FLP-MEDIATED CONDITIONAL LOSS OF AN ESSENTIAL GENE TO FACILITATE COMPLEMENTATION ASSAYS

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Commonly, when it is desirable to replace an essential gene with an allelic series of mutated genes, or genes with altered expression patterns, the complementing constructs are introduced into heterozygous plants, followed by the selection of homozygous null segregants. To overcome this laborious and time-consuming step, the newly developed two-component system utilizes a site-specific recombinase to excise a wild-type copy of the gene of interest from transformed tissues. In the first component (the first vector), a wild-type version of the gene is placed between target sequences recognized by FLP recombinase from the yeast 2 μm plasmid. This construct is transformed into a plant heterozygous for a null mutation at the endogenous locus, and progeny plants carrying the excisable complementing gene and segregating homozygous knockout at the endogenous locus are selected. The second component (the second vector) carries the experimental gene along with the *FLP* gene. When this construct is introduced, FLP recombinase excises the complementing gene, leaving the experimental gene as the only functional copy. The *FLP* gene is driven by an egg apparatus specific enhancer (EASE) to ensure excision of the complementing cDNA in the egg cell and zygote following floral-dip transformation. The utility of this system is being tested using various experimental derivatives of the essential sucrose-proton symporter, *AtSUC2*, which is required for photoassimilate transport.

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CHAPTER 1

INTRODUCTION

1.1 The Role of Phloem and Phloem Anatomy in Transport

The phloem is a plant vascular tissue that plays an important role in the transport of various organic and inorganic nutrients, signal molecules and hormones. The products of photosynthesis (photosynthate) from mature leaves, which are the areas of photosynthate supply called sources, are translocated to areas of growth and storage in the plant, called sinks, via the phloem. The photosynthate (sugars) from mesophyll cells moves to sieve elements, which are phloem cells that translocate sugars and other organic materials throughout the plant. Sieve elements are associated with densely cytoplasmic parenchyma cells called companion cells, which provide the necessary proteins and metabolites for sieve element function and maintenance (van Bel and Knoblauch, 2000). The sieve elements and companion cells together form the sieve element-companion cell complex (SE-CCC). The sieve elements and companion cells are connected by numerous plasmodesmata (plasma membrane-bound tubules). Fig. 1 gives an illustration of a sieve element and companion cell in the SE-CCC.

There are three different types of companion cells: (1) ordinary companion cells, (2) transfer cells, and (3) intermediary cells, based on the type of plant species. All three types of companion cells have abundant mitochondria and dense cytoplasm. The ordinary companion cells have abundant connections with their sieve elements but very few plasmodesmata between other surrounding cells and are involved in solute uptake from the cell wall space. They have well-developed thylakoids in chloroplasts, and smooth inner cell wall surface. The transfer cells have fingerlike cell wall ingrowths, which increase the plasma membrane surface area and are

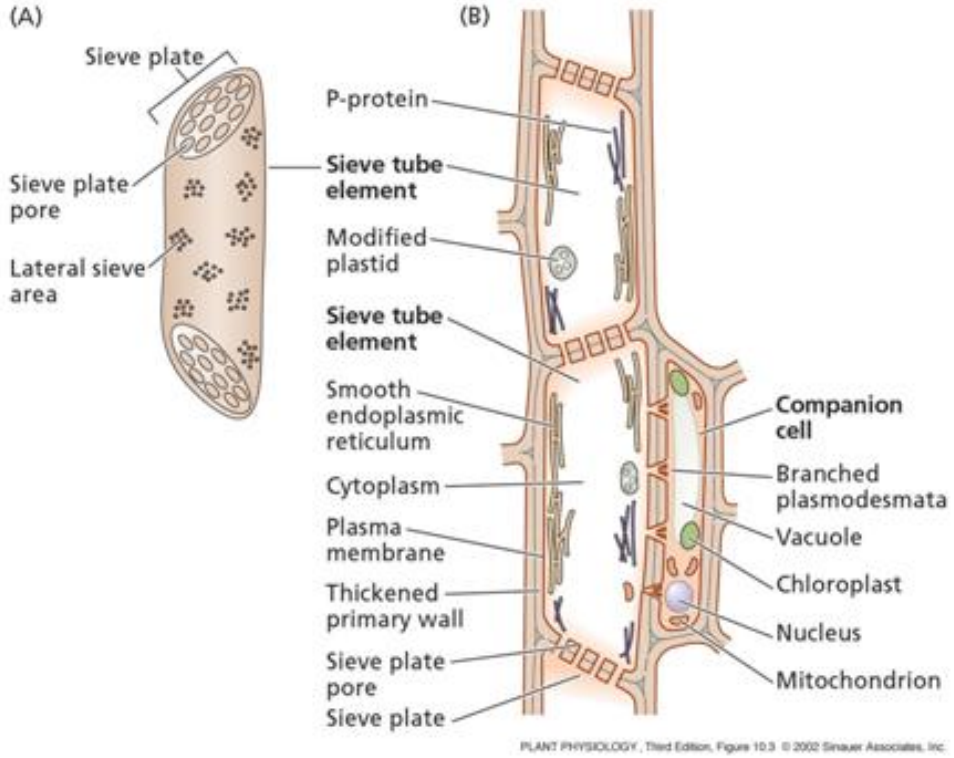

Figure 1: Diagram showing the sieve element-companion cell complex (SE-CCC) (Taiz and Zeiger, 2002).
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also thought to facilitate solute uptake from the cell wall space (Wimmers and Turgeon, 1991). The intermediary cells have abundant plasmodesmata connections with surrounding cells and are suitable for solute uptake via cytoplasmic connections (Turgeon et al., 1993). Fig. 2 shows the anatomy of each type of companion cell.

1.2 Phloem Transport Mechanism

Transport of photosynthate from the source tissues to sink tissues via phloem is driven by osmotically generated pressure flow (OGPF). The pressure-flow model was proposed by Ernst Munch in 1930 (Munch, 1930). According to the pressure-flow model, a hydraulic pressure gradient is generated between the source and sink tissues due to a difference in their solute potentials. This results in the mass flow of water and dissolved photosynthate in the phloem. The processes of phloem loading and unloading seem to control the rate of phloem transport.

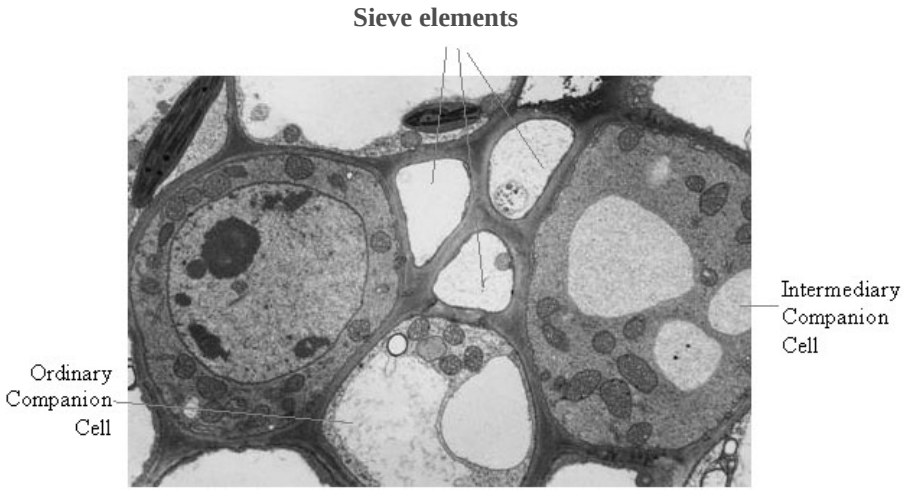
1.3 Phloem Loading

The process of phloem loading refers to the movement of photosynthate from the mesophyll cell to the sieve elements to establish a high solute concentration in the phloem. Phloem loading can be either through the symplastic route or through the apoplastic route. The symplast lies within the plasma membrane and is connected from cell to cell via plasmodesmata. The apoplast lies outside the plasma membrane and is comprised of the cell walls and xylem conducting cells.

1.3.1 Symplastic Phloem Loading

Symplastic phloem loading operates in species that translocate sugars belonging to the

(a)



(b)

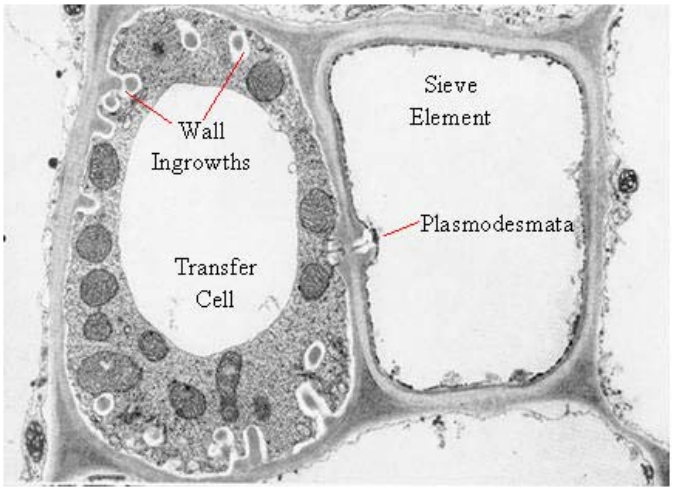


Figure 2: Electron micrographs showing anatomy of different types of companion cells: (a) ordinary companion cell and intermediary cell (Turgeon et al., 1993) (b) transfer cell (Brentwood and Cronshaw, 1978) (source: Taiz and Zeiger, 2002).
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raffinose family of oligosaccharides (RFOs), such as raffinose and stachyose, in addition to sucrose. Plants that load via the symplast have intermediary companion cells. Symplastic phloem loading can be explained by the "polymer trap" model proposed by Robert Turgeon (Turgeon and Gowan, 1990; Turgeon, 1991). According to the polymer trap model (Fig. 3) sucrose diffuses from mesophyll cells into the intermediary cells (specialized companion cells) via specialized plasmodesmata, gets converted into larger oligosaccharides (raffinose and stachyose), thus keeping the sucrose concentration low in the intermediary cells and allowing for continued diffusion of sucrose. The size exclusion limit of the plasmodesmata between the bundle sheath cells and intermediary cells allows for diffusion of sucrose into the intermediary cells but does not allow raffinose and stachyose to diffuse in the opposite direction due to their larger size. The RFOs then enter the sieve elements via large plasmodesmata connecting these cells.

1.3.2 Apoplastic Phloem Loading

In apoplastic phloem loading, photosynthate from the mesophyll cells is first released into the apoplastic space, and is then loaded into the sieve elements. Loading of sugars from the apoplast into the sieve elements and companion cells is an active process carried out by transporters located in the plasma membrane of these cells. Sucrose is the predominant transport sugar in the apoplast, which enters the apoplast at the boundary between the phloem parenchyma and SE-CCCs. Sucrose-H^+ symporters mediate its transport from the apoplast into companion cells and the sieve elements of the leaves (Fig. 4). The H^+ concentration gradient (i.e., the proton force) generated across the plasma membrane between the apoplast and symplast energizes transport of sucrose by the sucrose-H^+ symporter (Taiz and Zeiger, 2002). Sovonick et al. in

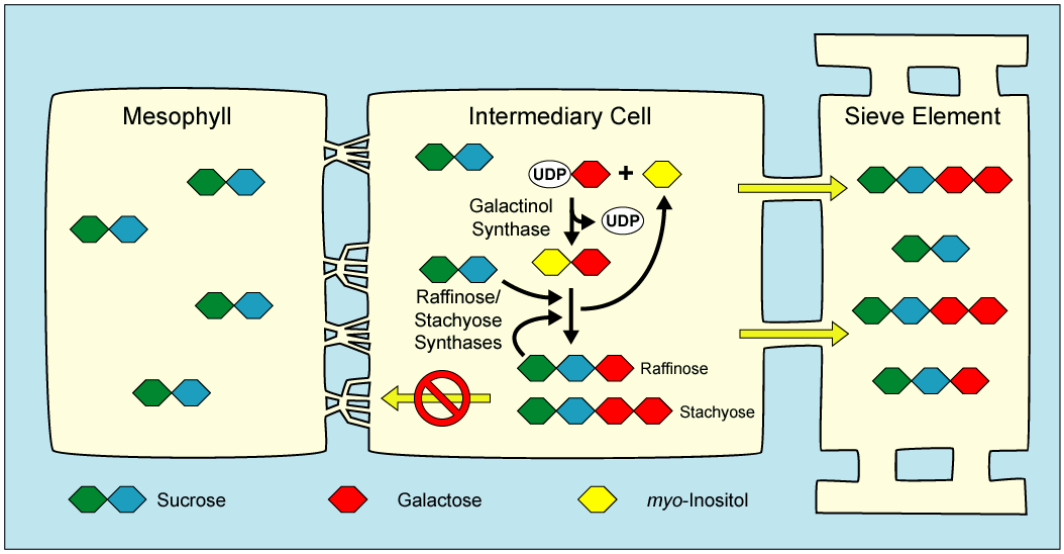


Figure 3: The ‘polymer trap’ model for symplastic phloem loading.

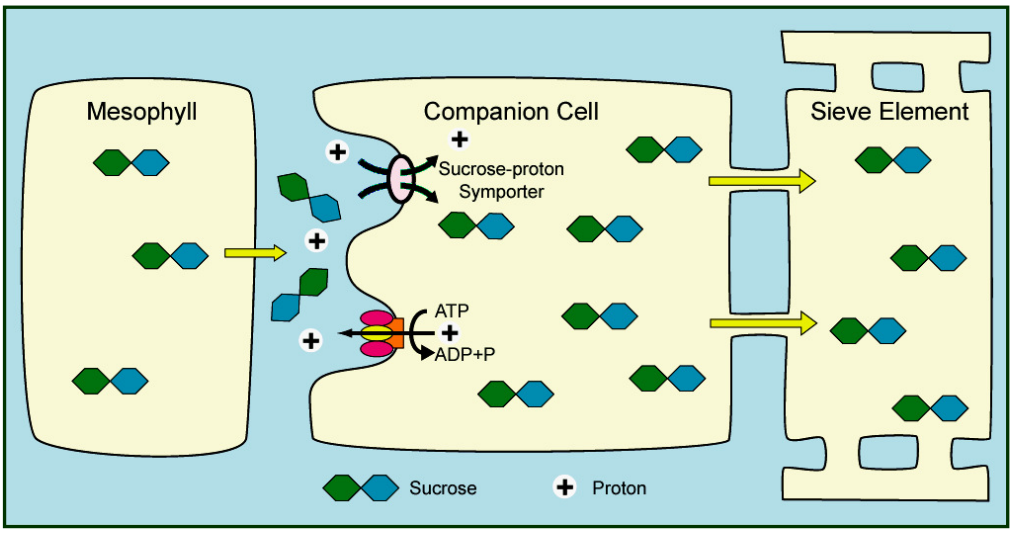


Figure 4: Apoplastic phloem loading mediated by the sucrose-proton symporter.

1974 showed for the first time that sucrose could enter the phloem via an energy-dependent plasma membrane localized sucrose- H^+ symporter in sugar beet. The first sucrose carrier was cloned from spinach by heterologous expression in yeast (Riesmeier et al., 1992). Inhibition of sucrose transporters by application of p-chloromercuribenzene-sulfonic acid (PCMBS) further demonstrated their importance in apoplastic phloem loading (Giaquinta, 1983).

1.4 *AtSUC2*: *Arabidopsis thaliana* Sucrose Symporter

The *AtSUC2* gene in *Arabidopsis thaliana* encodes an essential sucrose- H^+ symporter, which is found (in the companion cells) throughout the phloem (Stadler and Sauer, 1996). It functions in loading sucrose (Truernit and Sauer, 1995) from the apoplast into the SE-CCC (DeWitt and Sussman, 1995). *AtSUC2* is essential for phloem transport in *Arabidopsis* and mutations in the *AtSUC2* gene result in inability to transport sugars. Plants homozygous for a knockout mutation in the *AtSUC2* gene show severely stunted growth and develop more slowly. These plants do not set flowers and do not produce any viable seeds (Gottwald et al., 2000). Their mature leaves show anthocyanin and starch accumulation, which are indicative of an inability to transport the products of photosynthesis from source to sink tissues.

1.5 The FLP Site-specific Recombinase

The *AtSUC2* gene is essential and it is difficult to work with plants homozygous for a mutation in the endogenous *AtSUC2.* It would be desirable to create mutants with conditional expression in specific tissues or at specific stages of development by use of a site- specific recombinase.

Site-specific recombinases are enzymes that recognize specific DNA sequences and

catalyze recombination between them. FLP from the 2µm *Saccharomyces cerevisiae* plasmid (Broach and Hicks, 1980), Cre from the bacteriophage P1 (Austin et al., 1981) and R from the pSR1 plasmid of *Zygosaccharomyces rouxii* (Araki et al., 1985) are all site-specific recombinases that catalyze recombination between two 34 bp long target sites (McLeod et al., 1986). The sites recognized by FLP are referred to as FLP recognition target sites (*FRTs)* and the Cre recognition sites are referred to as *loxP* (locus of crossing over, x, P1) sites. DNA between the recombinase target sites is inverted if the target sites are in opposite orientation (Broach and Hicks, 1980) and excised if the target sites are arranged in the same orientation (Vetter et al., 1983). Fig. 5 shows the possible recombination events using FLP-mediated recombination as an example. Site-specific recombinases have been used to excise DNA sequence between two recombinase target sites, to bring about marker gene expression from a promoter. The marker gene is expressed only after excision upon site-specific recombination but not before recombination.

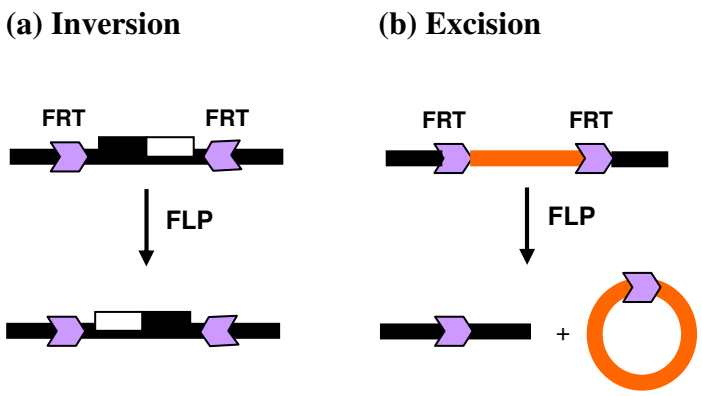


Figure 5: FLP-mediated recombination between two *FRT* sites. (a) Inversion of interlying DNA with *FRT* sites are in opposite orientation. (b) Excision of interlying DNA with *FRT* sites in same orientation.

CHAPTER 2

TWO-COMPONENT VECTOR SYSTEM

A two-component vector system was established to generate "on-demand" knockout plants of specific genes. The two-component vector system comprises two vectors: the first vector is pART-P450-ecSUC2-BAR harboring the excisable complementing *AtSUC2* (ec*SUC2*) and the second vector is pCAM-Gent-TSpSUC2-EASE-FLP carrying the tissue-specific *AtSUC2* gene along with the *FLP* gene. The following sections discuss each in turn.

2.1 Establishment of Parent Lines Harboring the Excisable and Complementing *AtSUC2* cDNA

In order to establish a parent line carrying the excisable and complementing *AtSUC2* cDNA that could be removed when desired, the FLP-mediated recombination system was employed. The plasmid pFLP-SWITCH was obtained from James A.H.Murray, University of Cambridge (Davies et al., 1999) and used as parent vector with modifications for constructing the first vector pART-P450-ecSUC2-BAR of the two-component system. In pFLP-SWITCH (Fig. 6) the *GUS* reporter gene is flanked by the FLP recognition target sites (*FRTs*) and separates the gene of interest from the constitutive cauliflower mosaic virus 35S promoter thus blocking expression of the gene of interest. The *GUS* gene is expressed from the 35S promoter prior to FLP-mediated recombination but after FLP-mediated recombination the *GUS* gene between the *FRT* sites is excised and the gene of interest becomes adjacent to, and is expressed from, the 35S promoter. The T-DNA region of the first vector pART-P450-ecSUC2-BAR is designed to completely complement the endogenous *AtSUC2* T-DNA mutation prior to excision by the FLPrecombinase, and also designed with markers to ensure excision after recombinase

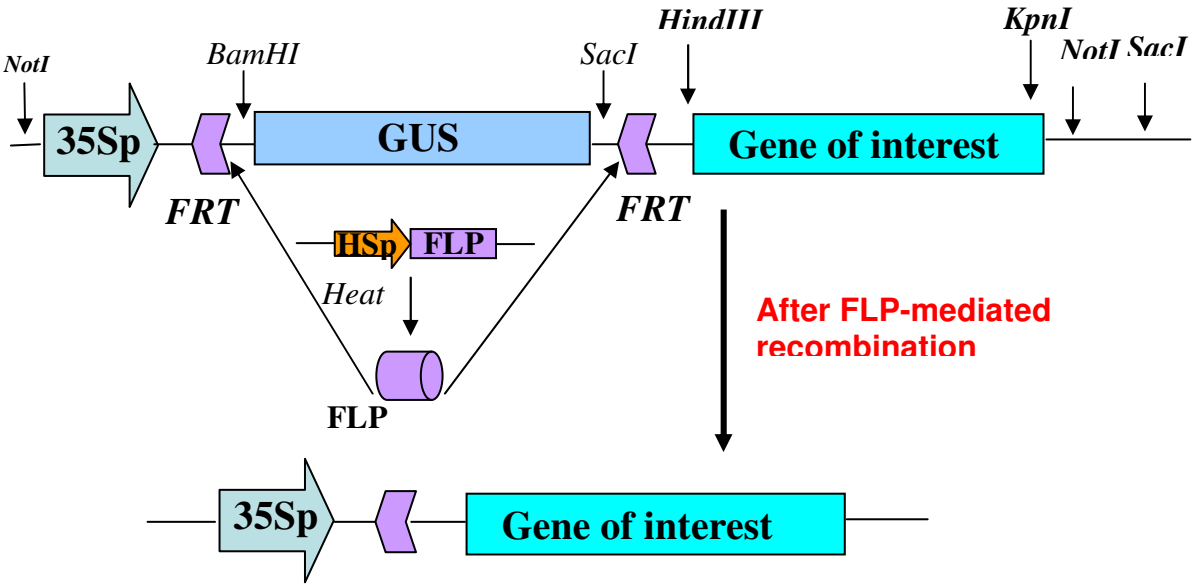


Figure 6: Strategy of using pFLP-SWITCH, with the 'gene of interest' inserted downstream of the *GUS* gene, using *HindIII/KpnI* sites. (*35Sp*: Cauliflower mosaic virus 35S promoter, *HSp*: Heat shock promoter, *FRTs*: FLP recognition target sites).

induction (Fig. 7). The vector T-DNA components include (1) *Bar* gene, conferring glufosinate ammonium herbicide resistance, (2) *P450* gene, a counter selection marker, (3) cDNA of the *AtSUC2* gene, (4) *nptII* gene, encoding kanamycin resistance, and (5) Left and right borders for *Agrobacterium tumefaciens* mediated plant transformation. The *P450* and *Bar* components are the safeguards confirming the excision event upon *FLP* induction. The vector pART-P450-ecSUC2-BAR was created using the plasmid pFLP-SWITCH obtained from J.H.Murray and binary vector pART27 (Gleave, 1992).

2.1.1 *Bar* Gene

The *Bar* gene from the bacterium *Streptomyces hygroscopicus* encodes the enzyme phosphinothricin acetyltransferase (PAT), which confers resistance to phosphinothricin (PPT; also glufosinate ammonium). Phosphinothricin (PPT) is a potent inhibitor of glutamine synthetase, which plays a central role in ammonia assimilation and regulation of nitrogen metabolism in plants (Skokut et al., 1978). Glutamine synthetase is the only enzyme in plants functioning in ammonia detoxification and its inhibition leads to plant cell death due to ammonia accumulation and toxicity (Tachibana et al., 1986). Expression of the *Bar* gene in tobacco, potato and tomato plants confers resistance to high doses of commercial sources of PPT, such as Basta or Finale (Block et al., 1987) and is commonly used as a selectable marker in Arabidopsis. In the plasmid pART-P450-ecSUC2-BAR, the *Bar* gene is separated from the 35S promoter (Fig. 7) before FLP-mediated recombination and its expression is minimized. Upon FLP-mediated recombination, the *Bar* gene will be adjacent to, and become expressed from, the 35S promoter. Resistance of transgenic plants to glufosinate ammonium treatment (100 mg/L) will indicate successful excision and thus, provide an effective selectable marker.

2.1.2 *$P450_{SU1}$* Gene

The *$P450_{SU1}$* gene from *Streptomyces griseolus* encodes a cytochrome P450 monooxygenase capable of metabolizing sulfonylurea herbicides (O'Keefe et al., 1988). The expression of *$P450_{SU1}$* results in the metabolism of the proherbicide R7402 to a highly phytotoxic metabolite. Thus, plants expressing the *$P450_{SU1}$* are killed by R7402 treatment that is harmless to plants without *$P450_{SU1}$* expression. The *P450* cassette between the two *FRT* sites (Fig. 7) is expressed before FLP-mediated recombination but will not be expressed after FLP-mediated recombination due to excision. The *$P450_{SU1}$* is therefore a highly effective counter-selectable marker as plants resistant to R7402 treatment (100 µg/L, Tissier et al., 1999) will indicate successful excision of the gene cassettes (*P450* and *ecSUC2*) between the *FRT* sites.

2.1.3 *AtSUC2* Gene

The cDNA of the *AtSUC2* gene is driven by the 2kb *AtSUC2* promoter, and will complement the homozygous (*Atsuc2/Atsuc2*) T-DNA insertion mutation in Arabidopsis. The excisable complementing *AtSUC2* (ec*SUC2*) cDNA between the *FRT* sites of plasmid pART-P450-ecSUC2-BAR (Fig. 7) will be excised upon FLP-mediated recombination. Thus, ec*SUC2* will complement the homozygous mutation only before FLP recombination and complementation of homozygous Arabidopsis plants after FLP recombination will be from the *AtSUC2* gene under control of a tissue-specific promoter, delivered by the second vector (See Fig. 8).

