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Tissue and organelle targeted transgene expression in plants

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Declaration

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Acknowledgements

Abstract

This research develops systems for tissue and organelle targeted transgene expression in commercial strawberry. Two approaches were taken to achieve this. I) the development of a root-specific expression system for nuclear transgenes and II) the development of plastid transformation methodologies for strawberry.

FavRB7, a root-specific, RB7 tonoplast intrinsic protein homologue was isolated from strawberry root tissue using reverse transcriptase-polymerase chain reaction (RT-PCR). A 2.8 kb region of promoter sequence associated with the *FavRB7* gene was isolated and cloned upstream of the β-glucuronidase (*gusA*) reporter gene. Transgenic strawberry and tobacco were produced harbouring the *gusA* gene under control of the *FavRB7* gene promoter. Promoter activity in these lines was characterised using histochemical, fluorometric and RNA molecular assays. *FavRB7* promoter activity was shown to be strong and near root-specific in strawberry. However in contrast, in the heterologous species tobacco, *FavRB7* promoter activity was shown to be constitutive.

A series of novel plastid transformation vectors, harbouring selectable and scorable marker genes under control of strawberry plastome regulatory elements, were created to enable plastid transformation in strawberry and tobacco. Plastid transformation methodologies were developed for strawberry utilising the aminoglycoside 3'-adenyltransferase (*aadA*) gene and spectinomycin selection. Additionally, plastid transformation using a non-antibiotic selection system was investigated utilising the xylose isomerase (*xylA*) gene and D-xylose selection. Heteroplasmic plastid transformed strawberry lines were recovered using both selection systems and expression of the soluble modified green fluorescent protein (*smGFP*) scorable marker gene visualised specifically in strawberry chloroplasts of three individual lines. Additionally, heteroplasmic plastid transformed tobacco lines harbouring transgenes under control of strawberry plastome regulatory elements were generated using spectinomycin selection.

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Chapter 1

General introduction

1.1 The Strawberry

1.1.1 Distribution and physiology

There are numerous species of strawberry distributed throughout the temperate regions of the world. The cultivated strawberry, *Fragaria x ananassa*, is grown world wide. Annual fruit production exceeded 3 million metric tonnes in 2002 from 226, 554 hectares in production worldwide. In the United Kingdom alone almost 34, 000 metric tonnes of fruit were produced from 3, 557 hectares in production during the same period (FAOSTAT database at www.apps.fao.org).

Strawberry is a herbaceous perennial which has a central shortened stem or crown from which trifoliate leaves, roots, stolons and inflorescences emerge (Figure 1.1). Stolons generally consist of two nodes, the second node produces a 'daughter plant' whilst the first node either remains dormant or develops into another stolon (Hancock, 1999) . Vegetative clonal propagation through stolons is often used to preserve and multiply specific genotypes. However, meristem culture is often employed to eliminate viruses prior to mass propagation (Adams, 1972; Jain and Pehu, 1992).

1.1.2 Breeding and genetics

The modern cultivated strawberry *F. x ananassa* is a hybrid octaploid resulting from a cross of two New World octaploid species *F. chiloensis* and *F. virginiana* (Darrow, 1966). Formal strawberry breeding was initiated in England in 1817 by Thomas A. Knight who used both *F. chiloensis* and *F. virginiana* in his crosses to
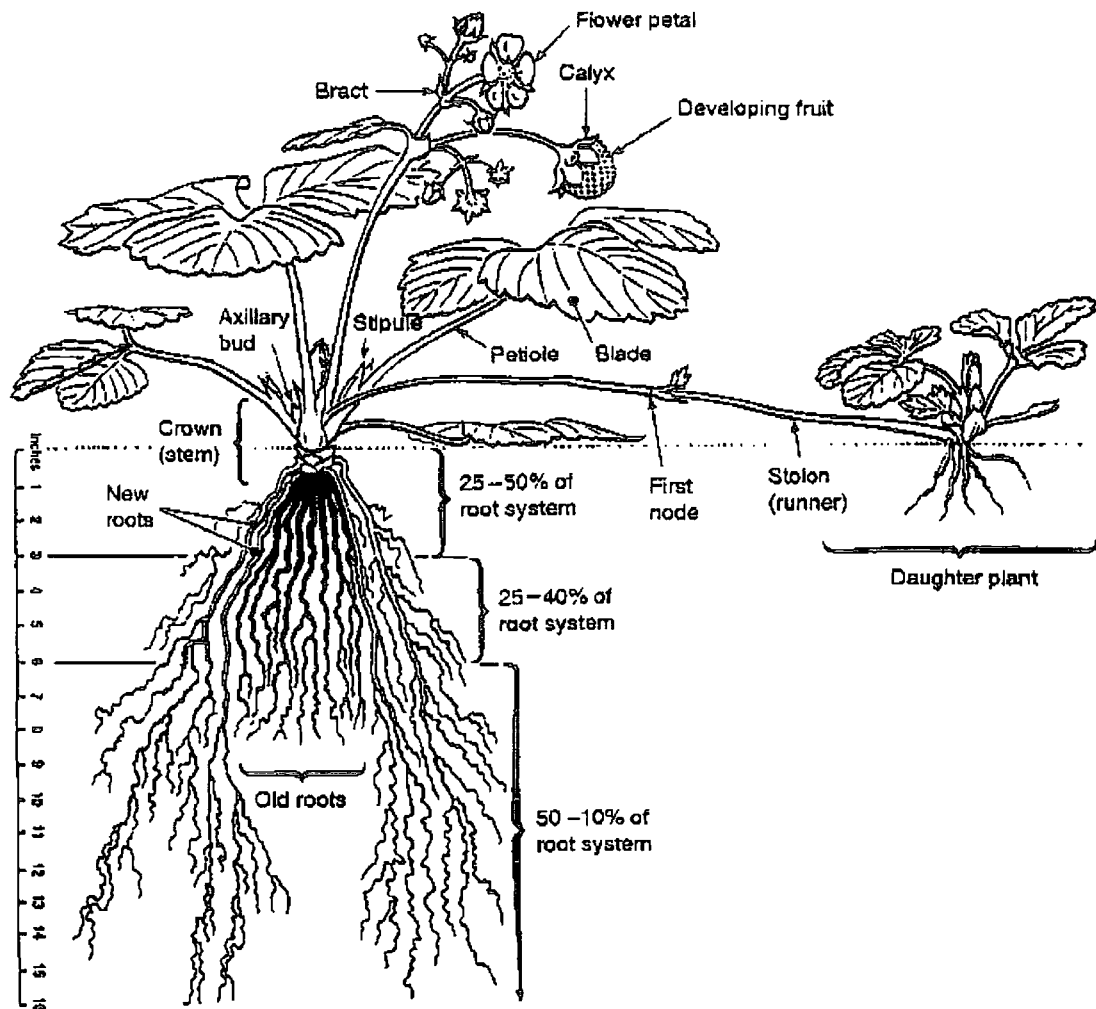
Figure 1.1 Structural characteristics of a strawberry plant

Diagram taken from Hancock 1999, after Strand, 1994. The various features that characterise strawberry plant structure are indicated.

improve the vigour, hardiness and fruit quality of *F. x ananassa* cultivars, which form part of the genetic background of many of today's modern cultivars (Darrow, 1966).

Breeding programmes for elite strawberry varieties across the world have concentrated on improvements to plant vigour, fruiting habit, fruit quality, winter hardiness, frost tolerance and in developing pest and disease resistance (Galletta *et al.*, 1997; Simpson *et al.*, 2002). The majority of genes in modern strawberry are derived from seven nuclear and ten cytoplasmic sources (Hancock *et al.*, 2001), which has resulted in a narrow germplasm base. Sources of resistance to pests and diseases available to breeding programmes are often limited, either due to the narrow germplasm base of *F. x ananassa* or due to the requirement for interploid crosses, which are often problematic. Introduction of resistance traits from exotic gene sources, whilst maintaining the complex set of characteristics required of elite commercial varieties, can therefore involve lengthy back crosses using conventional breeding methods.

1.1.3 Pests and diseases of strawberry

Strawberry is susceptible to a wide range of pests and diseases, which considerably reduce the quality and quantity of the crop. The major pest for the UK strawberry industry is vine weevil, *Otiorhynchus sulcatus* F. (Watt *et al.*, 1999). The larval stage of vine weevil (Figure 1.2B2) feed on the crowns and roots of strawberry, which restricts plant growth and often leads to plant death. Strawberry is commonly cultivated under protective polythene coverings to extend the fruiting season and it has been suggested that these systems have encouraged the increase in vine weevil populations in recent years (Moorhouse *et al.*, 1992). Genetic resistance to vine weevil has yet to be identified in commercial strawberry genotypes (Watt *et al.*, 1999), although there is a degree of resistance found in the beach strawberry *F. chiloensis* (Doss *et al.*, 1987).
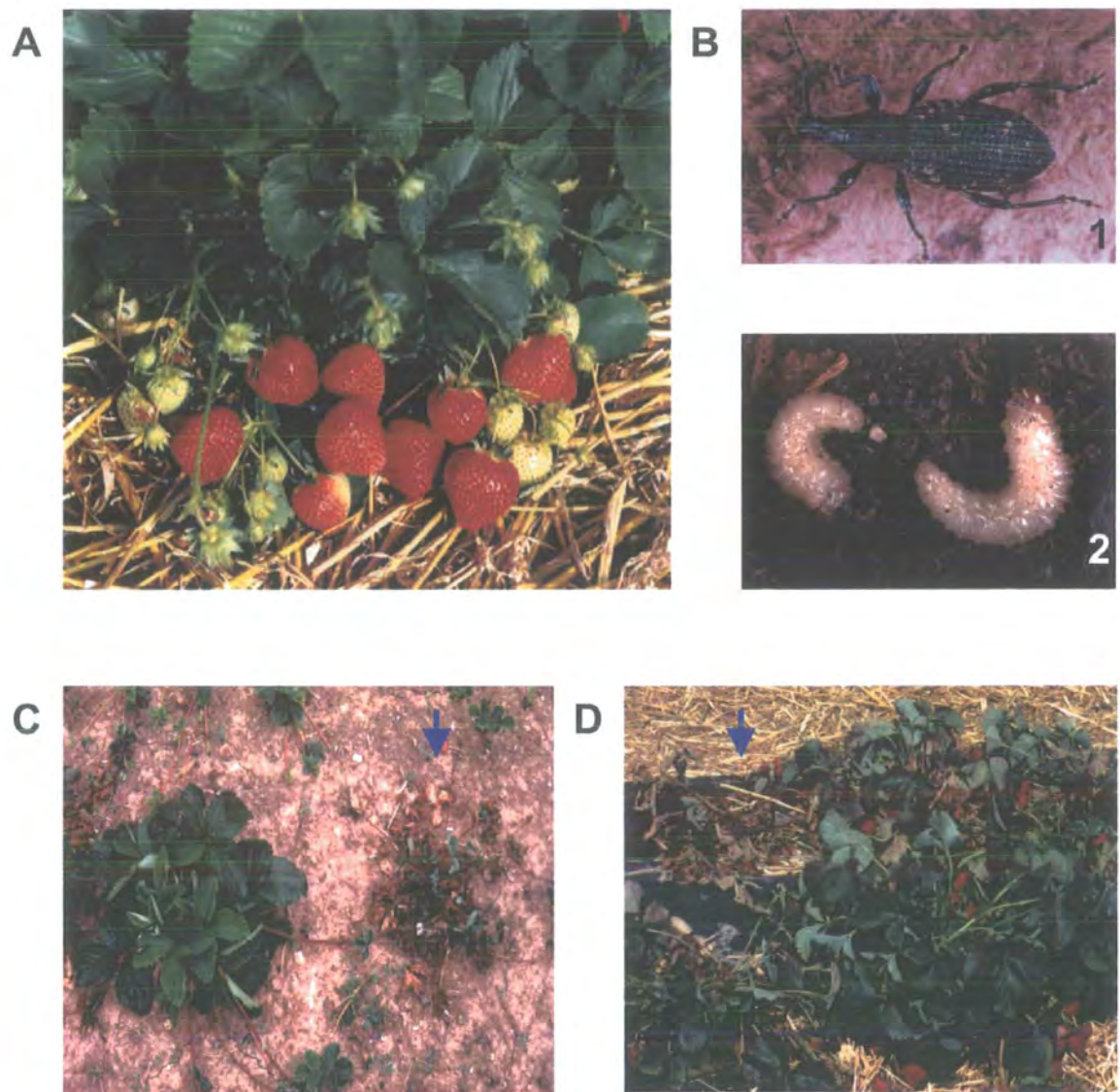
Figure 1.1 Examples of strawberry pests and diseases

Healthy field grown strawberry (A). Adult vine weevil (B1) and vine weevil larvae (B2). Infestation of field grown plants by *Verticillium dahliae* (C) and *Phytophthora fragariae* (D) are indicated by arrows.

Strawberry roots are subject to infection by several soil-borne fungi such as *Verticillium dahliae* (Harris and Yang, 1996) and *Phytophthora fragariae* (Pettitt and Pegg, 1994) (Figure 1.2C and D). In most cases infestation causes the plant to die. Race-specific resistance to certain fungal infections has been identified in a limited number of commercial cultivars (VandeWeg, 1997), however this often does not completely prevent infection.

Several cultivation methods are commonly employed in an attempt to alleviate the problems experienced by growers due to the lack of genetic resistance to pests and diseases in commercial strawberry. Soil sterilisation using methyl bromide is often undertaken prior to planting to reduce nematode, insect, fungal and bacterial populations. However methyl bromide has been identified as a significant ozone-depleting substance and is being withdrawn from commercial use (Thomas, 1996). An efficacious alternative to methyl bromide has not yet been discovered (Galletta, 1997; Martin and Bull, 2002). Many strawberry fungal pathogens can persist in the soil for many years and maintaining well drained soils and crop rotation are critical to discouraging fungal establishment (Hancock, 1999). Broad spectrum organo-phosphorus insecticides are often employed to control vine weevil, however these are only partially effective against the adult weevil and have little impact on the larval stages (Graham *et al.*, 1997). Repeated applications of broad spectrum insecticides often waste chemicals and can promote evolution of resistance in insect pests and reduce populations of natural predators (Cross *et al.*, 2001).

There are several candidate genes that could potentially confer resistance to some of the insect pests of strawberry. Some examples of these are the proteinase inhibitors, *Bacillus thuringiensis* (Bt) toxins and plant lectins. Proteinase inhibitors form complexes with proteinases inhibiting enzyme activity and thus disrupting digestion in insects that consume these compounds (Lawrence and Koundal, 2002). The Bt toxin gene product is a δ-protoxin that forms an active toxin during digestion by insects that consume the δ-protoxin (Gill *et al.*, 1992). The plant lectins act by

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binding to carbohydrates and also have a disruptive effect on insect metabolism (Gatehouse *et al.*, 1993).

Candidate genes which could potentially confer resistance to some of the fungal pathogens of strawberry are the endochitinases, which hydrolyse the chitin component of fungal cell walls (Jack *et al.*, 1995). There are also several antimicrobial peptides which have exhibited antimicrobial activity against bacteria and fungi when expressed in transgenic plants (Park *et al.*, 2000; DeGray *et al.*, 2001) and could be used to confer resistance to strawberry fungal pathogens. The beta-1-3-glucanase protein has been shown to confer a degree of resistance to several fungal pathogens in tobacco and wheat (Lusso and Kuc, 1996; Bieri *et al.*, 2003). A further class of proteins likely to confer resistance to strawberry fungal pathogens are the ribosome-inactivating proteins (RIPs). RIPs isolated from a variety of plant species have displayed varying antifungal and antiviral activities (Bieri *et al.*, 2000; Nielsen and Boston, 2001; Park *et al.*, 2002; Parkash *et al.*, 2002). These enzymes often serve as plant defence proteins and specifically inhibit protein synthesis through the depurination of the conserved alpha-sarcin loop of large ribosomal RNAs (Park *et al.*, 2002).

The lack of genetic resistance to major pests and diseases in strawberry and the problems associated with their chemical and cultural control could potentially be overcome by using genetic modification to directly transfer resistance traits to commercial strawberry. Genetic modification offers several benefits over traditional breeding with respect to the introduction of genetic resistance genes. The introduction of a specific gene or genes can be achieved without risk of phenotypic alterations occurring as a result genetic recombination during sexual crosses. The introduction of resistance genes directly to elite cultivars also eliminates the need for back crossing and greatly reduces the time taken to produce resistant varieties of commercial quality.

1.2 Genetic modification of the nuclear genome for crop improvement

Genetic modification for crop improvement can broadly be defined as the process whereby one or a few genes are introduced into an existing crop variety to confer novel character traits, whilst retaining the existing phenotype of the commercial variety.

1.2.1 Methods of nuclear gene transfer

The production of genetically modified (GM) or 'transgenic' plants is typically carried out using either *Agrobacterium*-mediated or direct gene transfer methods to insert genes of interest or 'transgenes' into the nuclear genome of crop plants (Lindsey and Jones, 1990). To ensure that transgenes are expressed in the transformed cells, nuclear transformation vectors contain promoter and terminator sequences flanking genes of interest. Promoters contain sequences for RNA polymerase and transcription factors to bind to for initiation of gene transcription, whilst terminator sequences contain signals for the termination of the transcription process and polyadenylation (Brown, 1995).

Agrobacterium-mediated gene transfer is the most common method for genetic transformation of crop plants and utilises the natural propensity of the soil phytopathogens *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* to transfer specific segments of their DNA to plant cells to induce gall or tumour formation (Zupan *et al.*, 2000). In natural systems the transfer of DNA (T-DNA) from the plasmid DNA of *A. tumefaciens* alters the biosynthetic make-up of plant cells, diverting the plant cell metabolism to produce substrates such as opines which only the bacterium can utilise (Schell *et al.*, 1979). The process by which *Agrobacterium* transfers DNA to a host plant cell has been characterised using molecular biological techniques. The bacterial tumour inducing (Ti) plasmid of *Agrobacterium* was found to contain two 25 bp direct repeats, known as the left and right borders, and a

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virulence region. Specific proteins produced from genes encoded in the virulence region covalently bind to single stranded copies of the DNA lying between the left and right borders forming the T-complex, which is imported into the plant cell nucleus where T-DNA becomes integrated into the plant chromosome (Zupan et al., 2000).

To enable introduction of foreign genes into the plant chromosome the natural process carried out by Agrobacterium is exploited, typically using a binary vector system involving two plasmids (Bevan, 1984). A nuclear transformation vector containing genes of interest, within regulatory elements, flanked by the Ti plasmid left and right border sequences is first created. The genes of interest and the regulatory elements accompanying them thus form a T-DNA region. This plasmid is then transformed into disarmed Agrobacterium harbouring a Ti plasmid containing the virulence region but from which the native tumour inducing DNA sequences have been removed. The separation of the virulence and T-DNA regions onto two different plasmids enables easier manipulation of the T-DNA plasmid but does not interfere with T-DNA transfer capability. Wounded plant tissues are then exposed to the modified Agrobacterium containing the binary vector and tissue culture methods used to recover whole plants from transformed cells containing stably integrated T-DNA.

There are several alternative direct gene transfer methods for incorporation of transgenes. Whereas in plasmid vectors designed for Agrobacterium-mediated transformation genes of interest are flanked by left and right border sequences, in direct gene transfer methods these sequences are not required since incorporation of foreign DNA occurs via DNA strand-break and repair (Ray and Langer, 2002).

Particle bombardment, 'biolistics', involves coating small particles of tungsten or gold with plasmid DNA and firing these into plant tissues under gas pressure. The particles penetrate the cell wall and nuclear membrane and the DNA is released within the nucleus and integrated into the nuclear genome (Sanford et al., 1993). This technique has been applied to over 80 different plant species and has become the second most commonly used technique for plant transformation (Luthra et al., 1997). Microinjection of transgenes into individual cells has also been used, however this

method is laborious and only one cell at a time may be manipulated (Kost *et al.*, 1995; Shen *et al.*, 2001). Electroporation and polyethylene glycol (PEG) -mediated treatments both permeablise cell membranes allowing nucleic acids to pass into the cell (Fisk and Dandekar, 1993). The major disadvantage of these methods is that they utilise protoplasts as donor material. This requires the development of protocols for whole plant regeneration from protoplasts, increases the length of time cell lines are kept in culture and increases the likelihood of somaclonal variation occurring (Nehra *et al.*, 1992). More recently biolistics has also been used to deliver *Agrobacterium* binary vectors into target cells (De Mesa *et al.*, 2000).

In addition to genes of interest, during transformation, selectable and scorable marker genes are also co-introduced, either on the same or on independent transformation vectors.

1.2.2 Selectable marker genes

Selectable marker genes produce proteins which confer resistance to specific substances which can be applied exogenously to regenerating plant tissues allowing a selective growth advantage to tissues that have been genetically modified. When co-transferred with genes of interest the selectable marker genes, and their encoded proteins, remain within the crop. Genes conferring resistance to a wide variety of antibiotics have been used as selectable markers for plant transformation. The antibiotics most commonly used are kanamycin, hygromycin, gentamycin, tetracycline, streptomycin and spectinomycin (Hellens and Mullineaux, 2000). A second category of widely used selectable marker genes confer resistance to herbicides such as glyphosate (Zhou *et al.*, 1995) and the phosphinothricin-based herbicides such as Basta ™ (De Block *et al.*, 1987).

The use of antibiotic and herbicide resistance genes as selectable markers in plant transformation has become one of the most contentious issues concerning genetic modification in recent years (Trewavas, 1999; Hohn *et al.*, 2001). There are several concerns among environmental groups and governmental authorities relating

to the use of these genes. In the case of the antibiotic resistance genes the concerns are that the final food product may be toxic to humans, that consuming antibiotic resistance genes will compromise antibiotic therapy in humans and that the spread of these genes will cause unacceptable damage to the environment (Flavell *et al.*, 1992; Kuiper *et al.*, 2001). In the case of herbicide resistance genes similar concerns exist relating to the potential toxicity / allergenicity of food products and the potential release of transgenic pollen and gene flow from transgenic plants to wild relatives of crop species resulting in the generation of 'superweeds' (Trewavas, 1999).

With regard to environmental risks, several studies have examined the potential for transgene transfer via pollen of GM plants to wild relatives in several species. In a field trial of GM tomato, over 2000 progeny were assessed to establish the frequency of spontaneous crosses occurring between the GM tomato and a field of non-transformed tomato. No transgene flow was detected in these studies (Ilardi and Barba, 2002). Whilst tomato is an insect pollinated species many crop species are wind pollinated which can increase the potential distance of transgene spread. For example in a study by Pleasants *et al.* (2001) it was determined that vast majority of maize pollen is deposited within 5 m of the field edge. However small quantities of GM maize pollen were detected up to 100 m from the field edge. In the case of oil seed rape, an insect and wind pollinated species, pollen dispersal has been detected up to 25, 000 m from the field edge (Smyth *et al.*, 2002). The potential for gene flow between GM and non-GM in oilseed rape was highlighted in 1999 when plants resistant to the herbicides Roundup, Liberty and Pursuit were discovered in Alberta, Canada. It was estimated that a triple resistant hybrid had been created through a variety of cross-pollinations in just two years (MacArthur, 2000). Thus it can be seen that the distances separating GM and non-GM plantings of different species have considerable influence over the potential for gene flow. Research carried out concerning the risks associated with the use of selectable marker genes in the environment has generated arguments for and against their use. However a

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perceived risk remains in the public perception requiring that alternatives to antibiotic and herbicide resistance markers are investigated (ACRE, 2000).

Several methods have been developed to remove antibiotic or herbicide resistance marker genes from transgenic plants. The *Cre/lox* system, using site-specific recombination, requires that the selectable marker used in the initial transformation is flanked by two 34 bp repeat sequences of the *Lox* gene. Introduction of the *Cre* recombinase gene into the transformed plant, either by sexual crossing or a second transformation procedure, results in the excision of the selectable marker since the *Cre* gene product catalyses recombination between the two *Lox* repeats (Brasileiro and Aragao, 2001). Further examples of gene excision systems which exploit site-specific recombinases are the Flp/FRT (Hohn *et al.*, 2001) and R/RS (Ebinuma and Komamine, 2001) systems. Both systems have been applied in a similar manner to that described for *Cre/Lox* to remove unwanted selectable marker genes from transgenic plants. A disadvantage of the recombinase systems is often the requirement for either a second transformation procedure or a sexual cross. However chemically induced expression using the R/RS recombinase system bypasses this requirement and reduces the time taken to produce a herbicide / antibiotic gene-free transgenic crop (Ebinuma and Komamine, 2001). Intrachromasomal recombination between bacteriophage λ phage attachment (attP) regions used to flank the scorable GFP marker gene and the *nptII* marker gene has also been used to excise unwanted genes (Zubko *et al.*, 2000). A further way of removing antibiotic selectable marker genes from GM plants is to employ co-transformation (Daley *et al.*, 1998). In this system the genes of interest and marker genes are introduced at separate loci of the recipient genome by introducing them on different plasmids. Sexual crossing can then be used to segregate the two loci resulting in marker-free progeny containing only the genes of interest (McCormac *et al.*, 2001).

Alternatively the use of antibiotic or herbicide selectable marker genes can be avoided altogether through the use of positive selection systems. These allow

transformed cells to metabolise compounds that are otherwise not metabolisable. This enables selection of transformed tissues without the death of non-transformed cells. Examples of positive selection systems exploiting hormone metabolism are the isopentyl transferase (*ipt*) system (Sugita *et al.*, 1999) and the *rol* gene system (Cui *et al.*, 2001; Puddephat *et al.*, 2001). The *ipt* gene from *Agrobacterium tumefaciens* catalyses cytokinin synthesis and promotes rapid proliferation in transformed lines containing the gene, thus enabling their selection. The *rol* genes of *Agrobacterium rhizogenes,* increase sensitivity to auxin and promote the proliferation of 'hairy roots' in transformed lines containing the genes, thus enabling their selection. Two examples of positive selection markers exploiting carbohydrate metabolism are the xylose isomerase (*xylA*) (Haldrup *et al.*, 1998a) and phosphomannose isomerase (PMI) (Joersbo *et al.*, 1998) selection systems. Xylose isomerase has been used in the starch processing industry since the 1960's and has been widely used in the food industry to catalyse the conversion of D-glucose to D-fructose during the production of high-fructose corn syrups, which are used as sweeteners in soft drinks and other products (Misset, 2003). Xylose isomerase also effects the isomerisation of D-xylose to D-xylulose and it is this activity which has been utilised as a selectable marker for plant transformation (Haldrup *et al.*, 1998a). Several plant species have been shown to be unable to utilise D-xylose as the sole carbon source during *in vitro* culture, but are able to utilise D-xylulose (Bojsen *et al.*, 1994). The *xylA* gene has been successfully used as a selectable marker enabling transformed plant lines of potato, tobacco and tomato to survive on D-xylose (Haldrup *et al.*, 1998b). PMI-transformed plant cells are able to convert mannose-6-phosphate into fructose-6-phosphate, which is incorporated into the plant metabolic pathway enabling transformed tissues to survive whilst non-transformed tissues starve (Reed *et al.*, 2001a). The use of a selectable marker such as *xylA* which has a long history of use in the food industry, which does not pose a risk to medical therapies and which does not confer characteristics likely to generate 'superweeds' should alleviate

several of the perceived problems associated with the production of genetically modified crops.

1.2.3 Scorable marker genes

As previously mentioned plant transformation vectors also typically include scorable marker genes, which provide a means by which transformed cells may be visually identified. The most commonly employed scorable marker gene is the *gusA* gene whose product, β-glucuronidase, catalyses the cleavage of a variety of β-glucuronides. When incubated with the substrate 5-bromo-4-chloro-3-indoyl-1 -glucuronide a blue stain is produced in *gusA* transformed plant tissues (Jefferson *et al.*, 1987). The *gusA* gene has been employed in a wide variety of plant species and is often linked to promoters of genes of interest to determine their spatial and temporal expression patterns in the host species (Gallagher, 1992). One disadvantage of the *gusA* system is that the assay is destructive.

A second scorable marker gene often used in plant transformation which can be assayed in living tissues, is the green fluorescent protein (GFP) gene isolated from jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994). GFP has the unique characteristic of emitting green light when exposed to ultraviolet (~395 nm) or blue light (~490 nm), enabling relatively easy monitoring of whole plant tissues without the need to add a substrate (Stewart, 1996). Several variants of GFP have been made in recent years to improve their application. This has involved modifications to improve expression levels (Pang *et al.*, 1996) and spectral modifications giving rise to red and blue shift variants (Heim *et al.*, 1994). Wild type GFP has been shown to form aggregates when expressed in higher plants which can lead to cytotoxic effects and reduced expression levels (Crameri *et al.*, 1996). To circumvent this a soluble modified GFP variant has been developed that does not form aggregates (Davis and Vierstra, 1998).

1.3 Transgene expression through the nuclear genome

1.3.1 Position effects and transgene copy number

Important considerations in GM crop plants are field performance and transgene stability (Fisk and Dandekar, 1993). In both *Agrobacterium*-mediated and direct gene transfer methods transgenes are integrated in random numbers of copies and at random sites in the recipient plant genome (Hobbs *et al.*, 1990; Hobbs *et al.*, 1993). In *Agrobacterium*-mediated transformation multiple T-DNA insertions are commonly observed and can take the form of direct, inverted and tandem repeats around the left and right border sequences (Jorgensen *et al.*, 1987). The number of copies of a transgene transferred to the recipient genome may or may not have a significant effect on the levels of transgene expressed (Hobbs *et al.*, 1993). Whilst multiple copies of the same transgene can increase expression levels the particular arrangement of transgene repeats may also have a 'silencing' effect by promoting localised heterochromatin formation (Ye and Signer, 1996). The position transgenes occupy in the recipient plant genome can also have a significant effect on their expression; either through the presence of endogenous regulatory elements in the vicinity of the integration site or by the surrounding chromosomal domain influencing the chromatin structure of transgenes (Weising *et al.*, 1990). Chromatin is formed by the association of a variety of proteins with nuclear DNA and aids the organisation of genes and regulatory elements into higher-order structures. In order for a gene to be expressed the surrounding chromatin has to be relaxed, as tight packaging of the chromatin restricts access to RNA polymerases thus inhibiting transcription (Kahl *et al.*, 1987). Specific DNA sequences known as scaffold or matrix attachment regions (MARs) mediate attachment of DNA to the nuclear scaffold (Breyne *et al.*, 1994) and have been shown to influence transgene expression levels. There are conflicting reports as to whether MARs stabilise gene expression. In some instances transgenes expression levels have been increased (Schoffl *et al.*, 1993; Mankin *et al.*, 2003) and in other reports no positive affects have been observed. Inadvertent insertion of

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transgenes into highly methylated regions of the recipient genome may also contribute to some of the position effects observed as increased methylation associated with transgenes has been shown to impair transcription (Hobbs *et al.*, 1990; Kilby *et al.*, 1992).

1.3.2 Regulatory elements and transgene expression

Regulation of gene expression can be either pre or post transcriptional or post-translational i.e. at the levels of transcript synthesis, transcript processing, protein synthesis and protein modification (Cohen and Mayfield, 1997; Tyagi, 2001). The expression of transgenes in GM plants is most commonly regulated at the transcriptional level through the use of promoter sequences placed upstream of transgenes in transformation vectors. Promoters generally comprise multiple *cis*-acting regulatory motifs which are required to initiate transcription (Dynan, 1989). Plant promoters typically contain the core promoter elements of a TATA box, implicated as the signal determining the site of transcription initiation, approximately 15-60 nucleotides upstream of the transcription start site and a more distal CCAAT element thought to be involved in stimulating transcription (Grierson and Covey, 1988). Transcriptional initiation is regulated by a complex set of interactions between specific *cis*-acting DNA motifs and sequence-specific *trans*-acting protein factors that bind to them (Weising and Kahl, 1991). RNA polymerase inspects the DNA by binding loosely to it for an instant, then releasing and moving on in a repeated fashion. General transcription factors bound to core promoter elements enable RNA polymerase to tightly bind to the DNA, thus enabling recognition of the site from which to initiate the process of transcription (Meshi and Iwabuchi, 1995). In addition to the core elements, promoters generally contain further enhancer and regulatory motifs which are recognised by regulatory factors that in turn affect the assembly of the general initiation factors (Roeder, 1996). Natural constraints, such as the availability of specific transcription factors, chromatin structure or negative co-factors, arising from the cellular environment (developmental stage, function, species etc.) in

which a gene is expressed further modulate the combinatorial effects of the factors regulating gene transcription.

3' untranslated terminator sequences are also incorporated into plant transformation vectors to facilitate termination of transcription and to stabilise the messenger RNA transcribed. Post-transcriptional processing of pre-mRNA is a fundamental step in gene expression, the 5' end of the primary transcript is capped, introns are removed and the 3' end is formed through endonucleolytic cleavage and subsequent polyadenylation (Rothnie, 1996). The formation of mature 3'-end mRNA is influenced by sequence motifs contained within the 3' untranslated region of the gene and has been implicated in determining the stability of messenger RNA (Abler and Green, 1996). The sequence motifs influencing the site of cleavage and polyadenylation addition are not tightly conserved within plants (Rothnie, 1996). However an 'AAUAAA' sequence motif associated with efficient 3' end mRNA formation is present in the 3' untranslated regions of many plant genes (Mogen *et al.*, 1990).

The terminator regions of several *Agrobacterium tumefiaciens* T-DNAs have been characterised and used as terminators for transgenes during genetic modification of crop plants, notably the nopaline synthase (*nos*) terminator (Bevan *et al.*, 1982). The 3' untranslated region of the cauliflower mosaic virus contains an AAUAAA motif 14 to 20 nt upstream of the polyadenylation site (Rothnie, 1996) and has also been widely used in plant transformation (Mitsuhara *et al.*, 1996).

1.3.3 Constitutive transgene expression

The promoter most commonly used to direct constitutive transgene expression in transgenic crops is the cauliflower mosaic virus 35S RNA (CaMV 35S) promoter. The intact CaMV 35S promoter confers dependably high levels of constitutive expression of transgenes in most plant species (Benfey and Chua, 1990). Expression of transgenes in a constitutive manner enables selectable marker

gene expression throughout all tissue types during the process of transformation and regeneration of plants in the presence of selective agents.

In recent years, concerns have been expressed relating to the use of the CaMV 35S promoter (Ho *et al.*, 1999). The discovery of a recombination hotspot within this promoter (Kohli *et al.*, 1999) has prompted the hypothesis that potentially mobile CaMV 35S sequences could be inserted next to dormant viral genes in the plant genome and reactivate the virus, or that these sequences could, upon ingestion in humans, insert next to an oncogene and thus cause cancer (Ho *et al.*, 1999). The hypothetical series of risks outlined by Ho *et al.* (1999) have been widely repudiated. Studies have shown that approximately 10% of cabbages and 50% of cauliflowers are naturally infected with CaMV prompting the observation that if consumption of DNA containing CaMV 35S were indeed dangerous then the public were likely to be more at risk from consuming CaMV infected plants than from transgenic ones (Morel and Tepfer, 2000).

An associated concern linked with the use of CaMV 35S is that of constitutive expression. The use of a strong constitutive promoter to drive transgene expression in crops means that foreign proteins will be produced in all tissues, including the consumed food product. This has prompted concerns relating to the potential risks associated with the consumption of transgene products and further complicates the issues concerning genetically modified foods. Consequently, the precautionary principle of reduced exposure and therefore reduced risk through avoidance of superfluous transgene expression has now been cited as a principal objective in genetically modified plant design (ACRE, 2000).

Constitutive transgene expression results in the presence of transgenic proteins in the pollen of transgenic crops. Transgenic pollen DNA and proteins were found to remain relatively intact and stable when incubated in honey for at least six weeks, suggesting that honey may be a potential vector for transgene spread into the wider environment (Eady *et al.*, 1994). The detrimental effects on non-target organisms of transgenic maize pollen expressing the *Bacillus thuringiensis* (Bt)

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insecticidal protein was demonstrated by dusting transgenic pollen onto milkweed leaves which were then fed to the larvae of the monarch butterfly, *Danaus plexippus* (Losey *et al.*, 1999). Monarch larvae fed on leaves dusted with the transgenic pollen suffered increased mortality and those individuals surviving the experiment exhibited reduced growth rates. A series of studies investigating the likely effect of Bt maize pollen on monarch larvae in the natural environment have refuted the likelihood of such high doses of transgenic pollen occurring in a field situation (Pleasants *et al.*, 2001). A risk assessment using the information from several U.S and Canadian studies concluded that the impact of Bt maize pollen from current commercial hybrids on monarch butterfly populations is negligible (Sears *et al.*, 2001). However the headlines generated from studies such as that of Losey *et al.* (1999) further exacerbate public concerns relating to genetic modification of crops.

1.3.4 Tissue-specific transgene expression

A means by which transgene expression can be eliminated from the consumed food product and the pollen of a transgenic crop is through the development and use of tissue-specific promoters. Tissue-specific expression is thought to be regulated by a complex interaction of enhancer and promoter regulatory motifs and general and tissue-specific factors (Weising and Kahl, 1991). Gene expression is regulated through a specific combination of transcription factors interacting with specific binding sites along the promoter (Aerts *et al.*, 2003). It is thought that the combination of specific factors available in different tissues as a result of cellular function, developmental status or external stimuli may give rise to tissue-specific expression (Dynan, 1987).

Many tissue-specific promoters associated with spatially or temporally regulated plant genes have been identified over recent years. The gene expression pattern conferred by plant promoters is often characterised through analysis of scorable marker genes, such as *gusA* or GFP, under the control of promoter regions in model plant systems such as Arabidopsis and tobacco (Holtorf *et al.*, 1995; Yang

et al., 2000). A comprehensive review of all the different tissue-specific promoters characterised will not be given here, instead the example of root-specific transgene expression will be used to highlight some of the considerations pertinent to tissue-specific expression with regard to crop improvement.

There are several benefits to localising transgene expression in the roots of crop species. As described for strawberry, there are many pathogens of crop species which particularly target the root, thus expression of insecticidal or anti-fungal proteins in this organ could resolve agronomically important problems. Restricting transgene expression to the roots of the plant may go some way to satisfying regulations regarding food safety and to alleviate public concerns over the consumption of genetically modified food.

Several root-specific promoters have been identified which are associated with genes representing the many different functions of the root. A group of genes found to be preferentially expressed in the roots of several plant species are the haemoglobin genes (Appleby *et al.*, 1988). In a large number of leguminous species haemoglobin expression has been linked with nodulation and nitrogen-fixation. However, these genes have also been isolated from non-nodulating species (Landsmann *et al.*, 1988; Taylor *et al.*, 1994) where it is thought that they may have a role in the respiratory metabolism of root cells (Bogusz *et al.*, 1988). The 1 kb *par* promoter associated with a haemoglobin gene from the non-legume *Parasaponia andersonii* has been used to confer transgene expression in a heterologous plant species, tobacco. It was claimed that the *par* promoter conferred root-specific expression, however low level transgene expression was also detected in the leaves by western blot analysis (Landsmann *et al.*, 1988). Later observations based on a large number of tobacco transformants using the same 1 kb *par* promoter revealed gross variation in expression levels of the *gusA* scorable marker gene including transgene expression in the leaves of 46 out of 140 transgenic lines (Van der Hoeven *et al.*, 1994). In contrast, when the 1 kb *par* promoter was used to drive transgene

expression in the leguminous species *Lotus corniculatus* expression was restricted to the root and nodular tissues (Bogusz *et al.*, 1990).

A further example of a root-specific promoter is that of the extensin A (*extA*) gene. Extensins are found in the cell walls of plant cells and it is thought that they strengthen the cell wall thus increasing resistance to pathogen attack and wounding (Shirsat *et al.*, 1996). A 1 kb *extA* gene promoter from *Brassica napus* (oilseed rape) has been used to control expression of the *gusA* scorable marker gene in tobacco as well as in rape. Whilst in rape *gusA* expression was only observed in the roots, in tobacco it was found that although transgene expression was not detected in the leaf tissues, activity was detected in both the stem and roots (Shirsat *et al.*, 1991). Further studies revealed four sets of positively and negatively acting *cis*-elements in the extA promoter that control wound inducibility, response to tensile stress and general expression levels (Elliot and Shirsat, 1998). Transgene expression conferred by the 1 kb *extA* promoter in apple (*Malus pumila*) revealed transgene expression in all tissues, with expression levels comparable to CaMV 35S in the stems. Using a histochemical assay particularly high levels of *gusA* staining were noted at nodal junctions where tensile stresses were likely to be highest (Gittins *et al.*, 2001).

A further example of a promoter found to be tightly regulated in the root tissues is that of the glutamine synthetase gene. Glutamine synthetase plays a central role in nitrogen metabolism in higher plants (Eckes *et al.*, 1989). A 3.5 kb root-specific promoter of the cytosolic glutamine synthetase gene isolated from soybean (Miao *et al.*, 1991) was shown to be root-specific and induced by the availability of ammonia in soybean. However when studied in tobacco the promoter was not root-specific and it was not ammonia-inducible.

A further example of lack of tissue specificity in heterologous systems is that of the pea metallothionein (*PsMT$_A$*) gene promoter. *PsMT$_A$* gene transcripts were shown to accumulate at much higher levels in the root than in aerial tissues of pea (Evans *et al.*, 1990). When the 814 bp pea *PsMT$_A$* gene promoter and 624 bp, 583 bp and 285 bp truncations of this promoter were used to drive expression of the

gusA scorable marker gene in Arabidopsis *gusA* expression was detected at high levels in the roots and to a lesser degree in expanding cotyledons, stipules and in several senescent tissues (Fordham-Skelton *et al.*, 1997). When the 814 bp pea *PsMT$_A$* promoter was used to confer *gusA* expression in transgenic apple the expression pattern observed was essentially constitutive (pers comm. J. Gittins).

Several other root-specific promoters have been reported, notably the promoters associated with the auxin-inducible GNT tobacco genes (Van der Zaal *et al.*, 1991) and the promoter associated with the *N*-methytransferase gene from tobacco whose expression has been linked with wounding responsiveness (Shoji *et al.*, 2000). Both of these promoters appear to be tightly regulated in root tissues in their host species; however their expression patterns in heterologous systems have not yet been tested.

The root-specific promoter studies so far described all illustrate that it cannot be assumed that spatial and temporal expression patterns observed in a host species will be reproduced when the promoter is used in a heterologous system. Since the lengths of promoter fragments used in both the native and heterologous systems are the same in the cases reported, then the differences in specificity observed in these promoter studies are likely to be due to the availability and interaction of trans-acting factors present in the different species. In this study, to circumvent potential problems of unpredicted specificity, the approach taken to achieve root-specific transgene expression in strawberry was to isolate a root-specific gene and its promoter from strawberry.

Further genes that display root-specific expression in different plant species are the aquaporins. One of the primary functions of the root is that of water uptake and transport to meet the requirements of the transpiring shoot (Steudle, 1994). The transport of water and solutes across biological membranes is a complex process and several pathways exist for the passive movement of water and solutes across the root cylinder. The three major routes are the symplastic and transcellular intra-cellular pathways and the apoplastic extra-cellular pathway (Steudle and

Frensch, 1996). Membrane intrinsic proteins (MIP) have been implicated in the facilitation of water transport through the intra-cellular pathways, plasma intrinsic proteins (PIP) associated with the symplastic route and tonoplast intrinsic proteins (TIP) associated with transcellular processes (Chrispeels, 1994). In Arabidopsis 23 members of the MIP family have been identified and, as noted in other species, these fall into three distinct classes (Weig *et al.*, 1997). These are the PIPs, TIPs, and nodulin-type MIPs. The MIP family of proteins share six putative transmembrane domains, with small hydrophilic loops connecting these regions (Chrispeels, 1994). Conserved N and C terminal amino acid sequences and loop B and loop E signature motifs have been used to classify the TIP and PIP classes (Schaffner, 1998).

Various isoforms exist within the TIP subfamily in plants. These are α, δ and γTIP and RB7 (Sarda *et al.*, 1999), which are found in the vacuolar membranes of a variety of tissue types. Several RB7 TIP genes have been identified which exhibit root-specific expression. It has been proposed that intracellular loop B and extra-cellular loop E interact between transmembrane helices to form a water-channel unit that is penetrated by water in both directions (Jung *et al.*, 1994). It is thought that the water channel proteins are involved in both cell expansion and vascular water permeability (Sarda *et al.*, 1997). It has also been shown that some TIP water channel proteins are differentially regulated under conditions of drought and salt stress and may be fundamental for the control of tissue water flux (Kirch *et al.*, 2000). The most fully characterised root-specific RB7 TIP identified is the tobacco gene *TobRB7,* isolated from a root mRNA library (Conkling *et al.*, 1990; Yamamoto *et al.*, 1991). *TobRB7* homologues have also been found to be expressed exclusively in root tissues in a number of other species; *Arabidopsis thaliana* (Yamamoto *et al.*, 1990), *Daucus carota* (Kawahara *et al.*, 1997), *Helianthus annuus* (Sarda *et al.*, 1999), *Petroselinum crispum* (Roussel *et al.*, 1997), *Mesembryanthemum crystallinum* (Kirch *et al.*, 2000) and *Solanum tuberosum* (Heinrich *et al.*, 1996). Since RB7 TIPs are expressed tightly in roots, they make good candidates for

isolation of their promoters to drive root-specific transgene expression in corresponding host species.

The promoter region associated with the *TobRB7* gene has also been well characterised (Yamamoto *et al.*, 1991). Deletion analysis of the *TobRB7* promoter suggests that the regulatory elements required for high level root-specific expression lie between – 636 bp to – 299 bp distal from the transcription start site. The *TobRB7* promoter was also found to contain regulatory elements invoking transgene expression in response to infection by root-knot nematode within the first 300 bp of the promoter (Opperman *et al.*, 1994). This region of the *TobRB7* promoter was used to confer low level root-specific expression of the hypersensitive-response linked avirulence gene (*hrmA*) in tobacco, which was shown to confer high levels of resistance to multiple bacterial, fungal and viral root pathogens (Shen *et al.*, 2000).

1.4 Plastid transformation

Whilst restricting transgene expression to specific tissue types may go some way towards reducing concerns relating to ubiquitous transgene expression and the effect it may have on both the environment and consumers, this technique does not address the issue of transgene containment. A newer technology, plastid transformation, which involves introduction of foreign genes into the genome of plastids (Svab *et al.*, 1990a) addresses this concern and has other benefits, which will be discussed later.

1.4.1 Plastid structure and function

Plastids are organelles which develop into several different forms from proplastids present in meristematic cells depending on the requirements of the differentiated cell (Grierson and Covey, 1988). Chromoplasts develop in the petals and fruits of many species and etioplasts develop in dark grown tissues. Leucoplasts develop in some epidermal and storage tissues, commonly as amyloplasts storing starch or as elioplasts responsible for lipid storage. The most common and best

studied plastid form is the chloroplast. Chloroplasts develop in green tissues and are specialised for photosynthesis. Photosynthetically active chloroplasts are characterised by high rates of transcription and translation allowing the synthesis of the large quantities of ribulose biphosphate carboxylase (Rubisco) and rapid renewal of electron transfer components required for efficient photosynthetic CO_2 fixation (Leister, 2003). Many biosynthetic reactions occur within the chloroplasts including the synthesis of amino acids, lipids and pigments and the assimilation of nitrogen, phosphorus and sulphur (Zerges, 2000).

A notable feature of all plastid types is that they possess their own genome, commonly referred to as the plastome. It has been estimated that in spinach up to 23% of the total cellular DNA may be accounted for by chloroplast DNA, with up to 13, 000 copies of the plastome present per cell (Bendich, 1987). Depending on the species, tissue type and developmental stage, cells can contain up to 100 chloroplasts and each of these can contain thousands of plastomes therefore the plastome may be represented ten of thousands of times per cell. The chloroplast genome is a circular molecule typically between 120-160 kb containing around 130 genes encoded on both strands (Figure 1.3).

It is generally accepted that plastids are remnants of what were free-living photosynthetic cyanobacterial progenitors that assimilated into a common ancestor of land plants and green algae (Zerges, 2000). Plastids have evolved over the past 1-2 billion years to become tightly integrated with the cellular processes of higher plants. As a result of their eubacterial origin the genetic processes of plastids still retain many prokaryotic characteristics. Many chloroplast transcription units tend to be polycistronic and organised in the same way as related *Escherichia coli* operons. Many chloroplast and *E.coli* coding sequences are homologous, the promoters and terminators of chloroplast genes are prokaryotic in nature and *E.coli* RNA polymerase can initiate accurate transcription of chloroplast genes *in vivo* and *in vitro* (Hanley-Bowdoin and Chua, 1987).

Figure 1.3 Gene map of tobacco chloroplast DNA

The genome consists of 86,686 bp of large single-copy region (LSC), 18,571 bp small single-copy region (SSC) and two inverted repeats (IR) s of 25,341 bp each. Genes shown on the inside of the circle are transcribed clockwise and genes on the outside are transcribed anti-clockwise. Asterisks denote split genes. Orf plus codon number represent open reading frames. Ycf plus designation number shows open reading frames of unknown function but common to plastomes. Adapted from Wakasugi (1998).

1.4.2 Plastid transformation methodologies

Plastid transformation is a relatively new technology and stable plastid transformed lines have only been generated for a small number of higher plant species (van Bel et al., 2001). There are several direct gene transfer methods by which plastid transformation has been achieved, most studies have been carried out in tobacco and have used particle bombardment (Bock, 2001). However PEG-mediated plastid transformation has also yielded stably transformed tobacco lines (Koop et al., 1996). Electroporation has also been used to introduce foreign DNA into isolated spinach chloroplasts (Daniell and McFadden, 1987) and microinjection used in tobacco (Knoblauch et al., 1999), however neither of these methods has yet yielded stably transformed lines.

Typically plastid transformation vectors contain a transcription unit, with plastid regulatory elements controlling expression of foreign genes, surrounded by plastome sequence acting as targeting sequence. Following biolistic or PEG-mediated insertion into the chloroplast, foreign DNA is released and integrated into the chloroplast genome via homologous recombination, which occurs between the plastome targeting sequence flanking the transcription unit and the corresponding native plastome sequences (Rochaix, 1997) (Figure1.4).

It is likely that initially only a single plastome copy will be modified in any transformation event (Bock, 2001). Selection pressure is routinely applied during plant regeneration to favour the sorting, replication and survival of transformed plastome copies over the wild-type during cell and organelle divisions. To obtain a stably transformed line a homoplasmic state must be achieved where all plastome copies in all cells contain the transgenes inserted. An extended period in tissue culture involving several repeat regeneration steps is often required before homoplasmy is achieved (Figure 1.5). In tobacco a homoplasmic state can be achieved within two to four regeneration procedures (Bock, 2001). Achieving homoplasmy in other higher plant species has proved more problematic. In potato
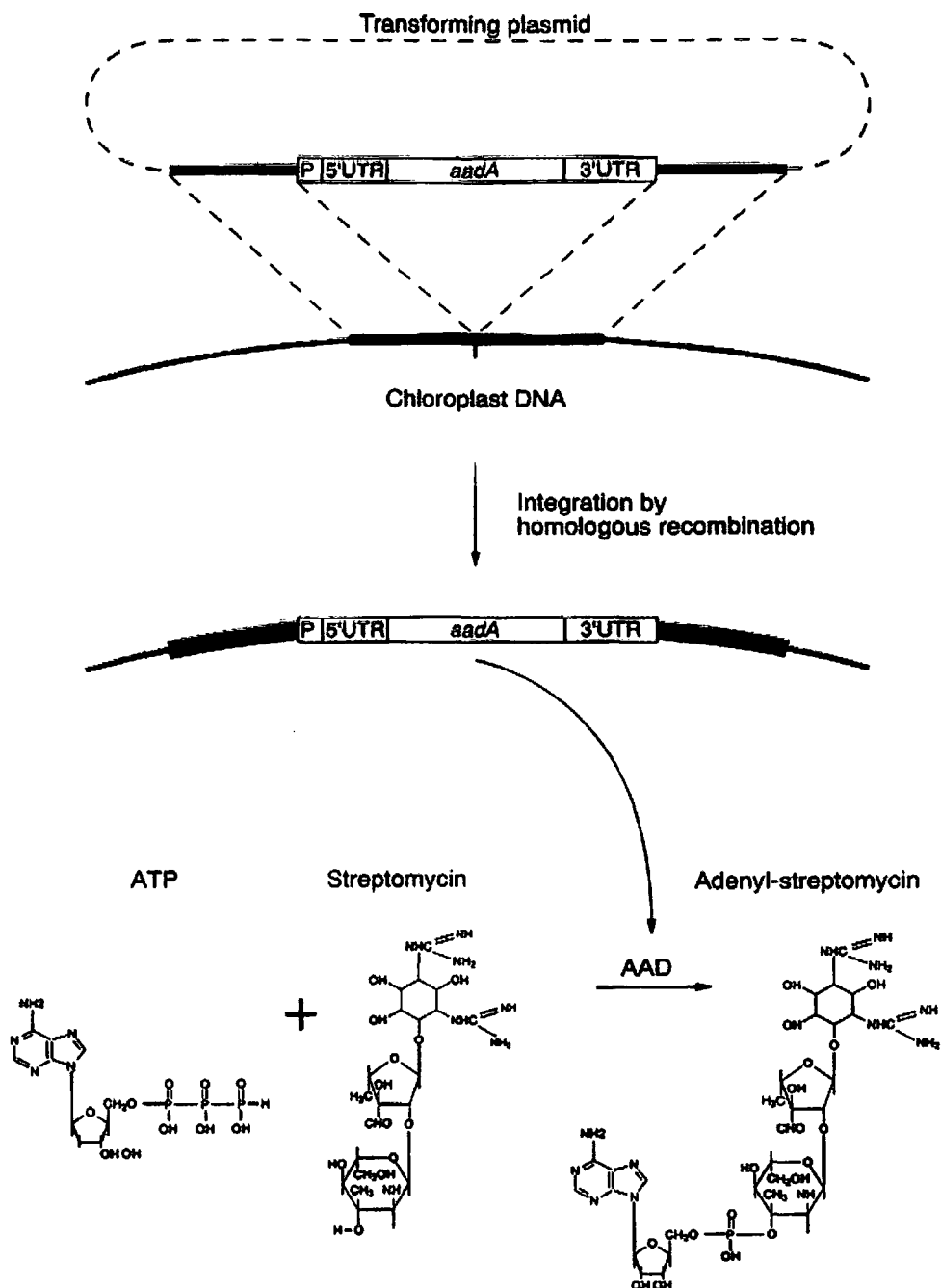
Figure 1.4 Integration of the *aadA* selectable marker gene into the plastome facilitated by homologous recombination.

Homologous recombination between plastome sequence flanking the *aadA* expression cassette in the transforming plasmid DNA and native plastome sequences facilitates the integration of an expression cassette containing the *aadA* selectable marker gene, a chloroplast promoter (P), 5' untranslated region (5'UTR) and 3' untranslated region (3'UTR) at a specific site within the plastome. Adenylation catalysed by the *aadA* gene product inactivates streptomycin / spectinomycin thereby conferring resistance to transformed tissues. Figure after Rochaix, 1997.

Biolistic transformation

Gold or tungsten particle coated with foreign DNA accelerated into the cell.
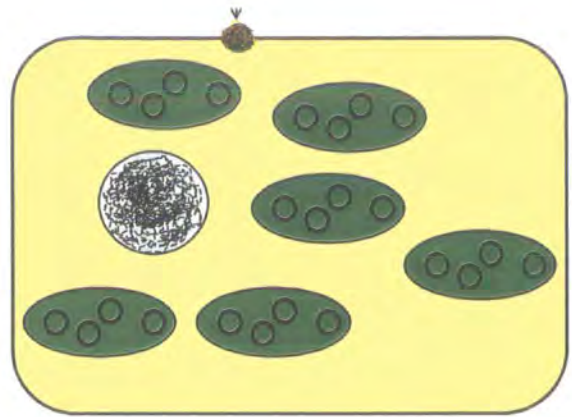
Primary plastid transformation event

Foreign DNA incorporated into a single plastome.
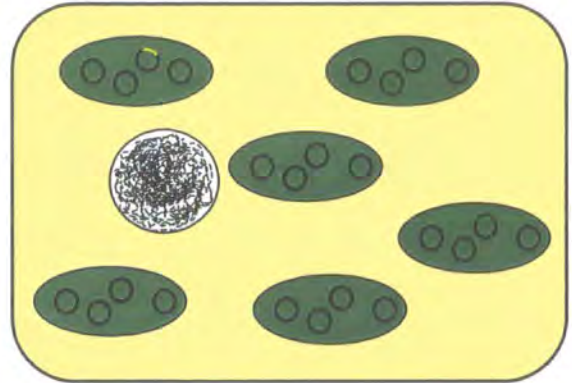
Heteroplasmic plastid population

Replication in favour of transformed plastomes containing resistance genes occurs during cell and organelle divisions in the presence of selective agent.
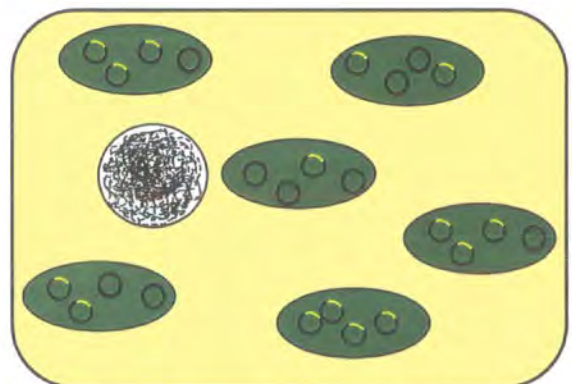
Homoplasmic plastid population

Repeated regeneration cycles in the presence of selective agent results in all plastome copies and all cells containing the foreign DNA and a stably transformed homoplasmic line.
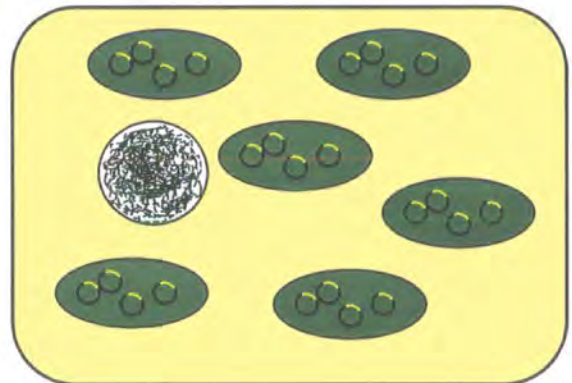
Figure 1.5 Progression to homoplasmy in transplastomic cells

Foreign DNA introduced into the chloroplast is represented by yellow sectors indicated in transformed plastome copies. Each leaf cell may contain several thousand plastid genomes, selection favours those containing the foreign DNA until all wild type plastomes are eliminated which results in a stable homoplasmic transformed line. Figure after Bock, 2001.

and petunia upwards of 8 months in culture was required before homoplasmy was attained (Schaaf *et al.*, 2000). In tomato low-light intensities and a drastic extension of the selection phase were required and it took almost two years to achieve fruiting homoplasmic lines (Ruf *et al.*, 2001). In rice (Khan and Maliga, 1999) and oilseed rape (Hou *et al.*, 2003) heteroplasmic lines have been reported with homoplasmy not being reached, despite repeated cycles of selection. Whilst extended selection cycles complicate the production of transgenic lines the benefits of plastid transformation (discussed later) more than compensate for the increased production times.

Several different regions of the plastome, all transcriptionally active, have been targeted for plastid transformation. A region often targeted in tobacco has been the RuBisCo large subunit (*rbcL*) and acetyl-CoA carboxlase beta subunit (*accD*) intergenic region (Carrer and Maliga, 1995; Staub and Maliga, 1995; Daniell *et al.*, 1998; Iamtham and Day, 2000) which lies in the large single copy region of the plastome. Recently, increased transformation efficiencies were reported when targeting the intergenic region between tRNA-Gly (*trnG*) and tRNA-fMet (*trnfM*) genes in tobacco and this region was successfully targeted for insertion of transgenes into the tomato plastome (Ruf *et al.*, 2001). Another commonly targeted region of plastome used in tobacco plastid transformation lies between the tRNA-Val (*trnV*) and ribosomal protein S12 (*rps12*) genes (McBride *et al.*, 1994; Zoubenko *et al.*, 1994; Khan and Maliga, 1999; Kuroda and Maliga, 2001; Ye *et al.*, 2001). This region lies within the inverted repeat of the plastome, thus transgenes inserted at this position are represented twice per plastome which may increase transgene expression levels. This region has also been targeted successfully in Arabidopsis and potato plastid transformation (Sikdar *et al.*, 1998; Sidorov *et al.*, 1999).

Due to the conserved nature of the plastome between higher plant species (Stoebe *et al.*, 1998) it has been possible to utilise plastome sequence derived from heterologous species to target specific regions of the plastome for transgene insertion. Multiple recombination events occurring between plastome targeting sequence derived from potato (*Solanum tuberosum*), carrying mutations conferring

spectinomycin and streptomycin resistance, and the same region of the tobacco plastome enabled efficient transformation of the tobacco plastome (Kavanagh *et al.*, 1999). Tobacco plastome targeting sequence has been successfully used in potato and tomato plastid transformation (Sidorov *et al.*, 1999; Ruf *et al.*, 2001) and the Arabidopsis plastome targeting sequence was used in *L. fendleri* plastid transformation (Skarjinskaia *et al.*, 2003).

1.4.3 Plastid regulatory elements and transgene expression

Plastid gene expression is regulated at the transcriptional level by two types of RNA polymerase, plastid-encoded RNA polymerase (PEP) and nuclear encoded RNA polymerase (NEP) (Maliga, 1998). NEP functions predominantly in proplastids in young tissues, whereas PEP preferentially transcribes the genes encoding proteins for photosysnthesis in mature tissues. Housekeeping genes involved in the genetic system and metabolism are thought to be transcribed by both polymerases (Hayashi *et al.*, 2003) A variety of promoters recognised by the two transcription systems exist in the plastome and have been utilised in plastid transformation.

The 16S RNA (*rrn16*) promoter, which contains both the NEP and PEP recognition sequences, has been widely used in plastid transformation because of the strong constitutive expression pattern it confers. The 16S RNA is a component of the small subunit of the ribosome and hence is required in all tissues at high levels (Sriraman *et al.*, 1998). The *rrn16* promoter isolated from tobacco has been used in tobacco plastid transformation to drive strong constitutive expression of transgenes (Svab and Maliga, 1993; Zoubenko *et al.*, 1994; Lutz *et al.*, 2001).The tobacco *rrn16* promoter has also been used to confer transgene expression in Arabidopsis (Sikdar *et al.*, 1998), *Lesquerella fendleri* (Skarjinskaia *et al.*, 2003), potato (Sidorov *et al.*, 1999) and tomato (Ruf *et al.*, 2001). Although here its use in heterologous plant species has been successful different types of *rrn16* promoter exist in some plant species and the requirement for species specific transcription activating factors has been highlighted (Sriraman *et al.*, 1998).

The photo-system II core protein gene (*psbA*) promoter, containing both NEP and PEP recognition sequences, has also been widely used in plastid transformation (Maliga *et al.*, 1993; Staub and Maliga, 1993; Reed *et al.*, 2001b; Huang *et al.*, 2003). The expression pattern conferred by the *psbA* promoter is not constitutive, expression increases at the early stages of chloroplast development and is maintained at high levels in mature chloroplasts reflecting the ongoing requirement of a functional photosystem II complex (Hayashi *et al.*, 2003). The *psbA* promoter was shown to confer high levels of *gusA* expression in light grown cotyledons, however *gusA* synthesis was inhibited when the plants were transferred to the dark (Staub and Maliga, 1993). The *psbA* promoter has also been shown to be functional in heterologous systems. The rice *psbA* promoter has been successfully used in tobacco, despite only 53% homology existing between the promoter regions of the two species (Reddy *et al.*, 2002). The tobacco *psbA* promoter has also recently been used in oilseed rape plastid transformation (Hou *et al.*, 2003).

In addition to plastid derived promoters, the bacterial T7 RNA promoter has also been used in plastid transformation. The T7 promoter requires T7 RNA polymerase to be present for transcription to be initiated. Plastid transformation of nuclear transformed lines expressing chloroplast targeted T7 RNA polymerase using the T7 promoter to drive *gusA* expression resulted in high levels of *gusA* expression in mature leaves, moderate expression in young leaves and petals and low level expression in stems and roots. *GusA* activity was not detected in plastid transformed lines lacking the nuclear encoded T7 RNA polymerase (McBride *et al.*, 1994). A further strategy has been to insert transgenes in close proximity to wild-type plastid promoters to create an artificial operon. Native *ycf3* regulatory elements were used to express the *aphA-6* gene conferring resistance to kanamycin, however these lines were found to be less tolerant to the antibiotic than lines expressing the same gene under control of the *rrn16* promoter (Huang *et al.*, 2002).

