systems were devised where transposition would be driven by induction of either the *alc* system or heat shock. These aimed to create gain-of-function mutations, after reinsertion of the transposon, as a consequence of aberrant expression from outward facing promoter constructs contained in the elements. In the first system, where transposition was induced by ethanol, the aberrant expression would be constitutive. In the second system, where transposition was induced by heat shock, outward expression would be controlled by the *alc* system.

Two genes, which encode the target enzymes of the herbicides glyphosate and the imidazolinones, were used in an attempt to analyse the potential of creating mutant phenotypes by aberrant expression, controlled by the *alc* system, and the potential of recovering mutants expressing lethal constructs. These experiments aimed to reduce the expression levels of the target genes by the production of aberrant antisense or partial sense transcripts.

The ethanol inducible expression of *alcA* constructs is reliant upon constitutive expression of the transcription factor AlcR. A homozygous transgenic line, constitutively expressing AlcR, that had previously been shown to exhibit ethanol inducible expression of *alcA* constructs by transient analysis, was used for the generation of all the experimental lines. Unfortunately, during the project, it was demonstrated that this line was prone to transgene silencing in later generations. Silencing resulted in the *alcR* transcript being reduced to undetectable levels. The silencing was not only targeted to the *alcR* construct, but also to the hygromycin selectable marker contained in the same T-DNA. The *alcA* constructs were therefore unable to exhibit ethanol inducible expression in either the transposon tagging or 'lethal' gene strategies. One abnormal phenotype was observed, after transforming the partial sense constructs into a second line of plants that were less prone to *alcR* silencing. However due to further silencing and a background phenotype, thought to be caused by the original T-DNA insertion, the mutant was difficult to characterise.

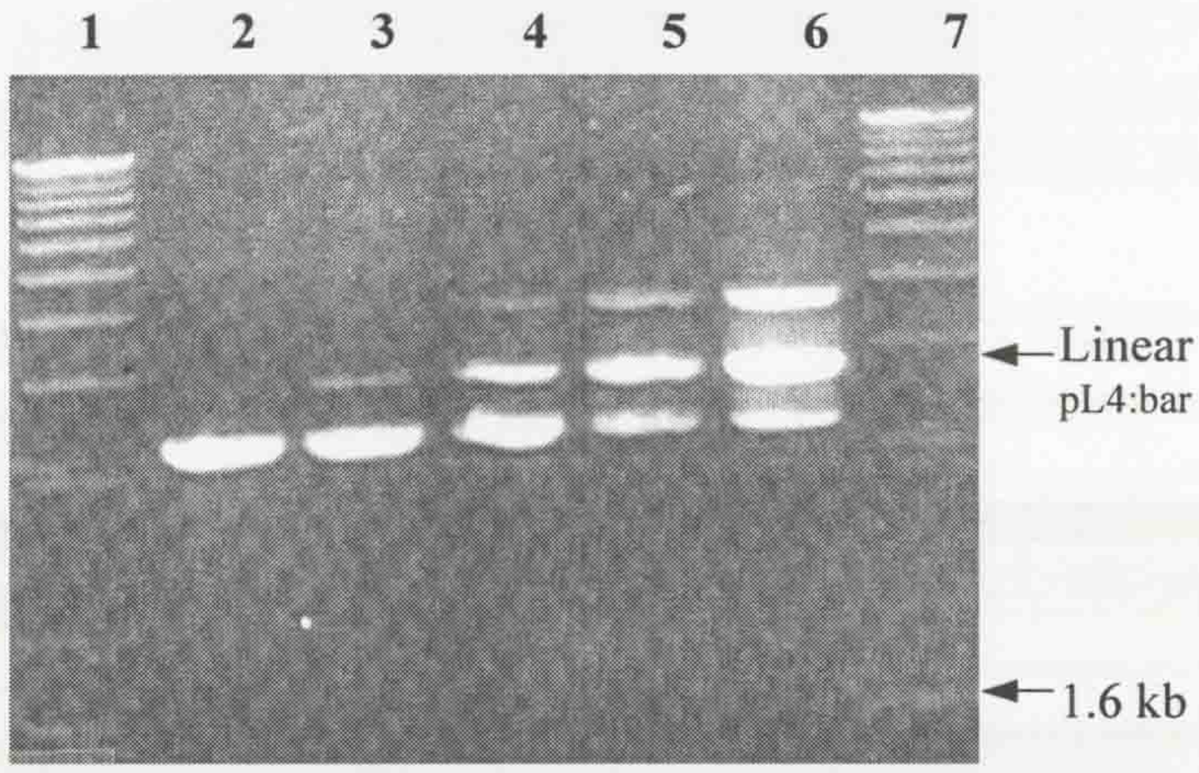
Figure 3.3 Generation of pL4::*nos*::bar

a.



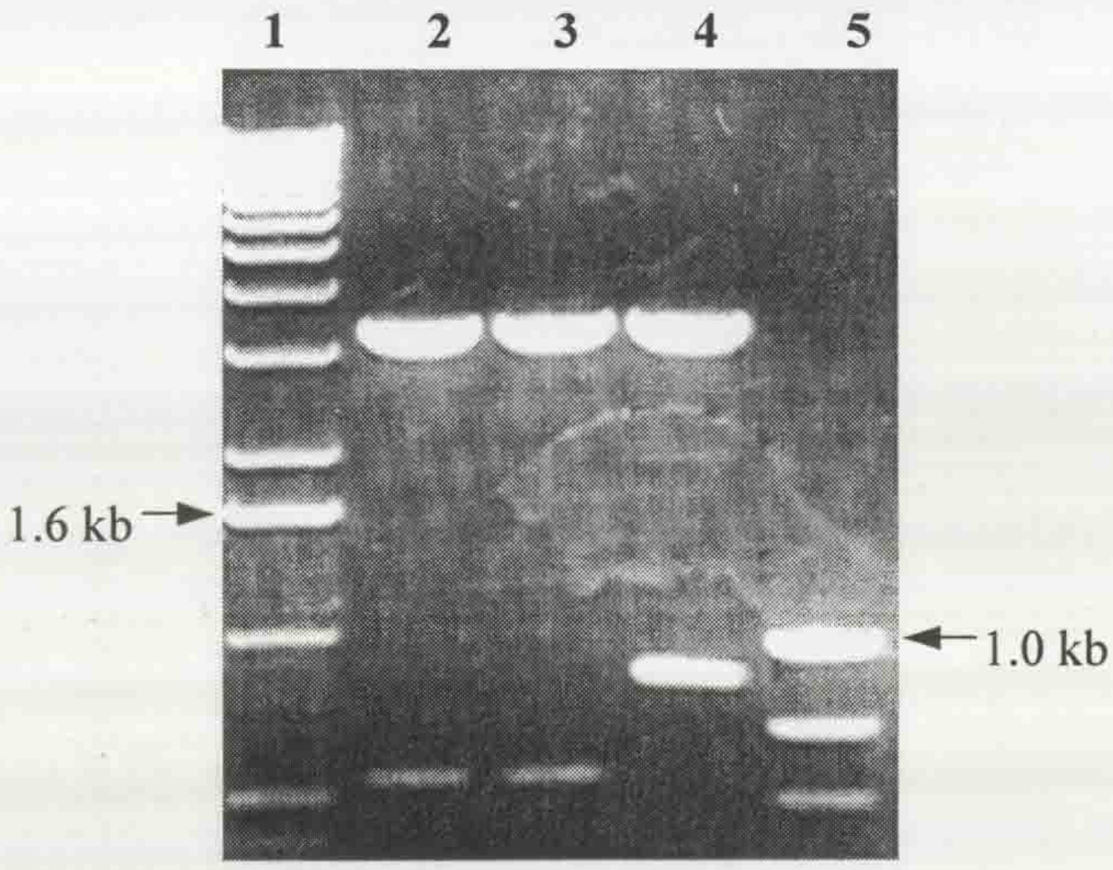
Schematic diagram of pL4: bar showing both the *Kpn*1 sites and the *Not*1 site.

b.



*Kpn*1 partial digest of pL4:bar. Lanes 1 and 7 contain ladder. Lanes 2-6 contain a series of digestions with decreasing enzyme concentration. An increase in the single cut 3.8 kb linear fragment and a decrease in the double cut 3.3 kb fragment can be seen as enzyme concentration decreases. The linear 3.8 kb band was extracted, re-digested using *Not*1 and the *nos* promoter cloned into the remaining 3.8 kb vector as a PCR fragment.

c.



Diagnostic digest of pL4::*nos*::bar. Lanes 1 and 5 contain ladder and molecular weight markers respectively and lanes 2-4 contain *Kpn*1 digested pL4::*nos*::bar. The increase in size of the 500 bp fragment to 800 bp in lane 4 indicates the cloning of the 300 bp *nos* promoter at the 5' end of the bar selectable marker.

restriction sites respectively. The 250 bp fragment was generated by proofreading PCR from pJJ43690, digested with *Not*1/*Sac*1 and purified by gel extraction. pL4:*Ac210:35S* was linearised by digestion with *Not*1/*Sac*1. Linear plasmid was recovered by gel extraction and ligated with the PCR product for 1 hour at 37°C. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pL4:*Ac210:35S::Ac250* (pL4::*35S:Ds*) by restriction analysis and PCR.

3.2d Generation of pL4*::nos::*bar

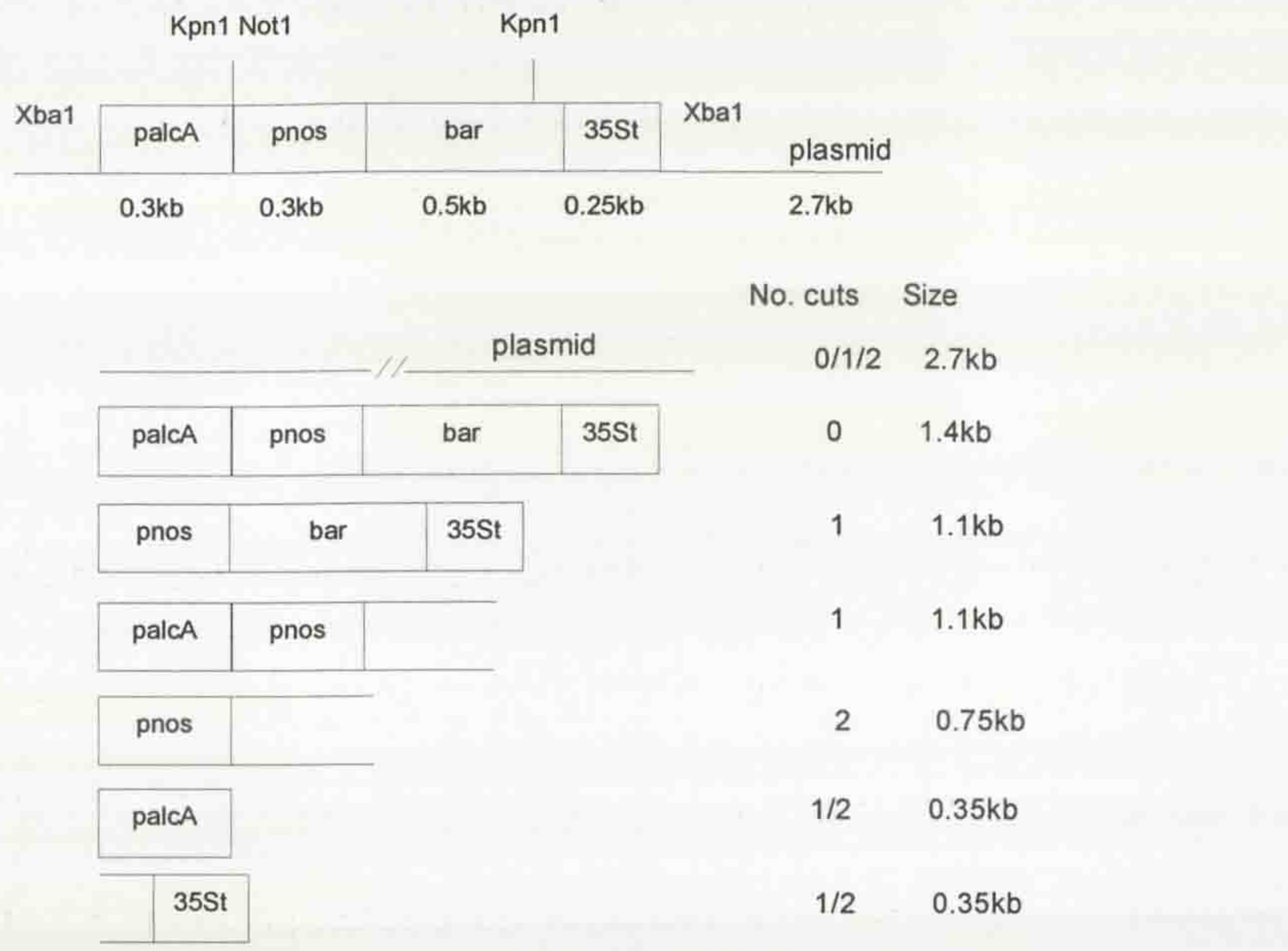
Primers were designed to amplify the *nos* promoter from pGreen 0029 (Hellens *et al*., 2000) (nosPfor/nosPrev2 Appendix A). The ends of the primers contained *Kpn*1/*Not*1 restriction sites respectively. The 300 bp fragment was generated by PCR, gel extracted and digested with *Kpn*1/*Not*1. pL4:bar (Roslan, 1999) was linearised by partial digestion with *Kpn*1 (Figure 3.3a/b). The linear plasmid was gel extracted and re-digested with *Not*1. Again the 3.8 kb linear plasmid was gel extracted and ligated with the *nos* promoter fragment at 8°C overnight. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pL4*::nos::*bar by restriction analysis (Figure 3.3c).

3.2e Generation of pL4:*35S:Ds::*bar

pL4*:35S:Ds* was linearised using *Not*1 and blunt ended using Klenow enzyme. The Klenow enzyme was heat-inactivated before re-digesting with *Kpn*1. pL4*:nos:*bar was digested with *Xba*1 in the presence of klenow enzyme and heat inactivated. A sample of this was checked to verify complete digestion and a partial digest was carried out using *Kpn*1. A fragment containing the *nos* promoter/*bar* coding

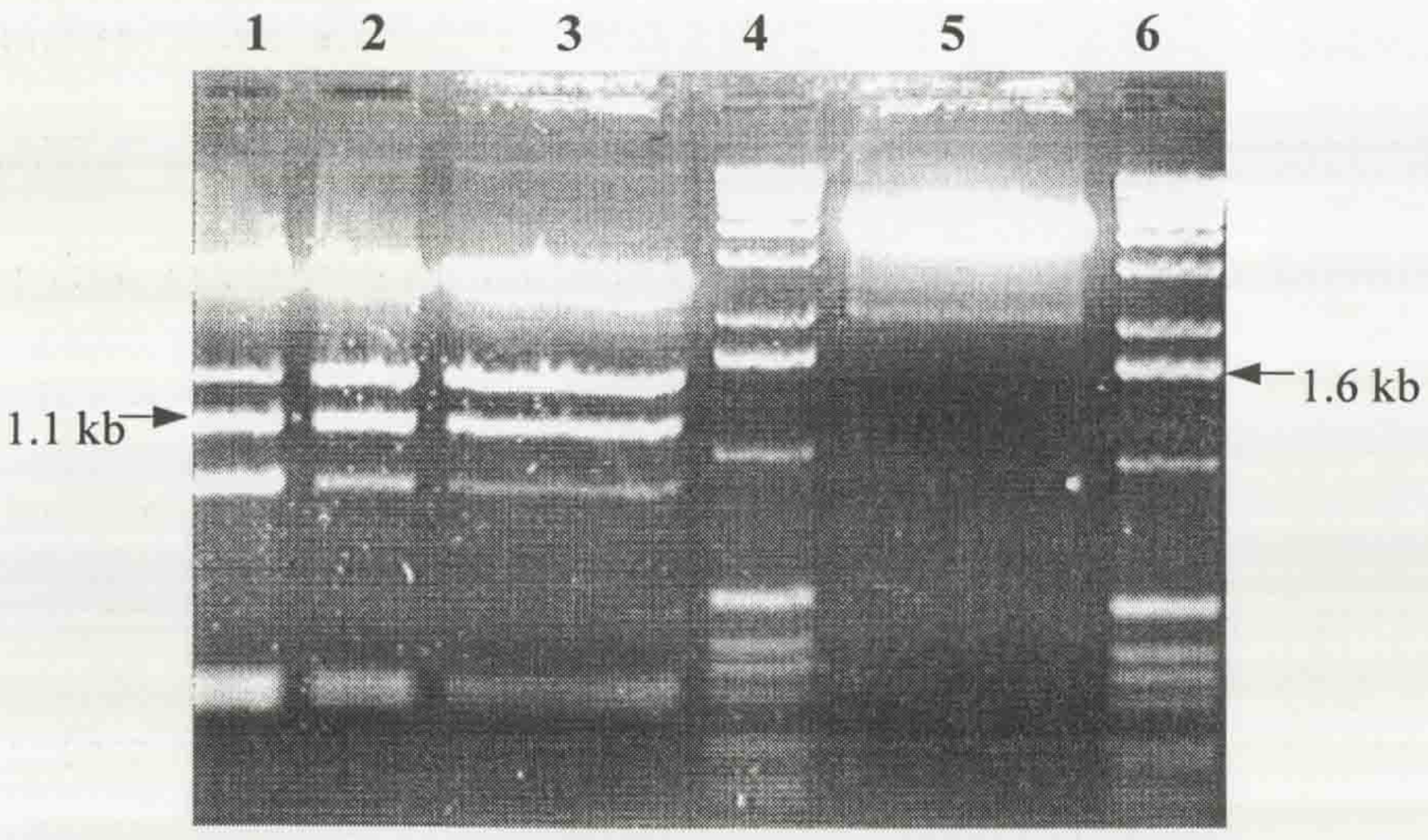
Figure 3.4 Generation of pL4:*35S:Ds*::bar

a.



Schematic diagram illustrating the various fragments seen after *Xba*1 digestion and *Kpn*1 partial digestion of pL4:*nos*:bar. There are two 1.1 kb fragments generated. Both of these were extracted together and can be seen in Figure 3.4b. Diagnostic digests were used to check the correct fragment was cloned.

b.



Restriction fragments for the generation of pL4:*35S*:*Ds*::bar. Lanes 1-3 contain pL4:*nos*:bar digested with *Xba*1 and blunt ended using Klenow enzyme before *Kpn*1 partial digestion. The concentration of *Kpn*1 decreases from lanes 1-3 resulting in an increase in the single cut 1.4 kb band and a decrease in the smaller (750 bp, 350 bp) bands resulting from multiple cuts. The 1.1 kb fragment was gel extracted and cloned into linear pL4:*35S*:*Ds* seen in lane 5. Lanes 4 and 6 contain ladder.

sequence/*35S* terminator was purified by gel extraction along with the linearised pL4*:35S:Ds* (Figure 3.4). Ligation was carried out over 3 days at 8°C. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pL4:*35S:Ds::*bar by restriction analysis (Figure 3.5).

3.2f Generation of pSLJ1491::*35S:Ds:*bar

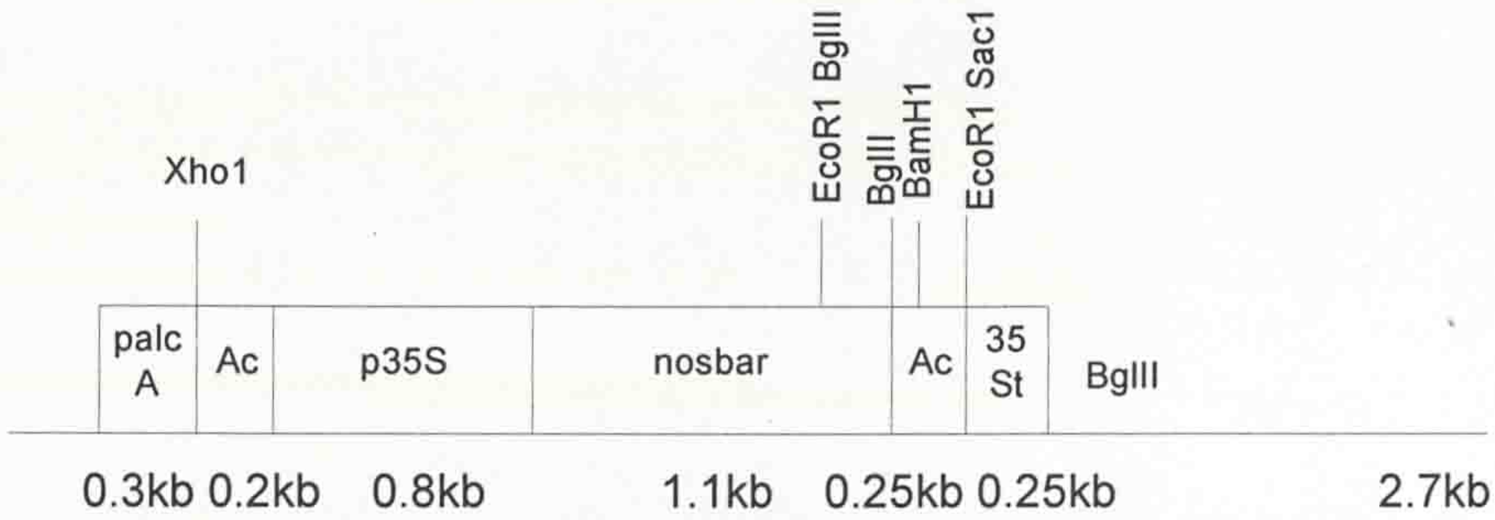
pSLJ1491 (Figure 3.6) was linearised by partial digestion with *Sac*1. The linear fragment was purified by gel extraction before digesting with *Xho*1. pL4:*35S:Ds*:bar was digested with *Sac*1/*Xho*1 and the *35S:Ds:*bar fragment purified, along with the linearised pSLJ1491 before ligating at 8°C for 48 hrs. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pSLJ1491::*35S:Ds:*bar by restriction analysis.

3.2g Generation of pG-/-*35S*:SPT

pG-/- (described later Chapter 4.2c and Figure 4.3) was linearised using *Hind*III and phosphatased to prevent re-ligation. The *35S*:SPT cassette was released as a *Hind*III fragment from pSLJ1491. The plasmid was cut into two 3.2 kb fragments so *Eco*R1 digestion was carried out to remove the *Hind*III site from the pUC119 fragment and prevent accidental cloning. The two 3.2 kb fragments of pSLJ1491 were purified along with the linear pG-/- by gel extraction and ligated overnight at 8°C. The ligation was transformed into *E. coli*, transformants were selected on LA(kan) and plasmid DNA was recovered. One sample was verified as pG-/-*35S*:SPT by restriction analysis.

Figure 3.5 Diagnostic digests of pL4:*35S:Ds*::bar

a.



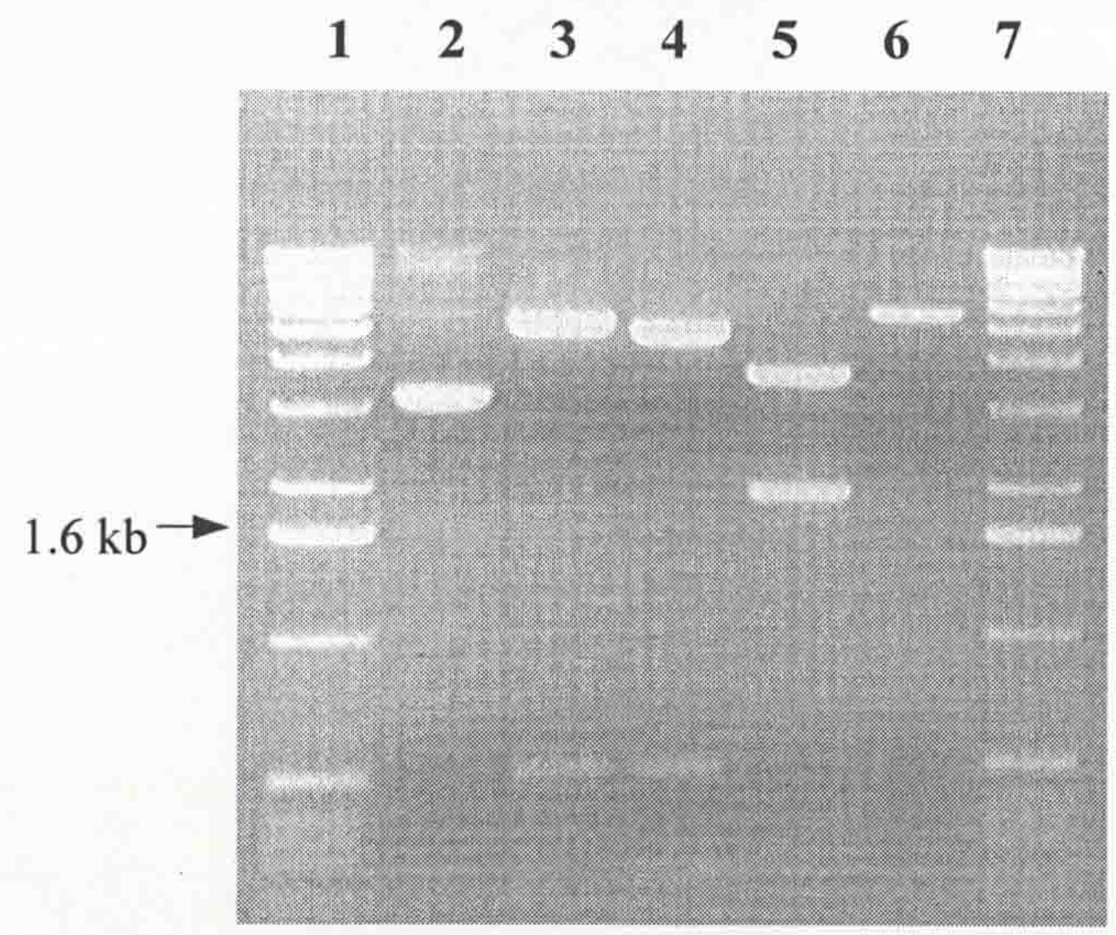
Schematic diagram of pL4:*35S*:*Ds*:bar. Restriction sites for the enzymes used in the diagnostic digests shown in Figure 3.5c are indicated.

b.

	***Eco*R1**	***Bgl*II**	***Bam*H1**	***Sac*1**
Size	5.0 kb 0.5 kb	4.9 kb 0.5 kb 0.1 kb	3.5 kb 2.0 kb	5.5 kb

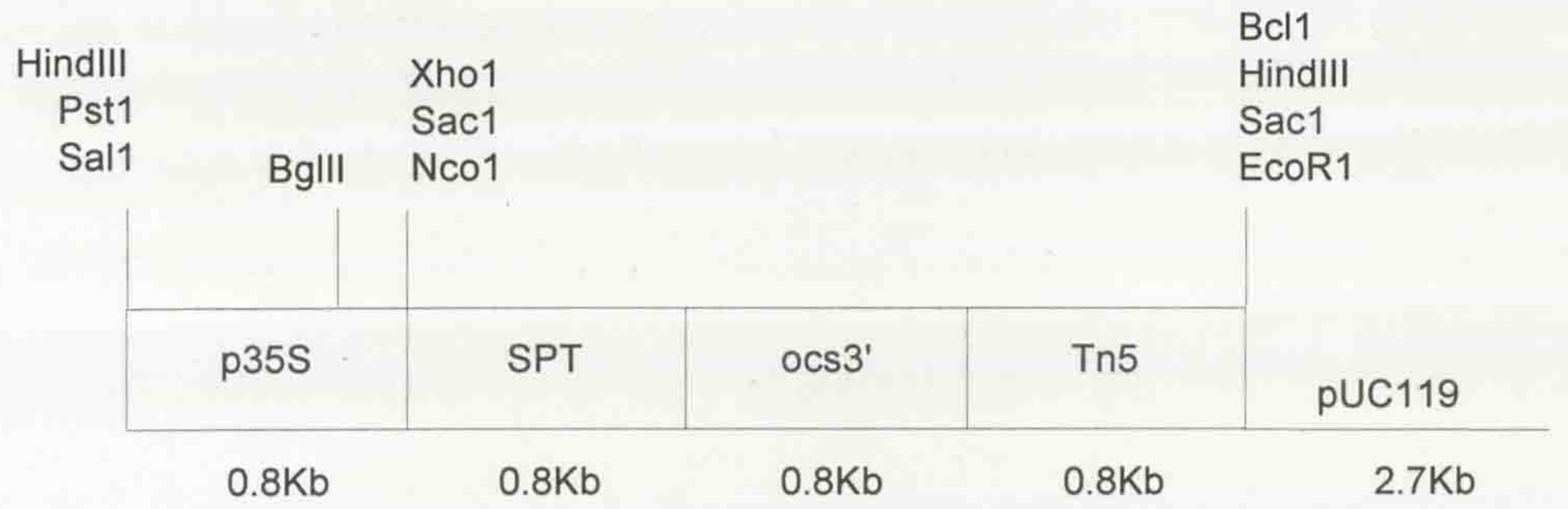
Expected fragment sizes generated by diagnostic digestion of pL4:*35S*:*Ds*:bar.

c.



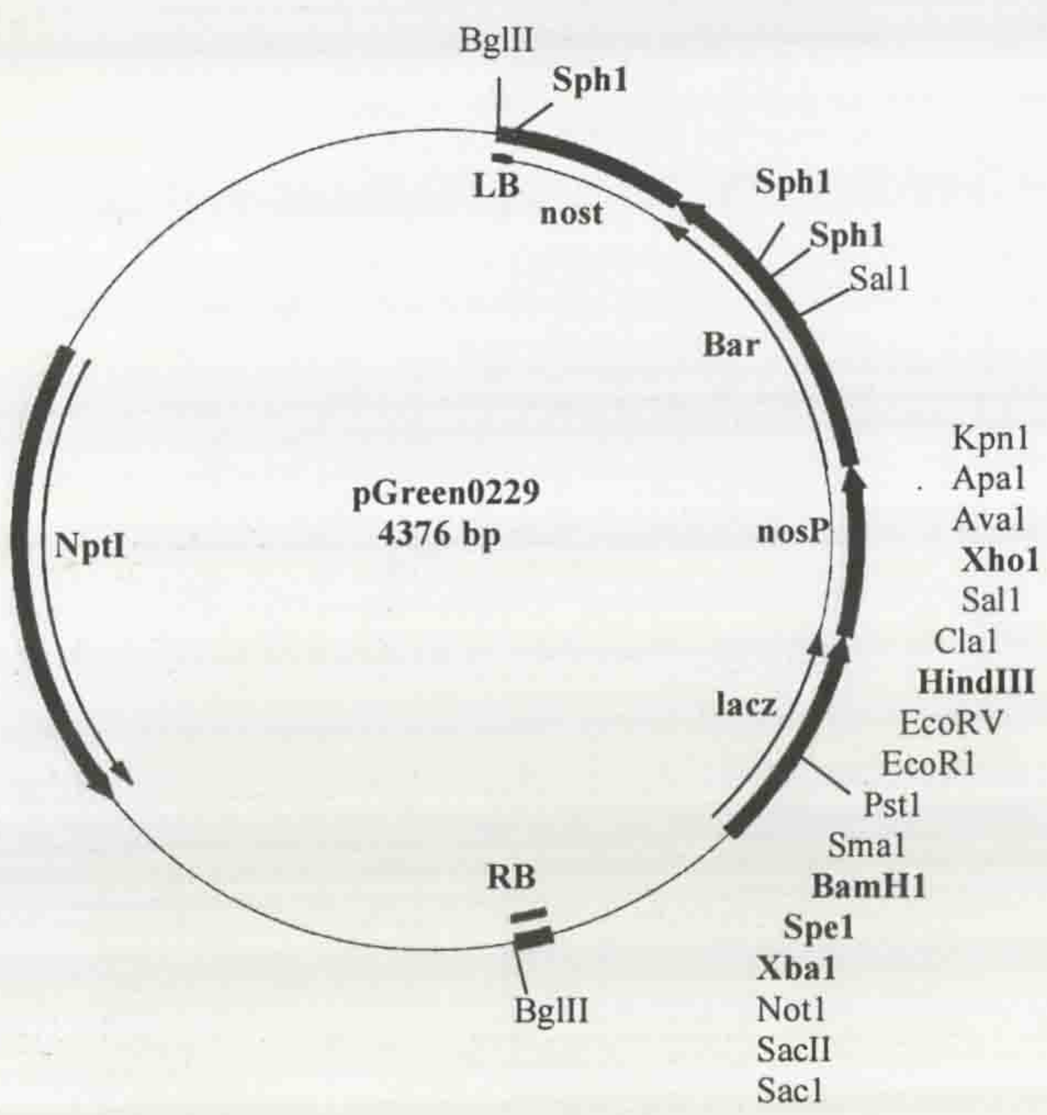
Diagnostic digests of pL4:*35S*:*Ds*:bar. Lanes 1 and 7 contain ladder. Lane 2 contains undigested pL4:*35S*:*Ds*:bar, lanes 3-6 contain pL4:*35S*:*Ds*:bar digested with *Eco*R1, *Bgl*II, *Bam*H1, and *Sac*1 respectively. Fragment sizes match those predicted in Figure 3.5b.

Figure 3.6 pSLJ1491



Schematic diagram of pSLJ1491. The plasmid contains a fusion of the *35S* promoter (p35S) and the Streptomycin Phosphotransferase (SPT) gene from *E.coli* Tn*5*. Transposon constructs are inserted in between the *35S* promoter and SPT. Transposition results in fusion of the promoter and SPT and can be visually scored by selection with streptomycin. The plasmid was kindly supplied by Jonathan Jones and is further described in (Jones *et al*., 1989.).

Figure 3.7 pGreen 0229



Schematic diagram of pGreen 0229. The plasmid contains Left Border (LB) and Right Border (RB) sequences from the Ti plasmid of *A.tumefaciens*. In between these are a selectable marker for glufosinate resistance (Bar) and a polycloning site. The restriction enzyme sites used for the generation of pGmini and the subsequent cloning steps are shown in bold. The plasmid was kindly supplied by Roger Hellens and is further described in (Hellens *et al*., 2000)

3.2h Generation of pGmini

The bar selectable marker was removed from pGreen 0229 (Figure 3.7) by digestion with *Sph*1/*Xho*1. Blunt ends were produced using Klenow enzyme. Plasmid was purified by gel extraction and re-ligated at 8°C overnight. The ligation was transformed into *E. coli*, transformants were selected on LA(kan) and plasmid DNA was recovered. One sample was verified as pGmini by restriction analysis.

3.2i Generation of pG*35S:35S:Ds:*bar:SPT

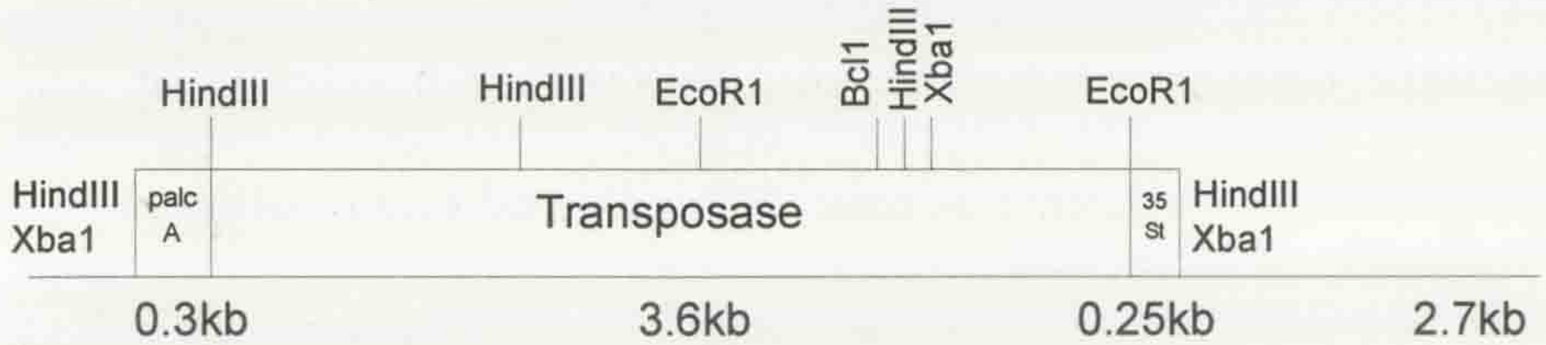
The *35S:35S:Ds:*bar:SPT cassette was released from pSLJ1491:*35S:Ds:bar* as a *Hind*III fragment by partial digestion and purified by gel extraction. pGmini was linearised using *Hind*III and phosphatased to prevent re-ligation. The two fragments were ligated at 8°C for 3 days. The ligation was transformed into *E. coli*, transformants were selected on LA(kan) and plasmid DNA was recovered. Two samples were verified as both orientations of pG*35S:35S:Ds:*bar:SPT by restriction analysis.

3.2j Generation of pAL2::*Tpase*

The complete *Ac* transposase coding sequence was released as a *Pst*1/*Pac*1 fragment from pACT6 (Figure 3.2). pAL2 (described in Chapter 4.2a, Figure 4.1) was linearised by digestion with *Pac*1/*Pst*1. Both fragments were gel extracted and ligated at 8°C for 6 days. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as the new pAL2::*Tpase* by both restriction analysis (Figure 3.8) and sequence analysis.

Figure 3.8 Diagnostic digests of pAl2::*Tpase*

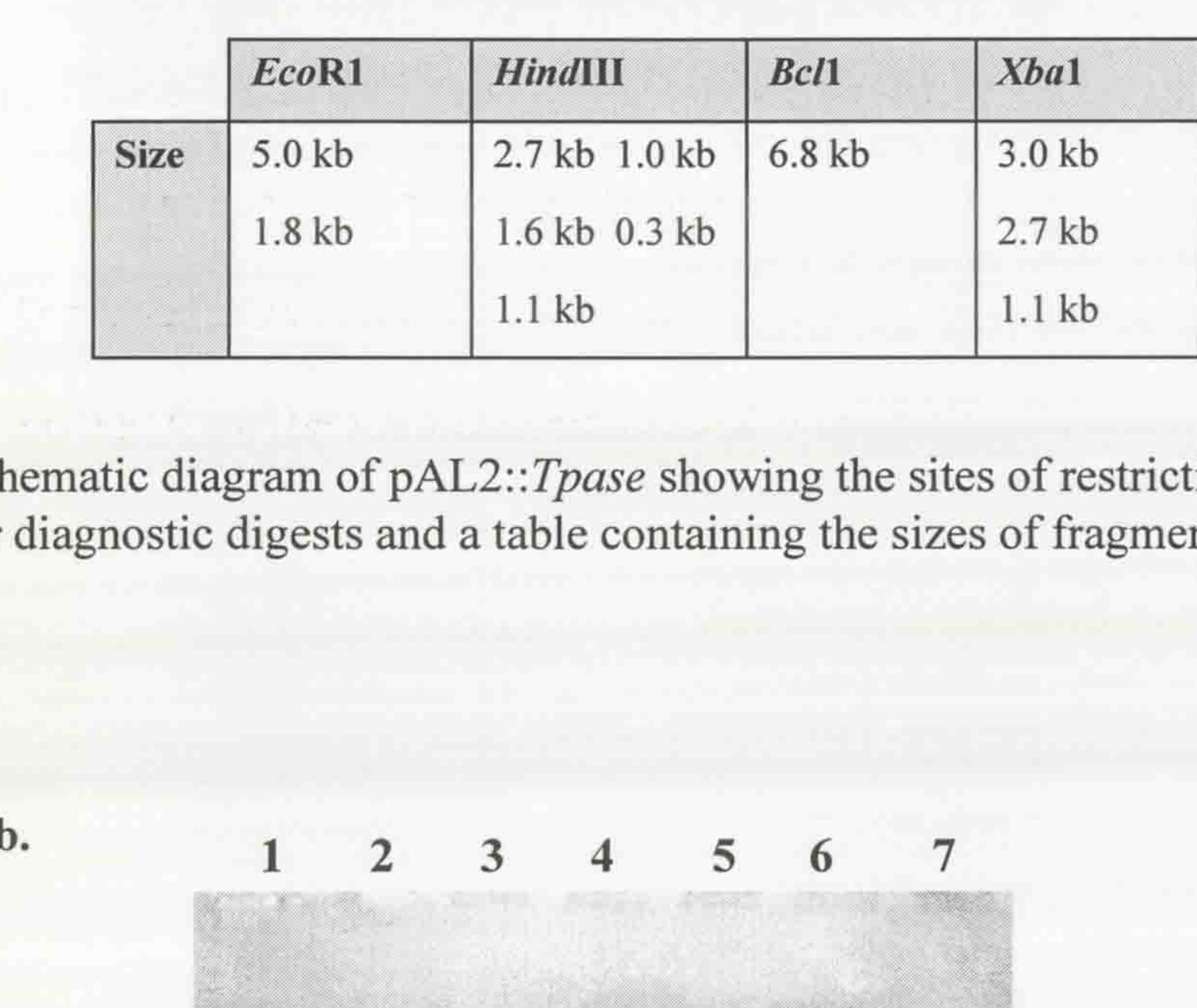
a.