2.1.4 *nptII* Gene

The *nptII* gene in the first vector's T-DNA (Fig. 7) is a plant selection marker, encoding

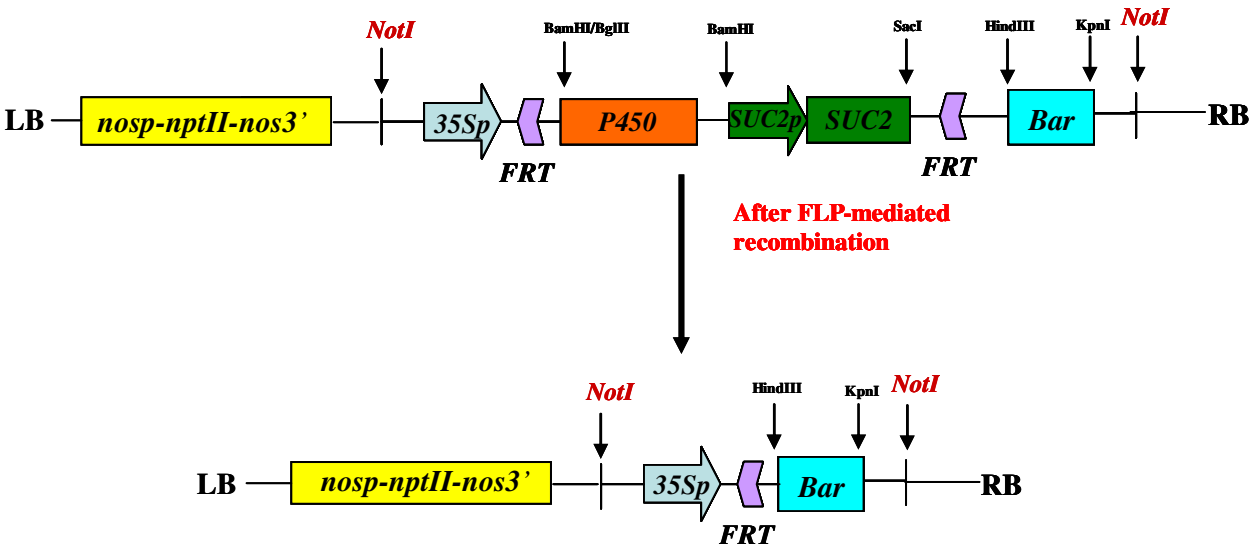


Figure 7: First vector pART-P450-ecSUC2-BAR T-DNA carrying the gene of interest and selection markers, with their unique restriction sites.*nosp- nptII-nos3'*: Gene encoding kanamycin resistance in transgenic plants expressed from the nopaline synthase promoter (*nosp*) and terminator (*nos3'*), *35Sp*: Cauliflower mosaic virus constitutive 35S promoter; *FRTs*: FLP recognition target sites; *P450*: Gene encoding counter selectable marker that makes plants sensitive to the proherbicide R7402, inserted upstream of the *AtSUC2*; *SUC2pSUC2*: cDNA of *AtSUC2* under control of its native promoter, replacing the *GUS* gene; *Bar*: Herbicide (glufosinate ammonium) resistance marker inserted downstream of *AtSUC2* cassette; LB: T-DNA left border; RB: T-DNA right border.

resistance to the antibiotic kanamycin. The *nptII* gene will aid in selection of transgenic plants, by conferring resistance to kanamycin in plants harboring the T-DNA of the first vector.

2.2 Second Vector: pCAM-Gent-TSpSUC2-EASE-FLP

The plasmid pCAM-Gent-TSpSUC2-EASE-FLP is designed to carry the tissue-specific *AtSUC2* cassette and also to deliver the *FLP* gene. The expression of *FLP* is required to remove the complementing and excisable cDNA copy of *AtSUC2* introduced by the first vector. Removal of ec*SUC2* renders the tissue-specific *AtSUC2* cDNA, introduced by the second vector, as the only functional copy The vector pCAM-Gent-TSpSUC2-EASE-FLP (Fig. 8) is created using pCAMBIA0390 plasmid as backbone (CAMBIA, www.cambia.org). The components of the second vector include, (1) *FLP* gene encoding the FLP recombinase, (2) Egg Apparatus-Specific Enhancer (EASE) driving expression of the *FLP*, (3) *aacC1* gene encoding gentamycin resistance, and (4) the *AtSUC2* gene under the expression of a tissue-specific promoter (*TSpSUC2*).

2.2.1 *FLP* Gene

The *FLP* gene encodes the site-specific FLP recombinase that recognizes the *FRT* sites and catalyses recombination between them (Fig 8). The FLP/*FRT* recombination system has been used in plants to excise genes lying between directly orientated *FRT* sites (Kilby et al., 1995). The FLP-mediated recombination system has been used to conditionally activate antibiotic resistance in transgenic tobacco plants (Davies et al., 1999). The production of hybrid seeds and plants from male-sterile plants has been demonstrated by use of FLP site-specific recombinase and restoration of fertility (Luo et al., 2000).

2.2.2 Egg Apparatus-Specific Enhancer (EASE)

The Egg Apparatus-Specific Enhancer (EASE) element with the CaMV 35S -45 minimal promoter will drive expression of the *FLP* gene (Fig. 8). The EASE element has egg-apparatus specific expression and also drives expression after fertilization in the zygote and early embryo (Yang et al., 2005) to ensure *FLP* expression in the very first cells of the embryo. Expression of the *FLP* gene from EASE is designed to prevent sectoring of expression from genetic mosaics.

2.2.3 *aacC1* Gene

The engineered *aacC1* gene (Fig. 8) encoding gentamycin acetyltransferase confers resistance to the antibiotic gentamycin. This enzyme in bacteria inactivates aminoglycoside antibiotics by acetylation. The use of *aacC1* gene as a plant selectable marker for transformation was first demonstrated by Hayford et al. in 1988 in a variety of plant species including Arabidopsis, tobacco (Carrer et al., 1991), tomato and petunia.

2.2.4 *TSpSUC2*

The *AtSUC2* gene under control of a tissue-specific promoter (e.g. *rolC, CoYMV, CmGAS1*) will replace, in function, the *AtSUC2* (ec*SUC2*) introduced by the first vector T-DNA after ec*SUC2* excision by the FLP recombinase (Fig. 8). The *rolC* promoter from *Agrobacterium tumefaciens* (Kuhn et al., 1996), the *CoYMV* promoter (Matsuda et al., 2002) from the Commelina Yellow Mottle Virus and the *CmGAS1* promoter (Ayre et al., 2003) from galactinol synthase gene of melon, all have phloem-specific expression. A near to wild-type growth restoration of homozygous Arabidopsis plants after excision of the ec*SUC2* cassette will indicate

effective expression of *AtSUC2* from the tissue-specific promoters. The expression of *AtSUC2* gene from the 2kb *AtSUC2* promoter (Wright et al., 2003) will serve as a positive control.

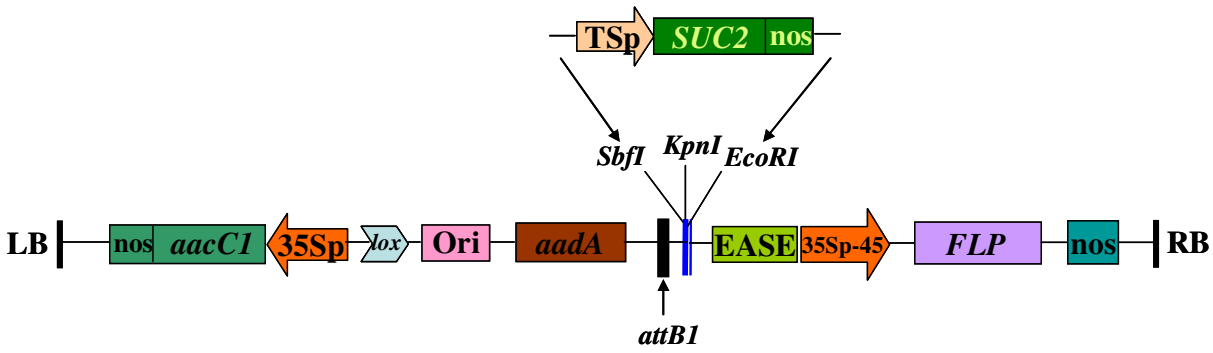


Figure 8: Second vector pCAM-Gent-TSpSUC2-EASE-FLP T-DNAdelivering the FLP recombinase. *aacC1*: Gene encoding gentamycin resistance under control of the 35S promoter and *nos* terminator, for selection of transgenic plants; *35Sp*: Cauliflower mosaic virus constitutive 35S promoter; *lox*: Cre recombinase recognition site; *Ori*: pBR322 bacterial origin of replication; *aadA*: Gene encoding kanamycin resistance for selection of transformed bacteria; *TSpSUC2*: Functional *AtSUC2* cDNA under the control of tissue specific promoter and *nos* terminator; *SbfI, KpnI, EcoRI*: Cloning sites for cloning the *TSpSUC2* cassette; EASE: Egg Apparatus-Specific Enhancer driving the expression of FLP recombinase; *35Sp*-45: Cauliflower mosaic virus 35S promoter -45 region; *FLP*: Gene encoding the site-specific FLP recombinase; *aatB1*: Gateway recombination site; LB: T-DNA left border; RB: T-DNA right border.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Molecular Techniques

All restriction digestions, ligation reactions and dialysis were carried out according to standard protocol (Sambrook et al., 2001). Restriction endonuclease enzymes were obtained from New England Biolabs (NEB, Beverly, MA). All DNA sequencing for sequence analysis was done at SeqWright DNA Technology Services (Houston, TX).

3.2 Media Preparation

Luria-Bertani (LB) medium was used for the cultivation of *Escherichia coli* and *Agrobacterium tumefaciens* strains (Sambrook et al., 2001). LB medium has the following ingredients per 1000 ml of distilled H_2O: 10 g tryptone, 5 g yeast extract and 5 g NaCl. The above ingredients were first dissolved in 850 ml of dH_2O. The pH was adjusted to 7.0 using 5 N NaOH and the volume was then adjusted to 1000 ml with dH_2O. 15 g of agar was added when making LB solid medium. The medium was sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle.

1% Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) with the appropriate antibiotic was used for screening transformed Arabidopsis seeds. MS medium has the following ingredients per 1000 ml of distilled H_2O: 10 g sucrose and 4.44 g MS Modified Basal Medium w/ Gamborg Vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS). The above ingredients were dissolved in 850 ml dH_2O. The pH was adjusted to 5.6 using 1 M KOH and the

volume was then adjusted to 1000 ml with dH_2O. 2.5 g of Gelrite gellan gum (Sigma, St.Louis MO) was added and the medium was sterilized by autoclaving for 20 minutes at 15 psi on liquid cycle. After autoclaving, the medium was allowed to cool to ~ 60° C. 200 mg/L Timentin (Ticarcillin, disodium salt / Potassium clavulanate mixture 15:1) (Research products International Corp., Mt. Prospect, IL) was then added to the medium to suppress *Agrobacterium tumefaciens* growth, along with the appropriate antibiotic, for selection of transformants.

3.3 Bacterial Transformation

The XL-1 BLUE strain of *E .coli* (Stratagene, La Jolla, CA) was used for all transformation procedures and was carried out by electroporation (Sambrook et al., 2001), using a Bio-Rad® gene pulser (Bio-Rad Laboratories, Inc. Hercules, CA). The competent cells were first thawed on ice. 4 µl DNA was added to 40 µl cells, mixed gently and incubated on ice for 1 minute. The cells were then transferred to a 2 mm electroporation cuvette and pulsed at 2.5 KV. 1 ml of sterile LB broth was added to them and the cells were cultured for 1.5 hrs at 37° C. Cells were plated on LB media with the appropriate antibiotic for plasmid selection. Transformation of *Agrobacterium tumefaciens* strain GV3101mp90 (Koncz and Schell, 1986) was also done as mentioned above.

3.4 Rapid Plasmid Isolation

Plasmid isolation was by an alkaline lysis miniprep procedure (Sambrook et al., 2001). Plasmid was extracted from a 2 ml overnight culture obtained from a single colony. The culture was vortexed briefly and transferred to a 2.0 ml microcentrifuge tube. The culture was centrifuged to harvest cells and the supernatant was aspirated off. The harvested cells were

resuspended in 200 µl of Solution I (50 mM Tris pH 8.0, 10 mM EDTA and 0.1 mg/ml RNAse) by vortexing. 200 µl of Solution II (200 mM NaOH and 1% SDS) was added and mixed gently by inversion. 200 µl of Solution III (60.0 ml of 5 M Potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml sterile dH_2O) was then added and mixed thoroughly by inversion. The sample was placed on ice for 5 minutes and then centrifuged for 10 minutes. The supernatant was transferred to a 1.5 ml microfuge tube containing 0.7 volumes of isopropanol and mixed well by inversion to precipitate the plasmid DNA and centrifuged at full speed for 10 minutes. The supernatant was aspirated off and the pellet was washed with 1 ml 70% ethanol. After washing, the supernatant was discarded; the DNA pellet was air-dried and resuspended in 50 µl sterile dH_2O.

3.5 Plant Genomic DNA Isolation

Genomic DNA from *Arabidopsis thaliana* plants was isolated either by CTAB method (Doyle and Doyle, 1987) with slight modifications or using the 'Extract-N-Amp' Plant PCR kit (Sigma, St. Louis, MO) with slight modifications to the manufacture's protocol.

For the CTAB method, 1-2 mg of plant leaf tissue was cut out, placed in a 1.5 ml microcentrifuge tube and ground. 300 µl of 2X CTAB buffer solution (2% (w/v) cetyl-trimethyl-ammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris HCl pH 8.0, 20 mM EDTA) was added and incubated at 65° C for 1 hour. The reaction contents were allowed to cool and 300 µl chloroform was added. The contents were vortexed thoroughly and then centrifuged for 1 min to separate the phases. The upper aqueous phase was transferred to a new microcentrifuge tube, 300 µl of isopropanol was added and mixed well. The samples were then spun in a microcentrifuge for 5 minutes to pellet DNA. The supernatant was aspirated and the pellet was washed with 500

µl 70% ethanol. The samples were spun in a microcentrifuge for 1 min, the supernatant was aspirated and the pellet was air-dried. 100 µl of Tris-EDTA buffer was added to the air-dried pellet and vortexed briefly to dissolve the pellet.

For the Extract-N-Amp protocol, 1-2 mg of plant leaf tissue was cut out and placed in a 2.0 ml collection tube. 70 µl extract solution was added, immersing the leaf tissue. The tube was then incubated at 85° C for 10 minutes. After incubation, the reaction components were allowed to cool for 2-3 minutes. 70 µl dilution solution was then added and vortexed briefly to completely dissolve leaf tissue.

3.6 Plant Genotyping

Plants were genotyped by touchdown PCR (Sambrook et al., 2001) using Taq DNA polymerase enzyme (NEB). The two gene specific primers PR1F (AACCGCAACCGCAGCCT-CTAAG) and PR1R (CCTAGGGAAAGTCCTGTGGAAGAA) were designed to amplify 800 bp of the *AtSUC2* gene. The T-DNA specific primer LB-SALK (GCGTGGACCGCTTGCTGC-AACT) and PR1R were designed to amplify a 450 bp mutant band. The touchdown PCR conditions optimized for the genotyping are listed in Table 1. A typical genotyping reaction contained 200 µM dNTPs, 200 nM each primer, 0.2 units Taq polymerase, 1 µL isolated genomic DNA and 2 µL of supplied buffer in a 20 µL reaction.

3.7 Arabidopsis Seeds

Seeds of three Arabidopsis lines SALK_087046, SALK_01331 and SALK_038124 (Alonso et al., 2003) with a T-DNA insertion in the *AtSUC2* gene were obtained from the Arabidopsis Biological Research Center (ABRC) as segregating T3 lines. Insertion mutant

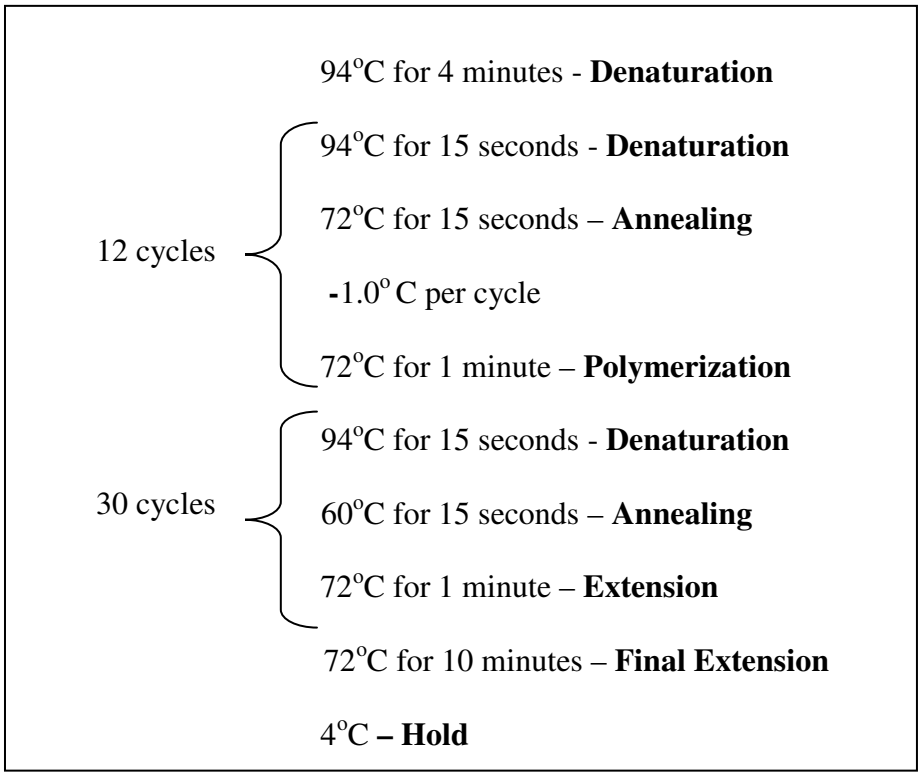


Table 1: Cycling conditions for touchdown PCR used for plant genotyping.

information was obtained from the SIGnAL website at http://signal.salk.edu. The SALK lines are T-DNA insertions from the binary vector pBIN-pROK2 (Baulcombe et al., 1986) in the Columbia-0 ecotype, generated at the The Salk Institute in California, USA.

3.8 Confirmation of T-DNA Location

Genomic DNA was amplified using the T-DNA specific primer set (PR1R and LB-SALK). The amplified PCR product was resolved on a 1.2% gel, and the 450 bp product corresponding to the T-DNA insertion site was cut out and purified using the Wizard SV Gel and PCR clean-up Kit (Promega, Madison, WI). The gel purified product was then ligated into the pGEM T-easy vector (Promega), the ligation product dialyzed and transformed. Plasmid DNA was isolated and analyzed by restriction digestion. Sequencing of the plasmid DNA further confirmed the presence and location of T-DNA in the second intron of *AtSUC2* gene.

3.9 Construction of pART-P450-ecSUC2-BAR

The construction of pART-P450-ecSUC2-BAR was carried out in four steps:

(1) Construction of pFLP-SWITCH-BAR by introduction of the *Bar* gene (encoding resistance to glufosinate ammonium herbicide).

(2) Construction of pGEM-ecSUC2-BAR by transferring the pFLP-SWITCH-BAR *NotI* cassette into pGEM T-easy (Promega), followed by introduction of the *AtSUC2* cassette in between *FRT* sites, rendering the *AtSUC2* gene excisable (*ecSUC2*) upon action of FLP recombinase.

(3) Construction of pGEM-P450-ecSUC2-BAR by introduction of the *P450* gene cassette (encoding R7402 herbicide metabolism).

(4) Transfer of the pGEM-P450-ecSUC2-BAR cassette as a *NotI* fragment into pART27 (Gleave, 1992), generating pART-P450-ecSUC2-BAR.

Detailed procedures follow.

3.9.1 Construction of pFLP-SWITCH-BAR

The plasmids pFLP-SWITCH (obtained from James A.H.Murray, University of Cambridge; Davies et al., 1999) and pGPTV-BAR (source of the *Bar* gene; Becker et al, 1992) were transformed individually into XL-1 BLUE *E.coli* cells using a heat shock method of transformation (Sambrook et al., 2001). The transformed cells were plated out on LB media containing 30 µg/ml of kanamycin antobiotic and 200 µg/ml of ampicillin antibiotic, for pGPTV-BAR and pFLP-SWITCH, respectively. Plasmid DNA was isolated and plasmid identity was confirmed by obtaining the expected fragment sizes after digestion with restriction endonucleases *EcoRI/HindIII* for pGPTV-BAR plasmid and *XbaI/NotI* for pFLP-SWITCH plasmid.

To facilitate sub-cloning of the *Bar* gene into the *HindIII/KpnI* sites of the pFLP-SWITCH backbone, an internal *KpnI* recognition site was mutagenized in the *Bar* gene by a mutagenic PCR approach (Fig. 9). The *Bar* gene from pGPTV-BAR was first amplified using the phosphorylated primers BARKpn3 (AGTAAGGTACCTCATCAGATTTCGGTGACG) and BARHind5 (TTACTAAGCTTAACAATGAGCCCAGAACGACG). Table 2 shows the PCR conditions for the first amplification. The amplified product was purified and ligated to itself for use as template for a mutagenic PCR round. The mutagenesis was done using the primers BAR-Kpnmut3 (ACGGGGCGGAACCGGCAGGCTGAAG) and BAR-Kpnmut5 (CCGGTCCTGCC-CGTCACCGAAATC). The mutagenized PCR product from this round of PCR was purified and

ligated to itself for use as template in a final round of PCR. The amplification of the mutagenized *Bar* gene was carried out using the primers BAR-Kpn3 and BAR-Hind5. Cycling conditions for the second and final amplification are listed in Table 3 and Table 4, respectively. The final amplified mutagenized *Bar* gene product was digested with restriction enzymes *HindIII* and *KpnI* and ligated into the pFLP-SWITCH backbone also digested with *HindIII* and *KpnI*. The ligated mixture was transformed into *E. coli* cells. Plasmid DNA was isolated and recombinant clones were tested for the presence of the insert by a *HindIII/KpnI* restriction digest. The mutagenesis of the undesirable *KpnI* restriction site in the *BAR* gene was also confirmed by sequencing. This plasmid is referred to as pFLP-SWITCH-BAR.

3.9.2 Construction of pGEM-ecSUC2-BAR

The pFLP-SWITCH backbone contained an inconvenient *SacI* restriction site. Therefore, the *NotI* fragment from pFLP-SWITCH-BAR that excluded the inconvenient *SacI* site was sub cloned into the *NotI* site of pGEM-T-easy cloning vector (Promega, Wisconsin, USA) creating the plasmid pGEM-BAR (Fig. 10). The ligation product was transformed into *E. coli* cells and positive clones identified by digestion with *HindIII* restriction endonuclease. (A *SacI* site in the pGEM-T-easy backbone was mutated prior to sub cloning by digesting with *SacI*, treating with T4 DNA polymerase to make the *SacI* digested ends blunt and religating). The *AtSUC2* cDNA under the control of its 2 kb native promoter was obtained as a *BamHI/SacI* fragment from the pGEM-SUC2p/SUC2 plasmid (Ayre et al., unpublished) and cloned into *BamHI/SacI* digested pGEM-BAR backbone, creating pGEM-ecSUC2-BAR. The insertion of the *SUC2pSUC2* cassette into the pGEM-BAR backbone was analyzed by restriction digestion with the restriction endonuclease *KpnI.* The desired fragment sizes of 6.5 kb and 2.5 kb were ontained, confirming

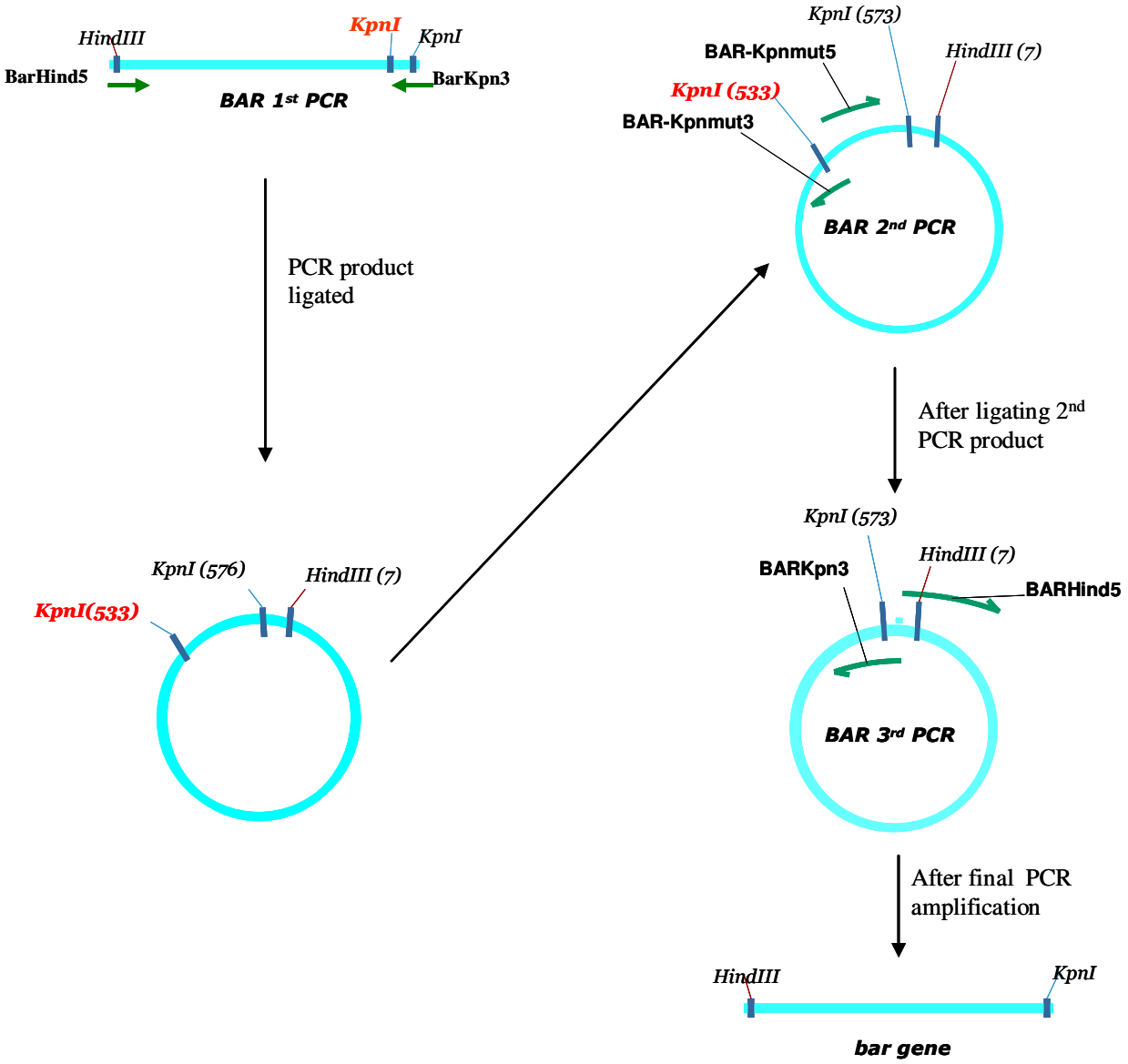


Figure 9: Schematic representation of *Bar* gene amplification and the mutation of an internal *KpnI* site. The internal, undesirable *KpnI* site is indicated in red and the *Bar* gene represented in blue. The primer binding sites are indicated in green arrows.