The structural function of rRNA molecules means that although constitutively transcribed these sequences remain untranslated (Watson, 1992). To ensure that

selectable and scorable marker genes under control of the *rrn16* promoter are translated a 5' untranslated region (5'UTR) containing sequence elements recognised by the ribosome as the correct site from which to initiate translation is often incorporated into plastid transformation vectors. The 5'UTR's of the *psbA, rbcL, atpB* and *rpl32* genes have all been used in plastid transformation (Eibl *et al.*, 1999; Khan and Maliga, 1999). The *rbcL* 5'UTR has been most extensively employed for plastid transformation and has resulted in high levels of expression of several transgenes (McBride *et al.*, 1995; Khan and Maliga, 1999; Skarjinskaia *et al.*, 2003). Synthetic ribosome binding sites have also been incorporated downstream of the *rrn16* promoter to initiate translation (Koop *et al.*, 1996; Huang *et al.*, 2002). Recent studies have also shown that substitution of the *rbcL* 5'UTR with the bacteriophage gene 10 leader (G10L), which shows increased complementarity to the *rrn16* anti-Shine-Dalgarno sequence, can dramatically increase transgene expression levels (Ye *et al.*, 2001). In conjunction with the *rrn16* promoter G10L has most recently been employed to express a subunit tetanus vaccine polypeptide at up to 25% of the total soluble cellular protein through the tobacco plastome (Tregoning *et al.*, 2003).

To ensure that stable transcripts are produced from the introduced transcription units 3' terminator regions (3'TR) are typically included in plastid transformation vectors downstream of the transgene termination codon. In plastid transformation the 3'TRs from the *rbcL* (Serino and Maliga, 1997; Tregoning *et al.*, 2003), *rps16* (Maliga *et al.*, 1993; Ye *et al.*, 2001), *rpl32* (Huang *et al.*, 2002) genes have all been used. However the most widely used 3'TR in plastid transformation has been that of the *psbA* gene (Iamtham and Day, 2000; De Cosa *et al.*, 2001; Reddy *et al.*, 2002). The *psbA* 3'TR has been shown to help stabilise plastid transcripts in tobacco (Eibl *et al.*, 1999) and has also been utilised in plastid transformation of several other species (Sikdar *et al.*, 1998; Sidorov *et al.*, 1999; Ruf *et al.*, 2001; Hou *et al.*, 2003; Skarjinskaia *et al.*, 2003).

Expression as individual units requires that each transgene is flanked by 5' and 3' regulatory elements. Plastid transformation vectors designed to express multiple transgenes in this way have often used different promoters for each transgene to avoid inadvertant excision of transgenes via homologous recombination occurring between repeated regulatory sequences (Sidorov *et al.*, 1999; Reed *et al.*, 2001b). However two copies of the *rrn16* promoter have been used within the same vector and both transgenes were successfully transferred to the recipient plastome (Hou *et al.*, 2003). Alternatively, multiple transgenes can be expressed as operons, as either transcriptional or translational fusions. In translational fusions transgenes are linked by adapters that eliminate the termination codon from all transgenes but the last in the transcription unit. This causes 'read through' and translation of a single transcript derived from multiple genes. This system has been used to express a 65 kDa *aadA*: GFP fusion protein in tobacco and *L. fendleri* plastids (Khan and Maliga, 1999; Skarjinskaia *et al.*, 2003). Chloroplast operons co-transcribed as polycistronic pre-RNAs can be either processed into shorter RNA units representing the individual genes, or remain as a single transcript from which individual proteins are translated (Sugita and Sugiura, 1996). In this way transcripts derived from the multiple genes present in a transcription unit are translated as separate units. In the case of transcriptional fusions the full coding sequence of transgenes, including the termination codon, are linked with adapters containing ribosome binding sites. Transcriptional fusion has been used to express multiple genes from a single promoter in several plastid transformation studies. The *aadA* gene and three genes of the *cry2Aa2* operon under control of a single *rrn16* promoter were successfully expressed through the tobacco plastome (De Cosa *et al.*, 2001). The *gusA, aadA* and *bar* genes were also individually expressed whilst under control of a single *rrn16* promoter, again in tobacco (Iamtham and Day, 2000).

1.4.4 Selection systems for plastid transformation

There are several selectable marker gene systems which have been used in plastid transformation. Point mutations in the 16S rRNA gene conferring resistance to the antibiotics spectinomycin and streptomycin (Fromm *et al.*, 1987) have been widely used to enable selection of plastid transformation events utilising both biolistic and PEG-mediated transformation methods in tobacco (Svab *et al.*, 1990a; Svab *et al.*, 1990b; Svab and Maliga, 1991; Golds *et al.*, 1993). It has been argued that these binding-type markers provide an efficient means of selection since only plastids carrying the 16S rRNA mutation are resistant to the antibiotic. This is in contrast to the situation where a gene is used to confer resistance and plastids carrying the resistance gene may transfer resistance to non-transformed plastids (Dix and Kavanagh, 1995). However relatively low transformation efficiencies were obtained using the binding-type markers which lead to the development of dominant selectable marker gene systems for plastid transformation (Bock, 2001).

The most commonly used dominant selectable marker gene in plastid transformation has been the aminoglycoside 3'-adenyltransferase gene (*aadA*) which confers resistance to the antibiotics spectinomycin and streptomycin (Svab *et al.*, 1990b; Carrer and Maliga, 1995; Koop *et al.*, 1996; Sikdar *et al.*, 1998; Maliga, 1999; Sidorov *et al.*, 1999; Hou *et al.*, 2003; Skarjinskaia *et al.*, 2003). The *aadA* gene and spectinomycin selection is particularly suited to plastid transformation since the antibiotic acts by binding to the 30S subunit of the plastid ribosome preventing association of the ribosome complex. This specifically disrupts plastid protein translation enabling green / white colour selection of resistant / non-resistant tissues through the inhibition of chlorophyll biosynthesis (Svab and Maliga, 1993; Zoubenko *et al.*, 1994; Bock, 2001).

The antibiotic kanamycin has also been used as a selective agent for plastid transformation. However, kanamycin resistance conferred by the *nptII* gene proved much less efficient than the *aadA* / spectinomycin system in tobacco (Carrer *et al.*, 1993; Maliga, 2002). It has recently been reported that kanamycin resistance

conferred through the aminoglycoside phosphotransferase gene *aph-6* provides a more efficient selection system for tobacco plastid transformation (Huang *et al.*, 2002). As described for nuclear transformation, herbicide resistance also provides a means by which plastid transformed lines may be selected. The glyphosate resistance gene, 5-endol-pyruvyl shikimate-3-phosphate synthase (EPSPS), from petunia was expressed through the tobacco plastome (Daniell *et al.*, 1998) as was the *arcA* gene, which also confers glyphosate tolerance (Datta and Daniell, 1996). The *bar* gene conferring resistance to phosphinothricin (PTT) based herbicides has also been expressed through the tobacco plastome (Lutz *et al.*, 2001).

As with nuclear transformation, in plastid transformation there has been a drive to either eliminate or avoid the use of antibiotic selectable marker genes. The *Cre/Lox* recombinase system has been applied in tobacco plastid transformation to excise marker genes (Corneille *et al.*, 2001; Hajdukiewicz *et al.*, 2001; Maliga, 2002). A further method, exploiting homologous recombination occurring between three short direct DNA repeat sequences used to surround the *aadA* selectable marker gene, was developed to introduce instability to the *aadA* gene resulting in its excision from a high proportion of plastid transformed tobacco lines (Iamtham and Day, 2000).

Selection systems avoiding the use of antibiotic or herbicide resistance selectable marker genes have also been developed for plastid transformation. A negative selection scheme using a cytosine deaminase (*codA*) gene from *E. coli* was developed for tobacco. Transformed chloroplasts expressing *codA* exhibit sensitivity to 5-fluorocytosine whereas non-transformed tissues remain unaffected, thus enabling selection of transformed lines (Serino and Maliga, 1997). A selection system using a betaine-aldehyde-dehydrogenase (BADH) gene from spinach, which confers tolerance to betaine aldehyde has been developed for tobacco plastid transformation. This system is reported to generate 25-fold higher transformation efficiencies in comparison to the *aadA* /spectinomycin selection system (Daniell *et al.*, 2001a).

1.4.5 Benefits of plastid transformation

One of the major benefits of targeting the plastome is that transgenes inserted into the plastome are represented many hundreds of times per cell which can result in extremely high transgene expression levels. In transplastomic tobacco expression of the Bt *cry2Aa2* operon lead to foreign protein accumulating at up to 45% of the total soluble protein in mature leaves (De Cosa *et al.*, 2001). Transgene expression levels have been regularly reported at levels of between 2 – 25% of total soluble protein using plastid transformation (McBride *et al.*, 1995; Kota *et al.*, 1999; Tregoning *et al.*, 2003).

The prokaryotic nature of the plastid transcription and translation machinery confers two main advantages when inserting transgenes into the plastome. Firstly several transgenes can be introduced through a single transformation event and expressed as a polycistronic unit under the control of a single plastid promoter. Secondly, the plastid genome is uniquely suited to accurately express genes of bacterial origin within a eukaryotic organism. For example when expressed through the nuclear genome the Bt protoxin *crylA*(c) was present in extremely low levels. It was suspected that this may have been a result of inefficient codon usage, incorrect transcription, aberrant mRNA splicing or poor mRNA stability (Murray *et al.*, 1991). However when expressed through the plastome the unmodified *crylA*(c) protoxin was expressed at high levels (3-5% of the total soluble protein) making the transgenic plants highly toxic to insect larvae (McBride *et al.*, 1995). Importantly, genes of eukaryotic origin have also been reliably expressed through the plastid genome, such as GFP (Khan, 2001) and human somatotrophin (Staub *et al.*, 2000).

A further benefit associated with plastid transformation derives from the use of homologous recombination to insert genes into the plastome. The specific site of transgene insertion into the plastome is determined by the plastome sequence used to flank the expression cassette. The use of homologous recombination firstly ensures that only specific fragments of the transformation vector are transferred to the recipient plastome. This greatly reduces the risk of so called 'backbone

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sequence' transfer occurring as can happen in *Agrobacterium*-mediated nuclear transformation when non T-DNA sequences are inadvertently transferred to the recipient genome (Dong *et al.*, 2001). Secondly, transgene insertion occurs at the same site for all transformation events, thus eliminating the need to evaluate many different transgenic plants. Thirdly, insertion of transgenes to specific intergenic regions of the plastome avoids potentially detrimental position effects, such as interruption of native gene function or insertion into transcriptionally inactive regions.

Additionally, plastid transformation offers a means of transgene containment. In the gymnosperms the plastome is paternally inherited (Mogensen, 1996). However, in the majority of flowering plant species the plastome is maternally inherited (Hagemann and Schroder, 1989). There are exceptions to this rule, notably *Pelargonium*, *Oenothera* and *Medicago*, which exhibit biparental inheritance of plastomes (Mogensen, 1996). However, the lack of plastid gene transfer or expression through pollen in many higher plant species has made plastid transformation an appealing target for 'environmentally friendly' genetic modification of crop species (Daniell *et al.*, 2002).

Over the course of plastid evolution many of the original cyanobacterial genes have been lost or transferred to the nucleus (Martin *et al.*, 1998). Evidence of transposition of a nuclear-specific transgene from the plastome to the nucleus has recently been reported in tobacco at a rate of ~1 event in every 16, 000 pollen grains (Huang *et al.*, 2003). It is important to note that the selectable marker gene under control of a plastid promoter which was incorporated along with the nuclear-specific gene in these studies was not expressed in the nucleus. The possibility of a broken fragment of a plastid transgene being transposed downstream of a functional nuclear promoter and thus expressed in pollen, assuming the transgene was not bacterial in origin and thus not transcribed through the nuclear genome, has been estimated at ~1 in 1.6 million pollen grains (Maliga, 2003). Therefore there is a very remote possibility that plastid incorporated transgenes may transfer to the nucleus. However,

the risk of transgene spread through pollen is significantly reduced using plastid transformation compared to nuclear transformation.

A further disadvantage of plastid transformation is that the gene products derived from transgenes inserted into the plastome are confined to the plastid, which may not always be appropriate. Additionally plastid transformation has proved extremely difficult to apply in many higher plant species. Over the past 12 years only six higher plant species have been stably plastid transformed and fertile progeny were only reported in three of these species, tobacco, tomato and *L. fendleri*.

1.4.6 Achievements in plastid transformation

Plastid transformation was first achieved in the unicellular green algae *Chlamydomonas* in 1988 (Boynton *et al.*, 1988). In 1990 plastid transformation was first achieved in a higher plant, tobacco, using biolistic transformation and 16S rRNA point mutations to confer resistance to spectinomycin (Svab *et al.*, 1990a; Svab *et al.*, 1990b). PEG-mediated plastid transformation was first achieved in tobacco using a 16S rRNA binding-type marker (Golds *et al.*, 1993). Low transformation efficiencies using the binding-type markers lead to the emphasis in plastid transformation shifting to expression of the *aadA* gene as a positive selectable marker, which resulted in the development of more efficient transformation systems (Svab and Maliga, 1993; Zoubenko *et al.*, 1994).

Eight years after plastid transformation was first achieved in tobacco the plastome of a second higher plant species, *Arabidopsis thaliana*, was successfully transformed utilising the *aadA* gene (Sikdar *et al.*, 1998). In Arabidopsis only two plastid transformed lines were generated, neither of which were fertile. In 1999 a third higher plant species, potato, was plastid transformed, using the GFP gene as a scorable marker (Sidorov *et al.*, 1999). In potato plastid transformation five transplastomic lines were regenerated, however transformation efficiencies were much lower, at approximately one transformation event in every 15 to 30 bombarded plates, compared to tobacco where approximately one transformation event per

bombarded plate has been reported (Svab and Maliga, 1993). Stable transformation of the Petunia plastome was reported in 2000. Two independent petunia lines were generated following at least four months of culture in the presence of spectinomycin and homoplasmy demonstrated by Southern blot analysis (Schaaf *et al.*, 2000). Plastid transformation was reported in a fifth higher plant species, tomato, in 2001 (Ruf *et al.*, 2001). In tomato six plastid transformed lines were generated from 60 bombarded leaf samples following a drastic extension of the primary selection phase to three to four months under extreme low-light conditions. The tomato plastid transformants were fertile and maternal inheritance of the plastome was confirmed in the F1 progeny. More recently in 2003, the successful plastid transformation of an oilseed Brassica, *L. fendleri,* using an *aadA* :GFP translational fusion, was reported (Skarjinskaia *et al.*, 2003). In *L. fendleri* two plastid transformed lines were generated from 51 bombarded plates following three cycles of regeneration on medium containing 400 mg/l spectinomycin. One of these lines proved fertile however seed progeny segregated for spectinomycin resistance indicating that the line remained heteroplasmic. Progress towards plastid transformation has also been made in rice. A GFP: *aadA* translational fusion was expressed through the rice plastome, however the lines generated remained heteroplasmic and homoplasmy was not achieved (Khan and Maliga, 1999). In oilseed rape plastid transformation was reported using both the GFP and *aadA* genes, however homoplasmy was not achieved and only 80% of plastomes were transformed in the sole transformed line produced (Oakes and Sumita, 2000). A more recent study reported plastid transformation in oilseed rape in four lines generated from 1000 bombarded cotyledon petioles. Both the *aadA* and *Cry1Aa10* genes were successfully integrated and expressed through the oilseed rape plastome. However, a homoplasmic state was again not reached for transplastomic oilseed rape (Hou *et al.*, 2003).

The development of efficient plastid transformation systems in tobacco has led to plastid transformation being utilised as a tool to further understand the biology of plastids. Investigations have used plastid transformation to disrupt open reading

frames to determine their function (Drescher *et al.*, 2000), and to knock out known genes to further characterise their role in plastid processes (Ruf *et al.*, 2000). It has also been used to study aspects of plastid gene transcription (Bock and Maliga, 1995b; Bock *et al.*, 1996; De Santis-Maciossek *et al.*, 1999). The regulatory elements conferring plastid gene expression and mRNA stability have also been widely studied using plastid transformation to improve the technology (Staub and Maliga, 1994; Shiina *et al.*, 1998; Eibl *et al.*, 1999; Kuroda and Maliga, 2001; Suzuki *et al.*, 2003).

Plastid transformation has been utilised to express several foreign proteins. These included reporter genes such as GFP (Khan and Maliga, 1999; Sidorov *et al.*, 1999; Khan, 2001; Skarjinskaia *et al.*, 2003) and *gusA* (Bock and Maliga, 1995a; Staub and Maliga, 1995; Iamtham and Day, 2000). Several genes conferring agronomically important traits have been expressed using plastid transformation in tobacco. Examples of these conferring herbicide resistance are the *arcA* (Datta and Daniell, 1996) and petunia EPSPS (Daniell *et al.*, 1998) genes, both of which confer resistance to glyphosate, and the *bar* gene that confers resistance to phosphinothricin (PTT) based herbicides (Lutz *et al.*, 2001). Other examples are the introduction and expression of insecticidal proteins. Various forms of the *Bacillus thuringiensis* crystal (*cry*) proteins have been expressed through the tobacco plastome to confer resistance to a number of Lepidopteran, Dipteran and Coleopteran insects (McBride *et al.*, 1995; Kota *et al.*, 1999; Zhang *et al.*, 1999; De Cosa *et al.*, 2001). It is important to note that plastid transformation enabled the introduction and expression of all three genes in the Bt *Cry2Aa2* operon through a single transformation. Foreign protein expression from the Bt *Cry2Aa2* operon accumulated at 45.3% of the total soluble protein in the mature leaves and conferred high levels of resistance to several insect pests. A further significant observation from this study was that milkweed leaves dusted with transgenic pollen from the Bt *Cry2Aa2* lines were not toxic to monarch larvae indicating that the foreign protein was not expressed in the pollen (De Cosa *et al.*, 2001). High level protection against several major fungal pathogens, *Pseudomonas syringae*, *Aspergillus flavus*,

Fusarium moniliforme, Verticillium dahliae and *Colletotrichum destructivum* has been achieved by expression of an antimicrobial peptide, MSI-99 (DeGray *et al.*, 2001). A degree of drought tolerance has also been conferred in plastid transformed tobacco expressing the yeast trehalose phosphate synthase (*TPS1*) gene. Transplastomic lines accumulated 15-25 fold higher amounts of trehalose, compared to nuclear transformed lines expressing the *TPS1* gene, and were able to recover after 24 days without water whereas control lines did not (Lee *et al.*, 2003).

A further application in plastid transformation is that of phytoremediation. The mercury ion reductase (*merA*) and organomercurial lyase (*merB*) genes have been used in nuclear transformation to obtain transgenic plants resistant to low levels of mercury and organomercurials, respectively (Bizily *et al.*, 2000). In an effort to increase expression levels to enhance the ability of transgenic plants to extract and detoxify methylmercury from the soil the *mer* operon has since been expressed via the tobacco plastome (Daniell, 2001; Ruiz, 2001).

The high levels of transgene expression resulting from plastid transformation have also made this technology highly amenable to the use of transgenic plants as factories for the production of polymers, biopharmaceuticals, antibodies and edible vaccines (Giddings *et al.*, 2000; Daniell *et al.*, 2001b). A synthetic biopolymer gene (EG121) product with medicinal applications was expressed in tobacco chloroplasts at levels 100 fold higher than those expressed through the nuclear genome (Guda *et al.*, 2000). Further studies have shown the potential for pharmaceutical protein production using plastid transformation with the production of a human therapeutic protein, human somatotropin, through the tobacco plastome (Staub *et al.*, 2000). Research has also been carried out to express cholera toxin subunits through the tobacco plastome (Daniell *et al.*, 2001c). The Guys 13 monoclonal antibody, which acts against the surface protein of *Streptococcus mutans*, the major cause of dental caries, has also been successfully expressed in transgenic chloroplasts (Daniell and Wycoff, 2001). Most recently a tetanus toxin fragment, which can be used as a

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vaccine against tetanus has been expressed at between 10 and 25% of the total soluble protein in tobacco chloroplasts (Tregoning *et al.*, 2003).

1.5 Progress in the genetic modification of strawberry

Tissue culture methods have long been established for strawberry using meristems as a means of virus elimination and mass propagation (Boxus *et al.*, 1977). Regeneration systems using leaf-derived callus and later leaf disks were first developed for commercial strawberry in the late 1980's (Nehra *et al.*, 1988, 1989) and nuclear transformation using *Agrobacterium tumefaciens* was first reported in 1990 (James *et al.*, 1990; Nehra *et al.*, 1990). Additionally, Mendelian inheritance of transgenes was demonstrated by James *et al.* (1990). Procedures were later developed to allow transformation of strawberry protoplasts using electroporation (Nyman and Wallin, 1992). Commercial strawberry has also been transformed using biolistics, by introduction of gold particle coated with *Agrobacterium* into leaf explants (De Mesa *et al.*, 2000). *Agrobacterium*-mediated transformation of the diploid *Fragaria vesca* has also been achieved using whole leaves as the explant source (Mansouri *et al.*, 1996). The most commonly used selectable marker gene in strawberry transformation has been *nptII* conferring resistance to the antibiotic kanamycin.

Relatively few genes of interest for crop improvement have been transferred to strawberry since 1990. The cow pea trypsin inhibitor (CpTi) has been shown to confer significant resistance to vine weevil predation under glasshouse conditions (Graham *et al.*, 1997). However, an earlier study concluded that the survival rate of vine weevil was not reduced by feeding on strawberry containing the CpTi gene (James *et al.*, 1992). The gene encoding S-adenosylmethionine hydrolase (SAMase) has also been transformed into commercial strawberry in an attempt to regulate ethylene biosynthesis (Mathews *et al.*, 1995). Mathews *et al.* (1995) also utilised the tomato fruit-specific promoter *E4* in strawberry, however *E4* did not confer

fruit-specific expression in strawberry. The tomato *E4* promoter is associated with an increase in ethylene during tomato ripening (Cordes *et al.*, 1989). Strawberry is a non-climacteric fruit and typically shows a decline in ethylene during ripening which may account for the inactivity of the tomato promoter in strawberry. However, ethylene plays a role in strawberry flower and fruit development. For instance when apple ACC synthase and ACC oxidase genes were placed under the control of the CaMV 35S promoter and transformed into strawberry the resultant transgenic flowers retained their petals for significantly longer times than non-transgenic controls, presumably through constitutive down regulation of ethylene induced abscission (D. James, pers Comm.). The petunia floral binding protein 7 (FBP7) gene promoter has also been used to drive expression of the *gusA* reporter gene in strawberry (Schaart *et al.*, 2002). In petunia the FBP7 promoter is active in floral receptacles, however in strawberry *gusA* transgene expression was observed primarily in floral and fruit tissues, but also in the roots and petioles.

Strawberry GM research has recently focused on the isolation and characterisation of ripening-related genes from strawberry (Manning, 1998). Screening of a fruit-specific cDNA library lead to the isolation of a cinnamyl alcohol dehydrogense gene from strawberry, whilst this gene presented a differential expression pattern in ripening fruit quantitative real time PCR studies showed that this gene was also expressed in the stolons, leaves and floral organs of strawberry (Blanco-Portes *et al.*, 2002). Two pectate lyase genes recently characterised in strawberry were shown to be expressed in a fruit-specific manner during ripening using quantitative reverse transcriptase PCR methods (Benitez-Burraco *et al.*, 2003). Two endo-β-1,4-glucanase genes, also associated with ripening, have been isolated from strawberry and their promoter regions analysed using the *gusA* reporter gene in a transient expression assay which involved injecting fruit at different developmental stages with *Agrobacterium* harbouring different plasmids containing various truncated forms of the promoter (Spolaore *et al.*, 2003). This study found that the longest promoter fragments used conferred the highest levels of *gusA* activity, however

expression was only studied in fruit tissues and stably transformed strawberry expressing *gusA was* not produced. To date there has been no report demonstrating tissue-specific expression of foreign genes in stably transformed strawberry.

1.6 Aims of the investigation

A major concern regarding the use of GM crops is that of safety and whether they will pose a risk to either human health or to the environment. The major aim of this study was to address some of these concerns associated with particular regard to the engineering of GM strawberry. The strategy taken was to refine the current technology by developing methodologies for the regulation and targeting of transgene expression in strawberry tissues and organelles. The strategies adopted to achieve more refined, regulated and predictable transgene expression to address the perceived risks are:-

1)   Isolation and characterisation of a root-specific promoter from strawberry for use in directing root-specific expression of transgenes in nuclear transformed GM plants.

2)   Development of plastid transformation in strawberry to achieve high-level plastid localised transgene expression, with transgene containment through restriction of escape and expression in pollen.

3)   Development of a non-antibiotic selection system using plastid transformation.

At the start of this project none of the methods described for removing or avoiding the use of antibiotic / herbicide resistance marker genes in plastid transformation had been published.

Chapter 2

Materials and methods

2.1 Growth of plant material

2.1.1 Establishment of *in vitro* strawberry proliferating shoot cultures

Cultivated strawberry, *Fragaria ananassa* cv. Calypso, was used for all strawberry experiments. Meristems were excised from newly formed strawberry runner tips under aseptic conditions these were then placed on 10 ml of SMI medium [Appendix 1] in sterile coulter pots [Ratiolab®] under artificial lighting (16 hr day) at 22°C. After ten weeks proliferating crowns were transferred to 100 ml of SC5 proliferation medium [Appendix 1] in sterile honey jars. At five-week intervals proliferating cultures were divided and transferred aseptically, three young crowns per jar, onto new medium to maintain healthy growth.

2.1.2 Establishment of *in vitro* rooted strawberry cultures

Individual crowns from five-week old proliferating cultures were isolated, their leaves removed, and placed onto R13 medium [Appendix 1] for three days. These were subsequently transferred, three per jar, to 100 ml R37 medium [Appendix 1] for five weeks, during which time roots form and fresh leaves are produced. Newly formed, but fully expanded, dark green trifoliate leaves were excised under aseptic conditions; the three leaflets separated and used either in regeneration or transformation experiments.

2.1.3 Establishment of *in vitro* tobacco shoot cultures

Tobacco (*Nicotiana tabacum*) cultivars, Ottawa and Petit Havana were used in tobacco experiments. Tobacco seeds were surface sterilised by washing five times in 10% [v/v] Domestos solution [Unilever] for two to three minutes, followed by six rinses

in sterile distilled water and a final rinse in 10% [v/v] Plant Preservative Mixture™ (PPM™) [Plant Cell Technologies]. Sterilised seed were germinated on 100 ml RM medium [Appendix 1] in magenta pots [Sigma] under artificial lighting (16 hr day) at 25°C. Excess sterilised seed were stored desiccated at 4°C for later use. After two weeks, seedlings were aseptically transferred, one per pot, to fresh RM medium. Seedlings were maintained in the juvenile phase by excising and transferring shoot tips to fresh RM medium every three to four weeks.

Uniformly green, spatulate leaves of at least 2.5 cm x 4 cm from three to four week old cultures were excised for use in regeneration and transformation experiments.

2.1.4 Acclimatisation and glasshouse growth of strawberry

Transgenic shoots from strawberry transformation experiments were aseptically excised and transferred to coulter pots containing 10 ml SMI shoot induction medium [Appendix 1] containing the appropriate selective agent for the transformation being undertaken. For shoots generated by nuclear transformation (section 2.5.2) using the pSCV1.6 and pSCVFavRB7 vectors (Figures 4.1 and 4.2) 25 mg/l kanamycin and 200 mg/l Claforan® were used. For shoots generated by plastid transformation (section 2.5.4) using the pFavAG and pFavXG vectors (Figures 5.12 and 5.14) xylose as the sole carbon source or 200 mg/l spectinomycin were used, respectively. After four weeks, large crowns were excised and rooted as described in section 2.1.2. Once rooted, plants were weaned onto sterilised compost and transferred to the glasshouse. Summer conditions were natural heat and light; supplementary heat and light were supplied during the winter to maintain 20°C ± 5°C under a 16 hr day. To promote rapid production of fruit, strawberry plants were transferred to a Sanyo controlled environment cabinet under a 16 hr day, with a 'day' temperature of 20°C and a 'night' temperature of 14°C.

2.1.5 Acclimatisation and glasshouse growth of tobacco

Transgenic shoots from tobacco transformation experiments were aseptically excised and transferred to Magenta pots containing 100 ml RM medium [Appendix 1] containing the appropriate selective agent for the transformation being undertaken; 300 mg/l kanamycin for nuclear transformation (section 2.5.3) using the pSCV1.6 and pSCVFavRB7vectors (Figures 4.1 and 4.2), xylose as the sole carbon source and 500 mg/l spectinomycin for plastid transformation (section 2.5.5) using the pNtAG and pNtXG vectors (Figure 5.10 and 5.11) respectively. After three to four weeks rooted plants were weaned onto sterilised compost and transferred to the glasshouse. Summer conditions were natural heat and light; supplementary heat and light were supplied as for strawberry.

2.2 Gel electrophoresis methods

2.2.1 Agarose gel electrophoresis

To visualise the products of genomic DNA (gDNA) extraction, PCR and restriction endonuclease digests of either plasmid or gDNA, DNA samples were typically size fractionated by electrophoresis through 0.8% [w/v] agarose / 0.5 x TBE (45 mM Tris-borate, 5 mM EDTA) gels containing 0.5 mg/ml ethidium bromide. However, for Southern blot hybridisation digested DNA was typically size fractionated by electrophoresis through 0.6% [w/v] agarose / 0.5 x TBE gels. One-sixth volume loading dye (15% [w/v] ficoll, 0.25% [w/v] bromophenol blue and 0.25% [w/v] xylene cyanol FF in sterile distilled water) was added to the samples prior to electrophoresis. PCR amplification products and fragments of restriction endonuclease digestion required for cloning were typically size fractionated by electrophoresis on 0.8% [w/v] agarose / 1 x TAE (40 mM Tris-acetate, 10 mM EDTA) gels containing 0.5 mg/ml ethidium bromide. DNA was visualised by UV illumination and photographed using a Polaroid MP4$^+$ instant camera system.

2.2.2 Polyacrylamide gel electrophoresis

To gel-fractionate DNA fragments less than 200 bp, precast 5% [w/v] polyacrylamide 1 x TBE gels (90 mM Tris-borate, 10 mM EDTA) [BioRad] were used. One-sixth volume loading dye (section 2.2.1) was added to DNA samples prior to electrophoresis. Gels were then stained in a solution containing 50 μg/ml ethidium bromide, with gentle shaking for 30 min. Gels were de-stained using distilled water for 30 min before visualisation by UV illumination.

2.2.3 Extraction of DNA from agarose and polyacrylamide gels

The required DNA fragments from either 1 x TAE agarose or 1 x TBE polyacrylamide gels were excised using a clean scalpel. DNA was extracted and purified from the gel slices using the QIAEX II kit [Qiagen] exactly following the manufacturer's instructions.

2.2.4 Electrophoresis and visualisation of RNA

Total RNA was quantified using a GeneQuant [Pharmacia] and fractionated through 1.2 % [w/v] agarose / 0.1x TPE (3.6mM Tris, 3 mM sodium di-hydrogen phosphate, 0.1 mM EDTA), 50 % [v/v] formamide gels. Total RNA was denatured at 65°C for 5 min in a 20 μl solution of 60 % [v/v] formamide in 0.1x TPE, placed on ice for 5 min and 3 μl sample buffer (50 % [w/v] glycerol, 0.1 % [w/v] bromophenol blue) added prior to loading. Gels were run at 20 mA for 2 – 3 hr in 0.1x TPE without submerging the gel. Post electrophoresis the gel was soaked in 2 μg/ ml ethidium bromide solution for 30 min, washed in distilled water for 30 min and then visualised on a UV transilluminator.

2.3 Standard cloning methods

2.3.1 Preparation of competent cells

Competent cells were either purchased as One Shot® TOP10 chemical or electro competent *E.coli* [Invitrogen], or prepared in the laboratory. To prepare competent cells a single colony of XL1 Blue MRF' *E.coli,* cultured on an LB agar (1.0% [w/v] tryptone, 0.5% [w/v] yeast extract, 1.0% [w/v] sodium chloride, pH 7.0, bacto agar 15 g/l) plate containing 10 μg/ml tetracycline, was used to inoculate 10 ml LB broth (1.0% [w/v] tryptone, 0.5% [w/v] yeast extract, 1.0% [w/v] sodium chloride, pH 7.0) and cultured at 37°C at 225 rpm overnight. A fresh 100 ml LB broth culture was initiated the following day using 1 ml of the overnight suspension. This was incubated at 37°C at 225 rpm for approximately 1 hr until an OD_{600nm} of 0.2 was reached. At this point 1 ml of 2 M magnesium chloride was added. Incubation was continued until an OD_{600nm} of between 0.45 – 0.55 was reached. The culture was subsequently incubated on ice for 2 hr, centrifuged in 50 ml falcon tubes [Sarstedt] at 5 500 rpm at 4°C for 5 min and the supernatant removed. The pelleted cells were resuspended in 50 ml Ca^2/Mn^{2+} solution (40 mM sodium acetate, 100 mM calcium chloride, 70 mM manganese chloride, pH 5.5), incubated on ice for 1 hr and then centrifuged at 3000 rpm at 4°C for 5 min. After removal of the supernatant the pelleted cells were resuspended in 2.5 ml Ca^2/Mn^{2+} solution containing 15% [v/v] glycerol and flash frozen in 150 μl aliquots using liquid nitrogen. Aliquots of competent cells were stored at -80°C.

2.3.2 Blunt-ending of DNA fragments

To facilitate cloning reactions it was occasionally necessary to remove protruding bases from DNA termini following restriction endonuclease digestion. Digested DNA fragments were gel excised as described in section 2.2.3 and 2.5 μg suspended in 20 μl reaction mix (1 x T4 polymerase buffer [Promega], 50 μg / ml Bovine Serum Albumen, 100 μM dNTP's, 7.5 U T4 DNA polymerase [Promega]). The reaction was incubated at 12°C for 20 min and then stopped by

incubating at 75°C for 10 min. Blunt-ended DNA was incubated on ice, or stored at - 20°C prior to gel fractionation using 1 X TAE gels as described in section 2.2.1. Gel fractionated fragments were extracted and purified as described in section 2.2.3 prior to use in ligation experiments.

2.3.3 Dephosphorylation of DNA

To prevent self-annealing of restriction endonuclease digested plasmid DNA during ligation, 5' phosphate groups were removed from DNA termini using Calf Intestinal Alkaline Phophatase (CIP) [New England Biolabs]. DNA was suspended in 1 x NEBuffer 3 at a concentration of 2.5 µg / 50 µl and 10 U of CIP added. For DNA with 5' protruding ends this mixture was incubated at 37°C for 1 hr. DNA with 5' recessed or blunt ends was incubated at 50°C for 1 hr. To stop the reaction and to prevent any CIP being carried over into successive reactions the incubated samples were passed through a QIAquick spin column [Qiagen] and the PCR purification kit protocol followed.

2.3.4 Ligation of DNA fragments

T4 DNA Ligase was used to clone DNA fragments into plasmid vectors. Relative amounts of vector and insert were quantified on agarose gels prior to ligation. Typically ligation reactions involved two vector: insert DNA ratios, 1: 3 and 1: 5. In thin walled 0.5 ml microfuge tubes, 30 fM of vector DNA and 90 fM or 150 fM of insert DNA, were added to 1 x T4 DNA ligase buffer solution [New England BioLabs] to a total volume of 18 µl. Following the addition of 2 µl of 400 U/µl T4 DNA Ligase [New England BioLabs] the reaction was mixed and centrifuged for 5 sec prior to incubation. DNA with cohesive ends and DNA with blunt ends were then incubated overnight at 24°C and 14°C respectively.

2.3.7 TOPO plasmid transformation

For rapid (5 min) cloning of PCR gel purified products into host plasmids the TOPO series of vectors [Invitrogen] were used. For blunt ended PCR products amplified using Platinum® Pfx DNA polymerase [Invitrogen] the pCR®-Blunt II-TOPO vector was used. For PCR products amplified using Hot Star Taq [Qiagen] or standard Taq [Invitrogen] the pCR®2.1-TOPO® vector was used. In both cases the manufactures instructions were followed exactly.

2.3.8 Transformation of *Escherichia coli*

Competent cells were thawed on ice and 50 µl transferred to a 1.5 ml microfuge tube containing 5 µl of ligation reaction. This mixture was incubated on ice for 30 min, incubated at 42°C for 90 sec and then returned to ice. After two minutes on ice, 150 µl of SOC medium at room temperature (2.0% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.05% [w/v] sodium chloride, 20 mM glucose, 2.5 mM potassium chloride, 10 mM magnesium chloride, pH 7.0) was added to each reaction and they were subsequently incubated at 37°C with gentle shaking for 1 hr. Following this the cells were plated out pre-warmed (37°C) LB agar plates (1.0% [w/v] tryptone, 0.5% [w/v] yeast extract, 1.0% [w/v] sodium chloride, pH 7.0, bacto agar 15 g/l) containing the appropriate antibiotic for the construct in use.

2.3.9 Oligonucleotide annealing to form synthetic adapters

Individual oligonucleotides were dissolved in sterile distilled water at 400 pmol/ µl and 5 µl added to 15 µl annealing buffer (40 mM Tris HCl, pH 7.5, 20 mM magnesium chloride, 50 mM sodium chloride). Individual reactions were heated to 70°C for 5 min and then pairs combined to form adapters. Reactions were held at 70°C for 2 min, allowed to cool slowly to room temperature and then stored at −20°C.

2.4 Extraction of nucleic acids

2.4.1 Genomic DNA extraction

Approximately 500 mg of young fully expanded leaf tissue (stored at -80°C) were ground to a fine powder in a mortar and pestle under liquid nitrogen and 5 ml of buffer 1 (50 mM Tris HCl, pH 8.0, 5 mM EDTA, 350 mM sorbitol, 0.3% [v/v] mercaptoethanol, 10% [w/v] polyethelene glycol 6000) added. The thawed homogenate was transferred to a sterile 15 ml tube and centrifuged at 5500 rpm for 20 min at 4°C. The aqueous layer was removed and the remaining pellet resuspended in 2.5 ml buffer 2 (50 mM Tris HCl, pH 8.0, 5 mM EDTA, 350 mM sorbitol, 0.3% [v/v] mercaptoethanol, 1% [w/v] sodium sarkosyl, 710 mM sodium chloride, 0.1% [w/v] CTAB) and incubated at 60°C for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added and the mixture gently shaken at room temperature for 15 min. The homogenate was then centrifuged at 5500 rpm for 30 min at room temperature. The aqueous phase was transferred to a fresh 15 ml tube and the chloroform: isoamyl alcohol extraction repeated. The resulting aqueous phase was transferred to a fresh 15 ml tube, 100 µg RNAse A added and the solution incubated at 37°C for 1 hr. Two-thirds volume of propan-2-ol at -20°C was added, the solution transferred to 2 ml eppendorf tubes and centrifuged at 12000 rpm for 20 min at 4°C. After removal of the supernatant the remaining pellet was washed in 70% [v/v] ethanol, air-dried and resuspended in 100 µl TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA). Genomic DNA was stored at -20°C until required for use.

2.4.2 Plasmid DNA extraction

A single bacterial colony harbouring plasmid DNA was used to inoculate 5 ml of LB medium (1.0% [w/v] tryptone, 0.5% [w/v] yeast extract, 1.0% [w/v] sodium chloride, pH 7.0) containing the antibiotics appropriate for the plasmid in use. The inoculum was shaken at 225 rpm at 37°C for 16 hr, transferred to microfuge tubes, centrifuged at

13,000 rpm for 10 min and the supernatant removed. Plasmid DNA was extracted from the pelleted cells using a QIAprep spin kit [Qiagen] following the manufacturer's instructions. Plasmid DNA was quantified using a GeneQuant II spectrophotometer [Pharmacia Biotech] and stored at -20°C.

2.4.3 RNA extraction

Total RNA was extracted from 200 mg of plant tissue, using the RNeasy kit [Qiagen] following the manufacturer's instructions, and stored at -80°C. Messenger RNA (mRNA) was extracted from either total RNA using Oligotex resin [Qiagen], or directly from 100 mg of plant tissue ground under liquid nitrogen using the Dynabeads® mRNA DIRECT™ kit [Dynal]. In both cases the manufacturers' instructions were followed and mRNA was subsequently stored at -80°C.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 Reverse transcriptase PCR (RT-PCR)

Messenger RNA extracted from plant tissues as described in section 2.4.3 was converted into complementary DNA (cDNA) using a reverse transcriptase kit [Omniscript] following the manufacturer's instructions. A 5 µl aliquot of mRNA was added to 15 µl reverse transcriptase reaction mix (1 x buffer RT [Omniscript], 0.5 mM dNTP's, 1 µM oligo-dT primer, 10 U RNAse inhibitor [Invitrogen], 4 U reverse transcriptase [Omniscript]). The reaction was mixed briefly and incubated at 37°C for 1 hr before inactivating the enzyme by heating at 93°C for 5 min. cDNA synthesised in this manner was stored at -20°C for up to one week.

RT-PCR was carried out using Platinum® Pfx DNA polymerase [Invitrogen]. In a 50 µl reaction (1 x Pfx amplification buffer, 0.3 mM dNTP, 2 mM magnesium sulphate, 2 µM forward and reverse primer, 2.5 U Platinum® Pfx) 2 µl cDNA served as template. PCR cycling conditions were generally, Stage 1: 94°C for 2 min; Stage 2: 94°C for 30 sec, 60°C for 1 min, 68°C for 1.5 min, for 30 cycles; Stage 3: 68°C for 10 min.

However if required the annealing temperature was altered to compensate for annealing temperature variation between different sets of primers. Amplification products were visualised by electrophoresis on 0.8% [w/v] agarose / 1 x TAE gels (section 2.2.1).

2.5.2 DNA isolation and modification using PCR

PCR was used to identify and amplify genes and regulatory elements from strawberry using a proof-reading polymerase. Primers were designed to introduce specific restriction enzyme recognition sites to the 5' and 3' termini of amplified sequences to enable cloning into transformation vectors. All modifications were carried out using Platinum[®] Pfx DNA polymerase [Invitrogen]. Both genomic and plasmid DNA were used as templates in these reactions where 1 µl of 10 pg – 100 ng /µl DNA was added to 49 µl Pfx reaction mix (1 x Pfx amplification buffer, 0.3 mM dNTP, 1 mM magnesium sulphate, 0.3 µM forward and reverse primer, 1 U Platinum[®] Pfx). PCR cycling conditions were generally, Stage 1: 95°C for 1 min; Stage 2: 94°C for 30 sec, 55°C for 30 sec, 68°C for 1 min, for 30 cycles; Stage 3: 68°C for 10 min. If required the annealing temperature and extension time was altered to account for primer annealing temperature variation and amplification product lengths of above 1 kb, respectively. Amplification products were visualised by electrophoresis on either 1 x TAE agarose or 1 x TBE polyacrylamide gels (section 2.2.1).

2.5.3 Screening of bacterial colonies for plasmids using PCR

A single bacterial colony was picked from a transformation plate and mixed into 25 µl PCR reaction mix (1 x PCR buffer [Invitrogen], 0.2 mM dNTP's, 1.5 µM magnesium chloride, 0.5 µM forward and reverse primer, 1 U Taq polymerase [Invitrogen]). PCR cycling conditions were generally, Stage 1: 95°C for 2 min; Stage 2: 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, for 30 cycles; Stage 3: 72°C for 10 min. If required the annealing temperature was altered to account for annealing temperature

variation between primers. Amplification products were visualised by electrophoresis on 0.5 x TBE agarose gels (section 2.2.1).

2.5.4 Screening of putative transgenic plants using PCR

Genomic DNA (gDNA) was extracted from putative transgenic tobacco and *in vitro* strawberry leaf material using a DNeasy kit [Qiagen] following the manufacturers' instructions exactly. Typically 0.5 µl of gDNA served as template in a 25 µl PCR reaction. Primers were designed to amplify foreign DNA inserted into either the chloroplast or nuclear genome. In PCR reactions using gDNA from putative plastid transformants Platinum® *Pfx* DNA polymerase [Invitrogen] was used, as described in section 2.5.2. For putative nuclear transformants *Taq* polymerase [Invitrogen] was employed, as outlined in section 2.4.3. Amplification products were visualised by electrophoresis on 0.5 x TBE agarose gels (section 2.2.1).

2.5.5 Genome walking using PCR

Clontech's Universal Genome Walker™ Kit was used to isolate nucleotide sequence upstream of the strawberry *FavRB7* gene. The manufacturer's instructions were followed using strawberry gDNA, isolated as described in section 2.4.1. Gene specific primers, TOBRB71A and TOBRB71B [Appendix 2] were designed and used with Platinum® *Pfx* DNA polymerase to amplify sequence upstream of the *FavRB7* gene by PCR.

Amplification products generated from the Genome Walker™ procedure were gel-purified as described in section 2.2.3 and cloned into *Sma*I cut, dephosphorylated pBluescript vector (EMBL: X52327). Ligation products were transformed into XL1 Blue MRF', as outlined in section 2.2.7. Following screening by restriction enzyme analysis, colonies shown to contain inserts of the anticipated size were sequenced.

2.6 Genetic transformation of plants

2.6.1 Transformation of _Agrobacterium tumefaciens_

Electro-competent cells of _Agrobacterium tumefaciens_ strain EHA101 were prepared according to the method outlined by Wen-jun and Forde (1989) and stored in 40 µl aliquots at – 80°C. Frozen cells were thawed on ice, 1 µl of a 300 ng/µl plasmid stock (pSCV1.6 or pSCVFavRB7, section 4.2.1) added and the mixture transferred to a pre-chilled 0.2 cm electroporation cuvette [EquiBio Ltd]. An electric pulse of 2.5 kV was applied to the cuvette using a BioRad _E.coli_ pulser and 1 ml of SOC medium at room temperature (2.0% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.05%[w/v] sodium chloride, 20 mM glucose, 2.5 mM potassium chloride, 10 mM magnesium chloride, pH 7.0) added immediately. The mixture was then transferred to a 1.5 ml microfuge tube and shaken at 29°C for 2 hr. Aliquots of 250 µl were plated out onto LB agar plates (1.0%[w/v] tryptone, 0.5%[w/v] yeast extract, 1.0%[w/v] sodium chloride, pH 7.0, bacto agar 15 g/l) containing 20 µg/ml gentomycin and 25 µg/ml kanamycin and incubated for two days at 28°C. Single colonies were streaked onto LB agar plates containing the same selective agents and incubated for two days at 28°C. Single colonies from streaked plates were subjected to PCR analysis as described in section 2.4.3 to confirm successful transformation of the plasmid into _Agrobacterium tumefaciens_.

2.6.2 Nuclear transformation of strawberry

Leaflets isolated from rooted strawberry plants (section 2.2.1) were placed adaxial side up on 35 ml ZN102 regeneration medium [Appendix 1] in 9 cm Petri dishes and cultured under 16 hr light at 25°C for 2 d prior to transformation. On the day of transformation each leaflet was cut into 6 – 8 pieces producing explants of 0.5 – 1.0 cm^2, which were placed, 50 per Petri dish, on ZN102 medium.

Single colonies of _Agrobacterium tumefaciens_ containing the pSCVFavRB7 and pSCV1.6 plasmids (section 4.2.1) were used to inoculate liquid LB medium (1.0%[w/v] tryptone, 0.5%[w/v] yeast extract, 1.0%[w/v] sodium chloride, pH 7.0), and grown to

log-phase of OD_{600nm} 1 - 1.5. The culture was then pelleted at 3 000 rpm for 15 min and resuspended to an OD_{600nm} of 0.25 in filter sterilised virulence induction medium (2% [w/v] sucrose, 4.4 g/l Murashige and Skoog powder including vitamins [Duchefa], 0.1 M betaine phosphate, 0.1 mM acetosyringone, pH 5.2) and cultured at 22°C with gentle shaking for 5 hr.

Following virulence induction, 20 ml of *Agrobacterium* suspension was applied to each Petri dish and infection of the explants continued for 15-30 min. Infected explants were then blotted on sterile filter papers and transferred to fresh ZN102 medium overlaid with 7 cm diameter filter paper wetted with 500 μl virulence induction medium. The explants were co-cultured with the *Agrobacterium* in the dark for 3 d at 25°C, after which they were transferred to filter sterile wash solution (500 mg/l Augmentin™[Duchefa], 200 mg/l Claforan® [Rousell]) and gently shaken at 22°C for 4 hr. Washed explants were blotted and placed adaxial side up, 10 per Petri dish, on ZN102 medium supplemented with 200 mg/l Claforan® and 50 mg/l kanamycin to induce callus formation from the wound sites. The explants were cultured in this manner under artificial lighting (16 hr day) at 25°C for five weeks, after which they were transferred to Z1 medium [Appendix 1] supplemented with 200 mg/l Claforan® and 50 mg/l kanamycin [Sigma], to induce shoot formation.

Transgene integration was confirmed in regenerated shoots using PCR as described in section 2.4.4, and by using the β-glucronidase (GUS) histochemical assay as described in section 2.7.1. Transgenic plants were then weaned and transferred to the glasshouse as described in section 2.1.4.

2.6.3 Nuclear transformation of tobacco

Nuclear transformation of tobacco was carried out essentially as described for strawberry (section 2.6.2) but with several key variations. Single leaves were excised from 3 – 4 week old *in vitro* tobacco cultures of Ottawa and Petit Havana, from which explants of ~ 0.5 cm^2 were excised on the day of transformation. These explants were placed on RMOP regeneration medium [Appendix 1] in Petri dishes.

Agrobacterium tumefaciens preparation, virulence induction, explant infection, co-cultivation and post co-cultivation washes were carried out as described in section 2.5.2 for strawberry. Washed explants were then blotted on sterile filter paper and placed abaxial side up, 10 per Petri dish, on RMOP medium, supplemented with 200 mg/l Claforan® and 300 mg/l kanamycin, to induce shoot formation and cultured in this manner under artificial lighting (16 hr day) at 25°C

Transgene integration was confirmed in regenerated shoots using PCR as described in section 2.4.4, and by using the β-glucronidase (GUS) histochemical assay as described in section 2.7.1. Transgenic plants were then transferred to the glasshouse (section 2.1.5).

2.6.4 Plastid transformation of strawberry

Four to five leaflets isolated from rooted Calypso strawberry plants, as described in section 2.2.1, were placed in the centre of a Petri dish containing regeneration medium ZN102 [Appendix 1]. Petri dishes were then stacked and sealed in a plastic sleeve and pre-cultured under artificial lighting (16 hr day) at 25°C for one or two days prior to biolistic transformation.

Plastid transformation was carried out using the BioRad Biolistic® PDS-1000/He System particle gun to bombard leaf tissue with gold particles coated with plastid transformation vector DNA.

Leaflets were bombarded with approximately 1.7 μg of plastid transformation vector, either pFavXG or pFavAG (section 5.3), precipitated onto 0.2 mg of 0.6 μm gold following the method described by (Maliga *et al.*, 1993). Controls were included where plant tissue was bombarded with gold not coated with DNA. Immediately after bombardment the Petri dishes were again stacked and sealed in a plastic sleeve and returned to the same growth conditions as previous.

Several experimental variables were evaluated. These included orientation of the explant (either abaxial or adaxial side up), length of pre-culture, overlaying pre-culture medium with sterile filter paper, bombardment pressure, position of the

target tissue in relation to the firing platform, the use of extra wounding prior to bombardment and osmotic treatment.

Two days after bombardment each leaflet was divided to yield explants of approximately 0.5 cm^2 in size. Explants were placed adaxial side up on ZN102 medium containing selective agents (20 mg/l spectinomycin for the pFavAG bombardments and medium containing 2.8% [w/v] xylose 0.2% [w/v] glucose for pFavXG bombardments). The plates were sealed and cultured at 25°C under low-level light intensities (1-3 µmol/m^2/s) to induce callus formation and shoot regeneration. Explants from 'control' bombardments were cultured on both selective and non-selective medium.

After five weeks explants were transferred to fresh ZN102 medium containing the selective agents, 200 mg/l spectinomycin for explants bombarded with pFavAG and 2.8% [w/v] xylose and only 0.2% [w/v] glucose selection for pFavXG bombardments. Thereafter explants were routinely sub-cultured on medium containing selection at five-weekly intervals.

Shoots regenerating from bombarded leaf tissue were excised once 3 – 4 trifoliate leaves had formed. The regenerated crown tissue with the two youngest leaves, the R1 line, was transferred to SMI medium [Appendix 1] containing selection. The older leaves were excised and subject to further rounds of repeat regeneration upon selective medium (500 mg/l spectinomycin for pFavAG bombardments and 2.9% [w/v] xylose and only 0.1% [w/v] glucose as the carbohydrate source for pFavXG bombardments) in order to promote homoplasmy in the subsequent R2 lines regenerated. A proportion of R1 leaves generated from pFavAG bombardments were subject to repeat regeneration upon ZN102 medium containing 200 mg/l streptomycin and 200 mg/l spectinomycin to screen for spontaneous mutations conferring spectinomycin resistance.

At each stage of regeneration, explants were screened visually for the presence of green fluorescent protein (GFP) and assessed using PCR to confirm transgene integration and evaluate progression towards homoplasmy. Repeat regeneration was

continued until a homoplasmic state had been achieved and confirmed at the molecular level.

2.6.5 Plastid transformation of tobacco

Uniformly green, spatulate leaves were isolated from 3 – 4 week old *in vitro* tobacco cultivars Ottawa and Petit Havana and placed abaxial side up in the centre of a Petri dish containing regeneration medium RMOP [Appendix 1] overlaid with 7 cm diameter sterile filter paper. Petri dishes were then stacked and sealed in a plastic sleeve and cultured under artificial lighting (16 hr day) at 25°C for 1 d prior to biolistic transformation.

Plastid transformation was carried out essentially as described for strawberry. Each leaf was bombarded with approximately 1.7 µg of plastid transformation vector, either pNtXG or pNtAG (section 5.3), precipitated onto 0.2 mg 0.6 µm gold particles following the method described by Maliga *et al.* (1993). Controls were included where plant tissue was bombarded with gold uncoated with DNA. Immediately after bombardment the Petri dishes were again stacked in a plastic sleeve and returned to the growth room for 2 d.

Two days after bombardment each leaf was divided to yield explants of approximately 0.5 cm^2 in size. Explants were placed abaxial side up, 10 per Petri dish, on RMOP medium containing selective agents, 500 mg/l spectinomycin for pNtAG bombardments and medium containing 3% [w/v] xylose as the sole carbohydrate source for pNtXG bombardments. To induce callus formation and shoot regeneration, explants bombarded with pNtAG were cultured at 25°C under 16 hr light conditions. Explants bombarded with pNtXG were cultured at 25°C in the dark. Explants from 'control' bombardments were cultured on both selective and non-selective medium.

After five weeks, explants were transferred to fresh RMOP medium containing selective agents and pNtXG bombarded explants were brought into the light to promote healthy shoot formation at this stage.

Shoots regenerating from bombarded leaf tissue were excised once 3 – 4 leaves had formed. The regenerated shoot tip with the two youngest leaves, the R1 line, was transferred to RM medium, in a Magenta pot, with selection. The older leaves were excised and subject to further rounds of repeat regeneration upon selective medium (500 mg/l spectinomycin for pNtAG bombardments and 3% xylose as the sole carbohydrate source for pFavXG bombardments).

Repeat regeneration was employed to promote homoplasmy in the subsequent R2 lines produced. A proportion of R1 leaves generated from pNtAG bombardments were subject to repeat regeneration on RMOP medium containing 500 mg/l streptomycin and 500 mg/l spectinomycin to screen for spontaneous mutations conferring spectinomycin resistance.

Repeat regeneration of pNtXG lines was carried out in the dark for the first five weeks to further inhibit shoot production and then transferred to the light to generate R2 lines.

At each stage of regeneration, explants were screened visually for the presence of GFP and assessed using PCR to confirm transgene integration and evaluate progression towards homoplasmy. Repeat regeneration was continued until a homoplasmic state had been achieved and confirmed at the molecular level.

2.7 Nucleic acid blot hybridisation

2.7.1 Southern blot hybridisation

a) Restriction endonuclease digestion and electrophoresis of genomic DNA restriction fragments

Genomic DNA was digested in a 150 µl reaction containing 10 µg gDNA and three to ten units of restriction enzyme per µg of DNA, in a 1 x enzyme reaction buffer supplemented with 0.3 mg/ml Bovine Serum Albumen (BSA) and 4 mM spermidine. Reactions were incubated at 37°C for 16 hr except for *Sma* I digests, which were

incubated at 25°C. Following digestion, 15 μl of 3 M sodium acetate (pH 5.5) and 450 μl absolute ethanol were added to each 150 μl reaction and the mixture incubated at -20°C for 30 min. The reaction was subsequently centrifuged at 13, 000 rpm for 30 min, the supernatant removed, and the pellet washed in 70% [v/v] ethanol. DNA pellets were dried *in-vacuo* and resuspended in 30 μl TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA). Prior to electrophoresis (section 2.2.1), digested DNA was heated to 65°C for 10 min and then transferred to ice. After addition of one-sixth volume loading dye (section 2.2.1), samples were allowed to settle in the wells for 30 min before initiating electrophoresis. Size fractionated, digested DNA was visualised by UV illumination.

b) Transfer of digested genomic DNA to nylon membrane

Post electrophoresis, gels were soaked in depurination solution (250 mM HCl) for 10 min and thoroughly rinsed in distilled water. Rinsed gels were then transferred to denaturation solution (0.5 M sodium hydroxide, 1.5 M sodium chloride) for 2 x 15 min washes and then to neutralisation solution (0.5 M Tris HCl, pH 7.5, 3 M sodium chloride) for 2 x 15 min washes. Digested genomic DNA was transferred from the neutralised gels to a positively charged nylon membrane [Roche Molecular Biochemicals] exactly as described by Sambrook *et al.* (1989). Transfer was allowed to proceed for 20 hr after which the membrane was baked at 120°C for 30 min to fix the transferred DNA.

c) Synthesis of Digoxigenin-II-dUTP (DIG) labelled probes

Digoxigenin-II-dUTP (DIG) was incorporated into double-stranded DNA probes, using plasmid DNA as template in PCR, according to the protocol outlined for the PCR DIG Probe synthesis kit [Roche Molecular Biochemicals]. PCR products were visualised on a 1% [w/v] agarose / 0.5 x TBE gel against control reactions (dTTP being incorporated instead of DIG).

d) Hybridisation and post hybridisation washes

Membranes were hybridised in rolling tubes in a Hybridising oven [Techne] according to the DIG system User's Guide for Filter Hybridisation [Roche Molecular Biochemicals]. Membranes were pre-hybridised in filter sterile DIG Easy Hyb buffer [Roche Molecular Biochemicals] for 2 hr at 42°C. The prehybridisation solution was then substituted for DIG Easy Hyb containing DIG–labelled DNA probe (section 2.6.1.c), denatured by boiling for 10 min, and hybridisation continued overnight. After hybridisation the probe solution was recovered and the membrane washed twice for 5 min in wash solution 1 (300 mM sodium chloride, 30 mM sodium citrate, pH 7.0, 0.1% SDS) at room temperature, then twice for 15 min in wash solution 2 (75 mM sodium chloride, 7.5mM sodium citrate, pH 7.0, 0.1% SDS) at 68°C and then twice for 15 min in wash solution 3 (15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0, 0.1% SDS) at 68°C.

e) Detection of Digoxigenin-II-dUTP (DIG) labelled probes

DIG-labelled DNA probes hybridised to the nylon membrane were detected exactly as described in the DIG system User's Guide for Filter Hybridisation [Roche Molecular Biochemicals] using CPD-Star™ [Roche Molecular Biochemicals]. The membrane was sealed between acetate sheets, placed in an autoradiograph cassette [GRI] and exposed to blue sensitive X-ray film [GRI]. The cassette was initially incubated at 37°C for 15 min and then incubated at room temperature. Multiple exposures were obtained from each membrane to optimise the degree of probe signal against the level of non-specific background.

2.7.2 Northern blot hybridisation

a) Extraction of total RNA

Total RNA was extracted from 80 mg – 300 mg plant tissue ground under liquid nitrogen. The homogenate was transferred to a 30 ml Oakridge tube using a baked spatula and 3 ml extraction buffer (50 mM Tris HCL, pH 8.9, 150 mM lithium chloride,

5 mM EDTA, 5 % [w/v] SDS) added. The mixture was vortexed for 2 min and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) added. Phases were separated by centrifugation at 8,000g for 15 min at 4°C and the upper phase transferred to a fresh tube and the phenol: chloroform: isoamyl alcohol extraction repeated. An equal volume of chloroform: isoamyl alcohol (24:1) was then added to the resulting aqueous phase in a fresh tube and the mixture vortexed for three min prior to centrifugation at 8,000g for 15 min at 4°C. The upper phase was then transferred to a 30 ml corex tube, 1/3 vol 8 M lithium chloride added and the sample incubated at −80°C overnight. The precipitate was collected by centrifugation at 12, 000g for 30 min at 4°C and the resulting pellet washed twice in 70 % [v/v] ethanol containing 0.15 M sodium chloride, each time centrifuging at 12,000g for 15 min at 4°C. The washed RNA pellet was air dried for 30 min, resuspended in 50 µl (diethyl pyrocarbonate treated) water and then stored at - 80°C.

b) Transfer of denatured total RNA to nylon membrane

Post electrophoresis (section 2.2.4), gels were soaked in 0.05 M sodium hydroxide for 30 min and then rinsed in distilled water for 5 min. Rinsed gels were then transferred to soak in 20x SSC for 45 min prior to transfer to positively charged nylon membrane [Roche Molecular Biochemicals] exactly as described in section 2.7.1b.

c) Hybridisation and post hybridisation washes

Membranes were hybridised in rolling tubes in a Hybridising oven [Techne] according to the DIG system User's Guide for Filter Hybridisation [Roche Molecular Biochemicals]. Membranes were pre-hybridised in filter sterile DIG Easy Hyb buffer [Roche Molecular Biochemicals] for 2 hr at 50°C. Hybridisation was then carried out exactly as described in section 2.7.1d using DIG–labelled DNA probes (section 2.6.1.c), After hybridisation the probe solution was recovered and the membrane washed three times for 5 min and once for 15 min in wash solution 1 (300 mM sodium

chloride, 30 mM sodium citrate, pH 7.0, 0.1% SDS) at room temperature, then twice for 15 min in wash solution 2 (75 mM sodium chloride, 7.5mM sodium citrate, pH 7.0, 0.1% SDS) at 68°C.

d) Detection of Digoxigenin-II-dUTP (DIG) labelled probes

DIG-labelled DNA probes hybridised to the nylon membrane were detected exactly as described for Southern analysis in section 2.7.1e.

2.8 Analysis of reporter gene activity

2.8.1 β-glucuronidase (GUS) histochemical assay

Tissues from putative transgenics containing the GUS (*gusA*) gene were excised and placed in X-Gluc solution (1 mM X-Gluc, 10 mM EDTA, 100 mM phosphate buffer (pH 7.0), 1 mM spermidine, 4 mM potassium ferricyanide, 0.1 % [v/v] Triton-X-100, 2 M ascorbate) and incubated at 37°C for 24 hr and then at 25°C for 24 hr. Chlorophyll was removed from the stained tissues by incubation in 70% ethanol with shaking at 25°C for 1-2 hr. The blue product synthesised as a result of GUS activity was observed by microscopy.

Histological studies were carried out using tissues subject to GUS histochemical assay. Tissues incubated in X-Gluc solution were fixed in 10 % [v/v] buffered formalin for 24 hr and then transferred to an ethanol series (70 %, 80 %, 95 %, 100 % [v/v]) for 24 hr each. Samples were then transferred to tertiary butyl alcohol (TBA) for three days at 25°C. Molten paraffin wax was then added to ~1/4 vol of the TBA and the solution held at 60°C for 24 hr, approximately half of the solution was then removed and replaced with molten paraffin wax and held at 60°C for a further 24 hr. This step was repeated to ensure all traces of TBA had been replaced by wax. Embedded sections were placed in a watch glass and submerged in fresh molten paraffin wax, allowed to cool and stored at room temperature prior to sectioning.

Sections were taken using a rotary microtome with the knife set at 10μm and angled at 15° to the sample block. Sections were transferred to slides coated with Haupts adhesive, overlaid with 4 % [v/v] buffered formalin and dried on a warming block until bound. Prior to visualisation, slides were cleared by three 10 min washes in Histoclear [CellPath Plc] followed by two min washes in a series of ethanol dilutions (100%, 70%, 50% and 30%) and four min in sterile distilled water. Slides were then washed for 20 sec in each ethanol series (30%, 50%, 70% and 100%) followed by a further three, 10 min washes in Histoclear. A drop of Histomount [CellPath Plc] was placed on each slide and a cover slip applied.

2.8.2 β-glucuronidase (GUS) fluorometric assay

GUS activity in strawberry tissue samples was quantified using the Fluorometric assay as described by Jefferson (1987) with a number of modifications. Frozen tissues were briefly ground in 1.5 ml microfuge tubes using a chilled plastic pestle [Berkhard Scientific Sales Ltd.] and 250 μl of GUS extraction buffer (50 mM sodium phosphate buffer, pH 7.0, 10mM Na_2 EDTA, pH 8.0. 0.1% [v/v] Triton X-100, 0.1% [w/v] sodium lauryl sarcosine, methanol 20% [v/v] 10mM β- mercaptoethanol). Grinding was continued until all tissues were homogenised. After centrifugation for 15 min at 13,000 rpm the supernatant was removed to fresh tubes. A volume of 150 μl was taken for GUS assay and 50 μl for protein assay.