	*Eco*R1	*Hind*III	*Bcl*1	*Xba*1
Size	5.0 kb	2.7 kb 1.0 kb	6.8 kb	3.0 kb
	1.8 kb	1.6 kb 0.3 kb		2.7 kb
		1.1 kb		1.1 kb

Schematic diagram of pAL2::*Tpase* showing the sites of restriction enzymes used for diagnostic digests and a table containing the sizes of fragments generated.

b.



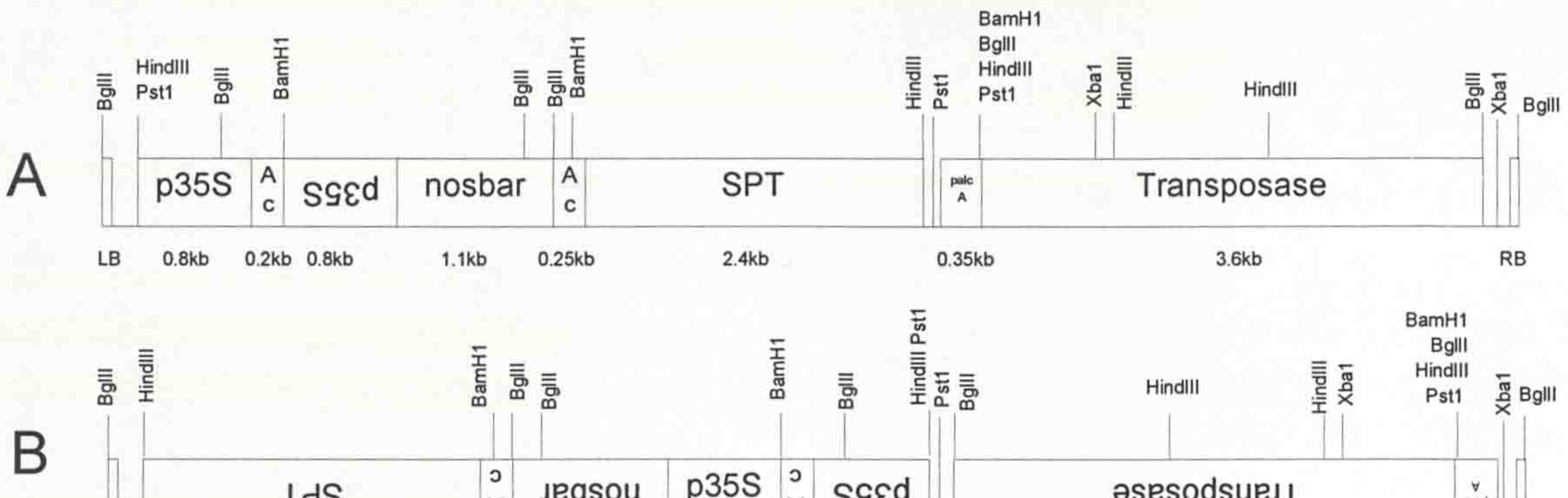
Diagnostic digests of pAL2::*Tpase*. Lanes 1 and 7 contain ladder. Lane 2 contains pAL2::*Tpase* undigested, lanes 3-6 contain pAL2::*Tpase* digested with *Eco*R1, *Hind*III, *Bcl*1, and *Xba*1 respectively. Fragment sizes match those predicted in Figure 3.8a. *Bcl*1 digestion has been unsuccessful. This is due to inactivity of the enzyme.

3.2k Generation of pG*35S:35S:Ds:*bar:SPT::*alcA:Tpase*

The *alcA:Tpase* cassette was released as an *Xba*1 fragment from pAL2Tpase by partial digestion. pG*35S:35S:Ds:*bar:SPT was linearised using *Spe*1 and phosphatased to prevent re-ligation. Both fragments were purified by gel extraction and ligated at 8°C for 4 days. The ligation was transformed into *E. coli*, transformants were selected on LA(kan) and plasmid DNA was recovered. Four samples were verified as the two possible orientations of pG*35S:35S:Ds:*bar:SPT along with the two possible orientations of *alcA:Tpase* by restriction analysis (Figure 3.9), and sequence analysis (Figure 3.9b). Sequences were checked by comparison to previous sequencing results and published sequences. The plasmids were re-named as pG*35S:Ds* A / B / C / D as seen in Figure 3.9 and are later referred to using these names.

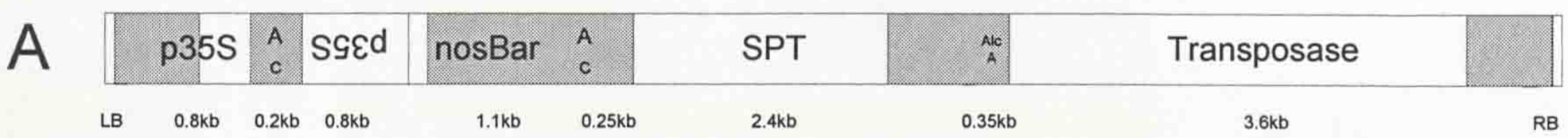
Figure 3.9 Verification of pG*35S*:*35S*:*Ds*:bar:SPT:*alcA*:*Tpase* (pG*35S*:*Ds*) A-D

a.



Schematic diagram of the four orientations of pG*35S:Ds* (A/B/C/D) showing the restriction sites used for diagnostic digests. All constructs are labelled reading 5'-3'. A table of the fragment sizes generated by diagnostic digests can be seen in Figure 3.9c. The results from these digests can also be seen in Figure 3.9d/e/f.

b.



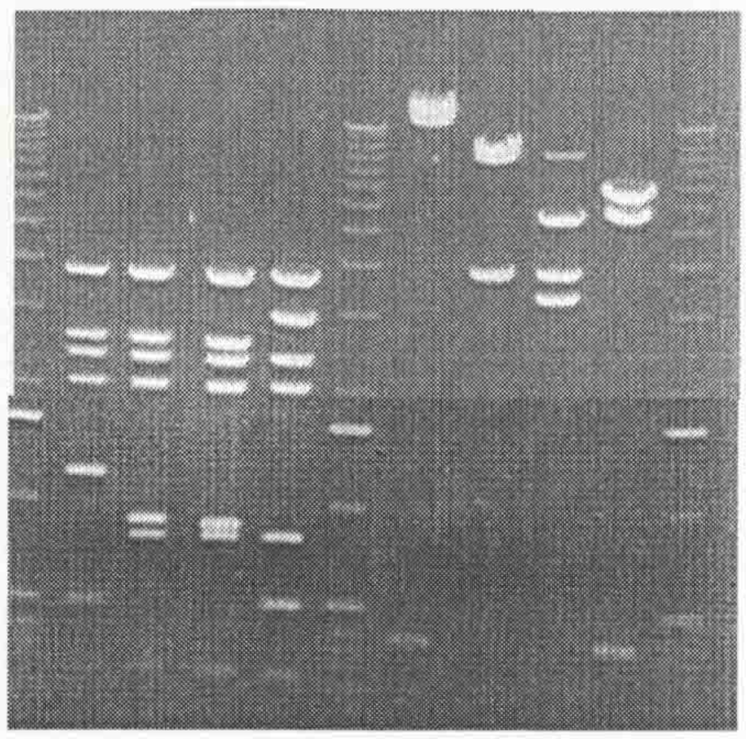
Schematic diagram of construct A. The areas in grey indicate those checked by sequencing.

c.

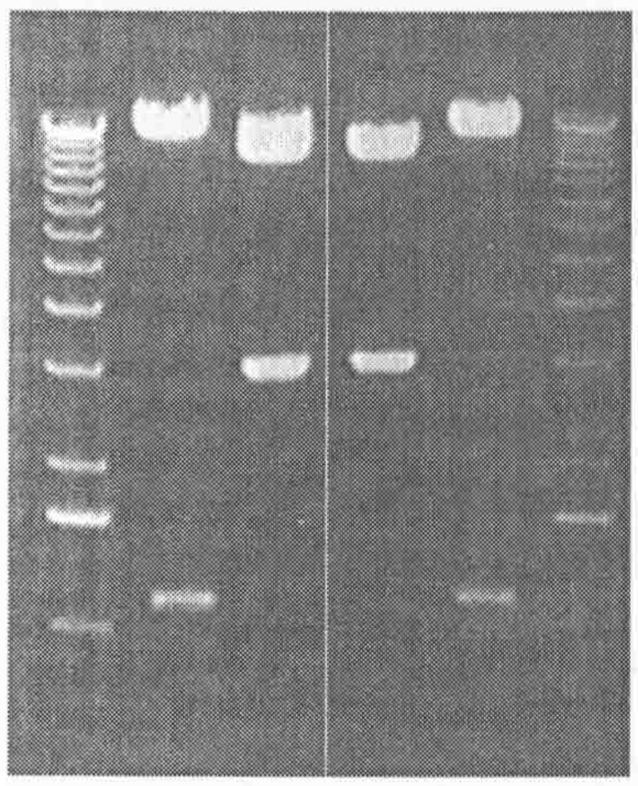
	*Bgl*II	*Pst*1	*Hind*III	*Bam*H1	*Xba*1
A	3.8 kb 3.0 kb 2.4 kb 2.0 kb 0.8 kb 0.2 kb 0.5 kb	6.8 kb 5.4 kb 0.4 kb	4.4 kb 4.1 kb 1.6 kb 1.1 kb 1.0 kb 0.4 kb	7.7 kb 2.5 kb 2.0 kb 0.4 kb	11.6 kb 1.1 kb
B	3.8 kb 2.7 kb 2.4 kb 2.0 kb 0.9 kb 0.2 kb 0.7 kb	8.8 kb 3.8 kb	4.4 kb 3.4 kb 1.6 kb 1.1 kb 1.1 kb 1.0 kb	5.8 kb 2.0 kb 3.8 kb 1.1 kb	9.6 kb 3.1 kb
C	3.8 kb 2.6 kb 2.4 kb 2.0 kb 0.9 kb 0.2 kb 0.8 kb	5.4 kb 3.8 kb 3.4 kb	4.4 kb 3.4 kb 1.6 kb 1.1 kb 1.1 kb 1.0 kb	4.3 kb 3.8 kb 2.0 kb 2.5 kb	9.6 kb 3.1 kb
D	3.8 kb 2.7 kb 2.4 kb 2.0 kb 1.1 kb 0.2 kb 0.5 kb	12.2 kb 0.4 kb	4.4 kb 4.1 kb 1.6 kb 1.1 kb 1.0 kb 0.4 kb	9.3 kb 2.0 kb 1.1 kb 0.4 kb	11.6 kb 1.1 kb

Expected sizes of fragments generated by restriction analysis of the four pG*35S:Ds* constructs A-D. Fragments generated in all the constructs are shown to the left. Fragments used for the identification of the orientation of constructs in each sample are shown to the right. Diagnostic digests using the enzymes can be seen in Figure 3.9 d/e/f.

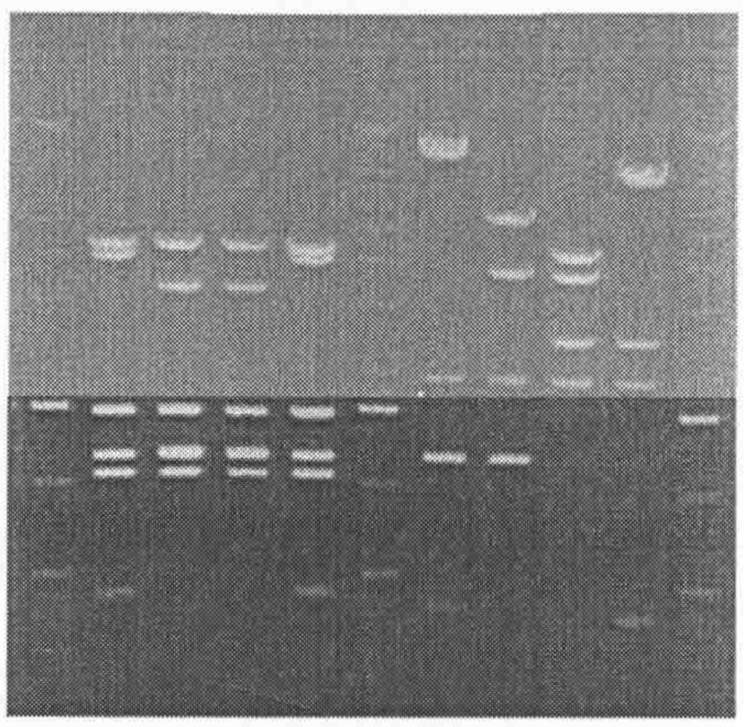
d.



f.



e.



d.-f. Diagnostic digests of pG*35S*:*Ds* A/B/C/D. Fragment sizes match those in Figure 3.9c. The figures contain areas of photographs at different exposure levels to aid the identification of the different size fragments.

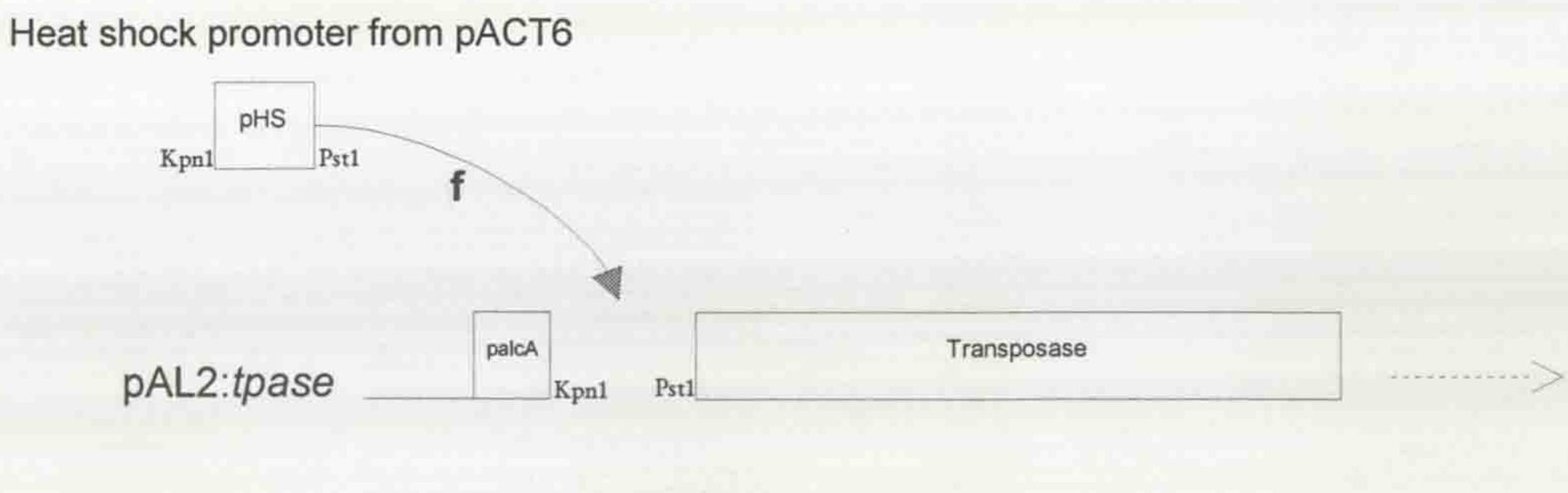
d. Lanes 2-5 contain constructs D/B/C/A digested with *Bgl*II respectively. Lanes 7-10 contain constructs D/C/B/A digested with *Pst*1 respectively. Lanes 1, 6 and 11 contain molecular weight ladder. *Pst*1 digestion is not complete in lane 9 resulting in an extra band of approximately 9 kb.

e. Lanes 2-5 contain constructs D/B/C/A digested with *Hind*III respectively. Lanes 7-10 contain constructs D/C/B/A digested with *Bam*H1 respectively. Lanes 1, 6 and 11 contain molecular weight ladder.

f. Lanes 2-5 contain constructs D/B/C/A digested with *Xba*1 respectively. Lanes 1 and 6 contain molecular weight marker

Figure 3.10 Cloning strategy for the generation of system2

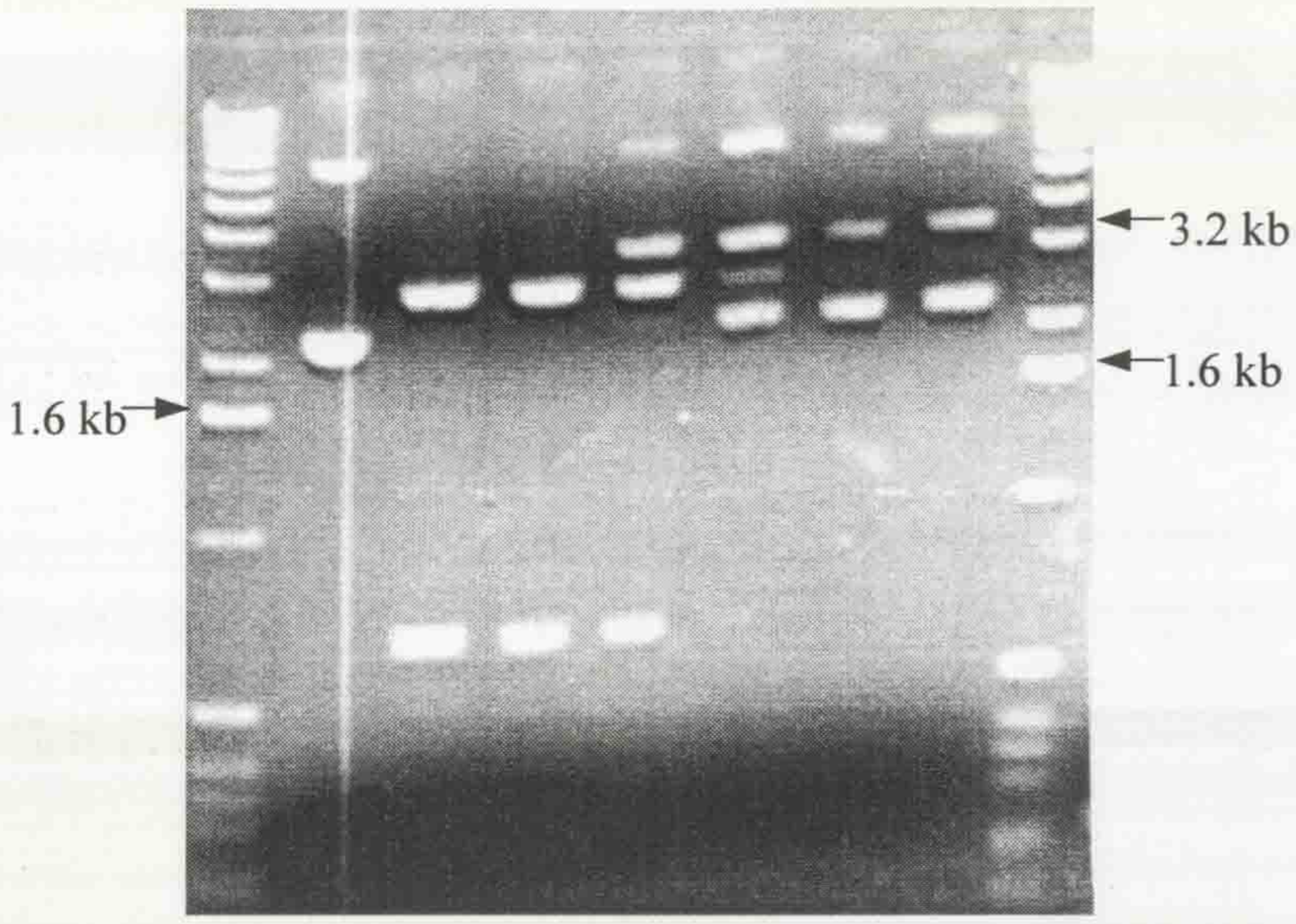
Schematic diagram illustrating the cloning strategy described in the following chapter. The enzymes used for the generation of fragments and cloning are shown. Each step is labelled to correspond to the appropriate description in Chapter 3.3. Promoter elements shown in inverse type indicate right to left promotion of transcription. Many of the plasmids are described in further detail later in the chapter.



Ac250 generated by PCR
Ac
Xba1
Asc1
a
palcA
Xba1
Asc1
pAL2
Ac
Sal1
Pst1
Ac210 generated by PCR
b
Ac
palcA
Sal1
Pst1
pAL2:Ac250
nosbar
Asc1
Asc1
nos:bar generated by PCR
c
Asc1
Ac
palcA
Asc1
Ac
Xho1
Sac1
pAL2:Ds
d
Xho1
p35S
HindIII
pSLJ1491
Sac1
SPT
HindIII
e
pGmini
HindIII
LB
HindIII
RB
pG35S:Al2Ds:bar:SPT
p35S
Ac
palcA
nosbar
Ac
SPT
LB
RB
pHS
Transposase
BamH1
Xba1
g
p35s
Ac
palcA
nosbar
Ac
SPT
BamH1
Xba1
LB
RB

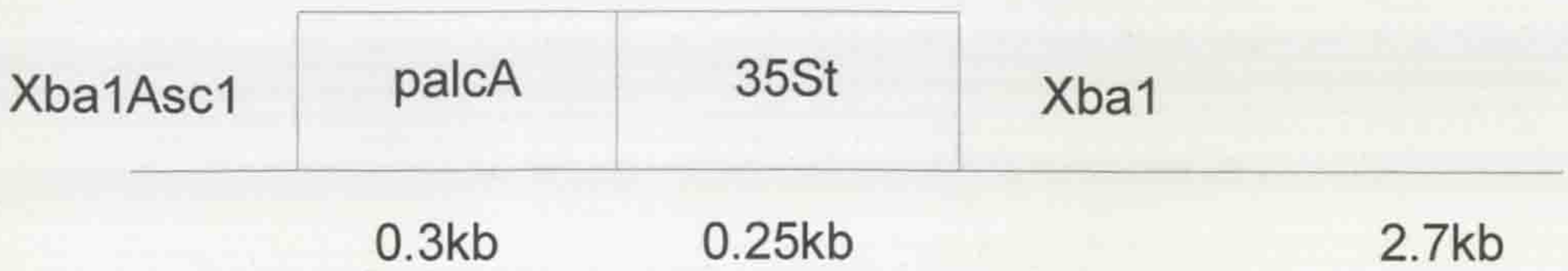
Figure 3.11 Generation of pAL2::*Ac250*

a.



*Xba*1 partial digestion of pAL2 for generation of pAL2::*Ac250*. The 3.2 kb linear fragment was recovered. Lanes 1 and 9 contain ladder. Lane 2 contains undigested plasmid. Lanes 3-8 contain a series of digestions with decreasing enzyme concentration. An increase in the linear 3.2 kb fragment and a decrease in the double digested 0.6 kb fragment can be seen as the enzyme concentration decreases.

b.



Schematic diagram of pAL2 showing the two *Xba*1 sites and the *Asc*1 site.

3.3 System 2: Heat shock inducible transposition creating ethanol inducible insertional mutations

3.3a Generation of pAL2::*Ac250*

Primers were designed to amplify the 3' end of the *Ds* transposon (Ac250in/Ac250out Appendix A). The ends of the primers contained *Xba*1 and *Asc*1 restriction sites respectively. The 250 bp fragment was generated by proofreading PCR from pJJ43690 (Figure 3.2a), digested with *Xba*1/*Asc*1 and purified by gel extraction. pAL2 (described in Chapter 4.2a, Figure 4.1) was linearised by partial digestion with *Xba*1 (Figure 3.11). Linear plasmid was recovered by gel extraction and digested with *Asc*1. The 3.2 kb linear plasmid was again recovered by gel extraction and the insert ligated for 1 hour at 37°C. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pAL2::*Ac250* by restriction analysis.

3.3b Generation of pAL2::*Ds*

Primers were designed to amplify the 5' end of the *Ds* transposon (Ac210in/Ac210out Appendix A). The ends of the primers contained *Pst*1 and *Sal*1 restriction sites respectively. The 210 bp fragment was generated by proofreading PCR from pJJ43690, digested with *Pst*1/*Sal*1 and purified by gel extraction. pAL2:*Ac250* was linearised by digestion with *Pst*1/*Sal*1. Linear plasmid was recovered by gel extraction and ligated with the PCR product for 10 days at 8°C. The ligation was transformed into *E. coli*, transformants were selected on LA(Amp) and plasmid DNA was recovered. One sample was verified as pAL2::*Ds* by restriction analysis.

3.3c Generation of pAL2:*Ds*::bar

A primer (nosPfor Appendix A) was designed to complement the 5' end of the *nos* promoter from pGreen 0029 (Hellens *et al*., 2000) and contained a 5' *Asc*1 site. A primer was designed for the 3' end of the *35S* terminator in pL4 (35Strev Appendix A), enabling the whole *nos*:bar:*35St* construct to be generated by PCR from pL4:*nos*:bar (Figure 3.4a) and cloned as an *Asc*1 fragment. The fragment was generated by proofreading PCR, digested using *Asc*1, and purified by gel extraction. pAL2:*Ds* was linearised using *Asc*1 and phosphatased to prevent re-ligation before gel extracting and ligating with the PCR fragment at 8°C for 48 hrs. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pAL2:*Ds*::bar by restriction analysis.

3.3d Generation of pSLJ1491::AL2:*Ds*:bar

pSLJ1491 (Figure 3.6) was linearised by partial digestion with *Sac*1. The linear fragment was purified by gel extraction before digesting with *Xho*1. The AL2:*Ds*:bar cassette was released as a *Sac*1/*Xho*1 fragment from pAL2:*Ds*:bar. Both fragments were purified before ligating at 8°C for 8 days. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pSLJ1491::AL2:*Ds*:bar by restriction analysis.

3.3e Generation of pG*35S*:AL2:*Ds*:bar:SPT

The *35S*:AL2:*Ds*:bar:SPT fusion was released as a *Hind*III fragment from pSLJ1491:Al2:*Ds*:bar. pGmini (described earlier in Section 3.2h) was linearised using *Hind*III and phosphatased to prevent re-ligation. Both pGmini and *35S*:AL2:*Ds*:bar:SPT were purified by gel extraction and ligated at 8°C overnight. The ligation was transformed into *E. coli*, transformants were selected on LA(kan)

Acknowledgements

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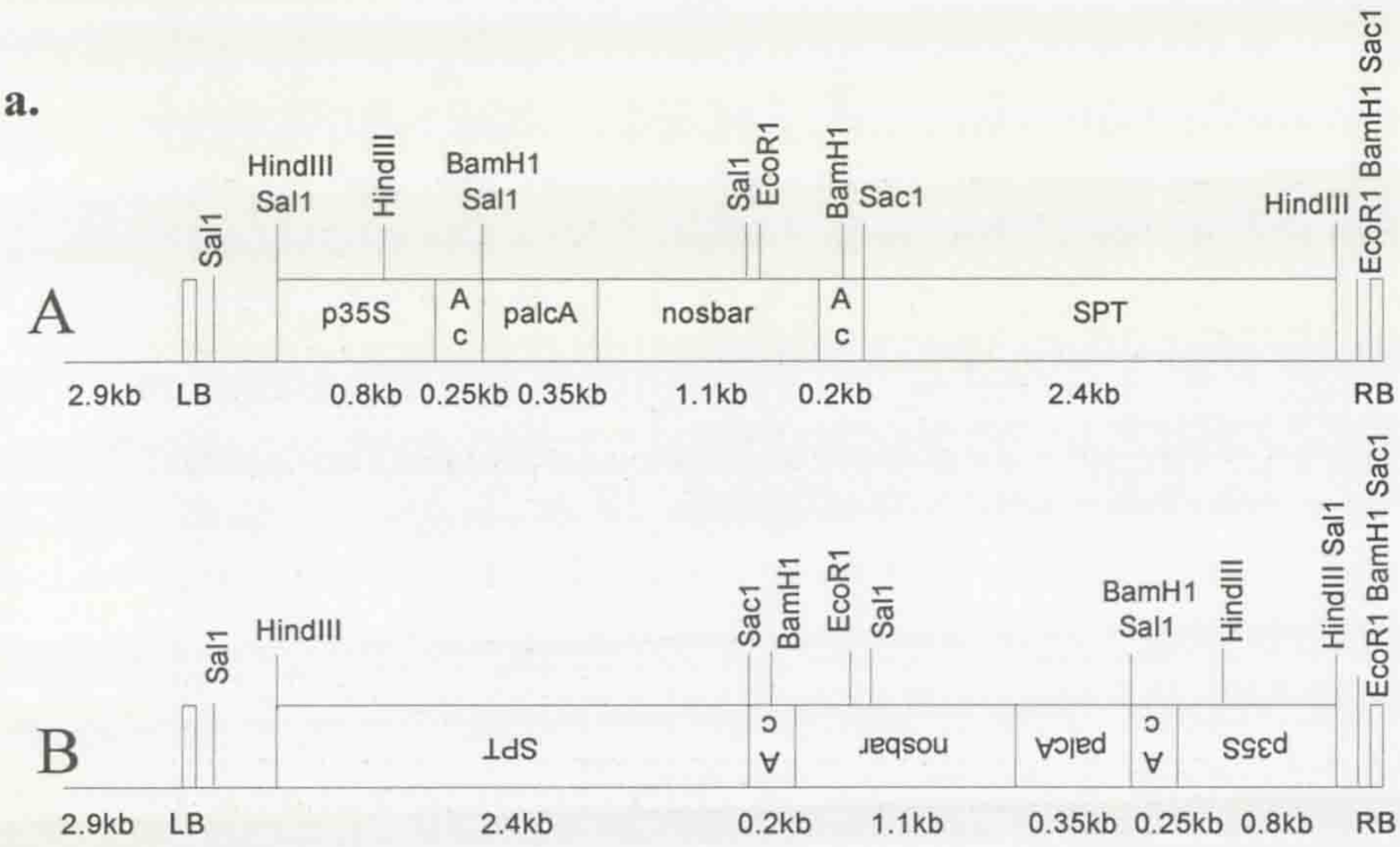
I have made many new friends during my time in Liverpool. I would like to thank them all, for making the time spent away from my studies so memorable. Finally I would like to thank my family for their constant support, encouragement, and understanding.

Figure 3.12 Diagnostic digests of pG*35S:*Al2*:Ds*:bar (pGAL2:*Ds*) A/B

a.



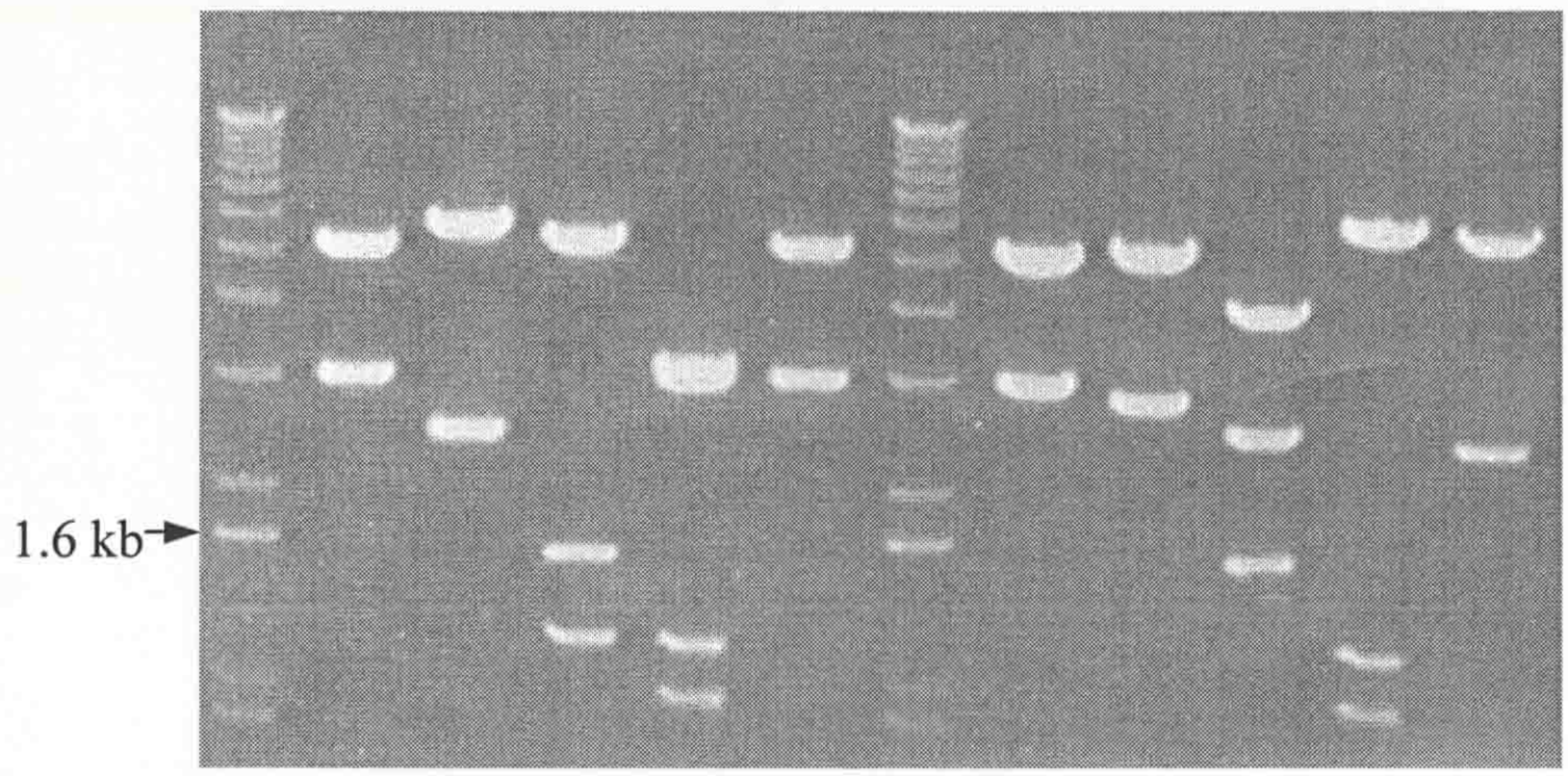
Schematic diagram of both orientations of pGAl2:*Ds* (A/B) indicating the restriction sites used for diagnostic digests in both constructs.

b.

	pGAl2:*Ds*A	**pGAl2:*Ds*B**
***Hind*III**	5.1 kb 2.9 kb	5.1 kb 2.9 kb
***Eco*R1**	5.4 kb 2.6 kb	5.5 kb 2.5 kb
***Bam*H1**	4.2 kb 2.4 kb 1.6 kb	5.5 kb 1.6 kb 1.1 kb
***Sal*1**	6.0 kb 1.1 kb 0.8 kb	2.9 kb 3.0 kb 1.1 kb 0.8 kb
***Sac*1**	5.9 kb 2.1 kb	5.3 kb 2.7 kb

Expected restriction fragment sizes for diagnostic digests of pGAL2:*Ds*

c.



Diagnostic digests of pGAL2:*Ds* A/B. Fragment sizes match those in Figure 3.12b. Lanes 2-6 contain pGAL2:*Ds* B digested with *Hind*III, *Eco*R1, *Bam*H1, *Sal*1, and *Sac*1 respectively. Lanes 8-12 contain pGAL2:*Ds* A digested with *Hind*III, *Eco*R1, *Bam*H1, *Sal*1, and *Sac*1 respectively. Lanes 1 and 7 contain ladder.

and plasmid DNA was recovered. Two samples were selected and verified as the two orientations of pG*35S*:AL2:*Ds*:bar:SPT (A/B) by restriction analysis (Figure 3.12). These were re-named pGAL2:*Ds* A / B as seen in Figure 3.12 and are later referred to using these names.

3.3f Generation of pAL2::*Hs::Tpase*

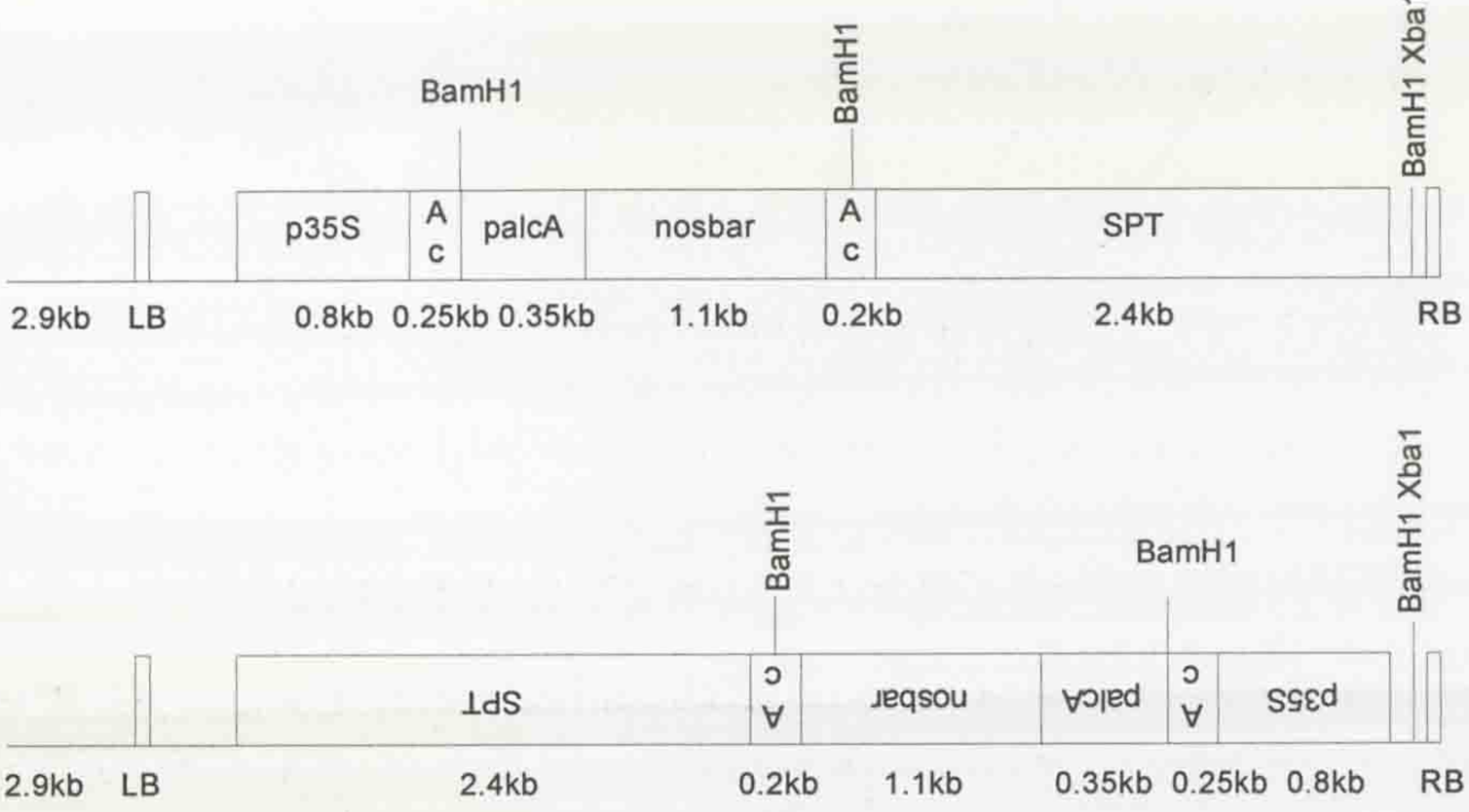
The Soybean *Gmhsp* 17.3-B heat shock promoter (Schöffl *et al*., 1984) was released as a *Pst*1/*Kpn*1 fragment from pACT6 (Figure 3.2c). pAL2:*Tpase* (Figure 3.8a) was linearised using *Pst*1/*Kpn*1. Linear pAL2::*Tpase* and the heat shock promoter were purified by gel extraction and ligated at 8°C for 14-28 days. The ligation was transformed into *E. coli*, transformants were selected on LA(Amp) and plated out on LA(Amp) for colony screening. One colony was selected after screening with a radiolabelled probe generated from a restriction fragment of the heat shock promoter. Plasmid DNA was recovered, further checked by restriction analysis and verified to be pAL2::*Hs::Tpase*.

3.3g Generation of pGAL2:*Ds::Hs:Tpase*

A number of attempts were made at cloning the *Hs:Tpase* cassette from pAL2:*Hs:Tpase* as an *Xba*1 partial/*Bam*H1 fragment into *Bam*H1partial/*Xba*1 linearised pGAL2:*Ds* A/B (Figure 3.13). This involved repeated generation and purification of the inserts, ligation using various ratios of insert to vector, and temperatures from 8-37°C, over periods of 24 hours – 4 weeks. Normal and high concentration ligase were used and transformation was done by both heat shock and electroporation. Unfortunately none of these were successful and the cloning strategy was abandoned.

Figure 3.13 Generation of pGAl2*:Ds::Hs:Tpase*

a.