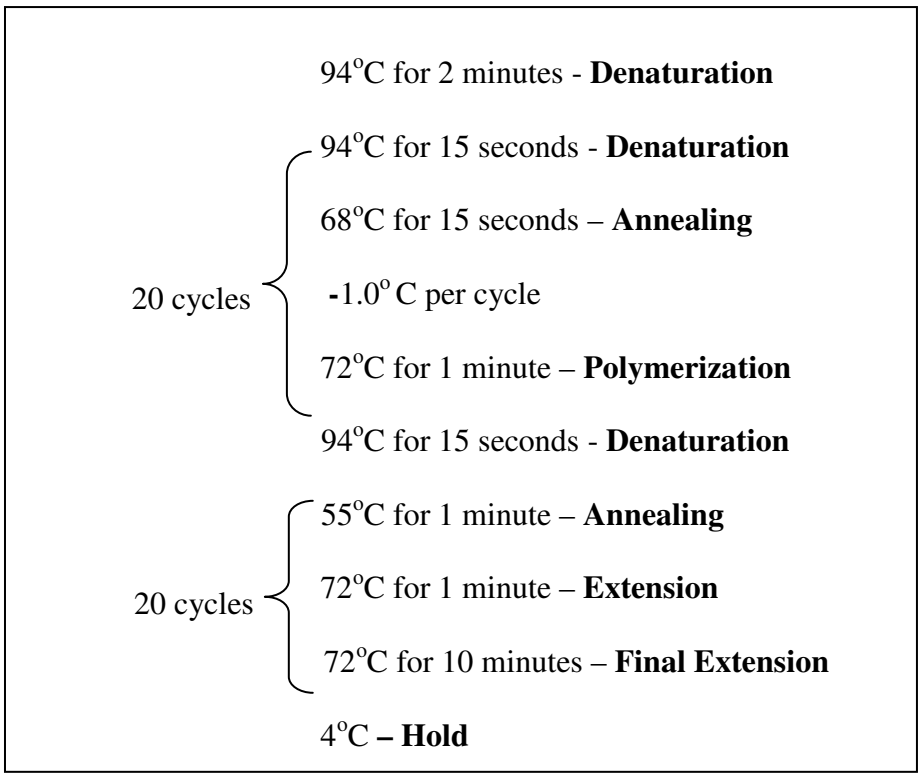


Table 2: Cycling conditions for the first PCR amplification of *Bar* gene from pGPTV-BAR.

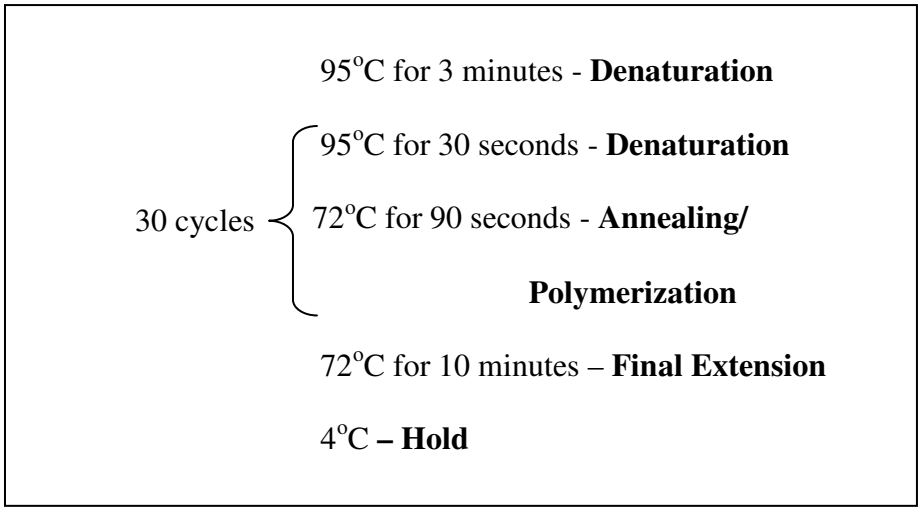


Table 3: Cycling conditions for the second mutagenic PCR amplification of *Bar* gene.

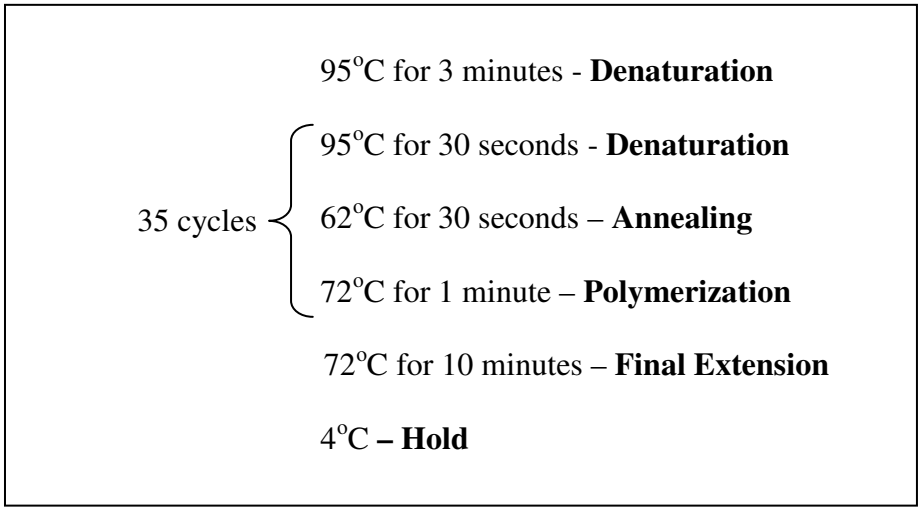


Table 4: Cycling conditions for the final round of PCR amplification of *Bar* gene.

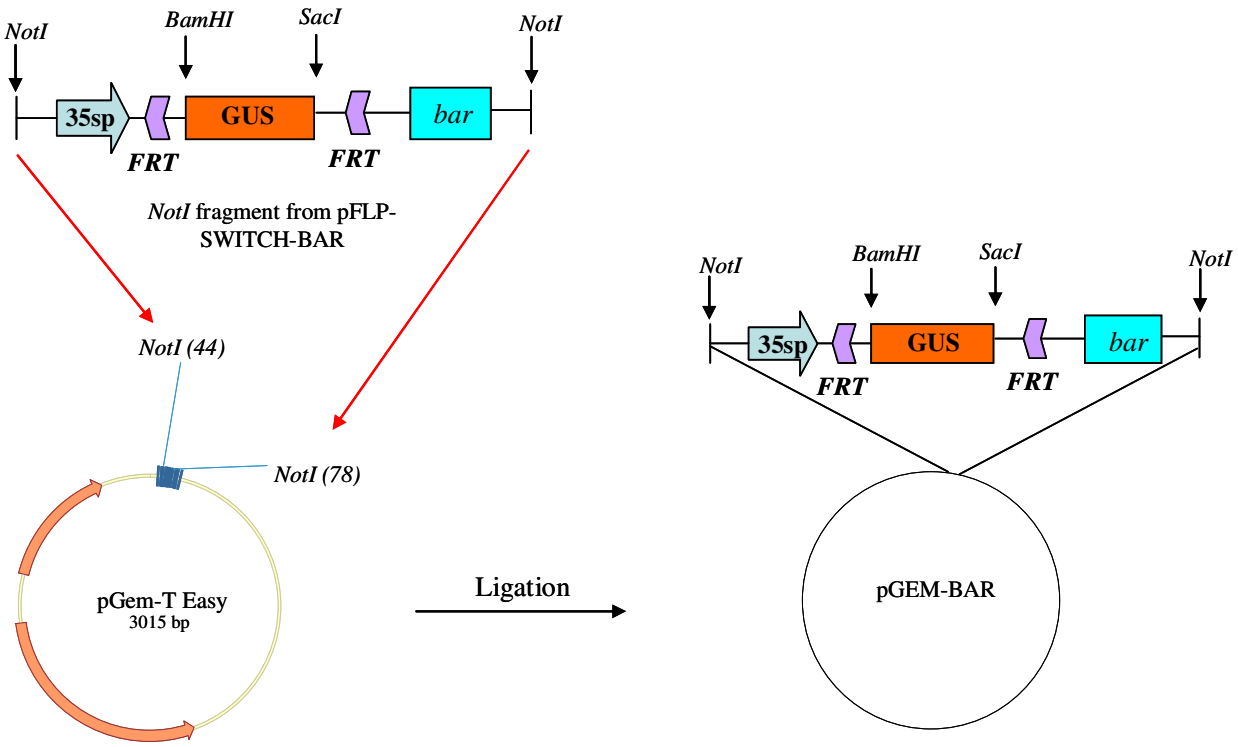


Figure 10: Schematic representation of steps involved in the construction of pGEM-BAR plasmid.

the positive clones.

3.9.3 Construction of pGEM-P450-ecSUC2-BAR

The *P450* gene cassette consisting of a promoter from the small subunit of rubisco, a chloroplast target sequence fused to the P450 ORF, and a polyadenylation signal from rubisco was obtained as a *BamHI* fragment in a pUC118 backbone from Daniel O'Keefe (Dupont, Wilmington; O'Keefe et al., 1994). The oligonucleotide PspOMImut R2496 (AATAACGGGG-CCCCCCCGCGATGTC) was designed to amplify the 3' portion of the cassette and to mutate an undesirable *NotI* restriction site and introduce a desirable *PspOMI* restriction site in its place. The *PspOMI* and *NotI* restriction sites have compatible overhangs, but both restriction sites are destroyed when ligated together. A *BglII* restriction site was incorporated at the 5'- end of the *P450* gene cassette by PCR approach using the oligonucleotide BglIImut F7518 (CATGATTAC-GAATTCTAGATCTTCTCTGC) for convenience in cloning. Table 5 shows the cycling conditions for the above PCR reactions. The mutagenized *P450* fragment PCR product digested with *EcoRI/PspOMI* was cloned into the *EcoRI/NotI* digested pUC118-P450 backbone and transformed into *E.coli* cells, creating the plasmid pUC118-P450mut (Fig. 11). Positive clones with mutation of the undesirable *NotI* restriction site, and the introduction of the desired *BglII* restriction site were confirmed using restriction analysis and sequencing.

The *P450* gene cassette from pUC118-P450mut was then digested with *BamHI/BglII* and ligated into *BamHI* digested pGEM-ecSUC2-BAR backbone, and transformed into *E.coli* cells by electroporation; generating pGEM-P450-ecSUC2-BAR. The desired orientation of the P450 gene cassette (one that would recreate the *BamHI* restriction site between the *P450* gene and ec*SUC2*) in pGEM-ecSUC2-BAR backbone was confirmed by a *BamHI/SacI* restriction

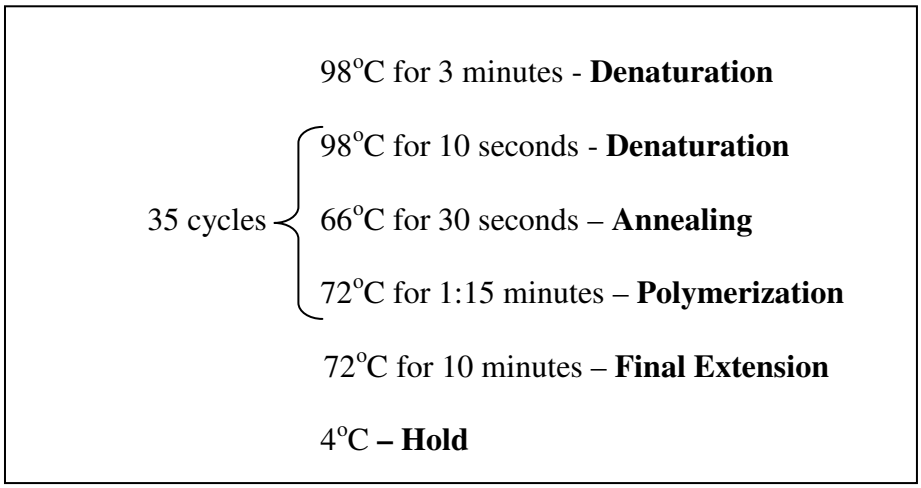


Table 5: PCR conditions for *P450* gene cassette amplification with desired mutations.

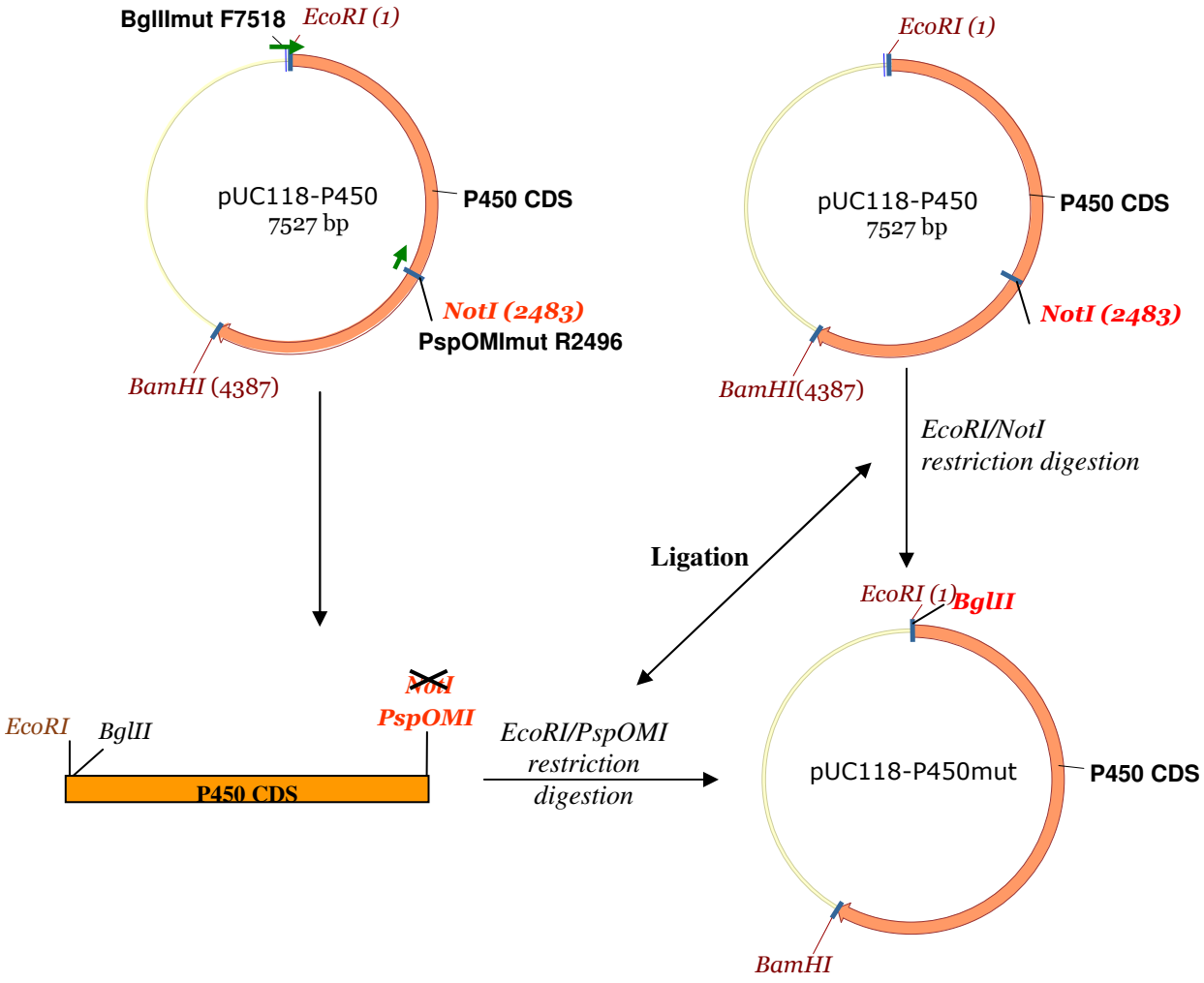


Figure 11: Schematic representation of steps involved in creating the pUC118-P450mut plasmid. The undesirable *NotI* and introduced restriction sites *PspOMI* and *BglII* are represented in red. The primer binding sites are indicated as green arrows.

analysis.

3.9.4 Construction of pART-P450-ecSUC2-BAR

The plasmid pGEM-P450-FRT-ecSUC2-BAR was digested with *NotI* to introduce the P450-ecSUC2-BAR fragment into the *NotI* digested pART27 binary vector (Gleave, 1992), generating the final vector pART-P450-ecSUC2-BAR. The ligation product was transformed into *E. coli* cells by electroporation. Restriction analysis confirmed two possible orientations of the insert in pART27 and one representative clone for each orientation was transformed into *Agrobacterium tumefaciens* strain GV3101mp90 by electroporation. Fig. 12 shows a schematic representation of pART-P450-ecSUC2-BAR construction.

3.10 Construction of pCAM-Gent-TSpSUC2-EASE-FLP

The construction of pCAM-Gent-TSpSUC2-EASE-FLP harboring the tissue specific *AtSUC2* copy (TSpSUC2) and rendering the *FLP* gene was carried out in four steps as follows:

(1) Construction of the synthetic fragment containing *attB1* site, multiple cloning site (MCS), EASE (Egg Apparatus Specific Enhancer), and the *CaMV* 35S -45 region, and cloning into pCAMBIA 0390 backbone creating pCAM-EASE.

(2) Amplification of *FLP* gene encoding the FLP recombinase and cloning into pCAM-EASE creating pCAM-EASE-FLP.

(3) Amplification of the *aacC1* gene cassette encoding gentamycin resistance and cloning into pCAM-EASE-FLP creating pCAM-Gent-EASE-FLP.

(4) Introduction of the TSp-SUC2 cassette into the *SbfI* and *EcoRI* restriction sites of the

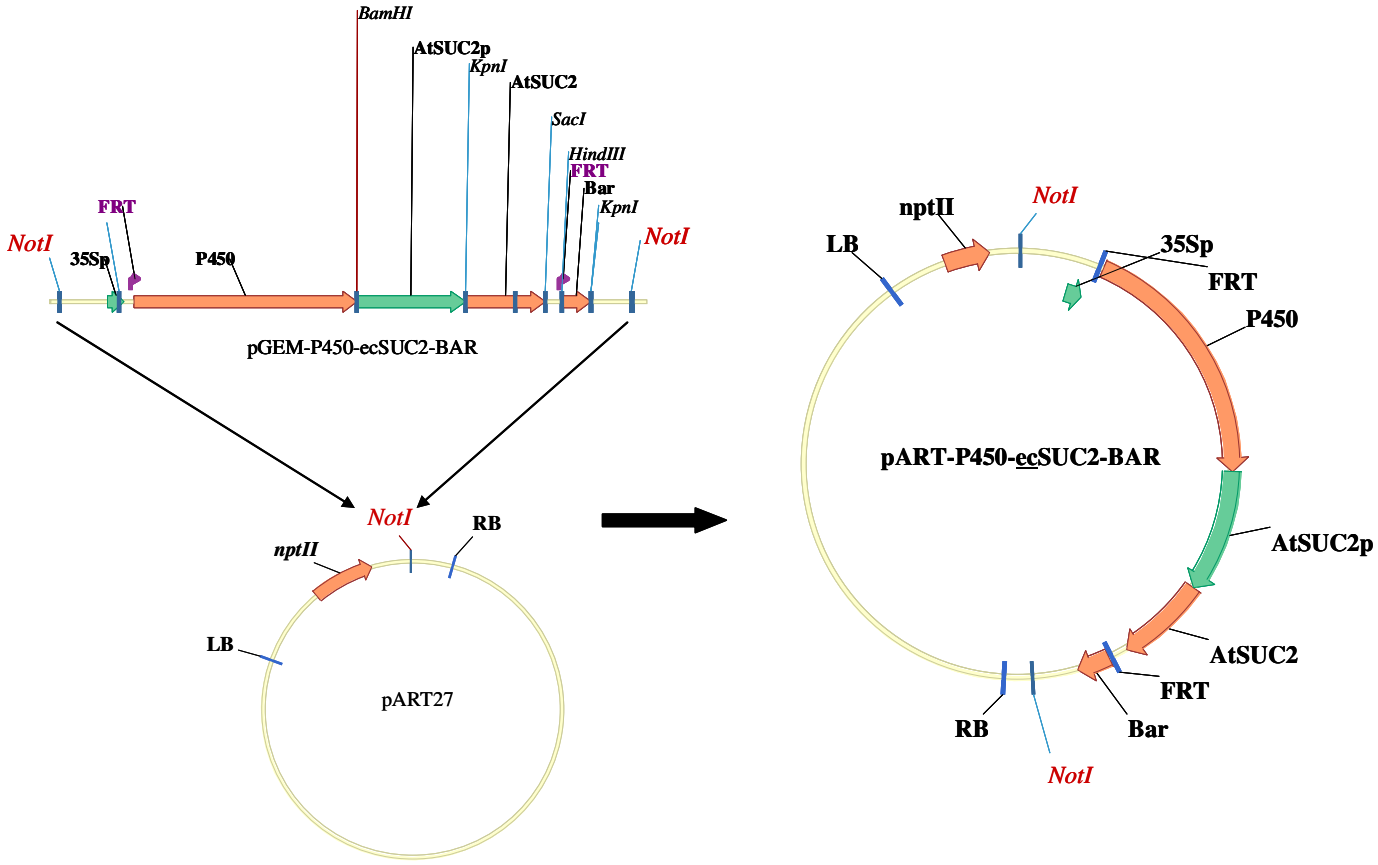


Figure 12: Schematic representation of construction of the first vector pART-P450-ecSUC2-BAR.

multiple cloning site of pCAM-Gent-EASE-FLP creating pCAM-Gent-TSpSUC2-EASE-FLP.

3.10.1 Construction of pCAM-EASE

A synthetic fragment consisting of the *attB1* gateway recombination site, a short multiple cloning site (*SbfI, KpnI* and *EcoRI)* and the EASE element fused to a *CaMV* 35S minimal promoter (-45 region) to provide a TATA box for transcription initiation, was constructed using a standard protocol (Ausubel et al., 2002) with slight modifications and using the Klenow fragment of *E. coli* (NEB). For construction of the synthetic fragment, two primer sets were designed: (1) attB1BglF1 (TGAATAAGATCTACAAGTTTGTACAAAAAAGCAGGCTCCT-GCAGGTCGGT) and EASER1 (GGTTACTTTTTTTAATAACGTTATCGATATATTTGCAT-CGTGGGAATTCGGTACCGACCTGCAGGAGC), and (2) EASEF2 (CGATAACGTTATTA-AAAAAAGTAACCGCATGATATATTCTCTTTCGTATGATATTAAGGCCGCAAGAC) and 35S-45NcoIR2 (GATCGCCCATGGCTCTCCAAATGAAATGAACTTCCTTATATAGAGGA-GGGTCTTGCGGCCTTAATATC). The primers attB1BglF1 and 35S-45NcoIR2 were designed to create *BglII* and *NcoI* sites, respectively, at the 5' and 3' end of the synthetic fragment for cloning into the *BclI/NcoI* sites of pCAMBIA 0390 (CAMBIA, www.cambia.org). Fig. 13 is a schematic representation of the steps involved in the construction of the synthetic fragment. The first half of the synthetic fragment was constructed using 1 µg each of primers attB1BglF1 and EASER1. The second half of the synthetic fragment was constructed using 1 µg each of primers EASEF2 and 35S-45NcoIR2. The primer annealing reaction conditions used are listed in Table 6.

The annealing reaction product from each primer set was extended using Klenow fragment of *E .coli,* column purified using Wizard SV Gel and PCR clean-up kit, and 1 µl of

each product was used as template in a single PCR amplification with primers attB1BglF1 and 35S-45NcoIR2, and Phusion polymerase (NEB). The PCR reaction conditions for the synthetic fragment amplification are listed in Table 7.

The synthetic fragment PCR product was digested with *NcoI/BglII* and ligated into the *NcoI/BclI* digested pCAMBIA0390 as the vector backbone, creating the plasmid pCAM-EASE. The *BclI* and *BglII* sites were destroyed after ligation. The ligation product was dialysed and transformed into *E. coli* cells by electroporation. Positive clones were identified by restriction analysis. Sequence analysis was done to confirm the sequence of the synthetic fragment. This cloning step deleted the left border of pCAMBIA0390.

3.10.2 Construction of pCAM-EASE-FLP

The *FLP* gene cassette (~ 1.2 Kb) was amplified from HSP-FLP plasmid (Kilby et al., 1995) using an overlap PCR approach to amplify the cDNA and remove an internal *EcoRI* site (Sambrook et al., 2001). Fig. 14 shows the primer binding sites for amplification of the *FLP* gene from HSP-FLP. Fig. 15 is a schematic representation of the overlap PCR approach. The first PCR amplification was done using the primer set FLPNcoIF1 and FLPmutR766, and the second PCR amplification was done with the primer set FLPmutF736 and FLPBamR1272, in two separate PCR reactions using Phusion polymerase (NEB). Table 8 shows the cycling conditions for the first and second PCR amplification rounds. The primers FLPNcoIF1 (AGTCTCCCATG-GCTATGCCACAATTTGGTATATTATGTAAAACACC) and FLPBamR1272 (AGACTTGG-ATCCTTATATGCGTCTATTTATGTAGGATGAAAGG) were designed to create 5'- *NcoI* and 3'- *BamHI* sites in the amplified product, respectively, for cloning into the *NcoI/BglII* sites of pCAM-EASE as backbone. The primers FLPmutR766 (GGACTGGTTCAGAGTTCCTCAAA-

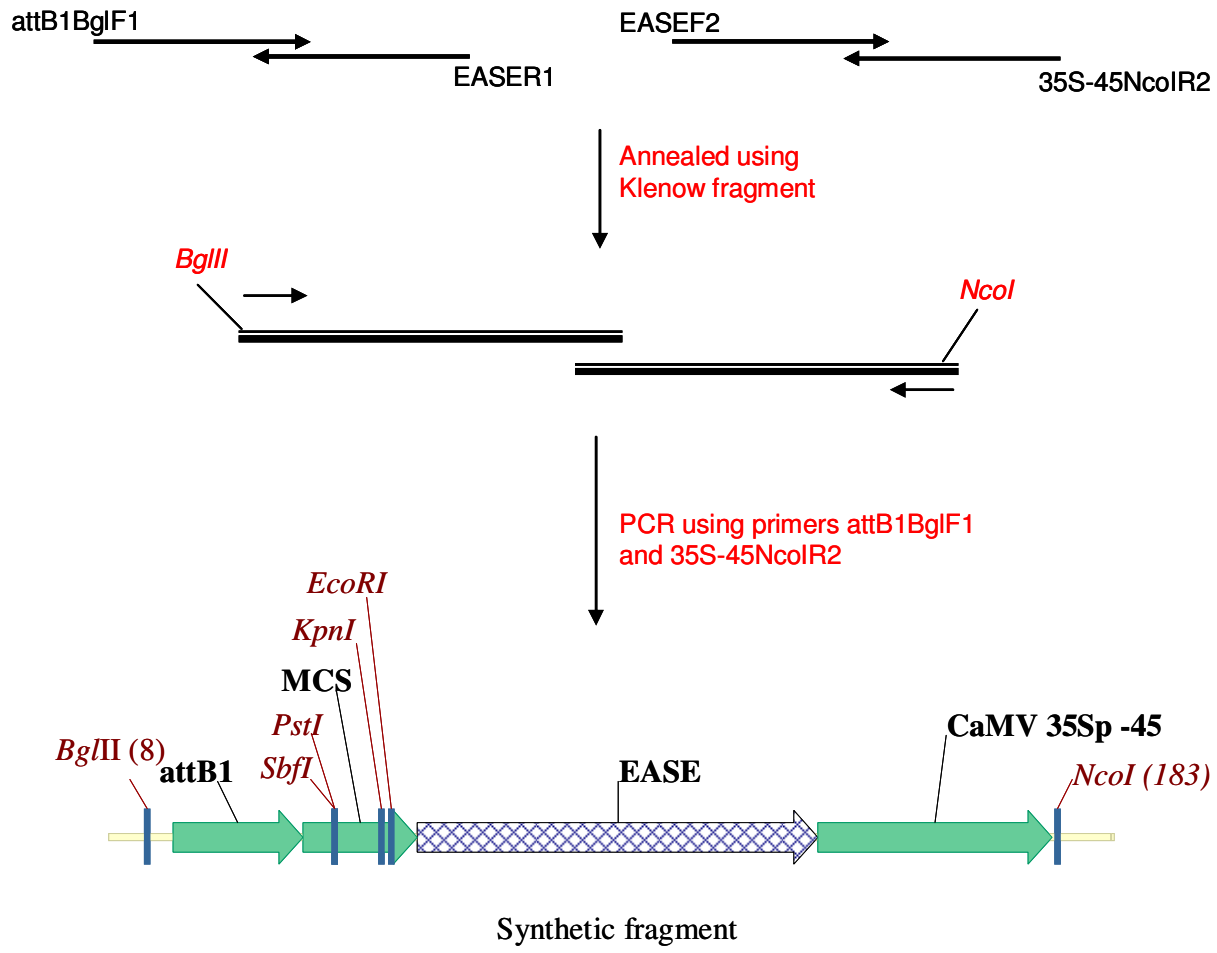


Figure 13: Schematic representation of steps involved in the synthetic fragment construction. *attB1*: Gateway recombination site; *MCS*: multiple cloning site comprised of restriction sites *SbfI, PstI, KpnI,* and *EcoRI*; EASE: Egg Apparatus-Specific Enhancer; *CaMV 35Sp* -45: Cauliflower mosaic virus constitutive 35S promoter -45 region.