For the GUS assay, the volume of the extract was raised to 500μl with extraction buffer and 200 μl MUG substrate (4mM 4-methylumbelliferyl β-D-glucuronide), dissolved in GUS extraction buffer) added to initiate the reaction at 37 °C. Background levels of GUS activity were determined by immediately transferring 200 μl of the reaction to a fresh tube containing 800 μl stop buffer (0.2M Na_2CO_3) to stop the reaction. The reaction tube was held at 37°C for 60 min after which 200 μl of the reaction was transferred to a fresh tube containing 800 μl stop buffer. The amount of 4-MU produced by the GUS enzyme in each reaction mixture was determined fluorometrically using a FLUOstar Galaxy multi-well plate reader with the excitation

filter set at 360 nm and the emission filter set at 460 nm. 4-MU activity was quantified against a series of dilutions in a mixture of two parts GUS extraction buffer and eight parts stop buffer to reproduce the stopped reaction mixture.

In parallel with the GUS assay, the protein content of each sample was determined using the protein assay II kit (BioRad) with BSA as the standard. Taking into account the dilution steps introduced during the GUS assay procedure and the concentration of protein, specific activity of the enzyme in nmoles 4-MU mg protein^{-1} min^{-1}, was calculated for each sample.

2.8.3 Visualisation of Green Fluorescent Protein (GFP) in plant tissues

GFP was visualised in plant callus and regenerating tissues using a Leica Wild MZ8 stereomicroscope fitted with a stereo fluorescence module. A GFP plant filter set [Leica] was used with the excitation filter at 470/440 nm and the barrier filter at 525/550 nm.

To visualise GFP at the sub cellular level, a Zeiss Axiophot microscope equipped with Neofluar lenses was used with an excitation filter at 450-490 nm combined with emission filters at 520 nm and 515 – 565 nm, in addition to excitation filter at 365 nm combined with emission at 420 nm. To visualise GFP expression in whole plants a hand held 254/365 nm UV GeneLamp [GRI] was utilised. Fluorescing cultures were photographed with a Nikon 'coolpix' 990 digital camera attached to the microscope.

Chapter 3

Isolation and characterisation of the strawberry root-specific gene, *FavRB7*, and its promoter

3.1 Introduction

The aim of this study was to identify a novel gene expressed solely in the root tissue in strawberry and to then isolate its promoter, which could be used to confer root-specific expression of foreign genes. The approach taken to identify the gene in strawberry was to identify well-characterised root-specific genes in other species and then to use their sequence information to isolate a homologue from strawberry.

As discussed in chapter 1 root tissues were selected as the target organ for tissue-specific transgene expression in strawberry for several reasons. Several pests and diseases particularly target the roots in strawberry and genetic modification could provide a means to introduce resistance traits directly into commercial cultivars without the need for extensive breeding efforts. The use of a strawberry root-specific promoter to express anti-fungal genes or insect resistance genes exclusively in the roots to address these problems may also go some way to satisfying regulations regarding food safety and to alleviate public concerns over the consumption of genetically modified food.

Several genes exhibiting root-specific expression were identified in the literature. The most fully characterised was the tobacco gene *TobRB7*, isolated from a root mRNA library, which was shown to be expressed exclusively in tobacco roots (Conkling *et al.*, 1990; Yamamoto *et al.*, 1991). *TobRB7* homologues have also been found to be expressed exclusively in root tissues in a number of other species; *Petroselinum crispum* (Roussel *et al.*, 1997), *Helianthus annuus* (Sarda *et al.*, 1999), *Arabidopsis thaliana* (Yamamoto *et al.*, 1990), *Daucus carota* (Kawahara *et al.*, 1997)

and *Solanum tuberosum* (Heinrich *et al.*, 1996). The presence of root-specific *TobRB7* isoforms in several plant species indicated that a homologous gene could exist in strawberry.

The *TobRB7* gene homologues all exhibit a strong likeness to the aquaporin family of proteins, which share six putative transmembrane domains with small hydrophilic loops connecting these regions (Chrispeels, 1994). Aquaporins often act as transmembrane proteins facilitating transport of water and solutes across biological membranes (Park, 1996). It has been proposed that intracellular loop B and extra-cellular loop E interact between transmembrane helices to form a water-channel unit that is penetrated by water in both directions (Jung *et al.*, 1994). In plants, aquaporins are primarily present as tonoplast intrinsic proteins (TIP) or plasma intrinsic proteins (PIP). Conserved N and C terminal amino acid sequences and loop B and loop E signature motifs distinguish the two classes of protein (Schaffner, 1998). There are various isoforms of TIP in plants, α, δ and γTIP and RB7 (Sarda *et al.*, 1999), which are found in the vacuolar membranes of a variety of tissue types.

A 1.8 kb region of 5' regulatory sequence associated with *TobRB7* (EMBL: S45406) has been well characterised (Yamamoto *et al.*, 1991). A series of repeats identified in this promoter were designated boxes A, B and C. Box A is a series of eight, 11 bp highly conserved repeats between positions - 1726 to - 1483. Box B is a pair of A/T rich regions, each of ~ 300 bp, at regions - 1290 to - 973 and - 501 to - 198 sharing 84% homology. Box C has two, 17bp perfect repeats at positions - 114 to - 98 and - 68 to - 52. Deletion analysis of the *TobRB7* promoter suggested that the regulatory elements required for root-specific expression are between positions - 636 to - 299. The *TobRB7* promoter has also been found to contain regulatory elements invoking transgene expression in response to infection by root-knot nematode (Opperman *et al.*, 1994).

To identify an RB7 TIP gene homologue in strawberry, sequence homology analysis was performed to identify conserved regions of sequence within the homologues of *TobRB7*. Degenerate primers were then designed to anneal in

conserved regions and used in PCR to isolate nucleotide sequence homologous to the *TobRB7* gene from strawberry. A PCR based approach, 'genome walking', was then used to isolate the regulatory sequence upstream of the strawberry RB7 homologue.

3.2 Identification and isolation of *FavRB7*, a root-specific gene from Strawberry

Homologues to *TobRB7* from other species were identified using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). Accession numbers are given in parentheses and refer to the EMBL database (Stoesser G., 2001). Sequence homology analysis of the gene sequences identified was carried out using the DNAstar Megalign (Lasergene) software. Primers were designed to anneal within conserved regions identified using Megalign and used to amplify corresponding regions within the strawberry genome. Primers were also designed to add restriction endonuclease sites at the 5' and 3' termini of amplified sequences to facilitate cloning.

PCR reactions were carried out using the proof reading Platinum® *Pfx* DNA polymerase as outlined in section 2.5.1. Gel isolation of amplification products and colony PCR was carried out as described in sections 2.2.3 and 2.5.3, respectively. TOPO plasmid transformation and preparation of plasmid DNA were carried out as described in sections 2.3.7 and 2.4.2, respectively. Restriction endonuclease digestion of plasmid DNA, blunt-ending, dephosphorylation and ligation of DNA fragments were all carried out as described in section 2.3

3.2.1 Identification of a *TobRB7* gene homologue in strawberry

A BLAST search carried out using the *TobRB7* gene sequences (X54855) identified seven homologous root-specific genes: *arabidopsis* (X54854), carrot (Ab000506), maize (Af057183), parsley (Z48232), potato (U65700), sunflower (X95953) and tomato (U95008). Pair wise comparisons at the nucleotide

level of the root-specific *TobRB7* homologues revealed lowest and highest homologies of 52.1% and 96.5%, respectively. However, no conserved regions suitable for degenerate primer design containing less than 5 points of degeneracy in 20 bp were identified.

The majority of the homologues identified are members of the *Asteridae* family. The arabidopsis (*Rosidae*) and maize (*Liliopsida*) sequences were found to be most divergent from the overall homology observed and were excluded from subsequent analysis. The potato and tomato sequences were found to be most divergent from the homologues identified within the *Asteridae* and were also excluded from subsequent analysis.

Pair wise comparisons at the nucleotide level of RB7 TIP homologues from tobacco, carrot, parsley and sunflower revealed lowest and highest homologies of 71.7% and 85% respectively, as well as regions of conserved sequence suitable for degenerate primer design. Degenerate primer pairs, TobRB71A and 1B and TobRB72A and 2B [Appendix 2] were designed to amplify 497 bp and 271 bp respectively, of a strawberry RB7 homologue, based on the *TobRB7* gene sequence.

RT-PCR was carried out as described in section 2.5.1, using cDNA generated from strawberry and tobacco leaf and root mRNA using Oligotex resin as described in section 2.4.3. RT-PCR amplification products were visualised on a 0.8% agarose 1 x TAE gel as described in section 2.2.1.

A fragment of the expected size (497 bp) was generated from strawberry and tobacco root cDNAs, but not the leaf cDNAs, using the degenerate primer pair TobRB71A and 1B indicating that a root-specific gene homologue to *TobRB7* existed in strawberry. The 497 bp amplification product generated from the strawberry root cDNA was gel excised, isolated and blunt-ended as described in section 2.3.4, ligated into *Sma* I digested pBluescript K/S+ and transformed into XL1-Blue MRF' cells as outlined in sections 2.3.6 and 2.3.8. Plasmid DNA was prepared from transformed colonies and sequenced using M13 forward and reverse primers [Appendix 2].

The 16 nucleotides representing the 5' end of the *FavRB7* gene, preceding the 497 bp of *FavRB7* gene sequence isolated using RT-PCR, were isolated during the genome walker™ procedure, which will be described in section 3.3.1.

Sequence homology analysis revealed that the 513 bp fragment isolated from strawberry root cDNA was between 75.8% and 79.1% homologous to comparable regions of the RB7 TIP homologues selected from within the *Asteridae* for the final alignment. This result strongly implied that a partial coding region for a strawberry RB7 TIP homologue had been successfully isolated (Figure 3.1). The isolated strawberry homologue was named *FavRB7*.

The predicted amino acid sequence, reverse translated from the *FavRB7* nucleotide sequence identified, was found to contain sequence motifs characteristic of plant TIPs. Sequence homology analysis revealed that the predicted *FavRB7* amino acid sequence contained the N-termini and loop B consensus motifs defined by Schäffner (1998) and observed in all of the RB7 homologues used in the initial alignment (Figure 3.2). This suggested that the putative water channel protein function ascribed to these proteins in other plant species may also apply in strawberry.

```
                              TobRB7 1a ----->
                              ttggyagcrtyggtgayyctttt
1 : ATGGTGAAGATAGCAATTGGTAGCATCGGTGATTCTTTCACTCATGGATC : 50
2 : ATGGTGAAGCTTGCAATTGGCAGCGTTGGTGACTCTTTCAGTGCAGTGTC : 50
3 : ATGGTGAAGTTGGCAATTGGTAGCATTGGTGACTCTTTGAGTGCCGGTTC : 50
4 : ATGGTGAGGATTGCCTTTGGTAGCATTGGTGACTCTTTTAGTGTTGGATC : 50
5 : ATGGTGAAGATTGGTTTTGGTAGCTTTGGTGACTCTTTCAGTGTTTGCTC : 50


1 : ACTCAAGTGTTACTTGGCTGAGTTTATAGCCACCCTTCTTTTTGTTTTTG : 100
2 : ACTCAAGTCTTATTTTGGCTGAGTTTATTGCCACTCTGCTCTTTGTTTTTG : 100
3 : AATCAAGTCTTACTTAGCCGAATTCATTGCCACACTTCTCTTCGTATTTG : 100
4 : ATTGAAGGCCTATGTAGCTGAGTTTATTGCTACTCTTCTCTTTGTGTTTG : 100
5 : CCTCAAGTCTTACCTAGCTGAGTTCATCGCCACCCTTCTTTTCGTGTTTG : 100


1 : CTGGAGTTGGATCAGCAATTGCTTTTAATAATCTCACAGCTGATGCAGGT : 150
2 : CTGGAGTTGGATCCGCCATTGCTTATGGGCATCTAACGGCTGATGCAGCA : 150
3 : CGGGTGTTGGGTCAGCTATTGCGTATGGTAAGCTTACTACAGATGCGGCA : 150
4 : CTGGGGTTGGGTCTGCTATAGCTTATAATAAATTGACAGCAGATGCAGCT : 150
5 : CTGGTGTTGGCTCTGCCATCGCTTATGATAAGCTGACTACAGATGCAGCT : 150


1 : TTGGATCCAGCTGGATTAGTAGCAATTGCAGTAGCTCATGCATTTGCTTT : 200
2 : TTGGACCCGGCTGGATTAGTGGCCATAGCCATAGCTCATGCATTTGCTCT : 200
3 : CTAGACCCTGCTGGACTAGTCGCAATAGCGATAGCCCATGCTTTTGCACT : 200
4 : CTTGATCCAGCTGGTCTAGTAGCAGTAGCTGTGGCTCATGCATTTGCATT : 200
5 : TTAGACCCTGCCGGCCTAGTTGCGGTGGCCGTAGCCCATGCATTTGCTCT : 200


1 : GTTTGTTGGAGTGTCCATAGCAGCCAACATCTCAGGTGGCCATTTAAATC : 250
2 : ATTTGTGGGAGTGTCCATGGCAGCCAACATCTCAGGTGGCCATTTGAACC : 250
3 : ATTTGTTGGGGTCTCAATGGCAGCAAACATATCGGGTGGCCATCTGAATC : 250
4 : GTTTGTTGGGGTTTCCATAGCAGCCAATTATTTCAGGTGGCCATTTGAATC : 250
5 : GTTTGTGGGGGTCTCCGTCGCAGCAAACATCTCCGGTGGCCATTTGAACC : 250
```

Figure 3.1 Alignment of partial *FavRB7* nucleotide sequence with RB7 TIP homologues within the *Asteridae*

Alignment of nucleotide sequences of RB7 TIP homologues from 1: parsley (Z48232), 2: carrot (Ab000506), 3: sunflower (X95953), 4: tobacco (X54855), 5: strawberry. Regions of conservation are highlighted in black or grey and divergence is highlighted in white. Degenerate primers TobRB71a and 1b used to isolate the strawberry homologue are indicated.

```
1 : CAGCTGTCACCTTTGGATTGGCTGTAGGCGGCCACATAACTCTCATAACT : 300
2 : CAGCTGTCACCCTCGGATTGGCCATCGGAGGCAACATCACCATCATTACT : 300
3 : CGGCAGTCACCTTTGGATTAGCTATTGGTGGTAACATCACCATCATAACC : 300
4 : CAGCTGTCACTTTTGGGATTGGCTGTTGGTGGAAACATCACCATCTTGACT : 300
5 : CGGCTGTCACTTTTCGGATTGGCTGTTGGAGGTAACATCACCATCCTAACC : 300

1 : GGATTTTTCTATTGGATTGCTCAATTGCTTGGTGCTACTGTTGCTTCCTT : 350
2 : GGACTCTTCTACTGGATTGCTCAGTGTCTTGGCTCCACTGTTGCTTGCTT : 350
3 : GGTCTATTTTACTGGATTGCCCAATGTCTTGGTTCCATTGTTGCATGCTT : 350
4 : GGCTTCTTCTACTGGATTGCCCAATTGCTTGGCTCCACAGTTGCTTGCCT : 350
5 : GGCATTTTTCTACTGGATTGCTCAACTCTTGGGCTCCATCGTCGCTTGCTT : 350

1 : TCTCCTCATATTTGTTAATGGTGGCAAGGCAGTTCCAATTCATGGAGTTG : 400
2 : CCTCCTTAAATTTGTCACTGCTGGCAAGGCCATCCCAACTCACGGAGTCG : 400
3 : TCTCCTTCAGTTTGTTACCGGTGGCTTGGCGTACCAACACATGGAGTTG : 400
4 : CCTCCTCAAATACGTTACTAATGGATTGGCTGTTCCAACCCATGGAGTTG : 400
5 : CCTCCTCAAGTTTGTCACCAGCGA---GAGTGTGCCAACCCATGGACTTG : 397

1 : GTGCAGGACTAGGTGCAGCTGAAGGTGTAGTGTTCGAGATCATCATCACC : 450
2 : GAGCCGGCTTGGGTGCAGCCGAAGGCGTAGTCTTTGAGATTGTTATCACA : 450
3 : CAGATGGCATGAATGGTGTTCAAGGAGTTGTGATGGAGATCATCATTACC : 450
4 : CTGCTGGGCTCAATGGATTACAAGGAGTGGTGATGGAGATAATCATAACC : 450
5 : CCTCTGGTGTAGGAGCTATTGAAGGAGTTGTGTTTGAAATCATCATAACC : 447

                                                    <--
                                                    ttccc
1 : TTTGCTCTGGTCTACACCTTCTACGCCACTGCTGCAGACCCCAAGAAGGG : 500
2 : TTTGCCCTTGTCTACACCGTTTACGCCACAGCCGCGGATCCCAAAAAGGG : 500
3 : TTTGCTCTAGTCTACACAGTTTATGCCACGGCCGTTGACCCTAAAAAGGG : 500
4 : TTTGCACTGGTCTACACTGTTTATGCAACAGCAGCAGACCCTAAAAAGGG : 500
5 : TTTGGTCTGGTCTACACTGTGTATGCCACAGCCGCTGACCCCAAGAAGGG : 497

      <--- TobRB7 1b
      gagtgawccktggtaa
1 : CTCACTTGGAACCATT : 516
2 : CTCACTAGGCACCATT : 516
3 : CTCACTTGGAACCATT : 516
4 : CTCACTTGGAACCATT : 516
5 : CTCACTAGGCACCATT : 513
```

Figure 3.1 continued. Alignment of partial *FavRB7* nucleotide sequence with RB7 TIP homologues within the *Asteridae*

```
1 : MVKIAIGSIGDSFTHGSLKCYLAEFIATLLFVFAGVGSAIAFNNLTADAG : 50
2 : MVKLAIGSVGDSFSAVSLKSYLAEFIATLLFVFAGVGSAIAYGHLTADAA : 50
3 : MPGIAFGSFDDSFSSSSIKAYIAEFISTLLFVFAGVGSAIAYAKLTADAA : 50
4 : MVRIAFGSIGDSFSVGSLKAYVAEFIATLLFVFAGVGSAIAYNKLTADAA : 50
5 : MVKIGFGSFGDSFSVCSLKSYLAEFIATLLFVFAGVGSAIAYDKLTTDAA : 50
    MxxxxxGxxxDxxxxxxLK
       N-termini

1 : LDPAGLVAIAVAHAFALFVGVSIAANISGGHLNPAVTFGLAVGGHITLIT : 100
2 : LDPAGLVAIAIAHAFALFVGVSMAANISGGHLNPAVTLGLAIGGNITIIT : 100
3 : LDPPGLVAVAVCHGFALFVAVCIAANICGGHVNPAVTLGLAVGGQITFLT : 100
4 : LDPAGLVAVAVAHAFALFVGVSIAANISGGHLNPAVTLGLAVGGNITILT : 100
5 : LDPAGLVAVAVAHAFALFVGVSVAANISGGHLNPAVTFGLAVGGNITILT : 100
                              NPAVTFGxxxGGxIT
                                   loop B

1 : GFFYWIAQLLGATVASFLLIFVNGGKAVPIHGVGAGLGAAEGVVFEIIIT : 150
2 : GLFYWIAQCLGSTVACFLLKFVTAGKAIPTHGVGAGLGAAEGVVFEIVIT : 150
3 : GLFYWIAQLLGSTVACFLLSFVTGGLAVPTHGVAEGVGTIQGVVFEIIIT : 150
4 : GFFYWIAQLLGSTVACLLLKYVTNGLAVPTHGVAAGLNGLQGVVMEIIIT : 150
5 : GIFYWIAQLLGSIVACFLLKFVTSE-SVPTHGLASGVGAIEGVVFEIIIT : 149

1 : FALVYTFYATAADPKKGSLGTIAPIAIGFIVGANILAAGPFSGGSMNPAR : 200
2 : FALVYTVYATAADPKKGSLGTIAPIAIGFIVGANILAAGPFSGGSMNPAR : 200
3 : FAMVYTVYATACDPKKGALGTIAPIAIGFIVGANILAAGPFSGGSMNPAR : 200
4 : FALVYTVYATAADPKKGSLGTIAPIAIGFIVGANILAAGPFSGGSMNPAR : 200
5 : FGLVYTVYATAADPKKGSLGT----------------------------- : 170
                              FxGGSMNPAx
                                 loop E

1 : HSGQLLLAETFLDTGSTGLVLSLEEA--LG--SYMVMFSLDHMLQSQPLR : 246
2 : SFGPAVASFDFSGH-WIYWVGPLIGGGLAGLVYGDVFIGSYAPIAEDYA- : 248
3 : SFGPAVASFDFSGH-WIYWVGPLVGGGLAGLIYPNVFIS-NEHIPLTNEY : 248
4 : SFGPAVVAGDFSQN-WIYWAGPLIGGGLAGFIYGDVFIGCHTPLPTSEDY : 249
5 : ------------------------------------------------- : -
    xFGPxVAxxxFxxx W              YxxxFIxxHxxLPxxEF
       loop E                        C-termini
```

Figure 3.2 Alignment of predicted protein sequences of RB7 TIP gene homologues

Alignment of predicted protein sequences of RB7 TIP homologues from 1: parsley (Z48232), 2: carrot (Ab000506), 3: sunflower (X95953), 4: tobacco (X54855), 5: strawberry. Black and grey shading denotes observed homology; white shading denotes divergence between the amino acid sequences examined. The N and C termini and loop B and E consensus sequences, as outlined by Schäffner, 1998, are highlighted. Single letter amino acid codes are indicated where consensus was found between all TIP sequences, x denotes positions where divergence was observed.

3.2.2 The *FavRB7* gene in strawberry

In tobacco *TobRB7* was originally thought to be part of a two member gene family (Conkling *et al.*, 1990; Yamamoto *et al.*, 1991). However it was later hypothesised that the proposed *TobRB7* gene family might simply be a gene duplication owing to the amphidiploid nature of tobacco (Conkling *et al.*, 1990; Yamamoto *et al.*, 1991). Southern analysis was carried out, as described in section 2.7.1, to further characterise the *FavRB7* gene in strawberry. Digoxigenin-II-dUTP (DIG) was incorporated into a double-stranded DNA probe during PCR using the primers FavRB73 and FavRB74 [Appendix 2] with plasmid DNA harbouring 495 bp of the *FavRB7* gene serving as template.

Genomic DNA from three species of strawberry, *F. x ananassa* (octaploid), *F. moschata* (hexaploid) and *F. vesca* (diploid), was digested with *Apa* I, *Bam* HI, *Bgl* II, *Eco* RI and *Sma* I, separated by agarose gel electrophoresis, blotted to a nylon membrane and hybridised with DIG labelled *FavRB7* probe (Figure 3.3).

Southern analysis revealed a complex pattern of hybridisation for *FavRB7*. *Apa* I and *Sma* I digestion did not yield discrete restriction fragments. The *Bgl* II digest yielded the simplest hybridising pattern in all three strawberry species; two bands in the diploid, three in the hexaploid and five in the octaploid. Both *Bam* HI and *Eco* RI digestion revealed further hybridising bands in all three species, three in the diploid, five to six in the hexaploid and seven to eight in the octaploid. This pattern suggests that there are at least two genes with close homology to *FavRB7* in strawberry. It is likely that the multiple hybridising bands visualised in the hexaploid and octaploid species are in part a result of the complex make-up of polyploid strawberry as well as restriction fragment length polymorphism affecting the size of restriction fragment surrounding the *FavRB7* gene homologues on different chromosomes.
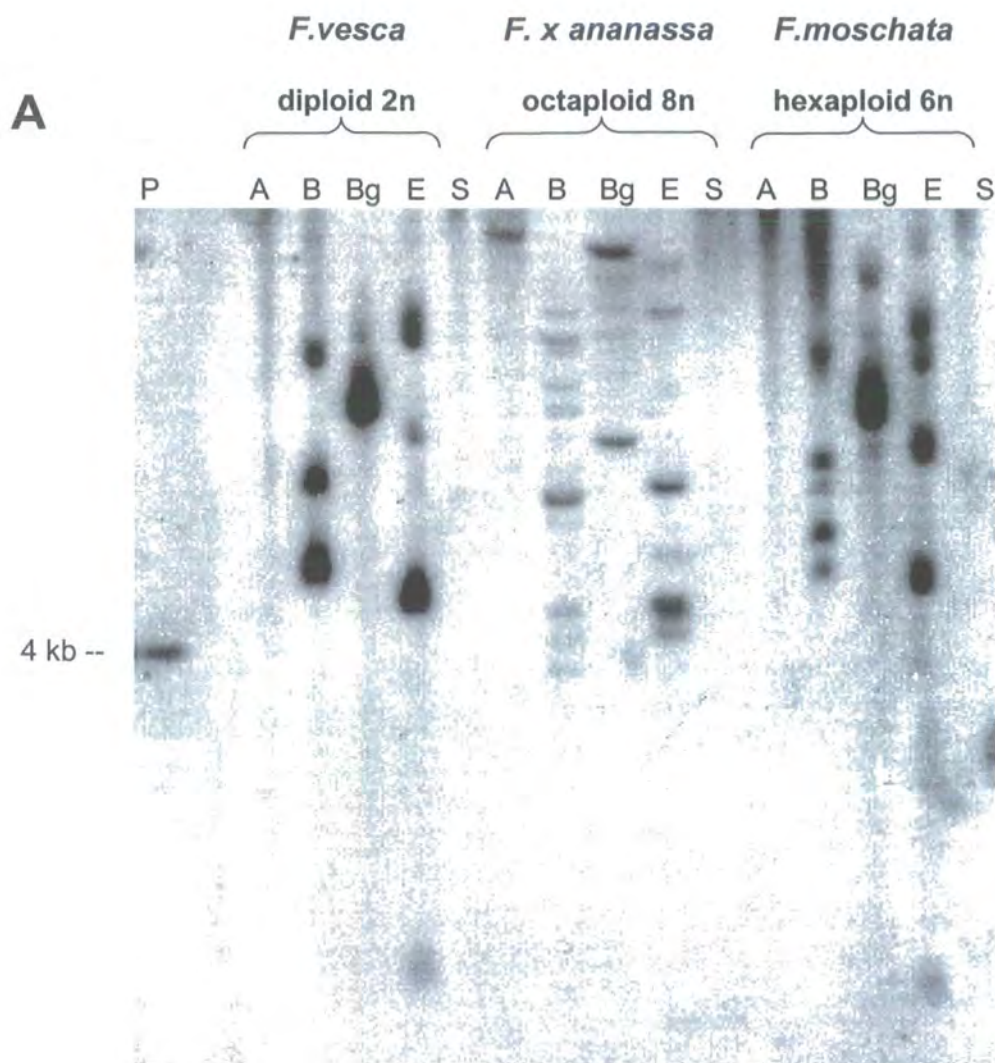
Figure 3.3 Genomic Southern blot of the *FavRB7* gene in *Fragaria vesca*, *F.moschata* and *F.ananassa*

Genomic DNA isolated from three strawberry species of varying ploidy was digested with five restriction enzymes; *Apa* I (A), *Bam* HI (B), *Bg*l II (Bg), *Eco* RI (E), *Sma* I (S) and hybridised with DIG labelled *FavRB7* probe. Linearised plasmid DNA harbouring the partial *FavRB7* coding sequence isolated (P) was also included. The ploidy of each species is indicated, commercial strawberry is represented by the octaploid; *F. x ananassa*. The membrane was washed under high stringency, (0.1 x SSC, 0.5% SDS, at 68°C) and visualised by autoradiography.

3.3 The spatial expression of *FavRB7* in strawberry

Prior to isolating the regulatory sequence controlling the expression pattern of *FavRB7* it was essential to verify that native *FavRB7* gene expression was root-specific. Two forms of analyses were undertaken to assess the expression pattern of *FavRB7* in strawberry; RT-PCR and northern blot hybridisation.

3.3.1 RT-PCR analysis of *FavRB7* expression

Specific primers *FavRB7*1 and *FavRB7*2 [Appendix 2] were designed against the known *FavRB7* sequence and used in RT-PCR as described in section 2.5.1. Messenger RNA isolated from strawberry crown, leaf, stamen, petioles, fruit and root were used as template in the RT-PCR reaction. Specific primers Ubq1 and Ubq2 [Appendix 2] were designed to anneal to the strawberry ubiquitin gene (EMBL: U02886), which is expressed in all tissues. A 300 bp amplification product generated using the ubiquitin primers in RT-PCR confirmed that the cDNAs were intact. RT-PCR using the *FavRB7* primers produced an amplification product of the expected size, 300 bp, from only the root cDNA (Figure 3.4). This result suggested that like *TobRB7*, *FavRB7* is expressed in a root-specific manner.

3.3.2 northern analysis of *FavRB7* expression

Northern analysis was carried out as described in section 2.7.2 using 30 µg total RNA extracted from strawberry root, leaf, floral organs, fruit and petioles as described in section 2.7.2. Digoxigenin-II-dUTP (DIG) was incorporated into a double-stranded DNA probe named FavRB7 II during PCR, using the primers FavRB73 and FavRB74 [Appendix 2], with plasmid DNA harbouring 497 bp of the *FavRB7* gene serving as template.

Prior to blotting denatured RNA was stained with ethidium bromide solution to confirm the integrity of the RNA and to ensure even loading, although all the RNA samples extracted were intact it was noted that the a greater quantity of RNA had
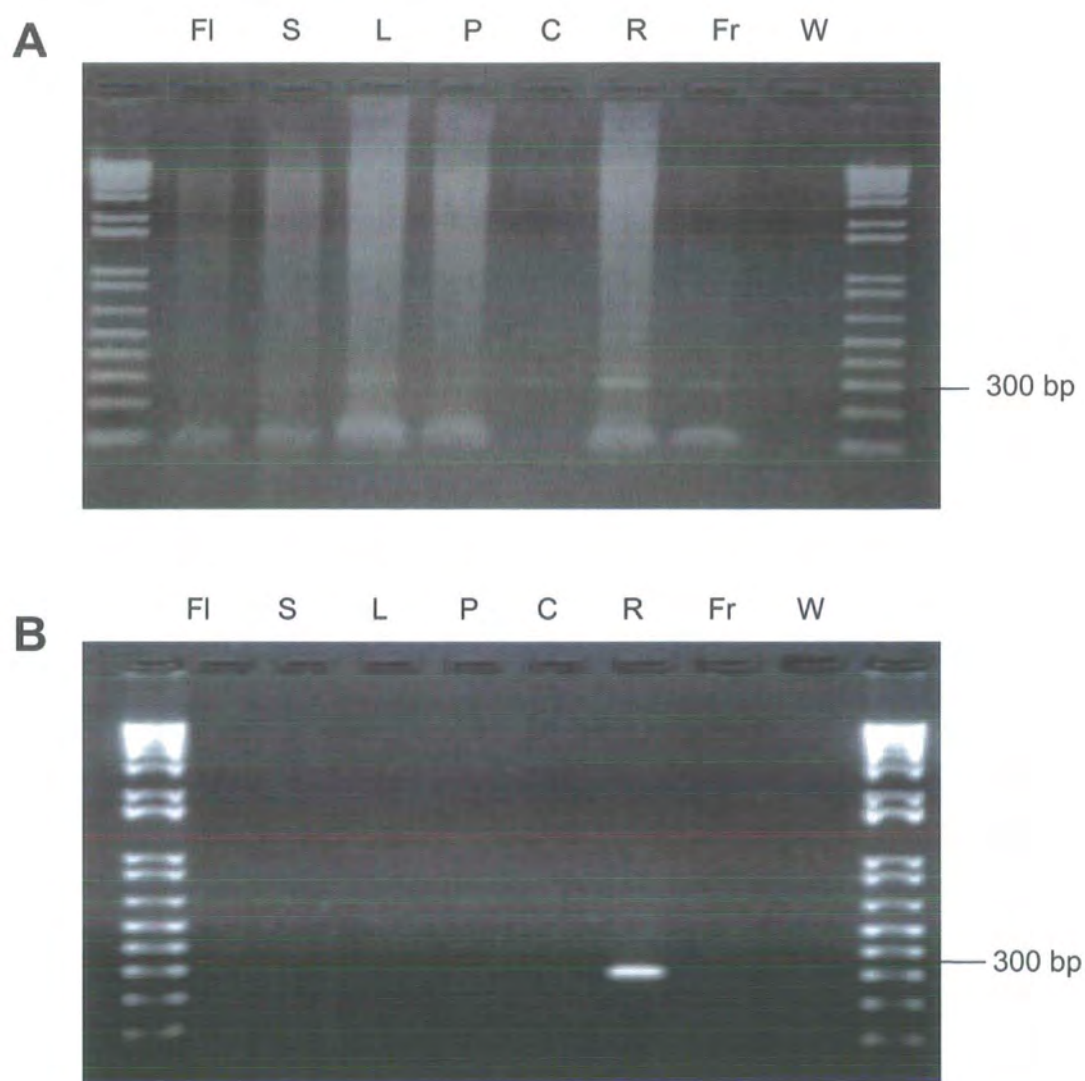
Figure 3.4 Detection of *FavRB7* in strawberry tissues using RT-PCR

RT-PCR was carried out using cDNA synthesised from several strawberry tissue types; floral organs (Fl), stamen (S), leaf (L), petioles (P), crown (C), root (R) and fruit (Fr). The integrity of the cDNA produced was confirmed using primers Ubq1 and Ubq 2 in PCR to detect expression of the strawberry ubiquitin gene (A). The same cDNA was also subject to RT-PCR using primers designed to detect expression of the *FavRB7* gene (B). *FavRB7* gene expression was detected in only the root tissues of strawberry.

been inadvertently loaded for the petiole sample (Figure 3.5A). Northern analysis revealed a strongly hybridising band in the root RNA sample and a much weaker band in the over loaded petiole sample (Figure 3.5B), indicating that whilst expressed strongly in the root tissues *FavRB7* may also be expressed to a much lesser extend in the petioles of strawberry. However as RT-PCR failed to detect *FavRB7* expression in the petiole this result may be an artifact of non-specific binding occuring to the high concentration of RNA loaded for this tissue type.

3.4 Isolation of regulatory sequences controlling *FavRB7* gene expression

3.4.1 Isolation of 5' regulatory sequence upstream of the *FavRB7* gene

To isolate the upstream regulatory sequence of the *FavRB7* gene a PCR based approach was employed, to 'walk' upstream from the gene into the genome.

Specific primers, FRB7GSP1 and FRB7GSP2 [Appendix 2], were designed to anneal at the 5' end of the *FavRB7* gene sequence and used in conjunction with the Universal GenomeWalker™ kit [Clontech] to isolate the promoter as described in section 2.4.5. GenomeWalker™ amplification products were visualised on 0.8% agarose 1 x TAE gels as described in section 2.2.1. Several fragments were amplified using GenomeWalker™ the largest of which was produced from the *Pvu* II library and was approximately 3 kb in length. Amplification fragments were gel excised, cloned into pCR-BluntII-TOPO and transformed into OneShot® Top10 cells. Plasmid DNA was prepared from transformed cells and sequenced using M13 forward and reverse primers [Appendix 2]. The plasmid containing the 3 kb fragment was named pFRB73.

Sequence analysis confirmed that the DNA isolated was a continuation in the 5' direction through the known sequence of *FavRB7* in all of the amplification fragments analysed. This identified 16 nucleotides upstream of the isolated *FavRB7* gene representing the 5' end of the gene including a proposed ATG initiation codon.
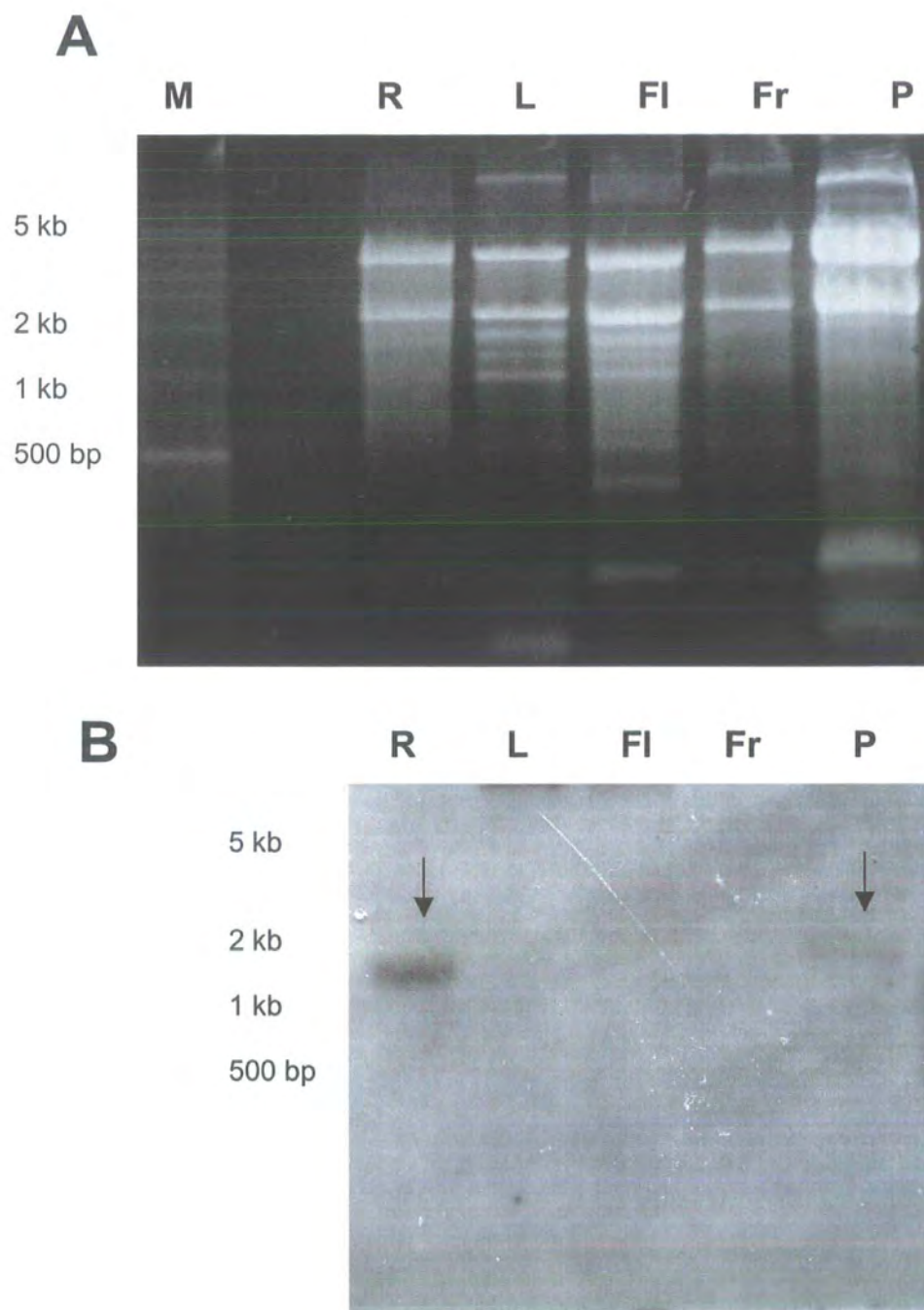
Figure 3.5 northern analysis of *FavRB7* expression in strawberry

Northern analysis was carried out using 30 µg total RNA extracted from strawberry root (R), leaf (L), floral organs (Fl), fruit (Fr) and petioles (P). The integrity and loading of RNA was checked by ethidium bromide staining (A) prior to blotting. Post hybridisation the membrane was probed using DIG labelled *FavRB7* II probe (B). Signals detected at approximately 1.5 kb in the root and petiole samples are indicated, the *FavRB7* II probe did not hybridise to any of the other tissue types assessed.

In plasmid pFRB73 a further 2843 bp upstream from *FavRB7* gene was isolated (Figure 3.6). This region was found to have a high A/T content of ~ 66%. A putative TATA box was identified at position – 22 and a putative AGGA box was identified at position – 115 suggesting that the *FavRB7* gene promoter sequence had been isolated.

The *FavRB7* gene promoter sequence was subject to a search for cis-acting regulatory elements using the PlantCARE database supplied at www.oberon.rug.ac.be. This search identified only one regulatory motif associated with root-specific expression, an as1 motif (Yamaguchi-Shinozaki and Shinozaki, 1994) at position - 2271. However several regulatory motifs linked to hormone response were identified; auxin response, an AuxRR-core element (Sakai et al., 1996) at - 215 and TGA elements (Liu *et al.*, 1994) at - 1975 and - 2271; an abscisic acid response, CE1 element (Shen and Ho, 1995), at - 198; ethylene response, ERE, elements (Itzhaki *et al.*, 1994) at – 542, - 935, 1184 and - 2113 and gibberellin response, P-box and TATC box, elements (Kim *et al.*, 1992; Washida, 1999) at - 785, - 1868, - 1904 and - 503.

Several regulatory motifs associated with environmental stresses were also identified; drought inducibility, MBS, elements (Yamaguchi-Shinozaki and Shinozaki, 1994) were found at - 68, - 2069 and - 2185; a low temperature response, LTR, element (Dunn *et al.*, 1998) at - 1557; heat responsive elements, HSE (Pastuglia *et al.*, 1997) at - 274, - 893, - 1009, - 1107, -1145 and - 2213; wound response, WUN, motifs (Pastuglia *et al.*, 1997) were found at - 38, - 544, - 815, - 1186, - 1343, - 1657, - 1734, - 2115, - 2281 and - 2617 and an anoxic specific inducible, GC-motif (Manjunath and Sachs, 1997) was found at - 126.

Several regions of repetitive sequence were identified in the *FavRB7* promoter (Figure 3.6), the longest of which is a direct repeat of 22/23 bases occurring at positions - 1798 / - 1819.   A direct repeat of 13 bp occurs at - 718 / - 772 and four 11 bp direct repeats were also identified. A 15 bp dyad repeat was identified at - 1883 / - 1416 and a 12 bp dyad repeat at – 1350 / – 942. Inverted repeats of 12 and 11 bp were found at – 2033 / - 537 and – 2429 / - 1817.

```
-2843                                             CGACGGCCCGGGCTGGTCTGCGAGTGACT

-2814   TTTATACCTCAAATCGTGTACCTTGATATTAAGTTTTCTTGTTTCTGTTATTGCTCTCAT

-2754   CATATCCTTTCTGATATTGGTCTTGTGATTGTAAGGTTTCGTATCAGTGTTCTATACTTT

-2694   TTGGATATTAATGGTTTGTTTCATCTGCAAAGATCGATTGGTTTCTGGTGTTTTTTTTTA

-2634   AAGAGGAAGGGAATGGTTCATTGCTTAATTTTATGTTCGATGGCAGGTTAATGTACTTAC
                         WUN

-2574   TGTTTCCATGTTTGCTCTTTTGTTGATTTGAATACAATGGTGTCAATTGATGTTGATTGT

-2514   AGCTAGTAAACTTGAGTGCTTACATGTACTACGTTTCGGTGTGTTCTTGTTGGTTTTGCT

-2454   TCCTAATGTGTGAGGTAAGTGTCTTGCCCAAAAAGAAAGAGATGAGGCTTTTAATTTATT
                                   INVERTED REPEAT (b)

-2394   TGGATTGATCTTGTTATGCTCTATTGATGTTCATGAATGTCATCGTACTCTTAAGGTTTC

-2334   TGAATATGCGATTAAGTACACTGGAAGAATTACTGATATTGTTGGCAATGTGCAAATTAC
                                                               WUN

-2274   CTATGACGTGACCATAAAGGGCACGATCAATGAGCTTAGGTAGCTCAGGCTGAAATAGCC
           As1/TGA

-2214   AAGAAAAGGTAAAAGGTTTGCAGTGGCATCAACTGATTGATAAGCTTGCAGCGGCAACTT
           HSE                          MBS

-2154   GGTTCTTTTAAGTGATTTTGATTGATCGTTGTGTTGATCTTATTTCAAAAGAGTGATTAT
                                                 WUN/ERE

-2094   GAGTTTTTATTTGTGTTTTTTTTTATCGGTCATTTGTTGTGGCAGATTTTATGAAAGATTA
                                    MBS

-2034   TACTTGGAAATGCTTAGTTAATAACGTATCCTCAATTCCTCAACCTGGTAGCCGTTTCTT
           INVERTED repeat (a)

-1974   GACGTTTTGATTGTTTCTATTATTATTCGGTATTTTCTTAAGTCACTCACTGAGCATTAG
           TGA element

-1914   TTTGCTCATCCCTTTTTGTGTTTTCTGTTTGCATGTCTGTTAGTTTCCTTTTCAGAAGTG
                   P-box                                    P-box

-1854   TTCGGGTTGTGTGTAGCTTAGAGTAGGATTTTCTTTTTGGTTCACTTGTTTAAGAGAAAG
                                 15bp DYAD (a)  |  DIRECT REPEAT (a)
                                 INVERTED REPEAT (b)

-1794   TGGTTCACTTGTTTAAAAGAAAGGGCTTTCTCTTATAAAACTGATTTGGATTTTATTTGT
           | DIRECT REPEAT (a)
```

Figure 3.6 Nucleotide sequence of the *FavRB7* gene promoter

Nucleotide positions are given distal to the proposed ATG *FavRB7* gene initiation codon. Putative regulatory motifs are indicated in bold:- As1- root specificity, AuxRR - auxin response, TGA – auxin response, CE1 - abscisic acid response, ERE - ethylene response, P-box and TATC boxes – gibberellin response, MBS - drought inducibility, LTR - low temperature response, HSE - heat responsive elements, WUN- wound response and GC-motif - anoxic inducible. Regions of repetitive sequence identified are underlined and highlighted in grey, the type of repeat is indicated and letters indicate pairs of repetitive sequence.

```
-1734  AAATTACTTCTGATGTGCTCATTTTCTCTACCTTTATATTTATAGAGAATTTACTTGCTT
       WUN
-1674  ATATCTTTTTGTATTTAGAATTTCATTTTTGAGATTTAATAATGCTATTGTCCATTTTGG
                       WUN
-1614  GACTGGGTCGTTTCATGCTTTATAGTCCAAAGCAGTCAAGATGGGTGTATTTATTACCCG
                                                                 LTR
-1554  AAATGAAAGGCCAACCTAGCTCTTAGTTCTTGGGAGATTGATATTAAGGTTGGATTATTC
-1494  AAGAAATGAAGGTTATTAATTGGAGTTCTTGTGTTAGCTCCTTTAATTTGAGAATAAAGC
-1434  AAACCGTGGCAGCTTTTCTCTTTTTCTTTTAGGATTTAGTATATGTTTAATGTTATGAAA
                             15bp DYAD(a)
-1374  AATGAATAATTTATTAATTAAAAGACAAATTTAATTACAAACCCTACAAACGTACTAAAG
                             12bp DYAD(b)WUN
-1314  AGCGTTGGTCACCAACTCAATCAAGATAGAAACAACGAGAGGAGACTGACAACTCATGTT
-1254  TGAGCTTGAGCTCGAGCATGATTTTGATATACATGGTTATATCATTGTTGATTGTTCATG
-1194  AGCGCCAAACATTTCAATTTTGTGACATGCTCTTGTCATCATTGTAGTGAGAAATTGTAG
               WUN/ERE                                  HSE
-1134  TAACAATCAAGGGACATGTGTAGAAGAATAAAATTTTCTTTCTAGCTAATTGTTACTTAT
                             HSE
-1074  AATGTTTTTTTATTGACTCTTACTCTCTTATATCCTTAGAGAGTTTTTAATGAAGACTTT
-1014  ATAATTAAAACTTTTGACTAAACGGTTGGATGACATGCATGTTGGAATTCAAAAACAGTA
             HSE
 -954  ACTAAATCTTATACCATTAATTTAAAAGTCCTCACTTAATCCTTAATGTAAAGTCCTTAT
                    12bp DYAD(b)ERE
 -894  TAGAAACTTTCCCATTATATCCTTACCTCTACATCAATACAACTCAAAATCCAAACTTGT
       HSE
 -834  TTACATTTACATGTTTACATGATTACATCCCTACCTCCTTTCAATATTCCTTTTCTTTTT
                             WUN                        P-box 13bp
 -774  TTTTTTTTCTTTTTTTTTAAGGGAATCTCCTTCCAATATCGTTGAGGCTCGTAAAAATACG
       overlapping direct repeat
 -714  ATAGCATACATCATCATAGGAGAGCTATGTTCTCGTCTTTTTTATTCATATCTTCCTAAC
 -654  TAAAGTGCTAATTCATAACCTGAAAACGCACTTTTGTATTCTGCTGGCAAGTATGTGCCA
 -594  TTATAAGAAGTAAATTTACATATCTACAAGTGCTTTTGACACTTCCAAAAGCATTTCCAA
                                                             WUN/ ERE
 -534  TACACAGGTGTCTTGATTCATATTGTTCTTATATCACACACCACCTACACCATATACAAA
                                     TATC box
 -474  GTTACAAGGAAAATGACATAATACCTCAAACTAATCACTAATGTAGTATAGTGTTCATGG
 -414  TCTCGACCATATGTATTCCATAAGAATTAACATGCTTATCTCCTAAGATTATAATACTCT
 -354  CATTGTCGGTTCATAATCAATACTAATTAAACTTAATTGTCCTATCTTCATAATGCCTAC
 -294  ATAATATAATCTCAACCCCAAAAAAAATATAGAAATGGCGGTTGGTCGGCGGCCAGTCGGA
                          HSE
 -234  CGATGACAACCCGTCCTTGGGTCCATCCTCTATGATTGCCGCCGGCTGAATTGGACGTTC
                                             CE1
 -174  TCACATAGTACTGATAGTACCTACTCTTACTAAGCACTATATATAGAAACCACGCTCAAA
                          AuxRR-core                    GC-motif
 -114  GGATGAGATGCACCTACCAAAACCTCGAGCTCTTCTAGTACTGTTTCAGTTGGCTTAGAA
       agga box
  -54  TTTAATTTGATTTGAATCATTGCCTTGGTGTTTATATTTTCCAGTGATCAAACAATG
                        WUN             tata box              FavRB7
```

Figure 3.6 continued. Nucleotide sequence of the *FavRB7* gene promoter

Sequence homology analysis between the *FavRB7* and *TobRB7* (EMBL: S45406) promoters revealed only 23% overall homology between the two sequences. However the sequence motif, CGAGCTCGATA, which forms the consensus of the *TobRB7* promoter A box, was present several times in the *FavRB7* promoter (Figure 3.7). A search to find other possible motifs present in both promoters was carried out using the MotifSampler program supplied at www.esat.kuleuven.ac.be. This revealed several sequence motifs found in similar positions in both promoters (Figure 3.8).
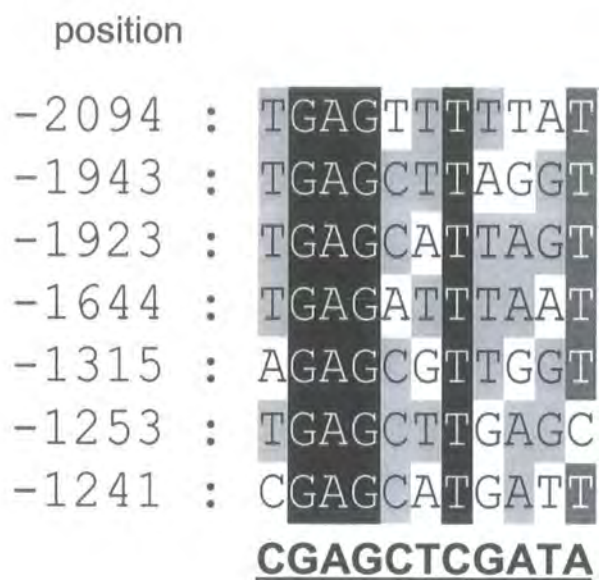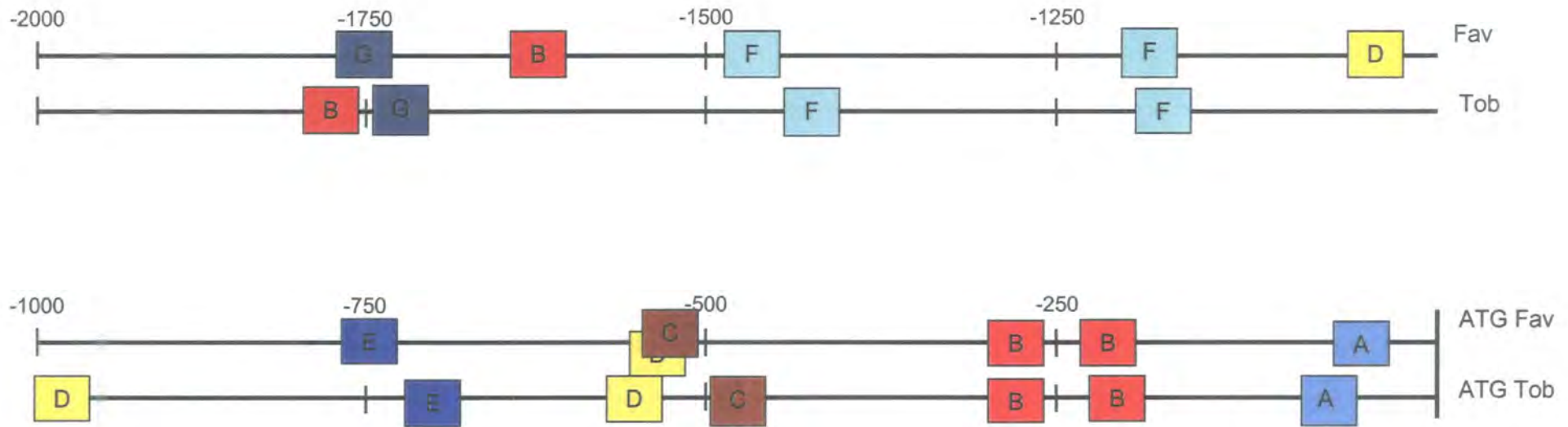
position

```
-2094 :  TGAGTTTTTAT
-1943 :  TGAGCTTAGGT
-1923 :  TGAGCATTAGT
-1644 :  TGAGATTTAAT
-1315 :  AGAGCGTTGGT
-1253 :  TGAGCTTGAGC
-1241 :  CGAGCATGATT
         CGAGCTCGATA
```

Figure 3.7 TobRB7 A box motifs homologues identified in the *FavRB7* gene promoter

Sequence positions are given distal to the proposed *FavRB7* gene initiation codon for sequence motifs identified in the *FavRB7* promoter homologous to the A box motifs identified in the *TobRb7* promoter. Regions of homology are indicated by black or grey shading; regions of divergence are highlighted in white. The *TobRB7* promoter A box consensus sequence is presented below the *FavRB7* sequence motifs in bold and underlined.

3.8 Graphical representation of sequence motifs identified in the *FavRB7* and *TobRB7* promoters

Promoter sequence positions are given distal to the proposed initiation codon for the *FavRB7* (ATG Fav) and *TobRB7* (ATG Tob) genes. Boxes represent the approximate position of sequence motifs found in both the *FavRB7* and *TobRB7* promoters. Consensus sequences are: A = GCTTAwAAyTT, B = GnnnsTnGGTCG, C = TTGTTCTT, D = GATTTTGAnTT, E = GnTAwGATTATA, F = TTsTTkyTTN, G = GATTTTGAnTT

3.5 Concluding remarks

A novel gene, FavRB7, exhibiting strong homology to the TIP family of water channel proteins was isolated from strawberry. Southern analysis suggests that FavRB7 may be part of a gene family in strawberry. RT-PCR analysis showed that the FavRB7 gene is expressed in a root-specific manner in strawberry, however northern analysis suggests that the gene may also be expressed at low levels in the petioles.

The 5' regulatory sequence upstream of the FavRB7 gene was isolated and its association with the FavRB7 gene previously isolated was confirmed. The 5' regulatory sequence upstream of the FavRB7 gene was found to contain several elements characteristic of plant promoters including sequence motifs identified in the tobacco TobRB7 promoter. The structural homology observed between the two promoters possibly suggests that they confer similar functionality. Several regions of repetitive sequence likely to affect the conformation of the promoter were identified in the FavRB7 gene promoter.

Several sequence motifs associated with regulatory elements identified in other species were found in the FavRB7 promoter. The significance of these elements is hypothetical, however factors such as heat, drought, anoxia, wounding and low temperature would all affect plant water relations and could therefore be linked with the expression of a water channel protein gene in the roots. Several regulatory motifs associated with hormonal responsiveness were also revealed. Auxin, gibberellin and ethylene all influence root growth and architecture which will in turn affect the water relations of the root and the requirement for water channel protein production. Responsiveness to abscisic acid has been linked to dehydration response; again it is possible that production of a water channel protein would be linked to this stimuli.

The following chapter will describe further analysis of the FavRB7 gene promoter to determine whether it is sufficient to drive root-specific expression of foreign genes in transgenic strawberry and in a heterologous system, tobacco.

Chapter 4

Characterisation of *FavRB7* promoter activity

4.1 Introduction

In chapter 3 the isolation of the 5' regulatory region associated with the novel root-specific strawberry gene *FavRB7* was described. The primary aim of this study was to determine whether the *FavRB7* gene promoter isolated was sufficient to confer root-specific expression of a foreign gene in strawberry. In addition, *FavRB7* gene promoter activity would be determined in a heterologous plant, tobacco.

The gene expression pattern conferred by plant promoters is often characterised using heterologous systems, often the model plant species arabidopsis and tobacco (Holtorf *et al.*, 1995; Yang *et al.*, 2000). However some promoters have not always exhibited the expected expression pattern in heterologous systems. For example *gusA* expression conferred by the *Agrobacterium rhizogenes rolC* promoter in transgenic aspen was found shift laterally from the phloem to include the cortex and pith during the onset of dormancy in response to starch accumulation within the cells (Nilsson *et al.*, 1996).

Promoters generally comprise multiple *cis*-acting elements (Dynan, 1989). Promoter activity is affected by many factors, but is largely mediated through sequence-specific DNA binding proteins recognising specific *cis*-acting elements in the promoter. Binding of specific proteins to the promoter can induce conformational changes in the DNA and allow further transcription factors to bind facilitating other components of the transcription machinery (Tyagi, 2001). It is likely that the transcriptional regulation conferred by a promoter depends on the presence of specific key factors present within certain tissues acting in a combinatorial manner with more ubiquitous factors (Meshi and Iwabuchi, 1995).

The approach taken to assess *FavRB7* gene promoter activity was to create a binary vector containing the *FavRB7* promoter linked to the *gusA* reporter gene (Jefferson *et al.*, 1987; Jefferson, 1989) and to use this vector to produce transgenic strawberry and tobacco.

The *gusA* gene encodes β-glucuronidase, which catalyses the cleavage of a variety of β-glucuronides. The presence and relative activity of the *gusA* gene in various plant tissues of transgenic lines is indicative of promoter activity. This activity can be analysed quantitatively though fluorometric analysis of β-glucuronide substrate cleavage and qualitatively by histochemical assay (Gallagher, 1992).

In addition, transgenic plants containing the *gusA* gene under the control of the cauliflower mosaic virus 35S RNA (CaMV 35S) promoter would be generated. The CaMV 35S promoter has been widely used in plant transformation since it confers dependably high levels of transgene expression and gene product accumulation in a wide variety of plant tissues in a wide variety of species (Fisk and Dandekar, 1993). These studies would allow determination of *FavRB7* gene promoter activity in strawberry and tobacco, a heterologous system, with comparison to the CaMV 35S promoter.

4.2 Experimental approach and results

Restriction endonuclease digestion of plasmid DNA, blunt-ending, dephosphorylation and ligation of DNA fragments and transformation of *Escherichia coli* were carried out as described in section 2.3. Gel isolation of the products of PCR amplification and restriction endonuclease digestion was carried out as described in section 2.2.3. Preparation of plasmid DNA was carried out as described in section 2.4.2.

4.2.1 Construction of the binary vector, pSCVFavRB7

The binary vector pSCV1.6 (Figure 4.1) harbours the *gusA* reporter gene driven by the CaMV 35S promoter next to the left T-DNA border and the neomycin phosphotransferase (*nptII*) gene also driven by the CaMV 35S promoter, next to the right T-DNA border. This vector was used to confer constitutive *gusA* expression, to serve as a comparison to the *FavRB7* gene promoter in these investigations.

To create a vector comparable to pSCV1.6, but with the *gusA* gene under control of the *FavRB7* promoter, the CaMV 35S RNA promoter controlling the *gusA* gene in pSCV1.6 was removed and replaced with the 2843 bp *FavRB7* gene promoter isolated from strawberry (section 3.4.1), creating the vector pSCVFavRB7. Primers, FavRB7SS and FavRB7N [Appendix 2], were designed to incorporate *Sph* I and *Sma* I sites at the 5' end of the promoter and an *Nco* I site across the initiation codon of the *FavRB7* gene to facilitate cloning. PCR was carried out using the high fidelity proof reading enzyme *Pfx* and pFavRB72.8 (section 3.4.1) as template. A 2860 bp amplification product incorporating the *FavRB7* promoter sequence and *FavRB7* gene 5' untranslated region up to the ATG initiation codon was gel isolated and transformed into the vector pCR-Blunt-TOPOII. Plasmid DNA was prepared from transformed colonies and sequenced to confirm the correct addition of restriction sites. The authenticated plasmid was named p*FavRB7P*.
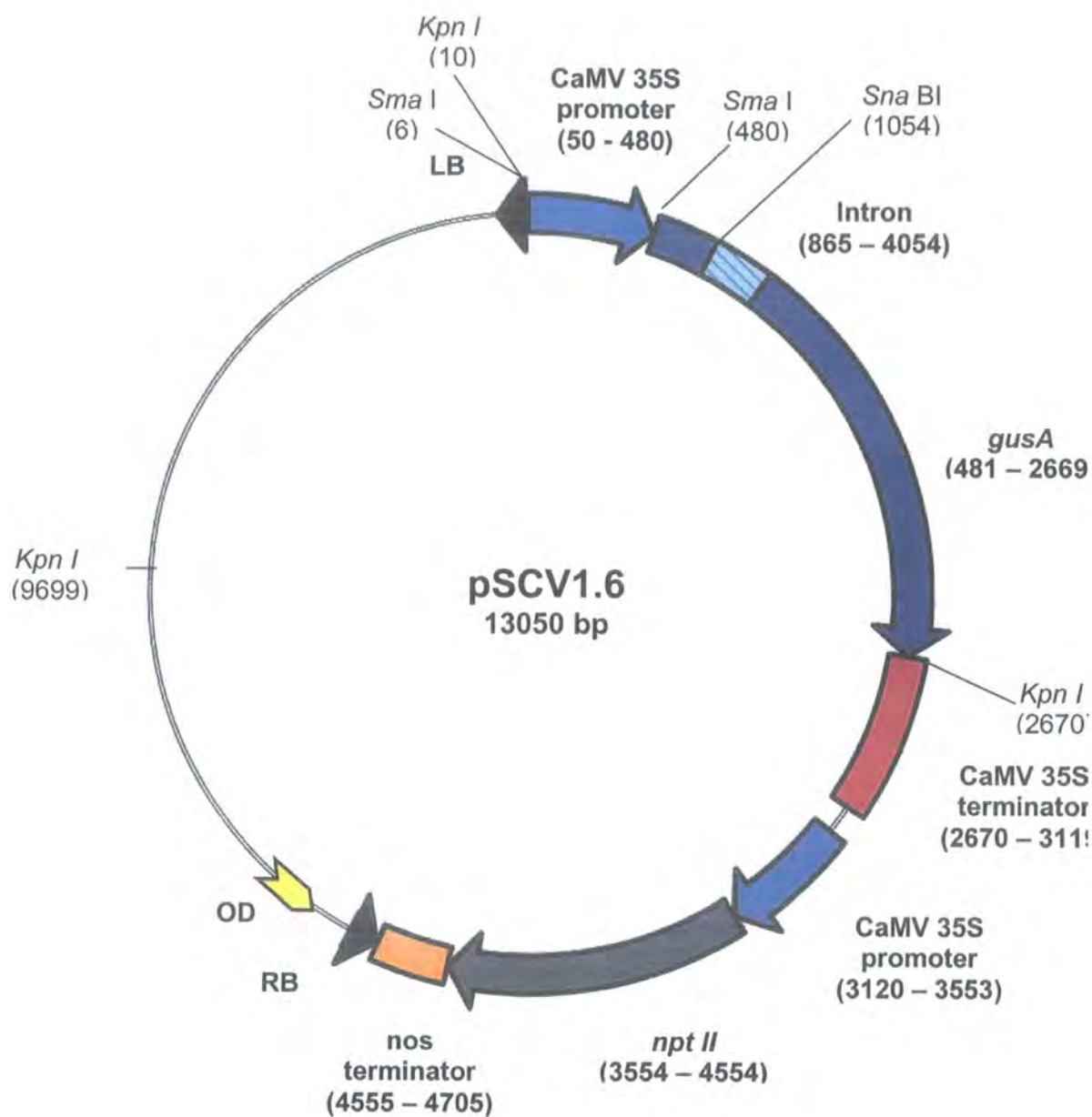
Figure 4.1 Binary vector pSCV1.6

Left (LB) and right (RB) T-DNA borders are indicated. OD indicates an overdrive T-DNA transfer enhancer. Arrows indicate the orientation of promoters and genes. Key restriction endonuclease sites are indicated and their positions noted.

The plasmid pFavRB7P was digested with *Sph* I and *Nco* I and the resulting 2856 bp restriction fragment ligated into *Sph* I and *Nco* I cut, dephosphorylated pGUS358-S. The plasmid pGUS358-S (EMBL: U02441) harbours the *gusA* gene with an *Nco* I site across the initiation codon. Correct linkage of the *FavRB7* gene promoter to the *gusA* gene in pGUS358-S was confirmed by sequencing and the authenticated plasmid named **pGFavRB7P**.