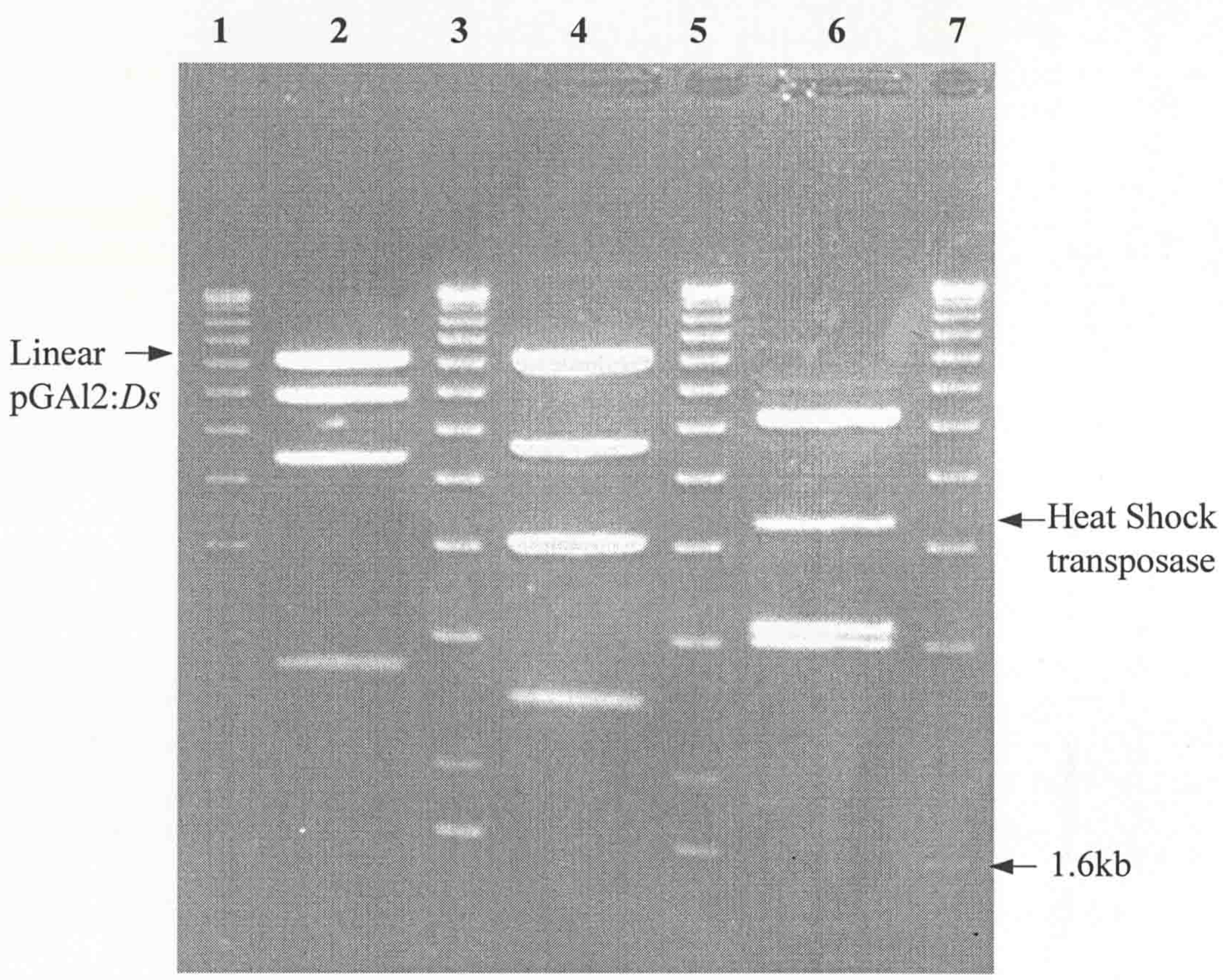


Schematic diagrams of pG*35S:*Al2:*Ds*:*bar*:SPT A/B indicating the restriction enzyme sites used for cloning in Figure 3.13c.

b.

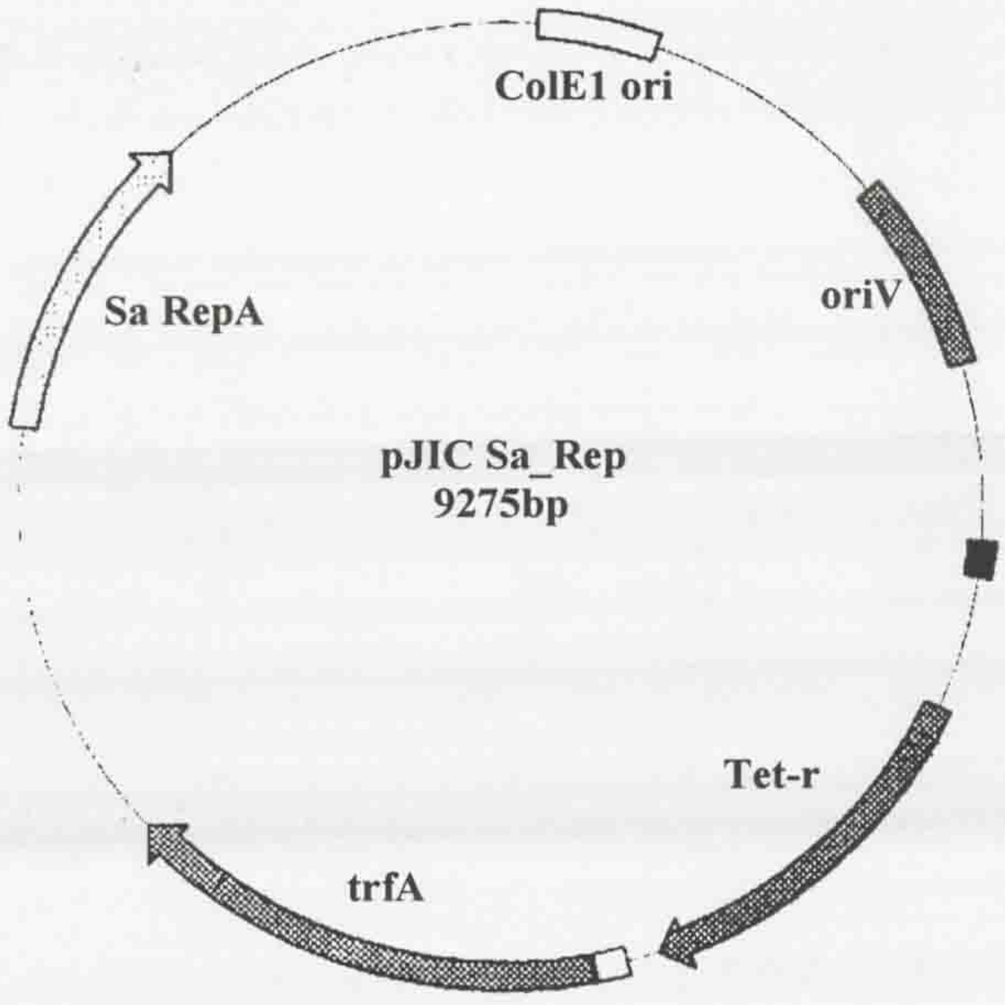
Schematic diagram of pAL2:*Hs*:*Tpase* indicating the restriction enzyme sites used for cloning in Figure 3.13c.

c.



Fragments generated by digestion for generation of pGAl2:*Ds::Hs:Tpase*. Lanes 2 and 4 contain pGAl2:*Ds* B/A linearised by partial digestion using *Bam*H1 then digested completely using *Xba*1. The resulting 8kb linear fragments were recovered. Lane 6 contains pAL2:*Hs*:*Tpase* linearised by partial digestion using *Xba*1 then digested completely using *Bam*H1/*Xmn*1. The 4.2kb *Hs:Tpase* fragment was recovered and used for attempted ligations. Lanes 1, 3, 5 and 7 contain molecular weight ladder.

Figure 3.14 pJICSa Rep (pSoup)



pJICSa Rep (pSoup) is the 'helper' plasmid in a two component system for generation of transgenic plants by *A. tumefaciens* transformation. The plasmid is based on pBin19 and the T-DNA region has been removed and replaced with Sa RepA which enables the replication of pGreen based plasmids in *A. tumefaciens*. The Tet-r gene replaces the *NPTII* gene and confers tetracycline resistance. It also contains trfA enabling its own replication in *A. tumefaciens*. The plasmid was kindly supplied by Roger Hellens and is further described in (Hellens *et al*., 2000).

3.4 Generation of transgenic plants for transposon tagging

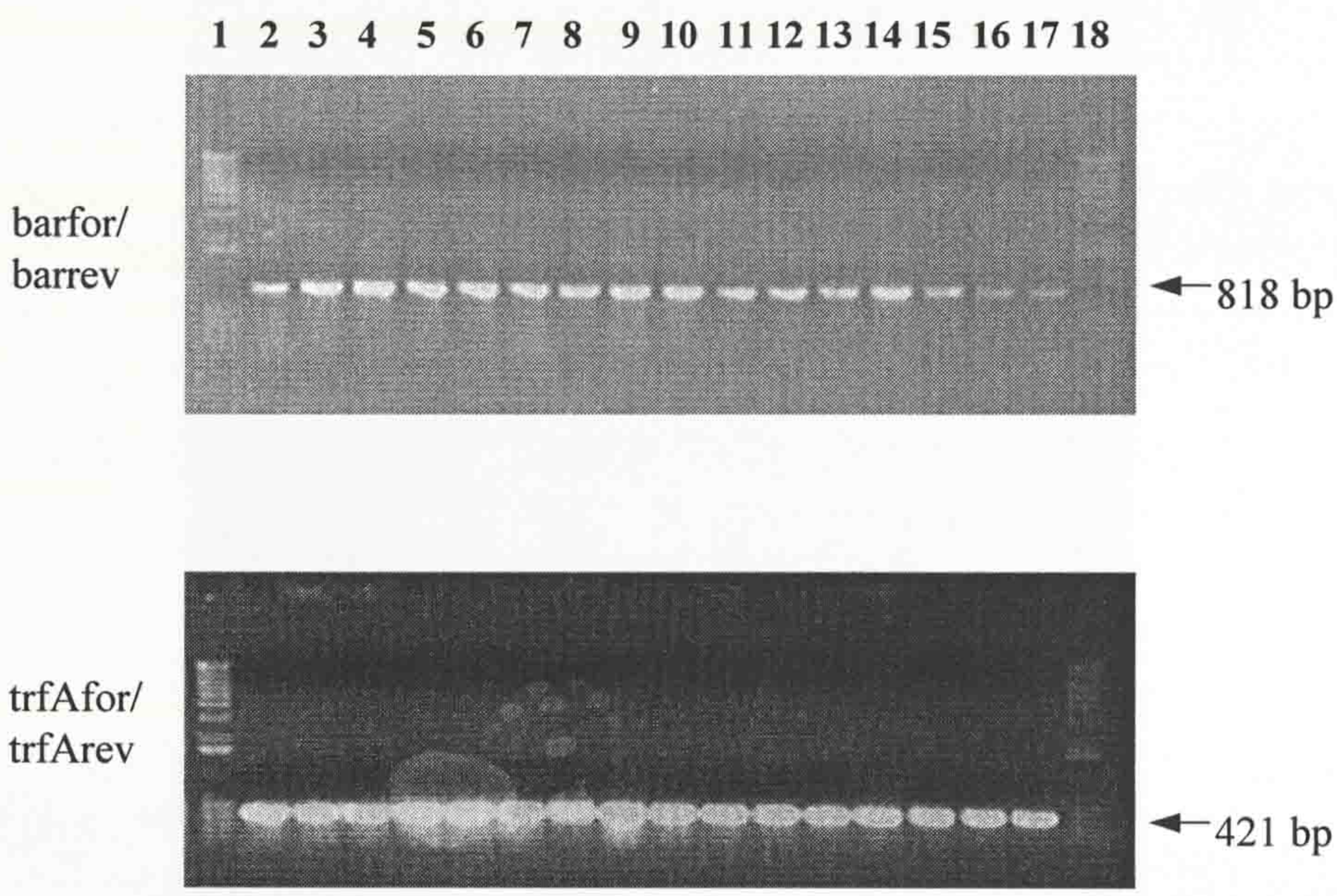
3.4.1 Transformation of constructs into *A. tumefaciens*

The four constructs from system 1 (pG*35S:Ds* A-D), the two constructs from system 2 (pGAL2:*Ds* A/B) and pG*35S*:SPT were individually co-transformed with pSoup (Figure 3.14) by electroporation into *A. tumefaciens*. Transformants were selected on LA containing tetracycline (10μg/ml), kanamycin (50 μg/ml), and rifampicin (100μg/ml). Fully resistant colonies were checked by PCR (Figure 3.15). Resistant PCR positive colonies were grown in liquid YT containing tetracycline, kanamycin, and rifampicin and used to transform *A. thaliana* using a floral dip protocol modified from Clough and Bent (1998) .

3.4.2 *A. thaliana* lines used for transformation

A transgenic line of *A. thaliana* was previously generated by Hairul Roslan. Wild type plants were transformed by vacuum infiltration using *A. tumefaciens* carrying a pGPTV-SRN4 plasmid containing a *35S* promoter, the *alcR* coding sequence and a *nos* terminator. The primary (T0) transformant was selected on media containing hygromycin and checked by diagnostic PCR using primers for the *alcR* sequence. The primary T0 plant was allowed to self fertilise and T1 seed was collected for segregation analysis using the hygromycin selectable marker in the T-DNA. The T1 family was called SRN1. T1 seed showed a resistant : sensitive ratio of 3 : 1 indicating the presence of a single T-DNA insert. The resistant plants were therefore assumed to consist of a mixture of hemizygous and homozygous plants in a 2 : 1 ratio. Hygromycin resistant plants were allowed to self fertilise and T2 seed was collected to identify a homozygous line. Constitutive *alcR* expression and the inducible control of the *alcA* promoter in one of these homozygous lines was

Figure 3.15 Diagnostic PCR of *Agrobacterium*



Diagnostic PCR of *A. tumefaciens* colonies. Each colony was suspended in 20 μl water and boiled for 10 min before pelleting the debris and using 2 μl of supernatant as a template for PCR. Samples were checked using primers for bar (top) and trfA (bottom). Positive bands of the correct size in both sets indicates the presence of both transformation and helper plasmid.

Lanes 2-5 contain pmABR:ALS, ALSAS, EPSPS, EPSPAS (described later in Chapter 4.3) respectively. Lanes 6 and 7 contain pGAL2:*Ds* A. Lanes 8 and 9 contain pGAL2:*Ds* B. Lanes 10 and 11 contain pG*35S*:*Ds* A. Lanes 12 and 13 contain pG*35S*:*Ds* B. Lanes 14 and 15 contain pG*35S*:*Ds* C. Lanes 16 and 17 contain pG*35S*:*Ds* D. Lanes 1 and 18 contain molecular weight marker.

Table 3.1 Segregation analysis of the SRN line to identify homozygous families

Line		Total	Resistant	Sensitive	Ratio	p
SRN 1 T1		60	41	19	3 : 1	> 0.2
T2	1	52	43	9	3 : 1	> 0.2
	2	59	40	19	3 : 1	> 0.2
	3	59	49	10	3 : 1	> 0.05
	4	123	105	18	?	
	5	175	173	2	1 : 0	
	6	103	76	27	3 : 1	> 0.8
	7	174	139	35	3 : 1	> 0.05
	8	76	57	19	3 : 1	> 0.99
	9	64	47	17	3 : 1	> 0.5
	10	143	125	18	?	
	11	168	54	114	?	
	12	178	177	1	1 : 0	
	13	100	66	44	?	
	14	103	88	15	?	
	15	182	42	140	?	
	16	159	117	42	3 : 1	> 0.2
	17	69	28	41	?	
	18	124	95	29	3 : 1	> 0.2
	19	191	189	2	1 : 0	
	20	189	177	12	1 : 0	

Segregation analysis was carried out on tissue culture media containing hygromycin (40μg/ml). Statistical analysis was performed (where possible) using the χ^2 test. The T1 seed was initially verified to segregate in a 3:1 (resistant : sensitive) ratio. Resistant plants were allowed to self-fertilise and T2 seed was collected. As the original transformant contained a single T-DNA insert the T2 families were expected to be either homozygous and entirely resistant, or mixed and segregate in a 3:1 (resistant : sensitive) ratio. The ratio of mixed to homozygous families was expected to be 2:1. Twenty T2 families were then analysed, again on hygromycin selective tissue culture media and four families, SRN1-5, SRN1-12, SRN1-19, and SRN1-20 were identified as homozygous. The families were used directly for transformation and to generate a stock of T3 homozygous seed for further experiments. The line had been further characterised previously by Hairul Roslan (Roslan, 1999). Seven of the families did not appear to segregate in either a 1:0 or a 3:1 ratio. These families were not further characterised but were not used in further studies.

checked by transient assays using high velocity particle bombardment. Constructs containing *alcA*:CAT were shown to express CAT upon induction (Roslan, 1999).

Seed from the SRN line was obtained from Hairul Roslan. T1 plants were verified as containing single inserts by segregation analysis on GM plates containing 40 μg/ml hygromycin (Table 3.1). Homozygous lines were identified amongst the T2 seed, again by segregation analysis on GM plates containing hygromycin (Table 3.1). SRN1-5, SRN1-12, SRN1-19 and SRN1-20 were all identified as homozygous lines. Seed from these lines was sown on soil for transformation using *A. tumefaciens*. Plants from these T2 homozygous lines were also grown to maturity on soil and allowed to self fertilise. T3 Seed was collected from all four homozygous lines and mixed. This mixed seed was then used for further transformations using *A. tumefaciens*.

Seed from two other homozygous T2 lines expressing constitutive *35S*:*alcR* and inducible *alcA*:GUS {AGS1-3 / AGS4-2 (Roslan, 1999)} were also obtained from H.Roslan. Plants from these lines were grown on soil and transformed again by *A. tumefaciens* with a number of the constructs.

3.4.3 Constructs transformed

The four orientations of pG*35S:Ds* (A/B/C/D) were transformed into homozygous SRN T2 and T3 plants. pG*35S:*SPT was also transformed into homozygous SRN T2 plants. Both orientations of pGAl2:*Ds* (A/B) were transformed into SRN and AGS lines with the view to cross in a transposase source at a later date. All the transformations were repeated numerous times to generate large amounts of T0 seed for primary selection.

Table 3.2 Segregation analysis of transposon tagging T1 lines

Line		Total	Resistant	Sensitive	p (3:1)
A	1	47	39	8	> 0.05
B	2	82	56	26	> 0.2
	4	68	47	21	> 0.2
	5	75	53	22	> 0.2
	6	64	43	21	> 0.05
	8	63	47	16	> 0.99
	9	25	18	7	> 0.05
	10	9	7	2	> 0.99
	12	88	76	12	> 0.05
	13	97	77	20	> 0.2
	14	82	67	15	> 0.05
	15	17	12	5	> 0.05
	16	13	10	3	> 0.99
C	3	32	24	8	> 0.99
	4	84	63	21	> 0.99
	5	22	16	6	> 0.5

Segregation analysis was carried out by spraying with Harvest™. Statistical analysis was performed using the χ^2 test. The table shows the results from the 16 T1 families that contained plants segregating in a 3:1 (resistant : sensitive) ratio indicating the presence of a single T-DNA insert. Resistant plants were retained form each line and allowed to self fertilise for the generation and identification of T2 heterozygous and homozygous plants by further segregation analysis (Table 3.3).

3.4.4 Selection of putative T0 transformants

Putative primary (T0) transformants were grown on soil and selected by spraying with Harvest™. Seedlings were grown for approximately 10 days until a lawn of plants appeared. Plants were sprayed a maximum of three times with six to nine day intervals between spraying. Putative transformants were identified after three weeks as healthy green plants amongst bleached non-transformants. A transformation frequency of between 1/2000 and 1/4000 seeds was typical. These survivors were grown to the 6-8 leaf stage and re-potted individually. Individual plants were kept apart using Arasystem tubes (obtained from Lehle seeds) and allowed to self fertilise. Once siliques had matured and begun to dry out, the plants were uprooted and placed on Whatman® 3MM paper to dry for seed collection. T1 seed was collected and sown on soil. T-DNA copy number was analysed by segregation analysis of the bar selectable marker by spraying with Harvest™.

3.4.5 Segregation analysis of T1 seed

T1 seed was sown individually on soil for segregation analysis of the bar selectable marker. For each line, 20-100 seed were sown and germinating seedlings selected by spraying with Harvest™. Plants were grown to the four-leaf stage before spraying with Harvest™ as before. All lines giving a ratio (resistant : sensitive) of approximately 3:1 (indicating single copy number) were selected, along with one line (SRN1-19 *35S*:SPT) which showed a 15:1 ratio indicating the presence of two segregating inserts. Any lines resulting in ratios, which differed significantly from 3:1 by χ^2 analysis, were discarded. Sixteen lines were selected containing the *35S:Ds* construct. Twelve of these contained construct B. One line was generated containing construct A, and three containing construct C (Table 3.2). The plants were grown to

Table 3.3 Segregation analysis of transposon tagging T2 families

Line		Total	Resistant	Sensitive	Ratio	p
A1	1	35	24	11	3 : 1	> 0.2
	2	42	41	1	1 : 0	
B2	1	39	30	9	3 : 1	> 0.5
	2	29	29	0	1 : 0	
B4	1	38	19	9	3 : 1	> 0.2
	2	27	20	7	3 : 1	> 0.99
B5	1	37	37	0	1 : 0	
	2	48	48	0	1 : 0	
	3	40	33	7	3 : 1	> 0.01
	4	37	24	13	3 : 1	> 0.5
B6	1	39	26	13	3 : 1	> 0.2
	2	34	32	2	1 : 0	
B8	1	33	23	10	3 : 1	> 0.2
	2	29	21	8	3 : 1	> 0.5
B9	1	35	25	10	3 : 1	> 0.5
	2	39	38	1	1 : 0	
B10	1	38	38	0	1 : 0	
	2	37	28	9	3 : 1	> 0.99
B12	1	23	19	14	3 : 1	> 0.01
	2	35	27	8	3 : 1	> 0.5
B13	1	36	26	10	3 : 1	> 0.5
	2	40	34	6	3 : 1	> 0.05
B14	1	36	31	5	3 : 1	> 0.05
	2	39	38	1	1 : 0	
	3	35	31	4	3 : 1	> 0.05
	4	31	31	0	1 : 0	
B15	1	29	18	11	3 : 1	> 0.05
	2	39	29	10	3 : 1	> 0.99
B16	1	38	38	0	1 : 0	
	2	37	27	10	3 : 1	> 0.2
A1	1	35	24	11	3 : 1	> 0.2
	2	42	41	1	1 : 0	
C3	1	37	29	8	3 : 1	> 0.2
	2	35	30	5	3 : 1	> 0.05
C4	1	43	43	0	1 : 0	
	2	39	39	0	1 : 0	
C5	1	39	27	12	3 : 1	> 0.2
	2	39	29	10	3 : 1	> 0.99

Segregation analysis was carried out by spraying with Harvest™. Statistical analysis was performed (where possible) using the χ^2 test. The T1 transformed lines had all been verified to contain single T-DNA inserts by segregation analysis (Table 3.2). T2 seed was collected from Harvest™ resistant T1 plants that were allowed to self fertilise. T2 families were therefore expected to contain either resistant and sensitive plants in a 3:1 ratio, or be entirely resistant. These two possible genotypes were expected to be present in a 2:1 ratio.

the eight-leaf stage and re-potted individually. Between six and eight resistant plants from each of the sixteen lines were re-potted and allowed to self fertilise for seed collection. T2 homozygous and hemizygous lines were identified amongst this seed, again by segregation analysis of the bar selectable marker (Table 3.3). Twenty plants were re-potted for line SRN1-19 *35S*:SPT and seed collected to verify the 15:1 ratio and identify families containing single and double inserts (Table 3.4).

Unfortunately 29 putative transformants were selected from early transformations. These contained AL2:*Ds* A and B in both SRN and AGS lines. Selection of these plants proved incorrect. All T1 plants from these lines were sensitive to harvest. Later selection of transformants proved more successful, however the AL2:*Ds* construct and the AGS lines were not re-used.

3.4.6 Segregation analysis of T2 seed

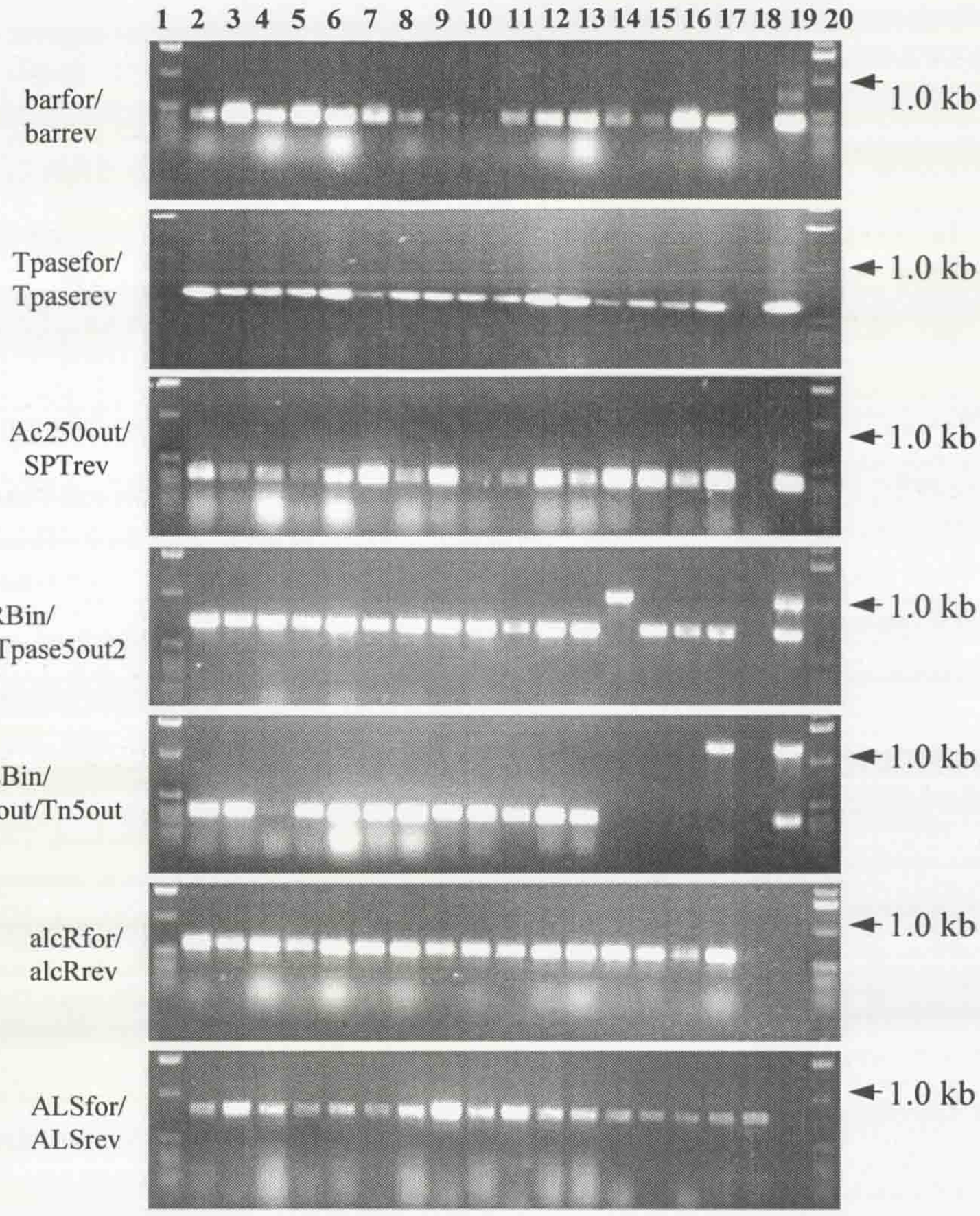
T2 seed from the sixteen *35S:Ds* single insert transformants was analysed again by segregation analysis. Between 30 and 50 seeds were sown on soil and grown to the four-leaf stage. Germinating plants were selected by spraying with Harvest™. Ratio's (resistant : sensitive) approximating to 3:1 or 1:0 were statistically verified where possible in most lines. This indicates that the lines contained single T-DNA inserts. The results of this analysis can be seen in Table 3.3. From this analysis segregating T2 lines from hemizygous T1 parents containing *35S:Ds* A, *35S:Ds* B and *35S:Ds* C were identified. These segregating lines were used in the initial transposon tagging strategy.

Table 3.4 Segregation analysis of the double insert line *35S*:SPT

Line		Total	Resistant	Sensitive	Ratio	p
35S:SPT T1		78	71	7	15 : 1	> 0.2
T2	6	48	37	11	3 : 1	> 0.5
	7	50	40	10	3 : 1	> 0.2
	8	48	46	2	15 : 1	> 0.5
	9	49	48	1	1 : 0	
	11	50	45	5	15 : 1	> 0.2
	16	49	49	0	1 : 0	
	17	49	49	0	1 : 0	
	18	51	51	0	1 : 0	

Segregation analysis was carried out by spraying with Harvest™. Statistical analysis was performed (where possible) using the χ^2 test. The T1 family showed a clear 15:1 ratio of resistant : sensitive plants suggesting the presence of two independent T-DNA inserts. 20 of the T1 resistant plants were retained and seed was collected to analyse the T2 generation. The expected result of segregation analysis of the T2 families was a ratio of seven families containing entirely resistant plants, to four families containing both resistant and sensitive plants in a 3:1 ratio, to four families containing resistant and sensitive plants in a 15:1 ratio. The T2 families that segregated in a 3:1 ratio would only contain one of the two T-DNA inserts. Eight of the T2 families were analysed by segregation analysis and two families that segregated in a 3:1 (resistant : sensitive) ratio were identified. The presence of all three ratios (1:0, 3:1, 15:1) amongst the T2 families further verified that the line contained two independent T-DNA inserts.

Figure 3.16 PCR analysis of transposon tagging lines



Genomic DNA from the transposon tagging lines was obtained using rapid DNA preps (Chapter 2.3.14). This was used as a template for the analysis of the lines by PCR. The lines were analysed using primers for a number of areas in both the transposon tagging and the SRN T-DNA constructs.

Lanes 2-17 contain samples from transformants B2-1, B4-1, B5-1, B6-1, B8-1, B9-1, B10-1, B12-1, B13-1, B14-1, B15-1, B16-1, A1-1, C3-1, C4-1, C5-1. Lane 18 contains wild type genomic DNA. Lane 19 contains pG*35S*:*Ds* plasmid template. Lanes 1 and 20 contain molecular weight ladder.

All the lines have produced bands using primers for the bar selectable marker (barfor/barrev), the *Ac* Transposase (Tpasefor/Tpaserev), the 5' *Ds* end leading into the SPT excision marker (Ac250out/SPTrev), the tagging insert right border T-DNA end (RBin/ NAK3S (construct B/C) /Tpase5out2 (construct A) and the *alcR* gene (alcRfor/alcRrev). Most of the samples produced bands using primers for the tagging insert left border T-DNA (LBin/ Ac210out (construct A/C) /Tn5out (construct B). Some lines appear not to contain the entire T-DNA and this follows the acceptance that T-DNA integration begins at the right border and carries on toward the left border. A positive control PCR was also carried out for the *csr1* gene (ALSfor/ALSrev). Wild type plants are used as negative controls and these were only PCR positive for the *csr1* PCR. The plasmid control in lane 19 contains bands for all the constructs in the transposon tagging T-DNA inserts. More than one of the original plasmids was used for the left and right border PCRs as the internal primers were different for the different lines. The bands generated using each different plasmid template were run together and these therefore contain two different bands.

3.4.7 PCR analysis of transgenic lines

All sixteen *35S:Ds* transgenic lines were analysed by PCR (Figure 3.16). DNA was isolated from T2 plants resistant to Harvest™. PCR was carried out using primers situated at a number of sites along the T-DNA insert and in the *alcR* gene in the SRN background. Positive bands of the expected size were seen in all plants from PCR using primers designed to sites in both the transposon tagging insert and the *alcR* insert. One of the *35S:Ds* B containing plants (B5), the *35S:Ds* A containing plant and two of the *35S:Ds* C containing plants (C3/C4) were PCR negative using primers located on the T-DNA left border. In the case of constructs A and C this would be likely to remove part of the *35S* promoter from the Streptomycin excision marker. In the case of construct B around 800 bp at the left border end constituted a portion of Tn*5* that was non-essential.

3.5 Discussion

The construction of plasmids for the investigation of the potential of two novel transposon tagging strategies has been described. Complex cloning strategies were utilised using numerous techniques. The fidelity of the plasmids was subsequently confirmed by both restriction analysis and sequencing.

Transgenic plants were generated to test the potential of one of the novel transposon tagging systems for generating transposon insertions. The system incorporates the *alc* switch to control transposase expression and therefore control transposition. Additionally the transposon contains a *35S* outfacing promoter expected to generate

Chapter 1

Introduction

1.1 *Arabidopsis thaliana* as a model organism

- **General features of *Arabidopsis* and its suitability as a model organism**

Arabidopsis thaliana is a small weed in the Cruciferae family and has been used as a tool in classical genetic studies for over fifty years. There are many characteristics that lend *Arabidopsis* towards use as a model organism. These have been described in detail and are the subject of a number of reviews (Redei, 1970; Redei, 1975; Meyerowitz and Pruitt, 1985; Meyerowitz, 1987; Meyerowitz and Somerville, 1994; Meinke, 1998). *Arabidopsis* is directly related to more economically important crops such as cabbage, broccoli and horseradish and can be found naturally across the continents of Europe, Asia, Africa, Australia and North America.

The original genetic studies were carried out by Friedrich Laibach. The haploid chromosome number was established as 5 in 1907 (Laibach, 1907). Laibach also collected many different ecotypes and performed the first mutagenesis experiments. Since then *Arabidopsis* has been the subject of many large scale mutagenesis studies using chemical mutagens and X-rays. Several thousand mutants have been obtained that show variations in morphology, development, metabolism, fertility, and signaling pathways. The Third International *Arabidopsis* Conference in 1987 is seen

dominant and semi-dominant mutant phenotypes. Experiments were carried out on these plants to assess the control of transposition by induction of the *alc* system, and to generate potential phenotypic mutants for the identification of novel genes. A separate transgenic line (SRN1-19 *35S:SPT*) was also generated. This was used as a positive control to aid in the selection of excision events by streptomycin selection.

Transformation rates using the pGreen based vectors were considerably lower than expected, based on reported findings (Clough and Bent, 1997; Bent, 2000). This could have been due to either the size or nature of the constructs transformed, the transformation procedure or the vectors used. No further analysis was carried out to assess this reduction.

A considerable difference in recovery of transformants from each of the four pG*35S:Ds* constructs was also noticed. pG*35S:Ds* B yielded far more transformants than A and C, and pG*35S:Ds* D yielded no transformants at all. Transformation and primary selection was adjusted with respect to this observation. In fact more seed was screened for transformants containing constructs A, C, and D than B.

Unfortunately the constructs for the second system proved impossible to complete. Attempts were made to transform plants with the pGAl2:*Ds* construct with a view to crossing in a transposase source at a later date. These also proved unsuccessful. The constructs were stored for potential transformation at a later date. To use this system two lines would be required. One containing the new Al2:*Ds* transposon and the other containing the *Ac* Transposase independently. These could be crossed together to mobilise the transposon.

Interestingly plasmid isolation of the pAL2:*HS:Tpase* construct often resulted in low yields. This problem was solved in *E. coli* by growing the cells at 28°C. This suggested that some cytotoxicity may be occurring at 37°C and may help to explain the apparent difficulty in cloning the construct into pGAl2:*Ds*.

Chapter 4

Expression of 'lethal' constructs using the *alc* system

4.1 Introduction

As described earlier in Chapter 3.1, the expression of an outward facing promoter from a transposon insertion could result in the production of either sense or antisense transcripts. It was also suggested that by using an inducible promoter to create this aberrant expression, potentially lethal phenotypes could be observed during induction and recovered upon the removal of the inducer. The aim of the following set of experiments was to investigate the potential of the transposon tagging strategies to generate and recover 'lethal' mutations.

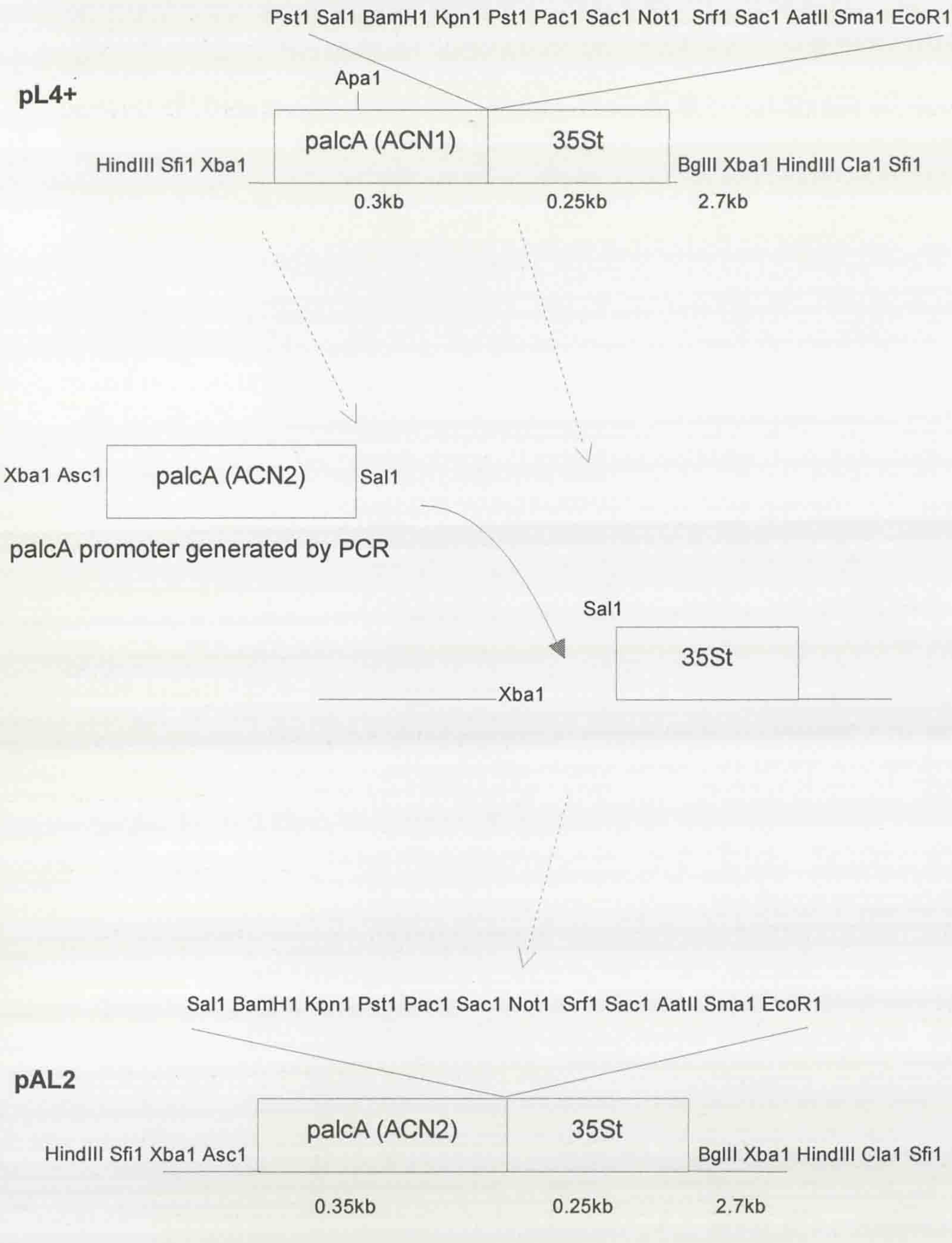
Two genes were chosen to test the system because of their essential nature. Both genes have been identified previously as the targets of herbicide action where chemical inhibition of the gene products is lethal. The gene *epsp* encodes 5-enol-pyruvylshikimate-3-phosphate synthase (EPSP synthase), the penultimate step in the shikimate pathway and the target enzyme of glyphosate. The gene *csr1* codes for acetolactate synthase (ALS), the initial enzyme in the biosynthesis of isoleucine, leucine, and valine, and the target of sulfonylurea, imidazolinone, and triazolopyrimidine herbicides. The products of these genes perform essential steps in the synthesis of amino acids and significantly reducing their expression would be expected to produce an observable and potentially a lethal phenotype.