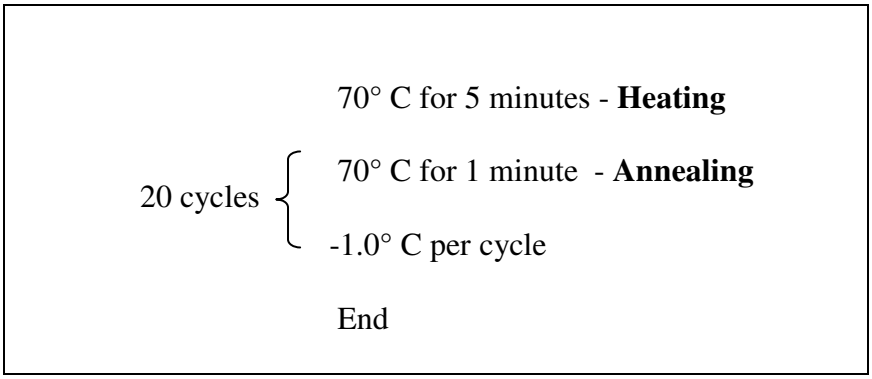


Table 6: Annealing conditions used for synthetic fragment construction.

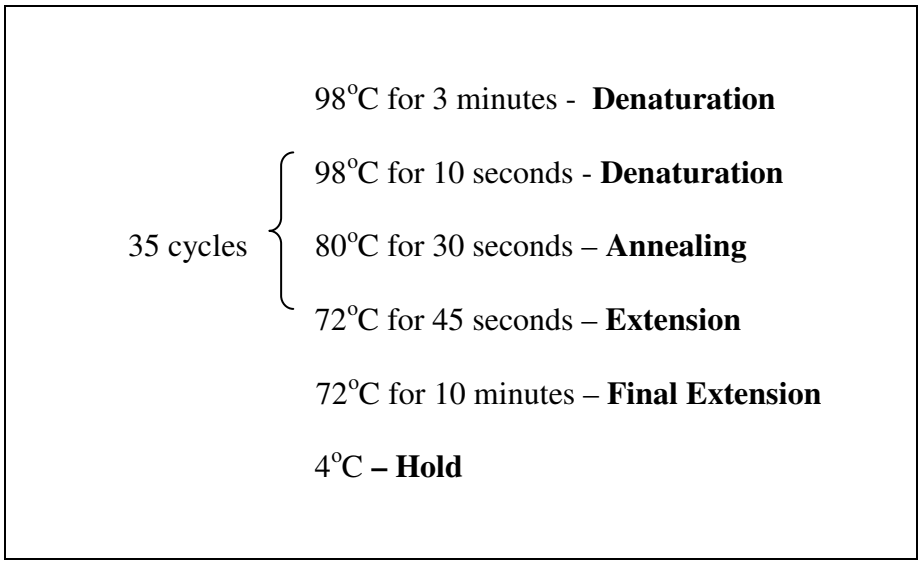


Table 7: PCR conditions for amplification of synthetic fragment.

AATTCATC) and FLPmutF736 (GATGAATTTTTGAGGAACTCTGAACCAGTC) containing a 30 bp overlap region were designed to mutate an *EcoRI* site in the *FLP* gene cassette, for convenience in cloning. The amplification products from the first and second PCR reactions were column purified using the Wizard SV Gel and PCR clean-up system and used as templates in a final PCR amplification step. The final round of PCR amplified the entire *FLP* cassette with the modifications incorporated in the first and second PCRs, using phosphorylated primers FLPNcoIF1 and FLPBamR1272. The cycling conditions for the final PCR amplification are shown in Table 9. 5 µl of the final amplification product was resolved on a 1% agarose gel confirming a ~ 1.2 Kb FLP PCR product. In order to sequence analyze the *FLP* cassette for incorporation of desired modifications, the column purified *FLP* PCR product was digested with *BamHI/NcoI* and ligated into the *BamHI/NcoI* digested pADH-MCS #6 vector (B.G.Ayre; unpublished), generating the plasmid pADH-FLP. The ligation reaction was transformed into *E .coli* by heat shock and transformed cells were selected on LB media with 50 µg/mL ampicillin. Plasmid DNA was isolated and a restriction analysis with *BamHI/NcoI* identified positive clones with the *BamHI* and *NcoI* restriction sites incorporated. Sequence analysis of the *FLP* cassette in pADH-MCS #6 backbone confirmed the mutation of *EcoRI* restriction site and the correct sequence of the entire cassette, including introduction of *NcoI* and *BamHI* restriction sites for cloning purpose.

The modified *FLP* cassette was obtained as an *NcoI/BamHI* fragment from the pADH-FLP plasmid and ligated into the *NcoI/BglII* digested pCAM-EASE backbone, generating the plasmid pCAM-EASE-FLP. The ligation product was dialyzed and transformed into *E. coli* cells by electroporation. Positive clones were identified by restriction analysis.

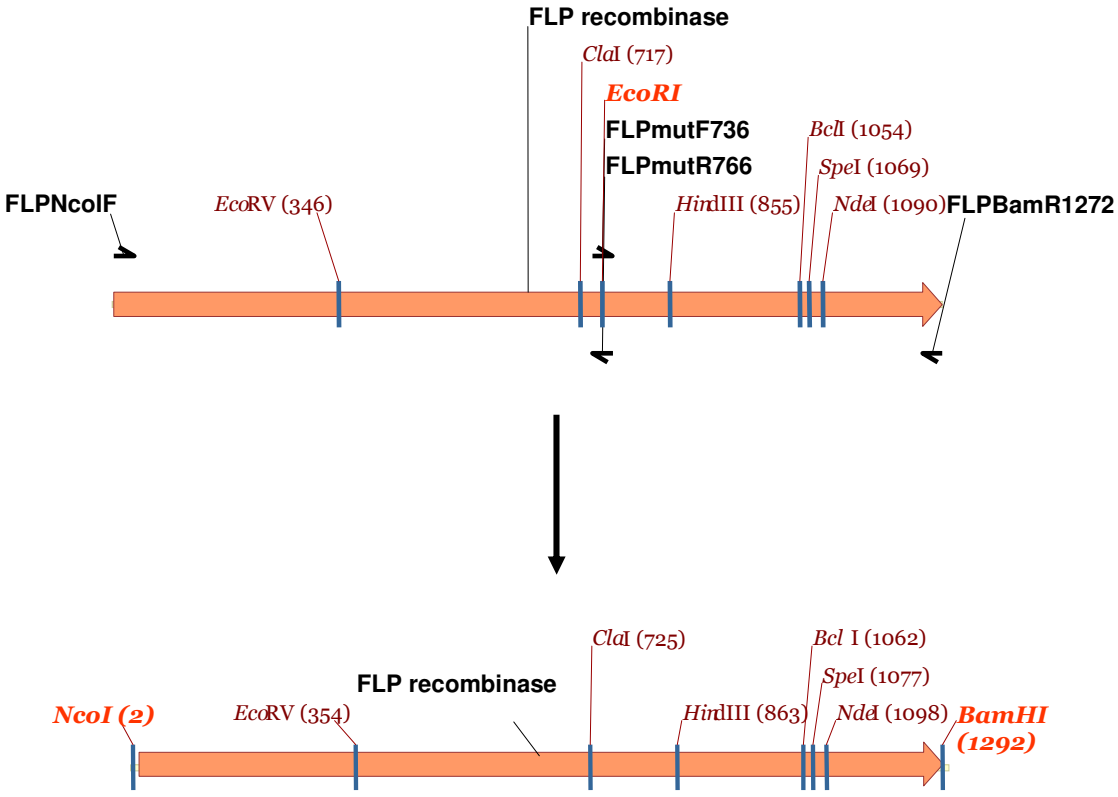


Figure 14: Diagram showing the primer binding sites for *FLP* gene amplification and the resulting product after a final PCR amplification.

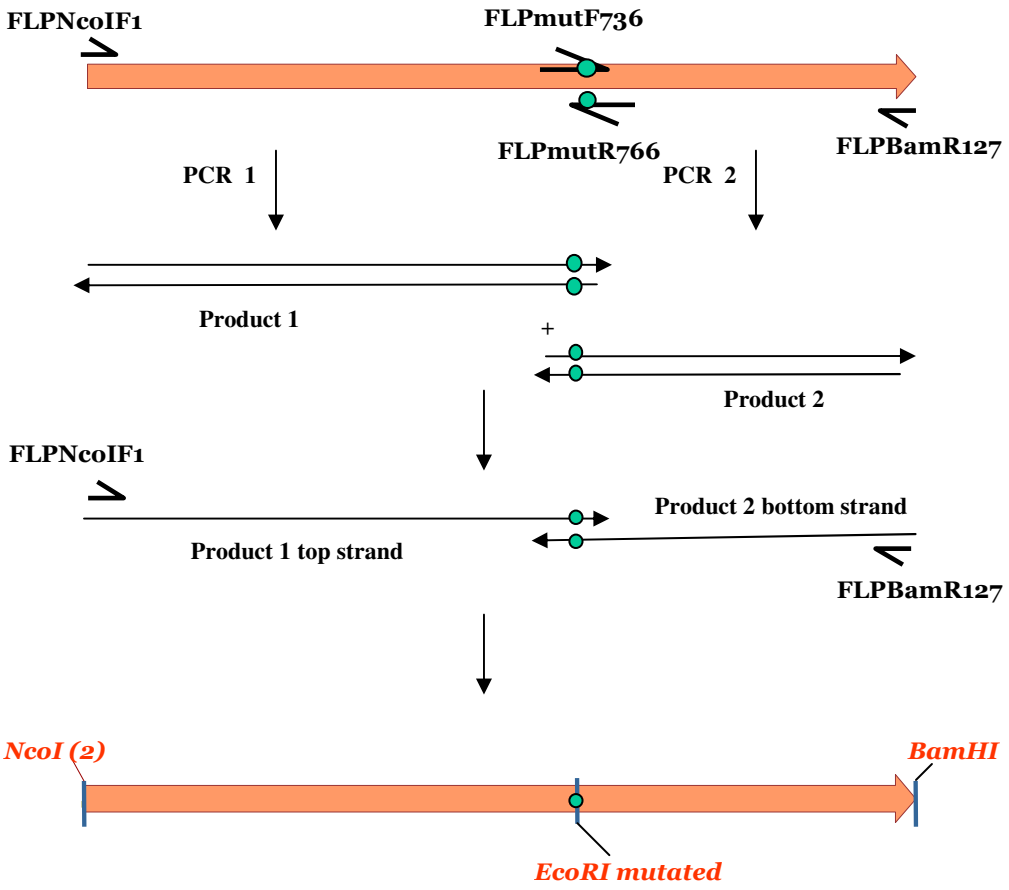


Figure 15: Schematic representation of overlap PCR approach for *FLP* gene amplification from HSP-FLP plasmid. The mutation of the undesirable *EcoRI* site is indicated by the filled circle.

	98°C for 3 minutes - **Denaturation**
35 cycles	98°C for 10 seconds - **Denaturation**
	66°C for 30 seconds – **Annealing**
	72°C for 1 minute – **Extension**
	72°C for 10 minutes – **Final Extension**
	4°C **– Hold**

Table 8: Cycling conditions for the first and second PCR amplification of *FLP* gene.

	98°C for 3 minutes - **Denaturation**
35 cycles	98°C for 10 seconds - **Denaturation**
	75°C for 30 seconds – **Annealing**
	72°C for 1 minute – **Extension**
	72°C for 10 minutes – **Final Extension**
	4°C **– Hold**

Table 9: PCR conditions for the final amplification of *FLP* gene.

3.10.3 Construction of pCAM-Gent-EASE-FLP

The *aacC1* gene cassette with Cauliflower mosaic virus 35S promoter (~ 1.4 Kb) was PCR amplified from pPZP121 (Hajdukiewicz et al., 1994) by using the primers GentLBNar (A-GTTCTGGCGCCTGATGGGCTGCCTGTATCGAGTG) and Gent35Slox (ACTAGAGGCGC-CATAACTTCGTATAATGTATGCTATACGAAGTTATGAAGACCAAAGGGCTATTGAGA C), and Phusion polymerase. The GentLBNar primer was designed to create a *KasI* restriction site followed by a T-DNA left border at the 5' end of the cassette and the primer Gent35Slox was designed to create a *lox* site (Cre recombinase recognition site) followed by a *KasI* restriction site at the 3' end of the cassette. The first round of PCR amplified the desired 1.4 Kb fragment along with a non-specific 1.6 Kb fragment. The first round PCR product was resolved on a 1% gel and the 1.4 Kb band was cut out and gel purified using the Wizard SV Gel and PCR clean-up system (Promega). The gel purified first PCR product was then used as template in a second round of PCR amplification with the same primers and polymerase. The cycling conditions for the PCR amplifications are listed in Table 10. The second round PCR product was digested with *KasI* restriction enzyme and ligated into the *KasI* digested pCAM-EASE-FLP backbone. A positive clone with the desired orientation of the *aacC1* insert was determined by restriction analysis and PCR with primers GentLBNar and GentR1. Sequence analysis further confirmed the desired orientation. Fig. 16 shows a schematic representation of the construction of pCAM-Gent-EASE-FLP. This cloning step reintroduces the left border sequence, and in doing so, places all sequences necessary for plasmid replication in *E. coli* inside the left and right borders.

3.10.4 Construction of pCAM-Gent-TSpSUC2-EASE-FLP

The *AtSUC2* gene under control of various tissue-specific promoters (*rolC*, *CoYMV*,

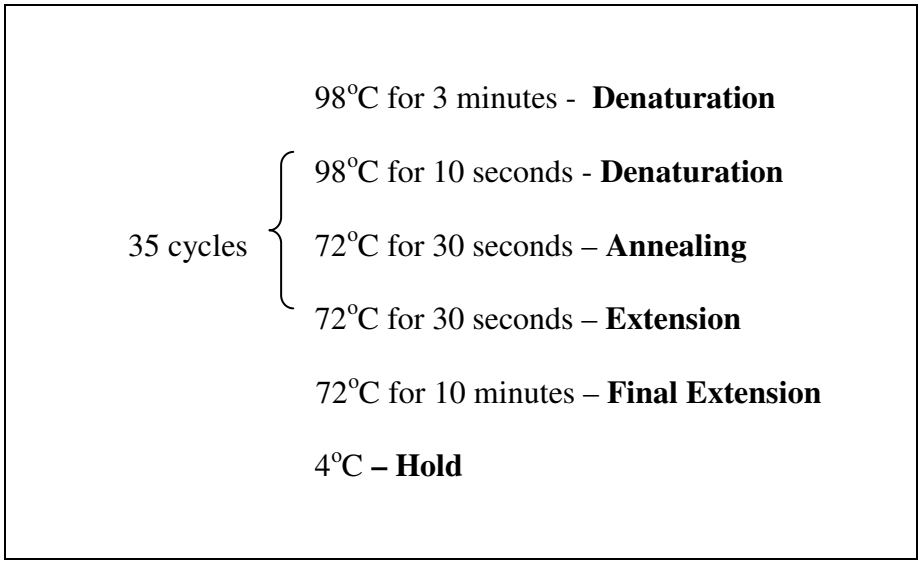


Table 10: Cycling conditions for the first and second round of PCR amplification of the *aacC1* gene cassette.

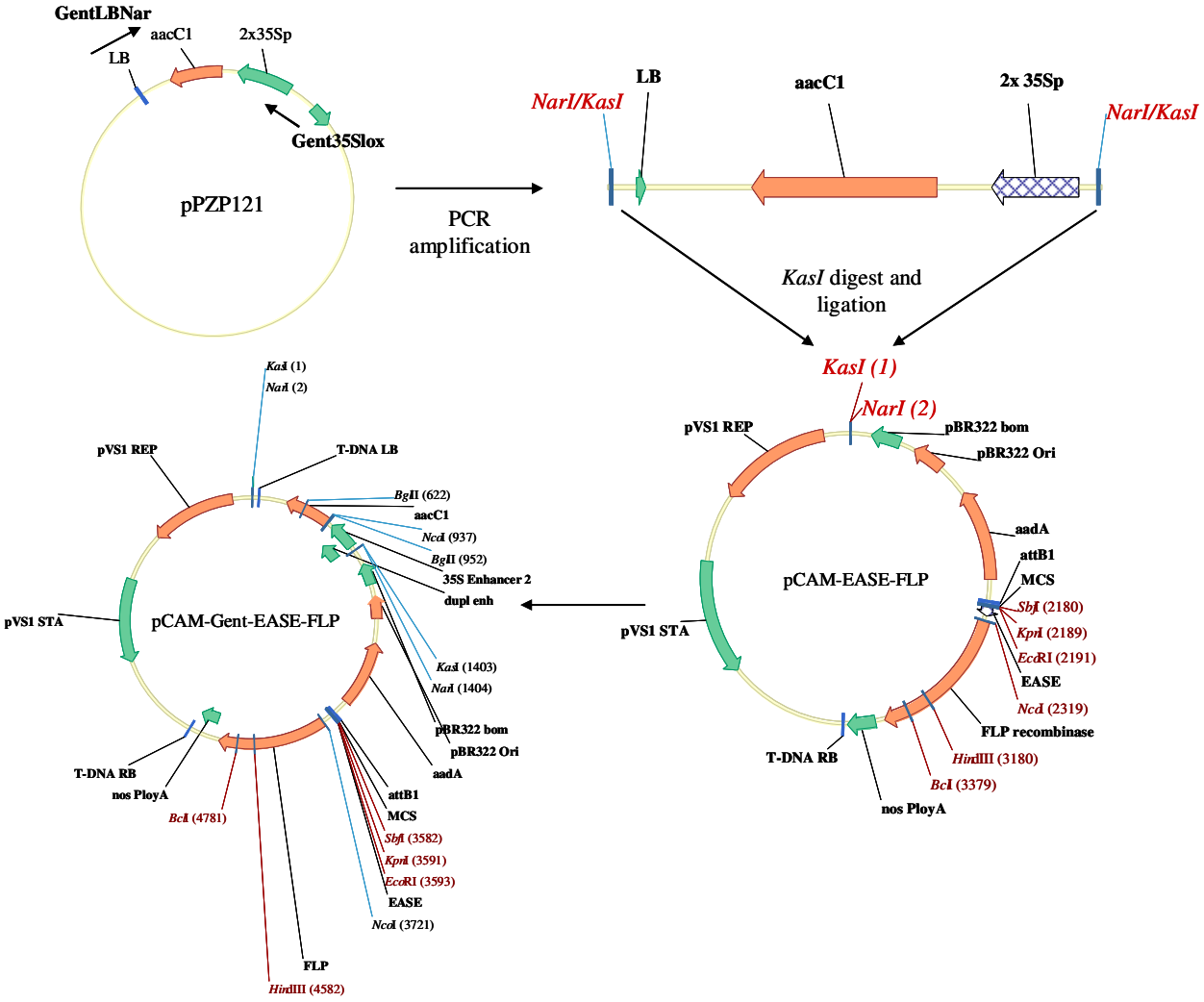


Figure 16: Schematic representation of construction of pCAM-Gent-EASE-FLP. The diagram shows amplification of *aacC1*gene cassette from pPZP121 plasmid and cloning into pCAM-EASE-FLP backbone, generating pCAM-Gent-EASE-FLP.

Cm*GAS1, AtSUC2*) was cloned into the *SbfI* and *EcoRI* sites of plasmid pCAM-Gent-EASE FLP, generating the series of plasmids pCAM-Gent-TSpSUC2-EASE-FLP. The plasmids pGPTV-RolCpSUC2, pGPTV-CoYMVpSUC2 and pGPTV-GASpSUC2 (Srivastava, Ganesan, Ismail, Ayre, manuscript in preparation) were digested with restriction enzymes *SbfI* and *EcoRI* generating the TSpSUC2 cassettes (promoter-*SUC2* cDNA-polyadenylation signal) (1) *RolCpSUC2*, (2) *CoYMVpSUC2*, and (3) *GASpSUC2*, respectively. Each of the above cassettes was then cloned into the *SbfI/EcoRI* digested pCAM-Gent-EASE-FLP as backbone, generating the vectors (1) pCAM-Gent-RolCpSUC2-EASE-FLP, (2) pCAM-Gent-CoYMVpSUC2-EASE-FLP, and (3) pCAM-Gent-GASpSUC2-EASE-FLP. The plasmids were transformed into *E. coli*, plasmid DNA was isolated and positive clones were identified by restriction analysis.

The vector pCAM-Gent-SUC2pSUC2-EASE-FLP with the *AtSUC2* gene under the control of its native promoter (*SUC2p*) was generated by digesting the plasmid pGPTV-SUC2pSUC2 with restriction enzymes *SbfI* and *KpnI* to obtain the *SUC2p* fragment. The *SUC2p* was then cloned into the *SbfI* and *KpnI* digested pCAM-Gent-RolCpSUC2-EASE-FLP as backbone, replacing the *rolCp*. The vector was transformed into *E. coli*, plasmid DNA was isolated and positive clones were identified by restriction analysis.The plasmids pCAM-Gent-RolCpSUC2-EASE-FLP, pCAM-Gent-CoYMVpSUC2-EASE-FLP, pCAM-Gent-GASpSUC2-EASE-FLP and pCAM-Gent-SUC2pSUC2-EASE-FLP were then transformed into *Agrobacterium tumefaciens* strain GV3101mp90 by electroporation.

3.11 Plant Transformation

Plant transformation was performed by the floral-dip method (Clough and Bent, 1998). *Arabidopsis thaliana* plants were grown in 3.5 inch square pots, in a plant growth chamber under

14-h white light / 10-h dark at 21° C. Plants of SALK_038124 line were genotyped and plants wild-type (*AtSUC2/AtSUC2),* heterozygous (*AtSUC2/Atsuc2*), and homozygous (*Atsuc2/Atsuc2*) for the *AtSUC2* T-DNA insertion mutation were identified (section 3.6). The first inflorescences were clipped and plants continued to grow until axillary inflorescences 2-4 cm in height were visible. The egg cells in the flowers were then transformed by inverting the pot and dipping the inflorescences into the *Agrobacterium tumefaciens* suspension. The inflorescences were then washed by spraying with water and the pots were placed under a 100% humidity chamber for 24 hours and were transferred to the growth chamber and grown to maturation. Seeds were collected roughly 4 weeks after transformation.

3.12 Seed Collection and Storage

Seeds from mature plants were harvested and stored according to standard protocol (Weigel and Glazebrook, 2002) with slight modifications. Seeds were released mechanically from dry siliques, passed through a sieve and collected into microcentrifuge tubes. The lid of the microcentrifuge tube was perforated with a needle. The seed containing tubes were placed in a desiccator at room temperature for 7 days and then moved into a -80°C freezer for 3 days to kill any insect eggs. After 3 days, seeds were removed from the freezer and immediately transferred back to a dessicator for 1 day. The dry seeds were then stored at room temperature in a seed box with desiccant for long-term storage.

3.13 Selection of pART-P450-ecSUC2-BAR Transformed Arabidopsis Seeds

Seeds collected from heterozygous plants (*AtSUC2/Atsuc2)* transformed with the first vector pART-P450-ecSUC2-BAR were surface sterilized with chlorine gas for 6 hours. 30 ml of

bleach was taken in a beaker and placed inside a bell jar. 2 ml of concentrated HCl was then added to the bleach beaker, generating chlorine vapors. The open eppendorf tubes containing the seeds were immediately placed inside the bell jar and covered with a lid. The seeds were held in chlorine vapor for 6 hours. Sterilized seeds were spread on 1% MS growth medium with 100 µg/mL kanamycin (~500 seeds per plate) and placed at 4° C in a cold room for 3 days for stratification. The seeds were then grown under illumination at room temperature. Transgenic seedlings resistant to the antibiotic kanamycin were then transferred to soil, covered with plastic wrap and transferred to a growth chamber under 14 h light / 10 h dark at 21° C. After 2 days, slits were made in the plastic wrap cover to harden off the seedlings and after 2 days the plastic wrap was removed, exposing the seedlings to the ambient environment.

3.14 Growth Analysis of *Atsuc2/Atsuc2* Homozygous Plants Complemented with ec*SUC2*

T1 seedlings (transformed generation 1) harboring the pART-P450-ecSUC2-BAR (as determined by resistance to kanamycin) were genotyped for zygosity at the *AtSUC2* endogenous locus (section 3.6). T2 Seeds were collected from T1 plants that were homozygous for the T-DNA mutation in the endogenous *AtSUC2*. The seeds of four independent transformed lines, SG 22-6, SG 22-7, SG 32-22, and SG 32-23, were put out on potting mix and grown under 14 h light / 10 h dark at 21° C. The SALK_038124 and Columbia-O ecotype seeds were put out as growth controls, and grown under same conditions. 21 d after germination, rosettes of plants from independent lines were photographed and the total rosette area was determined using the ImageJ software (Rasband, 1997). The values obtained were compared with the total rosette area of control plants.

3.15 Testing Glufosinate Ammonium Sensitivity in Independent Lines Harboring the pART-P450-ecSUC2-BAR T-DNA

Seeds of the four independent transgenic lines SG 22-6, SG 22-7, SG 32-22, and SG 32-23; the control lines SALK_038124, and Columbia-O ecotype, were put out on potting mix in 3.5 inch square pots. The seeds were stratified in a cold room at 4 ° C for 3 days and then grown under 14 h light / 10 h dark at 21° C. Several days after germination, the seedlings were sprayed with 50 mg/L glufosinate ammonium for 10 consecutive days, and sensitivity of seedlings to glufosinate ammonium was visually scored.