Plasmid pGFavRB7P was digested with *Sma* I and *Sna* BI to release a 3228 bp fragment representing the *FavRB7* promoter and the first 385 bp of the *gusA* gene. This fragment was then ligated into *Sma* I and *Sna* BI digested, dephosphorylated pSCV1.6 replacing the CaMV 35S promoter and the first 385 bp of the *gusA* gene. Correct linkage of the components was confirmed by sequencing and the authenticated binary vector containing the *FavRB7* promoter linked to the *gusA* gene named **pSCVFavRB7** (Figure 4.2).

Both pSCV1.6 and pSCVFavRB7 were transformed into electro-competent cells of *Agrobacterium tumefaciens* strain EHA101 (section 2.6.1) prior to use in nuclear transformation experiments.

4.2.2 *FavRB7* promoter activity in strawberry and tobacco transgenics

a) *Agrobacterium*-mediated nuclear transformation

Strawberry, cv. Calypso, and tobacco cvs Ottawa and Petit Havana were transformed (sections 2.6.2 and 2.6.3, respectively), using the binary vectors pSCV1.6 and pSCVFavRB7 in *Agrobacterium tumefaciens* strain EHA101. Shoots regenerating from transformation experiments for both strawberry and tobacco were maintained on selective medium for a minimum of 12 weeks prior to weaning.

Two replicates of each putative transgenic strawberry line were weaned and transferred to a controlled environment cabinet as described in section 2.1.4. After six weeks under standard growth conditions the plants were subject to a 14 d chilling
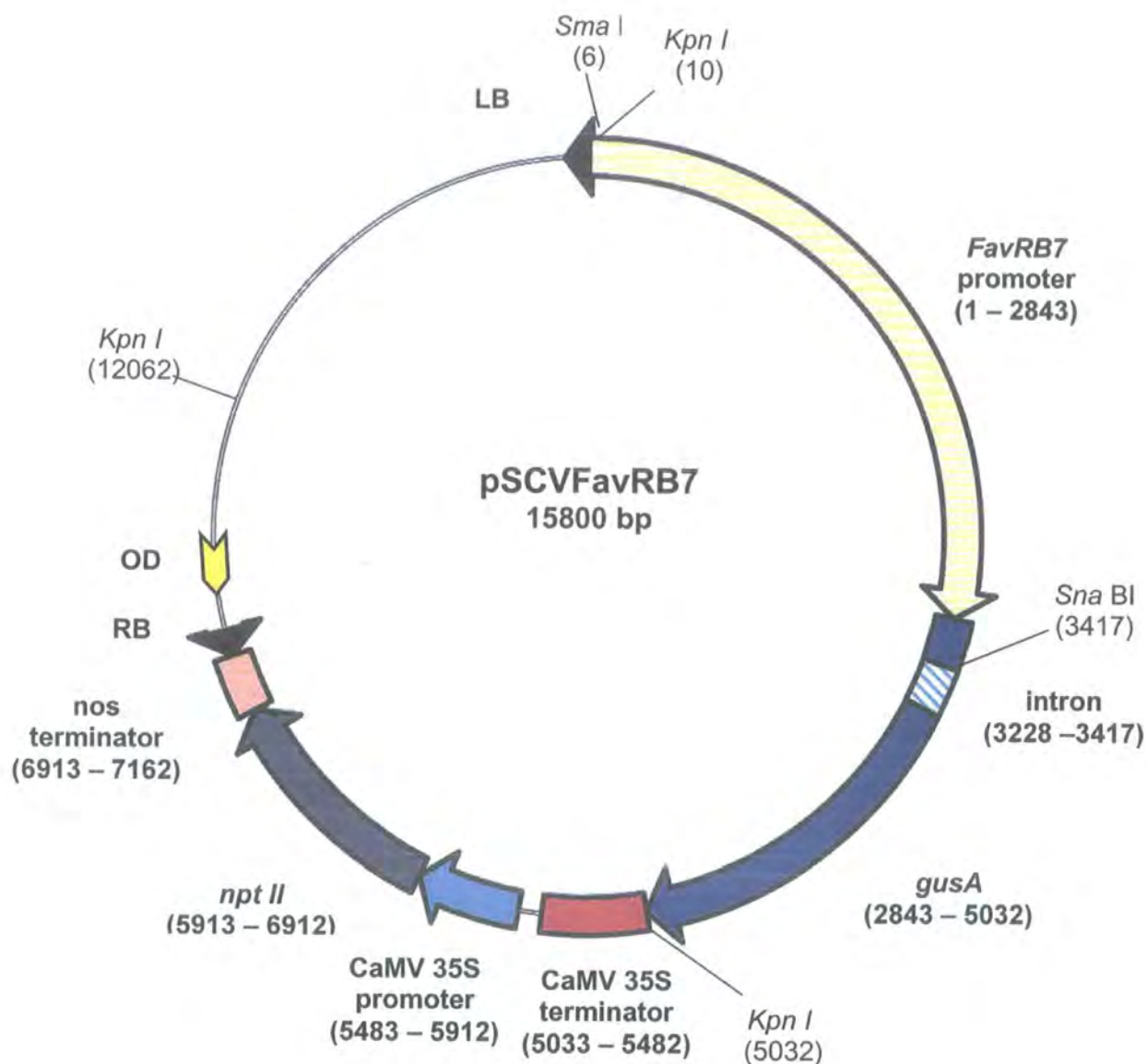
Figure 4.2 Binary vector pSCVFavRB7

The *FavRB7* gene promoter replaces the CaMV 35S promoter driving *gusA* in pSCV1.6 to create pSCVFavRB7. Left (LB) and right (RB) borders are indicated. OD indicates an overdrive T-DNA transfer enhancer. Arrows indicate the orientation of promoters and genes. Key restriction endonuclease sites are indicated and their positions noted.

treatment of 10 °C day and night to induce flowering. After a further six weeks under standard growth conditions the plants were transferred to the glasshouse under natural heat and light. All strawberry lines produced displayed the phenotype typical of Calypso (Figure 4.3A). Runners generated from the R0 lines were rooted and then detached to produce R1 lines. All R1 lines produced displayed the typical phenotype (Figure 4.3B).

Tobacco plants were weaned and transferred to the glasshouse as described in section 2.1.5. All of the tobacco lines produced displayed the typical phenotype for *Nicotiana tabacum* (Figure 4.4).

Primers FavPGUS1 and FavPGUS2 [Appendix 2] were designed to anneal in the *FavRB7* gene promoter and the *gusA* gene respectively, and were used in PCR (section 2.5.4) to confirm the presence of this region in pSCVFavRB7 transgenic lines of both strawberry and tobacco. Primers GUS27 and GUS392 [Appendix 2] were used to confirm the presence of the *gusA* gene in pSCV1.6 transgenic lines.

Thirty-two strawberry lines containing pSCVFavRB7 and one line containing pSCV1.6 T-DNA were generated. In tobacco, eight Ottawa lines containing pSCVFavRB7 and two lines containing pSCV1.6 T-DNA were generated. Eleven Petit Havana lines containing pSCVFavRB7 and two lines containing pSCV1.6 were also generated. PCR was used to detect the *FavRB7* gene promoter and *gusA* gene in these lines (Figures 4.5 and 4.6). Strawberry lines 50, 59a, 59b and 60, and tobacco lines 35 and 68 transformed with pSCVFavRB7 were additionally generated, however the *FavRB7* gene promoter: *gusA* region was not detected in these lines.
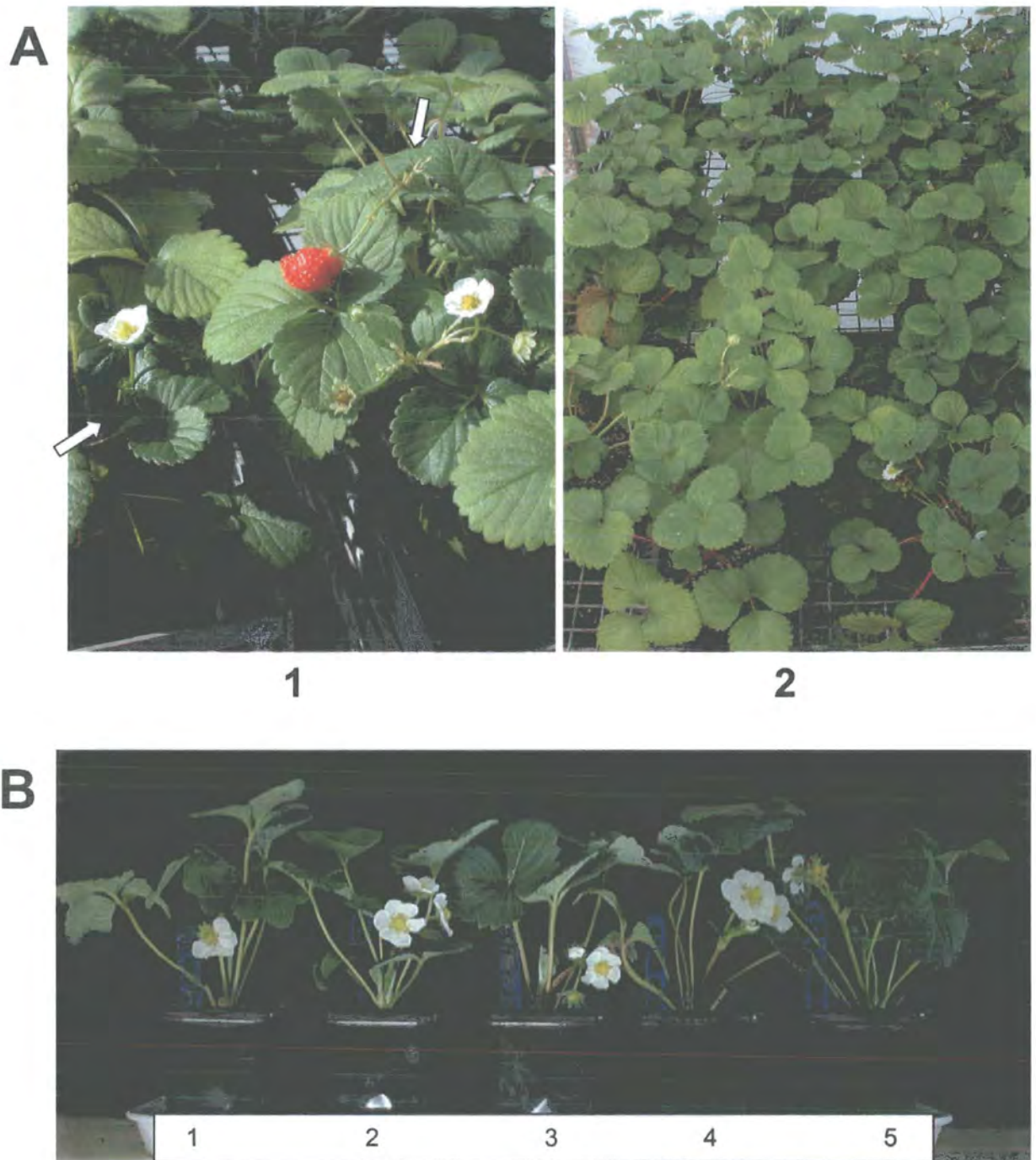
Figure 4.3 Glasshouse grown transgenic strawberry

R0 Calypso strawberry lines are shown (A) as well as R1 lines (B). Arrows indicate transgenic line 46 in flower and bearing fruit alongside a flowering non-transgenic control (A1). A range of R0 pSCVFavRB7 transgenic lines displaying the standard phenotype for Calypso are also shown (A2). Stolon propagated Calypso R1 'daughter' lines (B) representing non-transgenic control (1), pSCV1.6 (35S) line (2) and pSCVFavRB7 lines (3 – 5).
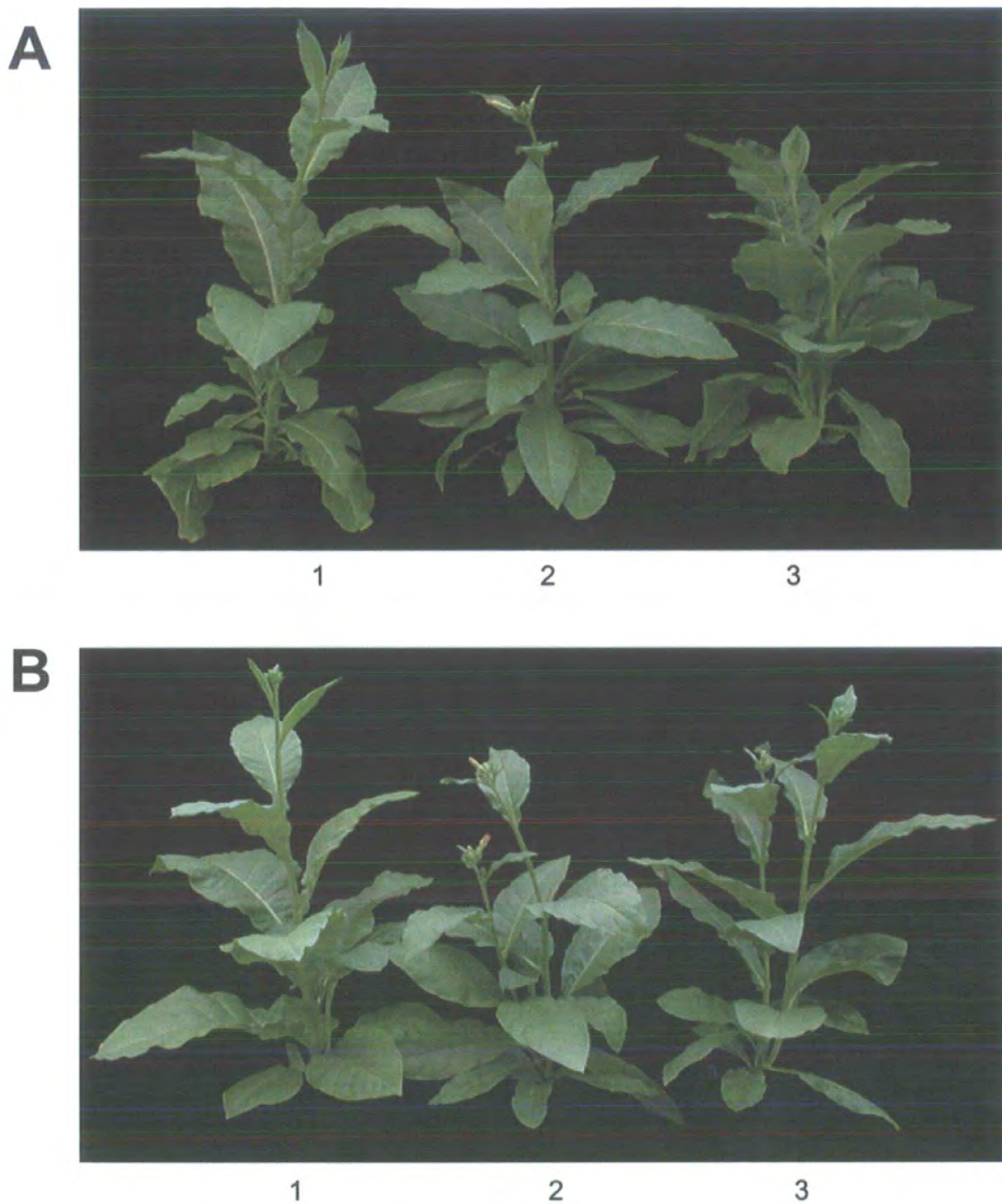
Figure 4.4 Glasshouse grown transgenic tobacco

A standard phenotype was observed in all the transgenic tobacco lines produced. Representative Petit Havana (A) and Ottawa lines (B) showing non-transgenic control (1), pSCVFavRB7 (2) and pSCV1.6 transgenic lines (3) are indicated.

Figure 4.5 PCR to confirm presence of *FavRB7* gene promoter: *gusA* region in transgenic strawberry

The amplification product present at approximately 1kb generated using the primer pair FavPGUS1 and FavPGUS2 in PCR confirms the presence of the *FavRB7* gene promoter controlling *gusA* in pSCVFavRB7 transgenic lines. The amplification product present at approximately 400 bp generated using the primer pair GUS27 and GUS392 in PCR confirms the presence of the *gusA* in the pSCV1.6 transgenic line (122). Non-transformed control (-) and positive plasmid controls (+) are included as is a water control (H₂O).
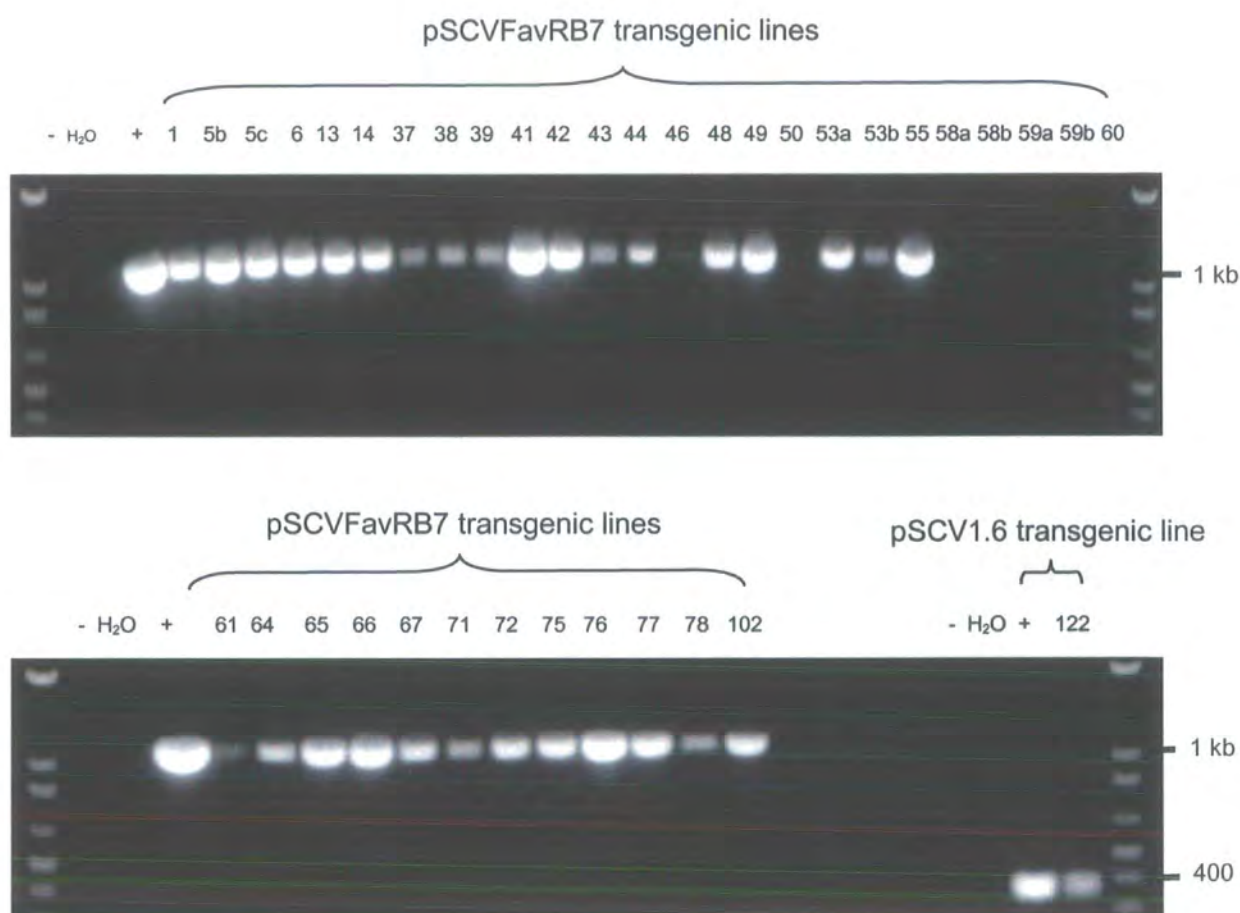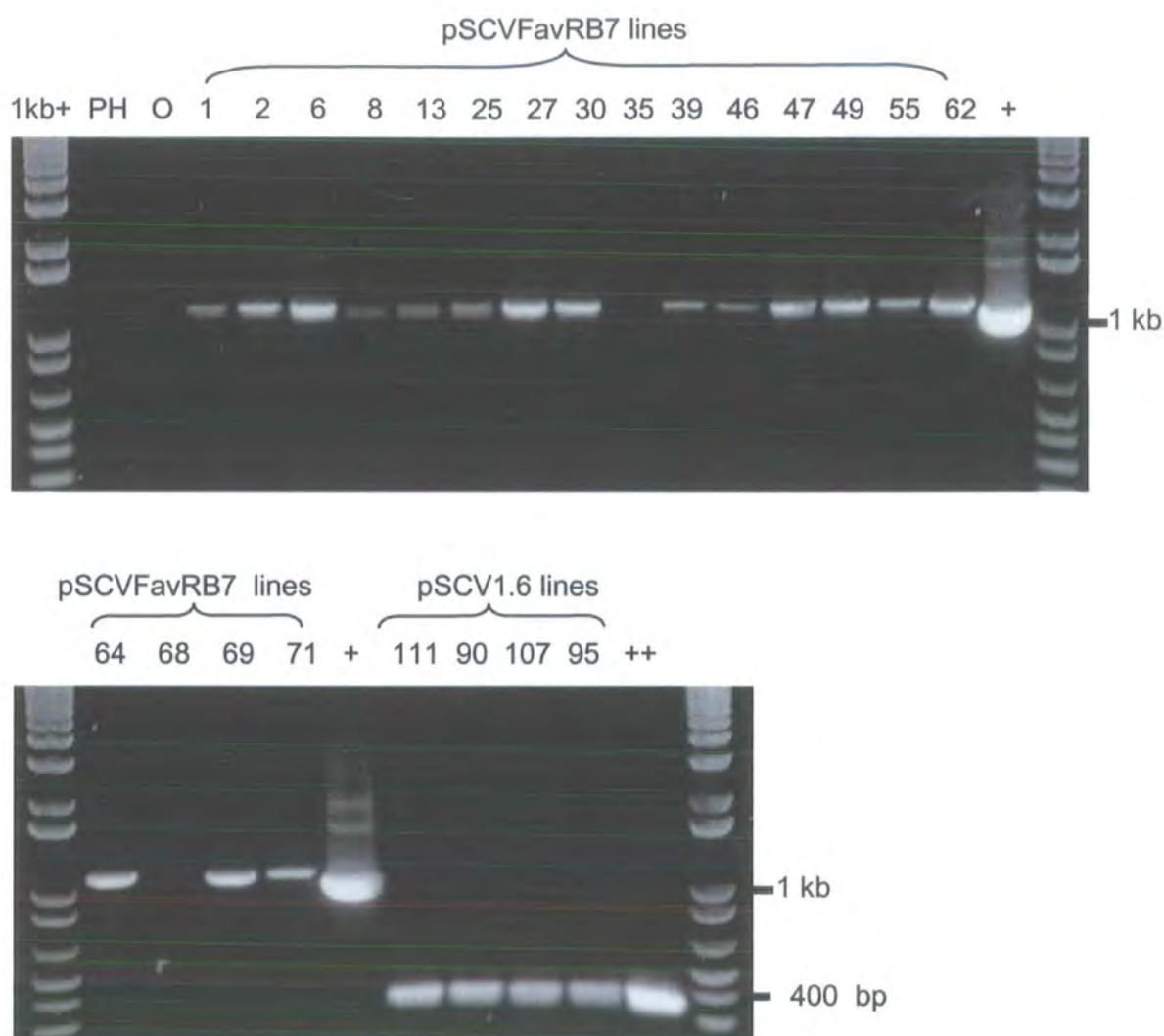
Figure 4.6 PCR to confirm presence of *FavRB7* gene promoter: *gusA* region in transgenic tobacco

The amplification product present at approximately 1kb generated using the primer pair FavPGUS1 and FavPGUS2 in PCR confirms the presence of the *FavRB7* gene promoter controlling *gusA* in pSCVFavRB7 transgenic lines. The amplification product present at approximately 400 bp generated using the primer pair GUS27 and GUS392 in PCR confirms the presence of the *gusA* in the pSCV1.6 transgenic lines. Negative lines of both petit Havana (PH) and Ottawa (O) are included as are plasmid controls pSCVFavRB7 (+) and pSCV1.6 (++).

b) Southern analysis of transgenic strawberry

Southern analysis (section 2.7) was carried out on 36 putative pSCVFavRB7 lines and one putative pSCV1.6 line of strawberry. The transgenic tobacco lines generated were not subject to Southern analysis due to time constraints.

To establish site integration number of the *gusA* and *nptII* genes and to confirm the presence of the *FavRB7* gene promoter:*gusA* gene region in transgenic strawberry lines, genomic DNA was digested with *Kpn* I and *Sma* I. Digoxigenin-II-dUTP (DIG) labelled *nptII* and *gusA* probes were synthesised using the primers NPTIIA and NPTIIB and GUS27 and GU392 [Appendix 3], respectively, using pSCV1.6 plasmid DNA as template in PCR. These probes were then used to evaluate the number of transgene integration sites.

Southern analysis of *Kpn* I digested genomic DNA revealed the number of integration sites for the *nptII* gene (Figure 4.7), which was confirmed through Southern using *Sma* I digested genomic DNA. Southern analysis of *Sma* I digested genomic DNA revealed the number of integration sites for the *gusA* gene.

The presence of a second *Kpn* I site within the T-DNA (Figure 4.2) meant that a 4.6 kb restriction fragment representing the *FavRB7* gene promoter:*gusA* gene region should be detected in all pSCVFavRB7 transgenic lines using the *gusA* probe. Identification of fragment greater than or less then 4.6 kb would imply that some manner of deletion or rearrangement has occurred within this region to remove a *Kpn* I site at either the 5' of 3' end of this region. In both cases it is important to note that the integrity of the promoter driving *gusA* cannot be guaranteed. Evidence of such alterations were observed in lines 41, 53a, 53b, 61, 67, 72 and 78 (Figure 4.8).
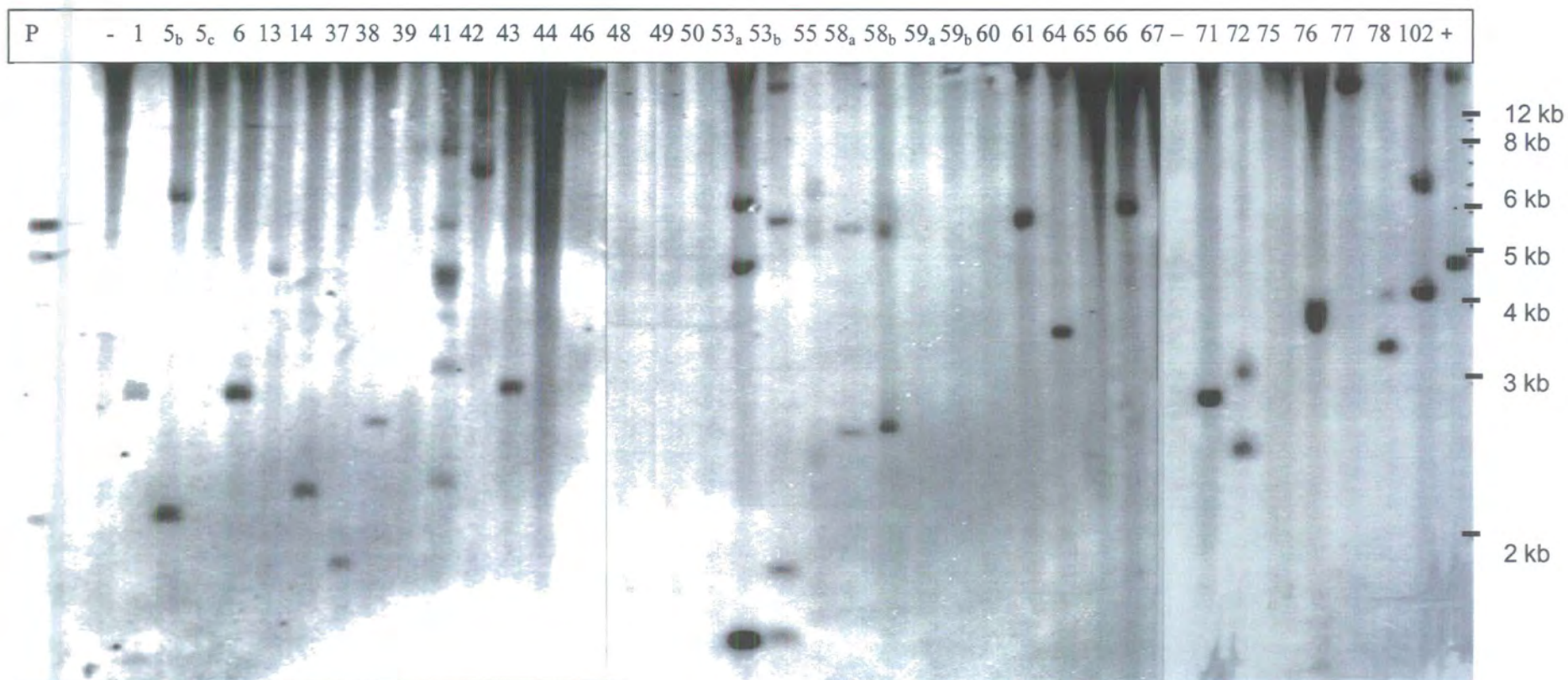
Figure 4.7 Southern analysis of transgenic strawberry

10 µg genomic DNA digested with *Sma* I was loaded per lane and probed with DIG labelled nptII probe. Individual pSCVFavRB7 lines are indicated above each lane. Plasmid control (P), non-transformed plant control (-) and a pSCV1.6 (+) transgenic line are also included.

Figure 4.8 Southern analysis of transgenic strawberry

10 μg genomic DNA digested with *Kpn* I and probed with DIG labelled *gusA* probe was loaded per lane. Individual pSCVFavRB7 lines are indicated above each lane. Plasmid control (P), non-transformed plant control (-) and a pSCV1.6 (+) transgenic line are also included. Lines with an additional fragment to the expected 4.6 kb fragment, representing some form of rearragement within the *FavRB7* gene promoter:*gusA* gene region, are marked with a red star.

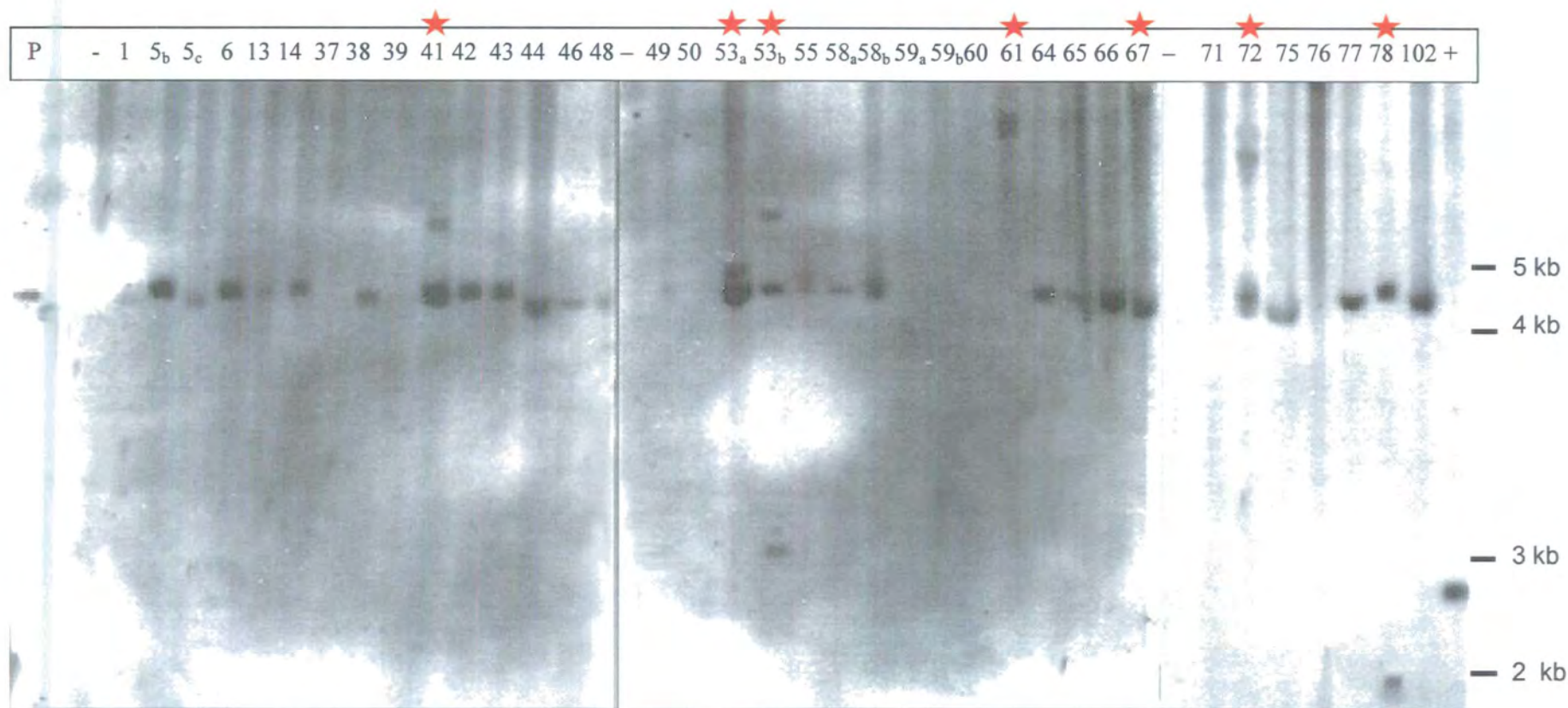Southern analysis established the number of integration sites for both *nptII* and *gusA* genes in the transgenic lines generated (Table 4.1). In the majority of the transgenic lines integration of both the *nptII* and *gusA* genes was found to have occurred at a single site, 20/34 and 27/34, respectively. Six lines (41, 53a, 53b, 67, 78 and 102) were found to contain multiple insertions of both genes. In four lines (41, 53a, 53b and 67) the integrity of the *FavRB7* gene promoter:*gusA* gene region could not be confirmed at all integration sites. Lines 58a and 58b were found to be clonal, having arisen from a single transformation event, and so only line 58a was subject to further analysis. Lines 50, 59a, 59b and 60 were found to contain neither the *nptII* or *gusA* gene, which supported the PCR observations (Figure 4.5) for these lines. These lines were not subject to further analysis.

Line	gusA	nptll	Line	gusA	nptll
-	0	0	55	1	2
1	1	2	58a	1	2
5b	1	2	58b	1	2
5c	1	1	59a	0	0
6	1	1	59b	0	0
13	1	1	60	0	0
14	1	1	61*	1	1
37	1	1	64	1	1
38	1	1	65	1	1
39	1	1	66	1	1
41*	2	7	67*	2	1
42	1	2	71	1	1
43	1	1	72*	2	2
44	1	1	75	1	1
46	1	1	76	1	1
48	1	2	77	1	1
49	1	1	78*	2	2
50	0	0	102	2	2
53a*	4	5	+	1	2
53b*	3	4			

Table 4.1 Number of integration sites observed for *gusA* and *nptll* in pSCVFavRB7 strawberry lines

The number of integration sites of each transgene in each pSCVFavRB7 strawberry line is indicated. Non-transformed control (-) and pSCV1.6 (+) lines are included. An asterisk denotes those lines highlighted in Figure 4.8, which may contain deletions or rearrangements within the *FavRB7* gene promoter:*gusA* gene region.

c) Histochemical analysis of transgenic strawberry

A β-glucronidase (X-Gluc) histochemical assay was carried out to assess expression of the *gusA* gene in a range of tissue types in the pSCVFavRB7 transgenic strawberry lines. Leaf, petiole and root samples were collected from all glasshouse grown lines. Not all lines flowered within the study period, however where possible floral organs (whole buds and open flowers) were also collected. All samples were stored at - 80°C prior to analysis.

Histochemical analysis was carried out as described in section 2.8.1. Blue staining was observed in the roots of all pSCVFavRB7 transgenic lines, at similar levels to that observed in the CaMV 35S line. No blue staining was observed for the *FavRB7* gene promoter lines in the leaves or floral organs, whereas intense blue staining was observed in the CaMV 35S line. Light blue staining was observed in the petioles of several of the *FavRB7* gene promoter lines, although not to the intensity of the CaMV 35S line, suggesting that *gusA* is also expressed to a lesser degree in the petioles of some *FavRB7* gene promoter lines (Figure 4.9). This suggests that the *FavRB7* gene promoter confers expression of *gusA* preferentially in the roots of strawberry.

Further samples of each tissue type were taken from non-transformed control, the CaMV 35S line and pSCVFavRB7 lines 37 and 53b and subject to histochemical assay. These samples were then fixed in 10% formalin prior to histological study, as described in section 2.8.2. To obtain detailed visualisation of *gusA* activity within the various tissue types, 10 µm sections were taken from the various tissues and photographed under magnification using the Zeiss axiophot microscope and a Nikon coolpix digital camera.

Intense staining as a result of *gusA* activity was observed in all tissues of the CaMV 35S line and was non-detectable in the non-transformed control line. Intense staining was only observed in the root tissues of the pSCVFavRB7 lines and no staining was observed in the leaf, floral organs or fruit of the pSCVFavRB7 lines.
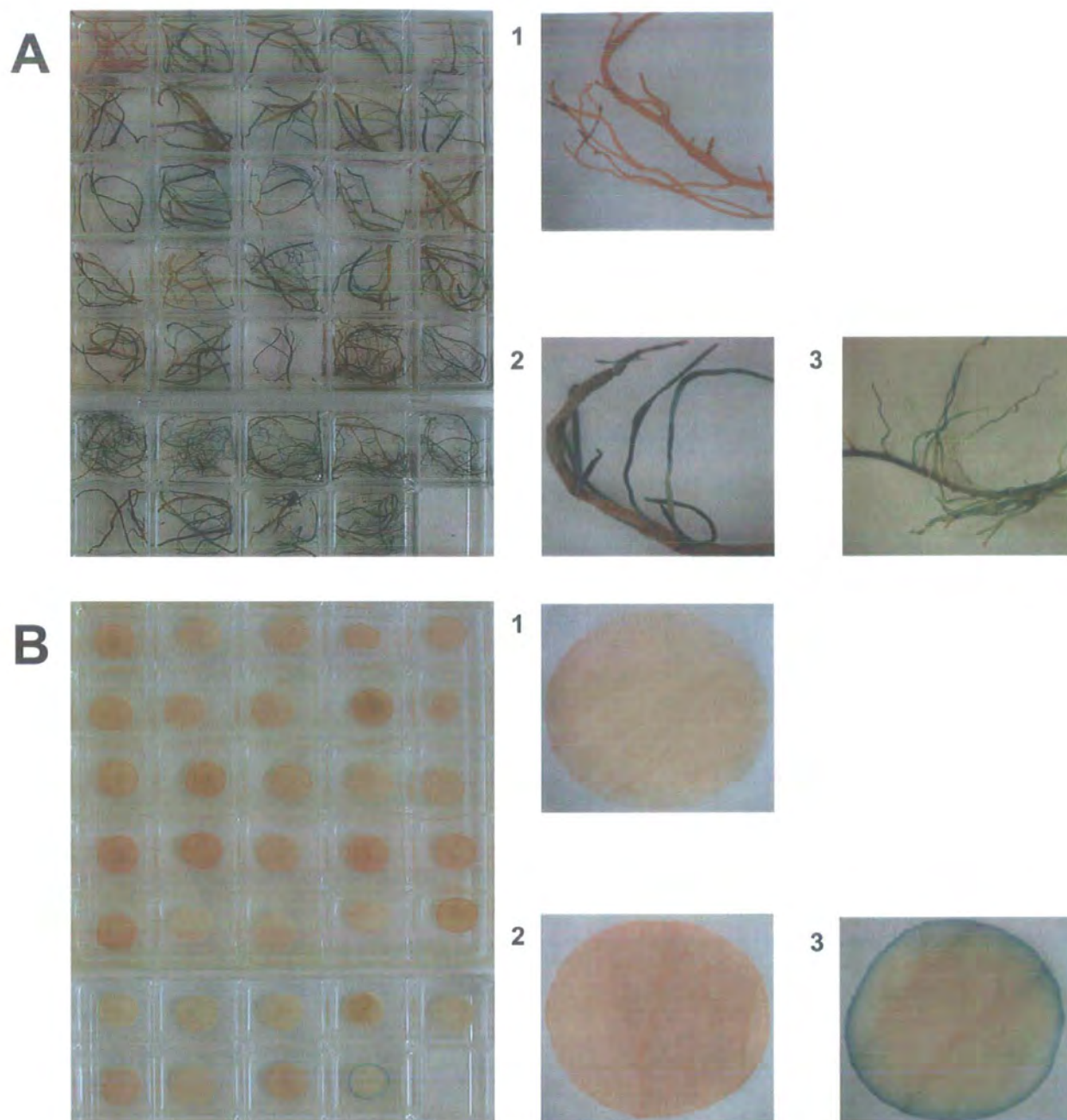
Figure 4.9 *gusA* histochemical analysis of transgenic strawberry lines

Various tissue types are shown for each line; root (A), leaf (B), floral organs (C) and petiole (D). Representative samples of negative control (1) pSCVFavRB7 (2) and pSCV1.6 constitutive control (3) lines are shown in detail. Lines represented in each block are indicated in the following table; + indicates pSCV1.6 constitutive control, - indicates negative control.

<u>Figure continued on next page</u>.

-	1	5b	5c	6
13	14	37	38	39
41	42	43	44	46
48	49	53a	53b	55
58a	61	64	65	66
67	71	72	75	76
77	78	102	+	
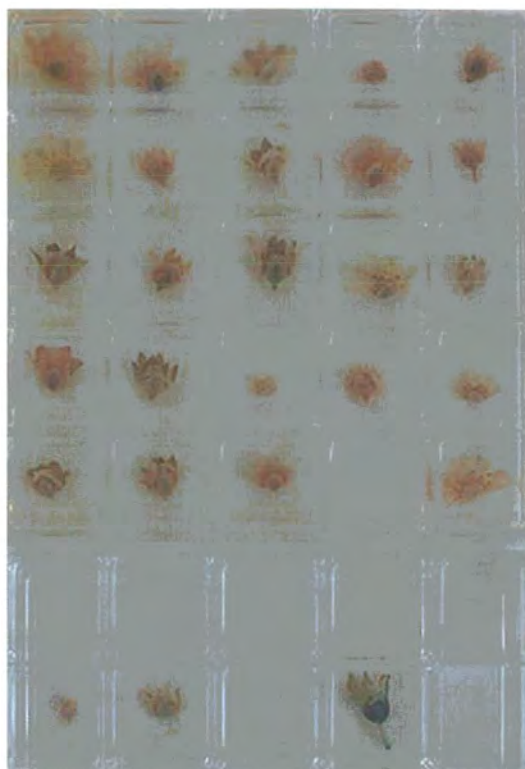
Figure 4.9 continued. *gusA* histochemical analysis of transgenic strawberry lines

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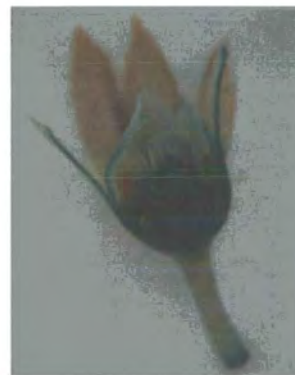However, slight staining was observed in the petioles. Microscopy revealed that *gusA* activity in the roots of the pSCVFavRB7 lines was highest in the central vascular region of the root and in the tip of the lateral root cap (Figure 4.10). Intense staining was often observed in the root tips, however it was thought that this may be a result of older root tissues being more highly suberised and less permeable to the β-glucuronidase substrate used in the histochemical assay rather than lack of activity in the older tissues.

To establish if the expression pattern conferred by the *FavRB7* gene promoter was maintained through vegative propagation in strawberry histochemical analysis was also carried out on a population of eight R1 lines, propagated from stolons of R0 pSCVFavRB7 lines. In all of the lines examined, the spatial expression pattern observed in the R0 lines of strong blue staining in the roots, weak staining in some petioles and no staining in the floral organs or leaves, was maintained in the R1 lines (Figure 4.11). This suggests that the transgene expression pattern conferred by the *FavRB7* gene promoter is maintained through vegetative propagation.

The pSCVFavRB7 transgenic tobacco lines were also subject to β-glucuronidase (X-Gluc) histochemical assay to assess the activity of the *FavRB7* gene promoter in a heterologous system. Tobacco tissue samples were collected from the leaf at the fourth internode, the petiole of this leaf, stem tissue above the fourth internode, young root tissue and floral organs. Comparable levels of blue staining were observed in all tissues for lines transformed with pSCV1.6 and pSCVFavRB7 (Figure 4.12). Although not quantitative, the histochemical assay showed that *gusA* expression levels were uniformly high in all tissues and not preferentially expressed in the roots of tobacco. This result clearly shows that *FavRB7* gene promoter activity differs significantly in tobacco compared to strawberry, behaving constitutively in the heterologous system.
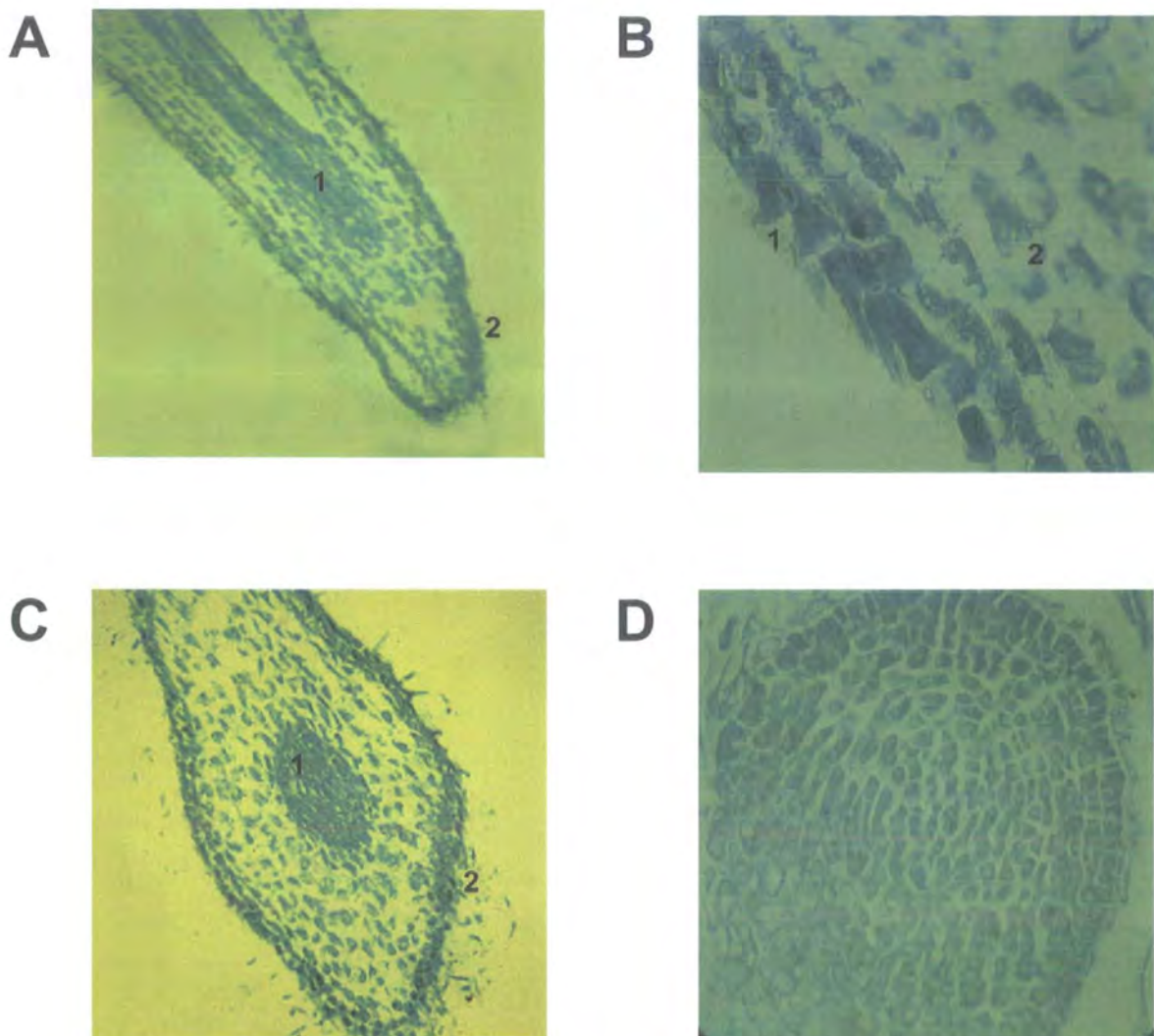
4.10 histological *gusA* histochemical analyses of transgenic strawberry roots

10 μm sections of root tissue taken from pSCVFavRB7 transgenic strawberry subject to histochemical assay are shown. *gusA* activity was observed throughout the roots, however longitudinal sections (A and B) showed *gusA* activity to be highest within the central vascular region of the root (A1) and the lateral root cap (A2), closer inspection of the epidermal layer revealed heaviest staining to be localised in the epidermis (B1) with lighter staining in the pericycle (B2). Transverse sections also showed highest *gusA* activity in the central vascular region (C1) and the epidermal layer (C2). Uniform staining was observed throughout the central vascular region (D).
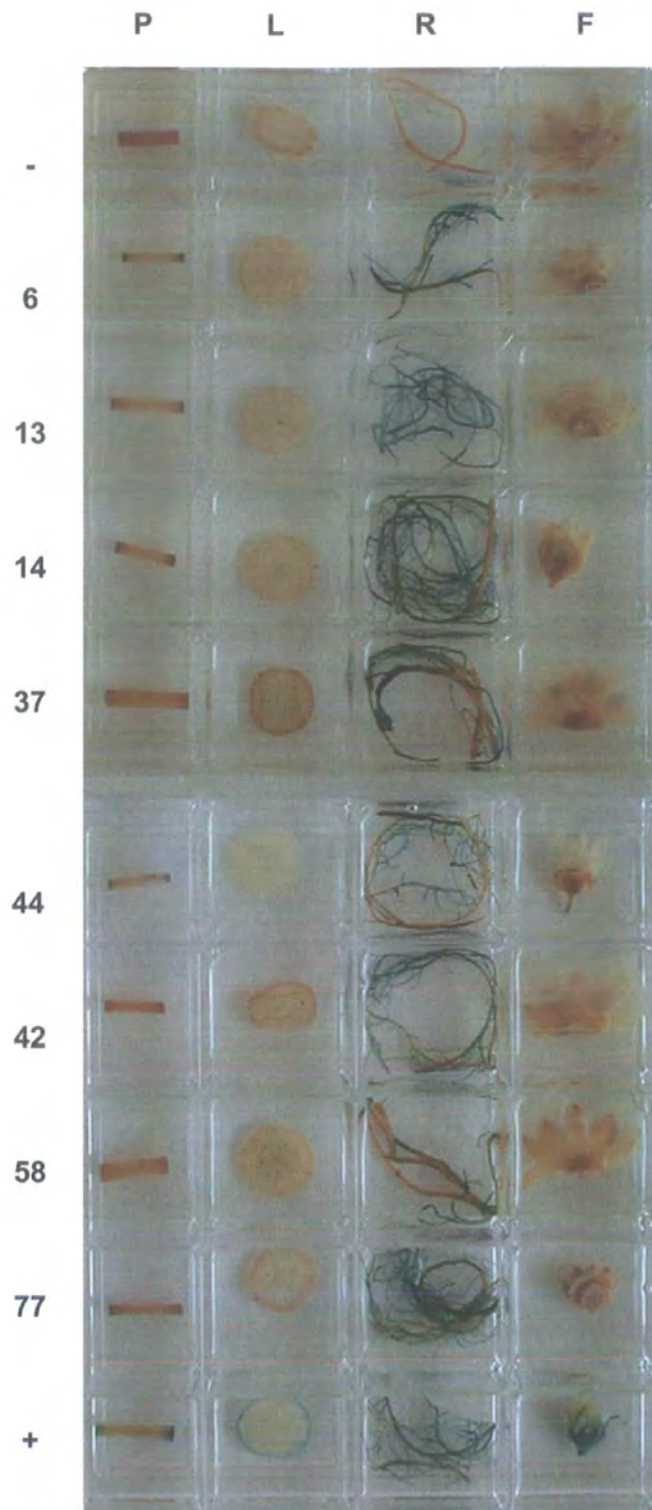
Figure 4.11 *gusA* histochemical analysis of transgenic R1 strawberry lines

Various tissue types subject to histochemical assay are shown for each R1 line; petiole (P), leaf (L), root (R), and floral organs (F). Independent pSCVFavRB7 R1 lines are indicated as are a non-transgenic control (-) and a pSCV1.6 constitutive expression line (+).
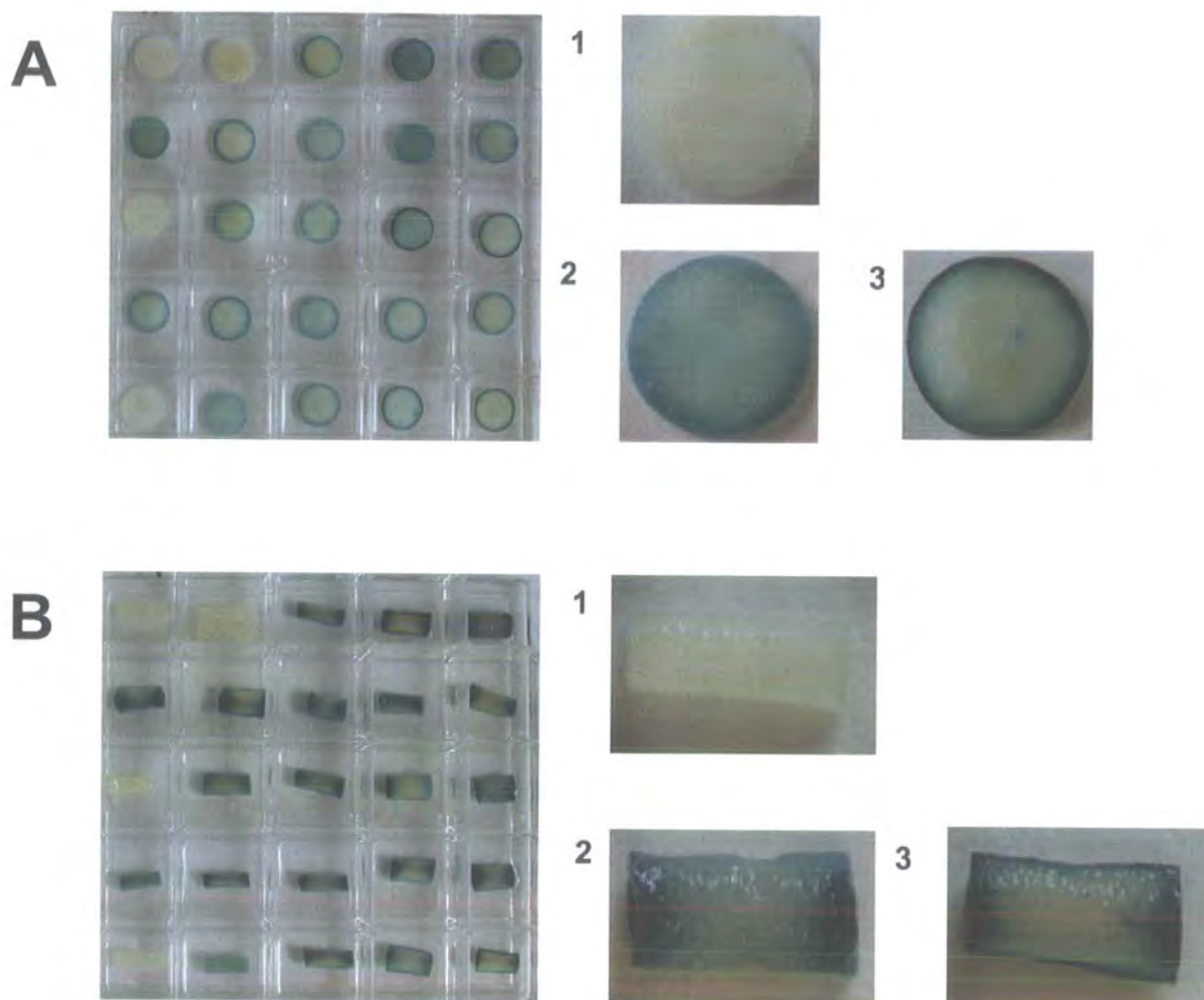
Figure 4.12 *gusA* histochemical analysis of transgenic tobacco lines

Various tissue types are shown for each clone; leaf (A), petiole (B), floral organs (C), stem (D) and root (E). Representative samples of negative control (1), pSCFavRB7 (2) and pSCV1.6 (3) transgenic lines are shown in detail.

Lines represented in each block are indicated in the following table; Petit Havana lines are represented in bold, Ottawa lines are in standard case, + indicates pSCV1.6 constitutive control, - indicates negative control.

PH-	O-	1	3	6
13	8	25	27	30
35	39	46	111+	90+
47	49	55	62	64
68	69	71	107+	95+
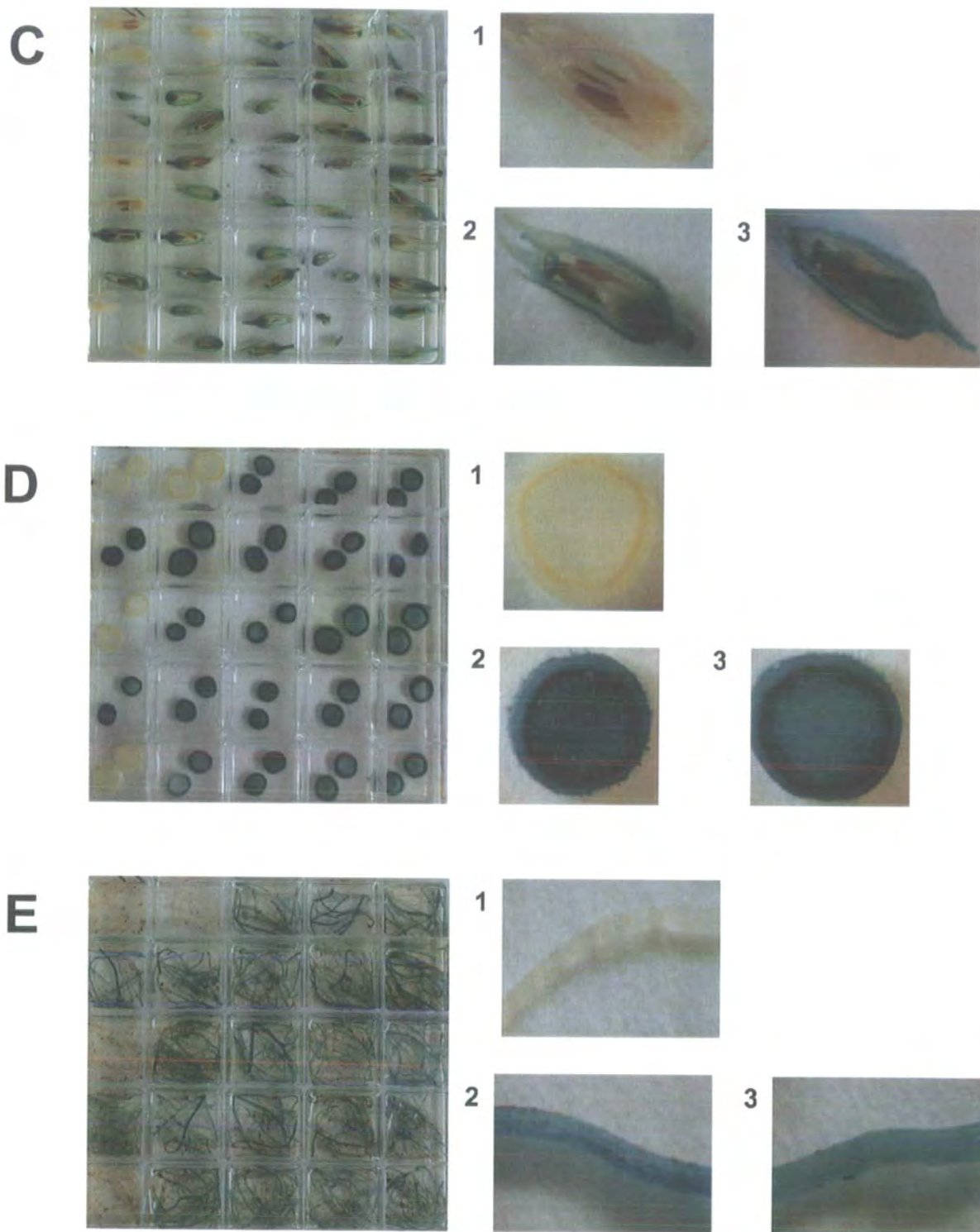
Figure 4.12 continued. *gusA* histochemical analysis of transgenic tobacco lines

d) GUS fluorometric analysis of transgenic strawberry

Fluorometric analysis was carried out to quantitatively assess the expression pattern of the *gusA* gene under control of the *FavRB7* gene promoter in strawberry. Leaf, petiole and root samples were collected from one pSCV1.6 and 32 pSCVFavRB7 lines of glasshouse grown transgenic strawberry. Not all clones flowered during the study period and no mature fruit was available for fluorometric analysis. However, floral organs, whole buds and flowers were collected from those clones producing flowers over a period of several weeks and stored at $-80^{\circ}C$.

To account for variation within the lines, samples were taken from 2 replicates of each line and a minimum of 6 replicates of each tissue type collected. For each line, leaves were excised using a 1 cm cork borer and petiole and root tissues were excised in sections of ~0.25 cm all tissues were immediately flash frozen in liquid nitrogen. Floral organs stored at $-80^{\circ}C$ were pooled prior to analysis. An aliquot of approximately 100 mg of each tissue type was taken for fluorometric assay, which was carried out as described in section 2.8.2

Fluorescence was quantified using a FLUOstar Galaxy multi well plate reader with the excitation filter set at 360 nm and the emission filter set at 460 nm. Each fluorescence reading was taken a minimum of four times and the average value calculated. Specific activity of *gusA* was quantified in terms of pmol 4-MU produced per min per mg total protein. Total protein was determined using the BioRad assay outlined in section 2.8.3 using the FLUOstar Galaxy multi well plate reader with the absorbance filter set at 595 nm. Each protein assay was read a minimum of four times and the average value calculated.

The fluorometric data generated from the 32 pSCVFavRB7 lines analysed revealed an average specific activity for *gusA* of 36,780 pmol 4-MU/min/mg in the roots and a comparable value of 40,482 pmol 4-MU/min/mg in the roots of the CaMV 35S line. By contrast, average *gusA* specific activities in the petioles, leaves and floral organs in the pSCVFavRB7 lines were found to be greatly reduced compared to the *gusA* expression in the roots (Figure 4.13). Average *gusA* specific
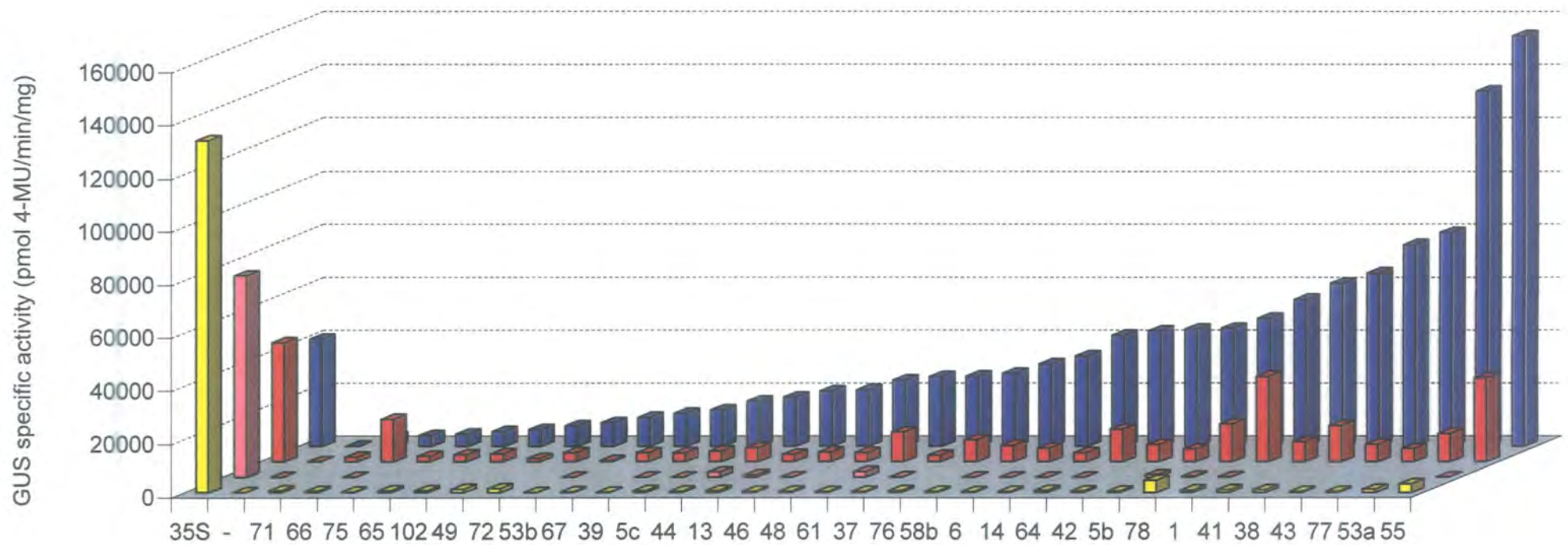
Figure 4.13 Specific activity of *gusA* driven by *FavRB7* and CaMV 35S promoters in transgenic strawberry

GusA specific activities are shown in pmol 4-MU/min/mg for a range of tissue types; root (blue), petiole (red), leaf (yellow) and floral organs (pink). Individual lines of pSCVFavRB7 transgenic strawberry are labelled. Specific activities for *gusA* conferred by the CaMV 35S promoter (35S) and non-transformed control (-) are also shown for each tissue.

activities for petiole, leaf and floral organs were found to be 7,417, 858 and 419 pmol 4-MU/min/mg respectively. For the same tissues CaMV 35S values were 44,908, 132,387 and 75,953 respectively. In pSCVFavRB7 lines the median *gusA* specific activity ratio between root: petiole was found to be 1: 0.3, root: leaf 1: 0.04 and root: floral organ 1: 0.009. These data corroborate the observations made using the histochemical assay that the *FavRB7* gene promoter confers expression of the *gusA* gene in a near root-specific manner.