Antisense expression of ALS in potato has previously been described and observed to cause phenotypic characteristics similar to those of imidazolinone treatment. The plants showed up to an 85% reduction in ALS activity and this impaired growth almost irreversibly. The affected phenotype included severe growth retardation, leaf chlorosis, the formation of necrotic lesions, and altered leaf morphology (Höfgen *et al.*, 1995). No completely inhibited plants were recovered in this experiment. Antisense expression was constitutive and therefore it was assumed that complete inhibition would be lethal. The use of an inducible promoter to express antisense ALS may possibly lead to generating mutants with increased inhibition upon induction, and may allow the observation of phenotypic reversal after the removal of induction.

To achieve this, part of the coding sequence of each gene was cloned in sense and antisense orientation, downstream of an *alcA* inducible promoter, contained in a plant transformation binary vector. The initial 801 bp of *csr1* and the initial 828 bp of *epsp* coding sequence were used. Transgenic plants constitutively producing the AlcR transcription factor were transformed using each construct. Transformants were selected and used to observe the effects of the production of partial sense and antisense transcripts, upon induction of the *alcA* promoter. Visual phenotypes were scored amongst induced and uninduced plants under normal growth conditions and after treatment with herbicides targeted to the same specific gene products as the aberrant expression.

The first step of the strategy involved generating a plant transformation vector containing the *alcA* promoter, a polycloning site, and a *35S* terminator. The pSoup / pGreen system (Hellens *et al.*, 2000) was chosen as this offered easier and more

Figure 4.1 Generation of pAL2



Schematic diagram of the cloning strategy used for the generation of pAL2. The pUC8 backbone, *35S* terminator and polycloning site of pL4+ were retained and the ACN1 *alcA* promoter was replaced with the *alcA* promoter from ACN2. The ACN2 promoter was generated by PCR using proofreading taq and cloned as an *Xba*1/*Sal*1 fragment in place of the ACN1 *alcA* promoter.

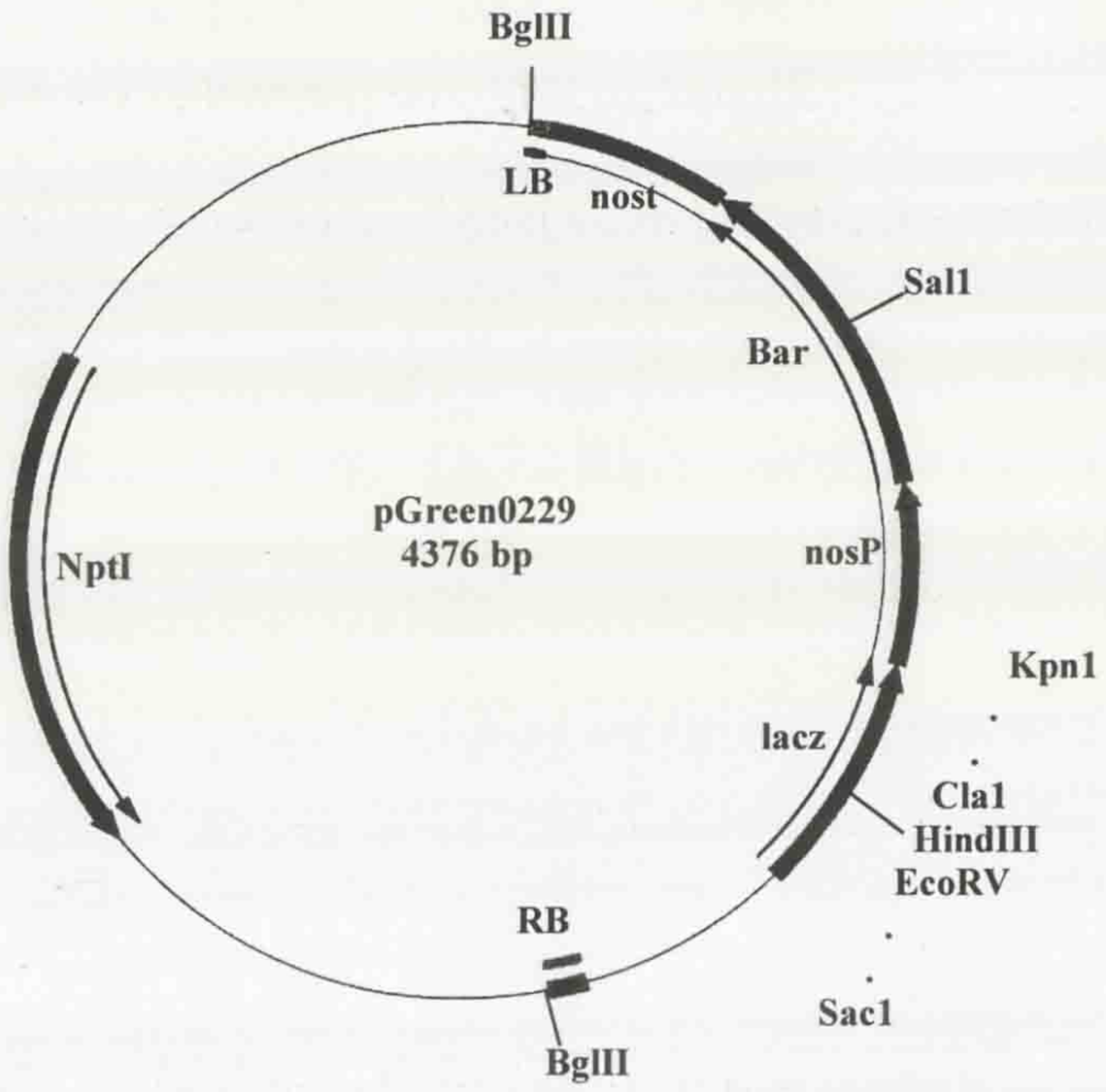
efficient cloning. Two plasmids were created, pmABF, and pmABR (Figure 4.5). The essential genes were cloned into pmABR downstream of the *alcA* promoter. Approximately 800 bp at the 5' end of each coding sequence was generated by PCR. The PCR fragment was cloned in both orientations into pmABR. The cloning strategies are described below along with the generation of a series of transgenic plants to analyse the effects of induction.

4.2 Step 1: Generation of pmABR/pmABF

4.2.a Generation of pAL2

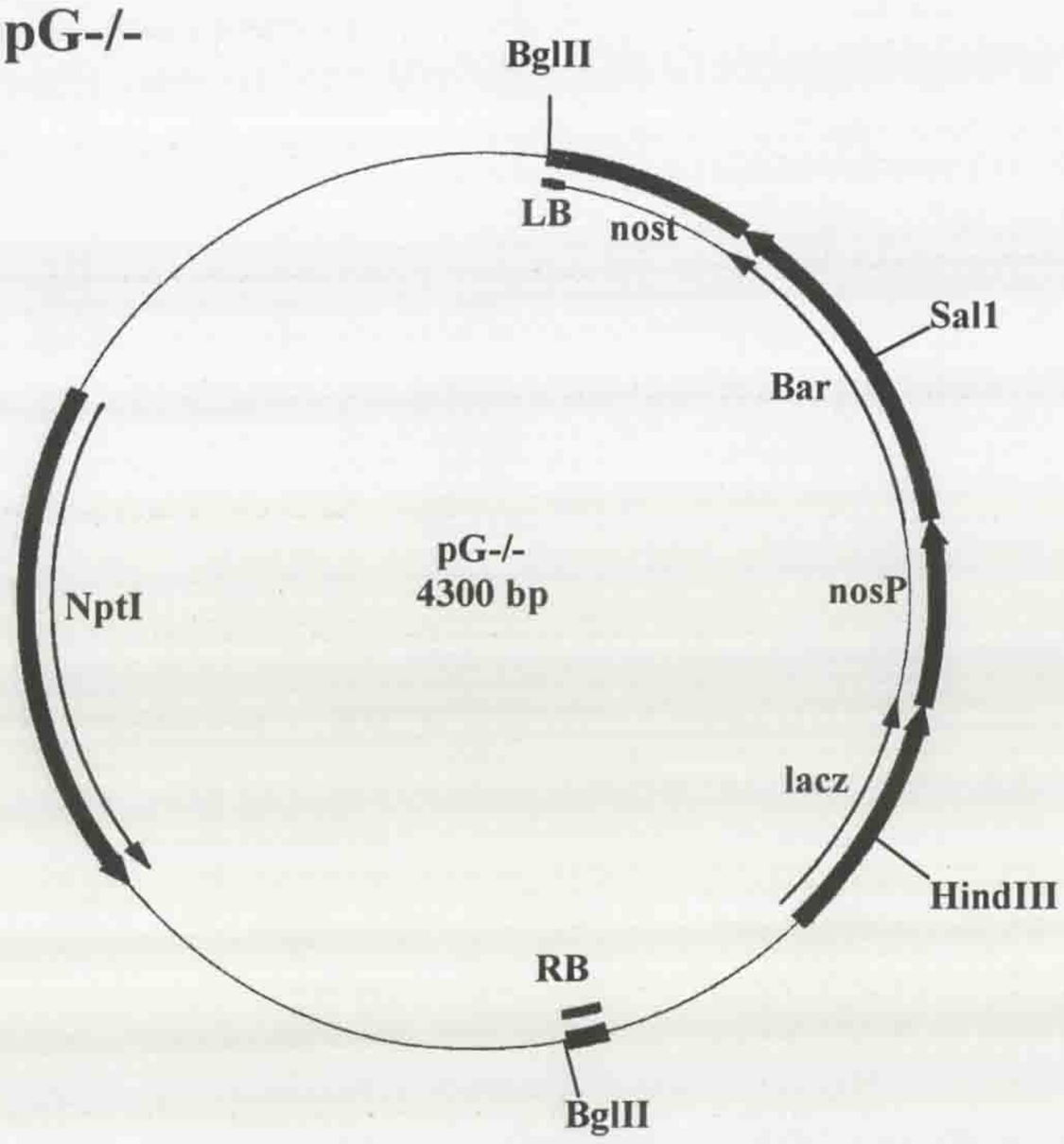
pAL2 was generated from pL4+ (Figure 4.1). The ACN1 *alcA* promoter in pL4+ was replaced by the native *alcA* promoter from pAÇN2 (supplied by Angela Tregova, unpublished), which was generated by proofreading PCR. The ACN1 promoter contains a minimal *35S* promoter sequence. The ACN2 promoter however contains the native TATA and upstream regions of the promoter and includes an additional AlcR binding site. ACN2 had previously been shown to give higher levels of expression in a higher percentage of transformants than ACN1 by transient analysis (personal communication, Tomsett *et al.*). Primers were designed to add *Xba*1 and *Asc*1 restriction sites at the 5' end of the promoter and a *Sal*1 restriction site at the 3' end (NAK5X/NAK3S Appendix A). pL4+ was linearised by partial digestion with *Xba*1. This generated two linear fragments and these were purified and digested with *Apa*1 (situated in the *alcA* promoter) to prevent ligation into the wrong fragment. The plasmid was then ethanol precipitated and the ACN1 promoter was removed by *Sal*1 digestion. *Xba*1/*Apa*1/*Sal*1 digested plasmid and *Xba*1/*Sal*1 digested PCR fragment

Figure 4.2 pGreen 0229



pGreen 0229 is one of the binary vectors from the pGreen / pSoup system (Hellens *et al*., 2000) and was used as the basis for generating pmABR/pmABF. The polycloning site is only partially shown. The *Kpn*1, *Cla*1, *Eco*RV and *Sac*1 sites were used to remove the polycloning regions surrounding the *Hind*III site as described in Chapter 4.2b/c to generate pG-/- below.

Figure 4.3 pG-/-



pG-/- is derived from pGreen 0229 by removing all but the *Hind*III site from the polycloning site. The *alcA* cassette from pAL2 was cloned into this *Hind*III site to create pmABR/pmABF shown in Figures 4.4/4.5.

were gel extracted and ligated overnight at 8°C. The ligation was transformed into *E. coli*, transformants were selected on LA(amp) and plasmid DNA was recovered. One sample was verified as pAL2 by restriction analysis/PCR and finally by sequencing.

4.2.b Generation of pG-

pG0229 (Figure 4.2) (Hellens et al., 2000) was digested with *Cla*1/*Kpn*1 to remove the polycloning region outside the *Hind*III site toward the left border T-DNA sequence. Blunt ends were generated using Klenow enzyme. The plasmid was purified by gel extraction and re-ligated at room temperature for 5 hours. The ligation was transformed into *E. coli*, transformants were selected on LA(kan) and plasmid DNA was recovered. One sample was verified to contain the new plasmid pG- by restriction analysis.

4.2.c Generation of pG-/-

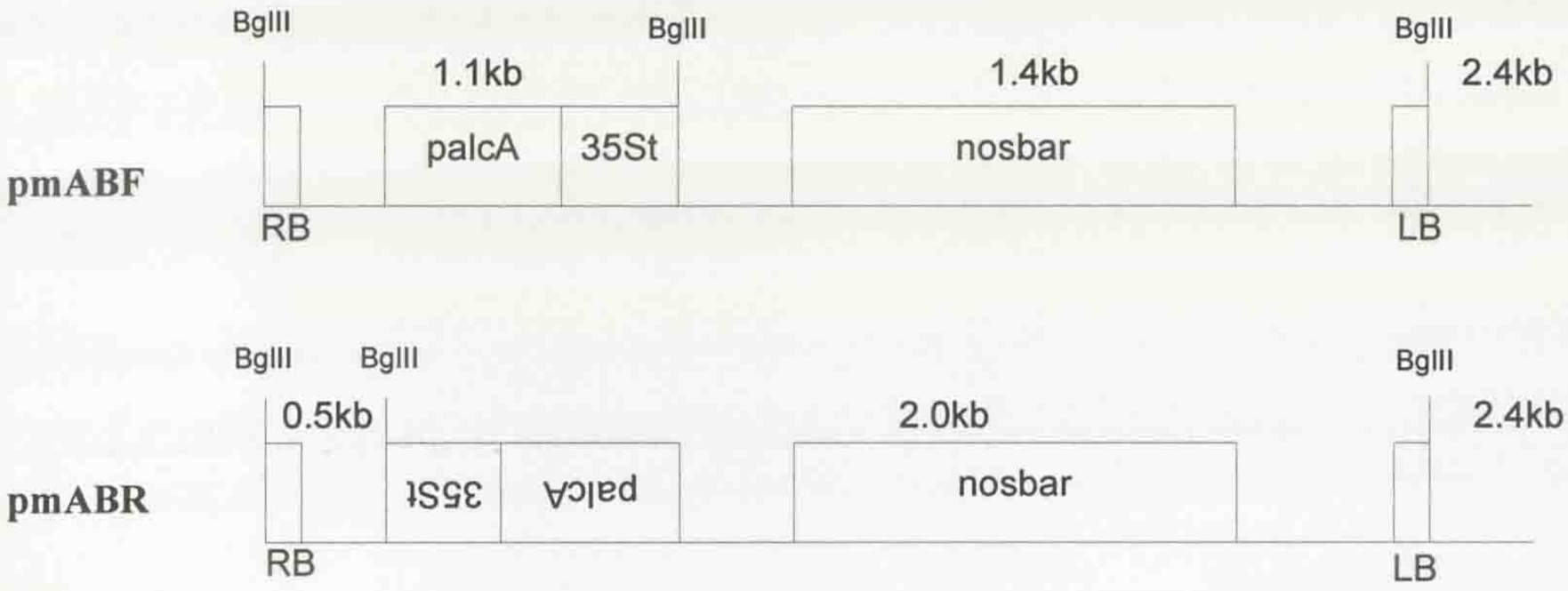
pG- was linearised by digestion using *Sac*1 and blunt ends produced using Klenow enzyme. The polycloning region outside the *Hind*III site toward the right border T-DNA sequence was removed from the linearised plasmid by digestion using *Eco*RV. The plasmid was purified by gel extraction and ligated at 8°C for 3 days. The ligation was transformed into *E. coli*, transformants were selected on LA(kan) and plasmid DNA was recovered. One sample was verified to contain the new plasmid pG-/- (Figure 4.3) by restriction analysis.

4.2.d Generation of pmABR/pmABF

pG-/- was linearised using *Hind*III and phosphatased to prevent re-ligation. The *alcA*:*35St* cassette was released as a *Hind*III fragment from pAL2. Linear pG-/- and *alcA*:*35St* were purified by gel extraction and ligated at 8°C for 3 days. The ligation

Figure 4.4 Diagnostic digests of pmABR / pmABF

a.



b.

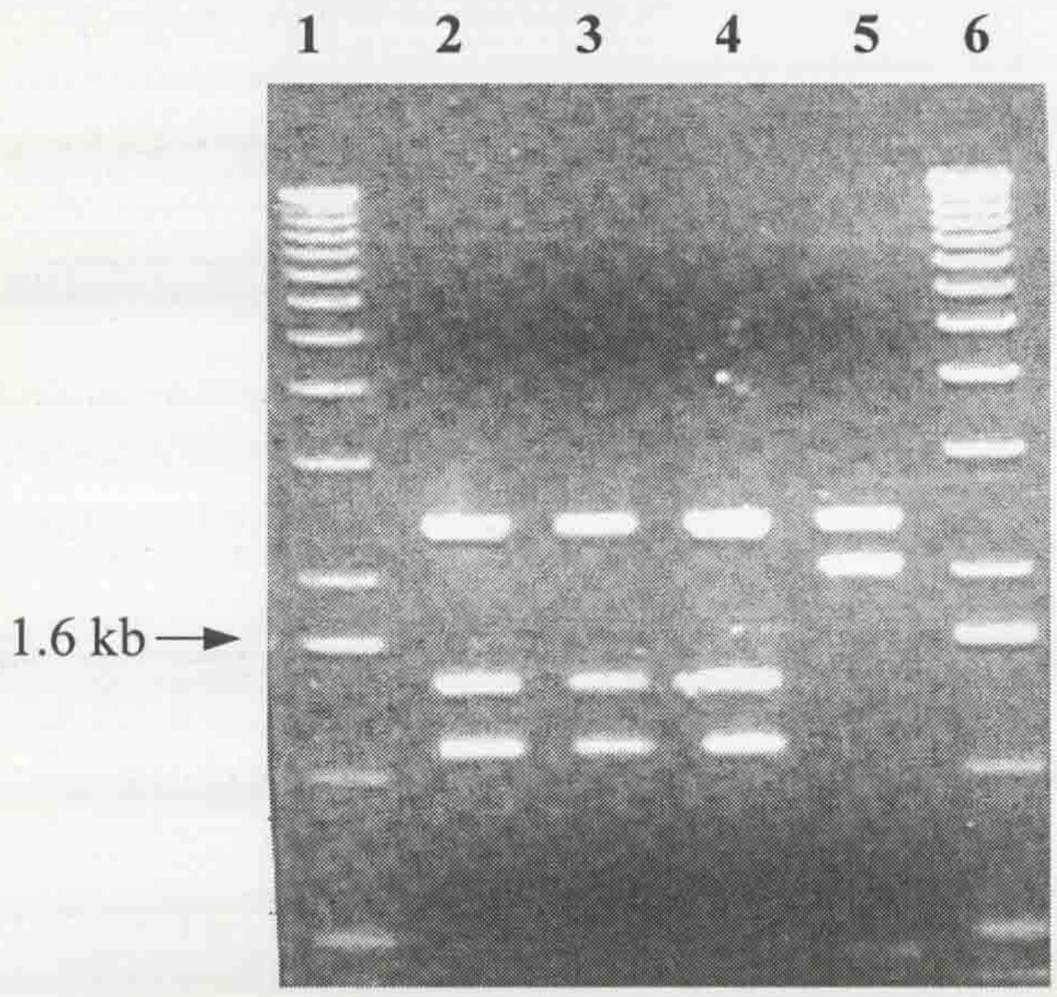


Figure 4.5a shows a schematic diagram indicating the two possible orientations of pmABF/pmABR including the *Bgl*II sites used for diagnosis. Figure 4.5b shows the results of the *Bgl*II digests. Lanes 2-4 contain the two bands of 1.1 kb and 1.4 kb predicted in the schematic diagram of pmABF. Lane 5 contains the bands of 2.0 kb and 0.5 kb predicted in the schematic diagram of pmABR. Lanes 1 and 6 contain molecular weight ladder.

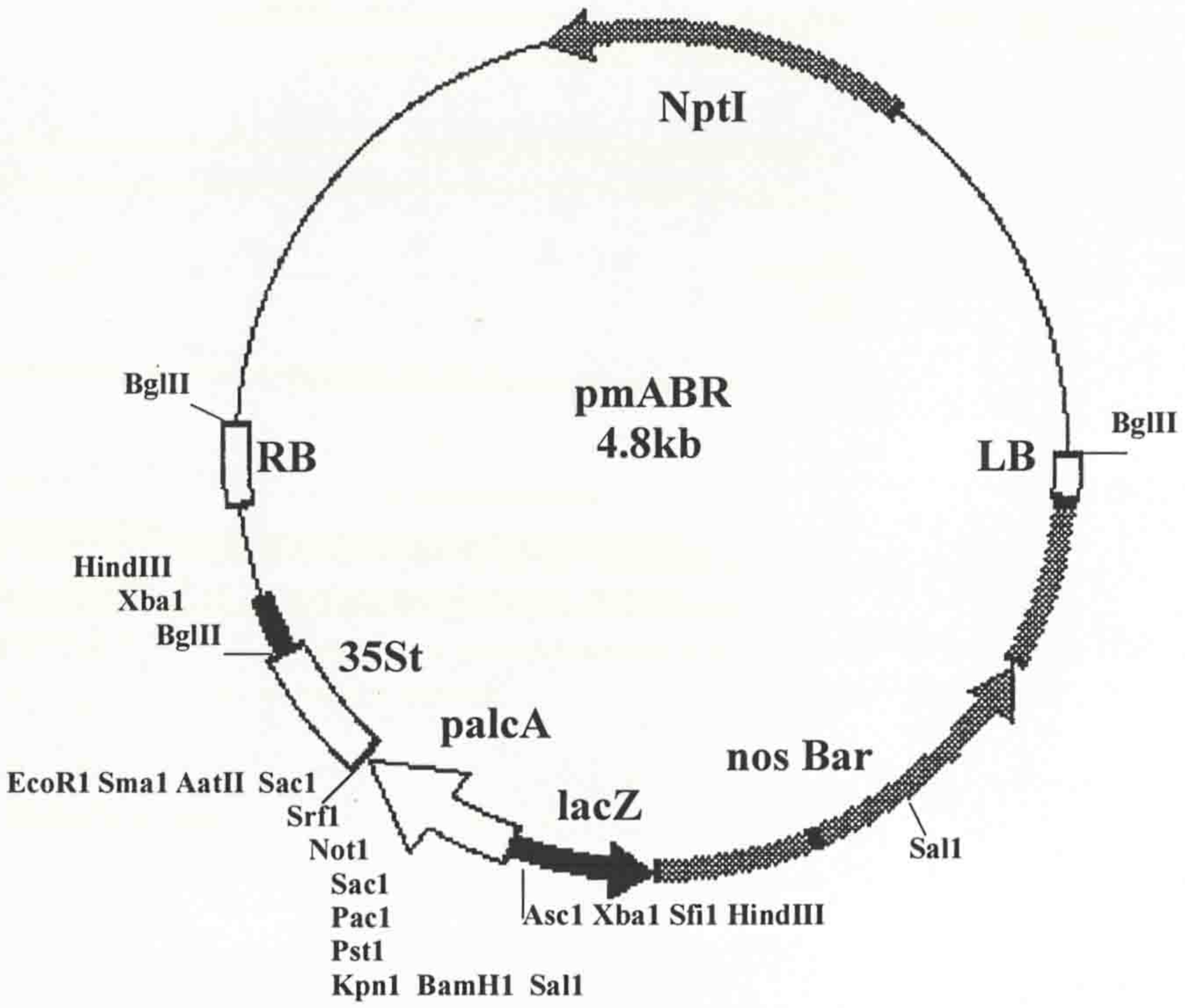
was transformed into *E. coli*, transformants were selected on LA(kan) and plasmid DNA was recovered. Two samples were verified by restriction analysis (Figure 4.4) and sequencing as the new plasmids pmABR and pmABF (Figure 4.5).

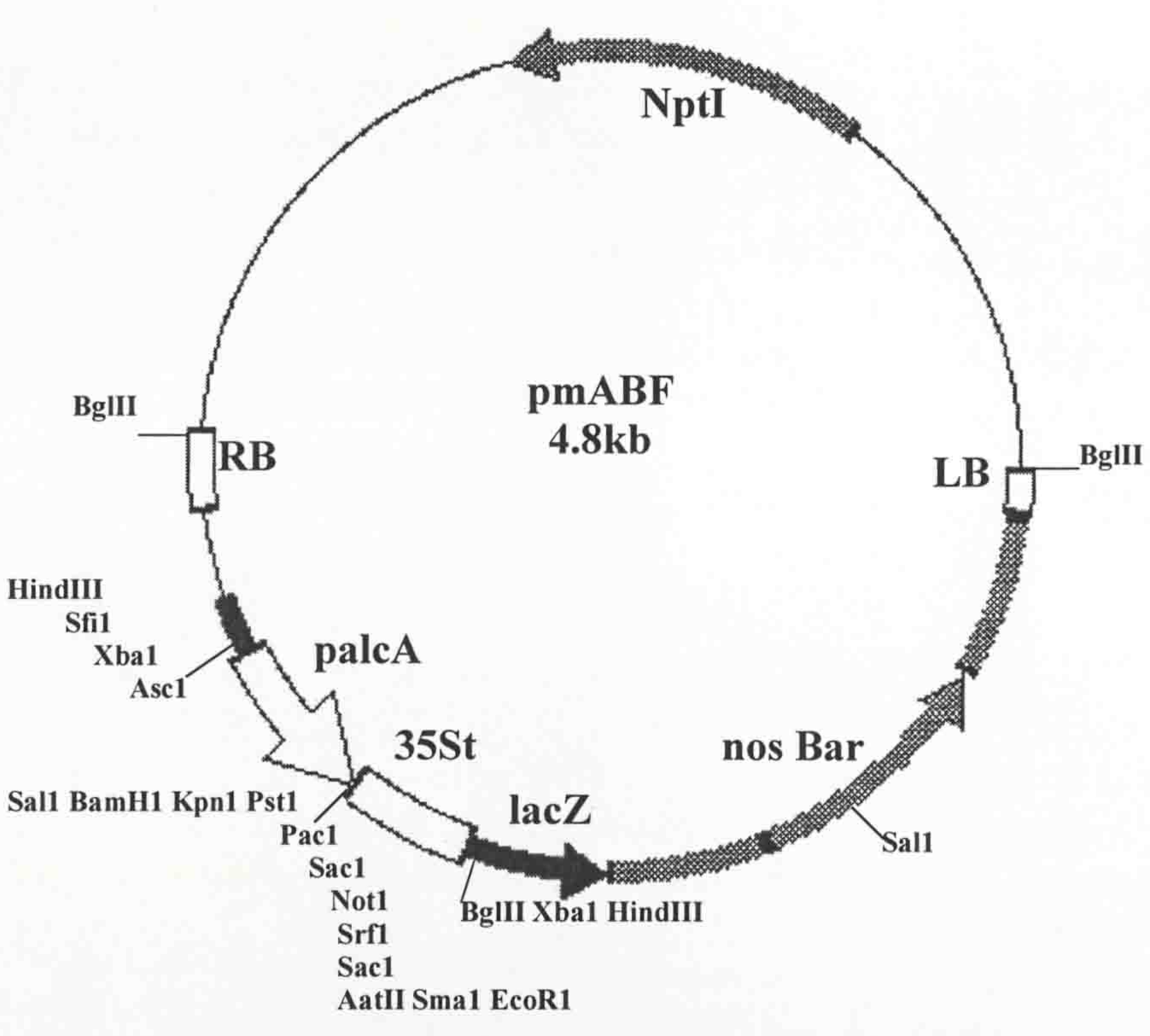
4.3 Step 2: Cloning the *csr1/epsp* gene sequences into pmABR

4.3.1 Cloning the *csr1* gene sequence

Primers were designed to amplify the first 828 base pairs of the coding sequence of *csr1*, the gene encoding acetolactate synthase in *A. thaliana* (ALSFOR/ALSREV Appendix A). PCR was carried out from *A. thaliana* genomic DNA and the product purified by gel extraction. The PCR product was cloned into pGEM®-T Easy (Promega) by TA cloning, transformed into *E. coli*, and transformants were selected by blue/white selection on LA(amp). White colonies were picked and plasmid DNA was recovered. One sample was verified to contain the insert by restriction analysis. The insert was released as an *Eco*R1 fragment from pGEM®-T Easy. pmABR was linearised using *Eco*R1 and phosphatased 'in gel' to prevent re-ligation. Ligations were set up 'in gel' and carried out at 8°C overnight. The ligation was transformed into *E. coli*, transformants selected on LA(kan) and plasmid DNA was recovered. Two samples were verified to contain the new plasmids pmABR:ALSS and pmABR:ALSAS by restriction analysis (Figure 4.6b), PCR (Figure 4.6c) and sequencing.

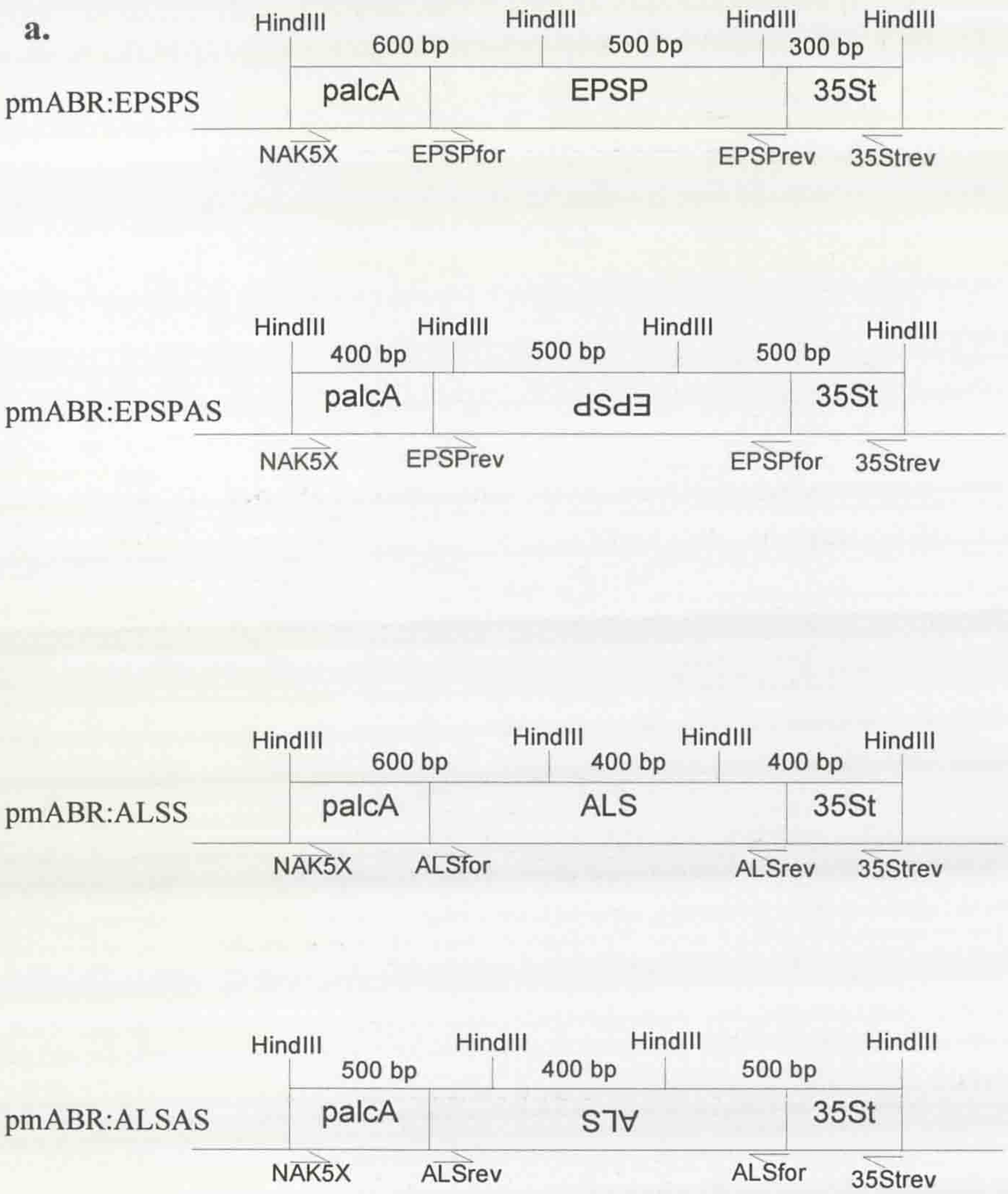
Figure 4.5 pmABR and pmABF





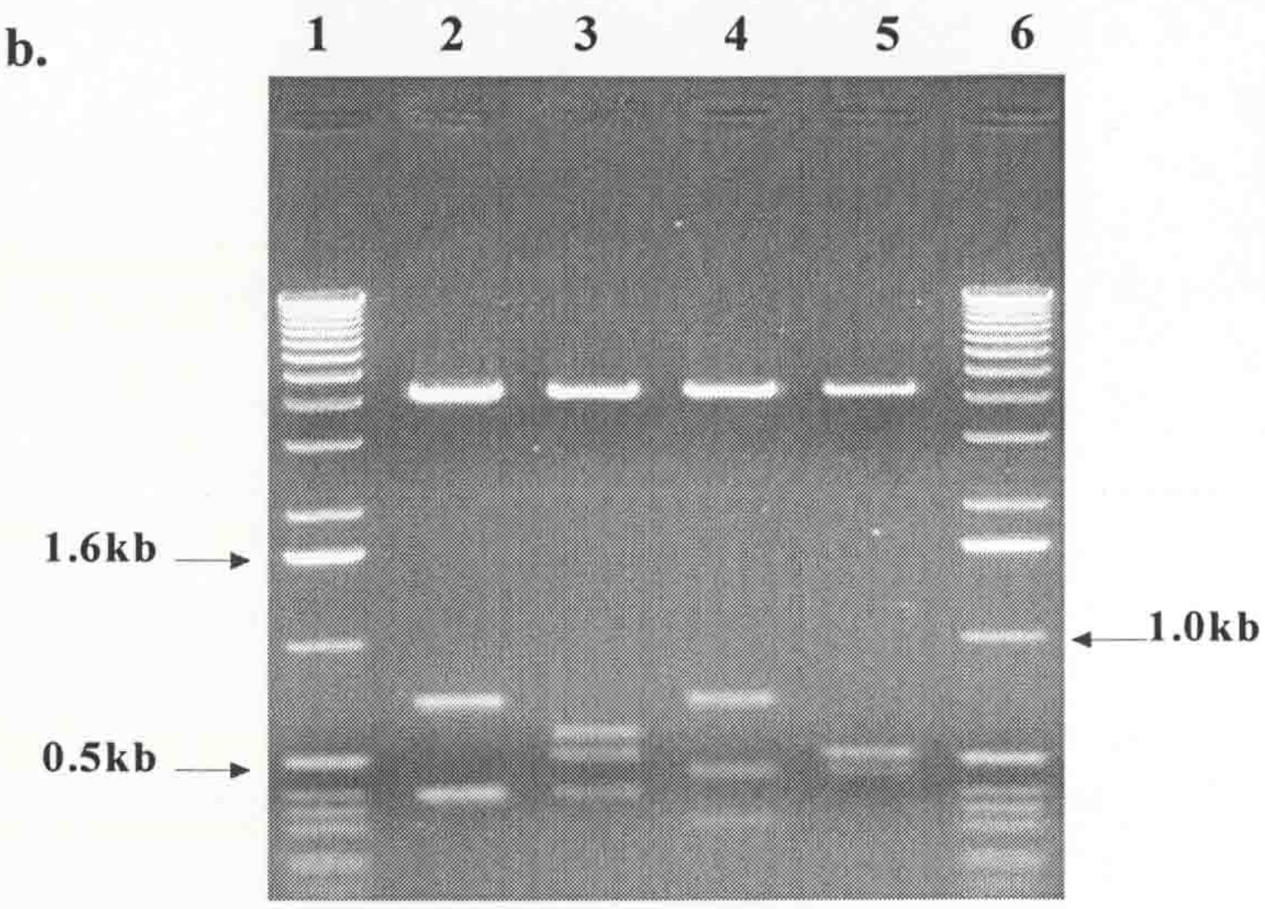
Both pmABR and pmABF originate from pG-/- with the *alcA* cassette from pAL2 cloned into the polycloning site. The orientation of the *alcA* cassette differs between the two plasmids. In pmABF, expression from *alcA* is in the forward orientation with respect to the bar selectable marker; in pmABR it is in the reverse orientation. The *alcA* cassette and polycloning site were checked by sequence analysis. pmABR was used in all further studies.

Figure 4.6 Diagnostic digests and PCR of pmABR:EPSP/ALS



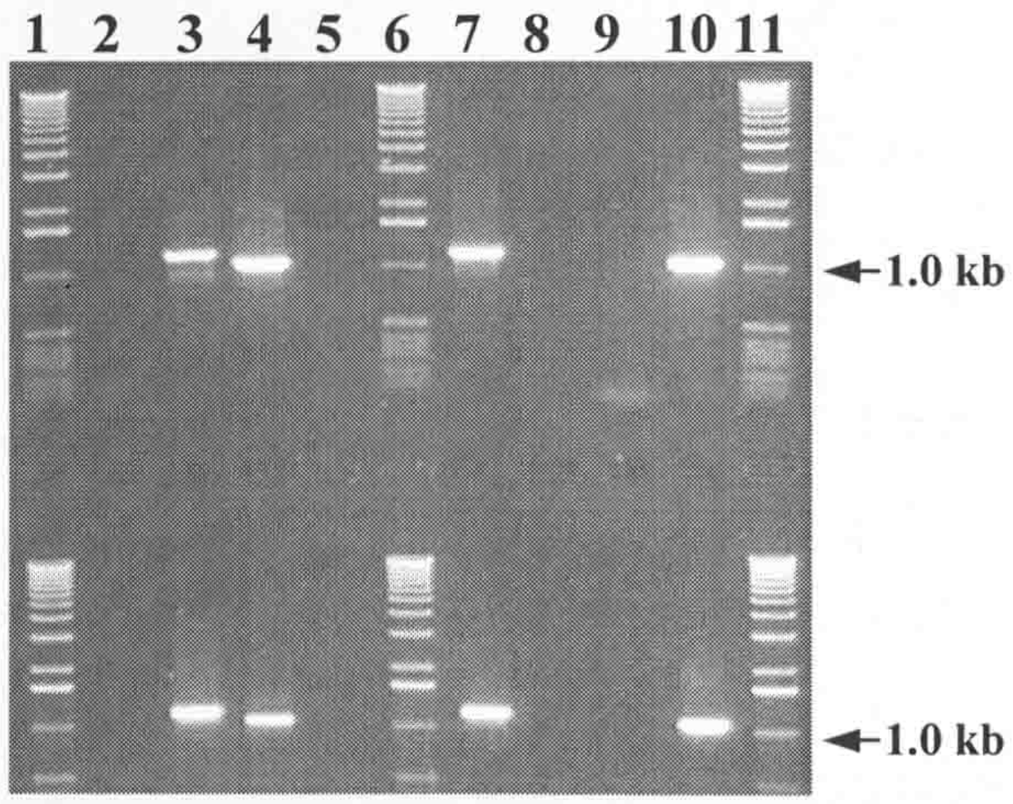
Schematic diagram illustrating the sizes of *Hind*III fragments generated by diagnostic digests. The upper diagram in each pair represents the partial sense construct and the lower diagram represents the antisense construct. The annealing sites of the primers used for diagnostic PCR are also shown. The results of diagnostic digests and PCR are shown in Figure 4.6b and c.

b.



The results of *Hind*III diagnostic digests of pmABR: EPSPS/ALS. Lane 2 contains pmABR:ALSS, lane 3 contains pmABR: ALSAS, lane 4 contains pmABR EPSPS and lane 5 contains pmABR: EPSPAS. Lanes 1 and 6 contain molecular weight ladder. The bands generated by digestion are the same sizes as predicted in Figure 4.6a.

c.



Diagnostic PCR of pmABR:EPSP/ALS. Lanes 2 and 7 contain the results of PCR using NAK5X and EPSPfor/ALSfor in the top and bottom half respectively. Lanes 3 and 8 contain the results of PCR using NAK5X and EPSPrev/ALSrev in the top and bottom half respectively. Lanes 4 and 9 contain the results of PCR using 35Strev and EPSPfor/ALSfor in the top and bottom half respectively. Lanes 5 and 10 contain the results of PCR using 35Strev and EPSPfor/ALSfor in the top and bottom half respectively. Primer annealing sites can be seen in Figure 4.6a. Lanes 1,6, and 11 contain molecular weight ladder. Both orientations of each gene have been successfully cloned.