3.16 Selection of Arabidopsis Seedlings Carrying the T-DNA of the Second Vector pCAM-Gent-TSpSUC2-EASE-FLP

Plants of the independent line SG 22-7, harboring the T-DNA of the first vector pART-P450-ecSUC2-BAR and homozygous (*Atsuc2/Atsuc2*) for the T-DNA mutation in the endogenous *AtSUC2,* were transformed with the T-DNA of the second vector series pCAM-Gent-TSpSUC2-EASE-FLP and seeds were collected from the transformed plants. Seeds were surface sterilized with chlorine bleach for 2 hours (section 3.13) and sterilized seeds were put out on 1% MS growth medium with 100 µg/mL gentamycin. MS plates containing seeds were placed in a cold room at 4° C for 3 days for stratification. The plates were then moved to a growth chamber and grown under illumination at room temperature.

3.17 Selection of Seedlings Positive for Excision

Seeds (T1 generation) from transgenic homozygous plants carrying the first vector and transformed with the second vector were sown on soil and grown under 14-h white light / 10-h

dark at 21° C. The seedlings were treated with a solution containing 50 mg/L glufosinate ammonium or 100 µg/L R7402, 5 days after germination. Spraying treatment was repeated every other day, for a total of 10 days with glufosinate ammonium and for a total of 5 days with R7402 pro-herbicide and allowed for selection to be clearly visible.

3.18 Molecular Analysis of Excision

The analysis for excision in transgenic seedlings at a molecular level was done by touchdown PCR using the primer set 35SpFwd; SUC2FRTF1; and Bar3-FRTRev in a single PCR reaction, and ExTaq DNA Polymerase (TaKaRa, Japan). The primers 35SpFwd (CTCGG-ATTCCATTGCCCAGCTAT) and Bar3-FRTRev (GTCCACTCCTGCGGTTCCTGCG) were designed outside the *FRT* sites of pART-P450-ecSUC2-BAR vector, whereas SUC2-FRT F1 (GCAACCGCAACCGCAGCCTCTA) is in the excisable and complementing *AtSUC2* cDNA (ec*SUC2*). The primers 35SpFwd and Bar3-FRTRev were designed to give a PCR product of 600-bp, if the DNA cassette between the *FRT* sites is excised by FLP-mediated recombination, and primer pair SUC2-FRT F1 and Bar3-FRTRev were designed to give a PCR product of 1089-bp if the DNA cassette between the *FRT* sites of the first vector is not excised by FLP-mediated recombination. The PCR cycling conditions optimized for the molecular analysis are listed in Table 11.

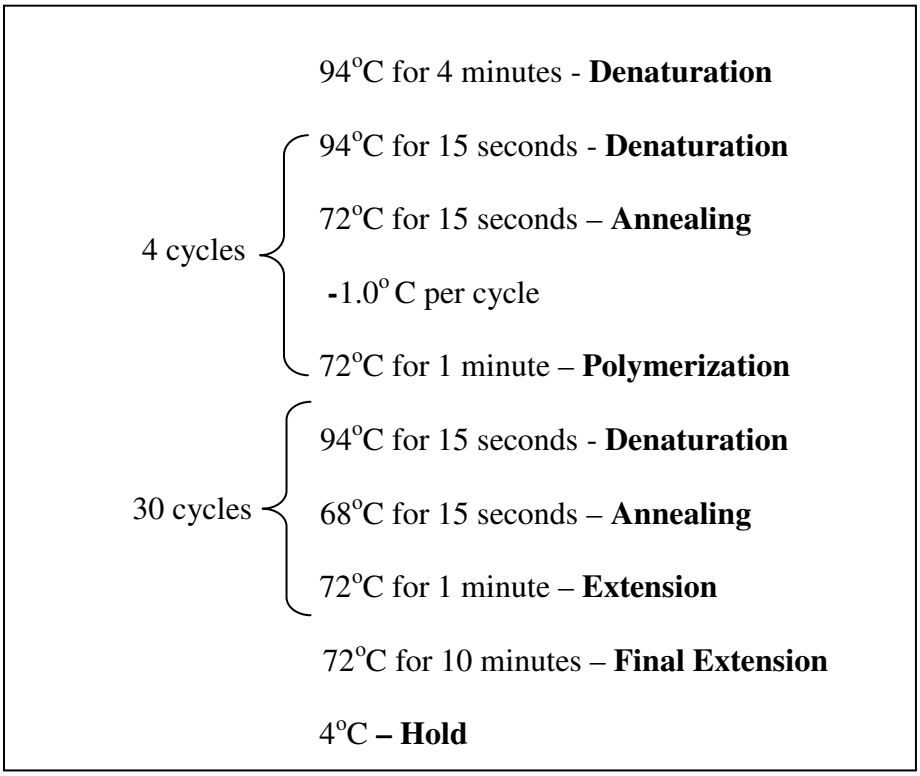


Table 11: Cycling conditions for molecular analysis of excision by PCR.

CHAPTER 4

RESULTS

4.1 Identification of Heterozygous (*AtSUC2/Atsuc2*) and Homozygous (*Atsuc2/Atsuc2*) Plants

A T-DNA insertional mutation in the *AtSUC2* sucrose-proton symporter of *Arabidopsis thaliana* was initially sought. Three potential candidates were identified in the pools created by Joseph Ecker and colleagues (Alonso et al., 2003), and are available through the Arabidopsis Biological Resource Center (ABRC, Columbus, OH). The SALK_087046 line has a T-DNA insert in the first exon of the *AtSUC2* gene, in the SALK_038124 line the T-DNA insert is in the second intron of the gene and in the SALK_01331 line the T-DNA insert is in the 3'-untranslated region (UTR) of the *AtSUC2* gene (Fig. 17).

Plants heterozygous (*AtSUC2/Atsuc2*) and homozygous (*Atsuc2/Atsuc2*) for the *AtSUC2* T-DNA insertion mutation were identified in the SALK_038124 line by PCR genotyping with two gene specific primers (PR1F and PR1R) and one T-DNA insertion specific primer (LB-SALK), in one PCR reaction. Fig. 18 shows the genotyping results obtained for a heterozygous (800 bp and 450 bp PCR product) and a homozygous (only 450 bp PCR product) plant. Phenotypically the heterozygous plants resemble the wild type in appearance, flower set and seed production. The homozygous plants are stunted in growth with anthocyanin accumulation in the mature leaves, and do not grow to set flowers and seeds (Fig. 19). Seeds from a single heterozygous *AtSUC2/Atsuc2* plant were harvested as a stock for all future experiments.

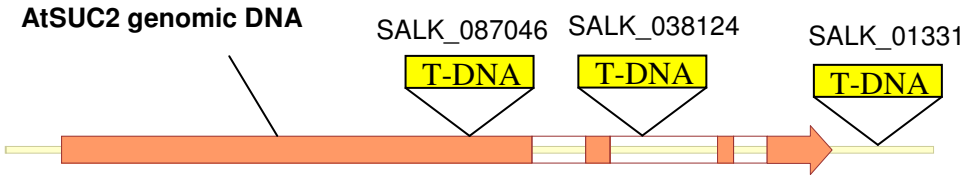


Figure 17: Positions of T-DNA insertion in the *AtSUC2* genome. Orange bars - exons, white bars – introns, yellow line – 5' UTR, 3' UTR.

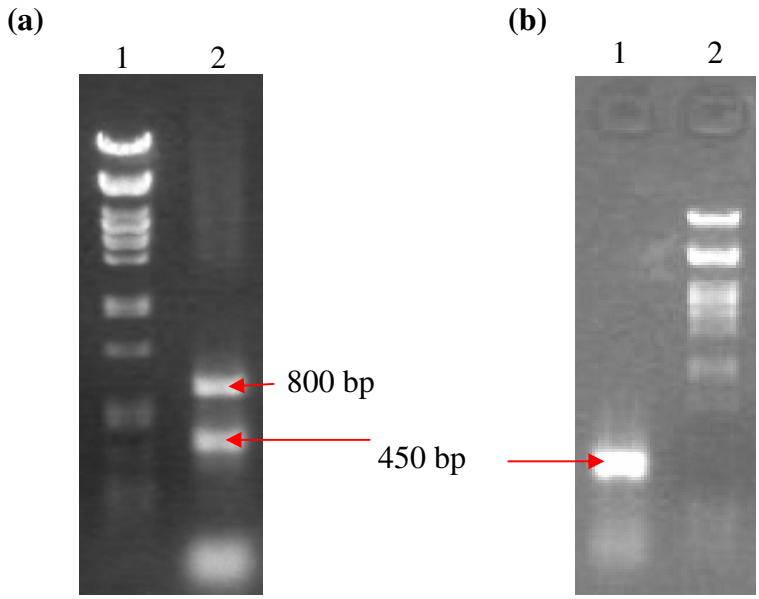


Figure 18: Genotyping results of the SALK_038124 using PCR. (a) Lane 1: lambda DNA ladder and Lane 2: 800 bp WT and 450 bp mutant bands confirming the heterozygous genotype. (b) Lane 1: A single 450 bp mutant band confirms the homozygous genotype and Lane 2: Lambda DNA ladder.

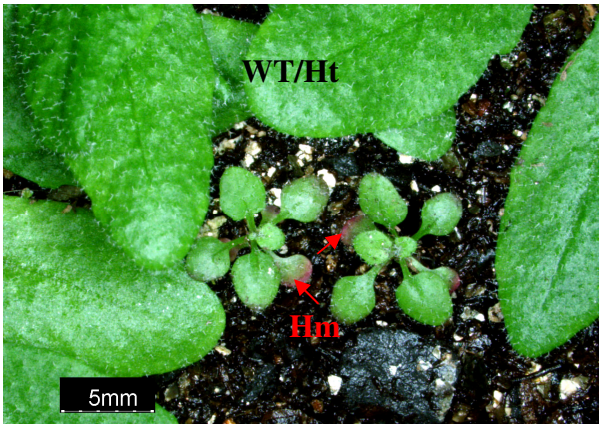


Figure 19: Phenotype of the homozygous (Hm) plants in comparison to the heterozygous (Ht) and wild type (WT). The homozygous plants show stunted growth. Arrows indicate anthocyanin accumulation in the leaves of homozygous plants.

4.2 Construction of the First Vector pART-P450-ecSUC2-BAR

Construction of the vector pART-P450- ecSUC2-BAR harboring the complementing and excisable cDNA copy of *AtSUC2* was carried out in four steps: (1) Construction of the plasmid pFLP-SWITCH-BAR, (2) Cloning the *NotI* fragment of pFLP-SWITCH-BAR into pGEM T-easy and construction of pGEM-ecSUC2-BAR, (3) Construction of pGEM-P450-FRT-ecSUC2-BAR, and (4) Construction of pART-P450-ecSUC2-BAR.

4.2.1 Construction of pFLP-SWITCH-BAR

The first step involved PCR amplification of the *Bar* gene with mutation of an inconvenient *KpnI* site and subconing into the pFLP-SWITCH vector to produce the plasmid pFLP-SWITCH-BAR. Fig. 20a shows the 600 bp *Bar* gene PCR product from the first PCR using primers BarKpn3 and BarHind5, from template pGPTV-BAR. Fig. 20b shows the amplified second PCR product using primers BarKpnmut3 and BarKpnmut5, which were designed to mutate the undesirable *KpnI* restriction site. Fig. 20c shows the third PCR product, which is the final amplification of the *KpnI* mutated *Bar* gene using primers BARKpn3 and BARHind5.

The mutation of an undesirable *KpnI* restriction site in the *Bar* gene and its subsequent cloning into the pFLP-SWITCH backbone was analysed by performing a restriction digestion using the restriction endonucleases *HindIII* and *KpnI* (Fig. 21a). The desired fragment sizes from the restriction digest (a 0.6 kb of *Bar* gene and ~13.0 kb of pFLP-SWITCH backbone) were obtained, confirming the mutated *Bar* gene cloning. The mutation of the *KpnI* restriction site was also confirmed by sequencing. Fig. 21b shows the sequencing result where the *KpnI* restriction

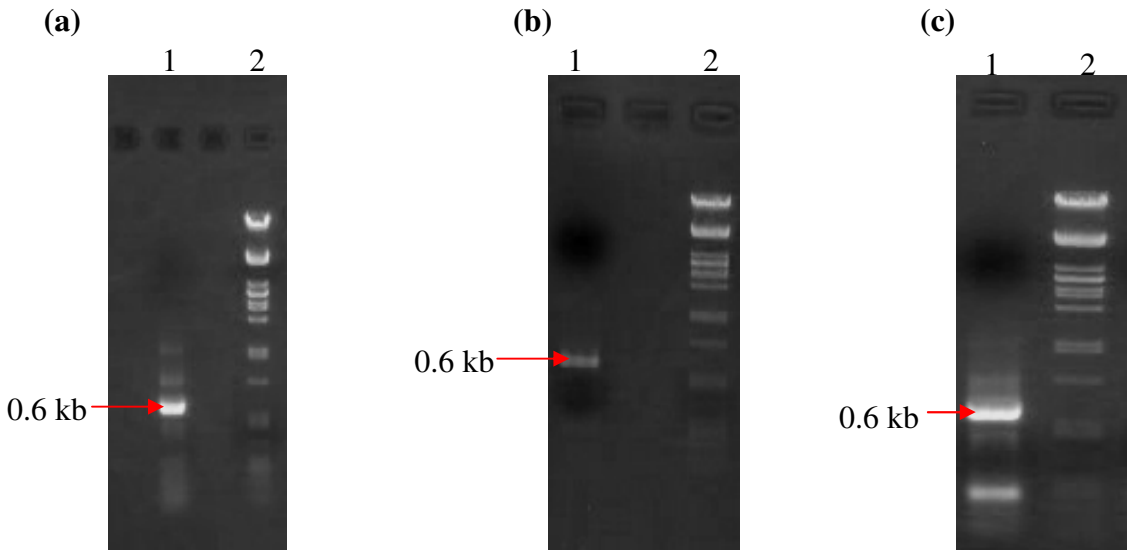
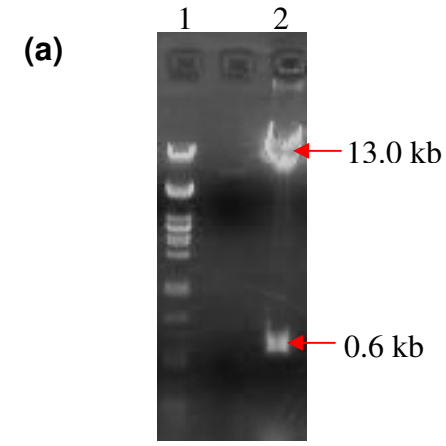


Figure 20: Ethidium bromide-stained agarose gel showing *Bar* gene PCR products. (a) First round PCR amplification using primers BARkpn3 and BARHind5 designed to amplify a 600 bp *Bar* gene from pGPTV-BAR. (b) Second round of mutagenic PCR using primers BAR-Kpnmut5 and BAR-Kpnmut3 to mutate an internal *KpnI* site. (c) Final round of PCR using primers BARKpn3 and BARHind5 and second round PCR product as template. Lane 1 shows the 600 bp *Bar* gene PCR product and lane 2 shows the the Lambda DNA ladder in (a), (b) and (c).



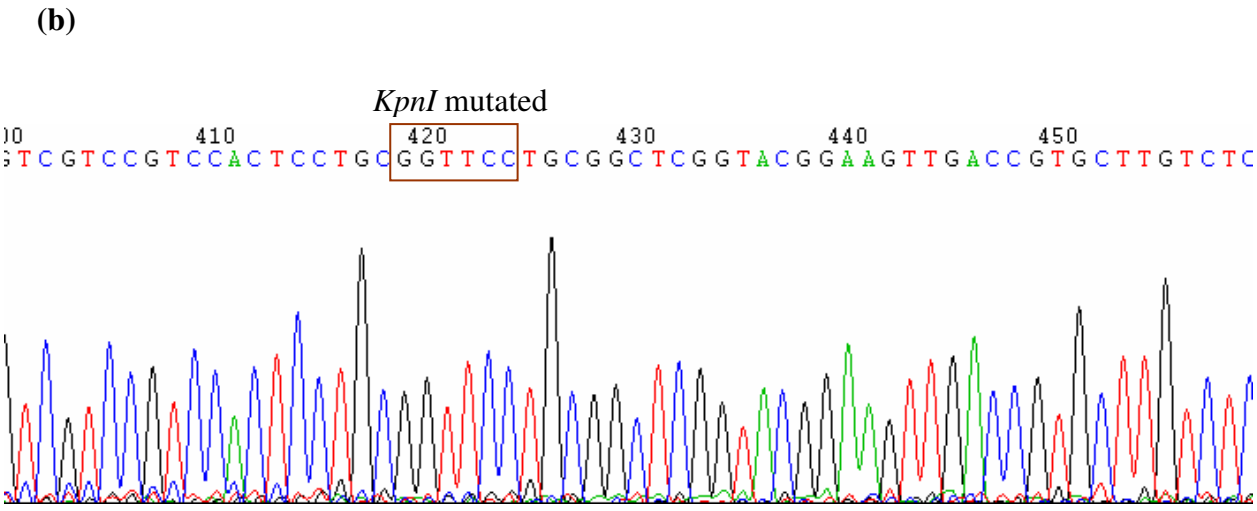


Figure 21: *Bar* gene cloning and sequence analysis. (a) Ethidium bromide-stained agarose gel showing in Lane 1: Lambda DNA ladder and Lane 2: products of *HindIII/KpnI* restriction digestion, a 0.6 kb *Bar* fragment and ~13.0 kb pFLP-SWITCH backbone. (b) Sequencing data showing the mutated *KpnI* restriction site in the *Bar* gene.

site is mutated to being a GGT***T***CC from GGT***A***CC.

4.2.2 Cloning the *NotI* Fragment of pFLP-SWITCH-BAR into pGEM T-easy and Construction of pGEM-ecSUC2-BAR

The pFLP-SWITCH vector backbone had an undesirable *SacI* restriction site and so the *NotI* fragment from pFLP-SWICTH-BAR was cloned into the pGEM T-easy vector. Before this cloning step, an undesirable *SacI* restriction site in the pGEM T-easy plasmid was mutated by a blunt-end ligation approach. The pFLP-SWITCH-BAR plasmid and pGEM T-easy (*SacI* mutated) were digested with the *NotI* restriction enzyme. The desired fragment sizes of 3.0 kb for pGEM T-easy and two bands, (5.0 kb and 3.0 kb) for pFLP-SWITCH-BAR were obtained (data not shown). The 5.0 kb band of pFLP-SWITCH-BAR was gel purified and ligated into the pGEM T-easy *NotI* digested backbone, generating pGEM-BAR. A desired fragment size of 8.0 kb (5.0 kb of pFLP-SWITCH-BAR and 3.0kb of pGEM T-easy) from the restriction digest with *HindIII* confirmed the positive clones.

The 3.7 kb *SUC2pSUC2* cassette was obtained as a *SacI/BamHI* fragment from pGEM-SUC2pSUC2 and cloned into the *SacI/BamHI* digested pGEM-BAR backbone, generating the plasmid pGEM-ecSUC2-BAR. Positive clones were identified by a *KpnI* restriction analysis (data not shown).

4.2.3 Construction of pGEM-P450-FRT-ecSUC2-BAR

The third step in the construction of the vector pART-P450-ecSUC2-BAR involved the construction of the plasmid pGEM-P450-ecSUC2-BAR by amplification and cloning of the *P450* gene cassette into pGEM-ecSUC2-BAR plasmid. An inconvenient *NotI* site in the *P450*

cassette was mutated and a convenient *PspOMI* site introduced in its place by PCR. Also, a *BglII* site was introduced at the 5'-end by PCR for convenience in cloning.

The mutation of an undesirable *NotI* restriction site in the P450 gene cassette was analyzed by restriction digestion with the restriction endonucleases *EcoRI* and *NotI* (Fig. 22a). A desired linear fragment of 7.5 kb was obtained, thus confirming the mutation. Fig. 22b shows the alignment result of the pUC118-P450 sequence with the pUC118-P450mut sequence using the VectorNTI software (Invitrogen, www.invitrogen.com), which also confirmed the mutation of the *NotI* restriction site from GG***C***GGCCGC to GG***G***GGCCGC.

The successful introduction of a *BglII* and *PspOMI* restriction sites in the P450 gene cassette was analyzed by doing a *BglII/PspOMI* restriction digest. Fig. 23a shows the desired linear 7.5 kb fragment (plasmid being digested only with *BglII* as PspOMI site lost after ligation) obtained after restriction digestion. The presence of the *BglII* restriction site in the pUC118-P450mut plasmid was also confirmed by sequence analysis (Fig. 23b).

The plasmid pUC118-P450mut was digested with *BamHI*/*BglII* and ligated into the *BamHI* digested pGEM-ecSUC2-BAR plasmid, generating pGEM-P450-ecSUC2-BAR.

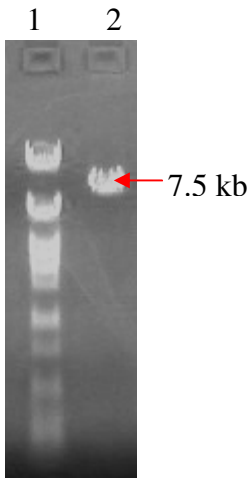
4.2.4 Construction of pART-P450-ecSUC2-BAR

The plasmid pGEM-P450-ecSUC2-BAR was digested with *NotI* and cloned into the *NotI* digested pART27 vector backbone, generating the final plasmid pART-P450-ecSUC2-BAR.

4.3 Construction of the Second Vector pCAM-Gent-TSpSUC2-EASE-FLP

The second vector pCAM-Gent-TSpSUC2-EASE-FLP was constructed in four steps as follows: (1) Construction of pCAM-EASE, (2) Construction of pCAM-EASE-FLP by cloning

(a)



(b)

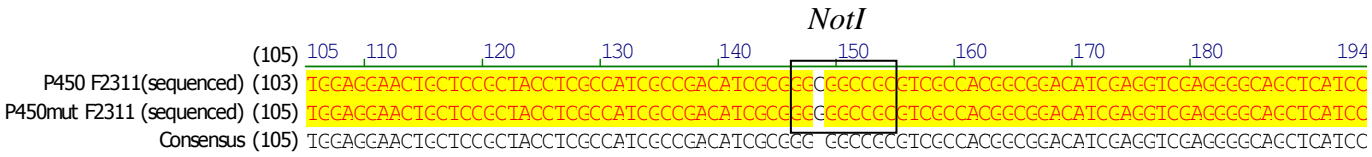
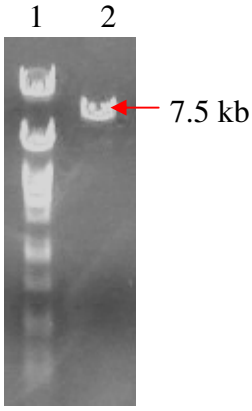


Figure 22: *P450* cassette restriction and sequence analysis. (a) Ethidium bromide-stained agarose gel showing Lambda DNA ladder in lane 1 and the 7.5 kb EcoRI/NotI restriction fragment of pUC118-P450mut in lane 2. (b) Alignment of the pUC118-P450 sequence (top sequence: P450 F2311) with the pUC118-P450mut sequence (bottom sequence: P450mut F2311). The boxed area represents the *NotI* restriction site sequence. The yellow highlighted region indicates homology between the two sequences and white highlight indicates the single-base mutation in the NotI site between the two sequences.

(a)



(b)

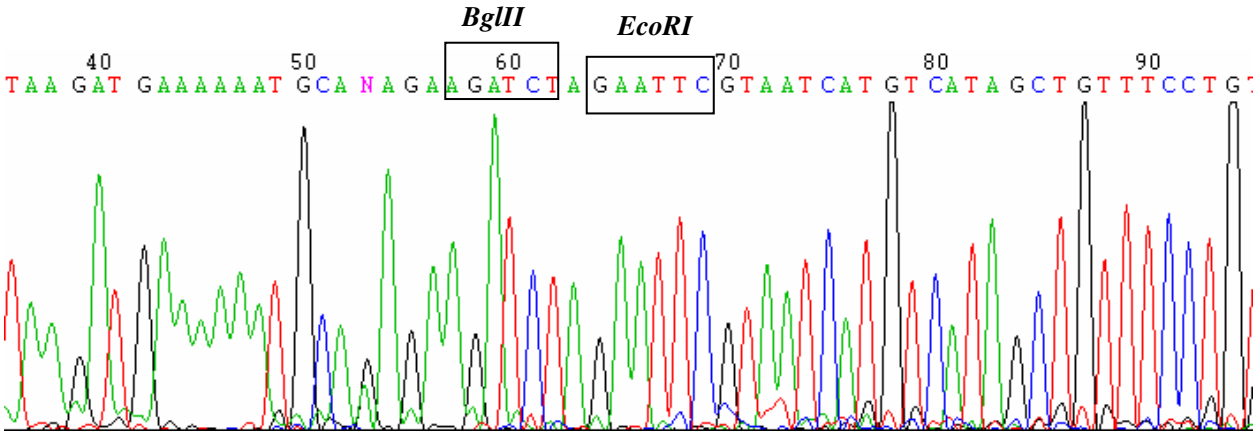


Figure 23: *P450* cassette restriction and sequence analysis. (a) Ethidium bromide-stained agarose gel showing Lambda DNA ladder in lane 1 and the 7.5 kb *BglII/PspOMI* restriction fragment of pUC118-P450mut in lane 2. (b) Sequence analysis data of pUC118-P450mut plasmid showing introduction of the *BglII* restriction site.

the *FLP* gene into pCAM-EASE, (3) Construction of pCAM-Gent-EASE-FLP by cloning the *aacC1* cassette into pCAM-EASE-FLP, and (4) Cloning the various *TSpSUC2* cassettes into pCAM-Gent-EASE-FLP backbone.

4.3.1 Construction of the pCAM-EASE Plasmid

The synthetic fragment was constructed using two primer sets, (1) attB1BglF1 and EASER1, and (2) EASEF2 and 35S-45NcoIR2. The primers were first annealed and extended using Klenow. The products from the Klenow extension of each primer set were used as templates in a PCR reaction, to construct the synthetic fragment. The desired ~ 250 bp PCR product was obtained (Fig. 24). The PCR generated synthetic fragment comprising of the *attB1*, multiple cloning site (MCS), EASE element, and *CaMV 35Sp* -45 region, was digested with *NcoI/BglII* and cloned into the *NcoI/BclI h*digested pCAMBIA0390 vector backbone, generating the plasmid pCAM-EASE. The synthetic fragment was sequenced after cloning it into pCAMBIA0390, to confirm correct sequence of the components.

4.3.2 Amplification of *FLP* Gene from HSP-FLP Plasmid and Construction of pCAM-EASE-FLP

The *FLP* gene was amplified from the HSP-FLP plasmid (Kilby et al., 1995) with mutation of an undesirable *EcoRI* site by using an overlap PCR approach (Sambrook et al., 2001). Fig. 25a shows the products of PCR 1 and PCR 2 in the overlap approach and Fig. 25b shows the final amplified ~ 1.3 kb *FLP* PCR product.