Further examination of the fluorometric data revealed that the majority (25/32) of *gusA* specific activities in roots were over 10000 pmol 4-MU/min/mg for the pSCVFavRB7 lines (Figure 4.14). The lowest and highest values observed in root tissue were 3,518 and 154,500 pmol 4-MU/min/mg, respectively. By contrast the majority (25/32) of *gusA* specific activities in leaves were beneath 900 pmol 4-MU/min/mg (Figure 4.15). The lowest value observed for leaf tissue was 100 pmol 4-MU/min/mg. The majority (23/32) of *gusA* specific activities observed in petioles were beneath 10,000 pmol 4-MU/min/mg (Figure 4.16). However lines 1 and 55 both showed specific activities of over 30,000 pmol 4-MU/min/mg, which although lower than the root specific activities observed in these lines, is comparable with root specific activities for *gusA* observed in other lines. Only 17 lines contributed to the data generated for *gusA* specific activity in pSCVFavRB7 floral organs, of these lines the majority (13/17) exhibited specific activities beneath 160 pmol 4-MU/min/mg (Figure 4.17).

No significant correlation could be made between the number of copies of the *gusA* gene present in each line and the expression levels observed, however the majority (4/6) of those lines containing two or more copies of the *gusA* gene ranked in the bottom 30 % for *gusA* root expression levels. By contrast the line shown to have the second highest level of *gusA* expression in the roots (53a) contains four copies of the *gusA* gene and was implicated as containing possible rearrangements in the *FavRB7* gene promoter: *gusA* gene region (Figure 4.8). Anomalous levels of *gusA* gene expression were noted in several of the other lines suspected to contain such

Figure 4.14 *gusA* specific activity driven by the *FavRB7* promoter in root tissue of transgenic strawberry

GusA specific activities are shown in pmol 4-MU/min/mg for root tissues. Individual lines of pSCVFavRB7 transgenic strawberry and a non-transformed control (-) line are labelled
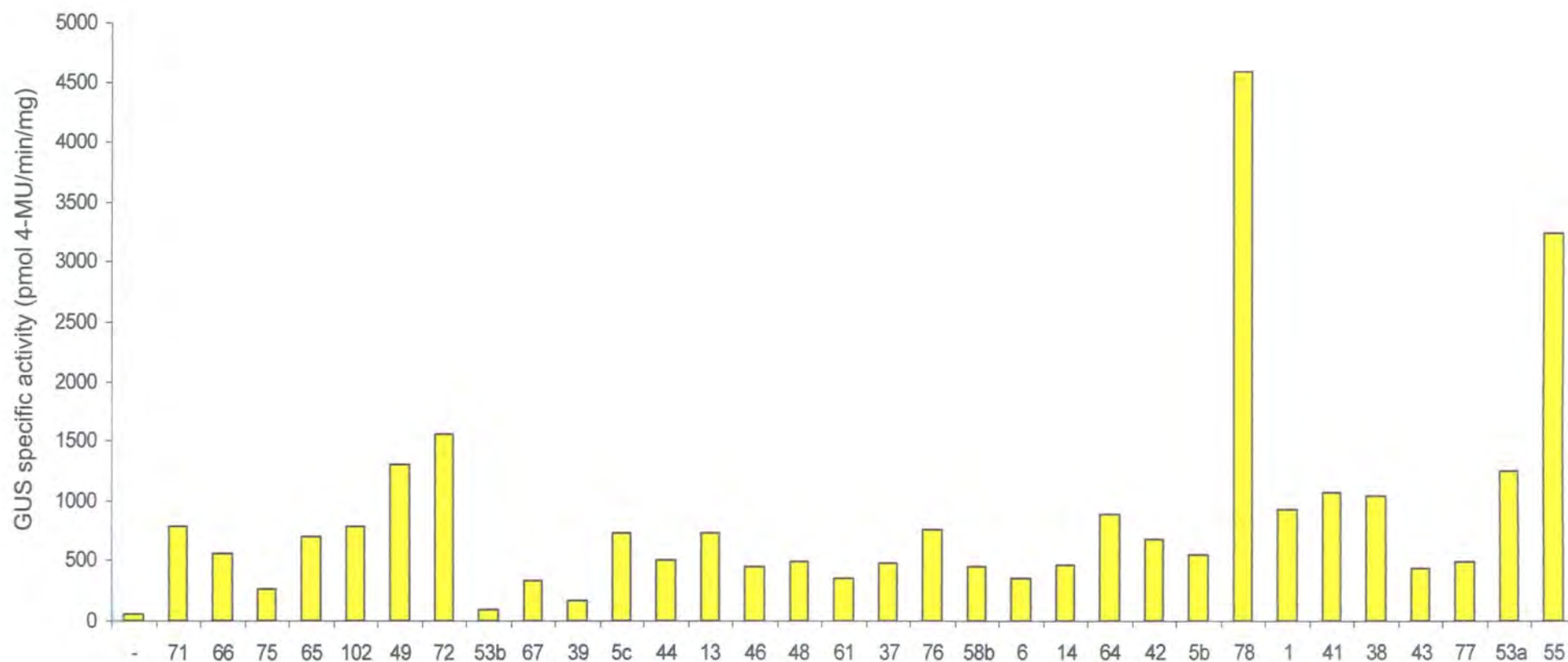
Figure 4.15 *gusA* specific activity driven by the *FavRB7* promoter in leaf tissue of transgenic strawberry

GusA specific activities are shown in pmol 4-MU/mg/min for leaf tissues. Individual lines of pSCVFavRB7 transgenic strawberry and a non-transformed control (-) line are labelled.

Figure 4.16 *gusA* specific activity driven by the *FavRB7* promoter in petioles of transgenic strawberry

GusA specific activities are shown in pmol 4-MU/min/mg for petiole tissues. Individual lines of pSCVFavRB7 transgenic strawberry and a non-transformed control (-) line are labelled.
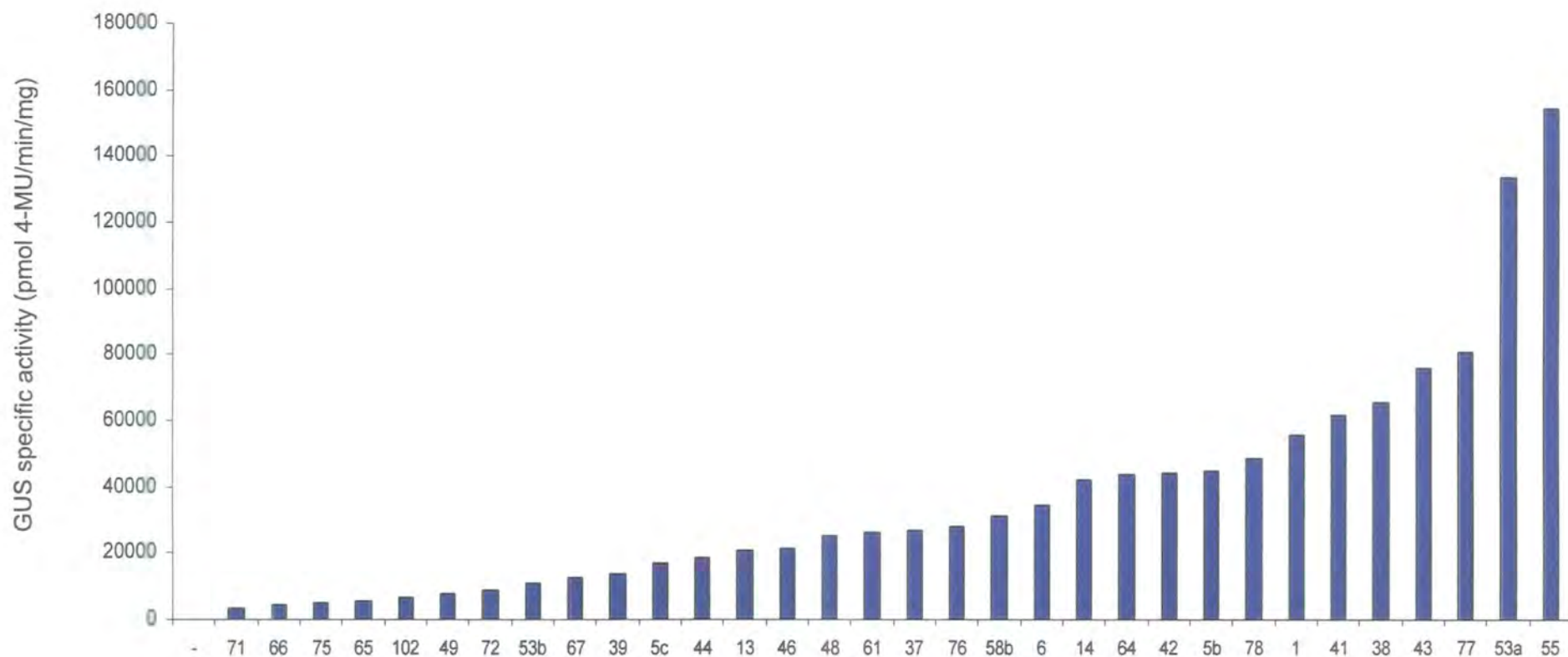
Figure 4.17 *gusA* specific activity driven by the *FavRB7* promoter in floral organs of transgenic strawberry

GusA specific activities are shown in pmol 4-MU/min/mg for floral organs. Individual lines of pSCVFavRB7 transgenic strawberry and a non-tra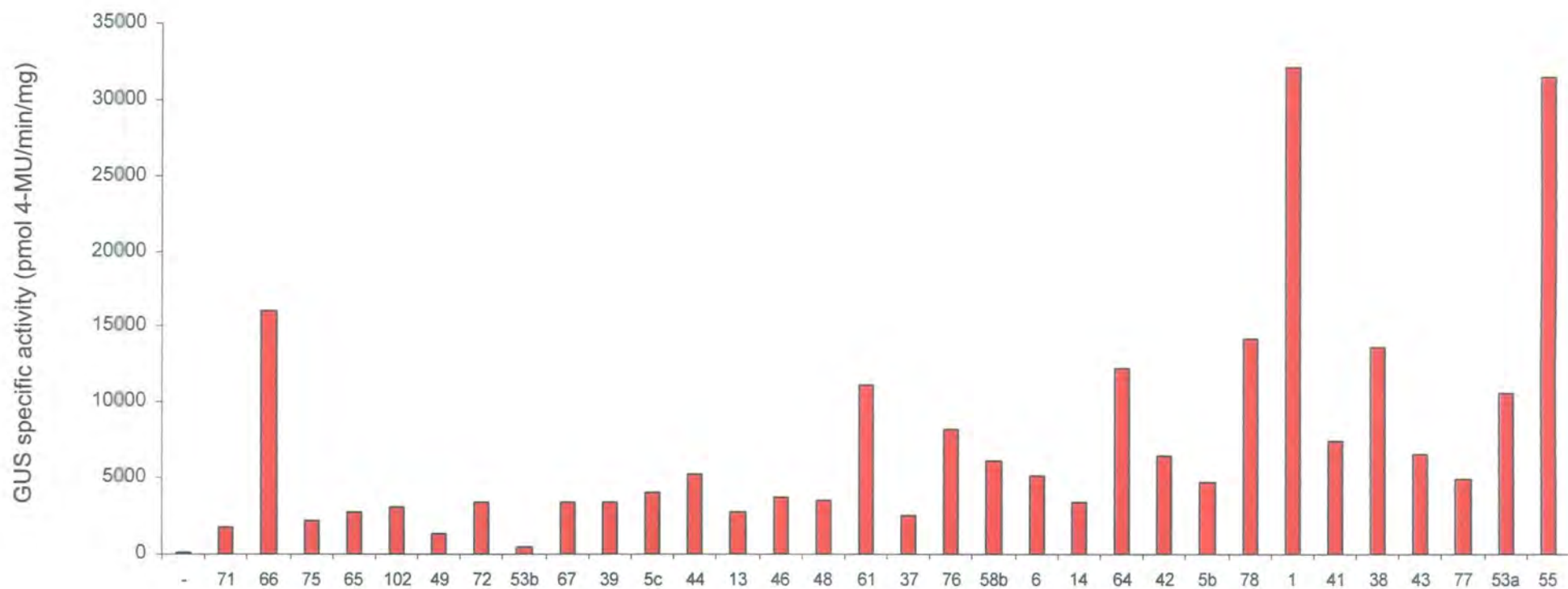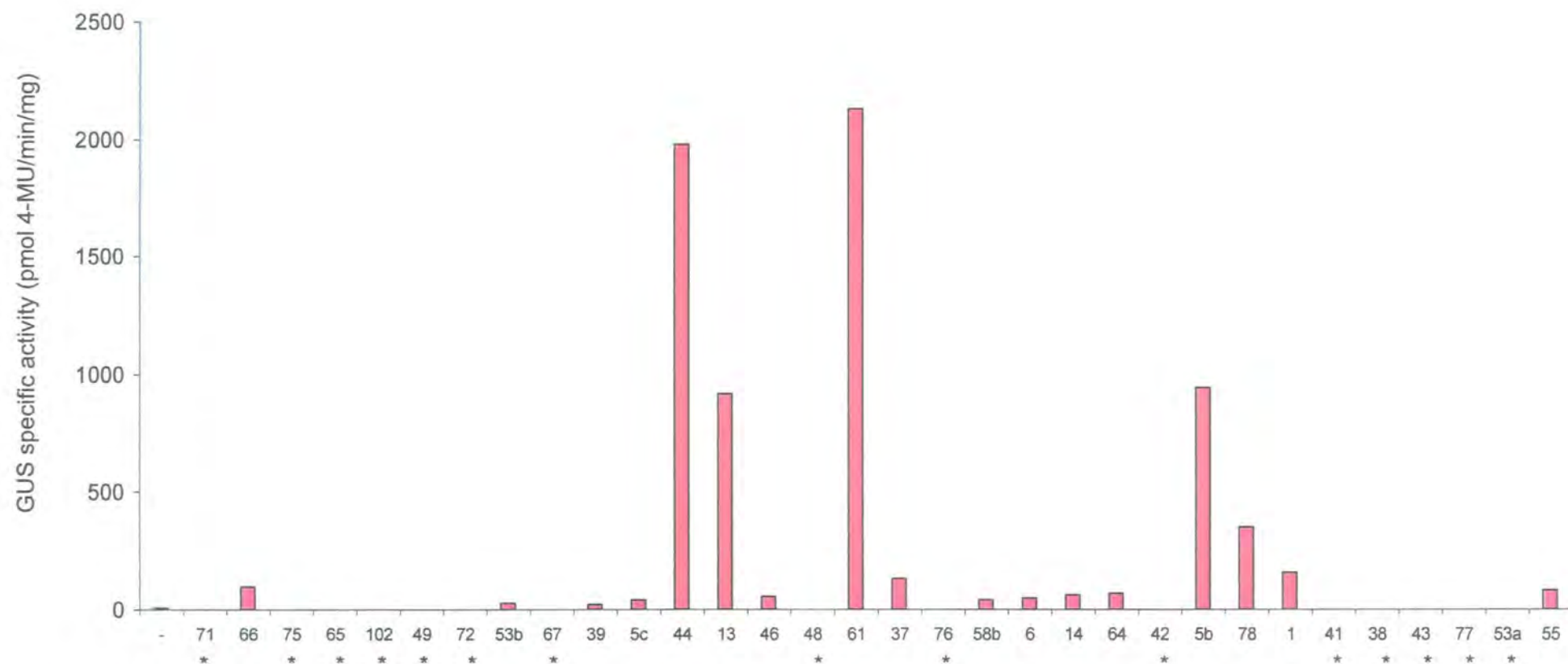nsformed control (-) line are labelled. An asterisk denotes lines where floral organs were not available for fluorometric assay.

rearrangements. Line 53b consistently displayed very low *gusA* expression levels in all tissue types other than roots, and lines 61 and 78 displayed the highest levels of *gusA* expression observed in floral organs and leaf tissues, respectively.

e) Reverse transcriptase PCR analysis of transgenic strawberry

Expression of the *nptII* and *gusA* genes were studied at the molecular level in both pSCVFavRB7 and pSCV1.6 lines of transgenic strawberry using reverse transcriptase PCR (RT-PCR). Messenger RNA extraction, cDNA synthesis and PCR were carried out as described in section 2.5.1. Primers NptIIA, NptIIB, GUS27 and GUS392 [Appendix 2] were used in a multiplexed RT-PCR reaction to detect expression of *nptII* and *gusA* transgenes in the root, leaf, petiole, floral organs and fruit. Lines 6, 14, 37, 46 and 55 were selected from the pSCVFavRB7 strawberry transgenics because fluorometric analysis had shown these lines to represent a range of *gusA* expression levels and samples were available for all of the tissue types examined in the fluorometric study. Lines 6, 46 and 55 also produced fruit during the study period. cDNA was synthesised from fruit tissues for these lines as well as the CaMV 35S constitutive control line.

Amplification fragments corresponding to the *nptII* and *gusA* genes were generated from all tissue types for the CaMV 35S line using RT-PCR (Figure 4.18). This confirmed that the assay was sensitive enough to detect the transgenes being expressed under control of the CaMV 35S promoter. Expression of the *nptII* gene under control of the CaMV 35S promoter was detected in all of the pSCVFavRB7 transgenic lines. The amplification fragment generated for the *gusA* gene under control of the *FavRB7* promoter was noticeably less intense than the *npt*II fragment generated in the pSCVFavRB7 lines. Only in the root tissues of line 55 was a *gusA* amplification fragment generated at similar levels to that of *nptII*. Although not quantitative this assay confirmed *gusA* activity conferred by the *FavRB7* promoter at the molecular level as was previously revealed through histochemical and fluorometric assay.
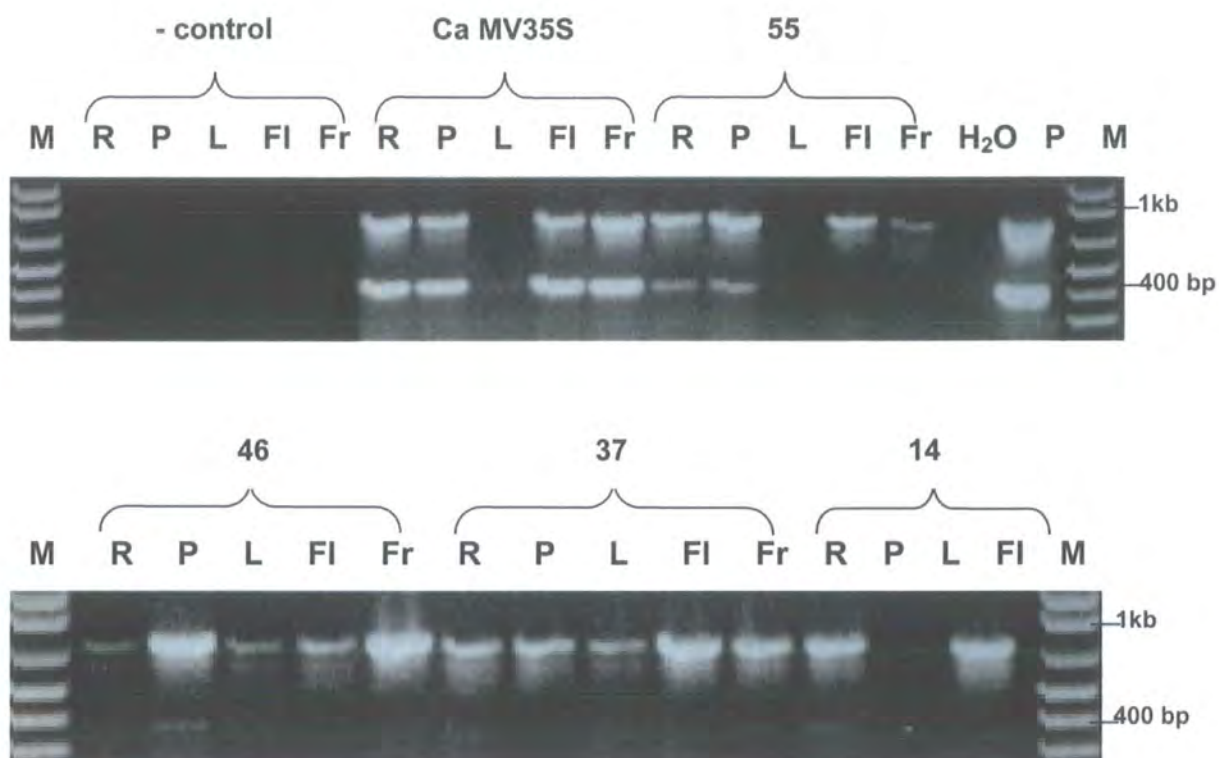
4.18 RT-PCR analysis of transgenic strawberry

RT-PCR was carried out using cDNA synthesised from various strawberry tissues; root (R), petiole (P), leaf (L), floral organs (Fl) and fruit (Fr). Expression of the *nptII* and *gusA* genes was revealed in PCR using the primer pairs NPTIIA/B and GUS27/392, respectively. Amplification fragments of ~800 bp represent *nptII* expression under control of the CaMV 35S promoter in all lines. Amplification fragments of ~360 bp represent *gusA* expression under control of the CaMV 35S promoter in the line labelled CaMV 35S and under control of the *FavRB7* promoter in pSCVFavRB7 lines 55, 46, 37 and 14.

4.3 Concluding remarks

In summary the *FavRB7* gene promoter isolated from strawberry was shown to act in a strongly root preferential manner in strawberry. Histochemical, fluorometric and molecular analyses revealed that its activity, as measured by activity of the expressed product of the *gusA* gene linked to the promoter, was low in tissues other than the root. In root however, *FavRB7* promoter activity was high and comparable to that of the CaMV 35S promoter.

Histochemical analysis showed that the *FavRB7* gene promoter imparts a very different spatial gene expression pattern in tobacco to that observed in strawberry. In tobacco the *FavRB7* gene promoter was found to act in a strong constitutive manner and accordingly may provide a useful alternative the CaMV 35S promoter in heterologous systems.

Chapter 5

Construction of plastid transformation vectors

5.1 Introduction

One of the main aims of this study was to achieve plastid transformation in strawberry to attain high levels of plastid localised transgene expression. Plastid transformation has most commonly been achieved in tobacco (*Nicotiana tabacum*) (Daniell *et al.*, 2002); it has yet to be achieved in strawberry. As outlined in chapter 1, there are many benefits to be gained through developing novel plastid transformation methodologies for a field grown perennial fruit. Strawberry was considered a suitable target for plastid transformation because of its high *in vitro* shoot regeneration and nuclear transformation efficiencies (James *et al.*, 1990).

This chapter describes the construction of a novel set of vectors designed to achieve plastid transformation in strawberry and tobacco. Tobacco has a relatively fast regeneration cycle of 3-4 weeks, as opposed to 8-12 weeks in strawberry, and plastid transformation has been routinely achieved using this species. To evaluate the relative efficiencies of the varying transformation methodologies applied using the novel transformation vectors, plastid transformation was also carried out in parallel with tobacco.

Typically plastid transformation vectors are comprised of a transcription unit, with plastid regulatory elements controlling expression of foreign genes, surrounded by plastome sequence acting as targeting sequence to direct homologous recombination (Figure 5.1). Plastome sequence isolated from both strawberry and tobacco was incorporated in plastid transformation vectors for this study to direct homologous recombination in strawberry and tobacco, respectively.

The plastome region selected for transgene insertion in these studies was the intergenic region between the tRNA-Val (*trn*V) and 3' ribosomal protein S12 (3' *rps*12)

133
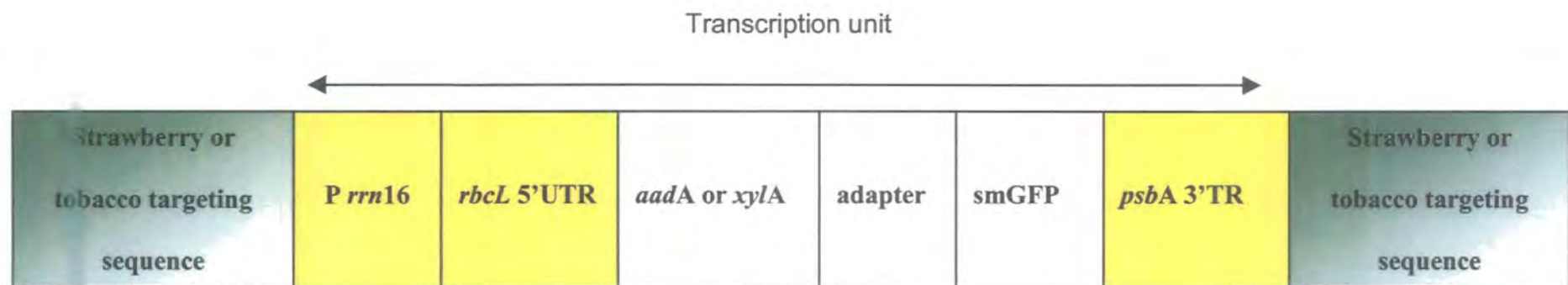
Figure 5.1 schematic showing the proposed composition of plastid transformation vectors

Plastome targeting sequence derived from either the *rrn*16 – *rps*7 plastome region of strawberry or tobacco surrounds the transcription unit. Selectable marker genes *aad*A or *xyl*A are linked by a synthetic adapter to the scorable marker gene, *smGFP*. Novel plastid regulatory elements, *rrn*16 promoter (P *rrn*16), *rbcL* 5' untranslated region (*rbcL* 5'UTR) and *psb*A 3' terminator region (*psb*A 3'TR) to be isolated from strawberry are highlighted in yellow.

genes. This region was chosen as it lies in the inverted repeat of the plastome. Therefore transcription units introduced at this site would be present as two copies per plastome and transgene expression levels could thus potentially be increased. This region is also transcriptionally active and has been used successfully several times in tobacco and has proved successful for arabidopsis and potato plastid transformation (McBride *et al.*, 1994; Sikdar *et al.*, 1998; Khan and Maliga, 1999; Sidorov *et al.*, 1999; Ye *et al.*, 2001).

Since plastid transformation has yet to be achieved in strawberry the transcription unit in the vectors designed for this study contained only selectable and scorable marker genes to serve as proof of concept for the methodologies developed.

It was decided to develop two selectable marker gene systems for strawberry plastid transformation. Firstly, antibiotic selection utilising resistance to spectinomycin conferred by the aminoglycoside 3'-adenyltransferase (*aadA*) gene was investigated. Spectinomycin selection has been successfully employed in plastid transformation of other species (Svab and Maliga, 1993; Koop *et al.*, 1996; Sikdar *et al.*, 1998; Khan and Maliga, 1999; Sidorov *et al.*, 1999; Ruf *et al.*, 2001) and was used in strawberry to test the hypothesis that the strawberry plastome can be amenable to transformation.

As previously discussed in chapter 1, it is desirable to avoid the use of antibiotic marker genes. The second choice of selectable marker gene system addresses this issue and utilises the xylose isomerase (*xylA*) gene, whose product reduces D-xylose to D-xylulose. Since xylulose is metabolisable and xylose is not transformed plant tissues have the selective agvantage and are able to grow in the presence of xylose (Haldrup *et al.*, 1998a, b; Haldrup *et al.*, 2001)

The *aadA* gene used in these studies was isolated from plasmid pZS208 (Zoubenko *et al.*, 1994) and the *xylA* gene, isolated from *Streptomyces rubiginosus*, was isolated from plasmid pVicxylAH (Haldrup *et al.*, 1998a).

For both selection systems a gene for a soluble modified form of the jellyfish (*Aequorea victoria*) green fluorescent protein (sm*GFP*) was introduced as a scorable marker in the plastid transformation vectors to enable visual screening of transformed lines and assist in the selection of homoplasmic tissues. The *smGFP* gene was isolated from the plasmid psmGFP (Davis and Vierstra, 1998), obtained from the Arabidopsis Biological Resource Centre (Columbus, Ohio, USA).

Plastid gene regulatory elements were required to control expression of the selectable and scorable marker genes, linked by an adapter, as an operon. It was decided to isolate novel plastid gene regulatory elements from strawberry to increase the likelihood of the transcription unit functioning in strawberry. Since plastome gene order is highly conserved between higher plant species (Stoebe *et al.*, 1998) a PCR based approach was used to isolate these regulatory elements from the strawberry plastome. The regulatory elements chosen are highlighted in Figure 5.2 alongside corresponding regions of the tobacco plastome.

The constitutive 16S rRNA (*rrn16*) promoter (Figure 5.2) was isolated from strawberry to drive expression of the selectable and scorable marker genes as a single operon. Ribosomal RNAs (rRNAs) are structural ribonucleic acids that form one of the major constituents of the ribosomes. In association with specific proteins the 16S rRNA (*rrn16*) forms the smaller, 16S subunit, of the ribosome and is constitutively produced in the plastids (Sriraman *et al.*, 1998). The structural function of rRNA molecules means that although constitutively transcribed these sequences remain untranslated (Watson, 1992).

Previous studies have implicated as little as 90 bp of plastome sequence within a transcription unit as sufficient to facilitate intermolecular recombination and cause the inadvertent excision of a reporter gene (Eibl *et al.*, 1999). Recombination occurring between repeated sequences integrated into the tobacco plastome was also exploited to excise selectable marker genes (Iamtham and Day, 2000). It was decided to express the genes under control of a single *rrn16* promoter to reduce the chance of recombination occurring between repetitive *rrn16* promoter sequences. To
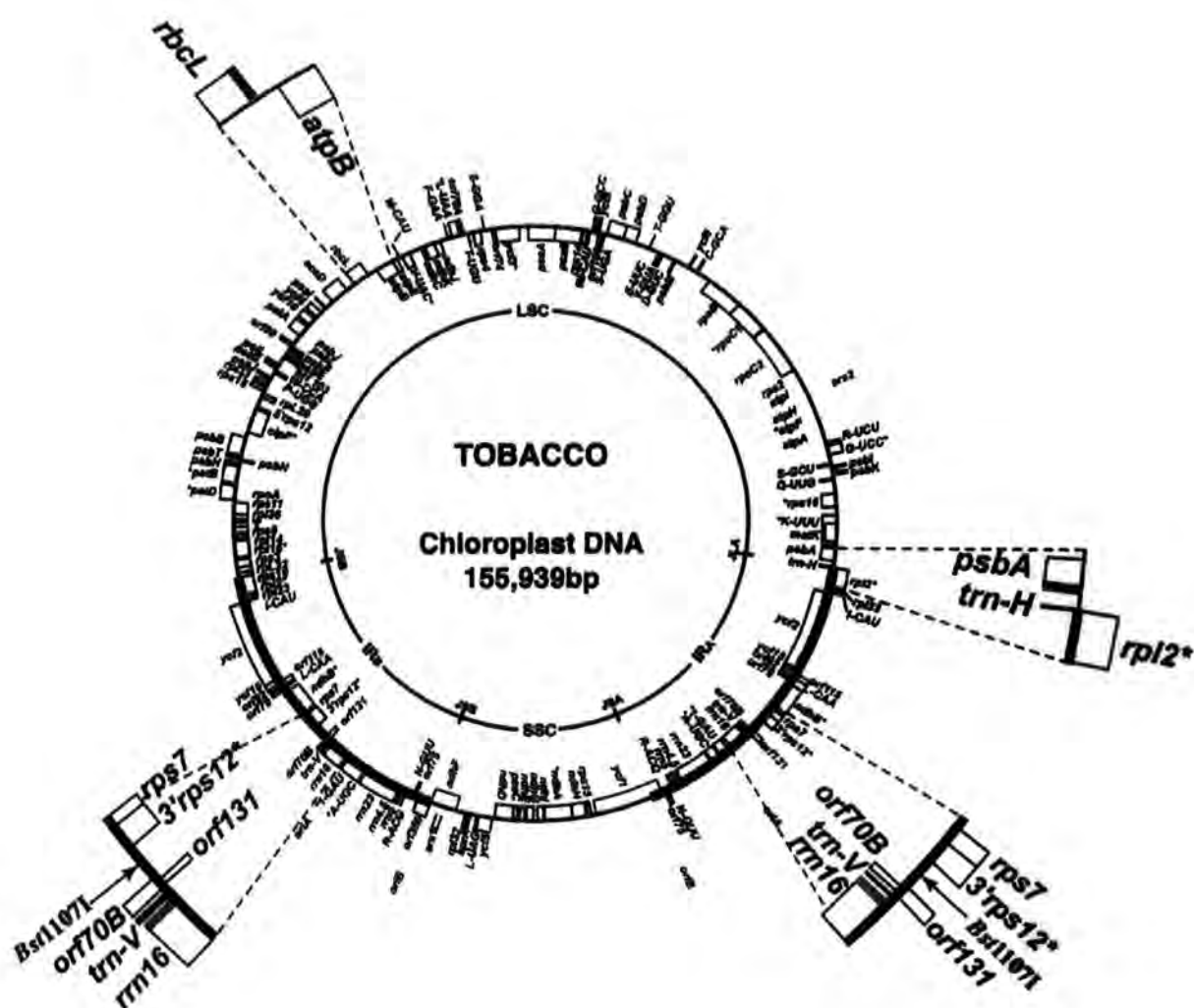
Figure 5.2 Gene map of tobacco chloroplast genome

Regulatory elements corresponding to those isolated from strawberry are shown in enlarged text; *rrn*16 promoter (red), *rbc*L 5' UTR (navy) and *psb*A 3' UTR (green). The *rps*7 – *rrn*16 gene region was chosen to act as targeting sequence in this study with transcription units inserted at the *Bst*1107I site present in the intergenic region between the *trn*-V and *rps*12 genes. The genome size is 155,939 bp and consists of 86,686 bp of large single-copy region (LSC) and 18,571 bp small single-copy region (SSC) and two inverted repeats (IR) s of 25,341 bp each. Genes shown on the inside of the circle are transcribed clockwise and genes on the outside are transcribed anti-clockwise. Asterisks denote split genes. Orf plus codon number represent open reading frames. Ycf plus designation number shows open reading frames of unknown function but common to plastomes. Adapted from Wakasugi 1998.

further reduce the likelihood of recombination occurring between the two promoter sequences the series of vectors designed for this study were created such that the native and introduced *rrn16* promoters were transcribed in opposing orientations.

To ensure that the selectable and scorable marker genes driven by the *rrn16* promoter would be translated it was necessary to incorporate sequence elements downstream of the promoter that would be recognised by ribosomes as the correct site from which to initiate translation. The strawberry RuBisCo large subunit (*rbcL*) gene 5' untranslated region (5'UTR) (Figure 5.2) was selected for this purpose and incorporated upstream of the selectable marker genes. In tobacco the *rbcL* 5'UTR contains signals that help to form a stable complex between the ribosome and mRNA, ensuring the following transcript is translated (Eibl *et al.*, 1999). The *rbcL* 5'UTR has been successfully used in conjunction with the *rrn16* promoter in stable tobacco plastid transformation (Maliga, 1999) and to transiently express GFP in both photosynthetic and non-photosynthetic plastid types (Hibberd *et al.*, 1998).

It has been suggested that translation of the second gene in an operon can be initiated *de novo* from the internal initiation codon (Staub and Maliga, 1995). However several other studies have shown that the presence of Shine-Dalgarno (SD) like sequences, present in the 5'UTR of chloroplast mRNA preceding secondary genes, act as ribosome binding sites and are essential for efficient translation (Betts and Spremulli, 1994; Hirose *et al.*, 1998; Esposito *et al.*, 2001). The SD element (GGAGG) found in the strawberry *rbcL* 5'UTR was therefore also incorporated in the adapter sequence upstream of the scorable marker gene.

To ensure that stable transcripts were produced from the introduced transcription units the strawberry photo-system II core protein gene (*psbA*) 3' terminator region (3'TR) (Figure 5.2) was introduced downstream of the *smGFP* termination codon. In tobacco the *psbA* 3'TR has been shown to help stabilise plastid transcripts (Eibl *et al.*, 1999). Most chloroplast transcription units contain short inverted repeats in their 3' terminator regions (3'TR). These sequences can potentially form stem-loop structures which are known to act as transcription

terminators in prokaryotes. However, it is thought that these repeats may act as RNA processing signals rather than terminators in the chloroplast (Stern and Gruissem, 1989). Examinations *in vitro* have indicated that these 3'TR structures are critical for the stability of RNAs in chloroplasts (Adams and Stern, 1990).

5.2 Isolation of plastid transformation vector components

Plastome gene and regulatory sequences entered in the major databases from different plant species were compared using the DNAstar Megalign (Lasergene) software. Primers were designed to anneal within conserved regions identified using Megalign and used to amplify corresponding regions of the strawberry plastome. Primers were designed to add restriction endonuclease sites to the 5' and 3' termini of amplified sequences to facilitate cloning.

PCR reactions were carried out using the proof reading Platinum® *Pfx* DNA polymerase as outlined in section 2.4.2. Gel isolation of amplification products and colony PCR were carried out as described in sections 2.5.3 and 2.2.3, respectively. TOPO plasmid transformation and preparation of plasmid DNA were carried out as described in sections 2.3.7 and 2.4.2, respectively. Restriction endonuclease digestion of plasmid DNA, blunt-ending, dephosphorylation and ligation of DNA fragments were all carried out as described in section 2.3

Sequences amplified from strawberry were compared to those in the major databases to confirm homologies, using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) provided by the National Centre for Biotechnology Information at www.ncbi.nlm.nih.gov.

All sequence positions shown for tobacco relate to the *Nicotiana tabacum* chloroplast genome DNA, (EMBL Z00044, Shinozaki *et al.* 1986).

5.2.1 Isolation of plastome targeting sequence from strawberry and tobacco

A 5508 bp region of the strawberry plastome was isolated by Dr Andrea Massiah. This represented the 5' end of the 16S rRNA gene (*rrn*16); *trn*V and 3'*rps*12 genes and the 5' end of the ribosomal protein S7 (*rps*7) gene. Of this sequence, 4495 bp were cloned into pBluescript, creating the vector pStraw4.5, corresponding to tobacco plastome sequence positions 138370 – 142865 in Inverted Repeat A (IRA). The respective (4421 bp), region of the tobacco plastome was cloned into pCR® Blunt II-TOPO, by Prof David James, creating the vector pTob4.4. The two vectors were kindly donated for use in these studies.

To facilitate PCR and Southern analyses of transgene integration into the native plastome, it was desirable to have known plastome sequence outside of that incorporated into the transformation vectors. For this reason the previously isolated plastome sequences were not used in their entirety.

The vector pStraw4.5, containing the isolated strawberry plastome sequence, was digested with *Stu* I and *Psi* I, releasing a 3179 bp blunt-ended fragment [SEQ 1, Appendix 4]. This fragment was gel isolated and ligated into *Sma* I cut, dephosphorylated pBluescript, to create the vector **pStraw3.2.**

The vector pTob4.4, containing the isolated tobacco plastome sequence, was digested with *Mbi* I and *Psi* I, releasing a 3351 bp blunt-ended fragment, corresponding to tobacco plastome sequence positions 139241 - 142592 [SEQ 2, Appendix 4]. This fragment was gel isolated and cloned into pCR®-Blunt II-TOPO, to create the vector **pTob3.3**.

Sequence homology analysis was carried out between the previously isolated tobacco and strawberry plastome sequences covering the *trn*V – 3'*rps*12 genes. This revealed an overall homology of 68.4% (Figure 5.3). Analysis, between sequence regions known to encode genes in the *trn*V – 3'*rps12* region of the tobacco
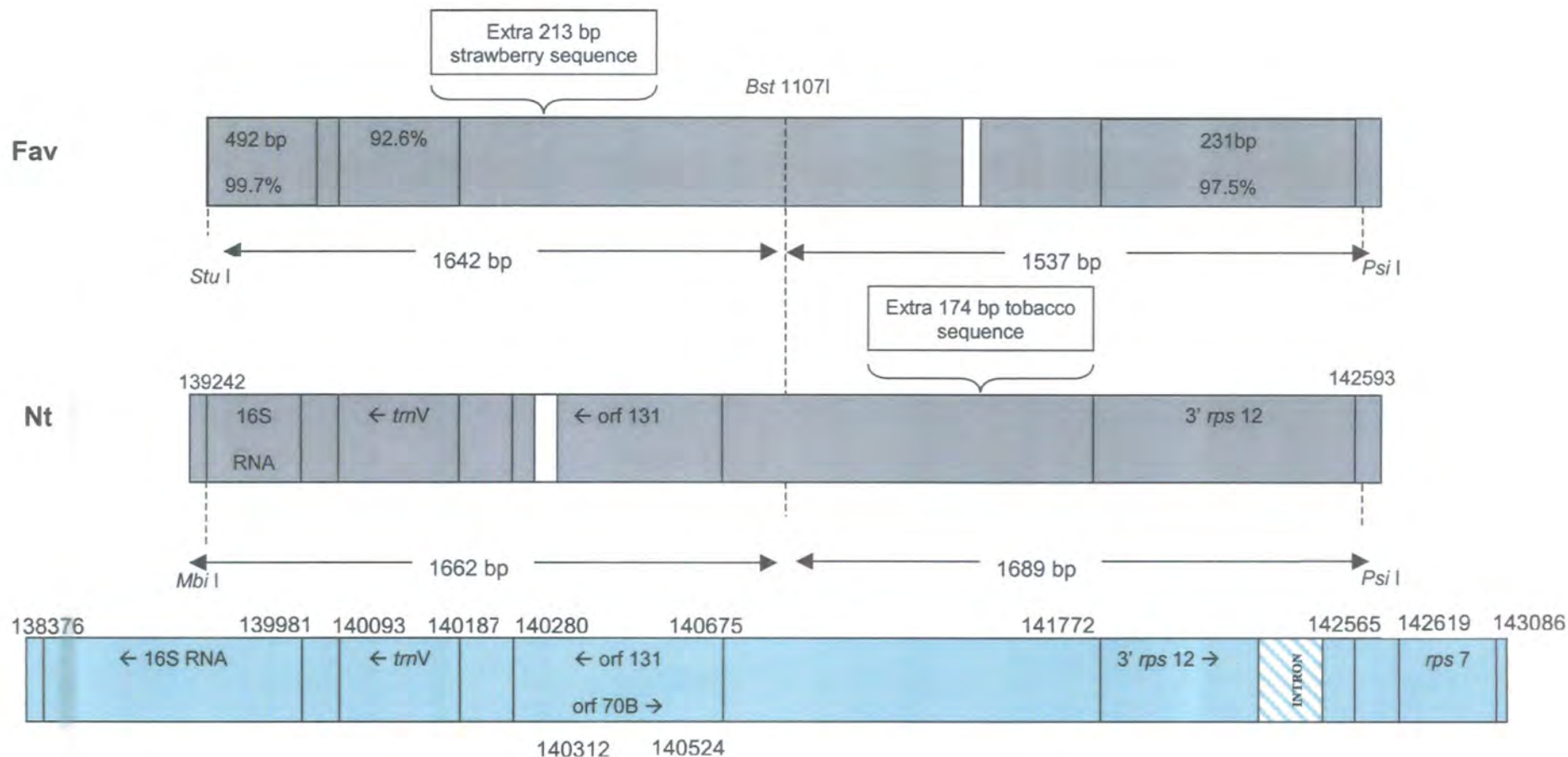
Figure 5.3 Alignment of strawberry and tobacco *trnV – 3'rps12* plastome targeting regions

Graphical representation of sequence homology analysis carried out using the clustal method between strawberry (Fav) and tobacco (Nt) plastid targeting sequences. Pale blue shading denotes reference tobacco plastome sequence, with gene positions indicated as described by Shinozaki *et al*. 1986. Grey shading denotes homologous sequence (overall homology = 68.4%), significant areas of divergence are indicated in white. Genes present in the tobacco plastome are labelled and their orientation indicated by arrows. Percentage homologies between regions of the strawberry plastome corresponding to known tobacco coding sequences are indicated in corresponding boxes. The *Bst* 1107I restriction enzyme sites used for insertion of transcription units and during cloning are also indicated.

plastome and corresponding regions in the strawberry plastome, revealed homologies of 92.6% to 99.5%. This provided strong evidence that the plastome gene order in this region is conserved between tobacco and strawberry.

A unique Bst 1107I site present in both plastome sequences was used to introduce the transcription units. This would result in 1642 bp and 1537 bp of plastome sequence either side of the introduced transcription unit for strawberry and 1662 bp and 1689 bp for tobacco in the vectors.

5.2.2 Isolation of selectable and scorable marker genes

a) Selectable marker gene: aminoglycoside 3'-adenyltransferase (aadA)

PCR primers, AADA PAG1 and AADA XHO 1 [Appendix 3] were designed to incorporate a Pag I restriction site across the initiation codon of the aadA gene from plasmid pZS208 and a Xho I site at the 3' end of the gene. PCR was carried out and the 808 bp amplification product gel isolated and transformed into the vector pCR zero-Blunt II-TOPO. Plasmid DNA was prepared from transformed colonies and sequenced to confirm the correct addition of Pag I and Xho I restriction sites [SEQ 5, Appendix 4]. The authenticated plasmid was named **paadA**.

b) Selectable marker gene: xylose isomerase (xylA)

The isolation of the xylA gene was carried out essentially as described for the aadA gene. A Pag I site was incorporated across the initiation codon and a Xho I site at the 3' end of the xylA gene using the primers XYLAPAGI and XYLAXHOI [Appendix 3] to enable later cloning. The 1319 bp amplification product was gel isolated, transformed into the vector pCR zero-Blunt II-TOPO and sequenced to confirm the correct addition of Pag I and Xho I restriction sites [SEQ 6, Appendix 4]. The authenticated plasmid was named **pxylA**.

c) Scorable marker gene: soluble modified green fluorescent protein (smGFP) and synthetic adapter to link selectable and scorable marker genes

An adapter was created to join the 3' termini of each selectable marker gene to the 5' termini of the *smGFP* gene in the transcription unit. The adapter was attached to the 5' end of the smGFP gene in the form of an oligonucleotide primer using the vector psmGFP as template in PCR. The primer used was *aadA/xylA: smGFP* (Figure 5.4). This primer contains additional stop codons to ensure efficient termination of *aadA/ xyl*A translation, a translation initiation site and *Pst* I and *Xho* I restriction sites to enable cloning. The second primer used in the reaction was SMGFP BGLAPA [Appendix 3], which introduces *Bgl* II and *Apa* I restriction sites at the 3' end of the *smGFP* gene.

The 762 bp amplification product was gel isolated and transformed into the vector pCR zero-Blunt II-TOPO. Plasmid DNA was prepared from transformed colonies and sequenced to confirm the correct addition of the adapter sequence and restriction sites to the *smGFP* gene [SEQ 7, Appendix 4]. The authenticated plasmid was named **pGFPad**.

5.2.3 Isolation of novel plastid regulatory elements from strawberry

a) Isolation of the strawberry plastid 16S rRNA (*rrn16*) promoter

The vector pStraw4.5 contains strawberry plastome sequence including the constitutive *rrn*16 promoter (Maliga, 1999). Sequence homology analysis was carried out between the tobacco *rrn16* promoter sequence, positions 139906 – 140021, inverted repeat A (IRA), and the corresponding strawberry sequence. This revealed a homology of 90.5% at the nucleotide level. Putative -10 'TATA', -35 'TTGACG' and rRNA upstream activator (RUA) 'GTGGGA' signals present in tobacco, found to be crucial for promoter activity in tobacco were also observed upstream of the proposed

5'   **3'**

Pst I   Xho I   1   2   3   SD   Pag I

atc, tgc, agc, tcg, aga, **tga**, c**ta, a**tt, a**g**a, agg, gag, gga, atc, ATG, AGT, AAA, GGA, GAA, GAA, CTT, TTC, ACT, G
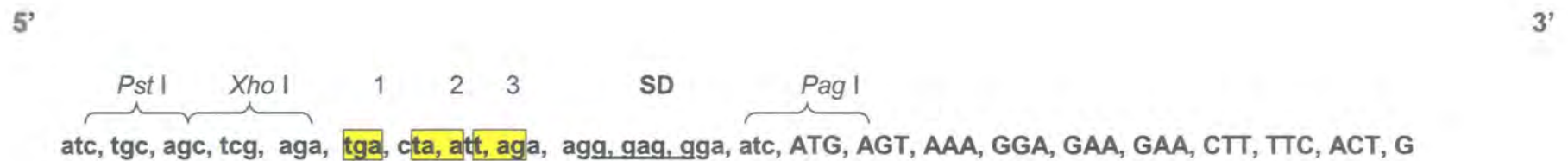
Figure 5.4 *aadA/xylA: smGFP* adapter primer

Nucleotides derived from the *smGFP* gene are in uppercase, introduced non-annealing sequence elements are in lowercase. Restriction enzyme sites introduced to enable cloning are bracketed. The *rbcL* 5'UTR Shine-Dalgarno (SD) translation initiation sequence is underlined. Termination codons to be introduced downstream of the selectable marker genes present in each reading frame are highlighted in yellow and numbered.

16S rRNA initiation codon in strawberry providing further evidence that this region represents the strawberry *rrn16* promoter (Figure 5.5).

PCR primers RRN16 KPNSGF and RRN19 SSP [Appendix 3] were designed to incorporate *Kpn* I and *Sgf* I restriction sites at the 5' end of the promoter and an *Ssp* I site at the 3' end. The 132 bp PCR amplification product was gel purified and transformed into the vector pCR zero-Blunt II-TOPO. Plasmid DNA was prepared from colonies transformed with the vector and sequenced to confirm correct addition of restriction sites [SEQ 3, Appendix 5]. The authenticated plasmid was named p*rrn*16.

b) Isolation of the strawberry plastid RuBisCo large subunit (*rbcL*) gene 5' un-translated region (5'UTR)

The *rbcL* 5'UTR in tobacco lies between the *rbcL* gene (position 57595...59028) and the ATP synthase β-subunit (*atpB*) gene (position [complement] 55281...56777), as is highlighted in Figure 5.2. It was assumed that this gene order would be conserved in strawberry. To amplify the *rbcL* 5'UTR from strawberry, the approach used was to identify conserved regions in *rbcL* and *atpB* homologues from different species and design primers against these regions and use them in PCR.

Pair-wise comparison of the *rbcL* genes from tobacco (Z00044), *petunia* (X04976), *capsicum* (U08610), *datura* (U08611), *ipomoea* (X60663), *anthocercis* (U08608), *physalis* (U08617), *salpiglossis* (U08618), tomato (L14403) and potato (M76402) revealed lowest and highest homologies of 93.1% and 98.7%, respectively. Degenerate primers, RBCL1 and RBCL2 [Appendix 5], were designed to anneal at 136 nt and 884 nt into the *rbcL* gene corresponding to positions 57726 and 58480 in tobacco.

Pair wise comparison of the *atp*B genes from tobacco (Z00044), *hedera* (AJ235489), *hydrangea* (AF093381), *petunia* (J236182) tomato (AJ236183) and *verbascum* (AJ236177) revealed lowest and highest homologies of 92.6% and

```
Fav :  TTTTCCATTTTTATTTGCTTCCCCGCCGTCATCGAATGAT  :   40
Nt  :  TTTTCTAGTTGGATTTGCTCCCCCGCCGTCGTTCAATGAG  :   40


Fav :  AATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGG  :   80
Nt  :  AATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGG  :   80
                         RUA      - 35

Fav :  GATGGCTATATTTCTGGGAGCGAACTCCAGGCGAATATG  :  119
Nt  :  GATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATC  :  119
              -10
```

Figure 5.5 Alignment of *rrn16* promoter regions of tobacco and strawberry

Sequence homology analysis was carried out using the clustal method between strawberry (Fav) and tobacco (Nt) *rrn16* gene promoter sequences (positions 139906 – 140021 in inverted repeat A, (Shinozaki *et al.* 1986). Putative -10 (TATA), -35 (TTGACG), RUA (GTGGGA) signals and ATG initiation codons are highlighted. Light grey shading denotes homologous sequence (overall homology = 90.5%), areas of sequence divergence are indicated in white.

98%, respectively. Degenerate primers, ATPB1 and ATPB2 [Appendix 3], were designed to anneal at 155 nt and 424 nt into the *atpB* gene corresponding to positions 56626 and 56353 in tobacco.

PCR was carried out using total strawberry DNA as template. The primer pair RBCL1: ATPB1 yielded an amplification product of the expected size of ~ 1100 bp based on the distances between the primer annealing sites in tobacco. Amplification products were gel isolated and transformed into the vector pCR zero-Blunt II-TOPO. Colony PCR was carried out to verify the presence of inserted DNA in the vector. Following this plasmid DNA was prepared from transformed colonies and sequenced.

Sequencing revealed that PCR had successfully amplified a region of the strawberry plastome spanning the *rbcL* 5'UTR segment of interest. Sequence homology analysis between this sequence and the comparable region of the tobacco plastome revealed an overall homology of 66.3%. The intergenic region between the *rbcL* and *atpB* genes accounted for the majority of the sequence disparity occurring between the two sequences. The homology between the sequences encoding the 5' ends of the *rbcL* and *atpB* genes, 136 bp and 156 bp, respectively, was found to be 94.9% and 83.9%, respectively. This confirmed that the gene order between the two plastomes is conserved in this region and enabled the isolation of the *rbcL* 5' UTR of strawberry.

The strawberry *rbcL* 5' UTR was found to be 86.9% homologous to the *rbcL* 5'UTR of tobacco, and a Shine-Dalgarno element (GGAGG), implicated as a ribosome binding site, was identifiable (Figure 5.6).

The strawberry rbcL 5'UTR was subsequently made synthetically by annealing two oligonucleotides. Specific restriction endonuclease recognition sites were incorporated to enable later cloning. Two separate *rbcL* adapters were made, *rbcL*1 and *rbcL* 2 using four oligonucleotides (Figure 5.7). Individual oligonucleotides were annealed as outlined in section 2.3.9.

Both adapters were designed to have *Apa* I compatible 5' termini and *Pag* I compatible 3' termini to enable cloning. The *rbcL*1 adapter contained an internal

                    5'   3'
Fav : TCAGTTGTAGGGGAGGGACACATG : 23
Nt   : TGAGTTGTAGGGGAGGGATCCATG : 23
            57574   57597

Figure 5.6 Alignment of strawberry and tobacco *rbcL* 5'UTR sequences

Sequence homology analysis was carried out using the clustal method between strawberry (Fav) and tobacco (Nt) *rbcL* 5'UTR sequences. Position in the tobacco plastome (Shinozaki *et al.* 1986) is denoted below the Nt sequence. Grey shading denotes homologous sequence, overall homology = 82.6%. Areas of divergence are indicated in white. A putative Shine-Dalgarno element is highlighted in blue. The ATG initiation codon of the *rbcL* gene is included at the 3' end of the sequences.

```
             5'                                             3'
rbcLa :   ----CTACGTAGTTGTAGGGAGGGACA----  :  23
rbcLb :   CCGGGATGCATCAACATCCCTCCCTGTGTAC  :  31
                Sna BI              SD

                    Adapter rbcL1

             5'                                             3'
rbcLc :   ----CAGGCCTGTTGTAGGGAGGGACA----  :  23
rbcLd :   CCGGGTCCGGACAACATCCCTCCCTGTGTAC  :  31
                Stu I               SD

                    Adapter rbcL2
```

Figure 5.7 Synthetic adapters representing the strawberry *rbcL* 5'UTR

Oligonucleotides designed to create synthetic *rbcL* 5'UTR adapters are labelled rbcLa to rbcLd. Annealing oligonucleotides rbcLa and rbcLb produces adapter rbcL1. Annealing oligonucleotides rbcLc and rbcLd produces adapter rbcL2. Restriction enzyme sites unique to each adapter are marked. 5' termini are *Apa* I compatible; 3' termini are *Pag* I compatible. A putative Shine Dalgarno (SD) sequence is shaded in black.

Sna BI site to enable cloning into the *aad*A series of vectors. The *rbcL* 2 adapter contained an internal *Stu* I site to enable cloning into the *xyl*A series of vectors.

c) Isolation of the strawberry plastid photo-system II core protein gene (*psbA*) 3' terminator region (3'TR)

The 3'TR following the UAA termination codon of the plastid *psbA* gene was isolated from strawberry using a PCR approach, assuming that the tobacco plastome gene order was conserved in strawberry. The *psb*A 3'TR in tobacco lies between the *psbA* gene (position [complement] 534...1595) and the ribosomal protein L2 (*rpL2*) gene (position 154,393...155,883), as is highlighted in Figure 5.2.

Pair-wise comparison of the *psb*A genes from tobacco (Z00044), *brassica* (M36720), cotton (X15885), *cuscuta* (X67512), *oenothera* (AJ271079), soybean (X00152), spinach (AJ400848) and alfalfa (X04973) revealed lowest and highest homologies of 91.3% and 96.1%, respectively. Degenerate primers, PSBA1, PSBA2, PSBA3 and PSBA4 [Appendix 3]; were designed to anneal at 689 nt, 988 nt, 776 nt and 642 nt into the *psb*A gene, corresponding to positions 906, 607, 819 and 953 in tobacco.

Pair-wise comparison of the *rpL2* genes from tobacco (Z00044), *arabidopsis* (AP000423), spinach (X00797), pea (X59015) and maize (X86563), revealed lowest and highest homologies of 87.8% and 96.9% respectively. Degenerate primers, RPL2(1) and RPL2(2) [Appendix 3], were designed to anneal at 489 nt and 586 nt into the *rpL2* gene, corresponding to positions 155549 and 155646 in tobacco.

PCR was carried out using total strawberry DNA as template, with combinations of the RPL2 and PSBA primer pairs. An amplification product of the expected size of 1397 bp based on the distances between the primer annealing sites in tobacco, was generated using primer pair PSBA3 and RPL2(2). Amplification products were gel isolated and transformed into the vector pCR zero-Blunt II-TOPO. Colony PCR was carried out to verify the presence of inserted DNA in the vector. Following this plasmid DNA was prepared from transformed colonies and sequenced.

Sequence homology analysis was carried out between the strawberry sequence isolated using PSBA3 and RPL2(2) and the comparable region of the tobacco plastome; an overall homology of 73.9% was observed. The majority of the disparity was accounted for in the intergenic region between the *psbA* and *rpL2* genes. The homology between the sequences encoding the 3' ends of the *psbA* and *rpL2* genes, 309 bp and 260 bp, respectively, was found to be 92.6% and 97.3%, respectively. This confirmed conservation of plastome gene order in this region and enabled the isolation of the strawberry *psbA* 3'TR.

The strawberry *psbA* 3'TR was found to be only 33% homologous, at the nucleotide level, to the corresponding region in tobacco (Figure 5.8). However it has several characteristics commonly associated with plant gene terminator regions. An 'AATAAA' sequence motif associated with efficient mRNA 3' end formation is present at position 178 – 183. An inverted repeat of the AATAAA motif is present at position 189 – 195 and in the context of the surrounding sequence a stable (-7.7 kCal/mol) stem-loop structure may be formed (Figure 5.9).

To create the 3'TR in the transcription units 222 bp of the downstream sequence from the *psbA* termination codon were isolated. This region included the AATAAA motif and potential stem-loop forming sequence elements. However, it excluded further downstream sequence that may include promoter elements of the *trnH* gene, which lies between the *psbA* and *rp2* genes.

PCR primers PSBABGL and PSBAECOSGF [Appendix 3] were designed to incorporate a *Bgl* II site at the 5' end of the strawberry *psbA* 3'TR and *Eco R*I and *Sgf* I restriction sites at the 3' end. PCR was carried out and the 228 bp amplification product gel isolated and transformed into the vector pCR zero-Blunt II-TOPO. Plasmid DNA was prepared from transformed colonies and sequenced to confirm the correct addition of restriction sites [SEQ 4, Appendix 4]. The authenticated plasmid was named p*psbA*3'.

```
Fav : TAAGACTTCCATCTA-TCAATGG-----------ATAAGG-- :  28
Nt  : TAAGATCCTGGCCTAGTCTATAGGAGGTTTTGAAAAGAAA :  40


Fav : -------------------CTT---------------TGATA :  36
Nt  : GGAGCAATAATCATTTTCTTGTTCTATCAAGAGGGTGCTA :  80


Fav : TTAGT-------------------------ATATATGAGT-- :  51
Nt  : TTGCTCCTTTCTTTTTTTCTTTTTATTTATTTACTAGTAT : 120


Fav : -----TCGTG-AAACTTTT------------GAAAGTAAAG :  74
Nt  : TTTACTTACATAGACTTTTTTGTTTACATTATAGAAAAAG : 160


Fav : GAGCAATACTAAATTTCTTGTTATTGTT-ATATCAAGAGG : 113
Nt  : AAGGAGAGGTTATTTTCTTGCATTTATTCATGATTGAGTA : 200


Fav : TTTTGTGTTGCTCCT----TTACTCTAATTA--AATTAG- : 146
Nt  : TTCTATTTTGATTTTGTATTTGTTTAAATTGTGAAATAGA : 240


Fav : ---TATTTTT--------TAGT---ACTATTTTTTTTTAG : 172
Nt  : ACTTGTTTCTCTTCTTGCTAATGTTACTATATCTTTTTGA : 280


Fav : ATT---------AAAATAAAAA----------GGTTTTTAT : 194
Nt  : TTTTTTTTTTCCAAAAAAAAAAATCAAATTTTGACTTCTTC : 320

                                                !
Fav : TTATGTTGTATTCTT-------TTTTAC-----AAGTAAT : 222
Nt  : TTATCTCTTATCTTTGAATATCTCTTATCTTTGAAATAAT : 360


Fav : GATA--AATGGTATAAA----------TAT-TTGTAATTT : 249
Nt  : AATATCATTGAAATAAGAAAGAAGAGCTATATTCGAACTT : 400


Fav : CTAT-TTTGATTTATTAATATAGTACAAGTTTATATAGTA : 288
Nt  : GAATCTTTTGTTTTCTAATTTAAATAATGTAAAAACGGAA : 440


Fav : CA-AGATAAT--ATGGGGCGG : 306
Nt  : TGTAAGTAGGCGAGGGGCGG  : 461
```

Figure 5.8 Alignment of strawberry and tobacco *psbA* 3'TR

Sequence homology analysis was carried out using the clustal method between strawberry (Fav) and tobacco (Nt) *psbA* 3'TR sequences. Sequences begin at a putative *psbA* stop codon, TAA, corresponding to position 534 in the tobacco plastome. A consensus 'AATAAA' signal and its inverted repeat found in strawberry are highlighted in black. The overall homology between the two sequences is 34.3%. The first 222 bp of the strawberry *psbA* sequence shown was included in the transcription units created in this study.

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Figure 5.9 Potential *psbA* 3'TR stem-loop formation

Nucleotides 160- 222 of the strawberry *psbA* 3'TR are shown. Nucleotides implicated in potential stable (-7.7kCal/mol) stem-loop formation are highlighted in grey. The AATAAA signal, position 178-184, and its inverted repeat, position 190-196, are linked by solid lines, other potential linkages are represented with broken lines.

5.3 Vector construction

A series of plastid transformation vectors were created using the component parts described in section 5.2. All gel isolation was carried out as described in section 2.5.3. Restriction endonuclease digestion of plasmid DNA, blunt-ending, dephosphorylation and ligation of DNA fragments and transformation of *Escherichia coli* were all carried out as described in section 2.3. Preparation of plasmid DNA was carried out as described in section 2.4.2.