4.3.2 Cloning the *epsp* gene sequence

Primers were designed to amplify the first 801 base pairs of *epsp* the gene encoding enol-pyruvyl-shikimate-3-synthase in *A. thaliana* (EPSPFOR/EPSPREV Appendix A). PCR was carried out from a cDNA pool generated by rtPCR first round synthesis by Dr KP Croft. The product was purified by gel extraction, cloned into pGEM®-T Easy (Promega) by TA cloning, transformed into *E. coli*, and transformants were selected by blue/white selection on LA(amp). White colonies were picked and plasmid DNA was recovered. One sample was verified to contain the insert by restriction analysis. The insert was released from pGEM®-T Easy as an *Eco*R1 fragment. pmABR was linearised using *Eco*R1 and phosphatased 'in gel' to prevent re-ligation. Ligations were set up 'in gel' and carried out at room temperature overnight. The ligation was transformed into *E. coli*, transformants selected on LA(kan) and plasmid DNA was recovered. Two samples were verified to contain the new plasmids pmABR:EPSPS and pmABR:EPSPAS by restriction analysis (Figure 4.6b), PCR (Figure 4.6c) and sequencing.

as the beginning of the modern era, whereby *Arabidopsis* became a major model organism for molecular and physiological research.

The suitability of *Arabidopsis* as a model organism is based upon a number of favourable characteristics. *Arabidopsis* has a small genome size, relative to the other flowering plants. The total genome is 125 Mb (The *Arabidopsis* Genome Initiative, 2000). This is substantially smaller than the other important model plant systems, for instance tobacco (1,600 Mb), maize (2,000 Mb) and wheat (5,900 Mb) (Meyerowitz and Pruitt 1985; Bevan, 1999). The small genome size makes map based cloning and the production of genomic libraries significantly easier. Korneef *et al*. (1983) used many of the known mutations to generate a genetic map containing linkage groups for each of the five chromosomes. Since then a number of restriction fragment length polymorphisms (RFLPs), simple sequence length polymorphisms (SSLPs), cleaved amplified polymorphic sequences (CAPSs) and expressed sequence tags (ESTs) have been added. The small genome size, along with the availability of previous physical and genetic mapping data lead to *Arabidopsis* being chosen as the first flowering plant to have its genome completely sequenced. Flowering plants diverged relatively recently and it is expected that sequence conservation across the angiosperms is still high. Genes identified in *Arabidopsis* will therefore also be present in many other more economically important species.

- **Practical considerations**

Arabidopsis has many characteristics that lend itself toward scientific study. The commonly used ecotypes Columbia and Landsberg were isolated as rapid flowering ecotypes from European populations. The plants can be grown on soil, tissue culture media and hydroponics. They can be grown under fluorescent lights or in

4.4 Generation of transgenic plants expressing 'lethal' genes

4.4.1 Transformation of constructs into *A. tumefaciens*

Both sets of constructs pmABR:ALSS/AS, pmABR:EPSPS/AS were transformed along with pSoup (Figure 3.14) into *A. tumefaciens*. Transformants were selected on on LA containing tetracycline (10μg/ml), kanamycin (50 μg/ml), and rifampicin (100μg/ml). Fully resistant colonies were checked by PCR (Figure 3.15). Resistant PCR positive colonies were grown in liquid YT containing tetracycline, kanamycin, and rifampicin and used to transform *A. thaliana* using a floral dip protocol (modified from Clough and Bent, 1998) .

4.4.2 Transformation into *A. thaliana*

Homozygous plants generated from the line SRN1 (described earlier in Chapter 3.4.1) were transformed along with AGS lines 1-3 and 4-2 and an *alcA*:Luciferase homozygous line. These lines were all generated and analysed previously by Hairul Roslan. All the lines were selected using a hygromycin selectable marker gene contained in the T-DNA (Roslan, 1999). pmABR:ALSS / ALSAS and pmABR:EPSPS / EPSPAS were transformed into SRN T2 and T3 plants and T2 plants from the AGS lines and *alcA*:Luciferase plants. Multiple transformations were undertaken in order to generate large amounts of T0 seed for primary selection.

4.4.3 Selection of putative T0 transformants

Putative primary (T0) transformants were grown on soil and selected by spraying with Harvest™. Seedlings were grown for approximately 10 days until a lawn of plants appeared. Plants were sprayed a maximum of three times with six to nine day

Table 4.1 Segregation analysis of 'lethal' knockout T1 lines

Line		Total	Resistant	Sensitive	p (3:1)
ALSS	6	89	71	18	> 0.2
	9	85	56	29	> 0.01
	10	77	51	26	> 0.05
ALSAS	1	86	64	22	> 0.99
	3	92	66	26	> 0.2
	4	9	7	2	> 0.99
	AGS1-3	58	37	21	> 0.05
EPSPS	3	87	64	23	> 0.8
	4	78	59	19	> 0.5
	5	38	27	11	> 0.5
	6	44	27	17	> 0.01
	7	44	32	12	> 0.5
	8	29	19	10	> 0.05
	11	76	55	21	> 0.5
	15	68	42	26	> 0.01
	19	78	49	27	> 0.01
	AGS4-2	87	82	5	> 0.99 (15:1)
EPSPAS	2	71	45	26	> 0.01
	3	12	7	5	> 0.05
	4	58	38	20	> 0.05
	9	29	22	7	> 0.99
	10	63	46	17	> 0.5
	AGS1-3	49	40	9	> 0.2
	*alcA*LUC 1	48	30	18	> 0.05
	2	38	29	9	> 0.5
	3	44	31	13	> 0.2

Segregation analysis was carried out by spraying with Harvest™. Statistical analysis was performed using the χ^2 test. The table shows the results from the 25 T1 families that were confirmed to contain plants segregating in a 3:1 (resistant : sensitive) ratio indicating the presence of a single T-DNA insert, and the single line AGS4-2 EPSPS that contained plants segregating in a 15:1 ratio, suggesting the presence of two independent T-DNA inserts. Resistant plants were retained from each line and allowed to self fertilise for the generation of T2 progeny. The AGS4-2 EPSPS T2 progeny were further analysed by segregation analysis (Table 4.2).

intervals between spraying. Putative transformants were identified after three weeks as healthy green plants amongst bleached non-transformants. A transformation frequency of between 1/2000 and 1/4000 seeds was typical. These survivors were grown to between the 6 and 8-leaf stage and re-potted individually. Individual plants were kept apart using Arasystem tubes (obtained from Lehle seeds) and allowed to self fertilise. Once siliques had matured and begun to dry out, the plants were uprooted and placed on Whatman® 3MM paper to dry for seed collection. T1 seed was collected and sown on soil. T-DNA copy number was analysed by segregation analysis of the bar selectable marker by spraying with Harvest™.

4.4.4 Segregation analysis of T1 seed

T1 Seed was sown individually on soil for segregation analysis of the bar selectable marker. For each line 20-100 seed were sown and germinating seedlings selected by spraying with Harvest™. Plants were grown to the four-leaf stage before spraying with Harvest™ as described earlier. All lines giving a ratio (resistant : sensitive) of approximately 3:1 (indicating single copy number) were selected, along with one line (AGS4-2 EPSPS) which showed a 15:1 ratio indicating the presence of two segregating inserts. Any lines resulting in ratios, which differed significantly from 3:1 by χ^2 analysis, were discarded. Three lines were selected containing ALSS, three containing ALSAS, nine lines containing EPSPS, and nine containing EPSPAS (Table 4.1). The plants were grown to the eight-leaf stage and re-potted individually. Between six and eight resistant plants from each of the 24 lines were re-potted and allowed to self fertilise for seed collection. Twenty plants were re-potted for line AGS4-2 EPSPS and seed collected to verify the 15:1 ratio again by segregation analysis (Table 4.2).

Table 4.2 Segregation analysis of the double insert line AGS4-2 EPSPS

Line		Total	Resistant	Sensitive	Ratio	p
AGS4-2:EPSPS T1		87	82	5	15 : 1	> 0.99
T2	1	41	41	0	1 : 0	
	2	47	41	7	15 : 1	> 0.01
	3	41	33	8	3 : 1	> 0.2
	4	42	42	0	1 : 0	
	5	44	33	11	3 : 1	> 0.99
	6	42	42	0	1 : 0	
	7	44	44	0	1 : 0	
	8	47	47	0	1 : 0	
	9	43	36	7	3 : 1	> 0.05
	10	46	42	4	15 : 1	> 0.5
	11	43	29	14	3 : 1	> 0.2
	12	42	42	0	1 : 0	
	13	32	28	4	15 : 1	> 0.05
	14	44	36	8	3 : 1	> 0.2
	15	33	30	3	15 : 1	> 0.2
	16	43	34	9	3 : 1	> 0.2
	17	39	39	0	1 : 0	
	18	45	45	0	1 : 0	
	19	32	32	0	1 : 0	
	20	23	22	1	15 : 1	> 0.2
	21	36	26	10	3 : 1	> 0.5

Segregation analysis was carried out by spraying with Harvest™. Statistical analysis was performed (where possible) using the χ^2 test. The T1 family showed a clear 15:1 ratio of resistant : sensitive plants suggesting the presence of two independent T-DNA inserts. 21 of the T1 resistant plants were retained and seed was collected to analyse the T2 generation. The expected result of segregation analysis of the T2 families was a ratio of seven families containing entirely resistant plants, to four families containing both resistant and sensitive plants in a 3:1 ratio, to four families containing resistant and sensitive plants in a 15:1 ratio. The table shows that the observed families could be classified as nine entirely resistant, seven segregating in a 3:1 ratio, and five segregating in a 15:1 ratio. This confirmed that the line contained two independent T-DNA inserts that were unlinked.

4.5 Discussion

The construction of a series of plasmids, for the investigation of the potential of generating, and recovering inducible lethal mutations has been described. The plasmids contain partial coding sequence from the genes of two known herbicide targets cloned in sense and antisense orientation. Also two binary plasmids (pmABF and pmABR) containing the *alcA*:*35St* cassette have been described. These plasmids provide the means to easily and rapidly clone genes and generate transformants exhibiting ethanol inducible expression. pmABR has the advantage of reverse orientation, minimising the chance of aberrant expression if the T-DNA inserts adjacent to promoter elements. The fidelity of the plasmids was confirmed by both restriction analysis and sequencing.

Transgenic plants were produced to test the potential of generating observable phenotypic mutants upon induction. The system incorporates the *alc* switch to control expression and therefore the mutant phenotype. Phenotypes are expected to result from decreased expression of the endogenous genes caused by either direct antisense or post-transciptional silencing. Experiments were carried out on these plants to assess phenotypic alterations upon induction of the *alc* system and these are described in Chapter 5.

Transformation rates using the pGreen based vectors were again considerably lower than expected based on reported findings (Clough and Bent, 1997; Bent, 2000). This could again have been due to either the size or nature of the constructs transformed, the transformation procedure or the vectors used. No further analysis was carried out to assess this reduction.

Chapter 5

Experimental Results

5.1 Transposon tagging experimental rationale

The generation, and analysis of a set of transgenic lines, containing constructs for ethanol inducible transposon tagging was described in Chapter 3. The following experiments were aimed at validating the inducible transposition system. Further experiments would be aimed at generating plants containing single insert transposition events, and the identification of phenotypic mutants. Genotypically mixed families of plants, originating from single insert transformants, were used to test the transposon tagging system.

The primary transformants (T0) had been allowed to self-fertilise and T1 seed collected. The T1 progeny constituted a mixed population, with the tagging inserts segregating in a 1:2:1 (homozygous : hemizygous : azygous) ratio. All the plants were homozygous for SRN. Azygous T1 plants were selected out using Harvest™ and resistant plants were allowed to self-fertilise for collection of T2 seed. Each batch of T2 seed was therefore either homozygous for the tagging construct, or contained a mixed population of plants with the tagging constructs again segregating in a 1:2:1 ratio. This was confirmed by segregation analysis and both homozygous and mixed families were identified for each line.

Hemizygous plants were preferred for use in the tagging studies as they were expected to produce fewer false positives during selection for transposition events. Homozygous plants would be expected to produce streptomycin resistant offspring that contain a mixture of either double or single excision events and this would complicate the identification of plants containing single re-insertion events. The initial experiments were designed purely to assess whether transposition occurred upon induction and both the homozygous and hemizygous plants in the mixed T2 families were used for this analysis.

Plants were grown on soil for the analysis of inducible germinal transposition events. In the initial experiments mixed populations of plants were used. Homozygous and hemizygous plants were selected by spraying with Harvest™. The survivors were expected to consist of a mixture of hemizygous and homozygous plants in a 2:1 ratio. The survivors were all induced and allowed to self fertilise. Seed from all the induced plants was pooled. This seed was used to analyse excision rather than to identify phenotypes and the fact that one third of the parent plants were homozygous was therefore unimportant.

Further experiments were designed to create a set of plants containing individual transposon insertions. Plants from the mixed populations were initially selected by spraying with Harvest™. Individual surviving plants were kept apart during induction and seed was collected from each plant separately. This allowed the seed from hemizygous parents to be selected and used for phenotypic screening.

Transposition was induced according to three profiles. Early transposition events in cells giving rise to the gametes would result in many offspring inheriting the same

transposon insert. Ideally transposition would occur in cells that give rise to the gametes, but would occur late enough in development, that progeny inherit individual transposon insertions. Plants were therefore induced from three individual time points between initial bolting and seed set. The plants were allowed to self-fertilise and seed was collected for screening on tissue culture media containing streptomycin.

The cell autonomous nature of the streptomycin excision marker allows the observation of early somatic excision events in cells resulting in the cotyledons. In an attempt to observe inducible somatic excision events, T2 seed was sown on streptomycin selective tissue culture media containing ethanol. The constant supply of ethanol was expected to induce early transposition events that would be observed as green sectors on the bleached cotyledons.

5.1.1 Induction of somatic transposition on tissue culture

T2 seed was sown on Lehle media containing 1% glucose, 200 μg/ml streptomycin, 0.05-0.1% ethanol. Seed from all 16 single insert lines containing the *35S*:*Ds* constructs was analysed. As the plants were only to be scored for successful excision events homozygous seed was used where possible. Somatic transposition events were expected to result in bleached cotyledons containing green sectors. Wild type seed and T2 seed from the *35S*:SPT transformed line was used as a control to aid the identification of streptomycin resistance phenotypes.

The induction of somatic transposition on tissue culture plates yielded no variegated plants. Both induced and uninduced plants showed a typical bleaching response indicating sensitivity to streptomycin (similar to that shown in Figure 5.1b). Between

200 and 400 germinating seeds were analysed from each line.

5.1.2 Induction of germinal transposition on soil.

Seed from the mixed T2 families was sown on soil and selected by spraying with Harvest™. The surviving plants contained a mixture (ratio 2:1) of hemizygous and homozygous plants. Primary bolts were removed from all the plants to induce secondary bolting. Transposition was induced in three groups of these plants at different time intervals. 'Early' induced plants were induced as soon as the primary bolts were removed. 'Middle' induced plants were induced as the secondary bolts reached around 5-10 cm. 'Late' induced plants were induced as the first siliques started to form. The plants were induced by watering with 1% ethanol every 4-5 days. Induction was continued until the initial siliques began to mature.

Seed was collected from the induced plants and pooled together for each line and each induction profile. The induction was repeated in a second set of mixed plants but seed was collected individually so that the progeny from induced hemizygous parents could be used for further study of single re-insertion events. All the seed was allowed to dry for 1-2 weeks before analysing excision events on streptomycin selective media.

The seed was sterilised and plated out on GM media containing 1-2% glucose, 200 μg/ml streptomycin. The seed was germinated under normal growth room conditions with a 16/8 light cycle. Successful induction of transposition was expected to result in green resistant plants amongst a background of bleached sensitive plants. T2 seed from the *35S*:SPT transformed line was also sterilised and plated out to help identify resistant phenotypes.

Figure 5.1 Streptomycin selection of induced transposon tagging lines

a.

b.

c.

Seed was sown on GM media containing 200 μg/ml streptomycin, 10 mg/ml glucose. Figure 5.1a shows wild type seed and illustrates the typical sensitive bleaching response. Figure 5.1b shows a typical plate for seed from the induced transposon tagging lines, in this case seed from B5-2 induced according to the 'early' profile. Figure 5.1c shows seed from *35S*:SPT 6. This line segregates in a 3:1 ratio with respect to the bar gene and so is expected to only contain a single copy of the *35S*:SPT T-DNA. It can also be seen to segregate in a 3:1 ratio with respect to streptomycin resistance.

The induction of transposition in soil grown plants yielded no streptomycin resistant progeny. Between 5,000 and 10,000 seeds were sown from each of the three induction profiles for each of the sixteen transformed lines. The seed from the *35S*:SPT line showed a resistant phenotype that segregated along with the Harvest™ resistance as seen from segregation analysis (Table 3.4). Figure 5.1 shows typical selective plates containing germinants from wild type, induced tagging lines, and the *35S*:SPT line.

5.2 Expression of 'lethal' constructs

The generation of a set of lines of transgenic plants, containing the 'essential' genes *csr1* and *epsp*, cloned in partial sense and antisense orientation, downstream of the *alcA* promoter, was described in Chapter 4. The following experiments aimed to use these lines to assess the potential of the *alc* switch for creating and recovering transgenics containing potentially lethal constructs.

The primary transformants (T0) had been allowed to self-fertilise and T1 seed collected. The T1 progeny constituted a mixed population with the *alcA* constructs segregating in a 1:2:1 (homozygous : hemizygous : azygous) ratio and this was confirmed by segregation analysis (Table 4.1). All the plants were homozygous for the SRN construct. Azygous plants were selected out during segregation analysis and resistant T1 plants were allowed to self-fertilise for collection of T2 seed. This T2 seed was therefore expected to be either homozygous for the *alcA* construct, or to

contain a mixed population of plants segregating in a 1:2:1 (homozygous : hemizygous : azygous) ratio. T2 seed was used in all the experiments described.

The ratio of mixed T2 families to homozygous T2 families was expected to be 2:1 as these would have come from parent plants either hemizygous or homozygous and these were in a 2:1 ratio in the T1 plants. The batches of T2 seed were not segregated for resistance to Harvest™ but were assumed to consist of a mixture of segregating and homozygous populations. For this reason a number of T2 families from each original transformed line were used.

Observable alterations in phenotype were scored relative to wild type plants grown in similar conditions. However there is a slight possibility that differences in seed quality may bias these results. Two thirds of the T2 families were expected to contain a segregation of homozygous / hemizygous / azygous plants and using these mixed populations provided an azygous internal control for some of the lines. It was expected that any observable phenotypes would follow the segregation of the transgenes and therefore the genotypically mixed families would be identifiable as containing either a 1:2:1 or a 3:1 phenotypic ratio.

Plants were assayed on both soil and tissue culture media. The inhibition of amino acid biosynthesis affects actively growing tissue and it was expected that the timing of induction may be crucial in identifying recoverable phenotypes. Consequently soil-grown plants were induced at two developmental stages. Plants on tissue culture media were grown in the continuous presence of ethanol. Disruption of *csr1* and *epsp* continuously from germination was expected to result in a severe or lethal phenotype. Plants were also grown on soil before being induced and scored for

greenhouses. Callus and cell suspensions can be generated and re-differentiated into plantlets.

Seeds germinate within a few days, after stratification. Mature plants can be obtained within four to five weeks. The mature plants are small and can be grown in close proximity. They have a minimum generation time of around six weeks and can produce over 10,000 seeds per plant. Once dried, the seeds remain viable for years. *Arabidopsis* self fertilises naturally but it is relatively easy to cross fertilise plants by hand.

Transformation protocols for *Arabidopsis* using the T-DNA from *Agrobacterium tumefaciens* were established by An *et al*. (1986); and Lloyd *et al*. (1986). These protocols relied on the transformation of explants and involved long tissue culture stages. Growth and regeneration of callus on tissue culture is costly and time consuming and can produce somaclonal variations. Feldmann and Marks (1987) described a protocol for the transformation of seeds, which avoided tissue culture stages. Germinating seed was co-cultivated with *A. tumefaciens* and transformants were identified amongst the progeny of the germinating plants after self fertilisation. In 1994, Chang *et al*. and Katavic *et al*. described *in planta* transformation procedures. Inflorescence shoots were removed and the wounds were inoculated with *A. tumefaciens* before allowing the plants to set seed.

In 1993, Bechtold *et al*. reported the transformation of whole plants using vacuum infiltration of an *A. tumefaciens* suspension. This dramatically increased the number of plants that could be inoculated and resulted in high frequencies of transformation. Vacuum infiltration became a standard procedure for the transformation of

increased / decreased sensitivity to herbicides targeted to the *csr1* and *epsp* gene products. The results of these experiments are described and discussed below.

5.2.1 Induction on soil

T2 seed from all 24 single insert lines was grown on soil. The seedlings were not selected for Harvest™ resistance. The T2 families were expected to consist either entirely of homozygous plants, or of a mixed population of homozygous / hemizygous / azygous plants. Seed from two individual T2 families was grown for each line. The plants were induced at two different developmental times. 'Early' induced plants were induced around the four-leaf stage (1-2 weeks), and 'Late' induced plants around the 6-leaf stage (2-3 weeks). The plants were induced by watering every 3-5 days with 1% ethanol. Induction was continuous in both cases for around three weeks. Induction of the transgene was expected to produce plants showing a range of stunted phenotypes with altered growth characteristics. Phenotypes were scored relative to wild type plants in similar conditions and the putative azygotes in the mixed populations. Plants were grown under normal growth room conditions and the experiments were repeated in both 16/8 and 24hr light cycles.

No significant difference in phenotype was seen between induced and uninduced plants, amongst the mixed populations, or between the transformants and wild type plants for any of the 24 lines. The experiments repeated on 16/8 and 24 hour light cycles produced similar results. Minor differences were observed in some instances between induced and uninduced plants but these were generally attributed to either a slight direct toxicity of the ethanol or the difference in growth conditions between induced and uninduced plants. (The induced and uninduced plants were grown in

different rooms to avoid accidental induction by ethanol vapour.)

5.2.2 Induction on tissue culture

T2 seed was sown on Lehle tissue culture media containing 0.05-0.1% ethanol. As this media contained no sucrose, seed sterilisation was not necessary. Again plants were not selected for Harvest™ resistance. Two individual families of T2 seed were analysed from each line. Continuous induction was expected to cause severe or lethal phenotypes. Phenotypes were scored relative to wild type plants in similar conditions and the presumed azygotes amongst the expected mixed populations. The plants were grown under normal growth room conditions on a 16/8 light cycle.

No observable difference in phenotype was seen amongst induced populations of any of the 24 transformed lines, or between the transformed lines and wild type plants.

5.2.3 Herbicide sensitivity

To assess whether abnormal phenotypes were not observed because the phenotypes were not sufficiently strong to be directly observable, the families of T2 plants were assessed for altered sensitivity to herbicides targeted to the specific gene products of *csr1* and *epsp*.

5.2.3a Glyphosate sensitivity of EPSPS/EPSPAS plants

T2 plants were grown to around the 4 to 6-leaf stage without selection and induced by watering with 1% ethanol. Induction was continued every 3-4 days for around three weeks. The plants were sprayed with glyphosate at 1/10, 1/20, 1/100, and 1/200 dilutions of recommended concentration (50 μg/ml) 4 days after the initial induction. These concentrations were chosen after spraying wild type plants with a series of

dilutions of glyphosate. The 1/100 dilution was the highest concentration to inhibit growth but not kill the plants immediately (data not shown). Increased sensitivity was scored relative to induced T2 plants untreated with glyphosate, uninduced T2 plants treated with glyphosate and uninduced, untreated T2 plants. All the plants were grown in normal growth room conditions on a 16/8 light cycle.

5.2.3b Ally® / Harmony® sensitivity of ALSS/ALSAS plants

T2 plants were grown to around the 4 to 6-leaf stage without selection and induced by watering with 1% ethanol. Induction was continued every 3-4 days for around three weeks. The plants were sprayed with Ally® or Harmony® (Kindly supplied by Dupont Agrochemicals) at the recommended concentration (Ally® 70 $\mu g/cm^2$, Harmony® 243 $\mu g/cm^2$) and 2x, 1/10, 1/50, and 1/100 dilutions 3-4 days after the initial induction. Increased sensitivity was scored relative to induced T2 plants untreated with herbicide, uninduced T2 plants treated with herbicide and uninduced, untreated T2 plants. The experiments were repeated and the herbicides applied by watering directly into the soil. All the plants were grown in normal growth room conditions on a 16/8 light cycle.

No observable differences in sensitivity to the herbicides were seen either amongst the T2 families of plants, induced or uninduced, or between families of plants and wild type plants, induced or uninduced. All the plants were noted to apparently be insensitive to both Ally® and Harmony® even at 4x the recommended concentration.

Figure 5.2 Initial phenotype in AGS4-2 EPSPS T2 plants

The abnormal phenotype identified in the AGS4-2 EPSPS line. The plants on the left are wild type, in the middle are AGS4-2 EPSPS 1-5, and on the right AGS4-2 EPSPS 1-3. All the plants have been grown under identical conditions and induced for around 14 days. The abnormal phenotype can be seen clearly in AGS4-2 EPSPS 1-5 and is identified as retarded growth and discolouring and curling of leaves. A weaker abnormal phenotype can be seen in AGS4-2 EPSPS 1-3. Both these plants segregate in a 3:1 ratio for Harvest™ resistance. They are therefore expected to contain only one copy from the two original inserts.

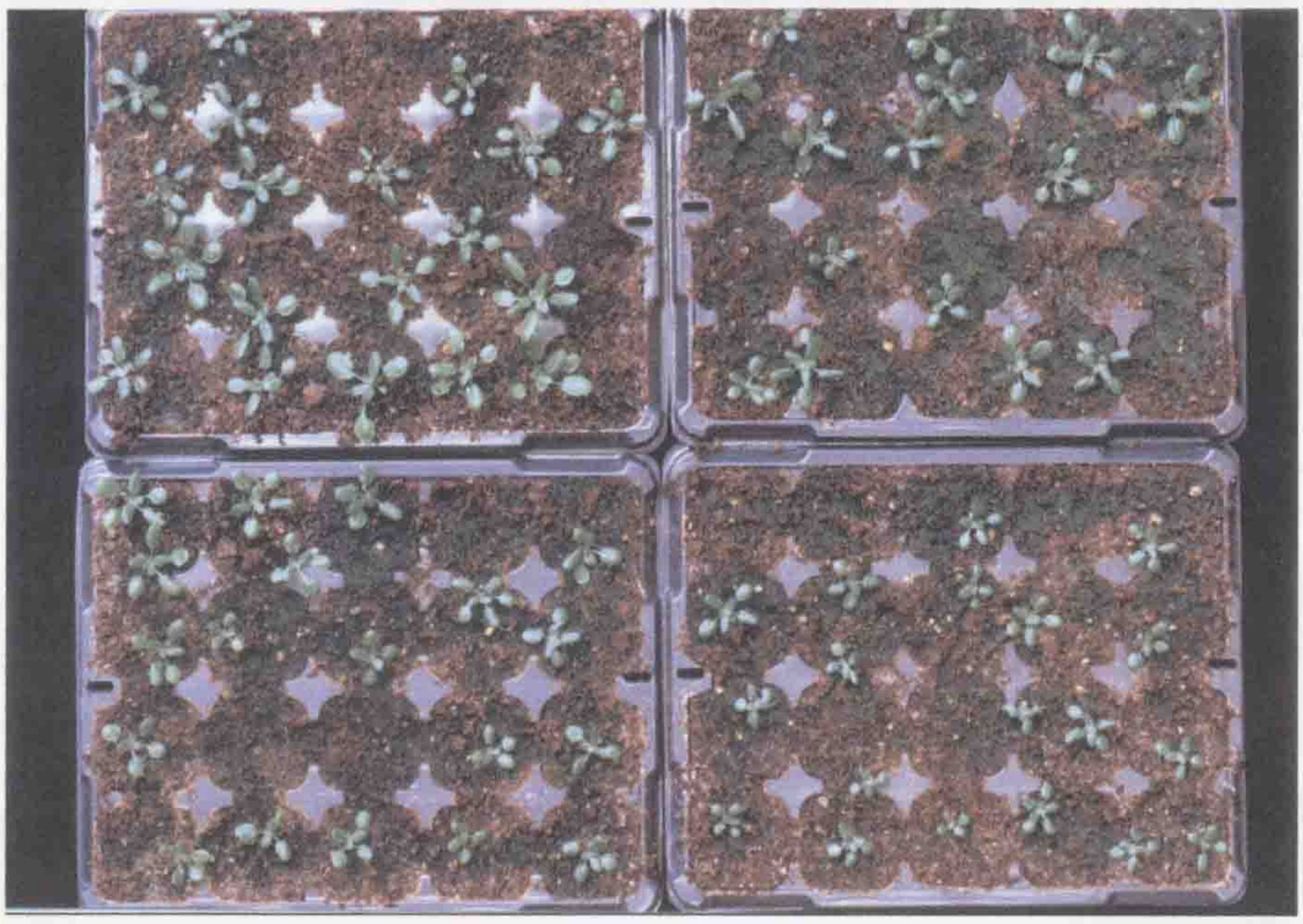
5.2.4 Possible phenotype in AGS4-2 EPSPS

A phenotypic difference was seen amongst induced T2 plants from the double insert line AGS4-2 EPSPS when compared to wild type (Figure 5.2). Growth was severely retarded, and leaves were curled and discoloured in AGS4-2 EPSPS 1-5 plants. A similar, but less extreme phenotype, with reduced growth and darkened heavy leaves was seen in AGS4-2 EPSPS 1-3 plants.

The original AGS4-2 EPSPS (T0) transformant probably contained two independent inserts of the pmABR:EPSPS construct as the T1 plants segregated for Harvest™ resistance in a 15:1 (resistant : sensitive) ratio. The abnormal phenotype was seen at different frequencies in different T2 families. These families represent a number of different genotypes due to the two T-DNA inserts in the T0 plant (for segregation analysis see Table 4.2). It was decided to analyse these further.

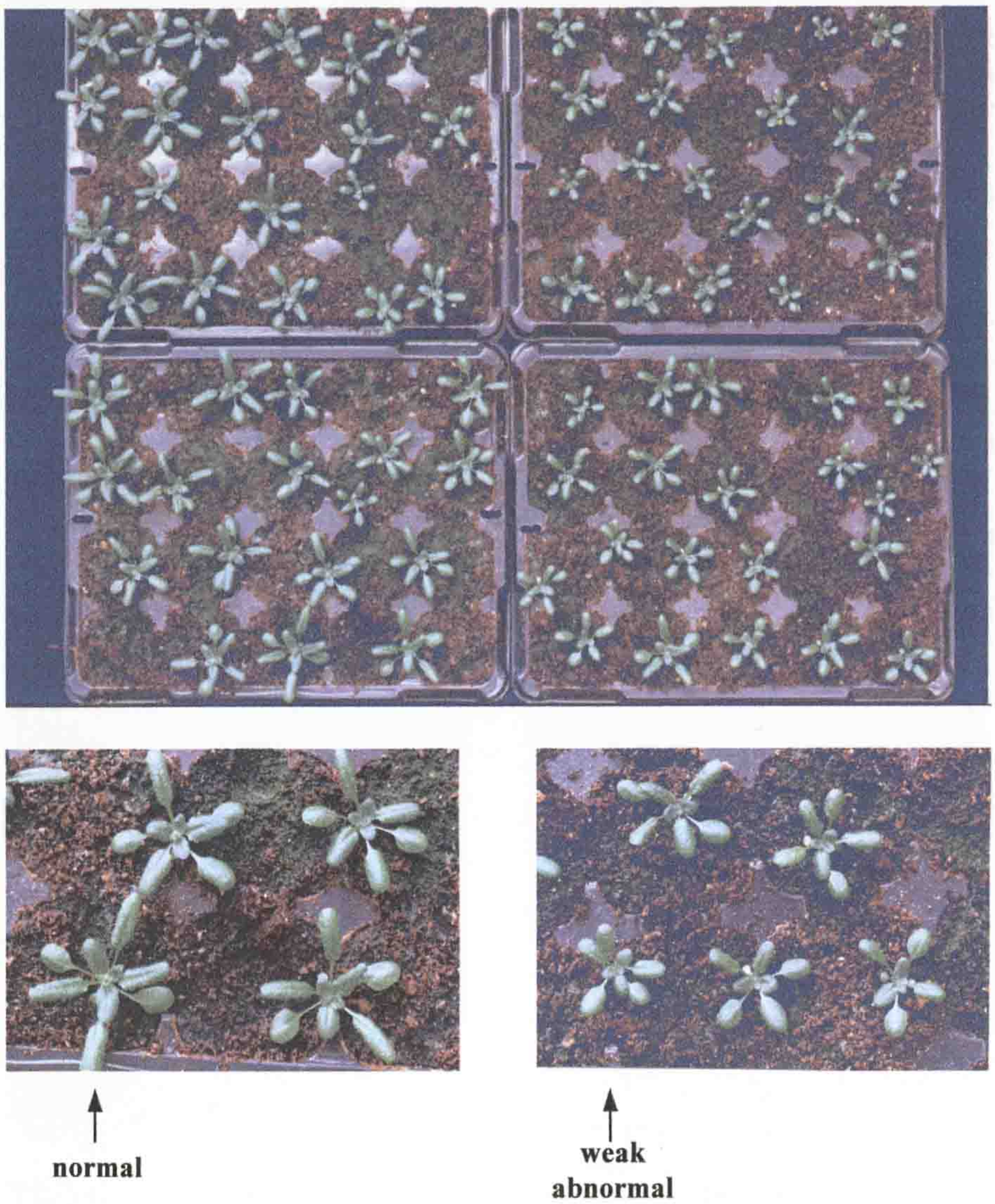
Further analysis however, suggested that the abnormal phenotype might be an artifact caused by GUS expression in the original AGS4-2 line. Induction of homozygous AGS4-2 (T3) plants not containing the EPSPS insert produced a mixture of normal and abnormal phenotypes. Figures 5.3-5.5 show AGS4-2 EPSPS (T2) families grown on soil and induced for around 10 days. A range of phenotypes including plants similar to wild type can be seen among the families. Phenotypically abnormal and normal plants can also be seen in the homozygous line AGS4-2 under identical conditions (Figure 5.6). As AGS4-2 is a homozygous line it was expected that all plants would be expressing GUS at similar levels. The observation of abnormal phenotypes in the AGS4-2 line suggests that the phenotype may be partly due to either GUS expression, or a mutation created at the insertion site of the T-DNA containing the GUS construct. Phenotypically abnormal and normal plants

Figure 5.3 Potential abnormal phenotype in AGS4-2 EPSPS 1-1



AGS4-2 EPSPS1-1 T2 plants were grown for around 3 weeks and induced by watering with 1% ethanol for 10 days. A range of normal and abnormal phenotypes are present. Phenotypically normal plants are paler and have the largest leaves. Weak phenotypically abnormal plants contain darker heavier leaves and show retarded growth.

Figure 5.4 Potential abnormal phenotype in AGS4-2 EPSPS 1-3

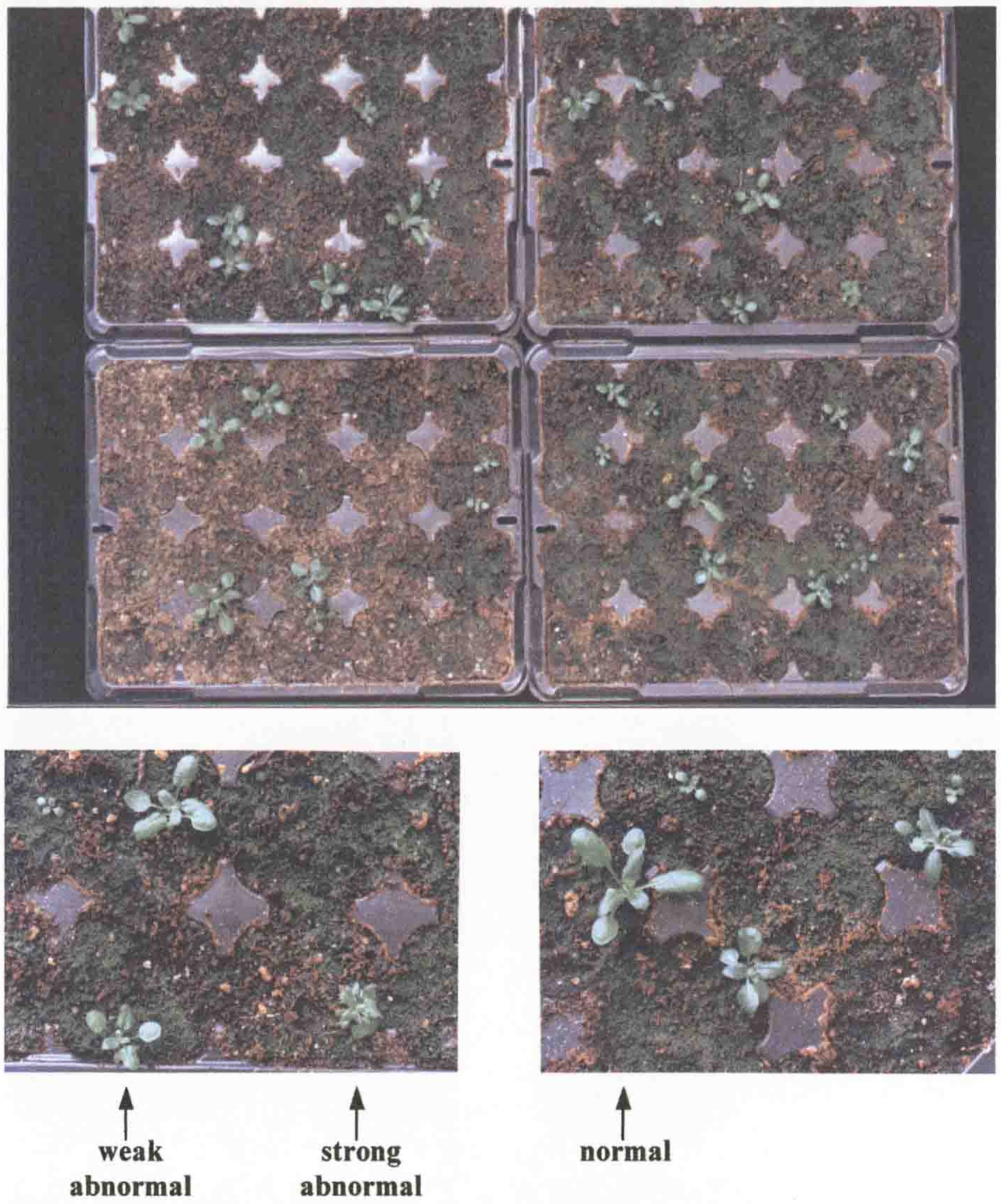


AGS4-2 EPSPS1-3 T2 plants were grown for around 3 weeks and induced by watering with 1% ethanol for 10 days. A range of normal and abnormal phenotypes are again present. Phenotypically normal plants are paler and have the largest leaves. Weak phenotypically abnormal plants have darker heavier leaves and show retarded growth.