The mutation of the *EcoRI* site in the *FLP* gene cassette by overlap PCR approach was confirmed by sequence analysis after cloning the *FLP* gene into pADH MCS #6 as vector

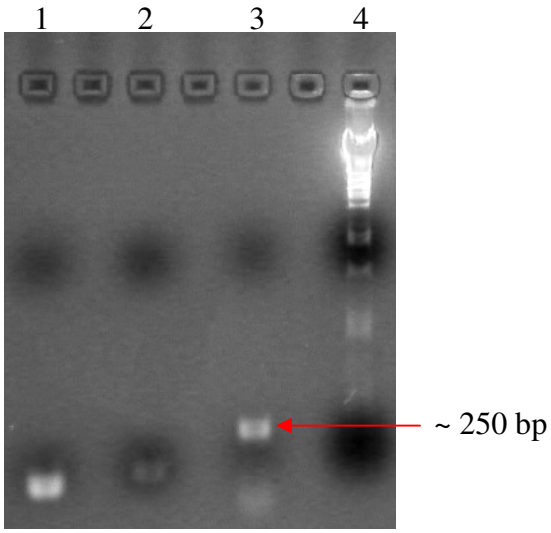


Figure 24: Syntheic fragment construction. Ethidium bromide stained agarose gel showing in, lanes 1 and 2: Annealed and extended reaction product using first primer set attB1BglF1 and EASER1, and second primer set EASEF2 and 35S-45NcoIR2; respectively, lane 3: Expected ~ 250 bp PCR product using annealed reaction products as template, and primers attB1BglF1 and 35S-45NcoIR2, and lane 4: Lambda DNA ladder.

backbone. Fig. 26 displays a portion of the sequencing data showing a single base mutation of the *EcoRI* restriction sequence (GAA***T***TC → GAA***C***TC). The mutation of the *EcoRI* site did not alter the amino acid sequence. Sequence analysis also confirmed introduction of the *NcoI* and *BamHI* restriction sites at the 5'-end and 3'-end, respectively. The *FLP* cassette was obtained as a *NcoI/BamHI* insert from pADH-FLP and cloned into the *NcoI/BglII* digested pCAM-EASE backbone, generating pCAM-EASE-FLP.

4.3.3 Construction of pCAM-Gent-EASE-FLP by Cloning the *aacC1* Cassette into pCAM-EASE-FLP

The *aacC1* gene cassette conferring resistance to the antibiotic gentamycin in transgenic plants was amplified from pPZP121 (Hajdukiewicz et al., 1994) using designed primers GentLBNar and Gent35Slox. Fig. 27a shows the products of the first PCR amplification, a non-specific 1.6 Kb fragment and the desired 1.4 Kb fragment. Fig. 27b shows the product of the second PCR where the desired 1.4 Kb fragment was amplified using the 1.4 Kb fragment from the first PCR as template. The PCR product was digested with *KasI* and cloned into the *KasI* digested pCAM-EASE-FLP backbone, generating the plasmid pCAM-Gent-EASE-FLP.

4.4 Generation and Characterization of Transgenic Arabidopsis Plants Transformed with the First Vector pART-P450-ecSUC2-BAR

The T-DNA region of plasmid pART-P450-ecSUC2-BAR was transferred to heterozygous (*AtSUC2/Atsuc2*) Arabidopsis plants by a standard *Agrobacterium tumefaciens* transformation technique. Transformed Arabidopsis seedlings carrying the P450-ecSUC2-BAR T-DNA were selected on 1% MS media containing the antibiotic kanamycin and resistant

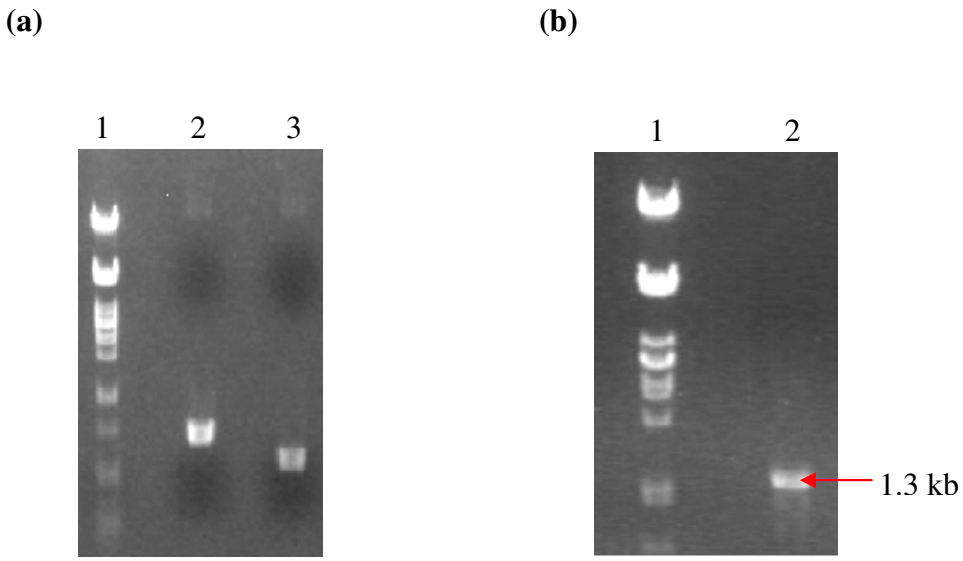


Figure 25: PCR products of *FLP* gene amplification. Ethidium bromide-stained agarose gel showing (a) Lane 1: Lambda DNA ladder, lane 2: Product of first PCR amplification using HSP-FLP plasmid DNA as template and designed primers FLPNcoIF1 and FLPmutR766, and lane 3: Product of second PCR amplification using HSP-FLP plasmid and primers FLPmutF736 and FLPBamR1272. (b) Lane 1: Lambda DNA ladder, and lane 2: ~ 1.3 kb PCR product obtained from final round of PCR amplification using first and second round PCR products as templates, and primers FLPNcoIF1 and FLPBamR1272.

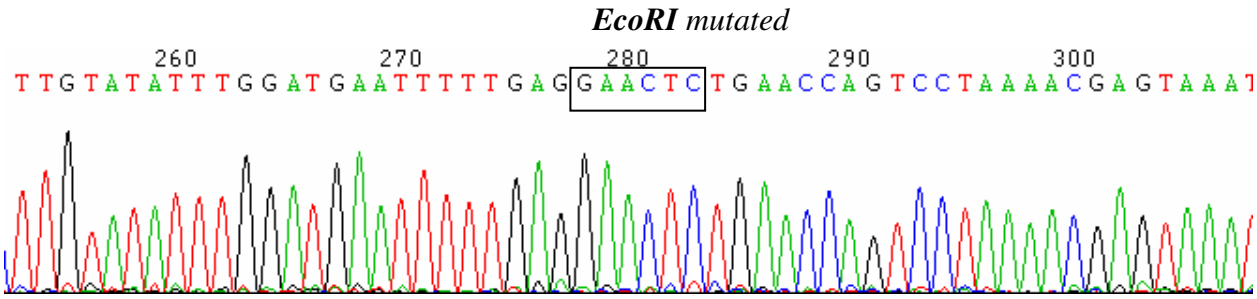


Figure 26: Sequence analysis data showing mutation of the *EcoRI* site in the *FLP* gene cassette.

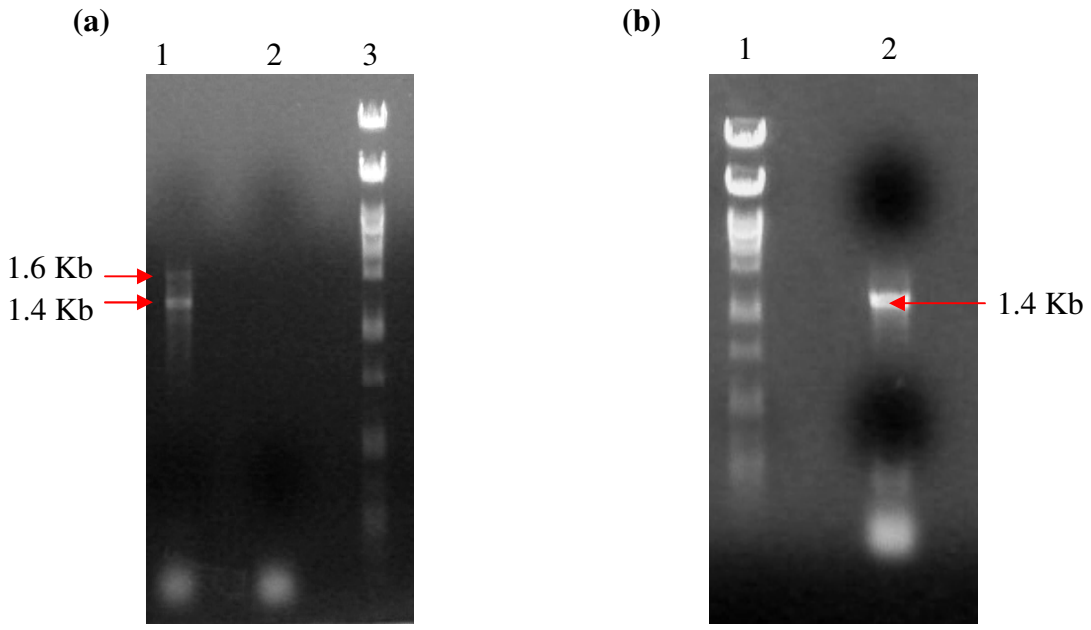


Figure 27: PCR amplification of *aacC1* gene cassette. (a) First round PCR amplification using primers GentLBNar and Gent35Slox designed to amplify a 1.4 kb PCR product. Lane 1: pPZP121 plasmid DNA used as template, lane 2: Negative control with no DNA template, lane 3: Lambda DNA ladder. (b) Second round PCR amplification using primers GentLBNar and Gent35Slox. Lane 1: Lambda DNA ladder, lane 2: Gel purified 1.4 kb product from first PCR round used as template. The expected 1.4 kb PCR product was obtained in both rounds of PCR.

seedlings were transferred to soil. The seedlings were grown under 14-h white light / 10-h dark at 21° C and the segregating population was genotyped for plants homozygous for a T-DNA insertion (knockout plants; KO) at the endogenous *AtSUC2* locus. The homozygous KO plants were effectively complemented by the ec*SUC2* cDNA. Fig. 28 shows a T1 generation transgenic (harboring ec*SUC2* cDNA) homozygous (*Atsuc2/Atsuc2*) plant phenotypically resembling a T1 generation transgenic heterozygous (*AtSUC2/Atsuc2*) plant. The transgenic homozygous KO plants had normal growth habit, showed no anthocyanin accumulation in their mature leaves, and also had wild-type levels of flowers and seeds. These homozygous KO plants were grown until maturity and seeds (T2 generation) were harvested. Fig. 29 shows the T2 generation transgenic homozygous plants in comparison to heterozygous and wild-type plants.

4.5 Quantitative Growth Analysis

The total rosette area of four independent transgenic lines SG 22-7, SG 22-6, SG 32-22, and SG 32-23 harboring the P450-ecSUC2-BAR T-DNA was measured using ImageJ software. The values obtained were compared with the total rosette area of SALK_038124 heterozygous and homozygous plants, and Columbia-O ecotype wild-type plants (Fig. 30). All four independent lines had a rosette area greater than the SALK_038124 homozygous knockout plants, which further shows effective complementation of homozygous plants with the *ecSUC2* cassette. However, growth in each line was less than wild-type and heterozygotes, indicating less-than-perfect complementation. The total rosette area of the primary transformed independent line SG 22-7 was the greatest among all other independent lines and was comparable to that of the SALK_038124 heterozygous plants.

Homozygous (*Atsuc2/Atsuc2*) **Heterozygous (*AtSUC2/Atsuc2*)**

Figure 28: Phenotypic comparison of transgenic plants. Shown is a T1 generation homozygous plant carrying the *ecSUC2* construct with a T1 generation heterozygous plant, transferred to soil after selection on 1% MS media with kanamycin.

(a)

(b)

(c)

Figure 29: Phenotypic comparison of the transgenic homozygous plants (T2 generation) with control plants. (a) Independent line SG 22-7, (b) SALK_038124 heterozygous plants, and (c) Columbia-0 ecotype wild-type plants.

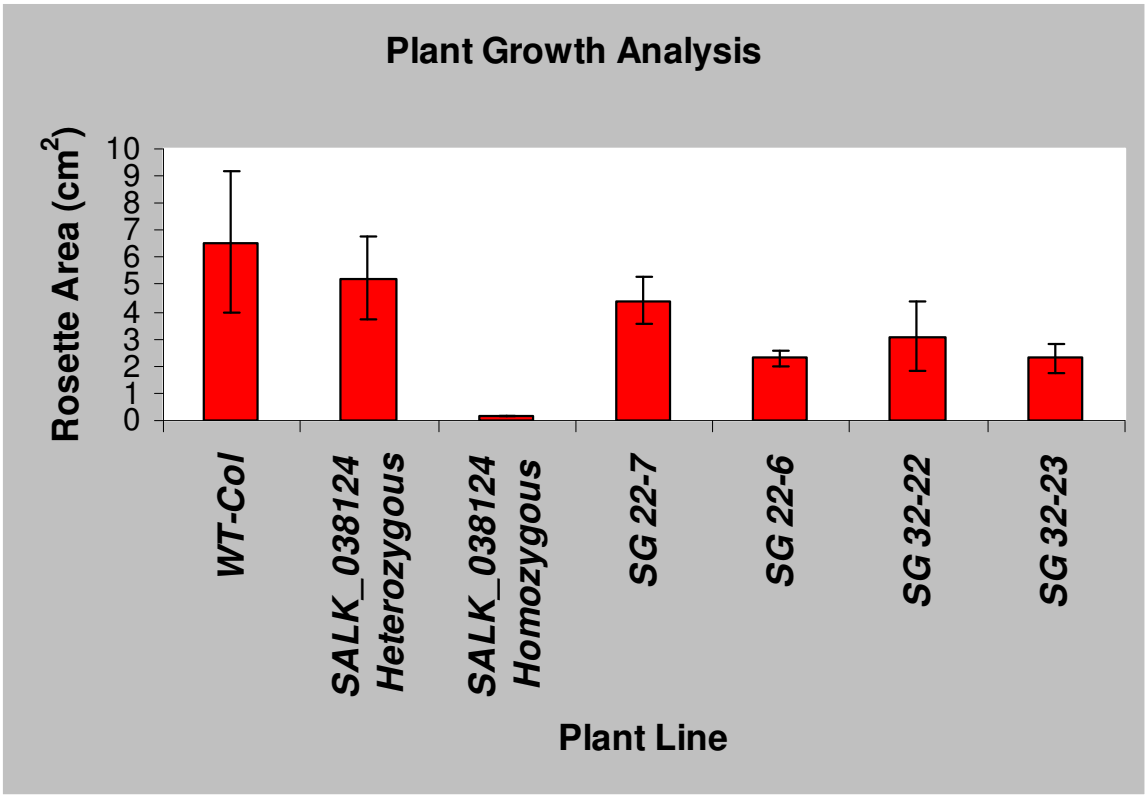


Figure 30: Quantitative growth analysis of transgenic plants. Comparison of total rosette area of plants from four independent transformed lines with SALK_038124 heterozygous and homozygous plants, and Columbia-O ecotype wild-type plants. n = 3 – 11; where 'n' is the number of plants used for rosette area measurement. WT-Col (n= 4), SALK_038124 heterozygous (n=3), SALK_038124 homozygous (n=3), SG 22-7 (n=8), SG 22-6 (n=8), SG 32-22 (n=11), and SG 32-23 (n=7).

4.6 Analysis of the *Bar* Gene as an Effective, Conditional Selection Marker in the P450-ecSUC2-BAR T-DNA

The expression of the *Bar* gene from the P450-ecSUC2-BAR T-DNA is blocked by the P450-ecSUC2 DNA cassette in between the *FRT* sites. In order to test the effectiveness of the DNA cassette in blocking *Bar* gene expression, the independent lines harboring the P450-ecSUC2-BAR T-DNA and showing complementation of homozygous knockout plants were treated with the herbicide glufosinate ammonium (50 mg/L). The SALK_038124 line and Columbia-O ecotype wild-type plants were used as glufosinate ammonium sensitive controls. Fig. 31 shows the glufosinate ammonium treated seedlings. The independent line SG 22-7 was as sensitive to the glufosinate ammonium treatment as the controls, whereas the other three independent lines tested were partially resistant to the herbicide. Increasing herbicide concentration to 100 mg/L overcame this partial resistance (data not shown).

4.7 Generation and Selection of Seedlings Harboring the T-DNA from pCAM-Gent-TSpSUC2-EASE-FLP and Test for Excision

T2 generation seeds from plants homozygous for a T-DNA mutation in the endogenous *AtSUC2* locus and also carrying the T-DNA from pART-P450-ecSUC2-BAR were sown on soil, and grown under 14-h light / 10-h dark at 21° C. The T-DNA region of the second vector pCAM-Gent-TSpSUC2-EASE-FLP harboring the *AtSUC2* cDNA under different tissue-specific promoters was transferred to transgenic (ec*SUC2*) homozygous knockout plants by the floral-dip method and seeds were collected. The seeds were put out on soil and also on 1% MS media containing the antibiotic gentamycin (100 mg/L) in order to establish seedlings harboring the T-DNA of the second vector. The seedlings on soil were treated with either 100 mg/L glufosinate

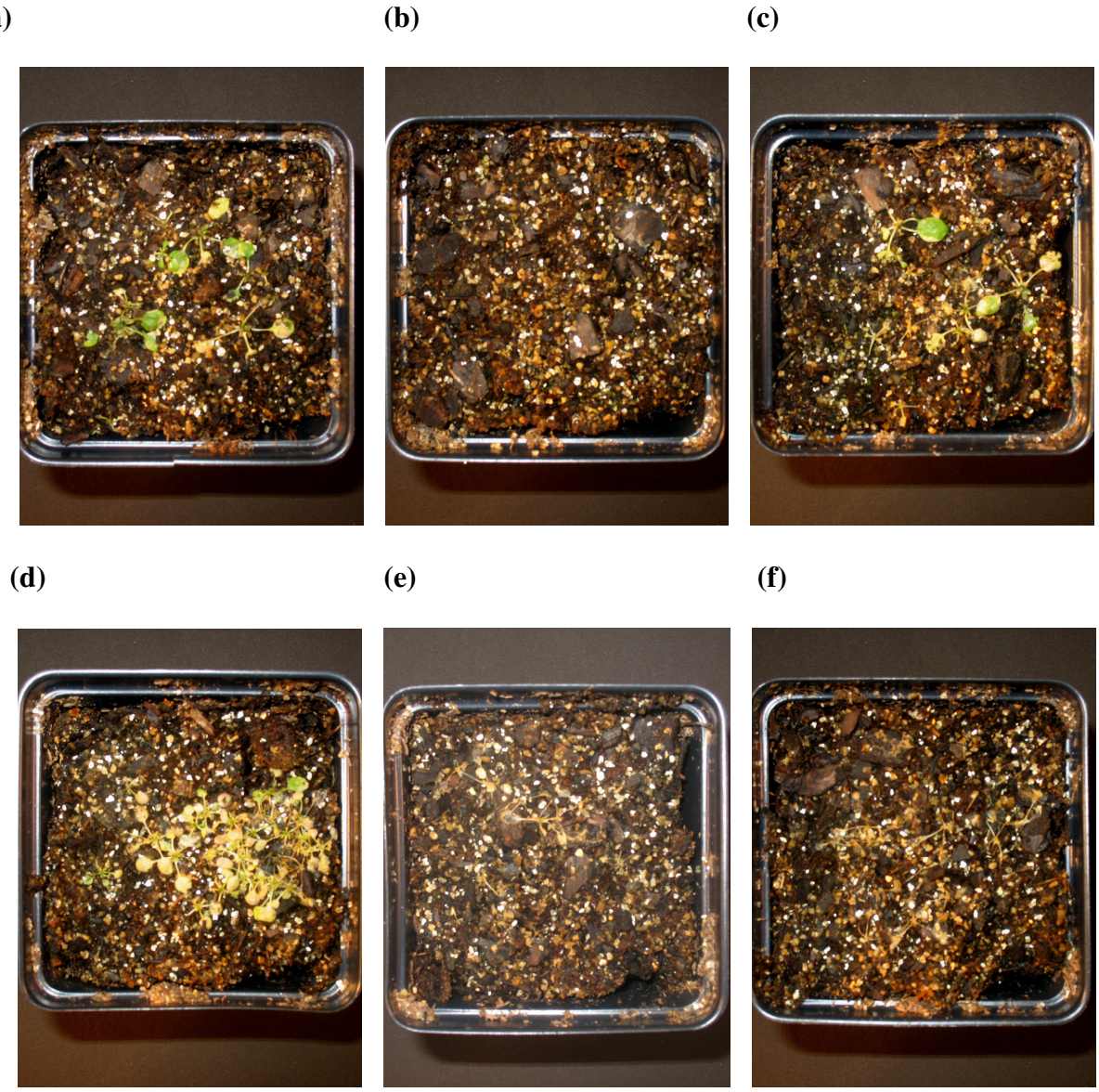


Figure 31: Independent lines treated with 50 mg/L glufosinate ammonium. (a) SG 22-6; (b) SG 22-7; (c) SG 32-22; (d) SG 32-23; (e) SALK_038124; and (f) Columbia-O ecotype wild-type. Seedlings were sprayed with glufosinate ammonium for 10 d, 2 weeks after germination.

ammonium or 100 µg/L R7402 pro-herbicide few days after germination. If the *FLP* gene is effectively expressed in the zygote from the Egg-Apparatus Specific Enhancer, FLP recombinase should bring about excision of the P450-ecSUC2 DNA cassette in between the *FRT* sites of the pART-P450-ecSUC2-BAR T-DNA. In this case, seedlings that harbor the T-DNA of the second vector and have undergone excision will be resistant to both glufosinate ammonium and R7402. On the other hand, in seedlings with no *FLP* gene expression or ineffective excision, the DNA cassette between the *FRT* sites of the pART-P450-ecSUC2-Bar T-DNA will not be excised and thus will be sensitive to both glufosinate ammonium and R7402.

Fig. 32 shows the glufosinate ammonium (100 mg/L) treatment on first-generation progeny of the secondary transformant line SG22-7-CoYMV-01 (pCAM-Gent-CoYMVpSUC2-EASE-FLP) in comparison to glufosinate ammonium sensitive control lines SALK_038124 and Columbia-O ecotype, and glufosinate ammonium resistant control lines JR111, Kd672 and WT1351 (Ayre and Srivastava, unpublished). Fig. 33 shows R7402 (100 µg/L) pro-herbicide treatment on first-generation progeny of the secondary transformant lines SG22-7-CoYMV-01, SG22-7-RolC-03, SG22-7-GAS-04, SG22-7-Empty-05, SG22-7-SUC2-06 with the *AtSUC2* under control of different tissue-specific promoters in comparison to the R7402 resistant controls SGSALK-SUC2-07, SALK_038124 and Columbia-O ecotype wild-type, and R7402 sensitive parent primary transformant line SG 22-7. The SG22-7-Empty-05 is a secondary transformant line of the SG22-7 parent line transformed with an empty second vector with no *AtSUC2* cDNA expression from a tissue-specific promoter (pCAM-Gent-EASE-FLP). The SGSALK-SUC2-07 line is a primary transformant line of the SALK_038124 heterozygous plants floral-dipped with only the second vector pCAM-Gent-SUC2pSUC2-EASE-FLP and therefore R7402 resistant. It was observed that the T1 generation seedlings of all the putative

secondary transformant lines were sensitive to treatment with glufosinate ammonium (100 mg/L) as well as R7402 (100 µg/L). This could be because they either did not carry the T-DNA of the second vector pCAM-Gent-TSpSUC2-EASE-FLP as would be the case if transformation efficiency was very low or the DNA cassette between the *FRT* sites of the first vector in these seedlings did not get excised, thus making them sensitive to both glufosinate ammonium and R7402. Thus far, no putative transgenics have been obtained with glufosinate ammonium and R7402 treatments for any of the secondary transformant lines.

A single, putatively resistant seedling was obtained from the seeds put out on 1% MS media with gentamycin selection. The putative transgenic seedling was transferred to soil and analyzed for excision using PCR. The primers 35SpFwd and Bar3-FRTRev were designed to give a band of 600 bp indicative of excision of the DNA cassette between the *FRT* sites of the first vector, whereas the primers SUC2FRTFwd and Bar3-FRTRev were designed to give a band of size 1089 bp indicative of no excision. The PCR analysis with the designed primers gave a 1089 bp product, which indicated that no excision had taken place (Fig. 34a). The seedling was also analyzed for presence of the gentamycin gene with PCR using designed primers Gent35Slox and GentLBNar (Fig. 34b). The putative transgenic seedling did not give the expected 1.4 kb size band. The PCR analysis with the gentamycin primers confirmed that the seedling did not harbor the T-DNA of the second vector and hence there was no excision of the DNA cassette between the *FRT* sites of the first vector. The above putative seedling was also treated with glufosinate ammonium (100 mg/L) for 5 consecutive days and it was observed that the seedling was sensitive to the treatment. This suggests non-expression of the *Bar* gene from the 35S promoter due to absence of the T-DNA of the second vector and hence non-excision of the DNA cassette

in between the *FRT* sites. These results suggest that the gentamycin selection needs to be more stringent.