5.3.1 Construction of a transcription unit containing the *aadA* and *smGFP* genes

A schematic of the cloning strategy employed to create a transcription unit containing the *aadA* and *smGFP* genes expressed as an operon under control of strawberry plastid regulatory elements is shown in Figure 5.10. Plasmid paadA (section 5.2.2a) was digested with *Pag* I and *Xho* I and the resulting 808 bp fragment ligated, along with the synthetic adapter *rbcL*1 (Figure 5.7) in a tri-partite ligation with pBluescript KS+ digested with *Apa* I and *Xho* I. The correct linkage of the *rbc*L 5'UTR and *aadA* gene was confirmed by sequencing and the authenticated plasmid named **pRA.**

Plasmid prrn16 (section 5.2.3a) was digested with *Kpn* I and *Ssp* I and the resulting 116 bp fragment, ligated into dephosphorylated plasmid pRA digested with *Kpn* I and *Sna* BI. Correct linkage of the components was confirmed by sequencing and the authenticated plasmid containing the *rrn16* promoter, *rbc*L 5'UTR and *aadA* gene named **pRRA.**

Plasmid ppsbA3' (section 5.2.3c) was digested with *Bgl* II and *Eco* RI and the resulting 222 bp fragment ligated into dephosphorylated plasmid pGFPad (section 5.2.2c) digested with *Bgl* II and *Eco* RI. Correct linkage of the components was confirmed by sequencing and the authenticated plasmid containing the *aadA/xylA: smGFP* adapter, *smGFP* gene and *psbA* 3'TR named **pAGP.**

Figure 5.10 Schematic outlining the construction of the *aad*A: *smGFP* transcription unit.

The various manipulations carried out using the component parts outlined in section 5.2 to create the *aad*A: *smGFP* transcription unit (contained in plasmid pAG) for plastid transformation vectors are shown. Red broken arrows indicate ligation steps. Black arrows relate to the path of the donor plasmid in the cloning strategy. Individual letters indicate the relative position of the restriction endonuclease sites used at each stage.

The complete transcription unit containing *aad*A and *smGFP* was created by ligating the 1029 bp fragment released by *Xho* I and *Eco* RI digestion of plasmid pAGP into *Xho* I / *Eco* RI digested, dephosphorylated plasmid pRRA. Correct linkage of the components was confirmed by sequencing and the authenticated plasmid containing the *rrn16* promoter, *rbcL* 5'UTR, *aadA* gene, *aadA/xylA: smGFP* adapter, *smGFP* gene and *psbA* 3'TR named pGA.

5.3.2 Construction of a transcription unit containing the *xylA* and *smGFP* genes

A schematic of the cloning strategy employed to create a transcription unit containing the *xylA* and *smGFP* genes expressed as an operon under control of the strawberry plastid regulatory elements described in section 5.2 is shown in Figure 5.11. Plasmid pxylA (section 5.2.2b) was digested with *Pag* I and *Xho* I and the resulting 1319 bp fragment ligated, along with the synthetic adapter *rbcL2* (Figure 5.7), in a tri-partite ligation with pBluescript KS+ digested with *Apa* I and *Xho* I. The correct linkage of the *rbcL* 5'UTR and *xylA* gene was confirmed by sequencing and the authenticated plasmid named **pRX**.

Plasmid prrn16 (section 5.2.3a) was then digested with *Kpn* I and *Ssp* I and the resulting 116 bp fragment ligated into dephosphorylated plasmid pRX digested with *Kpn* I and *Stu* I. Correct linkage of the components was confirmed by sequencing and the authenticated plasmid containing the *rrn16* promoter, *rbcL* 5'UTR and *xylA* gene named **pRRX**.

The complete transcription unit was created by ligating the 1029 bp fragment released by *Xho* I and *Eco* RI digestion of plasmid pAGP (section 5.3a) into *Xho* I / *Eco* RI digested, dephosphorylated plasmid pRRX. Correct linkage of the components was confirmed by sequencing and the authenticated plasmid containing the *rrn16* promoter, *rbcL* 5'UTR, *xylA* gene, *aadA/xylA: smGFP* adapter, *smGFP* gene and *psbA* 3'TR named **pXG**.
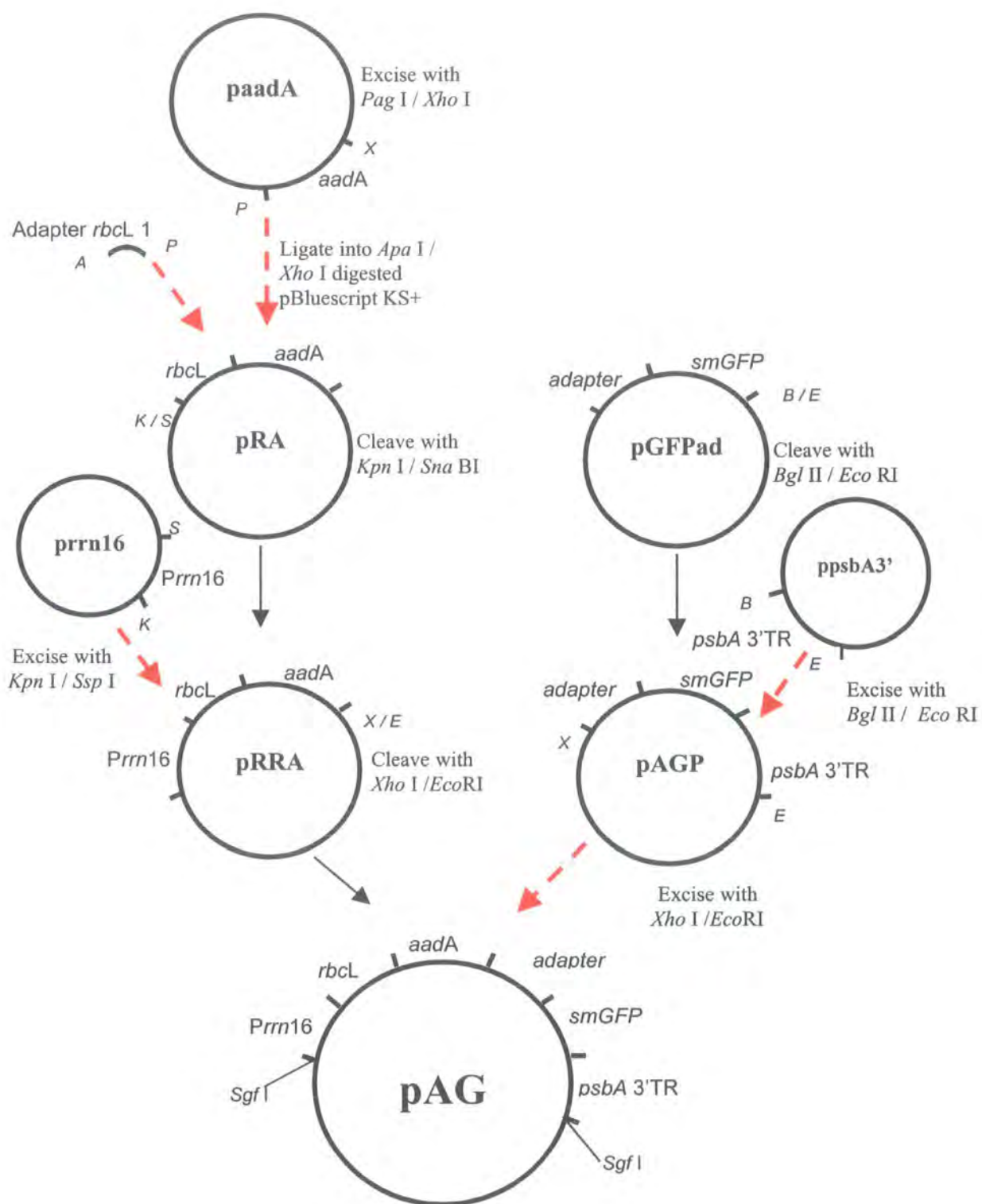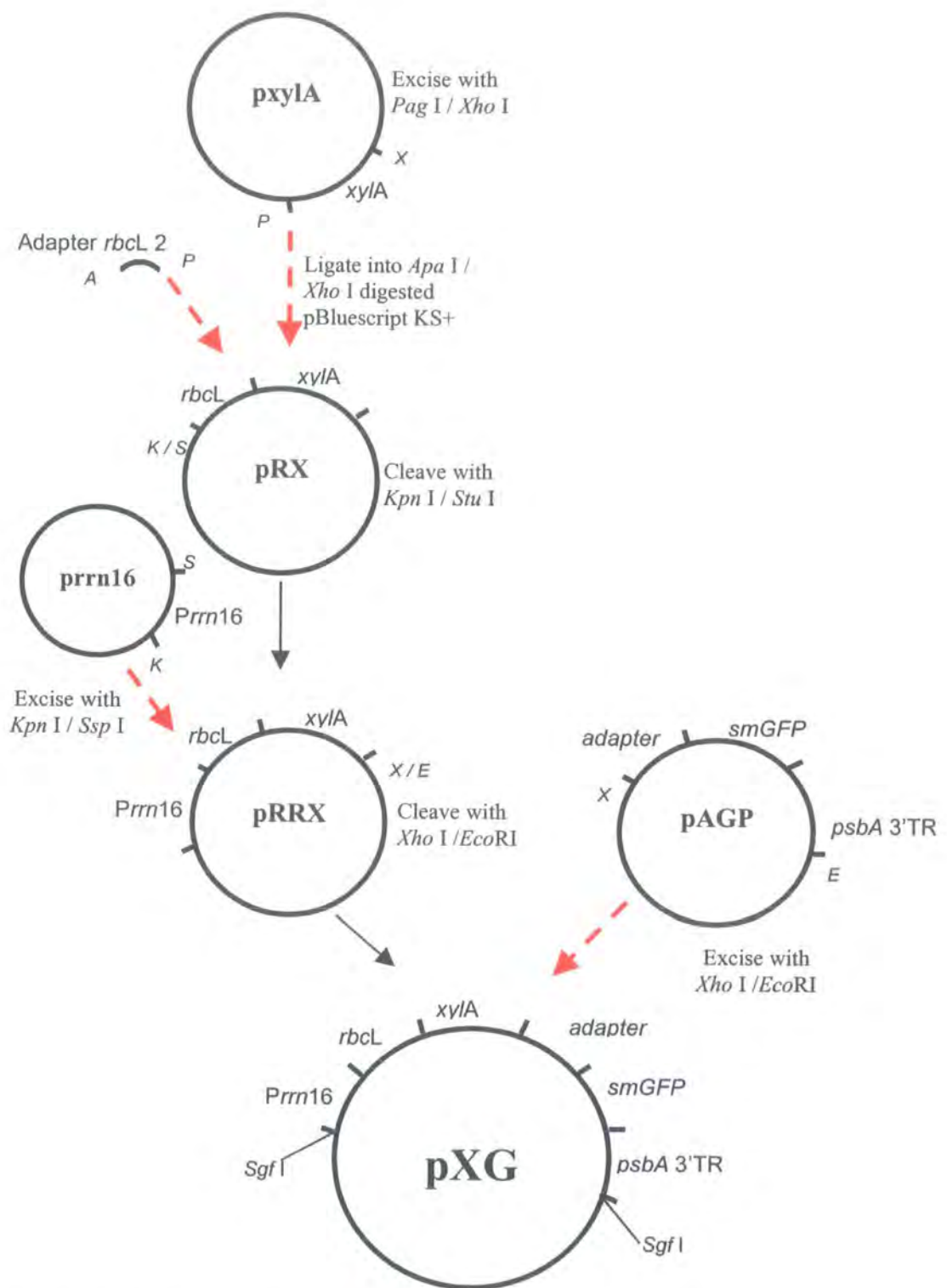
Figure 5.11 Schematic outlining the construction of the xylA: *smGFP* transcription unit.

The various manipulations carried out using the component parts outlined in section 5.2 to create the xylA: *smGFP* transcription unit (contained in plasmid pXG) for plastid transformation vectors are shown. Red broken arrows indicate ligation steps. Black arrows relate to the path of the donor plasmid in the cloning strategy. Individual letters indicate the relative position of the restriction endonuclease sites used at each stage.

5.3.3 Construction of plastid transformation vectors for spectinomycin selection

To create plastid transformation vectors containing the *aad*A and *smGFP* genes the entire, 1897 bp, transcription unit was released from plasmid **pAG** using *Sgf* I and blunt-ended. To produce a plastid transformation vector for strawberry this fragment was ligated into *Bst* 1107I cut, dephosphorylated plasmid pstraw3.2 (section 5.2.1) containing strawberry plastome sequence. To produce a plastid transformation vector for tobacco this fragment was ligated into *Bst* 1107I cut, dephosphorylated plasmid pTob3.3 (section 5.2.1) containing tobacco plastome sequence.

Restriction digest analysis and sequencing confirmed that the transcription unit had been placed into the plastome targeting sequence so that the native and introduced *rrn16* promoter regions were in opposing orientations. The authenticated strawberry plastid transformation vector was named **pFavAG** (Figure 5.12). The authenticated tobacco plastid transformation vector was named **pNtAG** (Figure 5.13).

Figure 5.12 Strawberry plastid transformation vector pFavAG

Strawberry plastid transformation vector designed for spectinomycin selection. Strawberry plastome surrounding the transcription unit is inserted at the *Sma I* site of pBluescript. The orientation of the native *rrn16* promoter present in the strawberry plastome and key restriction endonuclease sites are indicated.

Figure 5.13 Tobacco plastid transformation vector pNtAG

Tobacco plastid transformation vector designed for spectinomycin selection. Tobacco plastome surrounding the transcription unit is inserted at multiple cloning site of pCR-Blunt-TOPO II. The orientation of the native *rrn16* promoter present in the tobacco plastome and key restriction endonuclease sites are indicated.

5.3.4 Construction of plastid transformation vectors for xylose selection

To create plastid transformation vectors containing the *xyl*A and *smGFP* genes the entire, 2424 bp, transcription unit was released from plasmid pXG using *Sgf* I and blunt-ended. To produce a plastid transformation vector for strawberry this fragment was ligated into *Bst* 1107I cut, dephosphorylated plasmid pstraw3.2 (section 5.2.1) containing strawberry plastome sequence. To produce a plastid transformation vector for tobacco this fragment was ligated into *Bst* 1107I cut, dephosphorylated plasmid pTob3.3 (section 5.2.1) containing tobacco plastome sequence.

Restriction digest analysis and sequencing confirmed that the transcription unit had been placed into the plastome targeting sequence so that the native and introduced *rrn16* promoter regions were in opposing orientations. The authenticated strawberry plastid transformation vector was named pFavXG (Figure 5.14). The authenticated tobacco plastid transformation vector was named pNtXG (Figure 5.15).
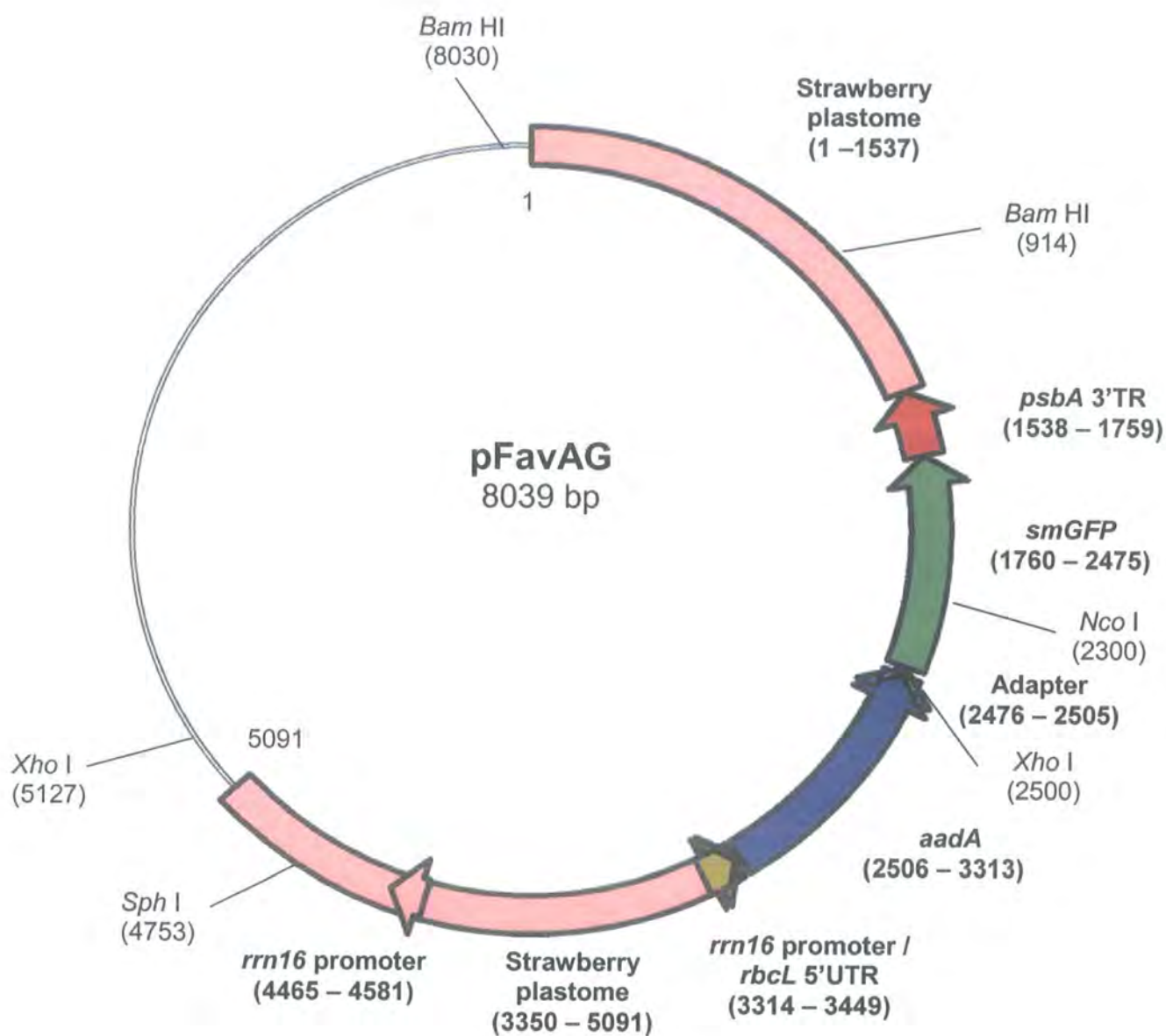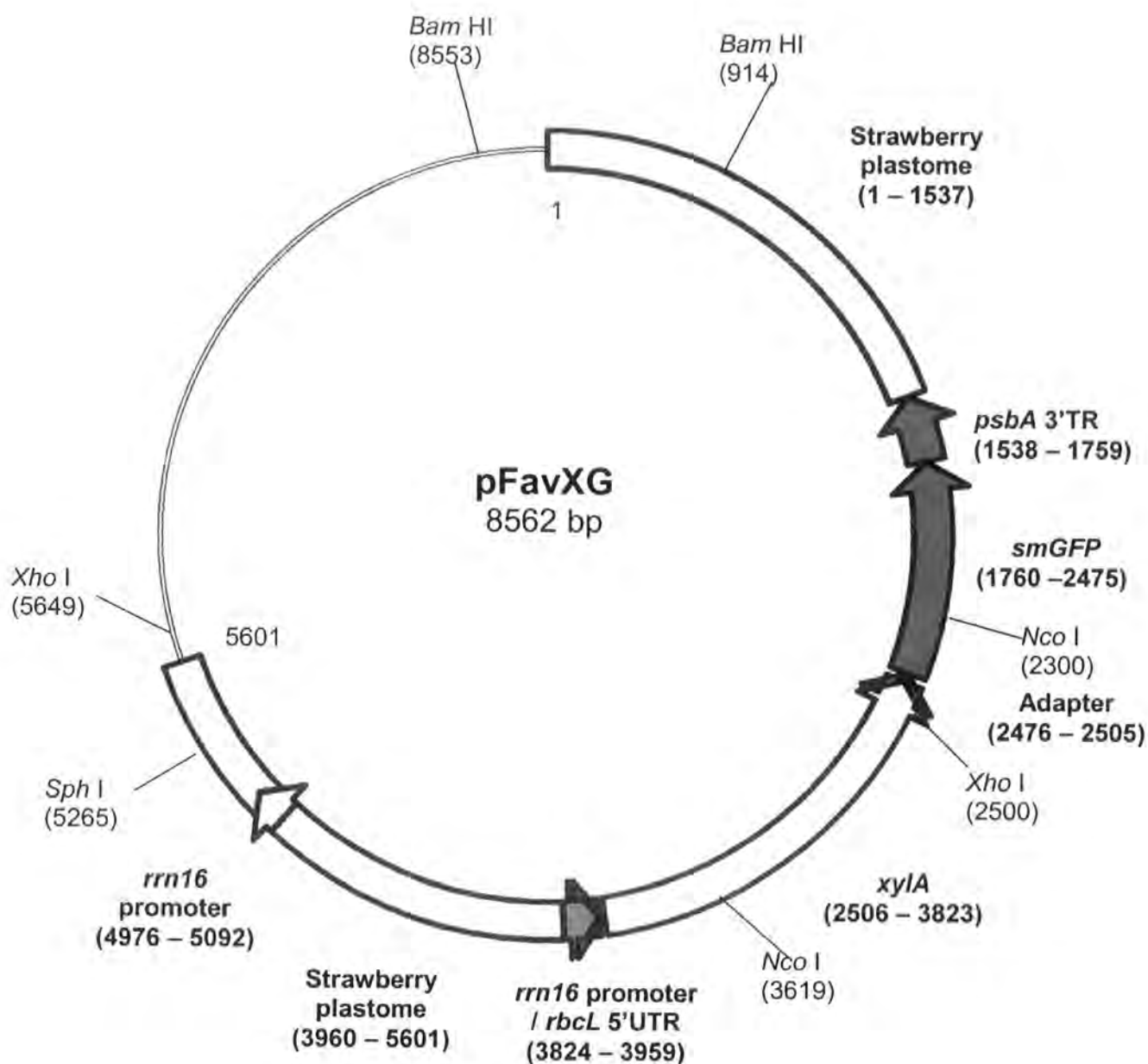
Figure 5.14 Strawberry plastid transformation vector pFavXG

Strawberry plastid transformation vector designed for xylose selection. Strawberry plastome surrounding the transcription unit is inserted at the *Sma I* site of pBluescript. The orientation of the native *rrn16* promoter present in the strawberry plastome and key restriction endonulease sites are indicated.
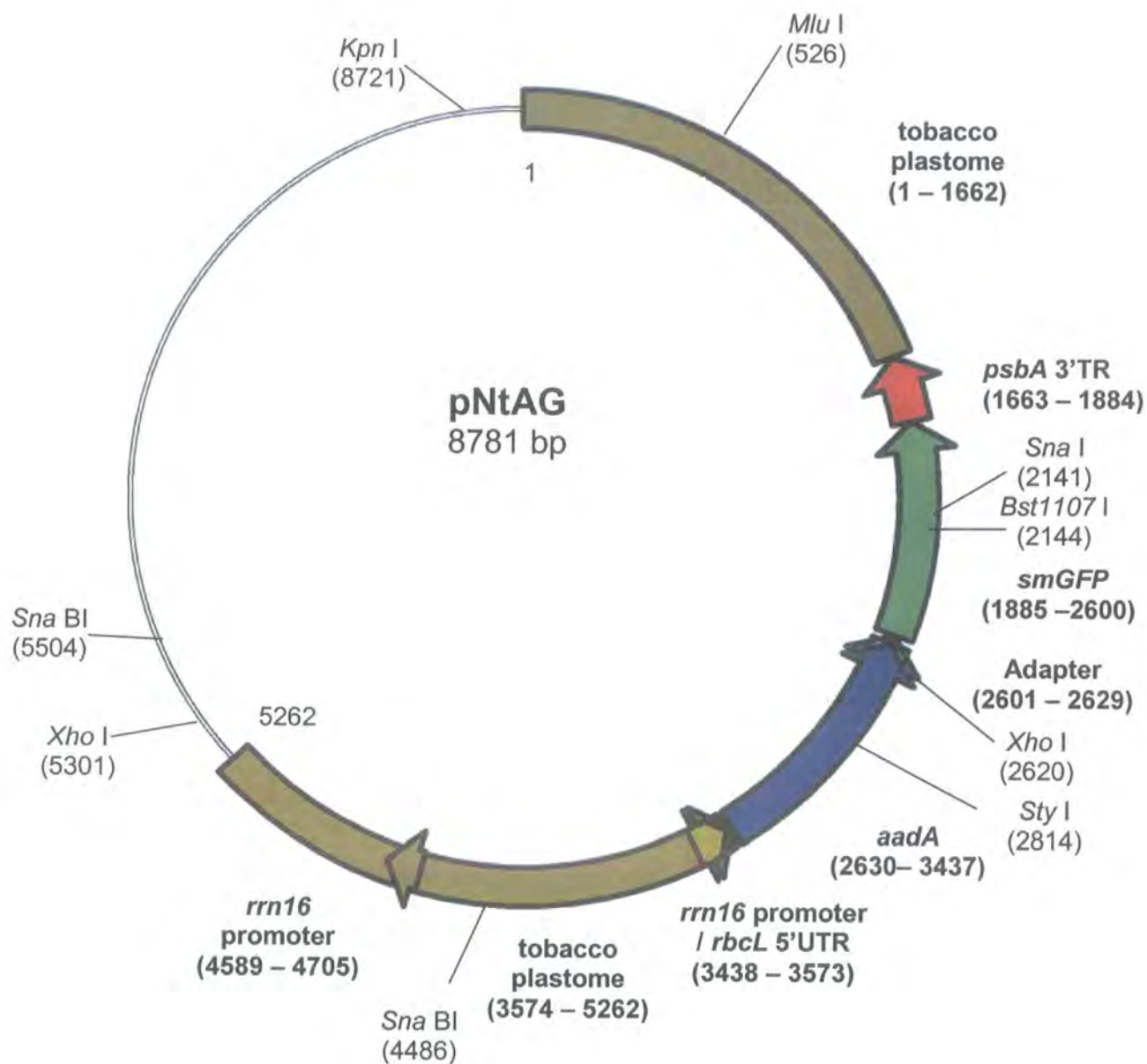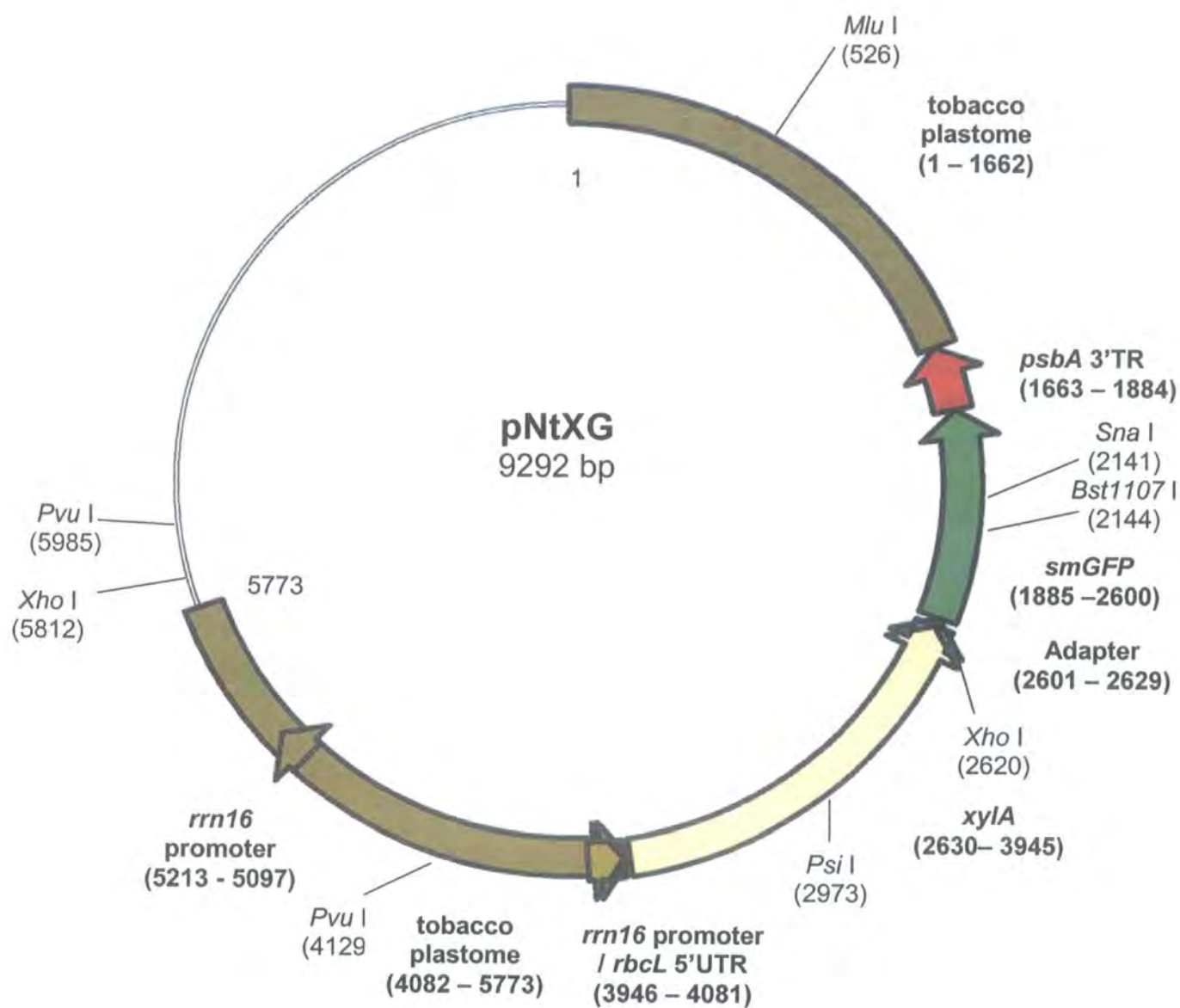
Figure 5.15 Tobacco plastid transformation vector pNtXG

Tobacco plastid transformation vector designed for xylose selection. Tobacco plastome surrounding the transcription unit is inserted at multiple cloning site of pCR-Blunt-TOPO II. The orientation of the native *rrn16* promoter present in the tobacco plastome and key restriction endonuclease sites are indicated.

5.4 Heterologous expression in *Escherichia coli*

Having constructed and sequenced the novel plastid transformation vectors an expression study in *E.coli* was carried out using a stereomicroscope fitted with a stereo fluorescence module (section 2.7.3). GFP fluorescence was observed in *E.coli* harbouring all of the plastid transformation vectors (Figure 5.16). This confirmed that the vectors created were functional and that the strawberry plastid regulatory elements isolated function in *E.coli*.

5.5 Concluding remarks

In summary, novel strawberry plastome regulatory elements were identified and isolated. These were then incorporated into a series of plastid transformation vectors, pFavAG, pFavXG, pNtAG and pNtXG, designed for strawberry and tobacco plastid transformation using spectinomycin and xylose selection. Sequencing authenticated that the vectors had been made correctly.

The vectors were checked in a prokaryotic system and the scorable marker gene, *smGFP* was successfully visualised in *E.coli* as observed by fluorescence. This result confirmed that the strawberry *rrn16* promoter isolated was functional, that a transcript was being made and that translation of the second gene in the operon, *smGFP*, was being initiated. This also implied that the strawberry *rbc*L 5'UTR and *psb*A 3'TR regions cloned into the transcriptional units were functional in the heterologous system.

The application of the novel plastid transformation vectors outlined in this chapter will be described in the following chapter.
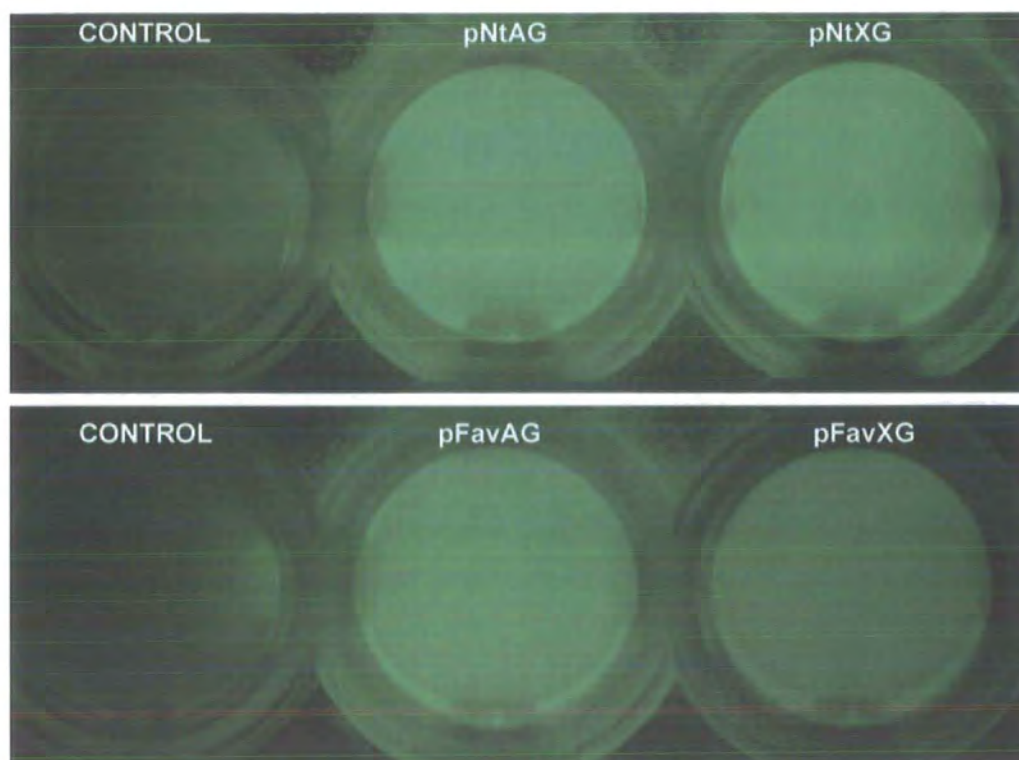
Figure 5.16 Expression of smGFP in *Escherichia coli*

Each well contains a 250 µl liquid (LB) suspension of *E.coli*, each harbouring a vector designed for plastid transformation as indicated. Control wells contain 250 µl liquid (LB) suspension of *E.coli* harbouring a plasmid lacking the *smGFP* gene. A Leica Wild MZ8 stereomicroscope fitted with a stereo fluorescence module with the excitation filter at 470/40 nm and the barrier filter at 525/50 nm was used for visualisation.

Chapter 6

Plastid transformation of strawberry and tobacco

6.1 Introduction

In chapter 5 the construction of a novel series of plastid transformation vectors for strawberry and tobacco was described. The vectors were constructed to enable insertion of either the *aadA:smGFP* or *xylA:smGFP* transcription units into the plastome *trnV* – *3'rps12* intergenic region, using antibiotic and non-antibiotic selection, respectively. The major aims of this study were to (i) develop methods for plastid transformation of strawberry and (ii) to develop a non-antibiotic based selection system for plastid transformation in either strawberry or tobacco utilising D-xylose as a selective agent.

Tobacco has a relatively fast shoot regeneration cycle of 3-4 weeks, as opposed to 8-12 weeks in strawberry, and plastid transformation has been routinely achieved using this species. For these reasons, to evaluate the relative efficiencies of the two selection systems, using the novel transformation vectors described in chapter 5, plastid transformation was additionally carried out in parallel with tobacco.

A schematic showing the main stages involved in plastid transformation is given in Figure 6.1. The process involves the introduction of gold particles coated with transformation vector DNA into donor leaf material using high pressure helium gas under vacuum. Following gold/ DNA introduction donor leaf material is cut into several smaller explants of ~ 0.5 cm^2 which are cultured in the presence of a selective agent. Initially only a very small number of chloroplasts in any one cell are likely to be transformed. However, exposure to the selective agent favours the multiplication of chloroplasts containing transformed genomes during subsequent cell and organelle divisions (Bock, 2001). Shoots from primary regenerants are therefore excised, cut and

A Pre-bombardment

Vacuum chamber

Disk holding DNA coated gold particles

Stopping screen

B Post-bombardment

Shock wave fires disk

Disk stopped by screen

DNA coated gold particles penetrate donor leaf material
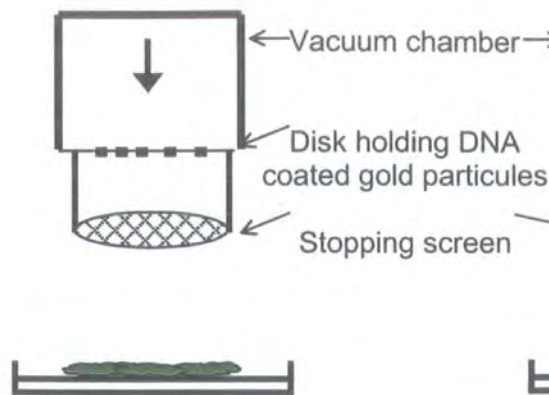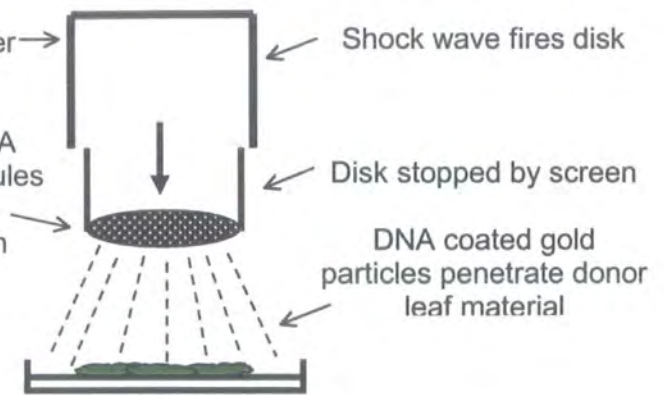
C

D
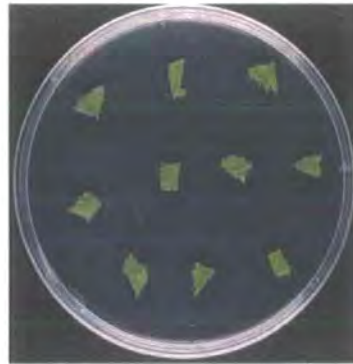
E

F
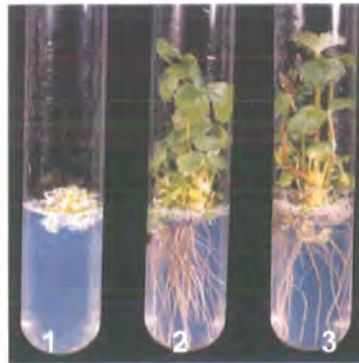
Figure 6.1 schematic showing key stages in plastid transformation

Introduction of plastid transformation vector DNA, on gold particles, into donor leaf material via biolistic transformation (A) and (B). Strawberry leaves arranged centrally on a Petri dish prior to bombardment (C). Strawberry leaves cut 2 d post bombardment to yield ~0.5 cm^2 explants for callus and shoot regeneration (D). Strawberry explants regenerating callus on selection 8 weeks post bombardment (E). Shoots regenerated from callus after 10-12 weeks are excised and the leaves subject to repeat regeneration on selective media to promote homoplasmy. Regenerated shoots are transferred onto proliferation media (F); non-transformed control cultured in the presence of 25mg/l spectinomycin (F1), non-transformed control cultured without selection (F2) and putative plastid transformed line cultured in the presence of 500 mg/l spectinomycin (F3).

placed on selective media to regenerate more shoots in a procedure termed here as repeat regeneration, until a homoplasmic state is achieved where the entire population of chloroplast genomes are transformed and a stable plastid transformed line is generated .

The first strategy used to develop plastid transformation methods for strawberry utilises the antibiotic spectinomycin as a selective agent. Spectinomycin resistance conferred by the *aadA* gene has been used successfully in the plastid transformation of several higher plant species (Svab and Maliga, 1993; Koop *et al.*, 1996; Sikdar *et al.*, 1998; Khan and Maliga, 1999; Sidorov *et al.*, 1999; Ruf *et al.*, 2001). Spectinomycin acts by binding to the 30S subunit of the plastid ribosome preventing association of the ribosome complex. This specifically disrupts plastid protein synthesis enabling green / white colour selection of resistant / non-resistant tissues through the inhibition of chlorophyll biosynthesis (Maliga, 1993).

Resistance to spectinomycin can occur through spontaneous mutation of the 16S rRNA gene (Fromm *et al.*, 1987; Dix and Kavanagh, 1995). In previous plastid transformation studies, between 60 % (Daniell *et al.*, 2001) and 90 % (Eibl *et al.*, 1999; Sidorov *et al.*, 1999) of the spectinomycin resistant clones produced have been a result of spontaneous mutation rather than integration and expression of the *aadA* gene. The *aadA* gene product inactivates streptomycin as well as spectinomycin, which enables plastid transformed lines carrying the *aadA* gene to produce green callus in the presence of both antibiotics. To discriminate between spontaneous acquired mutation and presence of the *aadA* gene in putative *aadA: smGFP* plastid transformed spectinomycin resistant lines, lines were screened on media containing both spectinomycin and streptomycin. Spontaneous mutation of the 3' end of ribosomal protein 12 (3'*rps12*) gene can also confer resistance to streptomycin (Dix and Kavanagh, 1995). However, the chances of selecting double mutants are greatly reduced.

The transformation procedure and level of spectinomycin to be used as a selective agent has been well documented for tobacco plastid transformation (Daniell,

1993; McBride *et al.*, 1995; Khan and Maliga, 1999; Maliga, 1999). This protocol was used for tobacco plastid transformation experiments using the vector pNtAG. There was no information in the literature relating to the sensitivity of strawberry to spectinomycin. Hence a series of preliminary investigations were carried out to assess strawberry callus and shoot regeneration sensitivity to spectinomycin prior to undertaking strawberry plastid transformation experiments using the vector pFavAG.

The second strategy developed in this study was the use of a non-antibiotic selection system for strawberry and tobacco plastid transformation utilising the xylose isomerase (*xylA*) gene. Expression of the *xylA* gene enables transformed plant tissues to utilise D-xylose as a carbohydrate source in tissue culture providing a selective advantage over non-transformed tissues (Haldrup *et al.*, 1998; Haldrup *et al.*, 2001). The *xylA* gene has been successfully used in nuclear transformation for several plant species (Haldrup *et al.*, 1998; Haldrup *et al.*, 2001), including tobacco. However there is no record of this selection method being used for strawberry nuclear transformation. A series of preliminary experiments were carried out to assess the sensitivity of strawberry regeneration to D-xylose prior to commencing strawberry plastid transformation experiments using the vector pFavXG. In tobacco plastid transformation experiments using the vector pNtXG, the level of D-xylose selection recommended in the literature for nuclear transformation of this species (Haldrup *et al.*, 1998) was initially used.

Strawberry nuclear transformation experiments have typically used sucrose as the carbohydrate source in regeneration media (James *et al.*, 1990; Mathews *et al.*, 1995; Mansouri *et al.*, 1996; Schaart *et al.*, 2002). However, preliminary investigations undertaken during the course of these studies showed that the number of shoots regenerating per explant could be increased by using glucose as the carbohydrate source in the media instead of sucrose. Shoot regeneration occurred over a greater surface area of the explant when using glucose. It was thought that this would increase the likelihood of shoots arising from callus formed on central regions of the explant around the point of DNA / gold entry following biolistic transformation.

Studies to develop plastid transformation in tomato cited the use of lower light intensities as a critical factor in the selection of transplastomic cells (Ruf *et al.*, 2001). Preliminary investigations revealed that strawberry would regenerate well under low light intensities. Low light intensities have also been implicated in intra-cellular relocation of chloroplasts. Under weak light, chloroplasts move towards a brighter area of the cell (Kagawa and Wada, 2002). Hence, in this study pre-bombardment culture of explants was carried out under low light intensities to align the chloroplasts towards the cell surface prior to bombardment.

The effects of several parameters were investigated to establish plastid transformation methodologies in strawberry. These included the determination of optimal concentrations of selective agent, either spectinomycin or D-xylose, to be used in regeneration medium, pre- and post-culture conditions and general bombardment parameters.

6.2 Plastid transformation utilising spectinomycin as a selective agent

6.2.1 Optimisation of strawberry shoot regeneration using spectinomycin selection

The concentration of spectinomycin to be used as a selective agent in strawberry plastid transformation using the vector pFavAG was determined empirically through a series of regeneration experiments. These assessed the sensitivity of Calypso explants to regenerate shoots in the presence of different spectinomycin levels. Two, 1600 explant regeneration experiments were carried out using different media to develop an optimised shoot regeneration procedure. The aim was to determine the highest spectinomycin level allowing shoot regeneration, where regenerated shoots were white and not green.

The regeneration experiments involved replicated culture of 50 explants, each on four different media with sucrose as the carbohydrate source; ZN102 contained 1 mg/l thiadiazuron (TDZ) and 0.2 mg/l napthal-acetic acid (NAA), ZD102 contained 1 mg/l TDZ and 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), ZD103 contained 1 mg/l TDZ and 0.3 mg/l 2,4-D and ZN202 contained 2 mg/l TDZ and 0.2 mg/l 2,4-D [Appendix 1]. Spectinomycin, ranging from 0 to 50 mg/l, was included in each type of medium. After five weeks of culture the explants were transferred to fresh media to maintain spectinomycin and hormone levels. Regeneration efficiency was calculated as the percentage of explants regenerating at least one shoot as a proportion of the total number of explants subject to each treatment. In experiment one callus formed on all explants on all media tested. However, shoot regeneration was severely inhibited by spectinomycin levels above 5 mg/l (Figure 6.1). Comparable results were generated in experiment two (data not shown). The few shoots that regenerated on medium containing in excess of 5 mg/l spectinomycin were bleached or mottled and had reduced vigour (Figure 6.2BIII). On media containing no spectinomycin shoot regeneration typically occurred between 10-12 weeks and was consistently highest on ZN102 media. The effect of spectinomycin on non-transformed proliferating strawberry cultures was also investigated. Spectinomycin levels of 25 mg/l reduced shoot vigour and bleached leaves in proliferating strawberry cultures (Figure 6.2C1).

Studies in parallel to this PhD study generated 756 putative plastid transformed strawberry lines from eight experiments using 5 mg/l spectinomycin selection. Only one of these lines was shown to be plastid transformed and was able to withstand levels of spectinomycin of 500 mg/l when grown as a shoot proliferating culture. On the basis of this observation and the potential benefits of growth in low light intensities and on glucose based ZN102 medium, explants in strawberry plastid transformation experiments were cultured on glucose based ZN102 medium, in low-level light (1-3 μmol/m^2/s) and in the presence of 20 mg/l spectinomycin for the first five to six weeks of the regeneration cycle. After which, subsequent rounds of regeneration were carried out using 200 mg/l spectinomycin.
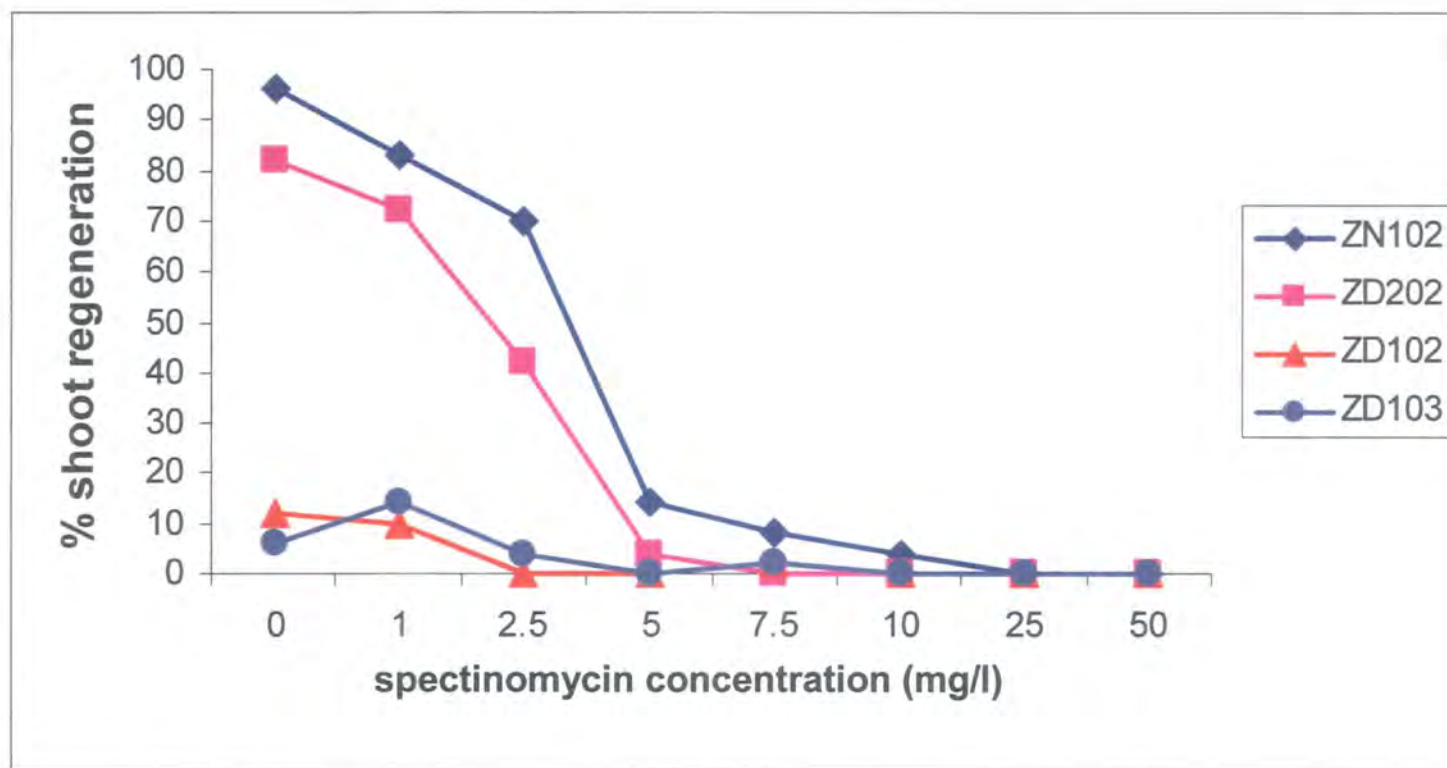
Figure 6.1 Sensitivity of strawberry shoot regeneration to spectinomycin

Percentage of strawberry, cv. Calypso, explants regenerating shoots in the presence of varying levels of spectinomycin are indicated for four media; ZD202 (2 mg/l TDZ, 0.2 mg/l 2-4D), ZD102 (1 mg/l TDZ, 0.2 mg/l 2-4D), ZD103 (1 mg/l TDZ, 0.3 mg/l NAA) and ZN102 (1 mg/l TDZ, 0.2 mg/l NAA). The data shown was generated from a single regeneration experiment. Each data point represents the average number of shoots regenerating from 50 leaf discs cultured in batches of 10 discs per Petri dish. The percentage of explants producing shoots was routinely highest using ZN102 media. Regeneration was severely inhibited on all media by levels of spectinomycin in excess of 5 mg/l.
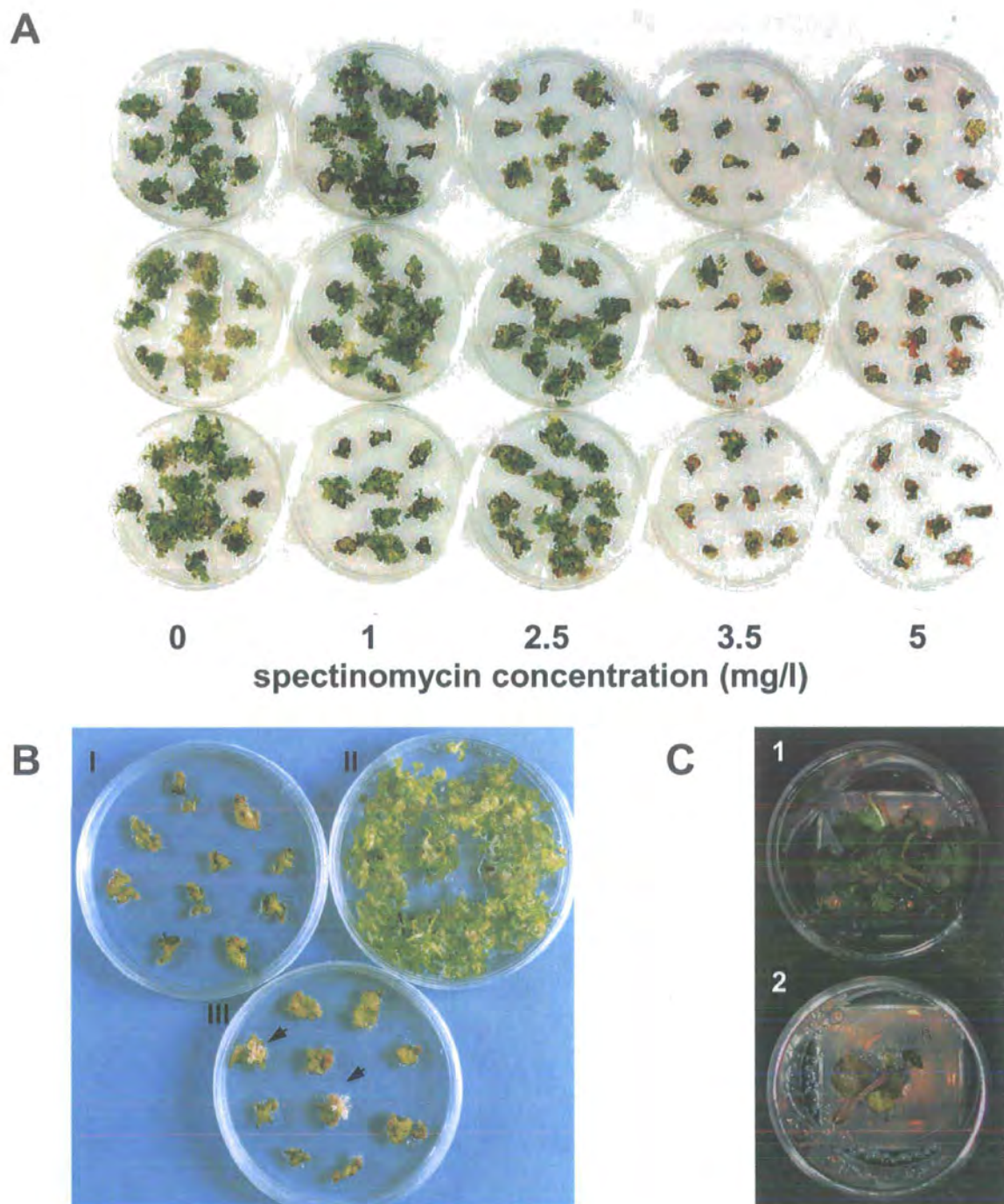
Figure 6.2 Strawberry regeneration in the presence of spectinomycin

Strawberry regeneration carried out in the presence of varying levels of spectinomycin (A). 100% explant regeneration observed using ZN102 media in the absence of spectinomycin (BII). Shoot regeneration severely inhibited in the presence of 5 mg/l spectinomycin (BI and BIII). White shoots regenerating on selection media are indicated. Non-transformed proliferating strawberry cv. Calypso cultured in the absence of spectinomycin (C1) and in the presence of 25 mg/l spectinomycin (C2).

6.2.2 Strawberry plastid transformation using the vector pFavAG

The basic structure for all plastid transformation experiments using pFavAG was as follows. Plastid transformation was carried out as described in section 2.6.4 using the BioRad Biolistic® PDS-1000/He System particle gun. Each plate containing four to five strawberry leaflets was pre-cultured for 1-2 d prior to bombardment using 1100 psi rupture discs. Two days post bombardment the leaflets were divided to yield explants of ~0.5 cm^2. Explants were placed, ten per plate, on ZN102 medium [Appendix 1] containing 20 mg/l spectinomycin and cultured at 25°C under low-level light intensities (1-3 µmol/m^2/s) to induce callus formation and shoot regeneration. After six weeks the explants were transferred to fresh media containing 200 mg/l spectinomycin. After a further 10 weeks explants were again transferred to fresh media containing 200 mg/l spectinomycin and returned to culture under standard light intensity (58 µmol/m^2/s).

Four strawberry plastid transformation experiments utilising the vector pFavAG with spectinomycin selection were carried out to study the effect of several experimental parameters. The parameters studied were pre-culture time, pre-culture medium, donor leaf orientation and donor leaf distance from the firing platform. These variables were tested to either optimise the condition of the donor material prior to bombardment or to aid the recovery of those cells penetrated by gold/DNA. A summary of these four experiments, which yielded six green callus lines and one green shoot line, is provided in Table 6.1.

In all experiments (pFavAG 1-4) pre-culture treatments were imposed by maintaining donor leaf material in Petri-dishes on ZN102 medium at 25°C under low-level lighting for 1-2 d prior to bombardment. This was carried out to achieve orientation of the chloroplasts towards the surface of the donor leaf material hence increasing the chance of DNA uptake. Additionally for pFavAG 1and pFavAG 4, pre-culture included mild desiccation of the donor material. This was achieved by maintaining the leaflets on half of the plates on sterile-filter paper in experiment

Experiment number	No. plates bombarded	Pre-culture time	Pre-culture medium	Orientation and position of donor material	No. explants generated	No. lines regenerated
FavAG 1	42	1d	ZN102, half plates overlaid with sterile filter paper	Replicated either adaxial or abaxial side up, 7.5 cm or 11 cm below firing platform.	1260	1 shoot 1 callus
FavAG 2	14	1d	ZN102	Replicated either adaxial or abaxial side up, 7.5 cm or 11 cm below firing platform.	420	1 callus
FavAG 3	14	2d	ZN102	Replicated either adaxial or abaxial side up, 7.5 cm or 11 cm below firing platform.	420	2 calluses
FavAG 4	18	1d	ZN102, half plates substituted with 0.2M mannitol	Replicated either adaxial or abaxial side up. All 11 cm below firing platform.	540	2 calluses

Table 6.1 Summary of strawberry plastid transformation experiments utilising spectinomycin selection.

Each plate containing 4 – 5 strawberry leaflets was bombarded at 1100 psi with approximately 1.7 µg of plastid transformation vector, pFavAG, precipitated onto 0.2 mg of 0.6 µm gold. Each experiment included two control plates bombarded with gold free of DNA. Following bombardment, leaf material was left on the same medium as pre-culture for 2 days , each leaf cut into ~0.5 cm^2 pieces and then transferred to ZN102 medium containing 20 mg/l spectinomycin. Following subsequent culture on medium containing 200 mg/l spectinomycin, resistant regenerated material was assessed.

pFavAG 1 or through the addition of 0.2M mannitol to half the plates in pFavAG 4 to reduce the osmotic potential. The purpose of these treatments was to reduce the chances of cell rupture on particle bombardment. In pFavAG 4 in addition to mannitol pre-treatment, leaf material was also wounded with garnet paper 3-4 hr prior to bombardment whilst submerged in liquid ZN102 supplemented with 0.2M mannitol to promote callus formation across the whole leaf surface. In all four experiments material on half the plates were cultured, pre- and post-bombardment, either adaxial or abaxial side up and distances of the donor material were either 7.5 cm or 11 cm from the firing platform. Therefore, for each experiment, there were equal numbers of plates representing all combinations of the different parameters being tested.

All putative plastid transformed regenerated lines, whether callus or shoot, were given a line name and the suffix RI. New lines arising from R1 leaf material subject to repeat regeneration were given the suffix RII and lines regenerating from RII leaf material referred to as RIII etc. The same original line name was maintained throughout the repeat regeneration procedure; however a further suffix (a, b, c etc.) was given if more than one shoot was regenerated and maintained for a single line at the same stage of regeneration. This system was adopted for all plastid transformation experiments.

The regenerated green shoot line, Sa1 RI, was generated from donor material bombarded adaxial side up on sterile filter paper 7.5 cm from the firing platform in experiment pFavAG 1. To promote homoplasmy, leaves from line Sa1 RI were subjected to repeat regeneration on ZN102 medium containing 200 mg/l spectinomycin. The Sa1 RII lines produced did not initially form elongating shoots and so were transferred to Z1 medium [Appendix 1] lacking auxin and containing 100 mg/l spectinomycin. Healthy elongating Sa1 RII shoots were produced on this medium and transferred to either SMI media [Appendix 1] containing 500 mg/l spectinomycin to achieve a proliferating shoot culture or subject to a further cycle of repeat regeneration on ZN102 medium containing 500 mg/l spectinomycin. Additionally, line Sa1 RII was screened for spectinomycin resistance arising from spontaneous mutation through

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culture on ZN102 medium containing 200 mg/l spectinomycin and 200 mg/l streptomycin. Line Sa1 RII shoots cultured on SMI media containing 500 mg/l spectinomycin remained green and proliferated and rooted at comparable rates to non-transformed control tissue cultured in the absence of the antibiotic (Figure 6.4A). The majority of line Sa1 RIII shoots regenerated on 500 mg/l spectinomycin were green, however several white shoots were also produced indicating that line Sa1 was not homoplasmic (Figure 6.4B). The leaf material of line Sa1 RII cultured in the presence of streptomycin remained green and regenerated small amounts of green callus. Non-transformed control tissues bleached and did not produce callus in the presence of streptomycin (Figure 6.4C), thus indicating that the resistance observed in line Sa1 was not due to a spontaneous mutation of the 16S rRNA gene and was due to the presence of the *aadA* gene.

A fourth round of repeat regeneration was initiated from the Sa1 RIII shoots using spectinomycin at 500 mg/l and 5000 mg/l. Green callus and green shoots regenerated under both of these conditions however, the Sa1 RIV shoots produced were not large enough to be analysed within the timeframe of this study.

In addition to the green shoot line, Sa1, six green callus lines, Sa2-7, were produced. Line Sa2 was produced from experiment FavAG 1 from material bombarded abaxial side up on sterile filter paper 11 cm from the firing platform. Lines Sa3-6 were produced in experiment FavAG 3, all of which were subject to 2 d pre-culture. Lines Sa3, Sa4 and Sa5 were bombarded adaxial side up, line Sa6 adaxial side up. Lines Sa3 and Sa6 were 7.5 cm from the firing platform and lines Sa4 and Sa5 11 cm from the firing platform when bombarded. Line Sa7, was generated from donor material bombarded abaxial side up 11 cm from the firing platform in experiment FavAG 4.

Throughout the regeneration cycles regenerated shoots and callus of putative transplastomic lines were repeatedly screened using a stereomicroscope fitted with a stereo fluorescence module (section 2.8.3) to visualise GFP fluorescence. The screening for GFP fluorescence within these lines proved problematic. Often control tissues appeared to auto-fluoresce at similar intensities to those observed in the
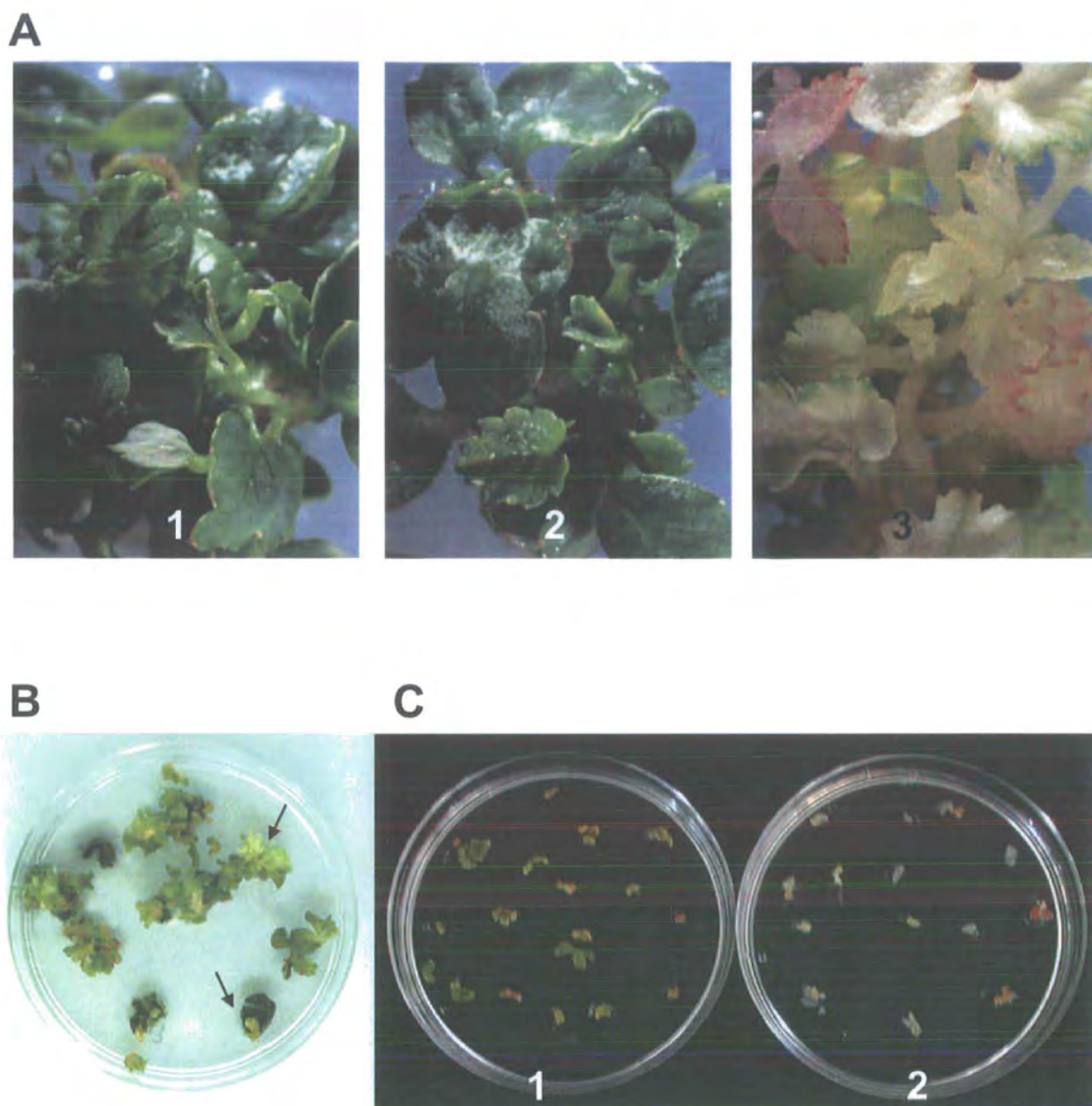
Figure 6.4 *In vitro* culture of putative transplastomic strawberry line Sa1 bombarded with the vector pFavAG

Proliferating *in vitro* strawberry lines on SMI medium (A); line Sa1 RII on medium containing 500 mg/l spectinomycin (A1), non-transformed control cultured in the absence of spectinomycin (A2) and non-transformed control cultured on medium containing 25 mg/l spectinomycin (A3). Line Sa1 RIII shoots regenerating from Sa1 RII leaf material on medium containing 500 mg/l spectinomycin (B); white shoots indicative of heteroplasmy in this line are indicated. Sa1 RII leaf material cultured in the presence of 200 mg/l spectinomycin and streptomycin (C1) and non-transformed leaf material cultured under the same regime (C2).

putative transplastomic lines making it difficult to unambiguously attribute fluorescence to GFP. Several of the callus lines appeared to fluoresce more intensely than the non-transformed control callus which may have been due to the presence of GFP. Examples are shown for callus lines Sa2 RI and Sa3 RI in Figure 6.5A2 and A3. Fluorescence was also observed in leaf tissues of line Sa1 RII having undergone one cycle of repeat regeneration (Figure 6.5B1-3).