Figure 5.5 Potential abnormal phenotype in AGS4-2 EPSPS 1-5

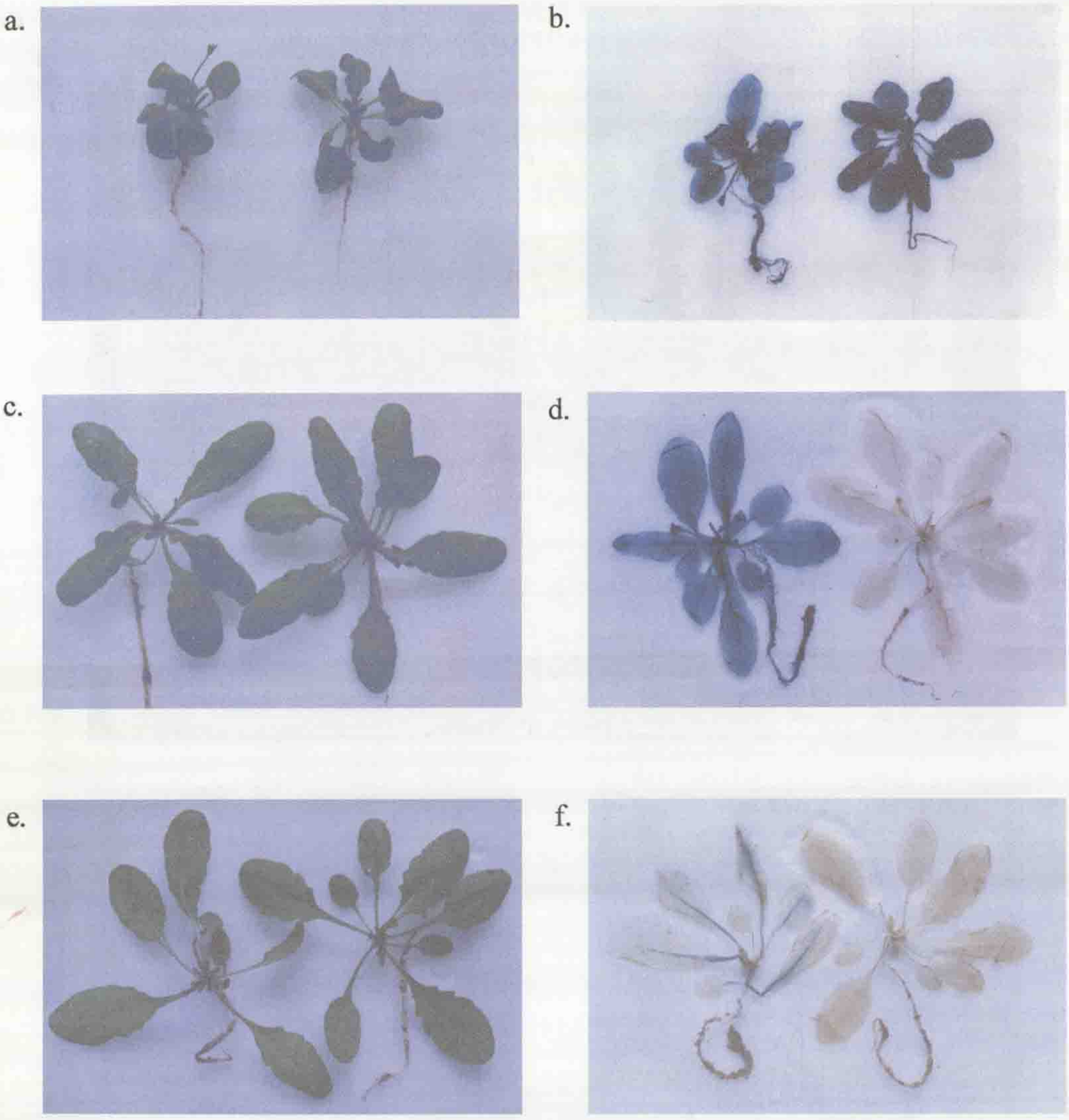
AGS4-2 EPSPS1-5 T2 plants were grown for around 3 weeks and induced by watering with 1% ethanol for 10 days. A range of abnormal phenotypes are present. Phenotypically normal plants are paler and have larger leaves. Weak phenotypically abnormal plants have darker heavier leaves and retarded growth and strong phenotypically abnormal plants have darker leaves and show severely retarded growth and curling of leaves.

Figure 5.6 Potential abnormal phenotype in AGS4-2 T3



AGS4-2 T3 plants were grown for around 3 weeks and induced by watering with 1% ethanol for 10 days. A range of normal and abnormal phenotypes are present. Phenotypically normal plants are paler and have the largest leaves. Weak phenotypically abnormal plants have darker heavier leaves and retarded growth and strong phenotypically abnormal plants have darker leaves and show severely retarded growth and curling of leaves.

Figure 5.7 Histological GUS staining of phenotypically abnormal and normal plants



Histochemical GUS staining of phenotypically abnormal and normal plants from AGS4-2 EPSPS. Plants were grown to the 4-6 leaf stage and induced constantly for 10 days. Photographs a. and b. contain typical strong phenotypically abnormal plants from AGS4-2 EPSPS1-5 (left) and AGS4-2 EPSPS1-11 (right) before and after staining. The phenotype can be identified by retarded growth and curling and darkening of the leaves. Both plants stain strongly for GUS and show high levels of expression throughout all tissues. Photographs c. and d. show induced phenotypically normal plants from the same families before and after GUS staining. Two interesting observations can be made. Firstly the GUS expression seen in the EPSPS1-5 plant (left) suggests that the phenotype is not entirely due to cytotoxicity of GUS. Secondly the EPSPS1-11 plant (right) shows no GUS expression. AGS4-2 is a homozygous line and all the plants should be homozygous for the *35S*:*alcR alcA*:GUS containing T-DNA construct. This lack of expression therefore represents the first observation of transgene silencing described during this study. Photographs e. and f. show uninduced plants before and after GUS staining. A low level of basal expression can be seen limited to the vascular tissue in the plant from EPSPS1-5. The lack of this expression in EPSPS1-11 could be due to either decreased leakiness of the *alcA* promoter or another instance of transgene silencing.

containing the *alcA*:*epsps* construct were further characterised.

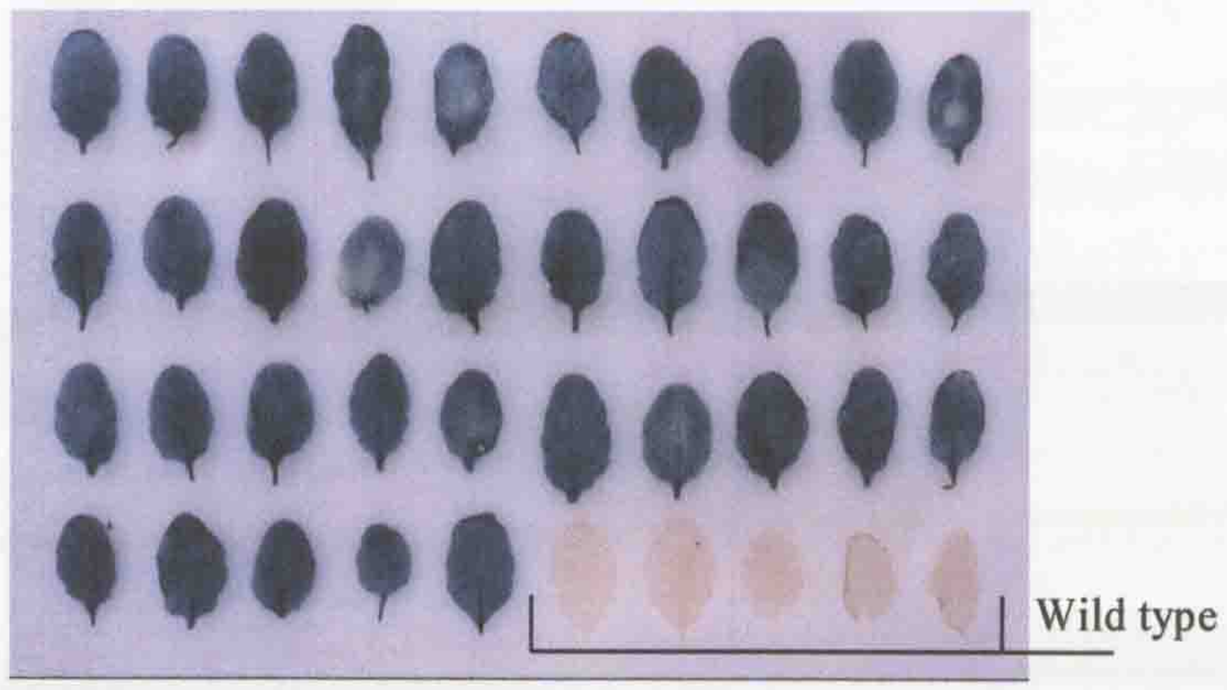
T2 plants from AGS4-2 EPSPS 1-5 and 1-11 both showed a mixture of strongly abnormal, abnormal and normal phenotypes upon induction. Induced plants with strongly abnormal phenotypes, induced normal plants, and uninduced normal plants were histochemically stained to analyse GUS expression (Figure 5.7). The results of staining show that whilst phenotypically abnormal plants have high levels of GUS expression, the phenotype cannot be entirely due to GUS toxicity. The plants from AGS4-2 EPSPS 1-5 with a near wild type phenotype also show high levels of GUS expression. The other interesting observation in this experiment was the observation of complete silencing of GUS expression. The phenotypically normal, induced plant from AGS4-2 EPSPS 1-11 shows no GUS expression. All these plants are homozygous for both the *alc* inducible GUS marker and the constitutively expressed *alcR* gene.

Attempts were made to analyse the phenotypic segregation of a number of the AGS4-2 EPSPS T2 families. The lines had previously been subjected to segregation for Harvest™ resistance (Table 4.2) and on this basis predictions were made regarding the individual genotypes contained in each family. The plants were induced and scored phenotypically. Segregation of the phenotypes was then compared with expected values based on the predicted genotypes. The expected ratios were generated on the assumption that either: i. Both of the inserted constructs would express and create a mutant phenotype independently, ii. Only one of the two inserted constructs would be able to express and create a mutant phenotype, iii. That the mutant phenotype was dependent upon the dosage number of the inserted constructs. Plants were grown to around the six-leaf stage before induction. One leaf

Figure 5.8 Segregation analysis of GUS expression and abnormal phenotype in AGS4-2 EPSPS

a.

b.



c.

Histochemical GUS staining of leaves from AGS4-2 EPSPS plants. All the photographs contain five identically stained wild type leaves in the bottom right corner as a negative control for GUS staining. Photograph a. contains leaves from AGS4-2 EPSPS1-5 which segregated for Harvest™ resistance in a 3:1 ratio indicating the presence of only one of the two original T-DNA inserts. Photograph b. contains leaves from AGS4-2 EPSPS1-15 which segregated for Harvest™ resistance in a 15:1 ratio indicating the presence of a mixture of combinations of both inserts. Photograph c. contains leaves from AGS4-2 EPSPS1-11 which again segregates for Harvest™ resistance in a 3:1 ratio. This line is homozygous for the *35S*:*alcR* / *alcA*:GUS construct and therefore silencing of the GUS gene can again be seen in AGS4-2 EPSPS1-11. Interestingly all the silenced plants showed normal phenotypes.

was removed from each plant and stained for analysis of GUS expression (Figure 5.8). Unfortunately the phenotypes appeared neither to segregate as predicted from bar segregation analysis (linked to the *alcA* 'lethal' constructs) or GUS expression (linked to the *alcR* and *alcA*:GUS constructs). Again silencing can be seen amongst the AGS4-2 EPSPS 1-11 family and it was noted that the silenced plants all showed normal phenotypes. These plants were not further analysed.

5.3 Discussion

5.3.1 Transposon tagging studies

The results of the transposon tagging experiments suggest that the transposition system is not functional. A number of hypotheses to explain this have been postulated, based on the elements used either in the tagging constructs themselves, or in the SRN plants used for transfomation. Problems caused by the tagging constructs could include the functionality of the transposable element and the regulation of transposition by the *Ac* transposase. The plants were selected and segregated by Harvest™ resistance, and had been shown to be PCR positive at a number of regions in the constructs. The functionality of the transposable element is reliant upon both secondary structure and primary sequence. The element had been generated by PCR amplification of the *Ds* end sequences. Inability of these ends to form appropriate secondary structures or errors in the sequence may have prevented the element from transposing. The transposable elements also contained a number of non-*Ac*-derived sequences. The interaction of these with the transposon ends may affect the ability of the element to transpose. To address this problem would require complete

sequencing of the transposon inserts in all the lines and the generation of lines of plants containing sequentially different elements. This investigation was not possible within the scope of the project.

The effective regulation of transposase expression and transposition by *alcA* is dependent on both the *alc* system and transposase function. A break down in the *alc* switching system would result in the absence of inducible transposase expression and could explain the lack of transposition. Sequence errors in either the *alcA* promoter or the transposase coding sequence could result in lack of expression or the production of a non-functional protein. The *alcA* promoter had been checked in pAL2 by sequence analysis and no sequence errors were identified. The promoter had been cloned from pAL2 as a restriction fragment and spontaneous mutation was unlikely. The transposase source had also been cloned by digesting from a plasmid that was used previously for transposon tagging by another group and no problems associated with the transposase expression had been reported. The switching system was also reliant upon constitutive expression of the transcription factor AlcR. Experiments are described in Chapter 6, which further address the functionality of the *alc* switching system in the transposon tagging plants.

5.3.2 'Lethal' gene studies

Similarly the apparent lack of abnormal phenotypes in the 'lethal' gene studies indicates either a problem in generating phenotypic mutants by the means tested, or a problem in the induction system. It is entirely possible that neither of the genes used are able to generate an alteration of phenotype in *A. thaliana* when expressed in either partial sense or partial antisense. However phenotypic mutants have previously been obtained by expressing *als* in antisense orientation in potato (Höfgen *et al.*,

1995). If the problem is situated in the functionality of the *alc* switching system, plants containing a non-functional expression system would be expected not to show mutant phenotypes.

The possible phenotype seen in AGS4-2 EPSPS raised some hope for the system. Whilst it was impossible to completely attribute the phenotypic alteration to the expression of the partial sense construct, it seemed that it was not entirely generated by either GUS or ethanol toxicity, or an effect of the insertion site of the AGS4-2 T-DNA insert. Interestingly all the silenced plants showed normal phenotypes. This would be expected if the phenotype was entirely due to the GUS expression. It would also be expected if *alcR* (which controlled expression of the *alcA*:GUS construct) is silenced, therefore also preventing the induction of *alcA:epsps* expression, or if a transcriptional silencing event had targeted the *alcA* promoter situated on both the GUS and the *epsp* constructs.

The abnormal phenotype was difficult to score and sensitive to changes in growth room conditions. The combination of the background phenotype caused by the AGS insert and the silencing of transgenes may have created sufficient deviation from any expected ratio that it was impossible to assess the true nature of the mutation.

Further study of this would require the use of Southern analysis to identify the copy number of the individual T2 families and to identify and possibly segregate the two inserts. Northern analysis could be used to follow the expression of *alcR*, GUS, and *epsps* in these lines and further verify the origin of the mutant phenotype.

Arabidopsis. The method described by Bechtold *et al*. (1993) involved uprooting plants and re-potting after infiltration. Clough and Bent (1998) reported a modified whole plant transformation procedure that produced a high frequency of transformants without requiring vacuum infiltration. Silwet L-77 is used as a surfactant to infiltrate the *Agrobacteria*. Whole plants are dipped in a suspension of *A. tumefaciens* containing 5% sucrose and 0.05% Silwet L-77. Transformation frequencies of 0.5-3% of progeny seed were reported. Modifications in the T-DNA vectors used for transformation have also lead to increases in the efficiency of generating transgenic plants and these are discussed later.

- **Genome sequence information and other resources**

The recent publication of the entire genome sequence of *Arabidopsis* (The *Arabidopsis* Genome Initiative, 2000) has provided an invaluable resource for further genetic and physiological study of this organism and all other flowering plants. The total number of genes has been estimated at 25,498. The average length of these genes is estimated at 2 kb and the gene density is approximately 4.4 kb per gene. Interestingly there are estimated to be less than 15,000 different genes in *Arabidopsis*. Large numbers of tandem duplications suggest that *Arabidopsis* has gone through two polyploid stages during its evolution. The extensive gene duplication means that functional redundancy must be taken into account when studying insertional mutagenesis. At least 10% of the genome is estimated to consist of transposable elements and these make up much of the heterochromatic regions surrounding the centromeres.

The information generated by the sequencing project and the many genetic studies of recent years have lead to the development of The *Arabidopsis* Information Resource

Chapter 6

Further analysis of transgenic plants

6.1 Introduction

One possible explanation for the results discussed in Chapter 5 is that one or more of the transgenes is not being properly expressed. Silencing of the *alcR* gene in the SRN lines would prevent both the expression of the inducible transposase in the transposon tagging plants and the inducible aberrant transcripts in the lethal gene knockout plants. Silencing in any other part of the transposon tagging system (eg. the *alcA* transposase or SPT excision marker) could explain the lack of streptomycin resistant progeny, but would not explain the lack of phenotypic mutants generated by the 'lethal' constructs. Transgene silencing was initially observed in the AGS4-2 EPSPS line, where GUS expression was completely suppressed. The line contained a constitutive *alcR* construct and an *alcA*:GUS construct. Silencing of either, or both of these constructs, would result in inhibition of GUS expression.

A number of experiments were carried out in an attempt to further understand whether a silencing event could explain the results discussed in Chapter 5. The transposon tagging plants and the SRN line used for their generation were both analysed to assess if transgene silencing had occurred. The expression of the transgenes in these plants was analysed, by northern analysis and antibiotic selection. Stably transformed plants were also generated, to assess the effects of transforming a

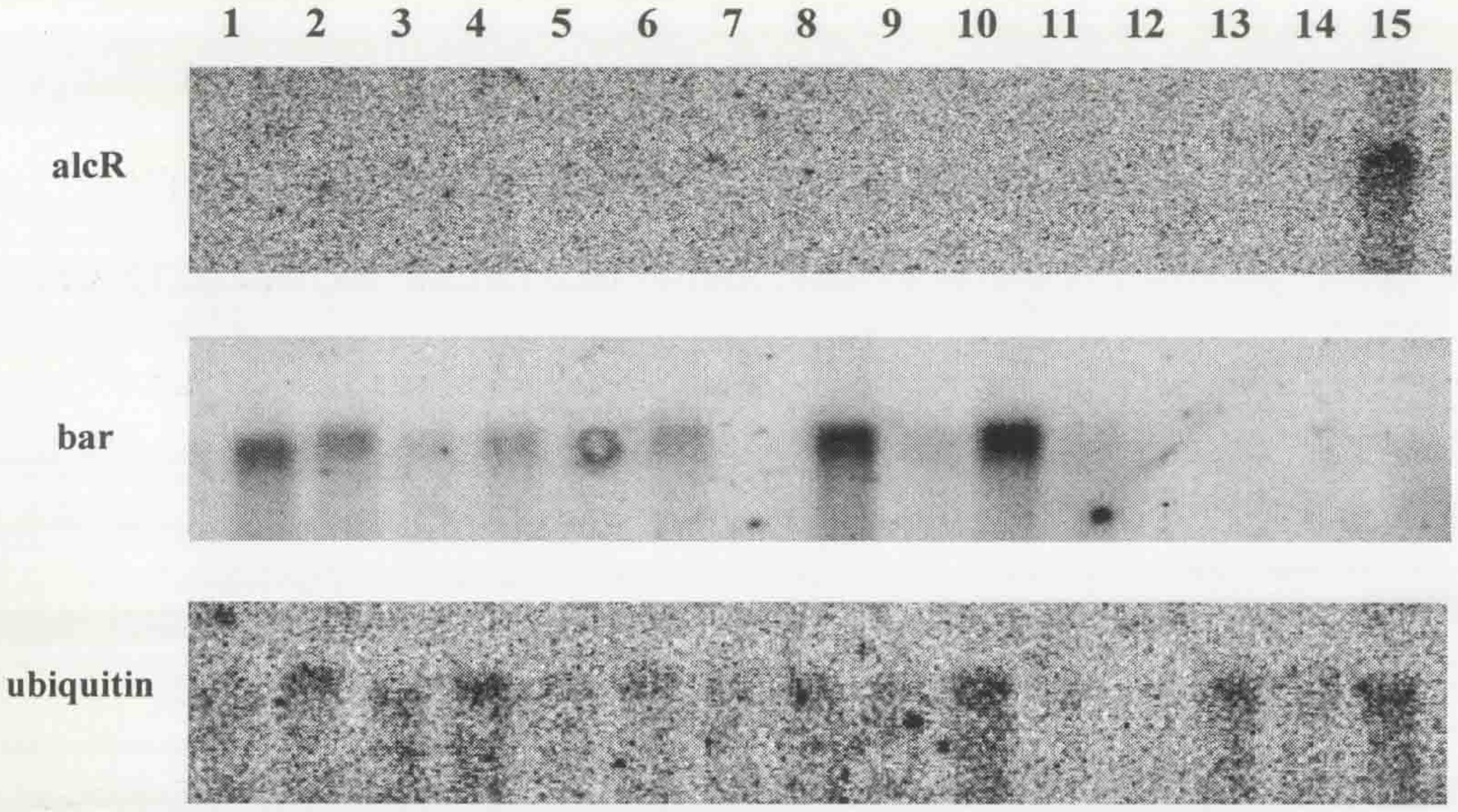
second T-DNA construct, containing the *alcA* promoter into the SRN lines. These contained the GUS reporter gene situated downstream of an *alcA* promoter and transformants were analysed by GUS staining. The hypothesis that one or more of the transgenes had been silenced was tested in all these cases and the experiments and results are described and discussed below.

The transposon tagging and 'lethal' gene lines were crossed with plants known to contain a functional copy of the *alc* system. A copy of *alcR* that has not undergone a silencing event may activate transcriptionally silenced plants. Post-transcriptionally silenced plants would be expected to silence this functional copy of *alcR* after crossing.

6.2 Northern analysis of transposon tagging plants

RNA was purified from induced transposon tagging (T2) plants. The plants were germinated on soil, induced by watering with 1% ethanol for 3-5 days and harvested. Plants were harvested at the 2 to 4-leaf stage as this was found to be optimal for RNA recovery. The expression of the bar, transposase, *alcR*, and hyg transgenes was analysed by northern blotting. Plants from all sixteen of the transposon tagging lines which had been shown to be PCR positive for the bar, transposase and *alcR* constructs (as demonstrated in Figure 3.16) were analysed. Homozygous AGS1-3 and AGS4-2 were known to contain both *alcR* and hyg constructs and were used as positive controls for *alcR* and hyg expression. Wild type plants were used as negative controls. No positive controls for either bar or transposase expression were available.

Figure 6.1 Northern analysis of the transposon tagging lines

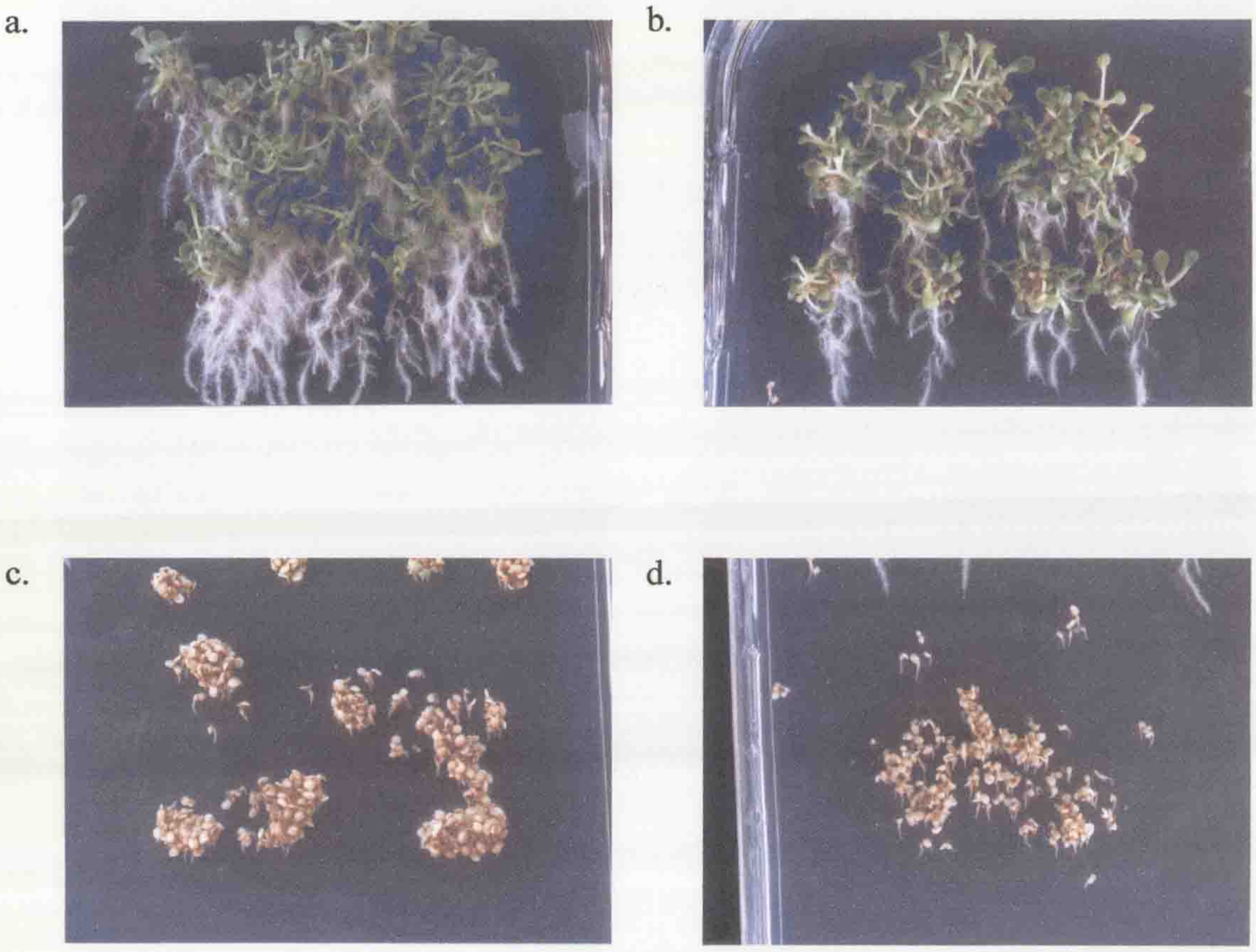


A selection of results from the analysis of northern blots are shown. Lanes 1-12 contain RNA from independent lines of the transposon tagging plants which were all PCR positive for both the *alcR* and bar construct (Figure 3.16). Lane 13 contains RNA from wild type plants. Lane 14 contains RNA from AGS1-3 plants and lane 15 contains RNA from AGS4-2 plants both of which contain the *alcR* construct but not the bar marker. The three blots are probed with radiolabelled fragments specific for the *alcR*, bar, and ubiquitin coding sequence from top to bottom respectively. No detectable expression of *alcR* is present in any of the tagging lines. The tagging lines do however express the bar gene. *alcR* RNA is only detectable in the AGS4-2 positive control. The AGS1-3 and wild type samples show no detectable *alcR* expression. All the lanes can be seen to contain RNA from the ubiquitin control.

The RNA was denatured and run on formaldehyde containing gels before transferring onto NEN Genescreen Plus® membrane. The RNA was then fixed to the membrane and analysed using radiolabelled probes generated from PCR fragments of *Ac* transposase, bar, *alcR*, hyg, and ubiquitin. Figure 6.1 shows a selection of the results obtained using *alcR*, bar, and ubiquitin specific probes. The AGS4-2 positive control was the only sample to show detectable expression of *alcR*. None of the transposon tagging lines showed any detectable *alcR* expression. Wild type plants, and interestingly the AGS1-3 plants, showed no detectable *alcR* expression. This fits with the previous observations that AGS1-3 plants are also prone to silencing resulting in the lack of GUS expression (personal communication Piyarat Parinyapong, Will Rowe).

The bar transcript was clearly detectable in all the transposon tagging transformants, as expected from selection and segregation analysis. Bar expression was not detectable in the wild type plants or the AGS lines. Ubiquitin RNA was detected in all the samples and was used both as a positive control for the probing and for comparison of the amount of RNA in each lane. No expression was detected in any of the lanes when the membranes were analysed using *Ac* transposase probes (data not shown), and an unclear result similar to the expression pattern of *alcR* was seen upon probing for expression of the hygromycin marker (data not shown). The results show clearly that the *alcR* gene is not expressing at any detectable level in the transposon tagging plants.

Figure 6.2 Hygromycin selection of SRN generations



a. SRN T2 seed from SRN1-5, one of the homozygous lines. All the germinating plants are resistant to hygromycin. b. A mixture of SRN T3 seed from the four homozygous lines SRN 1-5/12/19/20. This seed was used for the generation of the transposon tagging, and 'lethal' gene plants. Some of the seed is sensitive to hygromycin. Small brown sensitive seedlings can be seen in the background amongst the resistant plants. c. SRN T5 seed from transposon tagging T1 line B2. All the seed in this sample is sensitive to hygromycin (one green germinating seedling can be seen but this later showed a sensitive phenotype). d. wild type seed showing a fully sensitive phenotype.

6.3 Analysis of hygromycin resistance in the SRN line and the transposon tagging lines.

The hyg marker was part of the SRN T-DNA construct. Analysing the northern blots with probes for the hygromycin resistance marker gene yielded an unclear result suggesting that the gene was not being expressed in the transposon tagging plants. Subsequently plants were analysed for resistance to hygromycin. The original SRN1 transformants had been selected by hygromycin resistance and the line characterised as containing a single T-DNA insert by segregation analysis of the hygromycin resistant phenotype (Roslan, 1999). Homozygous T2 families had also been identified by segregation analysis on hygromycin selective media (Table 3.1) and used for transformation of the transposon tagging and 'lethal' gene constructs. Due to the complications of transplanting from tissue culture plates none of the further generations generated from these homozygous plants had been selected on hygromycin and were only assumed to be resistant.

Seed from one of the homozygous SRN T2 families (SRN1-5), the mixed T3 seed from the homozygous SRN lines used for transformation (SRN1-5, 1-12, 1-19 and 1-20), and T1 seed from the transposon tagging lines (which is T5 with respect to the SRN transformation) was scored for resistance to hygromycin. Wild type seed was used as a negative control to identify sensitive phenotypes. The results from this selection can be seen in Figure 6.2. The SRN 1-5 T2 plants were all fully resistant to hygromycin whilst the mixed SRN T3 seed contained a mixture of brown sensitive plants and healthy green plants. The T1 transposon tagging plants were all sensitive to hygromycin. The wild type plants were all sensitive as expected. The T3 seed

contained a mixture of seeds from four homozygous families. It was not possible to identify which seed came from which family but as this mixed seed was used for the generation of transformants it was expected that the transposon tagging transformants may contain lines originating from all four families. It was not investigated as to whether this hyg silencing was unique to one or more of the SRN T3 lines.

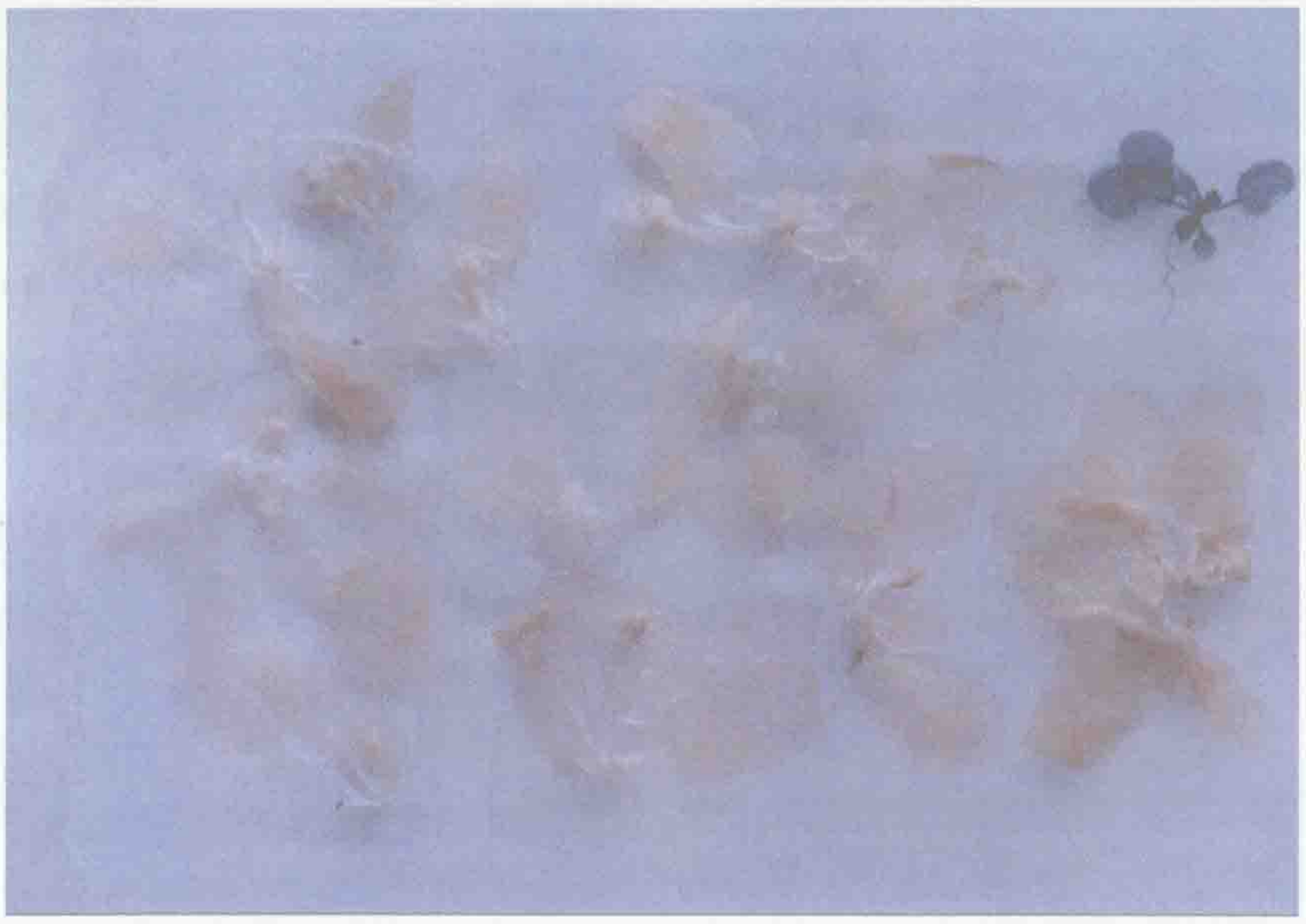
6.4 Generation of AGS lines of SRN1 plants

Plants from each of the four SRN1 T2 homozygous families were transformed with a T-DNA construct containing the *alcA* promoter upstream of a GUS marker gene. The *alcA*:GUS (AGS) construct was obtained as a *Hind*III fragment from pAGS (Salter, 1997). This was cloned into the plant transformation vector pG0229 (Figure 3.7). The new plasmid pG0229:*alcA:*GUS was transformed, along with pSoup, into *A. tumefaciens*. Transformants were selected on media containing kanamycin, tetracycline and rifampicin and checked by PCR before growing large cultures and transforming plants from the SRN1 T2 homozygous families by floral dip (modified from Clough and Bent, 1998). Primary (T0) transformants (with respect to the *alcA*:GUS construct) were selected by spraying with Harvest™. Putative transformants were allowed to self fertilise and segregation analysis was performed on the T1 progeny, again by spraying with Harvest™.

Progeny from two transformants (SRN1-12 AGS, and SRN1-20 AGS) gave a ratio of Harvest™ resistant : sensitive plants of 3:1 indicating the presence of a single T-DNA

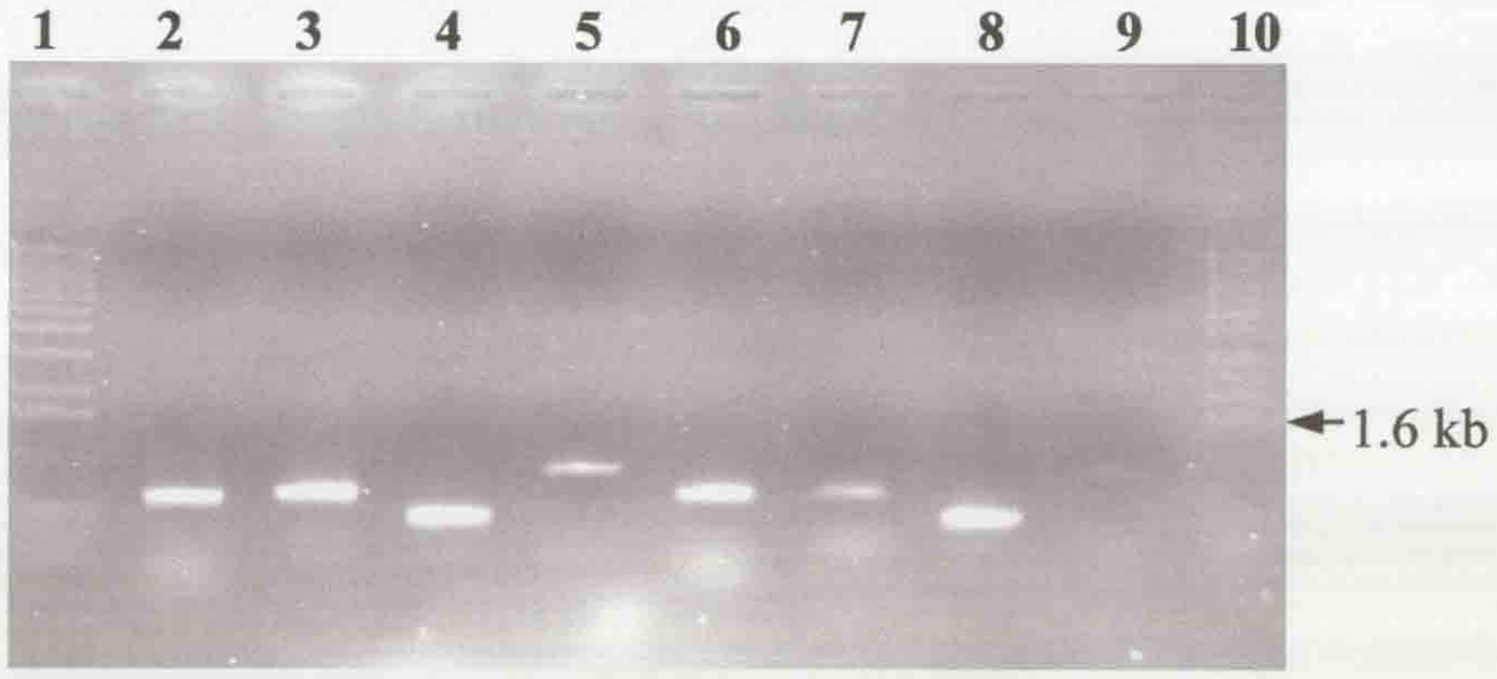
Figure 6.3 Analysis of SRN 1-12 / 1-19 AGS lines

a.



Plants were grown on soil and selected by spraying with harvest. Resistant plants were induced by watering with 1% ethanol for 5 days and histochemically stained for analysis of GUS expression. SRN 1-12 and SRN1-20 plants were stained separately and photographed together. A single AGS4-2 plant can be seen in the top right corner and this was used as a positive control for staining. None of the SRN AGS plants stain positively for GUS expression.

b.



DNA was extracted from groups of the AGS plants and analysed by PCR. Lanes 2-5 contain the results of diagnostic PCR of plants from SRN1-12 AGS and lanes 6-9 contain the results of diagnostic PCR of plants from SRN1-20 AGS. Lanes 2 and 6 contain the results of PCR using primers for the GUS construct. Lanes 3 and 7 contain the results of PCR using primers for the *alcR* construct. Lanes 4 and 8 contain the results of PCR using primers for the bar construct. Lanes 5 and 9 represent positive controls and contain the results of PCR using primers for the gene *csr1*. Lanes 1 and 10 contain molecular weight ladder.

insert. These plants were T1 with respect to the *alcA*:GUS transformation and T4 with respect to the SRN transformation. The Harvest™ resistant plants were presumed to be a mixture of 2:1 hemizygous : homozygous with respect to the *alcA*:GUS construct. They were induced by watering with 1% ethanol every 3 days for a week, before uprooting whole plants and histochemically staining to analyse GUS expression. Figure 6.3a shows the results of this staining. None of the plants stained positively for the expression of the GUS marker gene. Staining was assessed relative to a plant from the AGS4-2 homozygous T2 line, which was used as a positive control. The presence of the *alcR*, GUS and bar constructs was confirmed by PCR (Figure 6.3b).

6.5 Crossing the transposon tagging lines and AGS1-3

In an attempt to circumvent the problems of silencing in the SRN based tagging lines the plants were crossed with homozygous AGS1-3 plants which were known to contain a functional constitutively expressed *alcR* gene. Homozygous plants from the transposon tagging T2 families were used where possible, and grown on soil before crossing with plants from homozygous families of the AGS1-3 line. Mixed families of plants from the lines where no homozygous T2 families had been collected, were selected by spraying with Harvest™ to remove the azygotes. This meant that some of the plants used for crossing may be hemizygous and it was possible that their progeny, after crossing, would not contain the tagging construct. The transposon tagging plants were grown until around 5-6 bolts had developed and were up to 15

Figure 6.4 Transposon tagging plants crossed with AGS 1-3

a.

Diagnostic PCR of the progeny plants from crossing the transposon tagging lines with AGS1-3 plants. Lanes 2-16 contain samples from fifteen of the 16 transposon tagging lines. Lanes 1 and 17 contain molecular weight ladder. The plants were tested using primers designed to amplify the bar gene (top) and the *alcR* gene (bottom). All but one of the plants are positive for the bar gene PCR and all plants are positive for the *alcR* PCR.

b.

Histochemically stained leaves from the progeny plants obtained through crossing the transposon tagging lines and AGS1-3. From the staining pattern it can be clearly seen that some of these plants have undergone a silencing event. The AGS1-3 plant used for crossing was homozygous and stained positive for GUS expression. Progeny from fifteen of the sixteen transposon tagging lines are shown.

cm in length. The plants were then fertilised by hand using pollen from an AGS1-3 plant that had been shown previously to stain positively for ethanol inducible GUS expression by Will Rowe. The siliques were allowed to mature and seed was collected.

Seed was successfully collected from crosses involving fifteen of the sixteen transposon tagging lines. Nine of the fifteen had come from homozygous parent plants. The line that yielded no progeny was C5-1. This was assumed to be due to physical damage caused during the crossing procedure.