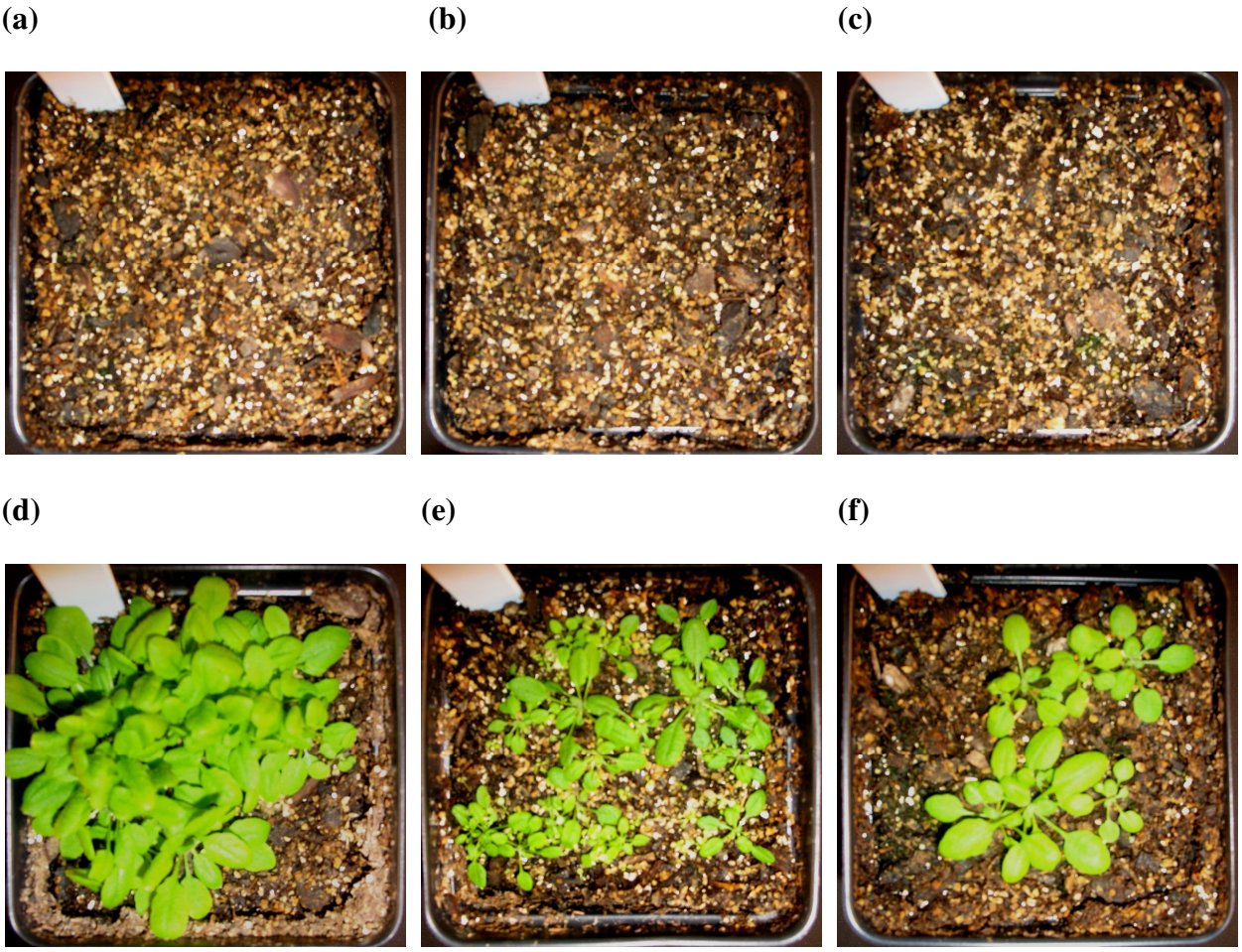


Figure 32: Arabidopsis seedlings treated with 100 mg/L glufosinate ammonium. (a) SG 22-7-CoYMV- 01, (b) SALK_038124, (c) Columbia-O ecotype wild-type, (d) JR 111, (e) Kd 672, and (f) WT 1351.

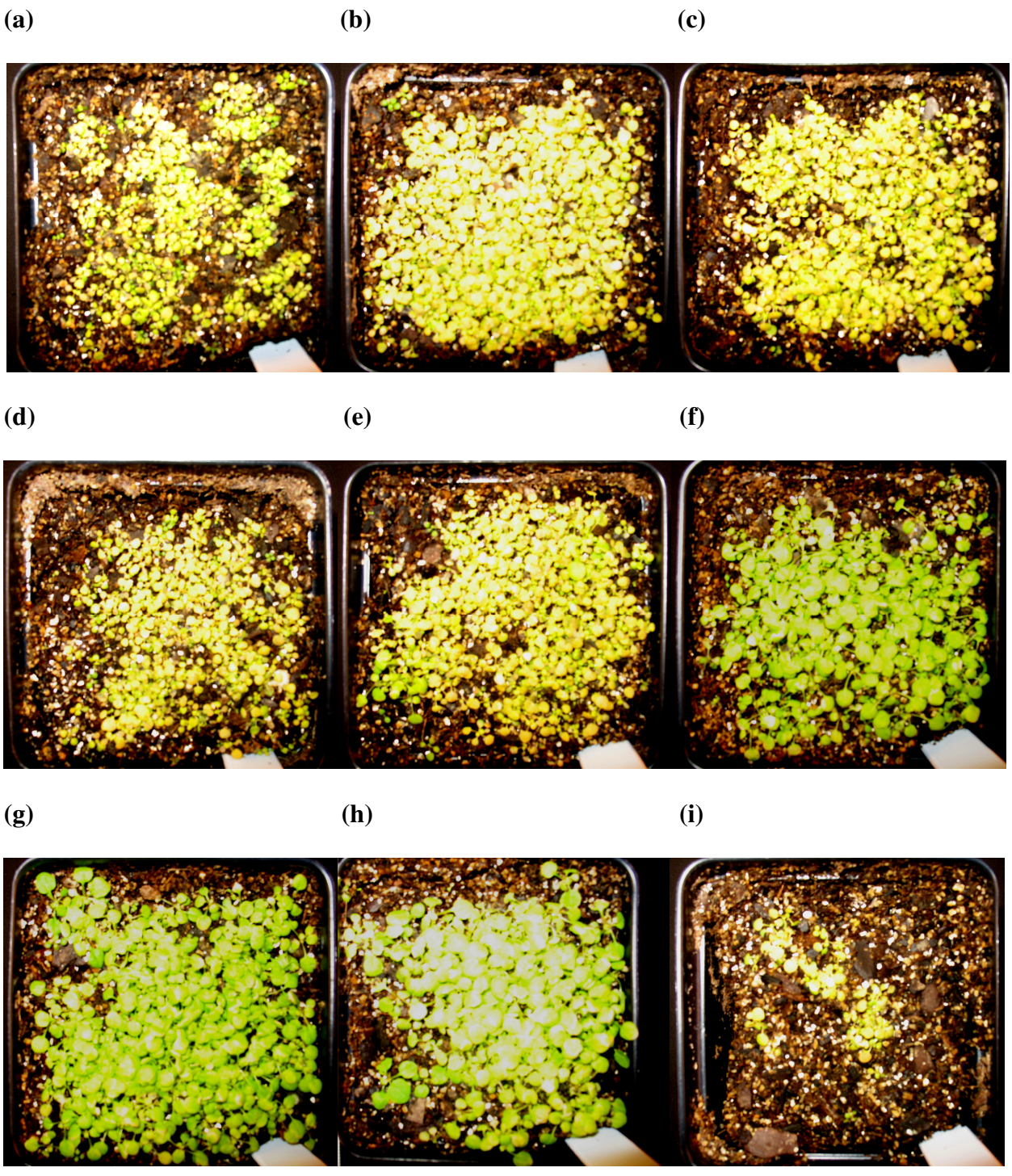


Figure 33: Arabidopsis seedlings treated with 100 µg/L R7402. (a) SG22-7-CoYMV-01, (b) SG22-7-RolC- 03, (c) SG22-7-GAS- 04, (d) SG22-7-Empty- 05, (e) SG22-7-SUC2- 06, (f)

SGSALK-SUC2-07, (g) SALK_038124, (h) Columbia-O ecotype wild-type, and (i) SG 22-7 parent. Treatment with R7402 done for 5 d, 2 weeks after germination.

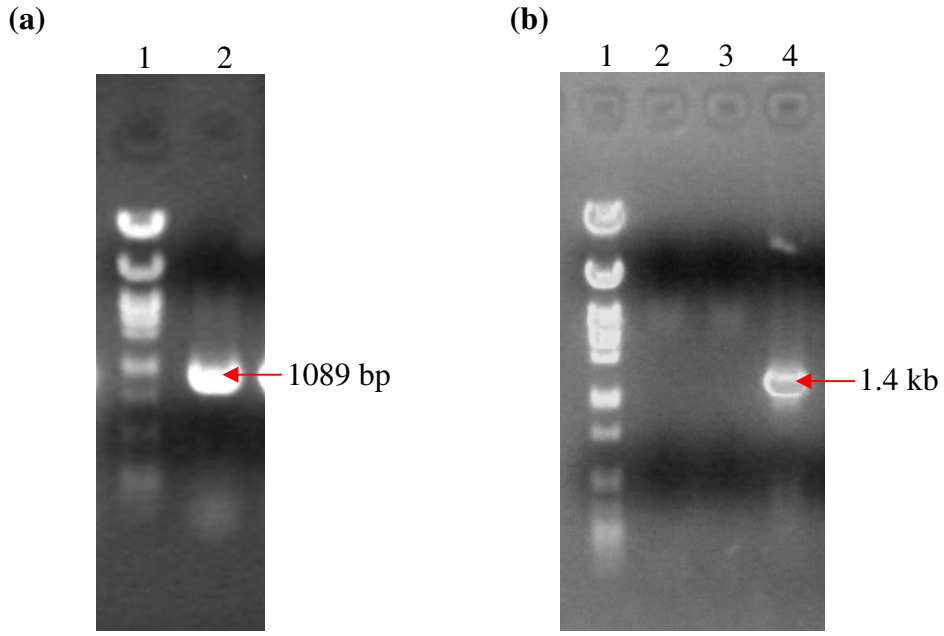


Figure 34: PCR analysis of excision event and second vector T-DNA in putative transgenic. (a) PCR analysis of excision event; Lane 1: Lambda DNA ladder, lane 2: DNA isolated from putative resistant transgenic was subjected to PCR analysis. Primers 35SpFwd, SUC2FRTFwd, and Bar3-FRTRev were designed to amplify either a 600 bp product indicative of FLP-mediated excision or a 1089 bp product indicative of no FLP-mediated excision. A PCR product of 1089 bp was obtained, indicating no excision. (b) PCR analysis of presence of second vector T-DNA. Lane 1: Lambda DNA ladder. DNA isolated from putative transgenic (lane 2), no DNA negative control (lane 3), and pPZP121 plasmid DNA used as positive control (lane 4) were subjected to PCR analysis with primers Gent35Slox and GentLBNar, designed to amplify a 1.4 kb gentamycin cassette. Absence of 1.4 kb PCR product in lane 2 indicates absence of gentamycin cassette and second vector T-DNA.

CHAPTER 5

DISCUSSION

Complementation assays and screening tools are an essential aspect of genetic studies, and aid in the identification and characterization of gene function. In *Arabidopsis thaliana,* the complementing transgene is usually introduced by the floral dip procedure (Clough and Bent, 1998), which requires healthy, robust plants with high fecundity. In cases where the homozygous mutation of a gene results in lethality or highly compromised growth habit, the complementing transgene has to be introduced to healthy heterozygous plants and homozygous knockouts identified among the segregating progeny. However, when a large number of complementing constructs are to be analyzed, screening for the homozygous plants in the segregating population of the progeny becomes time consuming and laborious.

Here I report the construction of two vectors, pART-P450-ecSUC2-BAR and pCAM-Gent-TSpSUC2-EASE-FLP, making up a two-component vector system to facilitate high-throughput complementation assays. The first vector pART-P450-ecSUC2-BAR was designed to complement the homozygous (*Atsuc2/Atsuc2*) T-DNA mutation in the endogenous *AtSUC2* gene in *Arabidopsis thaliana.* The T-DNA of the first vector was constructed in pART27 (Gleave, 1986) as the vector backbone. The T-DNA of the first vector comprises of the following components: (i) *P450* gene, a counter selection marker conferring sensitivity to R7402 pro-herbicide; (ii) cDNA copy of the *AtSUC2* gene under the expression of its native promoter; (iii) *Bar* gene, a plant selection marker conferring resistance to the herbicide glufosinate ammonium; (iv) Cauliflower mosaic virus constitutive 35S promoter; and (v) *nptII* gene, conferring resistance to the antibiotic kanamycin in transgenic plants. The *P450* gene cassette and the

AtSUC2 cassette are situated between *FRT* sites, and will get excised upon FLP-FRT recombination. The second vector pCAM-Gent-TSpSUC2-EASE-FLP was designed to deliver the *FLP* gene and the cDNA copy of the *AtSUC2* gene under the expression of different tissue-specific promoters (*CoYMVp, rolCp, CmGAS1p).* The T-DNA of the second vector comprises of the following components: (i) *aacC1* gene, conferring resistance to the antibiotic gentamycin in transgenic plants; (ii) Cauliflower mosaic virus constitutive 35S promoter, driving expression of *aacC1* gene; (iii) *lox*, Cre recombinase recognition target sequence; (iv) pBR322, bacterial origin of replication; (v) *aadA* gene, conferring kanamycin resistance in transformed bacteria; (vi) *aatB1*, gateway recombination site; (vii) *TSpSUC2*, various tissue-specific promoters expressing *AtSUC2* cDNA; (viii) EASE, Egg Apparatus-Specific Enhancer with CaMV 35S promoter TATA Box, driving expression of the *FLP* gene; and (ix) *FLP* gene, encoding the site-specific FLP recombinase. The *FLP* gene expression is driven by the Egg-Apparatus Specific enhancer ensuring FLP expression in the zygotic tissues, early in development and to avoid potential sectoring.

The first step to establish the two-component complementing system in *Arabidopsis thaliana* was to identify a line with a T-DNA insertion mutation in the *AtSUC2* gene. The SALK_038124 line was identified for plants heterozygous (*AtSUC2/Atsuc2*) and homozygous (*Atsuc2/Atsuc2*) for the T-DNA insertion mutation in the *AtSUC2* gene by genotyping as mentioned in Materials and Methods. The homozygous plants are stunted in growth, accumulate anthocyanin and show no inflorescence development. Our next step was to generate effectively complemented SALK_038124 homozygous (*Atsuc2/Atsuc2*) plants. We therefore transformed SALK_038124 heterozygous (*AtSUC2/Atsuc2*) plants with the first vector pART-P450-ecSUC2-BAR and the segregating progeny was PCR genotyped to identify homozygous plants.

Complementation of homozygous plants by the first vector T-DNA carrying the excisable and complementing cDNA copy of the *AtSUC2* gene was phenotypically visible. The homozygous plants were no longer stunted in growth, had no anthocyanin accumulation, developed robust inflorescences, and demonstrated high fecundity. The extent of complementation of homozygous plants was further analyzed by measuring the total rosette surface area, and comparing the results with Columbia-O ecotype and SALK_038124 heterozygous plants. It was observed that the complemented plants had a total rosette area comparable with that of the SALK_038124 heterozygous plants, while the Columbia-O ecotype wild-type plants had the greatest rosette area. This suggested less-than-perfect complementation of homozygous plants.

The independent transgenic homozygous lines established harboring the T-DNA of the first vector pART-P450-ecSUC2-BAR were also analyzed for *Bar* gene expression. The expression of the *Bar* gene from the Cauliflower mosaic virus constitutive 35S promoter in the first vector is blocked by the *P450-ecSUC2* cassette in between the *FRT* sites. (The DNA cassette in between the *FRT* sites will be excised upon FLP-FRT recombination and thus bring about expression of the *Bar* gene). Treatment of the transgenic seedlings with glufosinate ammonium herbicide demonstrated that expression of the selection marker was blocked effectively in the independent line SG 22-7 when 50 mg/L glufosinate ammonium was used. However, SG 22-7 seedlings treated with 20 mg/L glufosinate ammonium (a concentration sufficient to kill non-resistant plants) survived indicating that some leaky expression of the *Bar* gene is occurring (data not shown).

SG 22-7 plants were transformed with the T-DNA of the second vector pCAM-Gent-TSpSUC2-EASE-FLP series using the floral-dip method. Treatment of the progeny with 100

mg/L glufosinate ammonium and 100 ug/L R7402 did not yield any resistant seedlings, suggesting inefficient transformation or unsuccessful excision of the *P450-ecSUC2* cassette in between the *FRT* sites by the FLP recombinase. Selection of transformed seedlings on 1% MS agar containing gentamycin showed no positive transgenic seedling. Molecular analysis of the only "resistant" seedling obtained using PCR and primers designed specifically for the gentamycin gene suggested a false-positive transgenic seedling.

In conclusion, although no putative transgenic seedling harboring the T-DNA of the second vector series pCAM-Gent-TSpSUC2-EASE-FLP has been obtained, the two vectors of the two-component system were constructed successfully (Refer to Appendix for complete sequence analysis data). Floral-dips have been repeated on independent lines (SG 22-7, SG 22-6, SG 32-22, SG 32-23, SG 32-11, and SG 32-13) to generate more putative transgenic seedlings.

The selection for transgenics was tried with all three plant selection markers in the system, (i) *Bar* gene, conferring resistance to the herbicide glufosinate ammonium, (ii) *P450* gene, conferring sensitivity to the pro-herbicide R7402, and (iii) *aacC1* gene conferring resistance to the antibiotic gentamycin. False-positive results were obtained with glufosinate ammonium treatment at 50 mg/L probably due to leaky expression of the *Bar* gene, but increasing the glufosinate ammonium concentration to 100 mg/L was reliable. The glufosinate ammonium resistant control plants showed healthy growth and development even after treatment with 100 mg/L herbicide, suggesting that the high concentration of herbicide did not have any inhibitory effects.

The R7402 was used as a counter selection marker in the system, to eliminate possibility of any sectoring. For instance, sectors of plant tissue which do not express the *Bar* gene may survive glufosinate ammonium treatment due to effect of *Bar* gene expression in surrounding

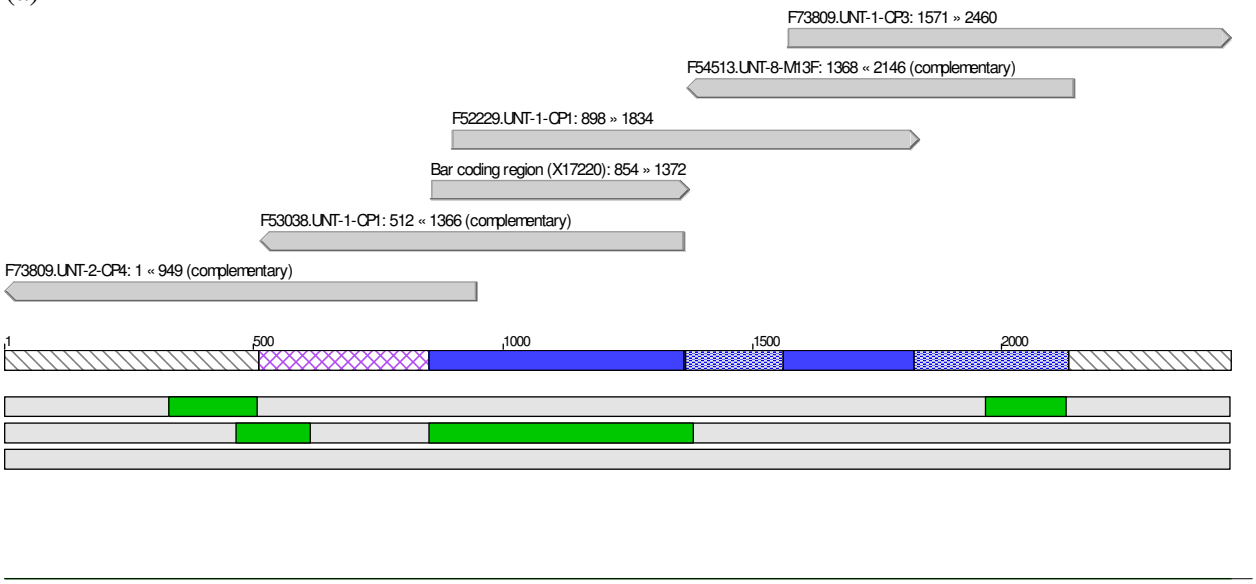
tissue sectors, but the same does not apply for R7402. Instead, sectors maintaing the *P450* gene will create a toxin and even destroy sectors not expressing the gene. The treatment of R7402 resistant controls with 100 µg/L R7402 pro-herbicide resulted in bleaching of leaf periphery, even though they were not expressing the *P450* gene. The seedlings expressing the *P450* gene were rapidly, and completely bleached and killed. The R7402 selection although effective in killing sensitive seedlings, was damaging on resistant plant tissue. However, treatment of plants with a lower concentration of R7402 pro-herbicide was not tested, and will likely resolve this issue. The selection for putative transgenics using the gentamycin marker (100 mg/L) was not very effective. The differentiation between the sensitive and putatively resistant seedlings was not clear, and gave false-positive results. Increasing the gentamycin concentration may effect better selection and needs to be tested.

The two-component system although established with the *AtSUC2* gene as the essential gene, is flexible to be replaced with any gene of interest. The system can also be modified to express the essential gene in different tissues of interest. The benefits of excision could be expanded for creating sectors in the plant tissue. For example, a heat shock promoter can be introduced in place of the EASE, which will express *FLP* only in heat shocked sectors and mediate excision in sectors. The broader application of the two-component vector system is in the discovery of phloem-specific regulatory sequences. The second vector in the system is designed to allow for incorporation of genomic libraries derived from different species; in place of the tissue-specific promoter. The library fragments with appreciable phloem-specific expression can be identified by a simple growth assay.

APPENDIX A

pART-P450-ecSUC2-BAR

(a)



(b)

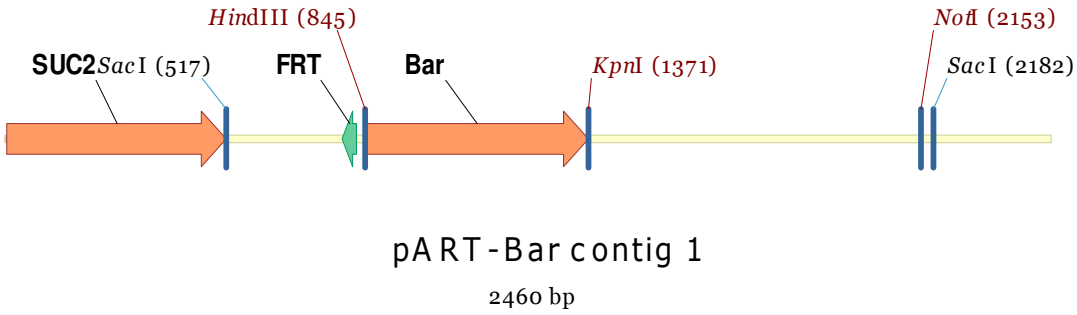
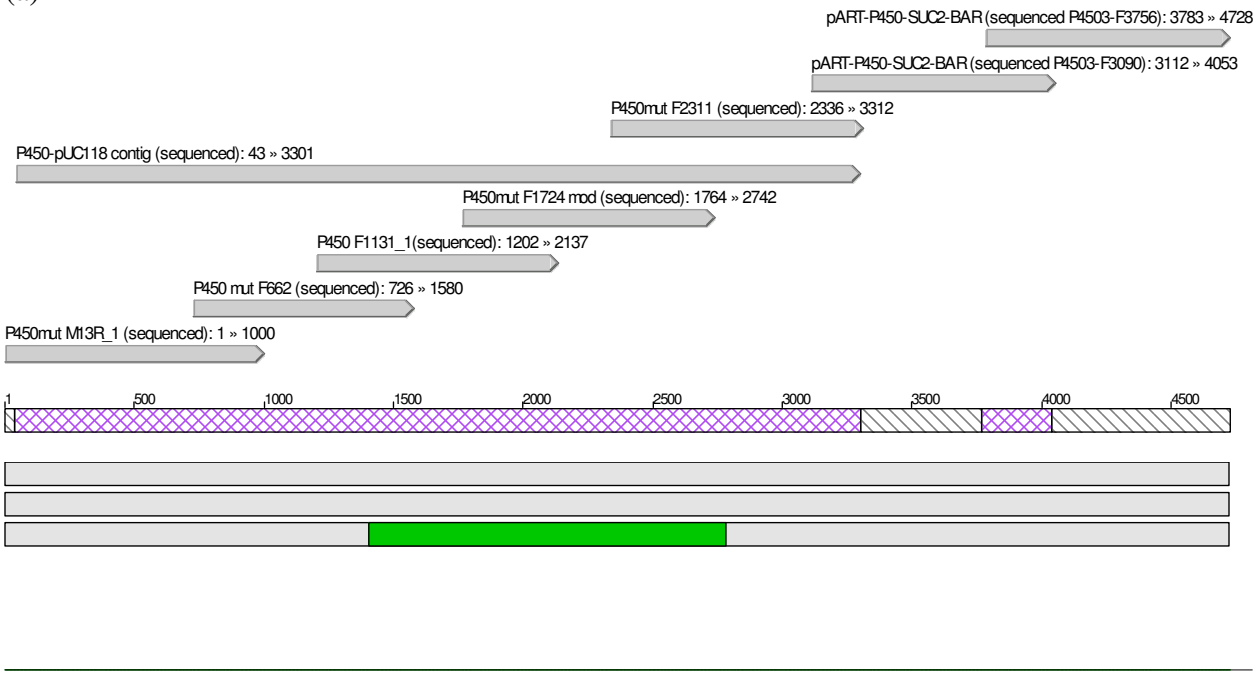
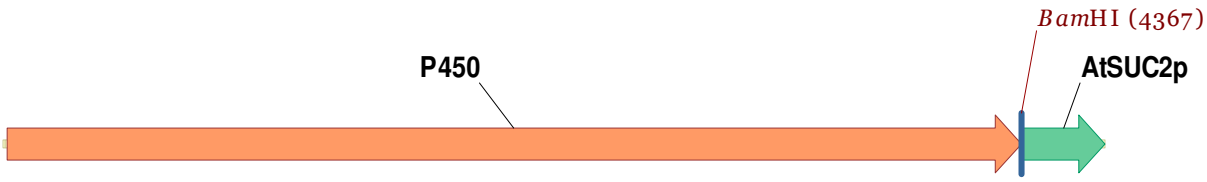


Figure 35: Sequence analysis data and sequence map of the *Bar* gene. (a) Contig assembly of the sequenced fragments. (b) Annotated sequence map of the pART-Bar contig assembly.

(a)



(b)



pART-P450 contig 2

4728 bp

Figure 36: Sequence analysis data and sequence map of the *P450* cassette. (a) Contig assembly of the sequenced fragments. (b) Annotated sequence map of the pART-P450 contig assembly.