It was decided to conduct a more detailed investigation of the fluorescence at the sub-cellular level using a Zeiss Axiophot microscope equipped with Neofluar lenses. Intense fluorescence attributed by GFP, was clearly localised to the chloroplasts in a small proportion of the cells examined in line Sa1 RII leaf material (Figure 6.5C). This result confirmed that the strawberry plastome had been successfully transformed using the vector pFavAG.

Material was taken for molecular analysis from all putative transplastomic lines produced during the strawberry plastid transformation experiments using the vector pFavAG.
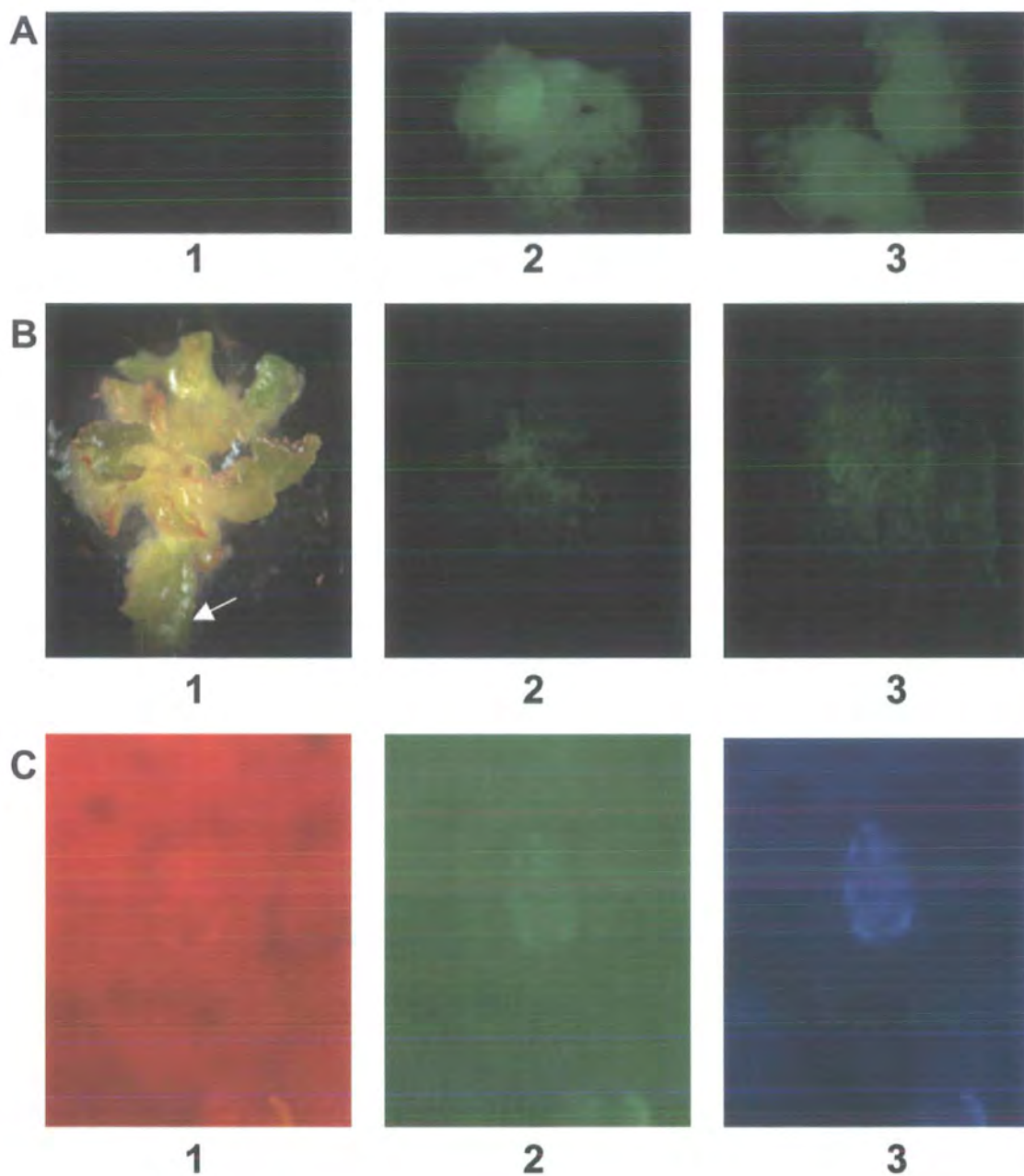
Figure 6.5 GFP expression in putative lines Sa1, Sa2 and Sa3 bombarded with the vector pFavAG

GFP visualised using a stereo fluorescence microscope in strawberry callus lines (A); non-transformed control (A1), putative pFavAG transformed RI lines Sa2 and Sa3 (A2 and A3). GFP visualised in Sa1 RII shoot material in bright field (B1) and using the stereo fluorescence microscope (B2) and x5 magnification of the leaf indicated in B1 (B3). GFP fluorescence visualised at the sub-cellular level in line Sa1 RII using a Zeiss Axiophot microscope; fluorescing chloroplasts visualised in a palisade cell are shown under auto fluorescence (C1), green filter (C2) and UV GFP (C3) filter sets.

6.2.3 Tobacco plastid transformation using the vector pNtAG

In conjunction with the strawberry studies two tobacco plastid transformation experiments using spectinomycin selection and the vector pNtAG were carried out (section 2.6.5). This vector contains the same transcription unit as pFavAG, however the transcription unit is flanked by sequence of tobacco plastome origin. Successful transformation of the tobacco plastome using the vector pNtAG would confirm that the transcription unit created was functional, with regulatory elements isolated from strawberry confering expression of both the selectable and scorable marker genes in tobacco.

Plastid transformation of tobacco was carried out as described in section 2.6.5. A total of thirty leaves, one per plate, were bombarded in two experiments. Eighteen plates were bombarded in the first experiment, NtAG 1, and 12 in the second, NtAG 2. Ten green shoot proliferating lines were generated from experiment NtAG 1, however no shoots were generated from NtAG 2. All of the green shoots produced were subject to repeat regeneration on RMOP media [Appendix 1] containing 500 mg/l spectinomycin. After two rounds of repeat regeneration all lines were split and half subject to regeneration on media containing both 500 mg/l streptomycin and 500 mg/l spectinomycin to screen for spontaneous mutation of the 16S rRNA gene and half maintained on 1000 mg/l spectinomycin RM medium. Of the ten lines subject to double selection only two lines Ta9 RIII and Ta10 RIII produced green callus indicating that the resistance in these lines was conferred by the aadA gene and not as a result of spontaneous mutation. Line Ta9 RIII regeneration in the presence of both antibiotics is shown in Figure 6.6B3 and B4.

Line Ta9 RIII and Ta10 RIII shoots cultured on RM media [Appendix 1] containing 1000 mg/l spectinomycin remained green and rooted at comparable rates to non-transformed control tissue cultured in the absence of the antibiotic (Figure 6.6A3-A4). A further cycle of regeneration on RMOP medium containing 5000 mg/l spectinomycin was initiated for both lines. Green callus and shoots have regenerated
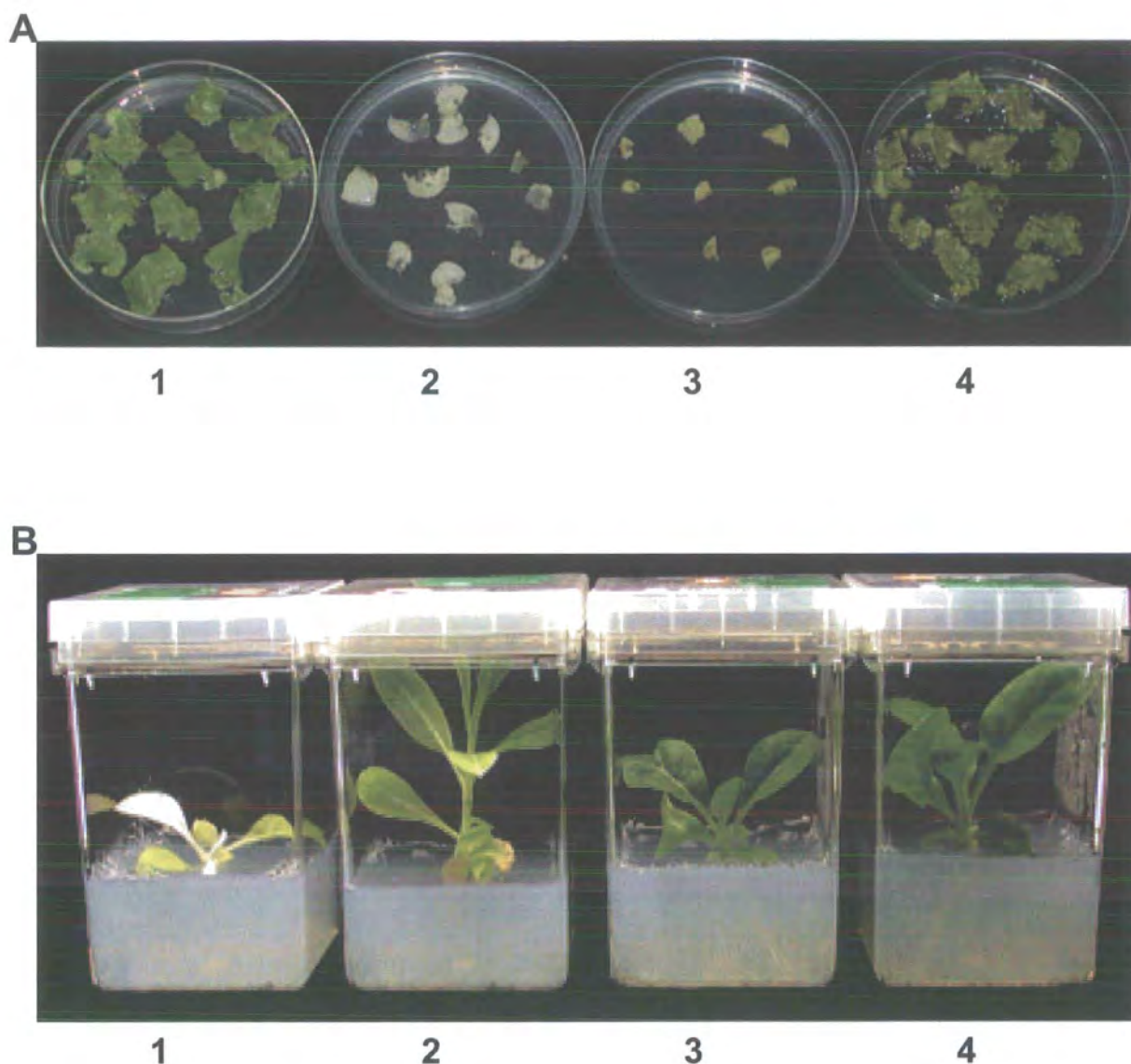
Figure 6.6 *In vitro* culture of putative transplastomic tobacco lines Ta9 and Ta10 bombarded with the vector pNtAG

Regenerating tobacco cultures on RMOP medium (A): non-transformed control cultured in the absence of spectinomycin (A1), non-transformed control cultured on medium containing 500 mg/l spectinomycin (A2). Line Ta9 RIII leaf cultured on medium containing 500 mg/l spectinomycin and 500 mg/l streptomycin (A3) and line Ta9 RIII leaf cultured on medium containing 500 mg/l spectinomycin (A4). Proliferating *in vitro* tobacco lines on RM medium (B); non-transformed control on medium containing 1000 mg/l spectinomycin (B1), non-transformed control cultured in the absence of spectinomycin (B2), RIII lines Ta9 and Ta10 on medium containing 500 mg/l spectinomycin (B3 and B4).

under these conditions; however the Ta9 and Ta10 RIV shoots produced were not large enough for molecular analysis within the timeframe of this study.

Visualisation of GFP fluorescence was carried out as described for strawberry. Visualisation of GFP fluorescence with the stereo fluorescence module was again complicated by auto-fluorescence in non-transformed tissues. This was particularly apparent in roots developing from callus and in necrotic regions of the leaves. High levels of fluorescence were not observed in the leaf tissue of any of the putative transplastomic tobacco lines using the stereomicroscope. However, intense fluorescence was observed in the callus of both lines Ta9 RII and Ta10 RII. The level of fluorescence observed in line Ta9 and Ta10 RIV regenerating callus cultures was significantly higher than in the RII lines suggesting that progression towards homoplasmy was occurring in the two spectinomycin and streptomycin resistant tobacco lines (Figure 6.7).

Leaf material was taken from both lines produced in experiment NtAG 1 and was subject to molecular analysis.

6.2.4 Molecular analysis of strawberry and tobacco putative transplastomic lines bombarded with pFavAG and pNtAG

Two forms of molecular analysis were carried out, PCR to determine whether the *aadA* and *smGFP* genes were present and Southern blot hybridisation analysis to confirm integration of the transgenes into the strawberry and tobacco plastomes.

PCR analysis

PCR was carried out as described in section 2.5.4. Primers were designed to identify lines containing the selectable and scorable marker genes. Primers aadAF and aadAR [Appendix 3] were designed to both anneal in the *aadA* gene and GA1 and GA2 [Appendix 3] designed to anneal in the *aadA* and *smGFP* genes, respectively. Primers Sf1 and Sf2 were designed to anneal within the plastome region surrounding the site targeted for transgene insertion. A schematic showing the relative annealing sites and predicted amplification product sizes for the primers is given in Figure 6.8.
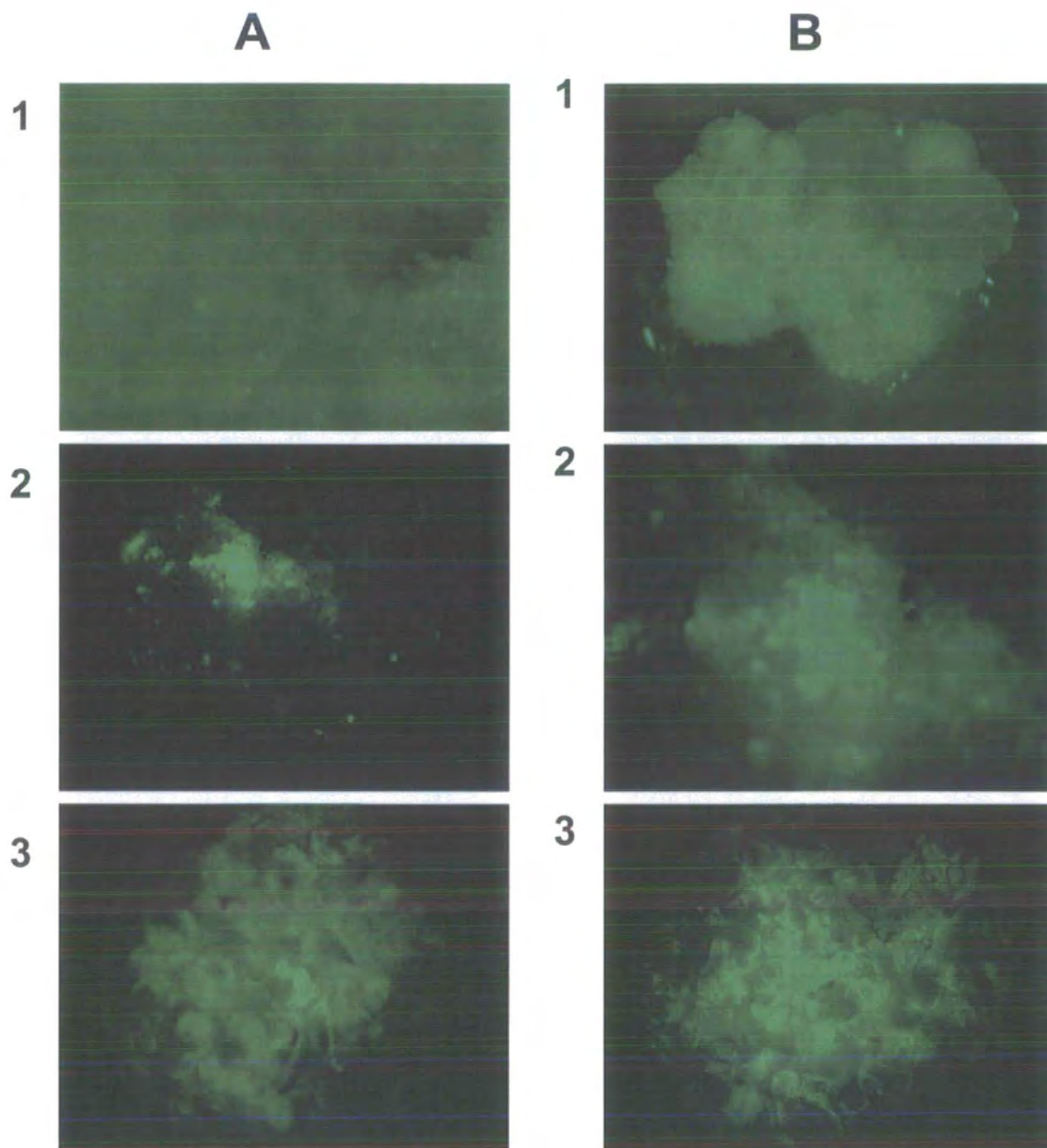
Figure 6.7 Fluorescence observed in tobacco lines Ta9 and Ta10

GFP visualised using a stereo fluorescence microscope in tobacco callus of lines Ta9 (A) and Ta10 (B). RII callus under bright field illumination (1). GFP visualised in RII callus (2) and in RIV callus regenerating shoots (3).
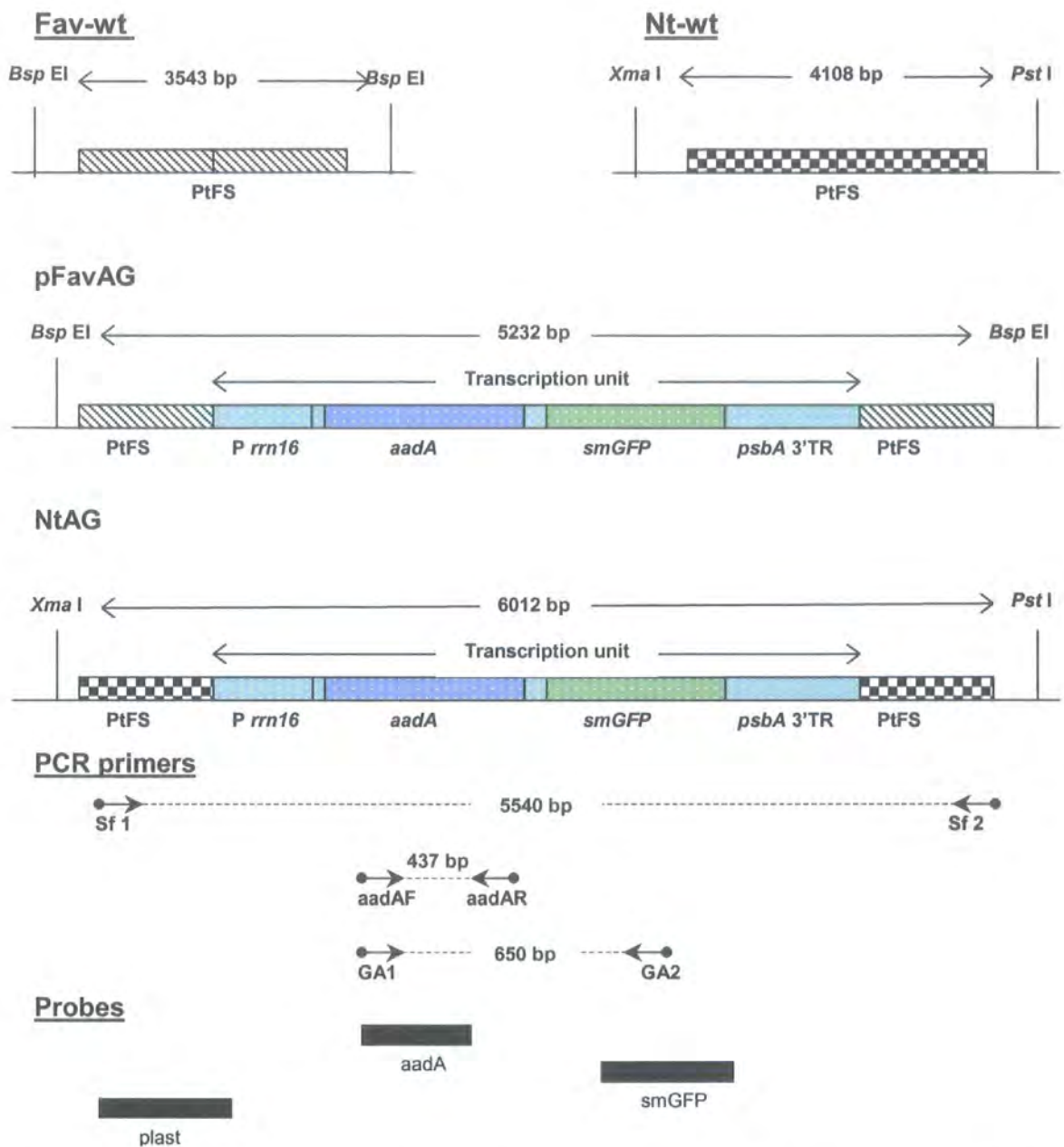
Figure 6.8 Schematic showing components relevant to the molecular analysis of pFavAG and pNtAG transgenic plants

Wild type plastome sequence is shown for strawberry (Fav-wt) and tobacco (Nt-wt), plastome sequence used to flank the transcription units is shaded, relevant restriction enzyme sites used in Southern analysis are labelled and the predicted restriction digest product size indicated. A schematic of the vectors pFavAG and pNtAG incorporated into the plastome is given, with the corresponding restriction enzymes sites and predicted restriction digest product sizes indicated. PCR primers used during the analysis of transgenic lines are labelled and their orientation and amplification product sizes indicated. The approximate region of hybridisation of DIG-labelled probes used in Southern blot hybridisation analysis is also indicated.

Strawberry RI callus lines Sa2-7 were not subject to PCR analysis. PCR using the primer pair aadAF and aadAR confirmed the presence of the *aadA* gene in lines Sa1 RII, Ta9RII, and Ta10 RII. Linkage of the *aadA* and *smGFP* genes within these lines was confirmed using the primer pair GA1 and GA2 (Figure 6.9). PCR using the primer pair Sf1 and Sf2 failed to amplify a product, indicative of insertion into either the strawberry or tobacco plastomes (Figure 6.9). However this result may be a false negative, indicative of low levels of transformed plastomes present in these lines and primer competition in PCR with high levels of wild type plastome copies, rather than lack of plastome transformation.

Southern blot hybridisation analysis

Southern blot hybridisation was carried out as described in section 2.7.1, using 5 µg total gDNA. Digoxigenin-II-dUTP (DIG)-labelled *aadA* and *smGFP* probes were synthesised using pFavAG plasmid DNA as template and the primer pairs aadA1 with aadA2 and gfp281 with gfp715, respectively [Appendix 3]. DIG-labelled Plast probe was synthesised using pStraw4.5 (section 5.2.1) plasmid DNA as template and the primer pair Plast1 with Plast2 [Appendix 3]. These probes were then used to evaluate integration of the pFavAG and pNtAG transcription units into the strawberry and tobacco plastomes in putative plastid transformed lines. A schematic showing the restriction sites and probes relevant to Southern blot hybridisation analysis is provided in Figure 6.8.

Strawberry gDNA was digested with *Bsp* EI which releases a plastome restriction fragment of 3543 bp from non-transformed strawberry. Integration of the *aadA: smGFP* transcription unit into the plastome region targeted should generate a 5445 bp restriction fragment upon *Bsp* EI digestion. Tobacco gDNA digested with *Xma* I and *Pst* I releases a plastome restriction fragment of 4108 bp from non-transformed tobacco and a fragment of 6012 bp if the pNtAG *aadA: smGFP* transcription unit is incorporated at the plastome region targeted.

Southern blot hybridisation was carried out on two RII and one RIV clones of tobacco line Ta9, two RII clones of tobacco line Ta10 and five RII clones of strawberry
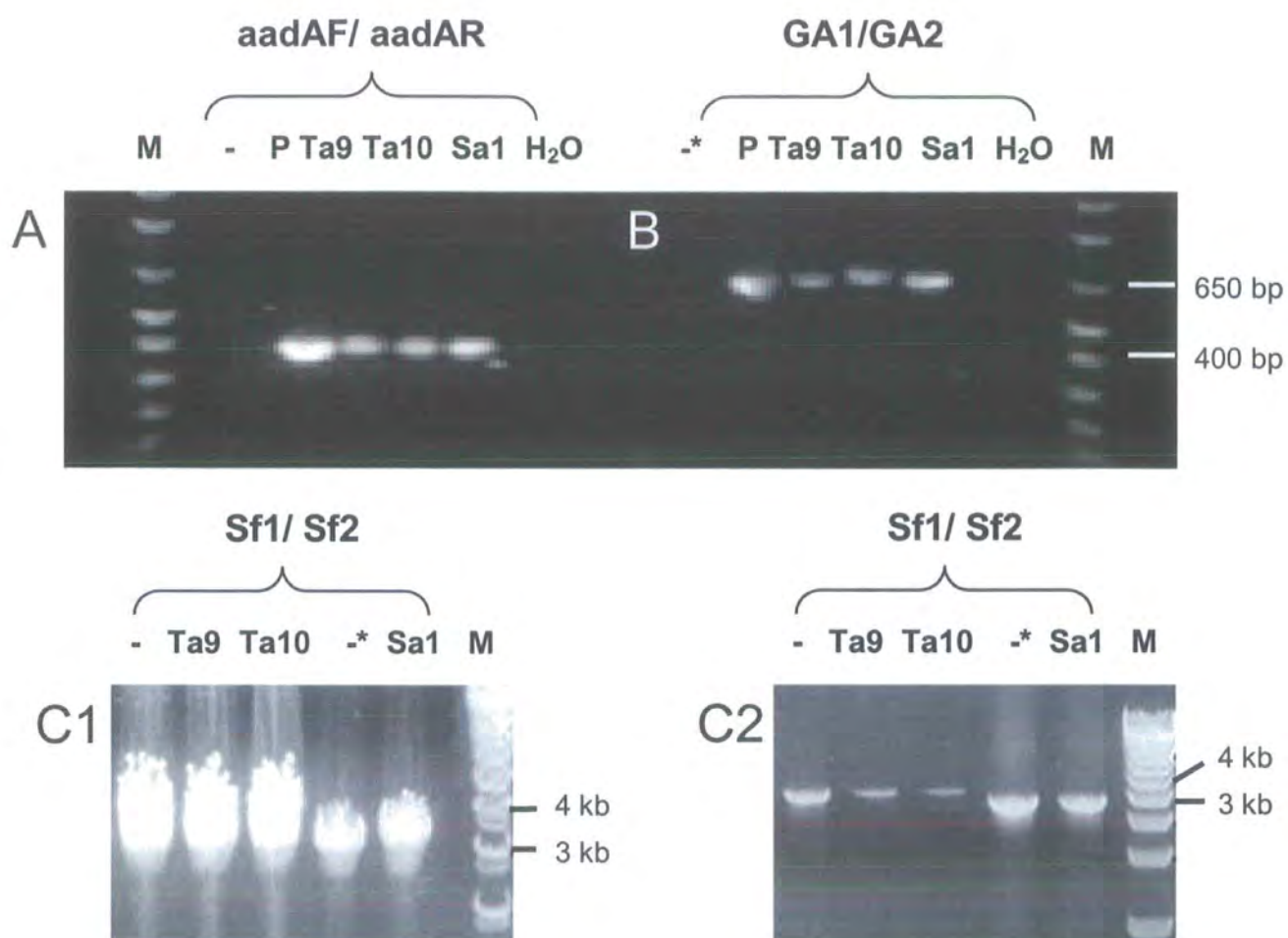
Figure 6.9 PCR Analysis of putative transplastomic strawberry and tobacco transformed with vectors pFavAG and pNtAG, respectively

PCR analysis of RII leaf material from tobacco lines Ta9 and Ta10 and strawberry line Sa1. pFavAG plasmid DNA (P) , water (H_2O) and non-transformed tobacco (-) and non-transformed strawberry (-*) served as controls. An amplification product of 437 bp generated using primer pair aadAF/R confirms the presence of the *aadA* gene in all three lines (A). An amplification product of 650 bp generated using primer pair GA1/2 PCR confirms the presence of the *aadA* gene linked to the *smGFP* gene in all three lines (B). Excessive amplification of wild type plastome using primer pair Sf1/2 (C1), 1:100 dilution of PCR product used to accurately visualise Sf1/2 amplification products (C2). The amplification product of approximately 3.5 kb generated using primer pair Sf1/2 is indicative of wild type plastome amplification (C1), an amplification product of ~5.5 kb indicative of integration of the pNtAG / pFavAG transcription units into the tobacco and strawberry plastomes, respectively, was not clearly identifiable.

line Sa1 (Figure 6.10). A hybridising band of approximately 8 kb was visualised using DIG-labelled *aadA* and plast probes in tobacco lines Ta9 and Ta10. The *aadA* probe hybridised in one strawberry clone at approximately 8 kb and 10 kb. Furthermore, the *aadA* and plast hybridising bands were much more intense in tobacco line Ta9 RIV when compared to the RII samples for this line and line Ta10. The *aadA* probe did not hybridise in any of the non-transformed DNA samples included as controls. Hybridising bands were noted at approximately 3.5 kb and 7 kb in all strawberry samples when using the plast probe. The 3.5 kb hybridising fragment represents the wild-type region of plastome targeted; the 7 kb restriction fragment is an artefact of non-specific probe annealing. A faint plast hybridising band was noted at approximately 5 kb in two clones of the strawberry line Sa.
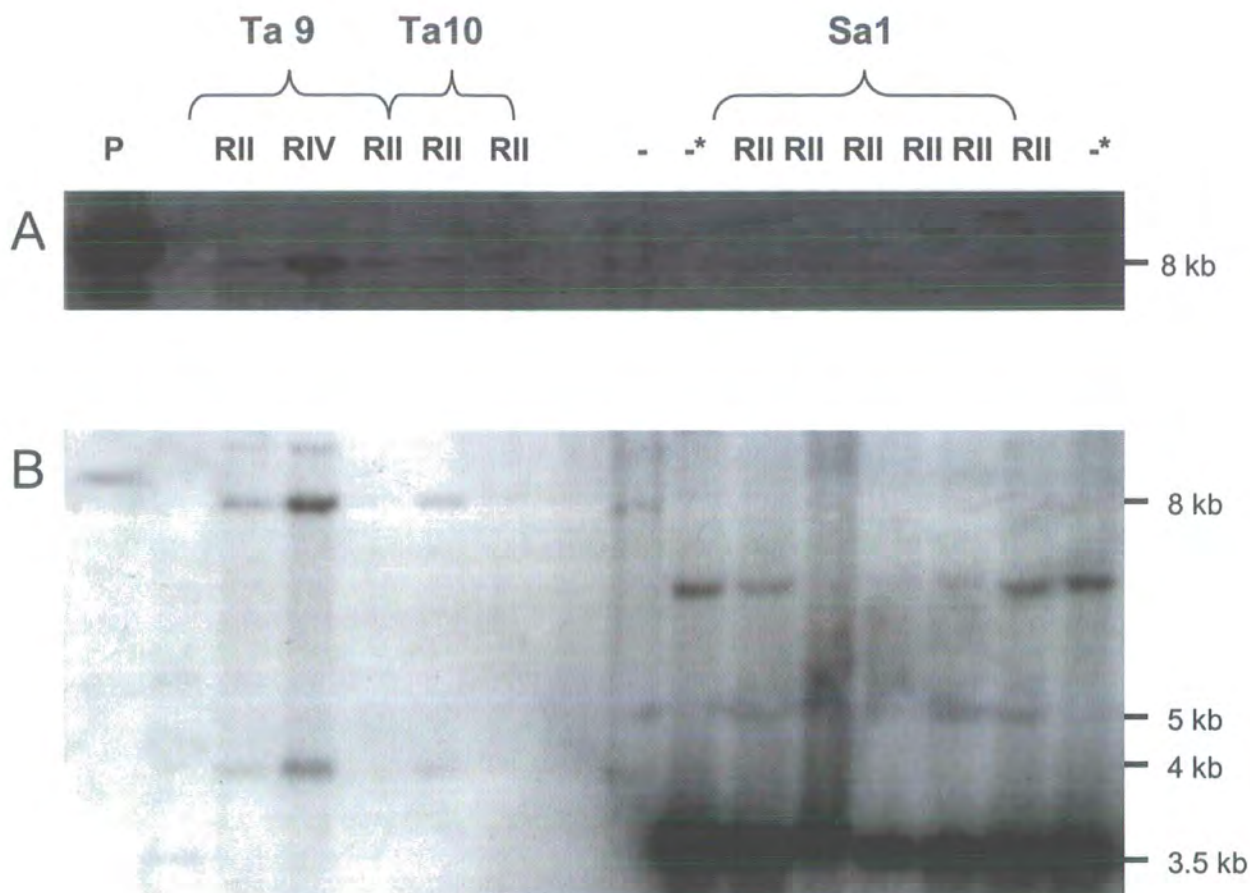
Figure 6.10 Southern blot hybridisation analysis of putative transplastomic strawberry and tobacco transformed with pFavAG and pNtAG, respectively

5 μg of Xma I/ Pst I digested tobacco or Bsp HI digested strawberry genomic DNA was loaded per lane and probed with DIG-labelled aadA probe (A) and DIG-labelled plast probe (B). The number of repeat regeneration cycles carried out for each clone is indicated for tobacco lines Ta9 and Ta 10 bombarded with the vector pNtAG as well as for strawberry line Sa1 bombarded with the vector pFavAG. Non-transformed tobacco control DNA (-) and non-transformed strawberry control DNA (-*) digests are indicated. Linearised plasmid pNtAG (P) is also indicated.

6.3 Plastid transformation utilising D-xylose as a selective agent

6.3.1 Optimisation of strawberry regeneration using D-xylose selection

The concentration of D-xylose to be used as a selective agent in strawberry plastid transformation, using the vector pFavXG, was determined empirically through a series of regeneration experiments. These assessed the sensitivity of Calypso shoot regeneration to different D-xylose concentrations and optimised regeneration conditions. For all regeneration experiments leaflets were excised aseptically from approximately seven-week-old *in vitro,* rooted Calypso plants and dissected into ~0.5 cm^2 explants. Ten explants were then placed adaxial side up on approximately 35 ml of media in 9 cm diameter Petri dishes for each media type being evaluated.

Initially three regeneration experiments were carried out using ZN102 media and varying levels of xylose as a percentage of the total 30 g/l carbohydrate supplied, with the remainder of the carbohydrate source in the media composed of sucrose. In all three experiments the percentages of xylose assessed were 0, 17, 34, 50, 66, 83 and 100%. Problems were experienced with regeneration in the control (100 % sucrose) plates in the first experiment, invalidating the data generated.

For the second and third experiments 100 explants were initiated for each xylose level evaluated and cultured under artificial lighting (16 hr day) at 22°C. After five weeks culture, half of the replicates were transferred to Z1 media (lacking the auxin component) and half maintained on ZN102 to investigate the effect of maintaining auxin in the media.

Explants were evaluated after a further 11 weeks and the number of explants producing callus and regenerating shoots on each plate recorded (Figure 6.11). When D-xylose accounted for 100 % of the carbohydrate source supplied callus and shoot production were severely restricted. Callus regeneration was observed on all media

Figure 6.11 Sensitivity of strawberry shoot regeneration to D-xylose under standard light growth regime

Regeneration was carried out under standard light regimes (16 hr day at 58 μmol/m^2/s) with varying proportions of the sucrose carbohydrate source in the media substituted with D-xylose. 100 explants were assessed at each xylose level for two media regimes; continuous culture on ZN102 media (ZN102) and five-weeks initial culture on ZN102 and then transfer to Z1 media (Z1), over the course of two replicated experiments. Regeneration frequency is given as a percentage of explants producing callus after 8 weeks (white) and shoots after 16 weeks (black). Error bars show the level of variation observed between replicate treatments.

where the D-xylose component was below 100 % of the total carbohydrate supplied. It was observed that where the auxin component was maintained in the medium (ZN102), the percentage of explants producing shoots was generally higher than in replicate treatments lacking auxin (Z1). A high amount of variation was observed within replicates and the percentage of explants regenerating did not always correlate to the proportion of D-xylose in the media. Although callus regenerated on all media containing less that 100 % D-xylose, the quality and quantity of callus produced was consistently lower than that produced on the 100 % sucrose media. It is also important to note that when proliferating crowns were cultured on 100 % D-xylose shoot proliferation was not greatly inhibited. It was thought that these cultures may be able to photosynthesise to a degree within the culture vessels and thus bypass their dependence on the carbohydrate supplied.

It was decided that whilst the 100 % D-xylose treatment was too severe to allow putative transformants to regenerate the next level of selection evaluated (83 % D-xylose) was inadequate to limit the number of non-transformed shoots surviving. As described in section 6.2.1, it had been observed that the substitution of sucrose with glucose had a beneficial effect on rates of strawberry regeneration. To optimise regeneration and to impose more stringent D-xylose selection it was decided to use glucose based media and to assess a range of media under dark growth conditions. It was thought that this approach would reduce photosynthesis and ensure explant dependence on the carbohydrate source supplied.

Replicates of 30 explants were cultured for each treatment in two large scale dark-grown regeneration experiments to evaluate four different media ZN102, ZN105, BN202 and BN205 [Appendix 1] with D-xylose accounting for 100, 98.3, 96.6, 91.6, 83.3, 75, 66.6 and 0% of the carbohydrate supplied. On average 40 % of the explants regenerated shoots on ZN102 media containing glucose as the sole carbohydrate source under dark growth conditions (Figure 6.12). Typically 80 – 100 % regeneration is achieved using this media under light growth conditions. Shoot regeneration was
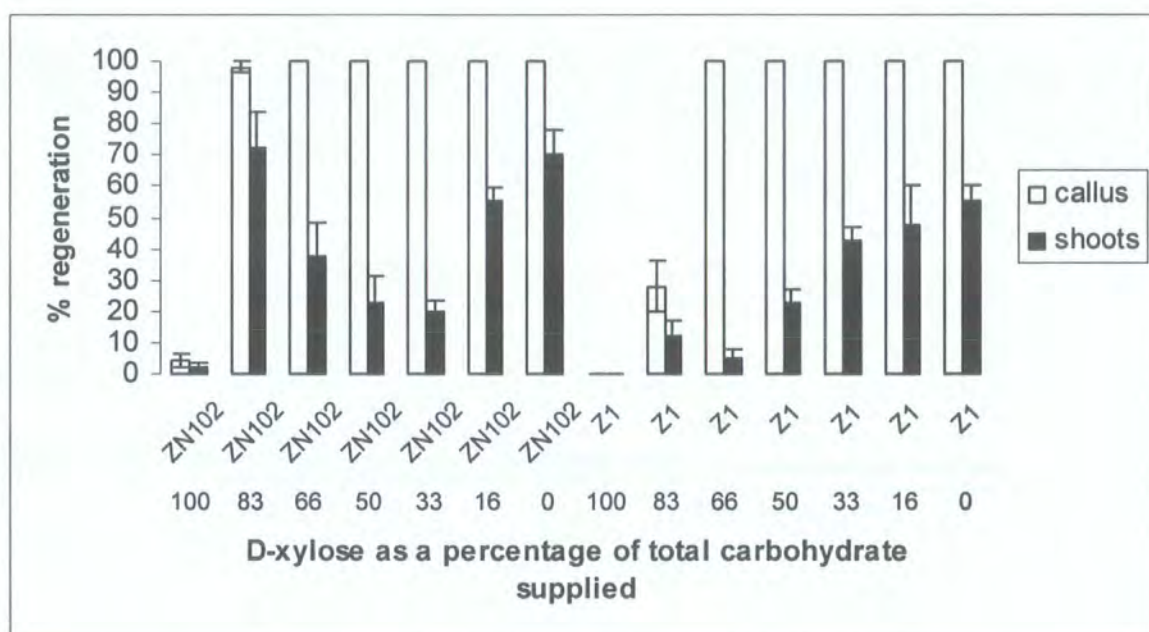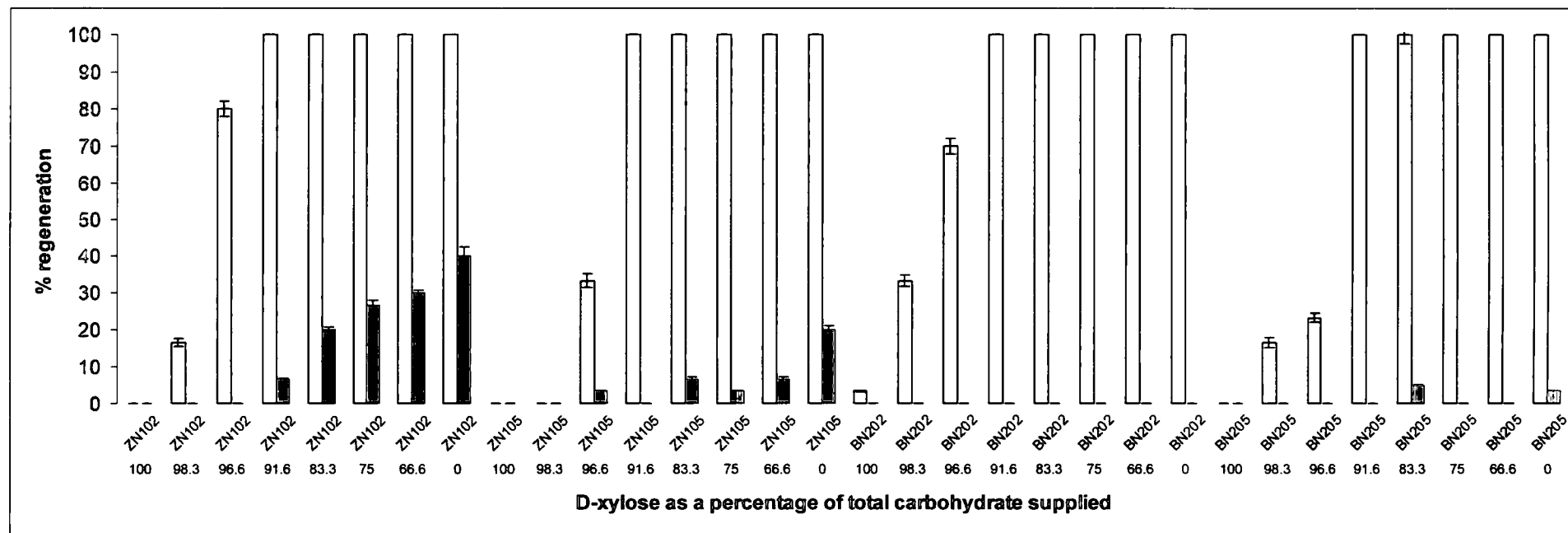
Figure 6.12 Sensitivity of strawberry shoot regeneration to D-xylose under dark growth conditions

Regeneration was carried out under dark growth conditions with varying proportions of the glucose carbohydrate source substituted with D-xylose. 30 explants were assessed at each xylose level for four different media; ZN102 (1 mg/l TDZ, 0.2 mg/l NAA), ZN105 (1 mg/l TDZ, 0.5 mg/l NAA), BN202 (2 mg/l BAP, 0.2 mg/l NAA) and BN205 (1 mg/l BAP, 0.5 mg/l NAA), over the course of two replicated experiments. Regeneration frequency is given as a percentage of explants producing callus after 8 weeks (white) and shoots after 16 weeks (black). Error bars show the level of variation observed between replicate treatments.

severely limited on the benzyl-amino purine (BAP) based media, BN202 and BN205. Callus formed on all levels of D-xylose assessed for all media, although to a much lesser degree when D-xylose accounted for in excess of 96.6% of the carbohydrate supplied. It was noted that under dark growth conditions shoot regeneration rates were inversely proportional to the amount of D-xylose in the medium. This was in contrast to the inconsistent relationship between the two factors observed under standard light regimes (Figure 6.11). Shoots regenerated under dark growth conditions were typically etiolated and often vitrified. A high proportion of dark grown shoots were unable to withstand transfer to proliferation media and culture under standard light regime.

On the basis of these observations it was decided to culture strawberry explants under low level light intensities rather than dark growth conditions on glucose based media with D-xylose accounting for 96.6% of the carbohydrate source

6.3.2 Plastid transformation using the vector pFavXG in strawberry

Strawberry plastid transformation utilising xylose selection was carried out essentially as described in section 6.2.1 however selection pressure was imposed by D-xylose accounting for 96.6% of the total carbohydrate supplied in glucose based ZN102 regeneration media. Explants were cultured at $25^{\circ}C$ under low-level light intensities (1-3 $\mu mol/m^2/s$) and transferred to fresh media with the same level of D-xylose at six-weekly intervals.

Five strawberry plastid transformation experiments were carried out to study the effect of several experimental parameters. As described for strawberry plastid transformation using spectinomycin selection (section 6.2.1) the parameters studied were pre-culture time, pre-culture medium, donor leaf orientation and donor leaf distance from the firing platform. These variables were intended to either optimise the condition of the donor material prior to bombardment or to aid the recovery of those cells penetrated by gold/DNA. A summary of the strawberry plastid transformation experiments carried out using the vector pFavXG with D-xylose selection is provided in table 6.2.

Experiment number	No. plates bombarded	Pre-culture time	Pre-culture medium	Orientation and position of donor material	No. explants generated	No. lines regenerated
FavXG 1	18	1 d	ZN102	Replicated either adaxial or abaxial side up, 7.5 cm or 11 cm below firing platform.	540	55
FavXG 2	18	2 d	ZN102	Replicated either adaxial or abaxial side up, 7.5 cm or 11 cm below firing platform.	540	7
FavXG 3	18	1 d	ZN102	Replicated either adaxial or abaxial side up, 7.5 cm or 11 cm below firing platform.	540	10
FavXG 4	18	2 d	ZN102	Replicated either adaxial or abaxial side up. All 11 cm below firing platform.	540	35
FavXG 5	18	1 d	ZN102, half plates substituted with 0.2M mannitol	All adaxial side up all 5 cm below firing platform.	540	3

Table 6.2 Summary of strawberry plastid transformation experiments utilising D-xylose selection.

Each plate containing 4 – 5 strawberry leaflets was bombarded at 1100 psi with approximately 1.7 µg of plastid transformation vector, pFavXG, precipitated onto 0.2 mg of 0.6 µm gold. Each experiment included two control plates bombarded with gold free of DNA. Following bombardment, leaf material was left on the same medium as pre-culture for 2 days, each leaf cut into ~0.5 cm^2 pieces and then transferred to ZN102 medium with D-xylose accounting for 96.6% of the carbohydrate source. Following subsequent culture, resistant regenerated material was assessed.

The five transformation experiments outlined in table 6.2 generated 110 putative transplastomic lines. Examples of plates with shoots regenerated on medium where D-xylose accounted for 96.6% of the carbohydrate source are shown in Figure 6.13A. In approximately 10 % of the pFavXG lines a white phenotype was observed in a small proportion of the leaves of each shooting cluster and in several cases the entire cluster was white (Figure 6.13B). All RI lines were transferred to SMI proliferation media containing 30 g/l D-xylose as the sole carbohydrate source. Examples of RI lines Sx1 and Sx2 are shown in Figure 6.13C. Leaf material was taken from all 110 lines for molecular analysis.

Putative transplastomic RI lines were screened for GFP expression using a stereomicroscope fitted with a stereo fluorescence module (section 2.8.3). The screening for GFP fluorescence within these lines proved problematic. As observed in the pFavAG lines, non-transformed control leaf tissues appeared to auto-fluoresce at similar intensities to those observed in the putative transplastomic lines making it difficult to unambiguously attribute fluorescence to GFP. The white leaves fluoresced uniformly.

It was decided to conduct a more detailed investigation of the fluorescence at the sub-cellular level in two promising RI lines, Sx1 and Sx2. Both lines resulted from experiment FavXG 1 and regenerated from donor material bombarded abaxial side up at 7.5 cm from the firing platform following 1 d of pre-culture. These were chosen since PCR screening revealed presence of both the *xylA* and *smGFP* genes (see later, section 6.3.4, Figure 6.16). Using a Zeiss Axiophot microscope equipped with Neofluar lenses intense fluorescence was clearly localised to the chloroplasts in a small proportion of the cells in the leaves examined in these lines (Figure 6.14). It was noted that often cells containing the most fluorescent chloroplasts appeared grey and abnormal under bright field illumination. This result confirmed that the strawberry plastome had been successfully transformed using the vector pFavXG.
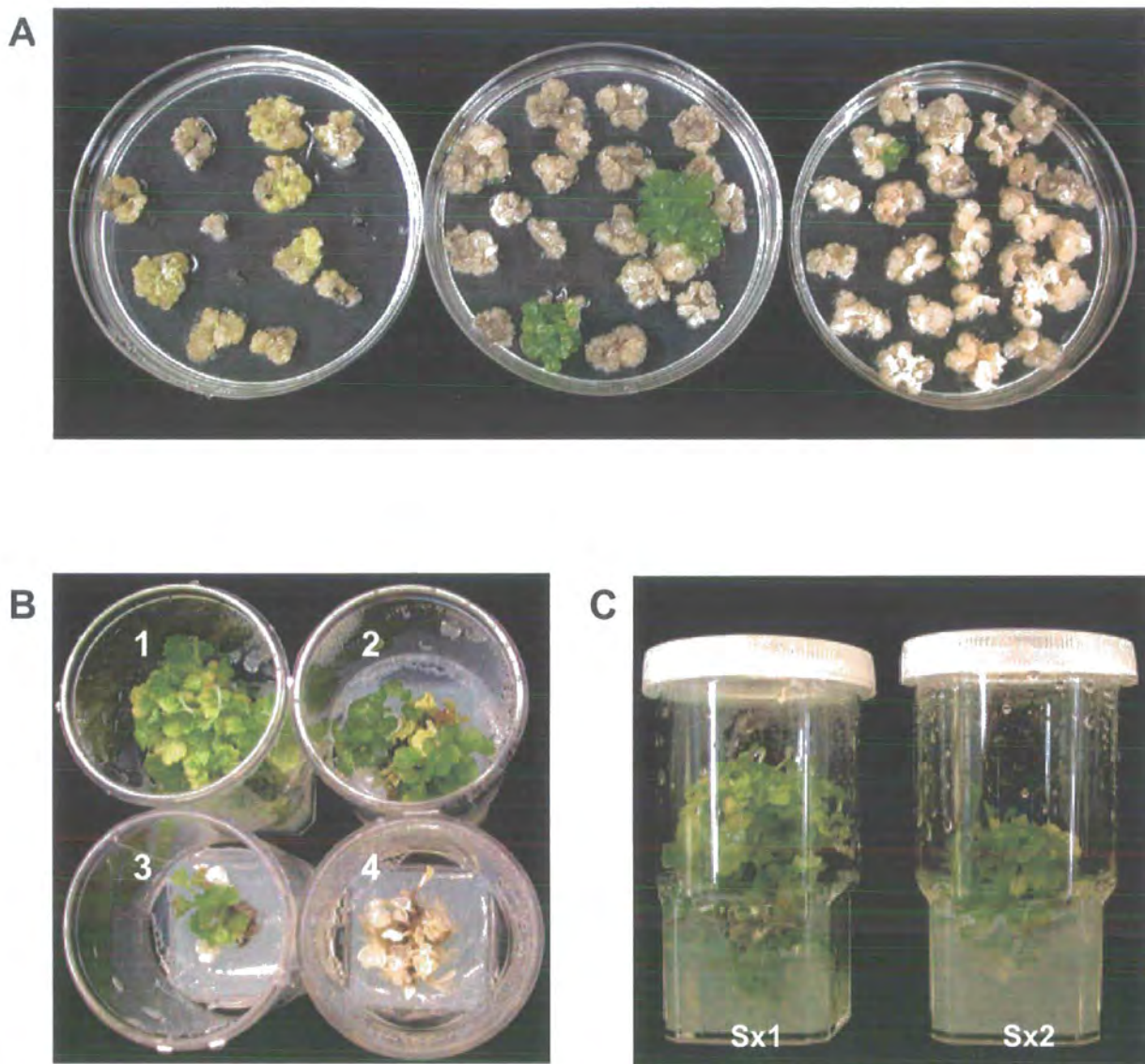
Figure 6.13 Putative transplastomic strawberry lines Sx1 and Sx2 bombarded with the vector pFavXG and cultured on D-xylose

Bombarded strawberry explants cultured under low light intensities (1-3 μmol/m²/s) on ZN102 media with D-xylose accounting for 96.6% of the total carbohydrate supplied (A). Range of phenotypes observed in shoot cultures of regenerated shoots (B); line Sx1 cultured on 100% D-xylose (B1), non-transformed control cultured on 100% glucose (B2), putative transplastomic lines cultured on D-xylose displaying a mixed green/white phenotype (B3), and entirely white phenotype (B4). Putative transplastomic RI lines Sx1 and Sx2 cultured on 100% D-xylose (C).

Line Sx1

1   2   3   4

Line Sx2

1   2   3   4
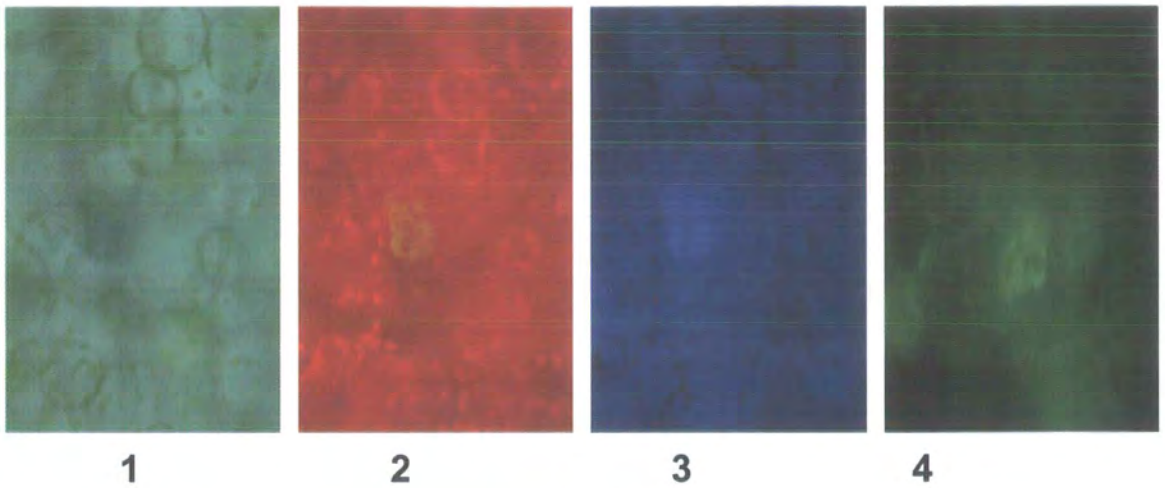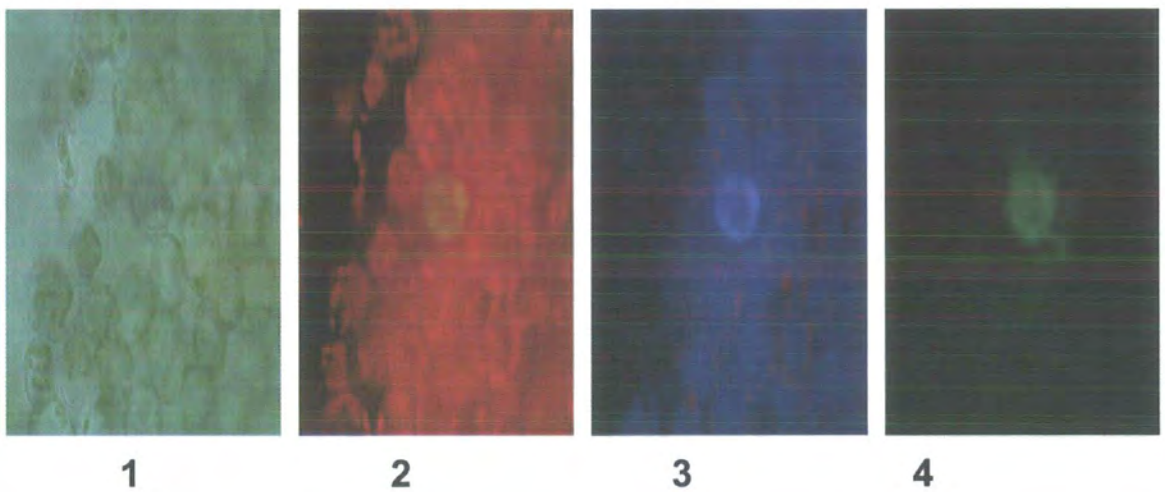
Figure 6.14 GFP expression in putative transplastomic strawberry lines Sx1 RI and Sx2 RI bombarded with the vector pFavXG

GFP fluorescence visualised at the sub-cellular level in leaf material of RI lines Sx1 (A) and Sx2 (B) cultured on SMI medium with D-xylose as the sole carbohydrate source. Using a Zeiss Axiophot microscope, fluorescing chloroplasts visualised in palisade cells are shown under bright field (1), auto fluorescence (2), green filter (3) and UV GFP (4) filter sets.

6.3.3 Tobacco plastid transformation using the vector pNtXG

In conjunction with the strawberry studies, three tobacco plastid transformation experiments using D-xylose selection and the vector pNtXG were carried out (section 2.6.5). This vector contains the same transcription unit as pFavXG, however the transcription unit is flanked by sequence of tobacco plastome origin. Successful transformation of the tobacco plastome using the vector pNtXG would confirm that the transcription unit created was functional, with regulatory elements isolated from strawberry confering expression of both the selectable and scorable marker genes in tobacco and that D-xylose selection was applicable to tobacco plastid transformation.

Plastid transformation of tobacco was carried out as described in section 2.6.5 and explants cultured on RMOP medium [Appendix 1].

The experimental parameters investigated were the level of D-xylose required to exert selection pressure and the growth conditions under which this was best achieved. Initially tobacco leaf explants were cultured on media where 80% of the carbohydrate supplied was composed of D-xylose and 20% sucrose. This level of D-xylose was reported to have successfully selected transformation events using the *xylA* gene and D-xylose selection for nuclear transformation of tobacco (Haldrup *et al.*, 1998). It was thought this could be applied to plastid transformation however this level of selection was found to be inadequate.

Two tobacco plastid transformation experiments totalling 30 bombarded leaves were initiated using 80% D-xylose as selection and growth under standard light regimes (~58 μmol/m^2/s). A mass of regenerated shoots were produced on all plates containing 80% D-xylose at similar frequencies to those observed on non-selective media. To increase selection stringency after initial culture for five weeks in light on 80% D-xylose, explants were transferred to media containing D-xylose at 100 % of the carbohydrate source. Half the regenerating cultures were transferred to the dark to inhibit any photosynthetic capacity the explants may have had.

It appeared that D-xylose as the sole source of carbohydrate and dark culture conditions inhibited the growth of the regenerating tissues, however by the time this treatment was applied over 3,000 putative transplastomic tobacco lines had been produced. Obvious fluorescence was not detected in any of the lines produced and it was decided that the number of lines generated made molecular analysis of the lines arising from the first two experiments untenable. No further analysis was carried out on these lines.

A third transformation experiment, in which 18 leaves were bombarded, was initiated with D-xylose as the sole source of carbohydrate and cultures were grown in the dark from the outset. Shoot regeneration was severely reduced in this experiment (Figure 6.14) and only 43 putative transformed shoot lines regenerated, all of which were etiolated and lacked vigour. After 16 weeks the dark grown lines were transferred to standard light culture on RM media [Appendix 1] containing D-xylose as the sole source of carbohydrate. The majority of these lines did not revert to the standard growth phenotype for tobacco under a standard light regime.

GFP visualisation was carried out as described for strawberry; however fluorescence was not apparent in any of the pNtXG lines. Leaf material was taken from the 12 regenerated lines large enough to withstand sampling and subject to molecular analysis.

6.3.4 Molecular analysis of strawberry and tobacco putative transplastomic lines

PCR was carried out to determine whether the *xylA* and *smGFP* genes were present in the pFavXG and pNtXG lines generated. However, Southern blot hybridisation analysis was not carried as insufficient DNA was recovered from the tissues available at the time of analysis.
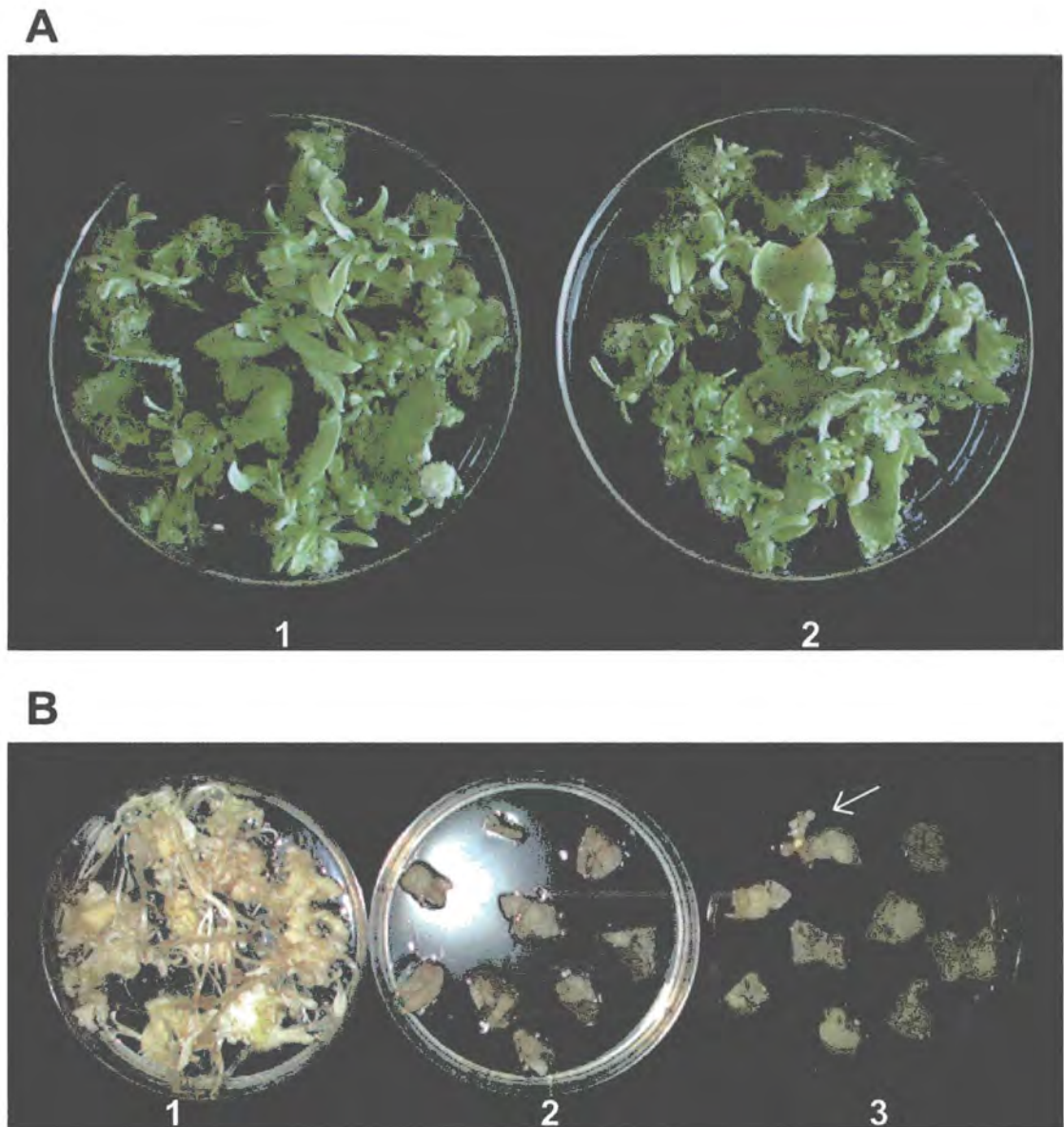
Figure 6.14 *In vitro* culture of tobacco explants in the presence of D-xylose

Light grown tobacco shoot regeneration (A), on medium with sucrose as the sole carbohydrate source (A1) and with D-xylose as the sole carbohydrate source (A2). Dark grown tobacco shoot regeneration (B) on medium with sucrose as the sole carbohydrate source (B1) and with D-xylose as the sole carbohydrate source (B2 and B3). A small etiolated shoot regenerated on D-xylose medium under dark growth conditions is indicated.