The progeny from the cross were grown on soil to around the six-leaf stage and analysed initially by removing leaves for PCR (Figure 6.4a). Leaves were taken from a number of plants and pooled for rapid DNA extractions. All but one of the sets were PCR positive for the bar gene in the tagging construct and all the sets were PCR positive for the *alcR* gene in the AGS construct. Interestingly, the PCR negative line (B9-2 x AGS1-3) had originated from a parent homozygous for the tagging construct. The PCR was not repeated but this line should in fact contain the construct.

The primary bolts were removed and the plants were induced by watering with 1% ethanol. Again leaves were removed for GUS staining to analyse the functionality of the *alc* switch in the plants (Figure 6.4b). One leaf was removed from each of two plants in each set (apart from B9-2 x AGS1-3 which only contained one plant) and each plant was labelled for future identification. The plants were all PCR positive for the GUS construct which was contained in the AGS T-DNA insert along with the *alcR* gene. Three out of twenty-nine plants showed no GUS expression and nineteen

of the remaining twenty-six showed reduced or weak GUS expression. The GUS staining pattern therefore indicated that silencing had again occurred in a number of the plants.

The plants were induced every 4-5 days with 1% ethanol until the siliques began to mature and seed was collected. The seed was dried, sterilised, and screened for streptomycin resistance on tissue culture media containing 200 mg/l streptomycin, 20 g/l glucose. Unfortunately no streptomycin resistant progeny were seen. All the germinants showed a typical sensitive bleached phenotype.

6.6 Crossing the 'lethal' gene lines with AGS1-3

In a similar attempt to circumvent the problems of silencing in the SRN based 'lethal' gene lines, the plants were again crossed with homozygous AGS1-3 plants which were known to contain a functional constitutively expressed *alcR* gene. Plants from the 'lethal' gene T2 families were grown on soil for crossing with the homozygous AGS1-3 plants. The T2 families of 'lethal' gene plants used for crossing had not undergone segregation analysis and so homozygous lines could not be specifically chosen. Azygous plants were removed from the mixed populations by spraying with Harvest™.

All the surviving plants were assumed to be either homozygous or hemizygous for the 'lethal' gene construct. Consequently progeny obtained after crossing was

expected to include some plants that would not contain the 'lethal' constructs. The plants were grown until around 5-6 bolts had developed and were up to 15 cm in length and were then fertilised by hand using pollen from an AGS1-3 plant that had been shown to stain positively for GUS expression by Will Rowe. The siliques were allowed to mature and seed was collected.

The progeny were grown on soil and induced by watering with 1% ethanol every 4-5 days. Plants with induced abnormal phenotypes were expected to exhibit retarded growth and discolouring and curling of the leaves. The progeny were expected to consist of a mixture of genotypes, some plants would be hemizygous for both the 'lethal' knockout construct and the AGS construct, and some plants would be azygous for the 'lethal' knockout construct and hemizygous for the AGS construct. This was expected to result in a range of phenotypes amongst the plants upon induction.

Unfortunately no abnormal phenotypes were observed upon induction. Growth was compared between the progeny obtained from the different lines and with wild type plants grown in identical conditions. No abnormal phenotypes were observed in any of the plants.

6.7 Discussion

The results of the northern blotting suggest that the *alcR* gene is silent in all the transposon tagging transformed lines. Bar and ubiquitin controls indicate that the lanes all contain RNA and that the bar selectable marker in the transposon tagging constructs is being expressed. No transposase mRNA was detectable in any of the samples (data not shown). Transposase expression is controlled by the *alcA* promoter, which in turn is reliant upon the AlcR transcription factor. Silencing of the *alcR* gene could be due to either a transcriptional or a post-transcriptional silencing event. Both transcriptional silencing of the *alcR* gene and post-transcriptional silencing of the *alcR* transcript would result in the prevention of inducible expression from the *alcA* promoter.

The results of hygromycin selection suggest that the hyg selectable marker is also silenced in all of the transposon tagging lines and in the generations of the SRN line from T3 onwards. This suggests that the silencing is likely to be transcriptional. The SRN and hyg constructs are situated in the same T-DNA and are therefore expected to be localised together in the genome. A chromatin based transcriptional silencing event could explain the loss of expression of both genes. If the silencing were post-transcriptional it is unlikely that both genes would be silenced.

The results of transforming the AGS construct into the SRN plants also suggest that *alcR* may have been silenced in the SRN line. The hygromycin selection of SRN T3 plants suggests that a silencing event has occurred in some of the homozygous T3 plants. The T1 AGS plants are T4 with respect to the SRN transformation and so if silencing begins to occur in the T3 generation it is entirely possible that *alcR* is silent

(www.arabidopsis.org), a database containing sequence, mapping and community information (Huala *et al*., 2001). Large numbers of mapped T-DNA and transposon insertion lines have been generated and are available from the Nottingham *Arabidopsis* Stock Centre (http://nasc.nott.ac.uk, reviewed in Parinov and Sundareson, 2000). Seeds and DNA samples from many of these are available for screening purposes. These stocks may also be used for directed transposon tagging strategies using lines containing transposon insertions genetically linked to a gene or region of interest.

1.2 Vector systems for the transformation of plants

- **Plant transformation during infection by *Agrobacterium tumefaciens***

A. tumefaciens is the soil phytopathogen responsible for the formation of crown gall tumours. Cells in the tumour tissue become stably transformed with DNA from the bacteria. This DNA encodes genes that cause the transformed cells to produce opines and plant growth hormones. Opines are small metabolic compounds which are in turn metabolised by the *Agrobacterium*. The plant growth hormones auxin and cytokinin stimulate cell growth and tumour formation. The mechanisms and processes involved in the development of crown gall tumours by *A. tumefaciens* have been the subject of a number of reviews (Drummond, 1979; Bevan and Chilton, 1982; Zambryski, 1988, 1992; Kado, 1991; Hooykaas and Schilperoort, 1992; Zupan *et al*., 2000; Gelvin, 2000). The virulence of *A. tumefaciens* was localised to a large plasmid, named the tumour inducing (Ti) plasmid (Van Larebeke *et al*., 1974;

in all these plants.

The hygromycin selection suggests that the silencing appears to increase down the generations beginning at the T3 generation. If this is true it is important to note that any transformants obtained from the SRN T2/T3 lines will be at generation T4/T5 by the time segregation analysis has been carried out for the secondary transformation and T5/T6 by the time homozygous lines have been generated.

The results of crossing the lines of transposon tagging plants and lethal knockout plants with the AGS1-3 line were inconclusive. No inducible transposition was detected in the progeny of induced transposon tagging crossed plants. Similarly no abnormal phenotypes were seen in the progeny of 'lethal' gene crossed plants. From GUS staining the transposon tagging crossed progeny it was noted that silencing had occurred in many of the plants. There were however, no observable inducible transposition events in either the silenced or unsilenced plants. The GUS staining may not show a direct reflection of the silencing state throughout the plant. Many of the leaves showed GUS staining only in the vascular tissue. The cells giving rise to the gametes, where transposition is required, to produce heritable transposition events, may have shown a different pattern of silencing.

Chapter 7

General Discussion

7.1 Transposon tagging strategies (Chapter 3/5)

Plasmid constructs have been described and used to transform plants to develop two novel transposon tagging strategies. The first strategy uses the *alc* switch to control ethanol inducible transposition of a *Ds* element, and will create dominant gain-of-function mutations as a consequence of aberrant expression from an outward facing *35S* promoter contained in the element (described in Chapter 3.2, 3.4). Transgenic plants were generated and analysed, before inducing with ethanol and analysing the progeny for successful transposition events. The second system uses the soybean *Gmhsp* 17.3-B heat shock promoter to control transposition and aimed to create ethanol inducible gain-of-function mutations due to aberrant expression from an outward facing *alcA* promoter contained in the transposable element (described in Chapter 3.3). Unfortunately no transgenic plants containing the second system were generated.

Plants containing the *alc* inducible transposition system were subjected to ethanol induction and examined for transposition, on the basis of excision from the streptomycin resistance marker (described in Chapter 5.1). Unfortunately no successful excision events were observed. This was later shown to be due to a gene silencing event in the SRN plants, into which the transposon tagging constructs were

transformed. *alcR* expression was undetectable by northern analysis (demonstrated in Chapter 6.2).

7.2 'Lethal' gene strategies (Chapter 4/5)

A series of plasmid constructs and lines of transgenic plants were generated that contained the *alcA* promoter controlling ethanol inducible expression of either partial sense or antisense copies of two 'essential' amino acid biosynthesis genes (described in Chapter 4). These were expected to create similar phenotypes, upon induction, to the effects of chemical inhibition of the gene products by glyphosate and the imidazolinone herbicides.

Upon induction the plants were scored for phenotypic alterations (described in Chapter 5.2). In the majority of lines, no difference was seen between the phenotype of induced and uninduced plants. For example the sensitivity of the plants to herbicides targeted to the specific product of each gene was unaltered upon induction. Most of these plants had originated from the same SRN lines in which silencing had been demonstrated during analysis of the transposon tagging plants. This may explain the absence of phenotypic effects.

One line (AGS4-2 EPSPS) showed a range of abnormal phenotypes upon induction (described in Chapter 5.2.4). Induced plants suffered retarded growth and discolouring of the leaves. Whilst this did, in part, correlate with the presence of the EPSPS T-DNA insert, which was expected to express a partial sense transcript of the essential gene *epsp*, the plants were again undergoing some transgene silencing

events (demonstrated in Figures 5.7, 5.8). Characterisation of the effect of the aberrant expression was further complicated by the abnormal phenotypes observed in the AGS4-2 plants (demonstrated in Figure 5.6). Because of this complex phenotypic background it was not possible to attribute any definite part of the abnormal phenotype to the expression of the EPSPS construct.

7.3 Further analysis of transgenic plants (Chapter 6)

Northern analysis of the plants generated for transposon tagging revealed that the *alcR* gene (as demonstrated in Figure 6.1) and therefore the inducible *Ac* transposase (data not shown) were not expressed at any detectable levels. Northern analysis of the hygromycin selectable marker, which was situated in the same T-DNA construct as the *alcR* gene, suggested that it was also not being functionally expressed (data not shown).

Analysis of the SRN plants for hygromycin resistance over a range of generations showed that the marker was not functional in homozygous plants older than T3 and in some plants at the T3 stage (Chapter 6.3, Figure 6.2). The generation of SRN plants containing an *alcA* inducible GUS reporter gene (Chapter 6.4, Figure 6.3) suggested that the *alcR* gene silencing was occurring in T4 generations independent of the transposon tagging T-DNA insert. This is important as any lines generated by secondary transformation would be at least in the T4 generation with respect to the initial SRN transformation before they could be used experimentally.

An attempt to rescue both the transposon tagging and the 'lethal' gene plants by

crossing with AGS plants, which contained a functional *alc* system was unsuccessful. Some of the AGS plants underwent a silencing event after crossing (as demonstrated in Figure 6.4b). It is not known if this silencing was related to the tagging or 'lethal' gene constructs as the AGS plants were themselves prone to silencing (Personal communication, Piyarat Parinyapong and Will Rowe).

7.4 Discussion of the results with respect to models of gene silencing

The issue of gene silencing is an important and well discussed subject, particularly amongst those using transgenic technology and especially in plants. The general acceptance is that there are two major forms of silencing, transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), each of which may involve a number of different sensing and response mechanisms, some of which are possibly shared by both systems.

TGS is thought to have developed as a method of controlling transposable elements and natural transgene inserts. The general mechanism involves targeting homologous promoter sequences and results in the methylation and inactivation of all homologous promoter sequences in the nucleus by structural factors including chromatin rearrangement.

PTGS is thought to have developed as a method of acquiring viral resistance and of controlling the chromosomal rearrangements resulting from both transgene insertion

and the movement of transposable elements. The mechanisms involved are complex and are thought to include the production of aberrant RNA, methylation of coding sequences, the action of RNA dependent RNA polymerases, and the production of antisense and double stranded RNA. The overall effect of these mechanisms is a reduction in steady state levels of the specific mRNA in the cytoplasm. There may be a number of different pathways involved in the post-transcriptional silencing of transgenes and this would explain the complexity of the evidence currently under discussion.

Many models and lines of evidence have been proposed for discussion to explain the processes involved in both transcriptional and post-transcriptional gene silencing (reviewed in Dougherty and Parks, 1995; Matzke and Matzke, 1995; Baulcombe, 1996; Meyer and Saedler, 1996; Depicker and Van Montagu, 1997; Stam *et al*., 1997; Wassenegger and Pélissier, 1998; Vaucheret *et al*., 1998; Covey and Al-Kaff, 2000; Fagard and Vaucheret, 2000; Marathe *et al*., 2000; Meyer, 2000; Muskens *et al*., 2000; Wassenegger, 2000; and references therein). The results observed and described in the previous chapters will be discussed with respect to some of these observations and proposals.

- **Methylation and chromatin rearrangement**

The observation that the hyg selectable marker gene is also silent in *alcR* silenced SRN plants, suggests that the silencing is likely to be created by a structural chromatin rearrangement. The chromosomal proximity of the genes suggests that the controlling factor is likely to be acting in the nucleus. A chromatin rearrangement or heavy methylation event could prevent transcription from both the promoter sequences of the *alcR* and hyg transgenes. PTGS results in cytoplasmic degradation

of mRNA. The probability of both these transgenes (which are located proximally in the genome and share no homologous sequences) being individually targeted for PTGS in the cytoplasm seems small.

- **Silencing appears to be heritable**

PTGS is not meiotically stable and is usually reactivated shortly after germination. TGS is often heritable. The tissue samples for northern analysis were collected after approximately ten days. Hygromycin selection was performed on plants germinating on tissue culture media containing hygromycin and sensitive phenotypes were scored again after approximately ten days. The timing of tissue collection for northern analysis and the nature of hygromycin selection suggest that the *alcR* and hyg genes are silenced at least within the first two weeks after germination. It is not known that this is because the silenced state is inherited but it is likely. The increase of silencing down generations also suggests that the silent state may be heritable.

- **There is no apparent spread of silencing to homologous promoter sequences**

TGS is thought to arise due to the presence of multiple gene copies and specifically homologous promoter sequences. The transgenic plants described contain a number of repeated sequences specifically in the promoter constructs used.

- ***35S* repeat sequences**

The silenced *alcR* gene in the SRN line contains a *35S* promoter sequence. Two copies of *35S* are present in homozygous plants. The transposon tagging construct also contains two other *35S* promoters (as demonstrated in Figure 3.9) and therefore hemizygous plants will contain four copies and homozygous plants will contain six copies. The additional *35S* promoters were situated in the *35S*:SPT marker, used for

the identification of excision events, and the transposon, for creating dominant mutations after transposition. Due to the lack of detectable transposition it is not known whether these promoters were still active in *alcR* silenced plants. It is possible that the *35S*:SPT excision marker was itself silenced and this could explain why successful excision events were not observed during streptomycin selection, although it is assumed that the lack of *alcR* and transposase transcripts (demonstrated by northern blotting Chapter 6.2) meant that no successful excision events would have occurred.

The SRN1-19 *35S*:SPT line was generated to act as a positive control for streptomycin selection and also contained the *35S*:*alcR* (SRN) T-DNA insert. The plants were shown to contain a double, unlinked *35S*:SPT T-DNA insert by segregation analysis (Table 3.4) each of which contained a *35S* promoter driving the SPT selectable marker. T2 families that segregated for Harvest™ resistance in a 3:1 ratio were expected to contain a mixture of plants, carrying only one of the two T-DNA inserts in either a homozygous, hemizygous, or azygous state. Families that segregated in a 1:0 ratio were expected to contain plants, either entirely homozygous for both T-DNA inserts, homozygous for only one of the two T-DNA inserts, or homozygous for one insert and containing the other insert segregating in a 1:2:1 ratio. These three possible genotypes were expected to occur in a 1:2:4 ratio. The plants from the families that were entirely resistant to Harvest™ were therefore expected to contain between two and six copies of the *35S* promoter sequence. The single insert containing (3:1 ratio for Harvest™ resistance) families segregated for streptomycin resistance in a 3:1 ratio (as demonstrated in Figure 5.1) suggesting that silencing is not targeted toward the homologous *35S* promoter sequences in

these plants. Only two of the 1:0 families were tested for streptomycin resistance and these were both entirely resistant to streptomycin (data not shown). The probability that these two samples were both from the class containing only one of the two inserts is less than 1/12 and so at least some of the plants tested were likely to contain up to six copies of the *35S* promoter sequence. These plants were not verified as being silent for *alcR* but were all silent for the hygromycin selectable marker gene (data not shown) as discussed below.

- **The *nos* promoter**

The hyg selectable marker in the SRN line contains a *nos* promoter. The *nos* promoter also drives expression of the bar selectable marker in all the transposon tagging lines, as well as the *35S*:SPT line. In all these lines the hyg marker is silenced (as demonstrated in Figure 6.2 for the transposon tagging plants, data are not shown for the *35S*:SPT line). The transposon tagging lines contain between two and four copies of the *nos* promoter and segregation analysis using the bar selectable marker indicates that the gene is properly expressed in both hemizygous and homozygous plants. Again the *35S*:SPT lines are expected to contain between two and six copies of this *nos* promoter sequence and segregation analysis using Harvest™ indicates that the bar selectable marker is properly expressed in all these plants (as demonstrated in Table 3.4).

A considerable reduction in transformation frequency relative to those previously reported was observed for all the transformations performed. Silencing of the *nos* promoter on the bar selectable marker gene could in part explain this, although if the silencing was able to target the bar construct it is expected that the marker would be likely to be silenced to some extent in the plants successfully recovered and this was

not observed.

- **Aberrant expression of the *alcR* and hyg genes by read-through expression**

Aberrant expression of the SRN construct from promoters or enhancers situated close to the T-DNA insert would be expected to result in post-transcriptional silencing of the *alcR* and hyg transgenes. Again this would also be expected to result in silencing of any other homologous sequences introduced in the later transformations. If the expression was from outside the construct this would include the homologous promoter sequences.

7.5 Summary of the silencing

From the results it is impossible to fit the pattern of gene silencing of the SRN construct to any one of the proposed silencing models. Generally the pattern suggests that the silencing occurs at a transcriptional level although it is not specific for the promoter sequence. Before the discovery of gene silencing it was accepted that position effects were often responsible for alterations in transgene expression. These were thought to be dependent upon the genomic scaffolding and are not necessarily sensitive to specific sequence elements. If the SRN insert is integrated into an area densely covered in chromatin then this could explain the seemingly sequence independent silencing. The initial segregation analysis of the SRN T1 family did not show any significant deviation from the expected 3:1 ratio for a single insert transformant (Roslan, 1999). However, segregation analysis of the T2 plants demonstrated that some families deviated from the expected ratios of 3:1 and 1:0

Watson *et al*., 1975). Chromosomal components are also required for the successful infection of plants by *A. tumefaciens*.

- **Ti plasmids and binary vector systems**

The Ti plasmid contains two genetic components required for the stable transformation of plant cells. The *vir*-region encodes about twenty gene products and is required for virulence (Iyer *et al*., 1982; reviewed in Zambryski, 1988). The T-DNA region is stably transferred into the genome of infected cells, carrying the genes for the production of the phytohormones and opines. This T-DNA contains imperfect 25 bp repeat sequences at both the left and right borders, which flank the transferred region (reviewed in Bevan and Chilton, 1982).

Binary vector systems are based on the ability to separate the T-DNA from the *vir*-region of the Ti plasmid. Hoekema *et al*. (1983) described two plasmids, one containing the Ti *vir*-region, the other carrying the Ti T-DNA region. Tumour induction was reported by an *A. tumefaciens* strain carrying the two plasmids. In 1984 Mike Bevan described a binary vector pBin19, designed for transformation of heterologous DNA to plants using *A. tumefaciens*. The T-DNA vector carried only the left and right border repeat sequences, which flanked a neomycin phosphotransferase gene, expressed from the nopaline synthase (*nos*) promoter, and a polycloning site. This enabled heterologous sequences to be cloned into the polycloning site and transformed plant cells containing the inserted sequence to be selected by kanamycin resistance. The T-DNA was mobilised by a set of *vir* genes carried on an engineered Ti plasmid which lacked the T-DNA region.

The plasmid pBin19 and its derivatives were used by many groups to transform

(demonstrated in Table 3.1).

Attempts were made to produce more lines of transformants constitutively expressing *alcR* but these were unsuccessful. Transformations into the AGS lines were also largely unsuccessful, although a few transformants were generated for the 'lethal' constructs. Again some gene silencing was observed in the AGS lines. This suggests that the silencing may be targeted to the T-DNA insert (the SRN and AGS lines were transformed using binary vectors that contained the same T-DNA backbone as well as SRN and hyg constructs). This could be related to any part of the insert and represents an area for further study.

7.6 Further work and experimental modification

Many groups are currently using the *alc* system for a range of purposes. The system is also used to control transgene expression in commercial crop varieties. Other groups have reported that gene silencing has occurred in lines containing *alc* controlled transgenes (personal communication, John Doonan, John Innes Centre). It is therefore important to understand the mechanism and cause of any silencing targeted to the system. If the silencing is specific to the *alc* system then strategies must be altered to accommodate this. Any opportunity to further understand the mechanisms behind transgene silencing would benefit researchers using transgenic technology at all levels.

Further experiments to analyse the silencing would initially be based around the SRN line. These could include isolating the SRN T-DNA insert and analysing the *alcR*

and hyg constructs using methylation sensitive restriction enzymes. Further analysis of the time course of silencing, by RT PCR for example, both during germination and down the generations of homozygous SRN plants, would assess whether the silencing is meiotically stable. Analysis of the four homozygous parent families individually could identify whether silencing is specific to one or more of the original T2 families. The T2 families which showed aberrant segregation patterns could be analysed genotypically to test whether the deviation from the expected ratios was due to silencing.

Further analysis of the abnormal phenotype identified in AGS4-2 EPSPS could include Southern analysis of each T2 family to identify copy number and genotype. The plants could be grown across a range of growth conditions, and with altered induction profiles to assess the contribution of the EPSPS T-DNA insert to the abnormal phenotype.

Generating more lines of plants, both for the transposon tagging and the 'lethal' gene strategies represents an obvious line of further investigation. If *alcR* silencing was responsible for the lack of inducible expression, then generating lines that express *alcR* efficiently, would create an opportunity to further study the two systems. The inclusion of scoreable markers such as GUS offers a relatively easy method of screening for silencing events, but the analysis of the AGS4-2 EPSPS line demonstrated that this may lead to complications in identifying the direct cause of any abnormal phenotypes. Ideally the plants would be free from any background phenotypic abnormalities and therefore any novel phenotypic effects, such as those seen in the AGS4-2 EPSPS line, could be attributed entirely to the novel construct.

Gene silencing has been a problem faced by those using transgenic technology ever since the technology was first developed. Traditionally many lines of transgenics are generated for each transformation and each line is individually characterised. By using this method it is often possible to select lines of plants where gene silencing is minimal or completely undetectable. Generating a number of *alcR* expressing lines, or adding the *alcR* construct to the same T-DNA as the other constructs, and analysing the transformants to identify lines where silencing does not occur, is therefore a favourable option.

Altering the expression pattern of *alcR* could also help to optimise the system. Although the results above suggest that the silencing was not specifically targeted to the *35S* promoter on the *alcR* construct, it is thought that high levels of constitutive expression can lead to transgene silencing. The use of developmentally regulated, or tissue specific promoters could represent a method for reducing *alcR* RNA levels in those tissues where functional *alc* switching is not required. This would be especially effective in the transposon tagging strategy where the aim was to utilise the tightly controlled timing of *alc* expression to induce transposition events in the cells that give rise to the gametes.

Limiting *alc* expression to specific tissues would, however limit the effectiveness of generating phenotypic mutants by the expression of potentially lethal constructs. Many of the constructs will be required to be expressed throughout the plant. Altering the time course of expression of *alcR* to coincide with the opportunity for induction could offer an alternative method for reducing expression levels. For instance, there is no real benefit in expressing *alcR* at high levels during germination if the plants are not induced until the four to six leaf stage.

The transposon tagging constructs contained a number of repeated *35S* promoter sequences. For future work it may be better to replace these with alternative constitutive promoter constructs. The presence of multiple copies of promoter constructs has been suggested as another trigger of gene silencing in transgenic plants.

7.7 Future work

The observation of an abnormal phenotype in one of the 'lethal' gene plants suggested that the *alc* system may represent a viable method of expressing potentially lethal constructs. This presents the opportunity for the identification of 'essential' genes, by methods including, or similar to the transposon tagging strategies. The expression of specific gene sequences using a reverse genetics approach can also be regulated using the *alc* system.

A number of groups have reported phenotypic alterations, created by the use of inducible gene expression systems for the expression of both sense and antisense constructs (Kumar *et al*., 1996; de Ronde *et al*., 2000; Potter *et al*., 2001; Lally *et al*., 2001). The *alc* system represents another method of regulating transgene expression for sensitive reverse genetic studies. The 'lethal' constructs used in this study were reliant upon reducing expression levels of their target genes by antisense or partial sense expression. This was, in part, to analyse the potential of generating abnormal phenotypes by aberrant expression from the outward facing promoters in the transposon tagging elements. Recently, methods for gene suppression have been

described that use the expression of inverted repeat sequences (Stam *et al*., 2000; Mette *et al*., 2000; Singh *et al*., 2000; Buck *et al*., 2001). The *alc* system represents a method of controlling the expression of such constructs.

In the world of genome sequencing and reverse genetics, the *alc* system offers a valuable tool for the control of genes, and gene functions in higher plants. Gene silencing represents one of the major limitations to the use of transgenic technology. The ability to understand, predict, and avoid the onset of silencing in the *alc* system and all other gene control systems will prove to be a major breakthrough in the field of biotechnology.

Appendix A Primers used for PCR and sequencing

Name	Position	Sequence 5'-3'
Ac transposable element from *Zea Mays* Accession No. X01380		
Ac210in	1-21	AACTGCAGCTCGAGTAGGGATGAAAACGGTC GGTA
Ac210out	211-192	ACGCGTCGACGGATCCTTTCATGTGTGATTTT ACCG
Tpaseout2	721-700	CTGTTAGGCGCTAGCTGCTAG
Tpasefor	1655-1675	GAAGCATGCTACAGCTAGTGC
Tpaserev	2280-2260	CATGTGAGGTGTGCTTGTCAC
Ac250out	4309-4330	GGCGCGCCGCGGCCGCTGGCCATATTGCAGT CATC
Ac250in	4565-4545	GCTCTAGAGCTCAGGGATGAAAGTAGGATGG G
TP3SEQOUT	3748-3768	CAGCTTGGTGCAATGGTGCTG
Bar resistance gene *pat* from *Streptomyces hygroscopicus* Accession No. X17220		
Barfor	98-118	CCATCGTCAACCACTACATCG
Barrev	518-498	AAACCCACGTCATGCCAGTTC
Barseqrev	586-567	GTCATCAGATCTCGGTGACG
Aspergillus nidulans alcA promoter Accession No. M16196		
NAK5X	4-12	GCTCTAGAGGCGCGCCTCGATAGTGTGATAG TTCC
NAK3S	286-266	ACGCGTCGACGGTTGATGATGTTGGTGAGAC
Arabidopsis thaliana csr1 gene for acetolactate synthase Accession No. X51514		
ALSFOR	341-361	TTCTTCGATCTCCTTCTCCAC
ALSREV	1169-1149	AGAATCTTCCGGAGGTTTAGG
Arabidopsis thaliana gene for EPSP Accession No. X06613		
EPSPSFOR	378-399	ATGGCGCAAGTTAGCAGAATC
EPSPSREV	1590-1570	AGAGCTAAGGGAGCAGACATG
Cauliflower mosaic virus genome Accession No. V00140.1 J02046		
35Strev	7654-7646	GGCGCGCCACGTTCTAGAGATCTGGAT
trfA gene from plasmid Bin 19 Accession No. UO9365		
TRFA3FOR	3215-3235	TACCAAGTACGAGAAGGACGG
TRFA4REV	4033-4013	AACGTGAAGGTGATCGGCTCG

Name	Position	Sequence 5'-3'
Agrobacterium tumefaciens nopaline synthase promoter Accession V00087 J01541		
nosPfor	285-304	GGGGTACCGGCGCGCCGATCATGAGCGGAG AATTAA
nosPrev2	583-564	GCCTGCGGCCGCAGATTATTTGGATTGAGA
E.coli transposon Tn*5* Accession No. U00004 L19385 / Plasmid pJJ43690		
Tn5out	3990-4010	ATCCTCACCGGAATCTGCTCG
SPTseqrev	2833-2815	GAGAGGGTGGTGAGCAGCTC
T-DNA end border sequences in plasmid pGreen Accession No.AJ007829		
LBin	540-559	TGGCAGGATATATTGTGGTG
RBin	1187-1168	GCAGCGAGTCAGTGAGCGAG
Aspergillus nidulans alcR gene Accession No. M24071		
AlcRfor	1044-1064	CGATATTCTCCTGCACACAGC
AlcRrev	1715-1695	ATCCGTAGTCTGCTCTTCCAG
GUS / *uidA* gene from *E.coli* genomic Accession No. S69414		
GUSfor	574-594	CATGTCGCGCAAGACTGTAAC
GUSrev	1176-1156	AATCGCCTGTAAGTGCGCTTG

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plants over the coming years. Use of the original pBin19 was limited by the fact that only a restriction map and an estimate of the size were available. This made cloning sequences into the plasmid inconvenient, reduced the number of unique cloning sites and made it incompatible with multiple stage cloning strategies. Modifications of the vector including reduction in size, inclusion of polycloning regions, and the development of a range of selectable markers increased the productivity of the generation of transgenic plants for research purposes.

- **Modification of transformation vectors**

T-DNA insertion begins at the right border sequence and continues toward the left border. However insertion is not always complete. The kanamycin resistance selectable marker in pBin19 is situated next to the right border sequence. Partial integration of this T-DNA can therefore generate kanamycin resistant plants that do not contain the cloned insert. To increase the frequency of selected transformants that do contain the cloned insert McBride and Summerfelt (1990) and Hajdukiewicz *et al*. (1994) generated transformation vectors with the selectable marker adjacent to the T-DNA left border. The pPZP vectors (Hajdukiewicz *et al*., 1994) were advantageous in a number of ways. They were smaller than pBin19, contained a pUC18 multiple cloning site and had been completely sequenced.

The complete sequencing of the pBin19 plasmid highlighted many superfluous regions contained in both transferred and non-transferred areas of the plasmid (Frisch *et al*., 1995). This enabled the development of transformation vectors that were considerably smaller in size and contained a series of selectable markers for the generation of transgenic plants. Xiang *et al*. (1999) described a series of transformation vectors based on pBin19. The non-essential sequences highlighted by

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Frisch *et al*. (1995) were removed. The basic plasmids are only 3.5 kb in length. A number of selectable markers and transit peptide sequences have been added, to generate a series of plasmids suitable for numerous applications. A transformation frequency of 1/50 transformants / progeny seed was obtained after transforming *Arabidopsis* by vacuum infiltration with *A. tumefaciens* containing a plasmid carrying a marker for glufosinate resistance.

The pGreen system was developed by Hellens *et al*. (2000). It consists of a series of binary vectors containing T-DNA left and right border sequences, carrying the selectable marker genes for glufosinate, hygromycin, kanamycin or sulfadiazine resistance and the scoreable marker genes for β-glucuronidase (GUS), green fluorescent protein (GFP) or firefly luciferase (LUC+). The vectors are small (4.3 kb), exhibit high copy number in *E. coli* and contain extensive multiple cloning sites. The pGreen vectors require a second plasmid, pSoup for replication in *A. tumefaciens*. The pGreen vector is based on pBluescript (Alting-Mees and Short, 1989). pSoup is based on pBin19 although the T-DNA region is removed. Both plasmids are co-transformed into *A. tumefaciens* by electroporation. The plasmids are publicly available and are further described at: www.pGreen.ac.uk. This system was chosen and used for the experiments described later.

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1.3 Transposon tagging

- **The identification of transposable elements in maize and other organisms.**

Transposable or 'controlling' elements were first identified and studied genetically in maize by Barbara McClintock (McClintock, 1951). McClintock identified the elements by studying mutable loci in maize. Loci for alleles such as *waxy*, *bronze* and *shrunken* were observed to alter through generations in a non-mendelian fashion. Chromosome rearrangements were also observed and McClintock attributed these to *Ds* elements.

By the 1970's transposable elements had also been identified in a wide range of organisms including the model systems of *Escherichia coli*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Antirrhinum majus* and were being extensively studied throughout (for review see Starlinger, 1984). Two autonomous maize elements, Activator (*Ac*) and Enhancer / Suppressor–mutator (*En/Spm*) were cloned and sequenced (Fedoroff *et al.*,1983; Mullerneumann *et al.*, 1984; Pohlman *et al.*, 1984; Periera *et al.*, 1986). Saedler and Nevers (1985) proposed a general mechanism for transposition, based upon sequence data and the observation of sequence disruptions caused by insertion and excision events. Putative genes required for transposition were identified by studying open reading frames contained in both *Ac* and *En/Spm* elements (Kunze *et al.*, 1987; Frey *et al.*, 1990). The suggestion that these transposases were essential for autonomous activity was backed by the observation that *Ds/dSpm* non-autonomous elements often contained internal deletions (Doring and Starlinger, 1986).

The potential of using transposable elements as tools for genetic study was realised and the elements have since been extensively characterised and used to develop gene tagging strategies by a number of groups (for reviews see Walbot, 1992; Osborne and Baker, 1995; Sundaresan, 1996).

- **Characteristics of the *Ac*/*Ds* family of transposable elements.**

The *Ac*/*Ds* system is the most commonly used transposition system in plant studies. *Ac* is an autonomous element 4565 bp in length, containing a long open reading frame of 3.6 kb, surrounded by a number of direct and inverted repeat sequences and terminated with 11 bp inverted repeats. The open reading frame encodes the *Ac* transposase. It contains 4 introns and a 600-700 bp AUG-free leader sequence (Kunze *et al*., 1987). The coding sequence is translated to produce a single protein 807 amino acids long that has been shown to interact with the subterminal sequences, but not the 11 bp terminal repeat sequences of *Ac* (Kunze and Starlinger, 1989). It is thought that some host encoded factors are also required for transposition.

Simple non-autonomous *Ds* elements show sequence similarities to *Ac* particularly in the repeat sequences and are thought to arise due to deletions in *Ac* that remove the ability to produce functional transposase (Doring and Starlinger, 1984; Doring *et al*., 1984). Aberrant *Ds* elements show less sequence similarity, but share the 11 bp terminal sequence and are trans-activated by *Ac*. These elements vary in size from a few hundred bases to thousands of bases and can carry sequence duplications (Doring and Starlinger, 1984). *Ds* elements create stable insertions and are trans-activated by active *Ac* elements. Many *Ds* elements exist naturally in maize (McClintock, 1951).

Transposable elements cause recessive insertional mutations in many of the genes in maize and the first gene to be successfully cloned using *Ac* as a target was the bronze locus by Fedoroff *et al*. (1984). Genomic DNA, from mutants containing an *Ac* insertion, was probed using an *Ac* fragment. This was used to identify the sequences flanking the insert in a maize genomic phage library and in turn clone the non-mutant allele.

- **Introduction of *Ac/Ds* elements into heterologous species.**

A major development in transposon tagging was the demonstration that *Ac* transposable elements were active in heterologous species. Baker *et al*. (1986) first observed successful transposition of *Ac* in tobacco. Whilst this result suggested that it was feasible to use *Ac* for genetic studies in heterologous species, the tetraploid nature of tobacco restricts the observation of recessive insertional mutations. Van Sluys *et al*. (1987) reported *Ac* transposition in *Arabidopsis*, and Yoder *et al*. (1988) in tomato. More recently Murai *et al*. (1991) have demonstrated transposition in rice, Yang *et al*. (1993) in lettuce and Finnegan *et al*. (1993) in flax. For a number of years strategies were developed to identify genes, by both random insertional mutagenesis and directed tagging, in these and other plants. Genes were successfully tagged and cloned in *Arabidopsis* (Bancroft *et al*., 1993; Long *et al*., 1993b), Petunia (Chuck *et al*., 1993), tobacco (Whitham *et al*., 1994), tomato (Jones *et al*., 1994) and flax (Ellis *et al*., 1995).

- **Characterisation of the *Ac/Ds* system and its potential for gene tagging**

Ac and *Ds* elements exhibit a number of favorable characteristics for use in transposon tagging strategies. Initial transposon tagging strategies relied upon insertional mutagenesis. Insertion of an element can either block transcription, create

truncated transcripts or alter expression patterns by disrupting control mechanisms. Splice sites in the *Ac* element can also result in altered processing of transcripts.

Ac and *Ds* elements create a transposon footprint upon excision. Insertion creates an 8 bp duplication flanking the element which is at least partly retained after excision (summarised in Doring and Starlinger, 1984; Saedler and Nevers, 1985). The 8 bp duplication is joined together during the excision process. The resulting junction often contains base substitutions or deletions.

Imperfect excision events from transcribed sequences can either result in phenotypic reversion events, or create allelic variation. Excision events from untranscribed or untranslated sequences typically result in phenotypic reversion. The effects of excision from translated sequences are dependent upon the footprint generated. This ability to generate mutations in coding regions is thought to be responsible for genomic evolution in transposon containing species such as maize. Phenotypic reversion and the generation of allelic variation have been used as evidence for successful gene tagging by transposable elements.

In maize, *Ac* elements have a tendency to transpose during DNA replication (Greenblatt, 1984; Chen *et al*., 1987). Germinal transposition events result in gametes, and therefore progeny, inheriting the transposon in a new genomic location. The autonomous activity of the *Ac* element in heterologous species limits its use in transposon tagging strategies. Somatic transposition can result in phenotypic reversion in non cell-autonomous mutations. Germinal excisions can result in loss of the gene tag or mutant phenotype. Stable elements are required to fully analyse mutations and follow mutant phenotypes through generations.

Two component systems provide a solution to this problem. *Ds* elements are mobilised in hemizygous plants, after crossing *Ds* homozygous and *Ac* transposase expressing homozygous lines. Transposase expressing *Ac* elements can be stabilised by removal of the end sequences creating a 'clipped wing' element (Altmann *et al*., 1992; Bancroft *et al*., 1992). Coupland *et al*. (1988) demonstrated the trans-activation of *Ac* deletion derivatives (*Ds* elements) in tobacco by both functional *Ac* elements and a fusion of the octopine 2' promoter from the T-DNA of *A. tumefaciens* (Velten *et al*., 1984) to the 3.5 kb *Ac* transposase transcript.

- **Characterisation and modification of *Ac***

Transposition of *Ac* and *Ds* requires only one *Ac* derived transcript, unlike *En/Spm* elements, which require an optimal ratio of at least two transcripts (Masson *et al*., 1991; Frey *et al*., 1990). In maize *Ac* shows a 'negative dosage' effect. Transposition frequency decreases and occurs later in development in plants with increased *Ac* copy number (McClintock, 1951). *Ac* transposase expression in maize containing two *Ac* elements however is higher than in those only containing one (Kunze *et al*., 1987).

Unlike maize a 'positive dosage' effect of transposase expression increases *Ac* and *Ds* excision in *Arabidopsis* similar to that seen in tobacco (Jones *et al*., 1989). Increased copy number of *Ac*, or the expression of *Ac* transposase from exogenous promoters with high expression levels has been demonstrated to increase *Ac* and *Ds* excision events (Swinburne *et al*., 1992; Keller *et al*., 1992; Grevelding *et al*., 1992; Honma *et al*., 1993; Bancroft and Dean, 1993a). *Ac* or *Ac* transposase insertion site has been observed to affect transposition frequency where *Ds* insertion site does not (Altmann *et al*., 1992; Smith *et al*., 1996). Position effects are widely accepted to

affect expression of transgenes and are likely to explain this observation.

Lawson *et al*. (1994) attributed the 'positive dosage' effect in *Arabidopsis* not to increased transcript levels, but to post-transcriptional regulation. Inefficient transcription and mRNA processing was suggested as a possible cause of low transposition rates in *Arabidopsis* by Grevelding *et al*. (1992); Keller *et al*. (1992) and Swinburne *et al*. (1992). Martin *et al*. (1997) demonstrated that inefficient transcription resulted in truncated copies of the transcript in *Arabidopsis*. Jarvis *et al*. (1997a) reported the isolation of two novel *Arabidopsis* mutant lines showing increased *Ac* activity. Further analysis of one of these mutants suggested that the increase was not related to increased transposase expression. A large number of transcripts in the mutant were incorrectly spliced (Jarvis *et al*., 1997b). An attempt to remove introns from an *Ac* element to obviate the requirement for correct splicing resulted in stabilisation of the element. The element was however shown to transpose autonomously in tobacco and when activated in *trans* by a genomic copy of *Ac* transposase in *Arabidopsis*.