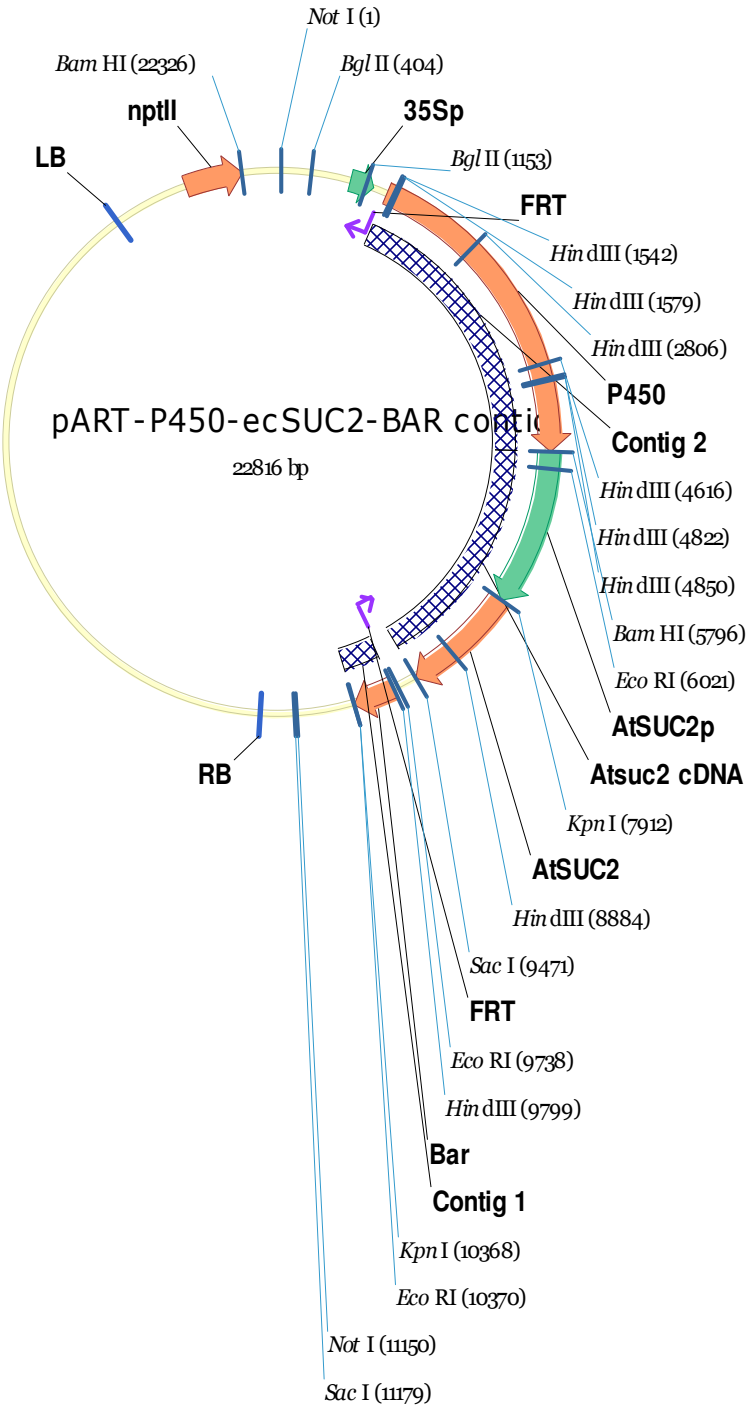


Figure 37: Vector map of pART-P450-ecSUC2-BAR constructed with the sequence analysed components and showing restriction sites used for cloning.

```
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2007
DEFINITION  Construction option #4 of pART-P450-ecSUC2-BAR.
SOURCE
  ORGANISM
COMMENT     This file is created by Vector NTI
            http://www.invitrogen.com/
COMMENT     VNTDATE|460240689|
COMMENT     VNTDBDATE|460241628|
COMMENT     LSOWNER|
COMMENT     VNTNAME|pART-P450-ecSUC2-BAR contig|
COMMENT     VNTAUTHORNAME|Demo User|
FEATURES             Location/Qualifiers
     CDS             9799..10367
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                     /label=Bar
     misc_recomb     complement(1384..1417)
                     /vntifkey="86"
                     /label=FRT
                     /note="FLP recognition target site from pFLP-SWITCH
backbone"
     misc_recomb     complement(9745..9778)
                     /vntifkey="86"
                     /label=FRT
                     /note="FLP recognition target site from pFLP-SWITCH
backbone"
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                     /label=AtSUC2p
     CDS             7912..9470
                     /vntifkey="4"
                     /label=AtSUC2
     promoter        937..1258
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                     /label=35Sp
     CDS             1450..5795
                     /vntifkey="4"
                     /label=P450
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                     /vntifkey="21"
                     /label=LB
     misc_feature    11634..11659
                     /vntifkey="21"
                     /label=RB
     CDS             21552..22330
                     /vntifkey="4"
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                     /vntifkey="14"
                     /label=Atsuc2\cDNA
                     /note="AtSUC2 cDNA inserted as a BamHI/SacI fragment
from pGPTV-SUC2pSUC2. Correct sequence established."
     insertion_seq   9799..10367
                     /vntifkey="14"
                     /label=Contig\1
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                     /note="PCR amplified Bar gene from pGPTV-BAR"
     insertion_seq   1450..5795
                     /vntifkey="14"
                     /label=Contig\2
                     /note="PCR amplified P450 cassette cloned as a
BglII/BamHI insert."
BASE COUNT     5763 a      5757 c      5481 g      5790 t        25 others
ORIGIN
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       61 ctctcagagc agaatcgggt attcaacacc ctcatatcaa ctactacgtt gtgtataacg
      121 gtccacatgc cggtatatac gatgactggg gttgtacaaa ggcggcaaca aacggcgttc
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13741 cctgttcagg aaccggatca aagagttcct ccgccgctgg acctaccaag gcaacgctat
13801 gttctcttgc ttttgtcagc aagatagcca gatcaatgtc gatcgtggct ggctcgaaga
13861 tacctgcaag aatgtcattg cgctgccatt ctccaaattg cagttcgcgc ttagctggat
13921 aacgccacgg aatgatgtcg tcgtgcacaa caatggtgac ttctacagcg cggagaatct
13981 cgctctctcc aggggaagcc gaagtttcca aaaggtcgtt gatcaaagct cgccgcgttg
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14101 cgccatccac tgcggagccg tacaaatgta cggccagcaa cgtcggttcg agatggcgct
14161 cgatgacgcc aactacctct gatagttgag tcgatacttc ggcgatcacc gcttccctca
14221 tgatgtttaa ctcctgaatt aagccgcgcc gcgaagcggt gtcggcttga atgaattgtt
14281 aggcgtcatc ctgtgctccc gagaaccagt accagtacat cgctgtttcg ttcgagactt
14341 gaggtctagt tttatacgtg aacaggtcaa tgccgccgag agtaaagcca cattttgcgt
14401 acaaattgca ggcaggtaca ttgttcgttt gtgtctctaa tcgtatgcca aggagctgtc
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16201 aaacaactgt aggttcgagt cgcgagatcc cccggaacca aaggaagtag gttaaacccg
16261 ctccgatcag gccgagccac gccaggccga gaacattggt tcctgtaggc atcgggattg
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16441 ccatggaaac cgcccccgcc aggcccgctg cgacgccgac aggatctagc gctgcgtttg
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16621 cgcctaccgt cgccgcgacc ccgcccggca ggcggtagac cgaaataaac aacaagctcc
16681 agaatagcga aatattaagt gcgccgagga tgaagatgcg catccaccag attcccgttg
16741 gaatctgtcg gacgatcatc acgagcaata aacccgccgg caacgcccgc agcagcatac
16801 cggcgacccc tcggcctcgc tgttcgggct ccacgaaaac gccggacaga tgcgccttgt
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16921 tgtaggcgcc gaatgccacg gcatctcgca accgttcagc gaacgcctcc atgggctttt
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19381 cagccagcgc tggcgcgatt tagccccgac atagccccac tgttcgtcca tttccgcgca
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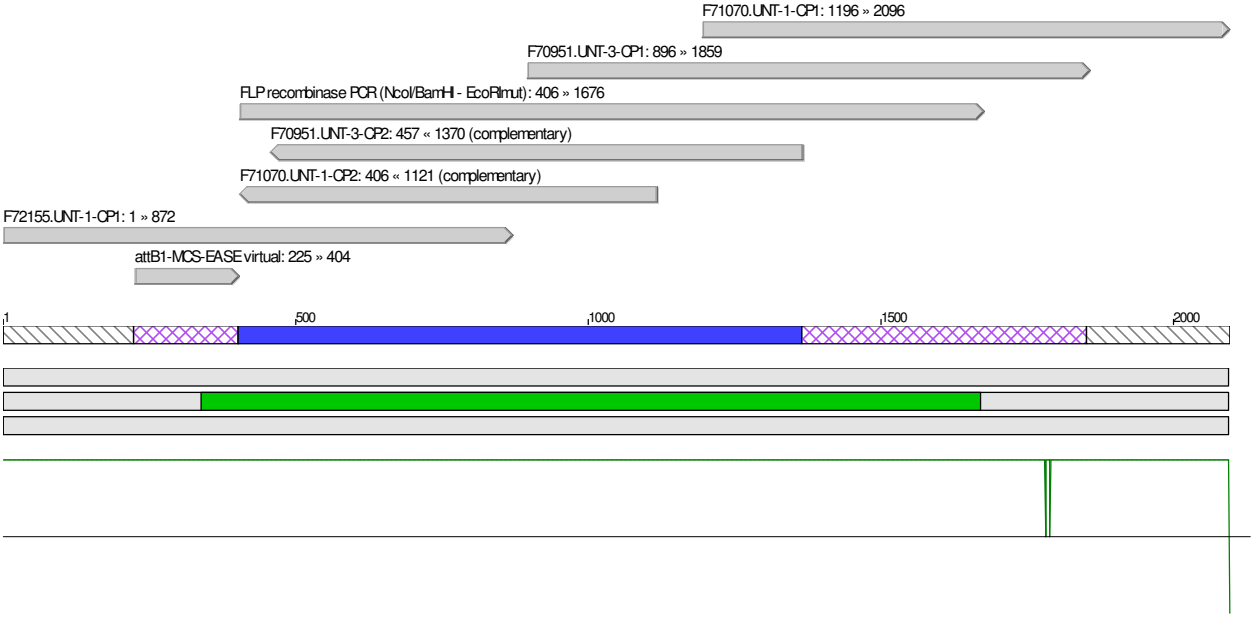
22681 tgggtaacgc cagggttttc ccagtcacga cgttgtaaaa cgacggccag tgaattgtaa
22741 tacgactcac tatagggcga attgggcccg acgtcgcatg ctcccggccg ccatggccgc
22801 gggatatcac tagtgc

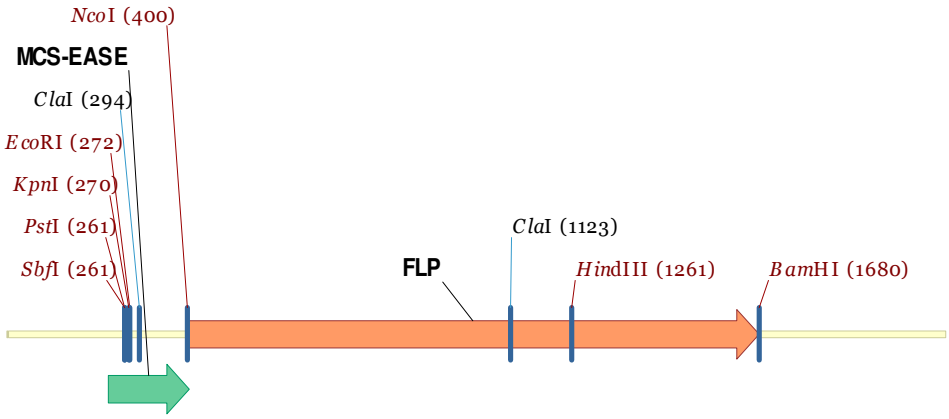
APPENDIX B

pCAM-Gent-TSpSUC2-EASE-FLP

(a)



(b)

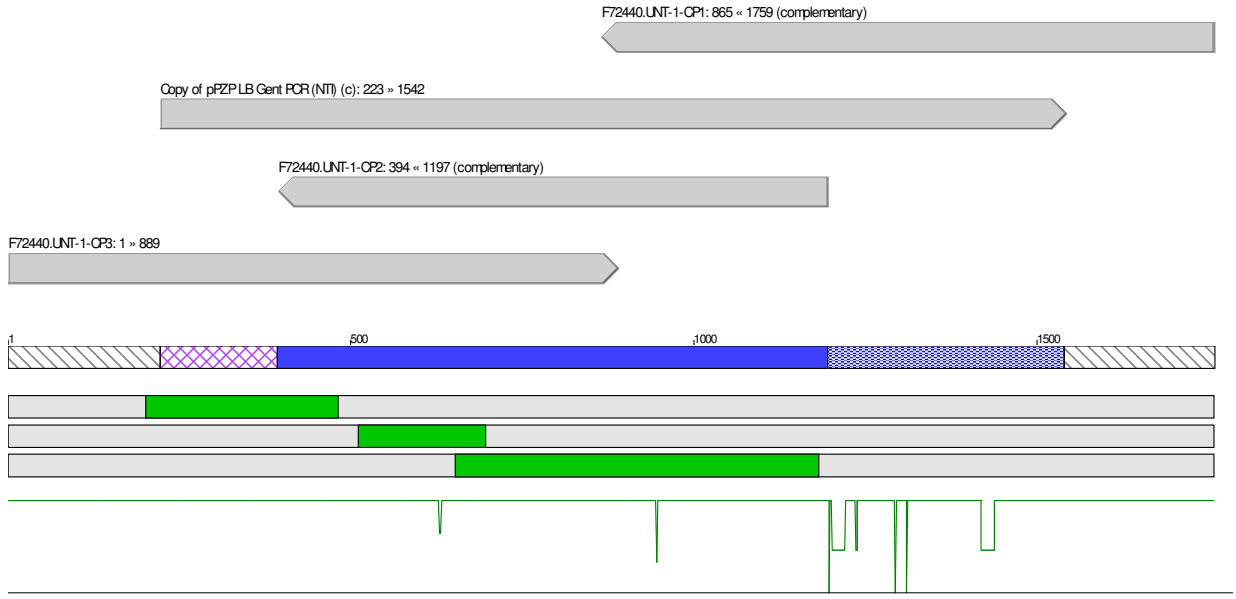


EASE-FLP (contig 1)

2096 bp

Figure 38: Sequence analysis data and sequence map of the *EASE-FLP* cassette. (a) Contig assembly of the sequenced fragments. (b) Annotated sequence map of the *EASE-FLP* contig assembly.

(a)



(b)

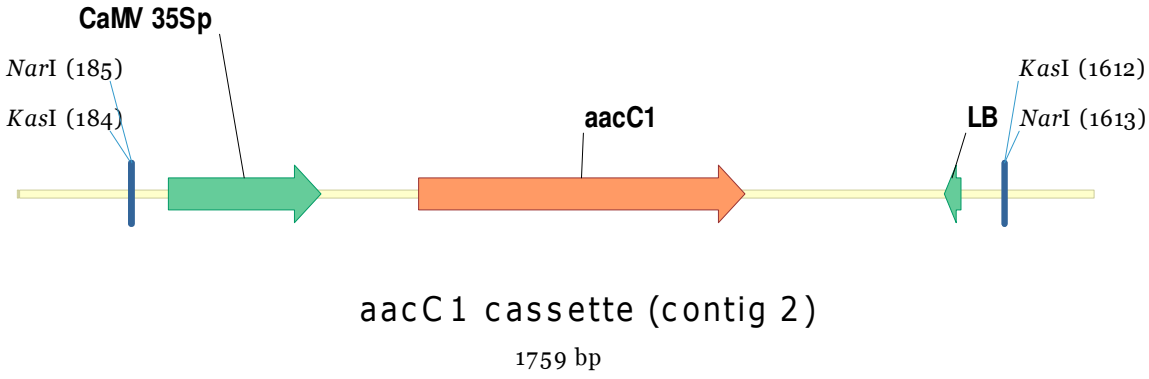


Figure 39: Sequence analysis data and sequence map of the *aacC1* cassette. (a) Contig assembly of the sequenced fragments. (b) Annotated sequence map of the *aacC1* contig assembly. Position of mismatches in the contig sequence relative to the virtual sequence: (1) Position 629-631: Outside ORF of the *aacC1* gene; not important, (2) Position 946: G→A substitution resulting in Arg→Lys change (not significant), and (3) Position 1198-1220: Outside ORF of *aacC1* gene; not important.

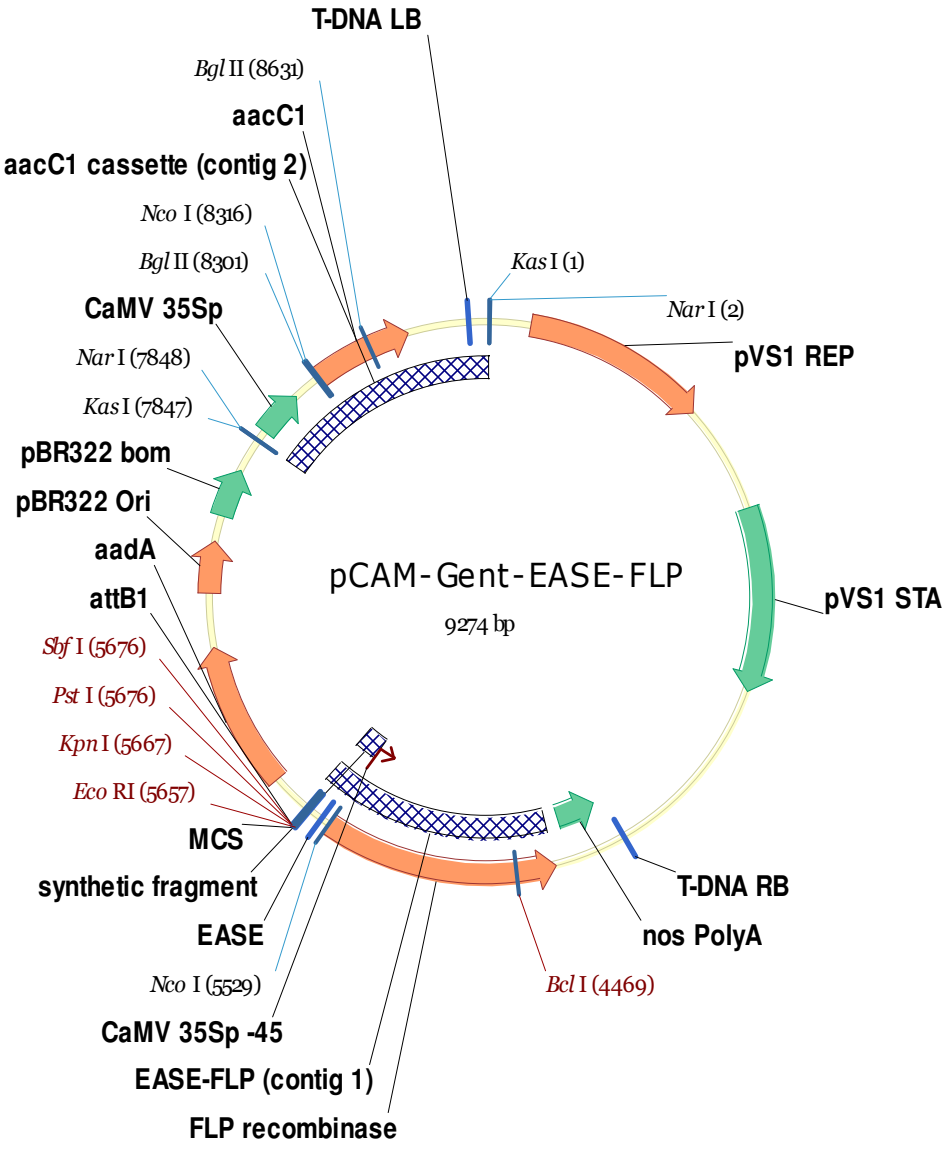


Figure 40: Vector map of pCAM-Gent-EASE-FLP constructed with the sequence analysed components and showing restriction sites used for cloning.

```
LOCUS       pCAM-Gent-EASE-F        9274 bp    DNA     circular     14-OCT-
2007
DEFINITION  Construction option #4 of pCAM-Gent-EASE-FLP 1.
SOURCE
  ORGANISM
COMMENT     This file is created by Vector NTI
            http://www.invitrogen.com/
COMMENT     VNTDATE|460214798|
COMMENT     VNTDBDATE|460215692|
COMMENT     LSOWNER|
COMMENT     VNTNAME|pCAM-Gent-EASE-FLP|
COMMENT     VNTAUTHORNAME|Demo User|
FEATURES             Location/Qualifiers
     misc_feature    complement(9178..9203)
                     /vntifkey="21"
                     /label=T-DNA\LB
                     /note="Left border T-DNA repeat"
     promoter        7907..8156
                     /vntifkey="29"
                     /label=CaMV\35Sp
     CDS             8317..8850
                     /vntifkey="4"
                     /label=aacC1
     insertion_seq   complement(5534..5702)
                     /vntifkey="14"
                     /label=synthetic\fragment
                     /note="PCR generated synthetic fragment . Correct
sequence established by sequence analysis."
     CDS             5895..6689
                     /codon_start=1
                     /product="aminoglycoside phosphotransferase"
                     /protein_id="AAF65320.1"
                     /db_xref="GI:7638044"
                     /vntifkey="4"
                     /label=aadA
                     /note="aadA (kanamycin resistance) gene amplified from
pIG121Hm"
     rep_origin      6980..7260
                     /vntifkey="33"
                     /label=pBR322\Ori
                     /note="pBR322 origin of replication"
     misc_feature    7400..7660
                     /vntifkey="21"
                     /label=pBR322\bom
                     /note="bom site from pBR322"
     rep_origin      224..1224
                     /vntifkey="33"
                     /label=pVS1\REP
                     /note="pVS1-REP; replication origin from pVS1"
     misc_feature    1817..2817
                     /vntifkey="21"
                     /label=pVS1\STA
                     /note="STA region from pVS1 plasmid"
     misc_feature    complement(3858..3883)
                     /vntifkey="21"
                     /label=T-DNA\RB
```

```
                     /note="right border T-DNA repeat"
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                     /label=nos\PolyA
                     /note="nos (nopaline synthase) 3'UTR (polyA signal)"
     promoter        complement(5534..5578)
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                     /label=CaMV\35Sp\-45
     misc_feature    complement(5656..5677)
                     /vntifkey="21"
                     /label=MCS
     enhancer        complement(5579..5655)
                     /vntifkey="9"
                     /label=EASE
                     /note="PCR generated synthetic fragment. Correct
sequence established by sequence analysis. "
     misc_feature    complement(5678..5702)
                     /vntifkey="21"
                     /label=attB1
     CDS             complement(4254..5525)
                     /vntifkey="4"
                     /label=FLP\recombinase
                     /note="PCR amplified FLP gene from HSP-FLP plasmid.
Correct sequence established by sequence analysis."
     insertion_seq   7847..9274
                     /vntifkey="14"
                     /label=aacC1\cassette\(contig\2)
                     /note="PCR cloned aacC1 cassette. Correct sequence
established by sequence analysis."
     insertion_seq   4254..5702
                     /vntifkey="14"
                     /label=EASE-FLP\(contig\1)
BASE COUNT     2126 a      2405 c      2357 g      2386 t
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       61 taggccagcc atttttgagc ggccagcggc cgcgataggc cgacgcgaag cggcggggcg
      121 tagggagcgc agcgaccgaa gggtaggcgc tttttgcagc tcttcggctg tgcgctggcc
      181 agacagttat gcacaggcca ggcgggtttt aagagtttta ataagtttta aagagtttta
      241 ggcggaaaaa tcgccttttt tctcttttat atcagtcact tacatgtgtg accggttccc
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      361 cccaatgtac gtgctatcca caggaaagag acctttttcga cctttttccc ctgctagggc
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      481 gttgcggtag cgcatgacta ggatcgggcc agcctgcccc gcctcctcct tcaaatcgta
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      601 tccggcgctg ccactgcgtt cgtagatcgt cttgaacaac catctggctt ctgccttgcc
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      721 atcggggtga accgtcagca cgtccgggtt cttgccttct gtgatctcgc ggtacatcca
      781 atcagctagc tcgatctcga tgtactccgg ccgcccggtt tcgctcttta cgatcttgta
      841 gcggctaatc aaggcttcac cctcggatac cgtcaccagg cggccgttct tggccttctt
      901 cgtacgctgc atggcaacgt gcgtggtgtt taaccgaatg caggtttcta ccaggtcgtc
      961 tttctgcttt ccgccatcgg ctcgccggca gaacttgagt acgtccgcaa cgtgtggacg
     1021 gaacacgcgg ccgggcttgt ctcccttccc ttcccggtat cggttcatgg attcggttag
     1081 atgggaaacc gccatcagta ccaggtcgta atcccacaca ctggccatgc cggccggccc
     1141 tgcggaaacc tctacgtgcc cgtctggaag ctcgtagcgg atcacctcgc cagctcgtcg
     1201 gtcacgcttc gacagacgga aaacggccac gtccatgatg ctgcgactat cgcgggtgcc
     1261 cacgtcatag agcatcggaa cgaaaaaatc tggttgctcg tcgcccttgg gcggcttcct
     1321 aatcgacggc gcaccggctg ccggcggttg ccgggattct ttgcggattc gatcagcggc
```

1381 cgcttgccac gattcaccgg ggcgtgcttc tgcctcgatg cgttgccgct gggcggcctg
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1501 ggatggtttg cgaccgtcac gccgattcct cgggcttggg ggttccagtg ccattgcagg
1561 gccggcagac aacccagccg cttacgcctg gccaaccgcc cgttcctcca cacatggggc
1621 attccacggc gtcggtgcct ggttgttctt gattttccat gccgcctcct ttagccgcta
1681 aaattcatct actcatttat tcatttgctc atttactctg gtagctgcgc gatgtattca
1741 gatagcagct cggtaatggt cttgccttgg cgtaccgcgt acatcttcag cttggtgtga
1801 tcctccgccg gcaactgaaa gttgacccgc ttcatggctg gcgtgtctgc caggctggcc
1861 aacgttgcag ccttgctgct gcgtgcgctc ggacggccgg cacttagcgt gtttgtgctt
1921 ttgctcattt tctctttacc tcattaactc aaatgagttt tgatttaatt tcagcggcca
1981 gcgcctggac ctcgcgggca gcgtcgccct cgggttctga ttcaagaacg gttgtgccgg
2041 cggcggcagt gcctgggtag ctcacgcgct gcgtgatacg ggactcaaga atgggcagct
2101 cgtacccggc cagcgcctcg gcaacctcac cgccgatgcg cgtgcctttg atcgcccgcg
2161 acacgacaaa ggccgcttgt agccttccat ccgtgacctc aatgcgctgc ttaaccagct
2221 ccaccaggtc ggcggtggcc catatgtcgt aagggcttgg ctgcaccgga atcagcacga
2281 agtcggctgc cttgatcgcg gacacagcca agtccgccgc ctggggcgct ccgtcgatca
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3121 cgggggccaa acttcacgcc tcgggccttg gcggcctcgc gcccgctccg ggtgcggtcg
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3781 ccctgtggtt ggcatgcaca tacaaatgga cgaacggata aacctttca cgccctttta
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9061 tccagtacta aaatccagat cacctaaagt ccctatagat cccccgaatt aattcggcgt
9121 taattcagta cattaaaaac gtccgcaatg tgttattaag ttgtctaagc gtcaatttgt
9181 ttacaccaca atatatcctg ccaccagcca gccaacagct ccccgaccgg cagctcggca
9241 caaaatcacc actcgataca ggcagcccca tcag

APPENDIX C

PLANT LINES AND VECTOR T-DNAs

Plant Line	Genotype (*AtSUC2* T-DNA)	Vector/T-DNA
SG 22-6	Homozygous	pART-P450-ecSUC2-BAR
SG 22-7	Homozygous	pART-P450-ecSUC2-BAR
SG 32-22	Homozygous	pART-P450-ecSUC2-BAR
SG 32-23	Homozygous	pART-P450-ecSUC2-BAR
SG 22-7-CoYMV-01	Homozygous	SG 22-7 parent floral-dipped with pCAM-Gent-CoYMVpSUC2-EASE-FLP
SG 22-7-RolC-03	Homozygous	SG 22-7 parent floral-dipped with pCAM-Gent-RolCpSUC2-EASE-FLP
SG 22-7-GAS-04	Homozygous	SG 22-7 parent floral-dipped with pCAM-Gent-GASpSUC2-EASE-FLP
SG 22-7-Empty-05	Homozygous	SG 22-7 parent floral-dipped with pCAM-Gent-EASE-FLP
SG 22-7-SUC2-06	Homozygous	SG 22-7 parent floral-dipped with pCAM-Gent-SUC2pSUC2-EASE-FLP
SGSALK-SUC2-07	Heterozygous segregating	SALK_038124 heterozygotes floral-dipped with pCAM-Gent-SUC2pSUC2-EASE-FLP

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