McClintock observed 'changes in state' and 'changes in phase' of *Ac* activity in maize. 'Changes in state' were defined by a spatial or temporal alteration in activity between generations (McClintock, 1951). 'Changes in phase' were recognised by inactivity of previously active elements which re-gain activity in later generations (McClintock, 1951). This is likely to be a result of methylation. Transposase expression and therefore *Ac* activity, is partly controlled in maize by the methylation state of the element in a CpG rich region of the transposase untranslated leader sequence (Kunze *et al*., 1988).

The untranslated leader sequence may contain a controlling region for transposase expression in *Arabidopsis*. Removal of the untranslated leader from *Ac* transposase has been demonstrated to result in an increase in activity of both *Ac* and *Ds* elements in *Arabidopsis* (Lawson *et al*., 1994; Bancroft *et al*., 1992). Insertion of *CaMV35S* enhancers in this region has also been shown to increase activity (Balcells and Coupland, 1994). Interestingly Keller *et al*. (1992) found that the methylation state of *Ac* elements did not effect activity in *Arabidopsis*.

- **Characterisation and modification of *Ds***

Simple *Ds* elements are internal deletions of *Ac* (summarised in Doring and Starlinger, 1986). The elements contain *Ac* termini but may contain non-*Ac* derived DNA between them. Coupland *et al*. (1988) constructed and tested a series of *Ac* deletion derivatives in tobacco. Internal deletions removing the transcribed sequences resulted in stable elements, which were trans-activated by a functional *Ac* element. Deletion of bases 44-92 and 75-181 abolished or strongly reduced excision rates. Further characterisation of deletion derivatives of *Ac* by Coupland *et al*. (1989) showed that around 200 bp of *Ac* sequence are required at both ends for excision at wild type levels. Excision levels higher than wild type were seen with elements containing only 238 bp at the 5' end and elements containing only 209 bp at the 3' end. Excision levels lower than wild type were seen in elements containing between 132-207 bp at the 5' end, or 101-130 bp at the 3' end. The termini were not interchangeable. This finding was consistent with the observation that *Ac* transposase interacts with sequences between bases 103-157 at the 5' end and bases 4451-4525 at the 3' end (Kunze and Starlinger, 1989).

The demonstration of successful transposition of *Ac* deletion derivatives created a number of opportunities for engineering *Ds* elements. Tagging strategies have been improved by generating *Ds* elements carrying markers to select for successful re-insertion events. Gene and enhancer trap elements carry scoreable markers often for *GUS* expression. Outward facing promoters have been inserted into *Ds* elements to create novel insertional mutants. Some of the strategies using these elements are described below and results obtained using such elements are described later in further detail.

Ds elements carrying markers for selection of re-insertion events using hygromycin, kanamycin, methotrexate and chlorsulfuron have been reported (Bancroft *et al*., 1992; Grevelding *et al*., 1992; Sundareson *et al*., 1995; Honma *et al*., 1993; Fedoroff and Smith, 1993). Both *Ds* selection and molecular analysis have lead to estimations of re-insertion frequency in *Arabidopsis* ranging from 29% to 58% (Bancroft *et al*., 1992; Dean *et al*., 1992; Keller *et al*., 1992; Altmann *et al*., 1992; Honma *et al*., 1993; Bancroft and Dean, 1993b). Similar re-insertion frequencies in the range of 50-60% were previously recorded in tobacco (Jones *et al*., 1990).

Gene trap and enhancer trap strategies were described for use in *Arabidopsis* by Fedoroff and Smith (1993). Gene trap *Ds* elements contain the coding sequence of the *GUS* gene and create novel GUS staining patterns upon insertion downstream of a promoter sequence. Enhancer trap *Ds* elements contain a minimal promoter sequence fused to the *GUS* gene and identify insertions in the region of enhancer elements. These elements show novel GUS staining patterns after insertion into, or close to actively transcribed genes. The studies have also produced data suggesting a tendency for re-insertion into actively transcribed regions in *Arabidopsis*.

Sundareson *et al*. (1995) reported that 50% of enhancer trap and 25% of gene trap insertions resulted in GUS expression. Maes *et al*. (1999) describe the potential for engineering gene tag elements in plants, which both identify novel expression patterns and allow direct selection for insertion into functional genes.

- **Monitoring excision events**

Excision events can be monitored by the insertion of *Ac*/*Ds* in between a constitutive promoter and a selectable or scoreable marker. Baker *et al*. (1987) first used the insertion of an *Ac* element into a kanamycin resistance cassette to select for excision in tobacco. Schmidt and Willmitzer (1989) showed *Ac* to have a low germinal excision frequency in *Arabidopsis* of 0.2-0.5% by monitoring excision from a kanamycin fusion cassette.

Jones *et al*. (1987) developed a streptomycin resistance marker based on the SPT gene from *E. coli* Tn*5* (Mazodier *et al*., 1983; Putnoky *et al*., 1983). Selection was improved by the use of a mutated SPT gene (Maliga *et al*., 1988), which increased resistance and allowed efficient selection of tobacco. Plants are germinated on tissue culture media containing streptomycin and glucose. Sensitive plants germinate and bleach but do not die if supplied with a carbon source. The selection is cell autonomous, which means that individual patches of cells containing excision events can be identified amongst a background of cells containing no excision events. This feature was fully utilised to visually detect somatic and germinal transposition events in tobacco (Jones *et al*., 1989). An *Ac* element was inserted into the untranslated leader sequence of a fusion between the octopine 2' promoter from *A. tumefaciens* T-DNA (Velten *et al*., 1984) and the SPT gene. Excision resulted in the fusion of the promoter and SPT creating streptomycin resistance. Plants resulting from germinal

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excision events were fully green, plants retaining the *Ac* element at the original location were bleached and plants containing somatic transposition events appeared as variegated with green patches on a white background.

The selection procedure was further modified by fusion of a Cauliflower Mosaic Virus 35S promoter (*35S*) to the SPT gene, resulting in higher SPT expression and a stronger resistance phenotype (Jones *et al*., 1990, 1991). Plasmids containing this construct along with a number of other transposon tagging oriented vectors have been described and made publicly available (Jones *et al*., 1992). The *35S*::SPT construct described by Jones *et al*. (1990) was shown to function as a cell autonomous excision marker for *Ac* and *Ds* elements in *Arabidopsis* (Bancroft *et al*., 1992; Dean *et al*., 1992; Keller *et al*., 1992; Swinburne *et al*., 1992).

- **Re-insertion events**

In maize *Ac* and *Ds* elements show a strong tendency for transposition to both genetically linked and active sites (Greenblatt, 1984; Dooner and Belachew, 1989; Sundareson, 1996). This observation has been used to generate directed tagging strategies. Launchpad lines containing mapped *Ds* elements can be used to saturate the surrounding genomic area with *Ds* insertions. Random mutagenesis has also been carried out using numerous launchpad lines in specific chromosomal locations.

Re-insertion frequencies as high as 68% to genetically linked sites have been reported in *Arabidopsis* (Bancroft *et al*., 1993, Bancroft and Dean, 1993b). Machida *et al*. (1997) reported 50% of *Ds* insertion to be within 1,700 kb and 35% to be within 200 kb. These observations have been used to generate mapped *Ds* insertions as launchpads for tagging strategies covering the entire genome. Over 550 *Ac*/*Ds*

insertions have been mapped onto all five chromosomes of *Arabidopsis* by a number of groups (Smith *et al*., 1996; Long *et al*., 1997; Li *et al*., 1999; Parinov *et al*., 1999). The availability of launchpad lines facilitates targetted tagging strategies for future functional studies based on genomic information.

Osborne *et al*. (1995) used the preference for linked transposition events to develop a system for creating chromosomal rearrangement using the bacteriophage P1 Cre-lox recombination system. Lox sites were inserted into both the T-DNA and a *Ds* element situated in the T-DNA. Transposition separated the two Lox sites and chromosomal rearrangement occurred on crossing with a plant expressing Cre recombinase.

Sundareson *et al*. (1995) described a novel selection method to identify unlinked insertion events for random mutagenesis strategies. The counter-selectable marker *iaaH* was included in the T-DNA constructs. A *Ds* element carrying a selectable marker for kanamycin resistance, was used for selection of primary transformants and successful re-insertion events. The *iaaH* gene confers sensitivity to NAM. Plants were selected that were both kanamycin resistant and NAM insensitive after transposition.

- **Practical considerations of two component systems**

Two component systems traditionally rely on the generation of two lines of transgenic plants. One line carrying the *Ds* element is crossed with a line containing a stable *Ac* element or *Ac* transposase source. Whilst this enables lines to be selected where transposase expression is optimal for transposition, it is labour intensive and often many lines must be crossed to generate sufficient transposition events for

mutagenesis studies. The transposase source must also be segregated away from the *Ds* element to stabilise the mutation. This must then be re-introduced for observation of phenotypic reversion events.

Selection methods using *GUS* and *iaaH* linked to the stable *Ac* element or *Ac* transposase construct have been reported. These make it possible to stabilise *Ds* mutations by removing *Ac* containing plants from populations after transposition (Bancroft *et al*., 1992; Honma *et al*., 1993; Osborne *et al*., 1995).

- **Summary of the results obtained using *Ac/Ds* in *Arabidopsis***

The characterisation of *Ac*/*Ds* activity in *Arabidopsis* and the development of strategies for transposon tagging have lead to a number of mutations being created and new genes being identified. Bancroft *et al*. (1993) and Long *et al*. (1993b) first identified the *DRL1* allele and the *albino* mutation after *Ds* insertion. Phenotypic reversions were shown in both cases upon re-activation of *Ds*. Novel genes and mutants have been identified and novel GUS staining patterns have been observed after activation of gene trap and enhancer trap elements (Springer *et al*., 1995; Klimyuk *et al*., 1995; Sundareson *et al*., 1995).

Large-scale studies (Long *et al*., 1993, 1997; Altmann *et al*., 1995; Bhatt *et al*., 1996) have reported the observation of 138 visible phenotypic mutations from plants containing approximately 2173 independent transposition events. 24 out of 67 of these have been shown to be linked to *Ds* insertions. Large numbers of plants containing independent transposon insertions could be simultaneously screened by PCR to select plants containing insertions in specific genes. Large scale tagging strategies can therefore create a useful resource for many groups of researchers.

In summary, many successful transposon tagging strategies using *Ac* and *Ds* have been developed and characterised in *Arabidopsis*. Both random and directed mutagenesis have been carried out. Modification of transposase expression has been used to optimise germinal transposition events. Excision markers, *Ds* markers, gene trap elements and enhancer trap elements have all been used to monitor transposition events. Numerous novel methods have been described. Many mutants have been successfully generated and genes have been identified and cloned.

- **Opportunities for improvement**

The development and use of transposon tagging strategies in *Arabidopsis* has highlighted a number of factors limiting the identification of mutants. Novel strategies have been designed to overcome the problems caused by the need for constitutive high-level expression of *Ac* transposase and the limits of insertional mutagenesis, some of these are discussed below.

- **Overproduction and constitutive expression of *Ac* transposase**

Increased *Ds* excision using *35S* driven *Ac* transposase has been suggested to lead to an increase in early excision events (Long *et al*., 1993a). Early excision events occur in cells which eventually give rise to the gametes. This can result in many progeny inheriting identical transposition events. Many plants must therefore be generated to obtain sufficient independent insertions for use in random mutagenesis studies. Grevelding *et al*. (1992) however reported that germinal transpositions controlled by *35S* transposase expression occurred late in development and offspring inherited individual transposition events. Constitutive high-level expression of *Ac* transposase has also been suggested to result in lower re-insertion frequency than that from native *Ac* transposase expression (Long *et al*., 1993a; Balcells and Coupland, 1994).

Firek *et al*. (1996) reported a fusion of the anther specific *Arabidopsis apg* promoter to *Ac* transposase. No somatic excision events were observed. Gametophyte specific transposase expression and germinal excision levels were increased in tobacco containing a *Ds* element.Balcells *et al*. (1994) demonstrated inducible *Ds* transposition by using the *Gmhsp17.3*-B soybean heat shock promoter to express *Ac* transposase in *Arabidopsis*. They found that transposition occurred in embryonic cells, which ultimately gave rise to the gametes and generated progeny containing individual germinal transposition events.

Transformants were produced containing a fusion of the heat shock promoter and the *Ac* transposase gene. Inducible transposase expression was determined in these transformants by RNase protection experiments. The heat shock promoter was induced by placing the plants at 42°C for 2 hours. Transposase mRNA levels were seen to increase over 100-fold after induction. Very low levels of transposase mRNA were detected in non-induced plants. Plants homozygous for the transposase fusion were crossed with plants carrying a homozygous *Ds* element inserted into a *35S*::SPT fusion.

Transposase expression was induced in the hemizygous progeny according to three different profiles. HS-veg plants were induced early after germination and until bolting occurred, HS-rep plants were induced only after bolting and until seed set, HS-veg/rep plants were induced early after germination and until seed set. Plants were induced at 42°C for 4 hours three times a week. Plants were then allowed to self-fertilise and progeny were screened for excision events using streptomycin.

Progeny from plants induced according to the HS-veg profile contained few excision events. Only a slight increase in the frequency of variegated offspring was seen from uninduced controls (0.2% uninduced to 0.5% HS-veg). No fully streptomycin resistant progeny were recovered. Progeny from plants induced according to the HS-rep and HS-veg/rep profile showed a high frequency (29%) of variegated phenotypes. Only 9/23 families contained fully green seedlings at a maximum frequency of 1.6%. Variegated plants were allowed to self fertilise and progeny contained a high frequency of fully green streptomycin resistant plants.

Transposition in these lines was successfully induced by heat shock controlled expression of *Ac* transposase. Few transposition events occurred in cells resulting in the gametes of mature plants. Transposition events were limited to the embryonic cells of the next generation. Transposition events were successfully induced in embryonic cells, which eventually gave rise to both the cotyledons and gametes. This pattern was attributed to the specificity of the heat shock promoter. Transposition in embryonic cells ultimately resulting in gamete formation results in many progeny inheriting the same transposition event.

An advantage of the relatively short period of transposase expression was proposed by the observation of an increase in re-insertion frequency from that reported using high levels of constitutive transposase expression (Long *et al*., 1993a).

Ideally transposition events would occur in the cells giving rise to the gametes in mature plants. The lack of activity in these cell lineages of mature plants and the activity in embryonic cells was attributed to specificity of the promoter. The *Gmhsp* 17.3-B promoter was observed to show non heat-shock related activity in embryo

cells of transgenic tobacco but not *Arabidopsis* (Prandl *et al*., 1995; Prandl and Schöffl, 1996). *Ds* re-insertion frequency was increased using this system. This was attributed to the short period of transposase expression. However transposase levels 25 times higher than uninduced plants were observed 8 hours after induction. This was mainly attributed to mRNA stability as heat shock proteins are undetectable in soybean within four hours of heat shock. Smart *et al*. (1991) recorded expression from the *Gmhsp* 17.3-B promoter to remain high for up to 8 hours and then fall substantially by 24 hours. A system that lacks the cell specificity of the *Gmhsp* 17.3-B promoter and shows similarly tight regulation of transposase expression, before and after induction, could potentially further increase the efficiency of a transposon tagging strategy.

- **Generation of gain-of-function alleles**

Insertional mutagenesis often results in recessive loss-of-function phenotypes, which further increases the labour involved because homozygous plants must be generated before any mutant phenotypes can be identified. Many genes are functionally redundant or only required at specific points in development. Some 'essential' genes may show no phenotypic effects in heterozygous plants but create lethal phenotypes in homozygous plants. Whilst these genes may be identifiable using the transposon tag, functional studies would be difficult to carry out. Dominant mutations created by increased or ectopic expression could identify genes that recessive loss-of-function mutation would be unable to identify.

Wilson *et al*. (1996) used a *Ds* element carrying a *35S* promoter facing outwards across one of the *Ds* ends to recover dominant gain-of-function alleles. They created and characterised a phenotypically visual semi-dominant mutant. The *tiny* mutant

was thought to result from increased or ectopic expression of a novel plant transcription factor.

A *Ds* element was constructed by inserting a hygromycin selectable marker into an *Ac* element. The *35S* promoter was then inserted facing outwards across 246 bp at the 5' end of *Ac*. This was inserted in both orientations into a *35S:*SPT fusion and transformants generated. The *35S* promoter was shown to create streptomycin resistance in plants where the element was oriented such that the promoter showed read-through into the SPT fusion.

Using plants containing the *Ds* element oriented in the opposite direction, transposon tagging was carried out. Homozygous plants containing the *Ds*(*35S*) construct were crossed with homozygous plants expressing *Ac* transposase. The heterozygous progeny were allowed to self-fertilise and offspring containing transformation events were selected using streptomycin and hygromycin. These plants were recovered and again allowed to self-fertilise. Mutant phenotypes were scored in the next generation. Of 33 mutants identified 29 were recessive and 4 dominant. One of these dominant mutants was further studied.

The *tiny* mutant showed a dwarfed phenotype. Progeny from plants heterozygous for the *Ds* insertion showed the phenotype segregating in a 1:2:1 (severely dwarfed : dwarfed : normal) ratio. Progeny from the selfed dwarfed plants in these populations showed an identical 1:2:1 ratio. Progeny from selfed normal plants all showed a normal phenotype and severely dwarfed plants were sterile. This was consistent with the generation of a semi-dominant mutation. Homozygous plants containing the *Ds* element showed a severe phenotype. Heterozygotes showed an intermediate

phenotype and azygous plants appeared normal.

The mutation was shown to be caused by the *Ds* insertion by phenotypic reversion of mutant plants crossed to an *Ac* transposase expressing line. Revertants were also shown to contain typical 8 bp repeat sequences seen after *Ds* excision. RNA studies identified that the mutant plants contained a transcript expressed from the *35S* promoter in the *Ds* element. The *Ds* insertion was mapped to the untranslated leader sequence of the *TINY* gene. The gene is expressed at low levels in wild type plants. The mutant phenotype was thought to result from either over-expression or ectopic expression.

Dominant mutations can be caused by the expression of genes from a *Ds* insert carrying an outfacing promoter. Dominant 'lethal' mutations may be recoverable by use of an inducible promoter. Phenotypic mutants could be identified upon induction of the promoter and therefore increased or ectopic expression. These could potentially be recovered after the removal of the inducer. Further mutants may also be recovered, caused by either partial expression or antisense expression of genes flanking the insertion site.

- **Further developments and the future potential of transposon tagging.**

Charng *et al*. (1997) developed an inducible transposon tagging technique in tobacco. An inducible promoter based on the PR1-a promoter was used to express *Ac* transposase after induction by salicylic acid. *Ds* excision was observed after induction. An inducible *Ac* element was developed carrying both the inducible transposase construct and a marker for hygromycin resistance. This was

demonstrated to act as an inducible autonomous selectable element in tobacco and tomato (Charng *et al*., 2000).

Phogat *et al*. (2000) described a four-element based tagging system. They used the tendency for *dSpm* elements to transpose to unlinked random sites to carry an inserted *Ds* element around the genome. The *Ds* element was then activated and used to create insertional mutations in the surrounding area.

Xiao and Peterson (2000) used a *Ds* element carrying the *GUS* gene to create intrachromosomal homologous recombination events in *Arabidopsis*. The *Ds* element was used to carry a partial duplication of the *GUS* gene to surrounding sites and recombination with a homologous region in the T-DNA insert was monitored by GUS expression.

Meissner *et al*. (2000) reported a promoter trapping system for use in tomato. The promoter trap resulted in expression of firefly luciferase. An advantage over GUS expression was the ability to observe the time course of novel expression patterns in tagged mutants.

Suzuki *et al*. (2001) describe a novel tagging system designed to generate gain-of-function mutations. A *Ds* element carries two *35S* promoters and/or four tandem repeats of the enhancer sequence of the promoter. These face out of each end of the element and increase the efficiency of creating mutations.

Heterogenous transposable elements have been shown to transpose in many of the important crop species. Murai *et al*. (1991) demonstrated *Ac*/*Ds* transposition and

Izawa *et al*. (1997) generated phenotypic mutants in rice. Meissner *et al*. (1997) reported activity of the *Ac*/*Ds* system in the miniature Micro-Tom tomato. Takumi *et al*. (1999) observed *Ds* transposition in wheat after activation by *Ac* transposase expressed from a *35S* promoter. Mutagenesis studies in these and other economically important species will provide a wealth of information for crop improvement.

Genome sequencing projects have provided access to the sequences of many putatively functional genes. The entire *Arabidopsis* genome sequence is available and EST and genome databases are available for many other important species. Putative functional genes can be studied by reverse genetic methods using directed tagging strategies. Specific areas of the genome can be saturated with mutations using mapped launchpad lines. This will allow functional mutants to be generated to study specific targets previously identified from the analysis of genome sequencing.

Native transposable elements have been identified in *Arabidopsis* (Klimyuk and Jones, 1997; Tsay *et al*., 1993). Tag1 has been identified as an autonomous element present in many ecotypes of *Arabidopsis* but not Columbia (Frank *et al*., 1997, 1998; Henk *et al*., 1999). Tag1 is thought to be trans-activated by *Ac* but also shows autonomous transposition in *Arabidopsis* ecotype Colombia, tobacco and rice (Frank *et al*., 1997; Liu and Crawford, 1998a, 1998b; Liu *et al*., 1999). Tag1 has also been activated by a T-DNA insertion containing a disabled dTag1 element (Liu and Crawford, 1998b). Characterisation of the element is currently being studied at a molecular level (Liu *et al*., 2001). This may provide encouraging results for developing new strategies using the endogenous transposable element.

1.4 Inducible promoter systems

- **Inducible promoters for use in transgenic plants**

The ability to generate transgenic plants has brought with it the need for control of transgene expression. In many cases strong constitutive promoters such as the Cauliflower Mosaic Virus 35S (*35S*) promoter (Odell *et al*., 1985) have been used to control high levels of transgene expression. The use of constitutive promoters to control expression limits the technology to the use of genes that show few, or no deleterious effects, when expressed constitutively at high levels throughout the plant. Developmentally regulated promoters have been used to control spatial or temporal expression in some cases (Kuhlemeier *et al*., 1987). These promoters hold an advantage over constitutive expression but are often still too insensitive for the expression of genes which result in lethality, or must be expressed at a range of specific points in development. This problem can be overcome by the use of inducible promoters to control the expression of transgenes.

Inducible promoters should ideally have low basal levels of expression, high levels of induced expression, high specificity for the inducer, fast response to induction, rapid switch-off, no physiological effects caused by induction and no endogenous homologues to either the inducer, or the induction system. Endogenous induction systems, such as heat shock systems, have been used to control transgene expression in plants and are discussed later. More applicable to the requirements of both academic study and industrial development are the range of heterogenous chemical-inducible systems.

A number of chemical-inducible systems have been described utilising tetracycline, dexamethasone, ethanol, RH5922 and β-estradiol to control transcription from heterogenous promoter constructs in transgenic plants (Gatz *et al*., 1992; Weinmann *et al*., 1994; Aoyama and Chua, 1997; Caddick *et al*., 1998; Martinez *et al*., 1999; Bruce *et al*., 2000). Various uses of these systems and their relative merits have been the subject of a number of reviews (Gatz, 1997; Gatz and Lenk, 1998; Zuo and Chua, 2000). Two inducible promoter systems, the ethanol inducible *alc* system (Caddick *et al*., 1998) and the soybean heat shock responsive system (Baumann *et al*., 1987) are used in the experiments described and these are discussed in detail below.

- **The *alc* system**

The *alc* system was initially described by Caddick *et al*. (1998) as an ethanol inducible gene switch for plants. The system is based on the *alc* regulator of *Aspergillus nidulans* (Felenbok, 1991). In *Aspergillus nidulans* the *alcA* gene encodes alcohol dehydrogenase I and is regulated by the AlcR transcription factor (Fillinger and Fellenbok, 1996). The AlcR transcription factor binds to specific sites within the *alcA* promoter region and responds directly to the inducer molecule (Kulmberg *et al*., 1992). A minimal system, consisting of the constitutively expressed *alcR* coding sequence and the *alcA* promoter, was demonstrated to produce ethanol inducible expression of the chloramphenicol acetyltransferase (CAT) reporter gene in stably transformed tobacco. Carbon metabolism was also manipulated by the ethanol inducible expression of yeast cytosolic invertase, again in tobacco.

- **Development and use of the *alc* system**

The *alc* system has been fully characterised in tobacco (Salter, 1997; Salter *et al*., 1998), tomato (Garoosi, 1998), and *Arabidopsis* (Roslan, 1999). It has been demonstrated to produce high levels of inducible expression, low levels of basal activity, and rapid switch-off. The system is induced by ethanol and concentrations as low as 0.01% are sufficient for induction. This sensitivity is advantageous as high levels of ethanol can cause cytotoxicity. Low levels of ethanol were seen to cause little or no observable physiological effects on the plants tested.

Caddick *et al*. (1998) reported that the *alc* system has a very low basal level of expression in tobacco and reaches induced levels equal to 50% of the expression seen from the *35S* promoter. Mike Salter (Salter, 1997; Salter *et al*., 1998) characterised the system in tobacco using expression of the CAT and GUS reporter genes. A variety of chemical inducers were analysed and ethanol was chosen as the optimal inducer. A threshold ethanol concentration of 0.5% was observed, which must be exceeded for induction of plants on soil. Increasing the concentration up to 7.5% had little effect although it was suggested that the threshold might in fact lie above 0.5%. Reporter gene expression was reported within 4 hours and a maximal expression level was reached 4 days after inducing plants on a hydroponics system. Soil grown plants showed induction for a longer period of time. This was attributed to the presence of ethanol for longer periods in the soil. Maximal levels of expression, equivalent to that seen from the *35S* promoter were recorded after 4 days. This expression fell to 50% after 8 days but the stability of the CAT protein made detailed analysis of the timing of switch-off difficult.

- **Characterisation of the *alc* system in *Arabidopsis***

Hairul Roslan (Roslan, 1999) characterised the *alc* system in *Arabidopsis* using a number of reporter genes. Transient expression was assayed after particle bombardment using constructs containing the CAT and GUS reporter genes. This verified that the *alc* system is functional in *Arabidopsis* and identified a line of plants carrying a single constitutively expressing *35S*:*alcR* insert.

Stably transformed plants containing *35S*:*alcR* and *alcA*:GUS reporter constructs were used to analyse the induction of plants on soil and agar. High basal levels of expression were observed in plants grown on agar. This was attributed to the anaerobic conditions of the roots. Concentrations of ethanol as low as 0.01% were seen to induce expression, with a maximum level of expression observed after induction with 2% ethanol. Induction using concentrations higher than 2% caused detrimental effects to the plants. Plants were also seen to be induced by ethanol vapour. This observation greatly affects experimental design.

After root drenching with 2% ethanol, expression was reported to increase from 4 hours, reaching a maximum level at 5 days. This returned to a very low level by day 15. Continuous induction was studied by root drenching plants with 2% ethanol every four days. A large increase in expression was seen on day 6 (2 days after the second induction) and maximal expression was seen on day 15 (3 days after the third induction). Toxicity was observed to cause detrimental effects in the plants after 13 days of constant induction.

Histochemical staining of whole plants showed that the *alcA*:GUS construct was expressed throughout all tissues including leaves, roots, inflorescences and flower

buds. The induction profile was further studied in *Arabidopsis* by real-time imaging of luciferase expression after the transformation of plants with an *alcA*:LUC construct. Expression was seen within 1 hour of induction. Expression was seen to spread up the plants when ethanol was applied to the roots and was observed in the leaves within 6 hours after induction. Induction from ethanol applied directly to the roots was maintained for 50 hours. Again this was seen to be longer in plants induced on soil presumably due to the ethanol staying in the soil for longer.

- **The *Gmhsp* 17.3-B soybean heat shock promoter**

Heat shock proteins and heat shock factors are identified as elements which are specifically expressed in response to heat stress. They have been identified and are highly conserved in many organisms (reviewed in Wu, 1995). Soybean possesses a gene family encoding low molecular weight heat shock proteins of 15-18 kd (Key *et al*., 1981; Schöffl and Key, 1982). A genomic fragment containing two of these genes was cloned and characterised (Schöffl and Key, 1983). One gene contained in this fragment *hs6871* encodes a protein 17.3 kd in size. The promoter region of this gene was identified (Schöffl *et al*., 1984) and has since been characterised by internal deletions and shown to function in transgenic tobacco (Baumann *et al*., 1987). Smart *et al*. (1991) used the *Gmhsp* 17.3 B promoter to express the *A. tumefaciens tmr* gene in transgenic tobacco. Phenotypic variations were observed after heat shock and the subsequent increase in cytokinin production.

The *Gmhsp* 17.3 B heat shock promoter was used to drive heat-shock inducible transposase expression, in an inducible transposon tagging system in *Arabidopsis* (Balcells *et al*., 1994). Transposition was observed after heat shock in embryonic cells, which ultimately gave rise to both the cotyledons and the gametes.

Interestingly *Gmhsp* 17.3 B shows heat-shock independent expression in seeds of transgenic tobacco but not in *Arabidopsis* (Prandl *et al*., 1995; Prandl and Schöffl, 1996).

1.5 Antisense and post-transcriptional silencing

- **Post-transcriptional gene silencing and co-suppression**

Gene silencing can occur at either the transcriptional level, or the post-transcriptional level. Transcriptional gene silencing is generally associated with the methylation of promoter sequences and an alteration in chromatin structure. It is thought to have arisen as a method to control the movement of transposable elements and transgene inserts. Post-transcriptional gene silencing (PTGS) is a less well-understood process. It is thought to originate as a defence mechanism to combat viruses and DNA integrating pathogens such as *A. tumefaciens*. A number of mechanisms are thought to be involved, all of which result in the rapid degradation of mRNA produced by the virus or transgenes. The subject of gene silencing is under constant discussion and many reviews are available (Baulcombe, 1996; Stam *et al*., 1997; Depicker and Van Montagu, 1997; Fagard and Vaucheret, 2000; Meyer, 2000).

Post-transcriptional gene silencing was originally identified in plants and described as co-suppression (Napoli *et al*., 1990; Smith *et al*., 1990; Van der Krol *et al*., 1990). Endogenous genes were silenced after transformation with constructs expressing identical or truncated copies of the endogenous coding sequence. Silencing can occur

in *cis*, whereby only the RNA from the silencing source (i.e. the transgene) becomes silenced, *trans*, whereby homologous endogenous genes are silenced, but the source is not, or *cis* and *trans* whereby all homologous RNA is degraded. PTGS can be activated by sense transgenes, antisense transgenes, and viruses (reviewed in Baulcombe, 1996; Ratcliff *et al*., 1999; Baulcombe, 1999; Marathe *et al*., 2000) For the purposes of this introduction only PTGS events resulting in *trans* silencing caused by sense or antisense expression will be discussed.

Many instances of PTGS have been reported and numerous mechanisms have been proposed. The silencing of endogenous genes by transgenes is related to the expression level of the transgene (Que *et al*., 1997) and can be instigated by highly expressed single copy number transgenes (Palauqui and Vaucheret, 1995; Que *et al*., 1998). In general PTGS is thought to be caused by the production of aberrant RNA molecules that resemble viral RNA (Metzlaff *et al*., 1997; reviewed in Depicker and Van Montagu, 1997; Vaucheret *et al*., 1998; Baulcombe, 1999). The production of aberrant RNA by endogenous genes has been proposed to be caused by the methylation of coding sequences in regions of similarity to the aberrant transgenic transcripts (Elmayan *et al*., 1998; Jones *et al*., 1998). Mechanisms involving the generation of double stranded RNA and antisense RNA have also been proposed and are summarised by various authors (Baulcombe , 1996; Fagard and Vaucheret, 2000; Stam *et al*., 2000; Wassenegger, 2000).

- **The basis and original identification of antisense**

The inhibition of gene expression by antisense mRNA transcripts, or antisense oligonucleotides, is a process that has been considerably more successful as a tool, than it has been understood as a process. The inhibition of gene expression by

antisense RNA is thought to involve different mechanisms to PTGS and is the subject of a number of reviews (Takayama and Inouye, 1990; Nellen *et al*., 1992; Nellen and Lichtenstein, 1993). In the simplest proposed mechanism double stranded RNA is thought to arise from the pairing of sense and antisense strands. This double stranded RNA is then targeted for degradation. Many viruses contain double stranded RNA. Many organisms specifically degrade double stranded RNA as a defence mechanism against double stranded RNA viruses. Inhibition of gene expression by antisense occurs in many diverse organisms and the processes leading to inhibition are thought to be highly conserved.

Antisense inhibition was first used as a method of inhibiting the expression of transgenes in whole plants by Rothstein *et al*. (1987). Smith *et al*. (1988) used antisense expression of a partial polygalacturonase gene fragment to suppress endogenous polygalacturonase expression and affect ripening in transgenic tomato fruits. Van der Krol *et al*. (1988) used antisense expression of chalcone synthase to alter flower pigmentation patterns in petunia and tobacco plants. Since these original demonstrations, antisense technology has become a standard tool for the study of gene products and pathways in plants. Antisense technology has been used to study the expression and inhibition levels of the target genes of many of the currently used herbicides (Temple *et al*., 1993; Höfgen *et al*., 1994; Höfgen *et al*., 1995). Novel herbicide targets could be identified, by antisense strategies leading to the inhibition of the specific steps in amino acid and photosynthetic pathways (Abell *et al*., 1993; Rendina and Abell, 1994).

1.6 Summary of the transposon tagging plan

Transposon tagging has been discussed as a useful tool for the identification of novel genes and the creation of mutant phenotypes in higher plants. *Arabidopsis thaliana* has been described as a suitable model for the study of plant genes and specifically for transposon tagging studies. Modified transposon tagging strategies have been described which increase the control of transposition events, by using inducible transposase expression and create gain-of-function mutations, by using transposon constructs containing outfacing promoters. The generation of sense transcripts from these modified transposons has been described to create over-expression phenotypes and the possibility of gene suppression caused by the creation of aberrant sense or antisense transcripts has been discussed. Inducible promoter systems and the *alc* system in particular have been described for the control of transgene expression patterns in *Arabidopsis*.

The project described in the following chapters involves the generation of constructs for the development of two novel transposon tagging systems. Both of the tagging systems are based on the *Ac*/*Ds* elements of maize. Both the systems employ inducible transposase expression to control the timing of transposition events and to create stable insertions that can be re-mobilised, upon activation of the transposase source. Both systems are designed to generate gain-of-function mutations, by outward expression from promoters contained in the transposon constructs.

The first system utilises the *alc* system to control transposition. The transposase source is expressed upon induction of the *alc* system by watering with ethanol. The transposon for use in this system contains a constitutive *35S* promoter facing

outwards across the transposon end and would be expected to create dominant gain-of-function mutations constitutively. The use of the *alc* system in this way is expected to provide a valuable method for controlling transposition events and therefore increase the efficiency and productivity of tagging strategies.

The second system described utilises the *Gmhsp* 17.3-B heat shock promoter to control transposition. The transposase source is expressed, upon induction of the system by heat shock conditions. The transposon contains an inducible *alcA* promoter facing outwards across the transposon end. This system would be expected to create inducible dominant gain-of-function mutations. The ability to create inducible gain-of-function mutations is expected to provide a method for identifying many novel genes whose mutant phenotype would normally result in lethality.

The development of the constructs and transgenic plants required for analysis of the systems is described, along with a number of experiments to analyse the efficacy of the novel strategies.

1.7 Herbicides and amino acid biosynthesis

- **Herbicide targets and amino acid synthesis**

The development of intensive farming practices has provided a requirement for the chemical control of weeds. The major targets for herbicide inhibition are involved in either the synthesis of essential compounds or photosynthesis. These processes are unique to plants and microbes and inhibitors are therefore potentially nontoxic to animals. The inhibitors of amino acid synthesis are systemic. The initial action occurs in the rapidly growing regions and inhibition in other tissues follows later. Low levels of herbicide are often sufficient, and plants die within days of spraying. Of the amino acid synthesis pathways targeted by herbicides, the major inhibitors are targeted to the pathways of branched-chain amino acid, aromatic amino acid, and glutamine synthesis. A number of reviews are available detailing studies of amino acid biosynthesis and the targeting of herbicides to the individual steps in the pathways (Kishore and Shah, 1988; Mazur and Falco, 1989; Schulz *et al*., 1990; Coruzzi, 1991; Lea and Forde, 1994; Abell, 1996).

- **ALS**

Acetolactate synthase (ALS) is the first common enzyme in the synthesis of the branched-chain amino acids, valine, leucine, and isoleucine. In the synthesis of valine and leucine, two pyruvate molecules are condensed to form 2-acetolactate, whilst in the synthesis of isoleucine, pyruvate is condensed with 2-ketobutyrate to produce acetohydroxybutyrate. The synthesis of branched chain amino acids and the targeting of herbicides that inhibit the pathway is the subject of a number of reviews (Stidham, 1991; Singh and Shaner, 1995). ALS is the target enzyme of three classes

of herbicides, sulfonylureas, imidazolinones and triazolopyrimidines.

ALS genes have been identified and isolated from tobacco and *Arabidopsis* (Mazur *et al*., 1987) and *Brassica napus* (Rutledge *et al*., 1991). The mature ALS protein is highly conserved. The genes are all nuclear-encoded and produce precursor proteins containing a transit sequence for chloroplast-localisation (Jones *et al*., 1985; Mazur *et al*., 1987).

Mutant ALS genes, which confer resistance to the sulfonylurea herbicides, have been discovered in a range of plants and used for the generation of herbicide resistant crops (reviewed in Schulz *et al*., 1990; Lea and Forde, 1994).

ALS was the subject of a study involving antisense expression in potato by Höfgen *et al*. (1995). An 85% reduction in ALS activity was reported to impair plant growth almost irreversibly. The phenotype observed in antisense plants was similar to that observed in imidazolinone treated plants and included severe growth retardation, leaf chlorosis, the formation of necrotic lesions, and altered leaf morphology. The antisense construct was constitutively expressed from a *35S* promoter. The constitutive expression was suggested to prevent the recovery of plants completely inhibited in ALS expression as complete constitutive inhibition would result in lethality.

- **EPSPS**

The aromatic amino acids, phenylalanine, tyrosine, and tryptophan are all end products synthesised by the shikimate pathway. The shikimate pathway involves seven metabolic steps beginning with the condensation of phosphoenolpyruvate and

erythrose 4-phosphate and ending with the synthesis of chorismate, and is the subject of a number of reviews (Bentley, 1990; Herrmann and Weaver, 1999). The pathway is found only in microbes and plants and is therefore a potential target for herbicide inhibition. The penultimate step of the pathway is catalysed by 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. EPSP is produced by the condensation of shikimate-3-phosphate and phosphoenolpyruvate. EPSP synthase is the target enzyme of the herbicide glyphosate (Steinrucken and Amrhein, 1980). Glyphosate is a potent inhibitor of EPSP synthase (Steinrucken and Amrhein, 1980).

EPSP synthases have been identified and cloned from petunia (Shah *et al*., 1986), *Arabidopsis* (Klee *et al*., 1987), tomato (Gasser *et al*., 1988), and *Brassica napus* (Gasser and Klee, 1990). The mature EPSP synthase protein is highly conserved. The genes are all nuclear-encoded and produce precursor proteins containing transit sequences for chloroplast localisation.

A number of researchers have shown that overexpression of EPSP synthase can confer glyphosate tolerance and these experiments are reviewed in Kishore and Shah (1988); Mazur and Falco, (1989); and Schulz et al (1990). Mutant EPSP synthase enzymes have been identified in a number of microbes (Kishore and Shah, 1988; Mazur and Falco 1989). These have been used to generate glyphosate resistant plants without the need for overexpression, which often results in reduced growth (reviewed in Kishore and Shah, 1988; Herrmann and Weaver, 1999). Transgenic crops resistant to glyphosate have been engineered, and contain an EPSP synthase plastid import sequence from *Petunia hybrida*, linked to a glyphosate-insensitive EPSP synthase, obtained from *A. tumefaciens* and expressed from a *35S* promoter (Padgette *et al*., 1991; Nida *et al*., 1996).

- **Antisense expression of ALS and EPSPS**

As described above, antisense expression of ALS in potato was reported to result in phenotypes similar to those generated by ALS inhibiting herbicides. Antisense technology is therefore a potential tool for the validation of herbicide targets. The previous experiments described by Höfgen *et al*. (1995) used constitutive expression of an antisense construct, controlled by a *35S* promoter. This was suggested to limit the recovery of plants to those only partially inhibited, due to the lethality of complete constitutive inhibition. Inducible antisense expression could potentially result in the selection of transformants whose inhibition was complete only upon induction. These would also be potentially recoverable after induction, or possible to bulk up sufficiently in the absence of induction for extensive analysis.

A similar approach could be attempted using EPSP synthase as a target for antisense inhibition. Again it is expected that complete constitutive inhibition would result in lethality and limit the recovery of transformants. Inducible expression of the antisense construct would be expected to improve this approach.

Inducible antisense inhibition of these 'essential' genes would improve the use of antisense technology in the elucidation of potential herbicide targets, and also provide evidence to assess the potential of a transposon tagging strategy, aimed at generating inducible dominant gain of function and antisense mutations.