PCR analysis

PCR was carried out as described in section 2.5.4. Primers were designed to identify lines containing the selectable and scorable marker genes. Primers xylA860 and xylA1241 [Appendix 3] were designed to both anneal in the *xylA* gene and GX1 and GX2 [Appendix 3] designed to anneal in the *xylA* and *smGFP* genes, respectively. Primers Sf1 and Sf2 were designed to anneal within the plastome region surrounding the site targeted for transgene insertion. A schematic showing the relative annealing sites and predicted amplification product sizes for the primers is given in Figure 6.15.

PCR using the primer pair xylA860 and xylA1241 confirmed the presence of the *xylA* gene in strawberry lines Sx1 and Sx2. Linkage of the *xylA* and *smGFP* genes within these lines was confirmed using the primer pair GX1 and GX2 (Figure 6.16). PCR using the primer pair Sf1 and Sf2 failed to amplify a product indicative of insertion into the strawberry plastomes (Figure 6.16). However this result may be indicative of low levels of transformed plastomes present in these lines and primer competition in PCR with high levels of wild type plastome copies rather than lack of plastome transformation. Insertion of the *xylA* and *smGFP* transgenes was not detected in any of the 12 tobacco lines generated in the third pNtXG experiment using PCR analysis (data not shown).
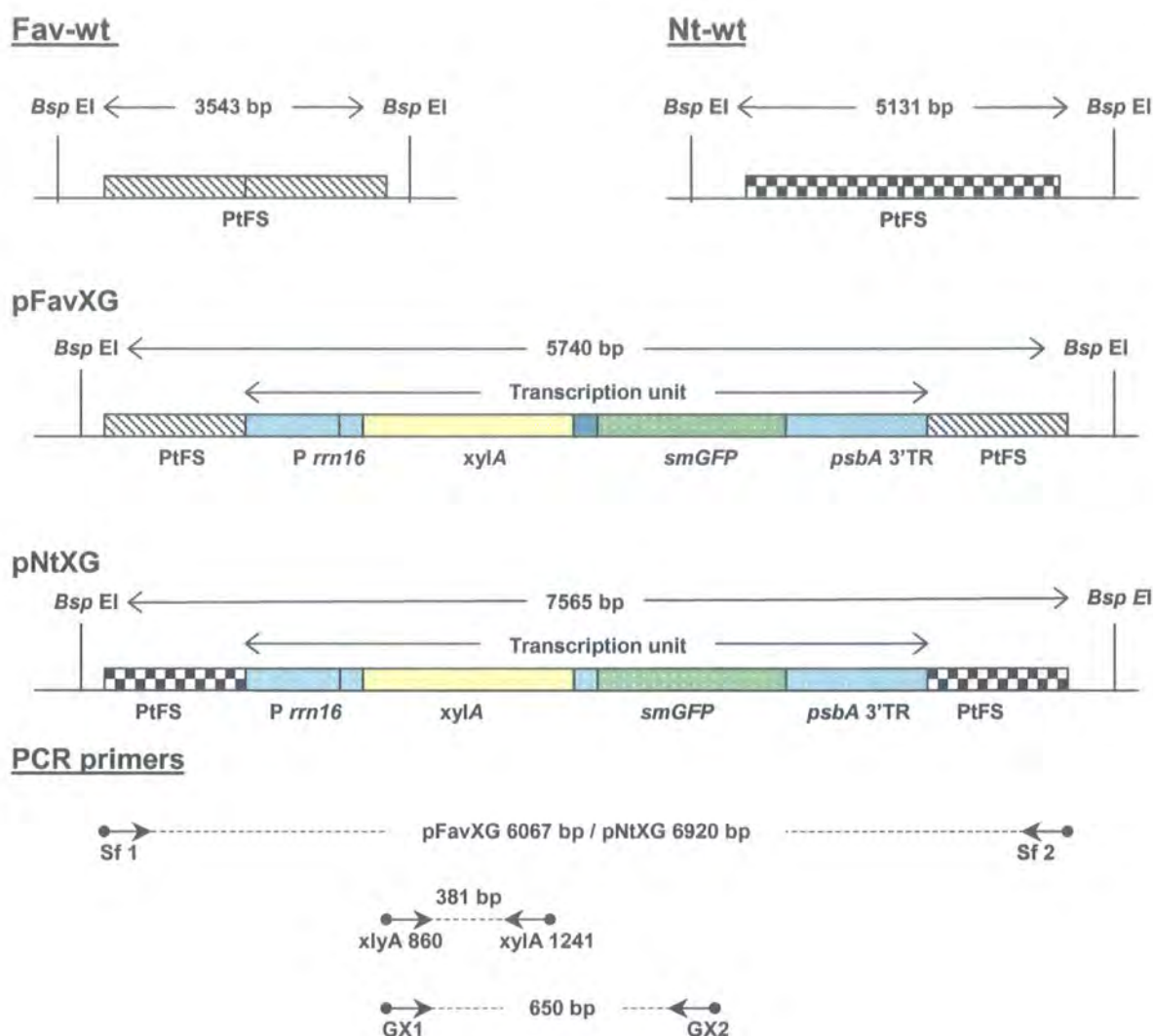
Figure 6.15 Schematic showing components relevant to the molecular analysis of pFavXG and pNtXG transgenic plants

Wild type plastome sequence is shown for strawberry (Fav-wt) and tobacco (Nt-wt), plastome sequence used to flank the transcription units (PtFS) is shaded, relevant restriction enzyme sites are labelled and the expected restriction digest product size indicated. A schematic of the vectors pFavXG and pNtXG incorporated into the plastome is given, with the corresponding restriction enzymes sites and expected restriction digest product sizes indicated. PCR primers used during the analysis of transgenic lines are labelled and their orientation and amplification product sizes indicated.
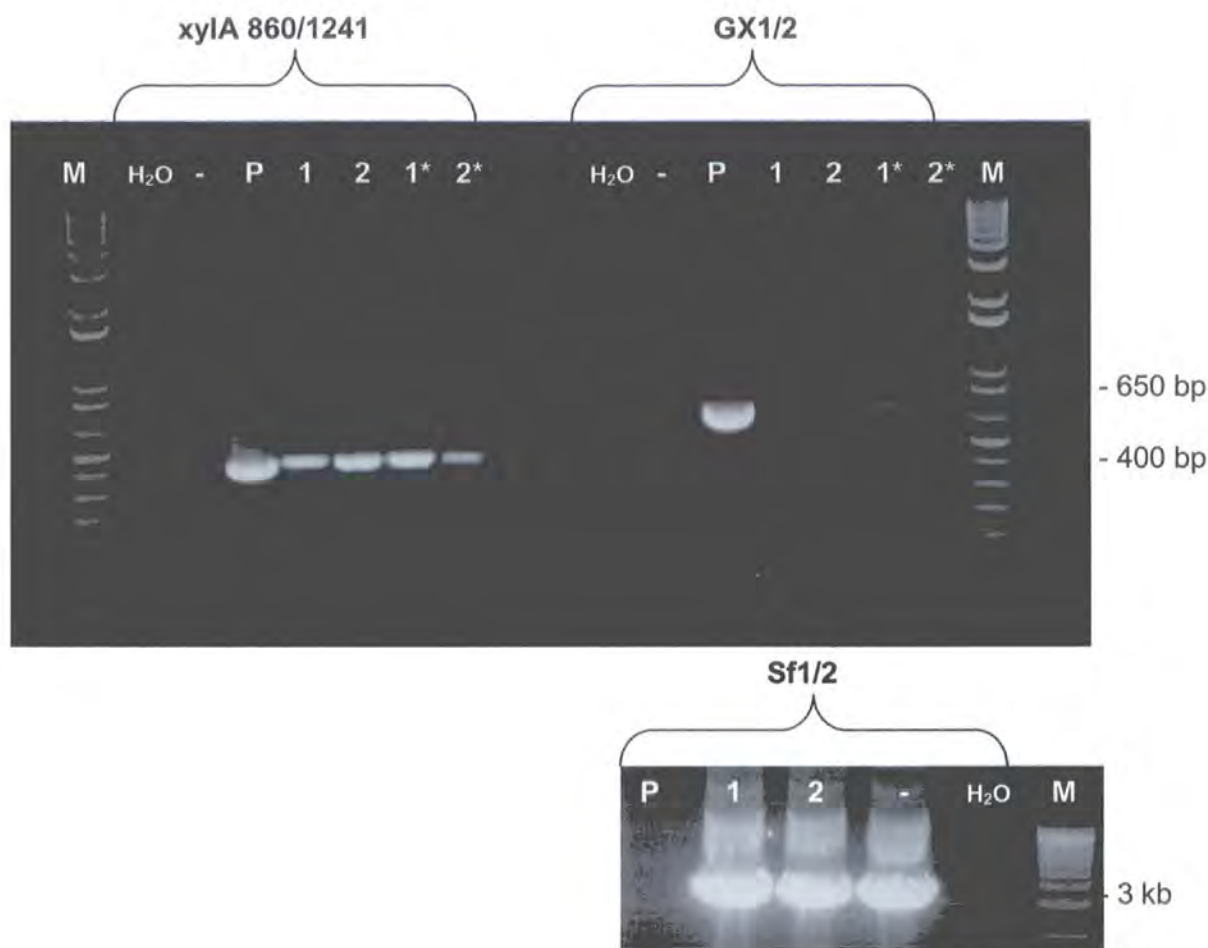
Figure 6.16 PCR analysis of putative transplastomic strawberry transformed with pFavXG

PCR analysis of RI strawberry lines Sx1 (1) and Sx2 (2) bombarded with the vector pFavXG. Asterisks denote 1:100 dilutions of DNA template used in PCR for the respective lines. pFavXG plasmid DNA (P), water (H_2O) and non-transformed plant DNA (-) served as controls in each reaction. An amplification product of 381 bp generated from the primer pair xylA 860/ 1241 confirms the presence of the *xylA* gene in both lines. An amplification product of 650 bp generated from the primer pair GX1/2 confirmed the presence of the *xylA* gene linked to the *smGFP* gene in both lines. An amplification product of approximately 3.5 kb generated from primer pair Sf1/2 in lines Sx1 (1) and Sx2 (2) as well as from non-transformed strawberry DNA is indicative of wild type plastome amplification, an amplification product of ~6 kb, indicative of integration of the pFavXG transcription unit into the strawberry plastome, was not clearly identifiable.

6.4 Concluding remarks and discussion

In summary plastid transformation of strawberry was achieved using both the vectors pFavAG and pFavXG created in this study to enable antibiotic and non-antibiotic selection, respectively. Plastid transformed line Sa1 containing the *aadA: smGFP* transcription unit was generated from experiment FavAG 1. Plastid transformed lines Sx1 and Sx2 containing the *xylA: smGFP* transcription unit were generated from experiment pFavXG 1. PCR confirmed the presence of the transcription unit in all three lines, although plastome integration was not confirmed. GFP fluorescence visualised specifically in chloroplasts of line Sa1, Sx1 and Sx2 confirmed plastid transformation and functional expression of the *aadA/ xylA: smGFP* transcription units. Additionally six putative plastid transformed green callus lines were obtained using the vector pFavAG. GFP fluorescence was visualised in the callus lines indicating that they were plastid transformed. Furthermore, two plastid transformed tobacco lines containing the *aadA: smGFP* transcription unit were generated from experiment NtAG 1.

Chapter 7

General discussion

7.1 Isolation and characterisation of the strawberry root-specific gene FavRB7 and its promoter

In chapter three the identification and isolation of a novel strawberry root specific gene, FavRB7, and its promoter was described. The approach taken to identify a root-specific gene was to identify well-characterised root-specific genes in other species and to use their sequence information to isolate a homologue from strawberry. The gene sequence was then used to isolate the gene's promoter. In chapter four a nuclear transformation vector linking the FavRB7 gene promoter to the gusA reporter gene was described and the transgene expression pattern conferred by the FavRB7 gene promoter in transgenic strawberry and tobacco determined.

7.1.1 Characterisation of the FavRB7 gene

A 513 bp strawberry RB7 TIP homologue named FavRB7 was identified using degenerate primers in PCR with strawberry root cDNA as template. The FavRB7 nucleotide sequence was subject to comparison with other RB7 TIP homologues and found to be between 75.8% and 79.1% homologous to the Asteridae RB7 TIPs, with highest similarity to the sunflower RB7 TIP (Sarda et al., 1999). Conserved N-termini $(Mx_{2-5}Gx_3[D/E]x_{6-7}[R/K])$ and loop B $(NPAV[T/.][L/F]x_3GGx[V/I][S/T])$ amino acid motifs have been cited as characteristic of plant TIPs (Schaffner, 1998). Sequence homology analysis carried out using the predicted amino acid sequence of FavRB7 and RB7 TIP homologues from parsley, carrot, sunflower and tobacco identified highly conserved N-termini and loop B amino acid motifs in all five sequences. However, divergence was observed elsewhere in the coding regions of the five homologues studied.

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The membrane intrinsic protein family TIP and PIP subfamilies have been classified using conserved N and C termini and loops B and E amino acid sequence motifs (Schaffner, 1998). Loops B and E are partially hydrophobic and it is thought that interactions between intracellular loop B and extracellular loop E within a tetrameric TIP aggregate lead to the formation of a water channel unit which is penetrated by water in both directions (Jung *et al.*, 1994). Whilst the specific sequence elements required to maintain TIP function remain conserved between different plant species it is likely that less critical sequence elements have been subject to alteration as plant families have diverged resulting in the heterogeneity observed in this study.

Time constrains did not allow for the isolation of the entire *FavRB7* coding region. The 513 bp *FavRB7* gene sequence isolated represents the 5' end of the gene and the *FavRB7* loop E and C termini motifs were not isolated. The coding region in the majority of RB7 TIP homologues is between 738 bp and 747 bp in length. Thus, it is likely that the *FavRB7* gene sequence isolated represents ~ 70% of the total gene length. The presence of highly conserved N termini and loop B motifs and homology of over 75% at the nucleotide level between *FavRB7* and other RB7 TIPs endorse the assertion that *FavRB7* represents a strawberry RB7 TIP.

The tissue distribution of *FavRB7* gene expression was assessed using RT-PCR and northern analysis. RT-PCR, using primers specific to the *FavRB7* gene and cDNA synthesised from mRNA extracted from strawberry floral organs, leaf, petioles, crown, root and fruit tissues, produced a strong amplification product from only the root tissue sample. RT-PCR carried out using ubiquitin primers for the same tissue types confirmed that intact mRNA had been extracted and cDNA synthesised from all tissues. This result strongly suggested that the *FavRB7* gene is expressed in only root tissues of strawberry.

Northern analysis carried out using total RNA extracted from strawberry root, leaf, floral organ, fruit and petiole tissues and hybridisation with a DIG-labelled *FavRB7* gene specific probe revealed a strongly hybridising band in the root RNA and also a much weaker band in the petiole RNA. During the northern analysis a much greater

quantity of RNA was inadvertently loaded for the petiole sample in relation to the other tissue types studied. The weakly hybridising band in the petiole sample may indicate that whilst expressed strongly in the root tissues, *FavRB7* may also be expressed to a much lesser extent in petioles. This result is anomalous to that observed with RT-PCR. Since the relative amount of gene expression is clearly less in the petioles than in the root, it is conceivable that the lack of an amplified fragment in the petiole sample in the RT-PCR could be due to operation below the sensitivity of the assay, with respect to the amount of template used. On the basis of these observations it was clear that the *FavRB7* gene is expressed almost exclusively in the roots, in a similar manner to RB7 TIPs found in tobacco (Conkling *et al.*, 1990; Yamamoto *et al.*, 1991), parsley (Roussel *et al.*, 1997), sunflower (Sarda *et al.*, 1999), Arabidopsis (Yamamoto *et al.*, 1990), carrot (Kawahara *et al.*, 1997) and potato (Heinrich *et al.*, 1996). The root-specific expression pattern observed for *FavRB7* adds further support to the assertion that this gene represents a root-specific RB7 TIP. Accordingly it was decided to isolate the regulatory sequence of this gene to test its efficiency at directing root-specific transgene expression.

To further characterise the *FavRB7* gene in strawberry Southern analysis was carried out using genomic DNA from three species of strawberry, *F. x ananassa* (octaploid), *F. moschata* (hexaploid) and *F.vesca* (diploid). It was not expected to see fragments representing gene numbers increasing proportionally with ploidy because of the complex make-up of polyploid strawberry. It has been proposed that significant introgressive gene flow from the diploids into the octaploids has occurred during the course of evolution in the octaploid progenitors of commercial strawberry; *F. virginiana* and *F. chiloensis* (Hancock, 1999). Naturally occurring pentaploid, hexaploid, enneaploid and decaploid progeny have been documented from crosses between *F. chiloensis* and *F. vesca* with varying combinations of reduced and unreduced gametes (Bringhurst, 1990). The proposed genome formula for octaploid strawberry is AAA'A'BBB'B' representing strict disomic inheritance of unreduced gametes from four separate diploid progenitors (Bringhurst, 1990).

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It is likely that the multiple diploids in the background of commercial strawberry and chromosomal divergence may have lead to the under representation of gene copies in relation to ploidy in the octaploid. As expected a complex pattern of hybridisation was observed in the hexaploid and octaploid species analysed. In addition to the complex make-up of polyploid strawberry, restriction fragment length polymorphisms surrounding the *FavRB7* gene on different chromosomes may also have contributed to the complex integration pattern observed. However, on the basis of Southern analysis in the diploid *F.vesca,* it seems likely that *FavRB7* is a member of a two to three member gene family in strawberry.

7.1.2 Characterisation of the *FavRB7* gene promoter

The 5' regulatory sequence associated with the *FavRB7* gene was isolated using the PCR-based GenomeWalker ™ to 'walk' upstream from the gene into the promoter region. Initial amplification products of approximately 800 bp were generated and sequenced. These products consisted of the first ~100 bp of *FavRB7* gene coding sequence and approximately 700 bp, which included the 5'UTR and some promoter sequence. In the promoter of the tobacco root-specific *TobRB7* gene it was concluded that the sequence elements required for root-specific gene expression were between positions -636 to -299 (Yamamoto *et al.,* 1991). However, several other sequence elements were identified in the *TobRB7* gene promoter at positions as distal as -1726 from the initiation codon (Yamamoto *et al.,* 1991). It was decided that 700 bp of promoter sequence associated with the *FavRB7* gene may be insufficient to confer root specificity. This necessitated further optimisation of the GenomeWalker ™ procedure to generate larger amplification products. Amplification products of approximately 1.8 kb, 2 kb and 3 kb were generated from three separate libraries. Several clones of each amplification product were analysed by restriction endonuclease digestion (data not shown) and the maps generated were comparable in all clones. Thus, it was concluded that all genome walker reactions generating promoter fragments had amplified the same target DNA. Sequence analysis of the 3 kb fragment showed that it comprised of

2843 bp promoter region linked to the 5'UTR and 96 bp of *FavRB7* gene sequence. The first 700 bp of the promoter sequence were also found to be identical to the smaller promoter fragment previously sequenced.

Southern analysis revealed the potential for a further gene with close homology to *FavRB7* existing in strawberry. Two clones sequenced during the isolation of the *FavRB7* gene were found to be identical. However, divergence between potential homologues may occur towards the 3' of the gene which was not isolated and hence not characterised, thus the possibility exists that the promoter of a homologous gene was isolated. However, this scenario seems unlikely since several promoter fragments were generated using the isolated *FavRB7* gene sequence and there were no disparities between the restriction digest maps generated from these.

Several regions of repetitive sequence were identified in the 2843 bp *FavRB7* promoter. A region of concentrated repeats was observed between positions -1771 to -1831 with corresponding sequences at -2429 and -1416 suggesting potential concatenation of the DNA in this region. Several putative regulatory motifs were also identified within the promoter sequence many of which lie within repetitive sequence. The regulatory motifs may function in response to specific stimuli and affect *FavRB7* promoter activity. However, their significance as regulatory regions in the *FavRB7* promoter remains hypothetical until further investigation of the effects of specific stimuli and mutation analysis of the motifs can be carried out.

Sequence homology analysis between the *FavRB7* and *TobRB7* promoters revealed only 23% overall homology between the two sequences at the nucleotide level. Comparisons between the *FavRB7* and *TobRB7* gene promoters revealed the presence of several sequence motifs common to the two, found in similar relative positions. The lack of overall sequence conservation between the *FavRB7* and *TobRB7* promoters may reflect physiological as well as phylogenetic distinctions between the two species. Strawberry is a member of the *Roseacae* and is a relatively slow growing, herbaceous, temperate perennial whereas tobacco is a member of the *Solanacae* and a fast growing, sub-tropic annual. It seems likely that the two

root-specific RB7 TIPs have similar functional roles and that conserved sequence motifs may facilitate the binding of conserved transcription factors to contribute towards this function. However, it is also likely that the presence and availability of species-specific transcription factors associated with different physiological water transport requirements and processes in the two species also influences the context in which transcription of the two RB7 TIPs occurs.

7.1.3 Characterisation of *FavRB7* promoter activity

The ability of the *FavRB7* promoter to confer root-specific transgene expression was assessed in strawberry and in a heterologous plant system, tobacco. The approach taken was to create a binary vector, pSCVFavRB7, containing the *FavRB7* promoter linked to the *gusA* reporter gene and to use this vector to produce transgenic strawberry and tobacco. The entire promoter 2843 bp sequence isolated was incorporated in the vector to ensure that all of the regulatory elements required to direct root-specific transgene expression were included. The binary vector pSCV1.6 containing the *gusA* reporter gene and the *nptII* selectable marker genes under control of constitutive CaMV 35S promoters served as a control in these investigations.

PCR, using forward and reverse primers to anneal in the *FavRB7* promoter and the *gusA* gene, respectively, confirmed the presence of the *FavRB7* gene promoter linked to the *gusA* gene in 32 strawberry and 19 tobacco phenotypically normal transgenic lines. Southern analysis of the 32 strawberry lines showed that the majority of the transgenic lines harboured the *gusA* and *nptII* genes integrated at single sites. However, seven strawberry lines were found to contain multiple copies of both genes and the integrity of the *FavRB7* gene promoter:*gusA* region was compromised in lines 41, 53a, 53b, 61, 67, 72 and 78. Multiple gene insertions resulting from *Agrobacterium*-mediated transformation are not uncommon (Jorgensen *et al.*, 1987; Hobbs *et al.*, 1993) and have been observed before in transgenic strawberry (A. Massiah and S. Vaughan, unpublished data). Of the seven lines likely to contain some form of deletion or rearrangement within the *FavRB7* gene promoter:*gusA* region

only line 61 contained a single copy of the *gusA* gene. In all of the other lines at least one intact copy of the *FavRB7* gene promoter:*gusA* region was also present. The activity of the *FavRB7* promoter was assessed through activity of the *gusA* gene linked to the promoter. All transgenic lines, both strawberry and tobacco were subject to histochemical assay. However due to time constrains only the strawberry lines were subject to fluorometric and molecular analysis.

A β-glucuronidase (X-Gluc) histochemical assay was carried out to assess expression of the *gusA* gene in a range of tissue types in the pSCVFavRB7 transgenic strawberry and tobacco lines. Several different tissue types were assayed for both species, however floral organ and fruit tissues were not available for all of the strawberry transgenic lines produced.

The pSCVFavRB7 transgenic strawberry tissues available for histochemical analysis were leaves, floral organs, petioles and roots. Blue staining indicating *gusA* activity and thus *FavRB7* promoter activity, was not observed in the leaves or floral organs of any of the lines. Faint blue staining was observed in the petioles of several of the *FavRB7* gene promoter lines, although not to the intensity of the CaMV 35S line, suggesting that *gusA* is also expressed to a lesser degree in the petioles of some *FavRB7* gene promoter lines. This result is in accordance with that of the northern analysis where native *FavRB7* gene expression was detected in petiole RNA. Intense blue staining was observed in the roots at similar levels to that observed in the constitutive CaMV 35S control in all 32 pSCVFavRB7 transgenic lines.

Floral initiation in commercial strawberry is affected by two main factors, photoperiod and temperature. Commercial strawberry cultivars can be classified as day-neutral or short-day depending on their photoperiodic requirements for flower bud initiation (Hancock *et al.*, 2002). Most cultivars require moderate temperatures for floral initiation to occur. The cultivar Calypso used in this study is reportedly weakly day-neutral, hence floral initiation occurs regardless of day length under moderate temperatures (Hancock, 1999). All transgenic strawberry lines were subject to six weeks under a 16 hr day, with a day temperature of 20°C and a night temperature of

14°C to promote floral bud initiation. Despite this treatment, not all lines flowered during the study period. The absence or lateness of flowers in many lines meant that it was not possible to assay *gusA* expression in fruit within the study period. However, fruit from two lines was subjected to histochemical assay (data not shown) and no blue staining was detected. This suggested that the *FavRB7* gene promoter is not active in strawberry fruits. However, analysis of fruit from several lines will be required for this to be confirmed.

The data provided suggest that the *FavRB7* promoter is able to confer high levels of transgene expression in a near root-specific manner in strawberry. A pSCVFavRB7 strawberry R1 population generated through stolon propagation was also subject to histochemical analysis. The expression pattern observed in the R0 lines was maintained in the R1 lines indicating that the near root-specific transgene expression conferred by the *FavRB7* promoter was stable through vegetative propagation. This is of significance for the commercial application of this technology as vegetative propagation is widely used to generate clonal stocks of elite strawberry selections for commercial purposes.

Microscopy revealed that *gusA* activity in the roots of the pSCVFavRB7 strawberry lines was highest in the central vascular region of the root and in the tip of the lateral root cap. This expression pattern was similar to that observed in studies using the *TobRB7* promoter in tobacco where *gusA* activity was localised to the root meristem and the immature central cylinder and absent from older root tissues (Yamamoto *et al.*, 1991). Yamamoto *et al.* (1991) proposed that *TobRB7* may be required in early root development. In contrast, histochemical visualisation revealed that *gusA* activity, under control of the *FavRB7* promoter, was present in the central cylinder of old as well as young root tissues in strawberry. The apparent difference in promoter activity between *FavRB7* and *TobRB7* in older root tissues may either be due to active *gusA* transcription or to strong initial expression of *gusA* resulting in high levels of *gusA* gene product persisting in older roots.

Histochemical analysis carried out in pSCVFavRB7 transgenic tobacco revealed strong *gusA* activity in the leaves, floral organs, petioles and roots of 17 of the 19 pSCVFavRB7 lines examined. Lines 35 and 68 survived *in vitro* kanamycin selection. However, both lines were PCR negative to verify the presence of the *FavRB7* promoter:*gusA* gene region. It is possible that these lines contain only a partial fragment of the pSCVFavRB7 T-DNA including the *nptII* gene conferring resistance to kanamycin but lack the *FavRB7* promoter: *gusA* gene region. It is also possible that these lines may be escapes and contain neither transgene. Seedling progeny, from selfing, of 8 of the tobacco pSCVFavRB7 lines were also subjected to histochemical analysis (data not shown). The *gusA* constitutive expression pattern observed in the parent lines was maintained in the T1 lines indicating stable transgene transmission and promoter activity in the sexual generation.

Histochemical analysis revealed a clear difference in *FavRB7* promoter activity in strawberry compared to tobacco. In strawberry strong root-preferential activity is displayed whereas in tobacco activity of the promoter is constitutive. This finding most likely reflects the interaction and availability of species-specific transcription factors that influence transcriptional processes in the two species. As discussed previously transgene copy number and positional effects may influence promoter activity. However, comparable *gusA* expression patterns were observed in 32 independent strawberry lines and 17 independent tobacco lines suggesting that promoter activity was the main factor influencing the expression patterns observed.

There are several disadvantages associated with the X-Gluc histochemical assay. Firstly, when the *gusA* reporter gene is under control of a strong promoter staining can 'leak' into neighbouring cells leading to an overestimation of promoter activity (Hull and Devic, 1995). Secondly, at the qualitative level *gusA* expression may appear comparable between independent transgenic lines. However, this can mask quantitative differences. For this reason fluorometric analysis was also undertaken to provide a quantitative assessment of *FavRB7* promoter activity in the pSCVFavRB7 strawberry lines.

Specific activity of the *gusA* gene product β-glucuronidase was quantified in strawberry tissue samples, in terms of pmol 4-MU produced per mg total protein per min, using the fluorometric assay as described by Jefferson (1987). All 32 transgenic strawberry lines were subjected to fluorometric assay. The mean specific activity for *gusA* in roots under control of the *FavRB7* promoter was found to be 36,780 pmol 4-MU/min/mg, which was comparable to the CaMV 35S promoter *gusA* activity in roots of 40,482 pmol 4-MU/min/mg. By contrast mean *FavRB7* promoter *gusA* specific activities for petioles, leaves and floral organs were 16.8%, 0.65% and 0.59% respectively, of the CaMV 35S promoter values for each respective tissue. Fluorometric analysis of the 32 pSCVFavRB7 transgenic lines revealed a mean *gusA* specific activity ratio between root: petiole/ leaf/ floral organs of 1: 0.3/ 0.04/ 0.009 which corroborated the root-preferential expression pattern observed through histochemical assay. Southern analysis suggested that the integrity of the *FavRB7* gene promoter:*gusA* region was compromised in lines 41, 53a, 53b, 61, 67, 72 and 78. However, PCR analysis confirmed that at least 600 bp of the *FavRB7* gene promoter linked to the *gusA* gene was present in all of these lines. All of these lines displayed root-preferential *gusA* expression, with three lines in each of the upper and lower quartiles and line 61 displaying median *gusA* root expression. The maintenance of root-preferential expression in these lines may be accounted for either through the presence of additional intact T-DNA copies in these lines or, especially in the case of line 61 which contains only one compromised copy, through minimal rearrangements in the *FavRB7* promoter:*gusA* gene region.

Detailed examination of the *gusA* specific activities in individual lines revealed wide ranging *FavRB7* promoter activity across the same tissue type. There was no strong correlation between transgene copy number and levels of *gusA* expression in individual lines. Specific activities in the strawberry root tissues ranged from 3,518 to 154,500 pmol 4-MU/min/mg with the majority of *gusA* specific activities over 10000 pmol 4-MU/min/mg. The majority of *gusA* specific activities observed in petioles were beneath 10,000 pmol 4-MU/min/mg, however two lines displayed *gusA* specific

activities of over 30,000 pmol 4-MU/min/mg. By contrast the majority of *gusA* specific activities in leaves were beneath 900 pmol 4-MU/min/mg. The lowest specific *gusA* activity recorded in strawberry leaf tissue was 100 pmol 4-MU/min/mg which was comparable to the non-transformed control 'background' level of 52 pmol 4-MU/min/mg. Only 17 lines contributed to the data generated for *gusA* specific activity in floral organs, of these lines the majority exhibited specific activities beneath 160 pmol 4-MU/min/mg with a lowest specific activity of 18 pmol 4-MU/min/mg recorded in line 39. The 'background' non-transformed control level recorded in floral organs was 9 pmol 4-MU/min/mg. The data presented clearly demonstrate near root-specific transgene expression in the majority of the strawberry lines examined. Fluorometric analysis revealed a range of *FavRB7* promoter activity within each tissue type which was masked using the qualitative histochemical assay. This suggested that positional effects may have influenced promoter activity as transgene copy number was not correlated to *gusA* expression levels.

To corroborate the histochemical and fluorometric analyses, expression of the *nptII* and *gusA* genes was studied at the molecular level in both pSCVFavRB7 and pSCV1.6 lines of transgenic strawberry using multiplex non-quantitative RT-PCR to detect expression of *nptII* and *gusA* transgenes in the root, leaf, petiole, floral organs and fruit. Amplification fragments corresponding to the *nptII* and *gusA* genes were generated for all tissue types for all lines assessed indicating that both were active in all tissues. This corroborates the fluorometric observations which revealed a degree of activity in all tissues, albeit very low for many leaf and floral organ tissues.

An average *gusA* specific activity of 36,800 pmol 4-MU/min/mg conferred by *FavRB7* promoter activity in strawberry roots contrasts with studies in tobacco using the promoter of the *TobRB7* gene homologue to confer *gusA* root-specific expression. Initially *gusA* specific activities of between 100 and 530 pmol 4-MU/mg/min in tobacco roots were reported (Conkling *et al.*, 1990). A later *TobRB7* study (Yamamoto *et al.*, 1991) created a series of promoter deletions to further characterise the *cis*-acting elements responsible for root-specific expression. In this study average *gusA* specific

activities were between 160 and 2242 pmol 4-MU/mg/min in the roots and between 3 and 55.7 pmol 4-MU/mg/min in the leaves. Comparisons between *TobRB7* and the CaMV 35S promoter were not made in either tobacco study and *gusA* expression was not evaluated in stem, petiole or floral tissues. Therefore *gusA* expression levels conferred by the *TobRB7* promoter in tobacco were considerably lower than those conferred by the *FavRB7* promoter in strawberry. However, studies using the CaMV 35S promoter to drive *gusA* expression in apple using the same vector background reported expression levels approaching 180, 000 pmol 4-MU/mg/min in leaf tissues (Gittins *et al.*, 2001) which is comparable the CaMV 35S *gusA* values observed in strawberry leaves during this study.

Membrane intrinsic proteins (MIP) facilitate water movement through three main pathways; apoplastic, symplastic and transcellular (Chrispeels, 1994). TIPs are associated with the transcellular route and are typically located in zones of elongation and cell growth, where it is thought their role may simply be the facilitated uptake of water during cell expansion (Schaffner, 1998). However there are many different isoforms of MIP present in higher plants. Arabidopsis alone contains at least 23 expressed MIPs, 11 of which are TIPs of which seven are expressed in roots (Weig *et al.*, 1997). Several TIPs have also been identified in sunflower (*Helianthus annuus*) (Sarda *et al.*, 1997; Sarda *et al.*, 1999). *SunRB7* is a root-specific TIP and homologous to *FavRB7* and *TobRB7*, however at least four other TIPs were shown to accumulate in sunflower roots. It has been postulated that different TIPs respond to different environmental stimuli. Drought stress experiments carried out to investigate the accumulation of sunflower TIP transcripts in roots showed that of the five sunflower TIPs identified, transcript levels of two of these increased in response to drought (Sarda *et al.*, 1999). In Arabidopsis expression of *AtγTIP* and *AtδTIP* in the roots was dramatically reduced by the application of osmotic stress (Weig *et al.*, 1997). It has been concluded by several authors that the expression of plant TIPs appears to depend on the complex regulation of a large gene family (Chrispeels, 1994; Weig *et al.*, 1997; Schaffner, 1998; Sarda *et al.*, 1999).

The difference in *FavRB7* and *TobRB7* promoter strengths could be attributed to the functional role of the genes they drive. Most MIPs appear to be excluded from meristematic tissues (Schaffner, 1998), however the exception to this is *TobRB7* (Yamamoto *et al.*, 1991). It is possible that *FavRB7* has a more generic role in transcellular water transport in the root compared to *TobRB7*, hence *FavRB7* promoter activity in older root tissues. The role played by additional TIPs in water and solute transport processes in strawberry and tobacco has yet to be studied. Combinatorial effects of non-RB7 type TIP accumulation and expression in the two species may also influence the expression patterns observed in the tobacco and strawberry RB7 TIP homologues.

Genetic modification using the *FavRB7* promoter to confer high levels of near root-specific transgene expression in the roots of strawberry may go some way towards reducing concerns relating to ubiquitous transgene expression and the affect it may have on both the environment and consumers. However to address the issue of transgene containment in commercial strawberry this study also investigated the development of plastid transformation methodologies for strawberry.

7.2 Plastid transformation in strawberry and tobacco

In chapter five the construction of a novel series of vectors to enable plastid transformation of strawberry and tobacco was described. The vectors created were pFavAG and pNtAG designed to enable plastid transformation using spectinomycin selection in strawberry and tobacco, respectively, and pFavXG and pNtAG designed to enable plastid transformation using D-xylose selection in strawberry and tobacco, respectively. In chapter six the application of these vectors in strawberry and tobacco plastid transformation and the production of plastid transformed strawberry and tobacco lines were described.

7.2.1 Plastid transformation vector construction

The plastome region selected for transgene insertion in these studies was the intergenic region between the tRNA-Val (*trn*V) and ribosomal protein S12 (*rps*12) genes. This region was chosen as it lies in the inverted repeat of the plastome. Therefore transcription units introduced at this site would be present as two copies per plastome and transgene expression levels could thus potentially be increased. This region is also transcriptionally active and has been used successfully several times in tobacco and has proved successful for Arabidopsis and potato plastid transformation (McBride *et al.*, 1994; Sikdar *et al.*, 1998; Khan and Maliga, 1999; Sidorov *et al.*, 1999; Ye *et al.*, 2001).

Several other regions of the plastome have been targeted for transformation. Perhaps most notably the *rbcL – accD* intergenic region which has been used as the site for transgene insertion by several laboratories in recent years (Carrer and Maliga, 1995; Guda *et al.*, 1995; Staub and Maliga, 1995; Daniell *et al.*, 1998; Kota *et al.*, 1999; Iamtham and Day, 2000). The main reason this region was not selected for transgene targeting in this study was that the *trnV – rps12* region from strawberry was previously isolated in our laboratory and had proved amenable to transgene insertion in earlier studies. Recent studies in tomato plastid transformation revealed that transformation frequencies varied in relation to the plastome region targeted (Ruf *et al.*, 2001). The isolation and targeting of several different strawberry plastome regions was not feasible within the scope of this PhD study hence only the *trnV – rps12* region was targeted.

Two selectable marker gene systems were investigated; the *aadA* gene conferring resistance to spectinomycin and the *xylA* gene conferring the ability to convert D-xylose to D-xylulose. For both selection systems a soluble modified form of the jellyfish (*Aequorea victoria*) green fluorescent protein (*smGFP*) gene was introduced as a scorable marker to enable visual screening of transformed lines and assist in the selection of homoplasmic tissues. It was decided to express the selectable and scorable marker genes as an operon under control of a single promoter. Previously

the *aadA* and *smGFP* genes have been expressed as a 65 kDa fusion protein in tobacco plastids (Khan and Maliga, 1999). However a translational fusion of *xylA* and *smGFP* would result in a fusion protein of approximately 80 kDa. It was not known if the larger size or indeed the translational fusion itself would interfere with the correct functioning of the *xylA* gene. Hence, a di-cistronic unit incorporating termination codons downstream of the first gene and ribosome binding signals upstream of the second gene in the operon was created to ensure that both cistrons would be translated as separate units.

To increase the likelihood of the transcription unit functioning in strawberry novel regulatory elements were isolated from the strawberry plastome rather than using those already isolated from tobacco. The 16S rRNA (*rrn16*) promoter was selected for use in this study primarily because of the strong constitutive expression pattern it confers (Sriraman *et al.*, 1998). The *rrn16* promoter isolated from strawberry was found to be 90.5% homologous to the tobacco *rrn16* promoter. Putative -10 'TATA' and -35 'TTGCACG' core promoter elements and the rRNA upstream activator sequence (RUA) 'GTGGGA' present in the tobacco *rrn16* promoter were also present in that of strawberry. Mutagenesis of the -35 core promoter element in tobacco severely reduced transcription levels (Suzuki *et al.*, 2003). The level of homology observed between the two species is not surprising. Generally plastome genes and gene order is highly conserved between higher plant species (Stoebe *et al.*, 1998). The core sequence elements essential for transcription of the structural 16S ribosome subunit are also present in the *rrnB* P1 promoter of *E.coli* although the RUA sequence is not conserved (Ross *et al.*, 1993). The *rrn16* promoter RUA, -35 and -10 sequence elements present in strawberry and tobacco are also highly conserved between rice, maize, spinach, carrot and Arabidopsis (Suzuki *et al.*, 2003). The fundamental requirement for the structural 16S ribosome subunit and conservation of the regulatory elements suggests a shared mechanism for the regulation of rRNA transcription has been preserved throughout higher plant evolution.

The RuBisCo large subunit (*rbcL*) gene 5'UTR was isolated from strawberry to ensure that transcripts initiated from the *rrn16* promoter were translated. The strawberry *rbcL* 5'UTR was found to be 86.9% homologous to the tobacco *rbcL* 5'UTR and a putative Shine-Dalgarno element was also identifiable. The tobacco *rbcL* 5'UTR has been used in conjunction with the *rrn16* promoter extensively for plastid transformation and has resulted in high levels of expression of several transgenes (McBride *et al.*, 1995; Khan and Maliga, 1999; Skarjinskaia *et al.*, 2003). In most studies a short, 20-30 bp, *rbcL* 5'UTR containing a putative Shine-Dalgarno (SD) 'GGAGG' sequence motif upstream of the transgene initiation codon has been used. Typically the sequence 'AGGAGGU' binds a complementary sequence on the 16S ribosomal subunit, helping to form a stable complex between the ribosome and mRNA (Stern *et al.*, 1997). Recent studies have shown that substitution of the *rbcL* 5'UTR with the bacteriophage gene 10 (G10L) leader sequence, which shows increased complementarity to the *rrn16* anti-SD can dramatically increase transgene expression levels (Ye *et al.*, 2001). The use of G10L in future strawberry plastid transformation vectors may well lead to enhanced expression levels.

To ensure that stable transcripts were produced from the introduced transcription units the strawberry photo-system II core protein gene (*psbA*) 3' terminator region (3'TR) was isolated from strawberry and introduced downstream of the *smGFP* termination codon. The *psbA* 3'TR was chosen primarily because it has been shown to help stabilise plastid transcripts in tobacco (Eibl *et al.*, 1999) and has been widely used in plastid transformation studies (Sikdar *et al.*, 1998; Sidorov *et al.*, 1999; Iamtham and Day, 2000; De Cosa *et al.*, 2001; Ruf *et al.*, 2001; Reddy *et al.*, 2002; Hou *et al.*, 2003; Skarjinskaia *et al.*, 2003).

The *psbA* 3'TR isolated from strawberry was found to be only 33% homologous at the nucleotide level to the corresponding region of the tobacco plastome. However, several characteristics commonly associated with efficient 3' mRNA formation were identified in the strawberry *psbA* 3'TR. An 'AATAAA' sequence motif associated with efficient mRNA 3' end formation was observed in the strawberry *psbA* 3'TR. An

inverted repeat of this motif was also observed in the surrounding sequence potentially allowing the formation of a stable stem-loop structure. Such sequences are a common feature of many plastid transcripts and appear to play a role in RNA 3'-end formation and stabilisation (Adams and Stern, 1990; Rott *et al.*, 1998). The strawberry *psbA* 3'TR was found to have a low G/C content (23.4%) which is again characteristic of gene terminator regions in plants (Hunt, 1994). It should be noted that an 'AATAAA' motif is not present in the tobacco *psbA* 3'TR and that the requirement of such sequences is not absolute and they are absent from many plant genes (Mogen *et al.*, 1990).

The translation of *psbA* is influenced by light which it has been suggested is mediated by nuclear encoded proteins binding to the 5'UTR of the *psbA* mRNA (Danon and Mayfield, 1991). Studies in tobacco have identified functional interactions taking place between the *psbA* 3'TR and the *psbA* 5'UTR (Staub and Maliga, 1994; Eibl *et al.*, 1999). It has been postulated that interaction between both transcript ends is a prerequisite for maximum but not basic level translation (Eibl *et al.*, 1999). Thus it can be seen that *psbA* translation is regulated by several factors. The apparent divergence between the tobacco and strawberry *psbA* 3'TRs may be in part explained through the different nuclear binding factors and 5' UTRs interacting with the 3'TR in the two species.

During the isolation of the strawberry *rbcL* 5'UTR and *psbA* 3'TR partial sequence information was obtained for the strawberry *rbcL*, *atpB*, *psbA* and *rpl2* genes. Sequence homology analysis revealed these strawberry genes to be 94.9%, 83.9%, 92.6% and 97.3% homologous, respectively, to comparable regions of the tobacco plastome. Additionally, the strawberry *trnH* gene, which lies between the *rpl2* and *psbA* genes, was identified during the isolation of the *psbA* 3'TR. Sequence homology analysis revealed the strawberry *trnH* gene to be 98.6% homologous at the nucleotide level to the tobacco, pea and rice *trnH* genes and identical to that of potato. These findings suggest that the conservation of gene structure and order observed between the plastomes of other higher plant species also apply to strawberry. This indicates that

a degenerate PCR approach could reliably be used to isolate further regions of the strawberry plastome.

GFP fluorescence was observed in *E.coli* harbouring the four plastid transformation vectors created for use in this study. Fluorescence observed in *E.coli* implies that transcription was successfully initiated from the strawberry *rrn16* promoter and that the strawberry *rbcL* 5'UTR was sufficient to initiate translation of the selectable and scorable marker gene operon in a prokaryotic system. Whilst GFP fluorescence indicates that the *smGFP* gene was translated, this assay does not determine whether the stop codons present in the first gene were recognised and hence that two independent translation products were produced with *de novo* ribosome entry rather than a translational fusion. Thus, the possibility of a fusion protein of *aadA:smGFP* or *xylA:smGFP* being translated could not be discounted. However, *smGFP* fluorescence in *E.coli* confirmed that functional transcription units using novel plastome regulatory elements from strawberry had been created in all four vectors.

7.2.2 Plastid transformation of strawberry and tobacco

a) Use of the *aadA* gene and spectinomycin selection

In chapter six the progress made towards achieving plastid transformation in strawberry and the development of a non-antibiotic selection system for strawberry and tobacco plastid transformation was described. Regeneration experiments carried out in strawberry on medium containing a range of spectinomycin concentrations revealed strawberry to be highly sensitive to spectinomycin. Spectinomycin concentrations of as little as 5 mg/l were sufficient to severely inhibit shoot regeneration and bleach the few shoots which were able to regenerate under this regime. By contrast, tobacco and Arabidopsis plastid transformation was carried out with spectinomycin levels of 500 mg/l in the regeneration media (Svab *et al.*, 1990; Sikdar *et al.*, 1998). Plastid transformation of potato used 40-400 mg/l spectinomycin (Sidorov *et al.*, 1999), tomato plastid transformants were selected using 300-500 mg/l spectinomycin (Ruf *et al.*,

2001) and *Lesquerella fendleri* using 400 mg/l spectinomycin (Skarjinskaia *et al.*, 2003).

A single strawberry plastid transformed shoot line containing the *aadA:smGFP* transcription unit and six putative plastid transformed green callus lines were generated from four experiments using the vector pFavAG and spectinomycin selection. The callus lines were not subject to molecular analysis due to time constraints. However significant levels of fluorescence were noted in these cultures using the stereo fluorescence module. Several parameters were assessed during the transformation process to optimise the bombardment process. The majority of the putative callus lines were produced from donor material bombarded adaxial side up, 7.5 cm from the firing platform. Experiments FavAG 2 and FavAG 3 were carried out in parallel, with pre-culture duration the only variable between the two. Four putative plastid transformed callus lines were generated from experiment FavAG 3 in which donor material was subjected to 2 d pre-culture. No lines were produced from experiment FavAG 2 in which pre-culture was only 1 d. The single confirmed plastid transformed shoot line was also produced from donor material bombarded adaxial side up at 7.5 cm from the firing platform. However, this line was only subjected to 1 d pre-culture. Although plastid transformation has not been confirmed in the callus lines at the molecular level and these lines remain recalcitrant in the callus phase it appears that bombardment of strawberry leaf tissue adaxial side up, at 7.5 cm from the firing platform, following 1-2 d pre-culture may increase the likelihood of gold/DNA integration into strawberry chloroplasts. In tobacco plastid transformation 1-2 d pre-culture under low light intensities to orientate chloroplasts towards the surface of the donor material is commonly practiced (J. Knapp, pers com.), however this is not discussed in the literature. Tobacco is typically bombarded abaxial side up (Svab and Maliga, 1993; Zoubenko *et al.*, 1994). In potato plastid transformation the use of an osmotic treatment was successfully incorporated into the transformation procedure (Sidorov *et al.*, 1999). It is perhaps significant that plastid transformed strawberry line Sa1 was generated from leaf material maintained on sterile filter paper prior to bombardment to induce mild

desiccation of the donor material. However, these inferences are hypothetical as the small number of putative transformants does not allow for statistical analysis.

Plastid transformation in the strawberry green shoot line Sa1 was confirmed by several analyses. Firstly, a streptomycin screen was carried out on RII leaf material which was shown to be resistant to up to 500 mg/l spectinomycin. Regeneration of green callus in the presence of 200 mg/l streptomycin indicated that resistance was conferred by the *aadA* gene as opposed to spontaneously acquired mutation of the 16S RNA (Fromm *et al.*, 1987; Dix and Kavanagh, 1995). It should be noted that no spontaneous mutation acquired spectinomycin resistance was observed in strawberry during this study or in a parallel project where 5 mg/l spectinomycin was used in the selection media and a large number of 'escapes' were produced. Furthermore, *smGFP* was successfully visualised in regenerating callus and specifically localised to the chloroplasts in leaf tissues of line Sa1. Cells containing fluorescing chloroplasts in line Sa1 appeared normal under bright field illumination (data not shown). This indicated that the *aadA:smGFP* transcription unit was successfully being transcribed in chloroplasts *in planta* under control of the strawberry plastid regulatory elements isolated during the course of this study. GFP was only clearly visualised in a small proportion of the cells examined in Sa1 RII leaf material. The small proportion of fluorescing chloroplasts observed may be a result of inadequate focusing through successive layers of the leaf. However, this observation may indicate that only a relatively small proportion of the chloroplasts contained transformed plastomes. Molecular analysis carried out in line Sa1 confirmed the presence of the *aadA* and *smGFP* genes in this line. However, PCR to confirm the integration of the transcription unit into the strawberry plastome failed to generate an amplification product indicative of plastome integration. This was unsurprising taking into account the small number of cells with fluorescing chloroplasts. However, GFP specifically visualised in chloroplasts in line Sa1 meant that nuclear insertion and expression of the *aadA:smGFP* transcription unit was improbable. A possible explanation for the failure to detect plastome integration in PCR is that primers annealed preferentially in the abundant wild

type plastome and that the smaller wild type amplification product was preferentially amplified. Thus, detection of the presence of a small population of transformed plastomes may have been obscured in this assay. A recent report has described the development of pulsed-field gel electrophoresis as a method for separating plastomes from gradient-purified plastid fractions to overcome the problems associated with PCR amplification from heteroplasmic DNA (Swiatek et al., 2003). In the future this method could be used to facilitate the confirmation of plastome integration in the lines generated in this study.

It seems likely that only a very small proportion of plastome copies or chloroplasts have been transformed in line Sa1. Further optimisation of PCR may enable the confirmation of plastome integration at the molecular level. However, further optimisation of the tissue culture regimes is also required to promote homoplasmy in this line. A fourth round of repeat regeneration was initiated from Sa1 RIII shoots, using spectinomycin at 500 mg/l and 5000 mg/l. Green callus and green shoots have regenerated on both levels of selection. However, the shoots produced were not large enough to be analysed within the timeframe of this study.

It has been postulated that a dominant selectable marker gene such as aadA in plastid transformation may provide cross protection to non-transformed plastids in a heteroplasmic population, thus reducing selection stringency and slowing progression towards homoplasmy (Dix and Kavanagh, 1995). Achieving homoplasmy using the aadA gene and spectinomycin selection in plastid transformed lines of several other species has taken considerable lengths of time; eight months in petunia (Schaaf et al., 2000) and almost two years in tomato (Ruf et al., 2001). In rice (Khan and Maliga, 1999) and oilseed rape (Hou et al., 2003) heteroplasmic lines have been reported with homoplasmy not being reached, despite repeated cycles of selection. Thus the heteroplasmy observed in the strawberry line Sa1 is not without precedent.

In parallel with the strawberry plastid transformation studies, tobacco plastid transformation experiments with the vector pNtAG and spectinomycin selection were also undertaken. Ten putative transformed tobacco lines were generated during this

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study. Streptomycin sensitivity revealed spontaneous mutation acquired resistance to spectinomycin in 80% of these lines. This result is in accordance with levels of spontaneous mutation observed in previous plastid transformation studies of between 60 % (Daniell *et al.*, 2001) and 90 % (Eibl *et al.*, 1999; Sidorov *et al.*, 1999). Intense fluorescence observed in RII regenerating callus of both spectinomycin and streptomycin resistant lines was markedly increased in RIV tissues, indicating that progression towards homoplasmy of transformed plastome copies was occurring. However, florescence was not observed in the leaf tissue of either line at any stage in the repeat regeneration process. A possible explanation for this may be that the strawberry plastome regulatory elements conferring expression of the transgenes are functional in tobacco proplastids in callus but not in tobacco chloroplasts. However transcription and translation of the *aadA* and *smGFP* genes would need to be assessed at the molecular level in the two tissue types using northern and western analysis, respectively, to confirm this hypothesis. PCR analysis confirmed the presence of the *aadA* and *smGFP* genes in both tobacco lines, however plastome integration could not be confirmed using PCR.

Southern analysis carried out using gDNA isolated from strawberry line Sa1 and tobacco lines Ta9 and Ta10 did not detect restriction fragment lengths representing the anticipated pattern of plastome integration. This may be a result of mutation of restriction enzyme recognition sequences during integration or of some manner of rearrangement occurring during homologous recombination. However, a fragment of the same size was identified in two independent tobacco lines suggesting that *aadA* integration had occurred at a similar site in the genomes of two separate lines. Furthermore, a probe designed to anneal in the plastome hybridised to the same restriction fragment suggesting that the *aadA* gene was incorporated into the tobacco plastome. Hybridisation of the *aadA* and plast probes was more intense in the RIV clone of tobacco line Ta9 when compared to RII material of the same line, suggesting that repeated cycles of selection may be promoting an increase the *aadA* gene copy number. These factors suggest integration of the *aadA:smGFP* transcription unit into

the tobacco plastome rather than the nuclear genome. The *aadA* probe did not hybridise to the same restriction fragment as the plast probe in DNA extracted from strawberry line Sa1. However, a weakly hybridising band at approximately 5 kb in two clones of line Sa1 may indicate plastome integration of the transcription unit. It may be that the number of transformed plastome copies in the Sa1 RII material was below the sensitivity of this assay.

As described for strawberry, increased selection during repeat regeneration cycles has been initiated for the two pNtAG tobacco lines and shoots are currently being regenerated on medium containing 5000 mg/l spectinomycin. Several authors report achieving homoplasmy in tobacco plastid transformation within two to four cycles of regeneration (Svab and Maliga, 1993; Maliga, 1999; Bock, 2001). Other reports have suggested that reaching homoplasmy in tobacco is not always a matter of routine. Reed *et al.* (2001) reported that in one tobacco plastid transformation experiment phenotypically normal tobacco shoots were never obtained although they were able to select for 'shooty' callus in which >99% of plastomes were transformed following two years of culture on spectinomycin containing medium. In a second experiment Reed *et al.* (2001) were able to regenerate phenotypically normal shoots, however after repeated selection for one year approximately two-thirds of the plastome copies remained non-transformed. Subsequent selfing of these lines and germination of seed followed by cotyledon regeneration, both on medium containing 5000 mg/l spectinomycin, resulted in the production of phenotypically normal lines with >99% transformed plastomes. This strategy could also be applied to lines Ta9 and Ta10 to achieve progression towards homoplasmy.

b) Use of the *xylA* gene and D-xylose selection

Alongside developing plastid transformation in strawberry using *aadA* and antibiotic selection, a non-antibiotic selection system utilising the *xylA* gene and D-xylose selection for plastid transformation was also investigated. Regeneration experiments to determine the optimal concentration of D-xylose to use as a selective

agent for strawberry revealed that under standard light regimes the percentage of explants regenerating did not always correlate to the proportion of D-xylose in the media and that strawberry crowns cultured on 100% D-xylose were able to proliferate. However, shoot regeneration rates inversely proportional to the amount of D-xylose in the medium were observed when regeneration was carried out under dark growth conditions. This result suggested that photosynthesis may provide a means by which explants can bypass their dependence on the carbohydrate source supplied in the medium. Growth of cultures in the dark promoted vitrification of shoots, many of which were unable to withstand transfer to proliferation media and culture under standard light regimes. It was therefore decided to culture strawberry explants under low level light intensities rather than dark growth conditions on glucose based media with D-xylose accounting for 96.6% of the carbohydrate source. D-xylose selection in nuclear transformation has been imposed by levels of D-xylose accounting for 33.3%, 80%, and 100% of the total carbohydrate source supplied for potato, tobacco and tomato explants, respectively (Haldrup et al., 1998b). Thus, it can be seen that determination of species-specific D-xylose selection levels is crucial in the application of this technology. Plastid transformation experiments carried out in tobacco using the vector pNtAG were initially carried out with D-xylose accounting for 80% of the carbohydrate supplied. This resulted in the production of excessive amounts of regenerating shoots, at rates comparable to regeneration in the absence of selection. This observation most likely reflects the different selective requirements of nuclear and plastid transformation. In nuclear transformation D-xylose selection favours the regeneration of transformed lines whilst allowing non-transformed tissues to grow at a reduced rate (Bojsen et al., 1994). Thus fast growing lines can be selected in favour of slow growing lines and slightly delayed regeneration and production of non-transformed shoots is irrelevant. In plastid transformation extended periods of tissue culture are required to achieve homoplasmy, thus all shoots arising have to be evaluated. Increased D-xylose selection stringency was achieved in tobacco through

dark growth conditions however this system was not optimised within the timeframe of this study.

Using D-xylose selection 110 putative plastid transformed strawberry lines and 43 putative plastid transformed tobacco lines, bombarded with the vectors pFavXG and pNtXG, respectively, were generated. PCR analysis revealed that two of the strawberry lines, Sx1 and Sx2, contained the *xylA* and *smGFP* genes. The transgenes were not detected in any of the tobacco lines analysed. It can be seen that D-xylose selection permitted a much greater number of escapes to survive the selection process compared to spectinomycin selection in both tobacco and strawberry. A xylose isomerase gene has been isolated from barley (Kristo *et al.*, 1996) and other reports suggest that the enzyme is expressed weakly in several plants species (Haldrup *et al.*, 1998a, b). Thus, it may be that that endogenous expression of xylose isomerase has contributed to the number of escapes observed in both tobacco and strawberry. The two *xylA:smGFP* strawberry lines were both produced from leaf material bombarded abaxial side up at a distance of 7.5 cm from the firing platform following 1 d of pre-culture. *SmGFP* was successfully visualised in regenerating callus and specifically localised to the chloroplasts in leaf tissues of both line Sx1 and Sx2. This indicated that the *xylA:smGFP* transcription unit was successfully being transcribed in chloroplasts *in planta*. However, as described for line Sa1, GFP was only clearly visualised in a small proportion of the cells examined in Sx1 and Sx2 RI leaf material. Again this may be either an artefact of the GFP visualisation method used or indicate that only a relatively small proportion of the chloroplasts contained transformed plastomes. As with line Sa1, PCR to confirm the integration of the transcription unit into the strawberry plastome in lines Sx1 and Sx2 failed to generate an amplification product indicative of plastome integration. Again it was thought that this may be the result of primer competition and wild type plastome abundance obscuring low amounts of plastome transformation. Whilst *smGFP* expression was specifically visualised in chloroplasts in lines Sx1 and Sx2 it was noted that the cells containing the fluorescing chloroplasts appeared grey and abnormal under bright field illumination. Furthermore, repeat regeneration cycles

initiated from these lines on medium containing D-xylose as the sole carbohydrate source have failed to produce shoots.

The use of xylose isomerase as a selectable marker gene for plastid transformation depends on the D-xylose substrate entering the plastid and the incorporation of D-xylulose into either the Calvin cycle or the pentose phosphate pathway. D-xylose is a monosaccharide pentose (aldose) sugar. The permeability of pea chloroplast membranes to three aldoses, D-xylose, L-arabinose and D-ribose, led to the postulation that a carrier exists to facilitate transport of these sugars (Wang and Nobel, 1971). Later studies demonstrated that several hexose and pentose sugars pass the inner membrane of spinach chloroplasts. The rate of D-xylose uptake was the highest of all sugars tested and D-xylose was also found to be a strong inhibitor of D-glucose uptake (Schafer and Heber, 1977). The order of uptake rate between several different hexose and pentose sugars and their relative inhibition of D-glucose was consistent, prompting the hypothesis that all the compounds were transported by the same carrier (Schafer and Heber, 1977). As a nuclear-encoded selective agent, xylose isomerase converts D-xylose into D-xylulose which is then phosphorylated by xylulokinase to xylulose-5-phosphate, which enters the pentose phosphate pathway. In plastid transformation the conversion of D-xylose to D-xylulose takes place within the plastid, hence D-xylulose is required to be phosphorylated within this organelle in order to enter the Calvin cycle. There is evidence that 1-deoxy-D-xylulose, which forms a precursor in the 1-deoxy-D-xylulose-5-phosophate pathway of isoprenoid biosynthesis in plastids is readily hydrolysed and phosphorylated in higher plants and algae (Lichtenhaler, 1999). Thus, in theory xylose isomerase should function as a selective agent within plastids. Indications that both glucose and maltose are exported from cauliflower bud amyloplasts during starch degradation (Neuhaus et al., 1995) and the observation that glucose uptake does not contribute to starch biosynthesis (Batz et al., 1994) have lead to the hypothesis that the function of the sugar transporters in plastids is that of export rather than import. Therefore, the possibility exists that D-xylulose

formed within a transformed plastid may be exported via facilitated diffusion and then be incorporated to the cytosolic pentose phosphate pathway.

The unhealthy appearance of fluorescing cells containing the *xylA: smGFP* transcription unit may indicate that either *xylA* gene product or D-xylulose accumulation in these cells has a toxic effect. Xylose isomerase also catalyses the isomerisation of D-glucose, D-allose, L-arabinose and L-rhamnose (Haldrup *et al.*, 2001). The inadvertent conversion of other sugars present in the plastid may have had an adverse effect. Inefficient phosphorylation, export or accumulation of D-xylulose may also be detrimental to the transformed cells. However, further analysis of the transgene expression levels and in these lines is required before such conclusions can be drawn. Sx1 and Sx2 explants did not produce shoots during repeat regeneration cycles. Hence, extended culture in the presence of D-xylose may also have adverse effects that were not observed in the preliminary regeneration experiments. An alternative mode of selection using the *xylA* gene may be to culture explants in the presence of L-rhamnose which *xylA* converts to L-rhamnulose (Korndorfer *et al.*, 2000). This method has been used successfully in potato nuclear transformation where D-xylose was found to be highly toxic (Haldrup *et al.*, 2001). In the future L-rhamnose can be investigated as a substrate for the further selection of lines Sx1 and Sx2 in an effort to achieve homoplasmy in these lines.

Further Southern analysis of both the *aadA* and *xylA* plastid transformed strawberry lines is required to confirm integration into the targeted region of the plastome at the molecular level. Northern analysis is also required to determine the nature of the transcripts produced *in planta*. Western analysis is also required to establish the levels of protein produced under control of the strawberry plastome regulatory elements in both tobacco and strawberry once homoplasmy is achieved.

There are several additional factors which may have resulted in the high level of heteroplasmy observed in both the *aadA* and *xylA* strawberry lines produced. Ploidy has been shown to have influence on plastome number and segregation, in tobacco haploid lines were found to have lower chloroplast densities and produce higher

number of plastomes with spectinomycin resistance mutation when selected with spectinomycin compared to diploid lines (Timmons and Dix, 1990). Increased efficiency of sorting wild type and mutated plastomes in the haploid was suggested as an explanation for the higher number of mutant plastomes observed. Strawberry is an octaploid, thus it may be expected that chloroplast densities will be high and therefore plastome sorting relatively inefficient. This may in part explain the high degree of heteroplasmy observed. The physiology of strawberry may also have contributed to the low levels of plastid transformation observed using both selection systems. Strawberry meristems lie within a central shortened stem, which often sits proud of the medium *in vitro*. Thus, selective agents are required to be transported through several whorls of petiole tissue before the developing leaf is exposed to selection. It may be that the physiology of strawberry thus reduces the stringency of section influencing plastome segregation in the developing meristem, thus maintaining heteroplasmy. For future studies several alternative culture methods should be investigated in an attempt to achieve homoplasmy. Preliminary investigations have shown that strawberry cultures can be induced to proliferate under static double phase media or in agitated liquid culture (data not shown). It is postulated that liquid application of selective agents may provide better coverage and penetration to developing leaves and crowns thus imposing more stringent selection. Meristem culture or isolated protoplast culture in the presence of high levels of spectinomycin could also be attempted to further increase selection stringency.

In tobacco, proplastid density and the number of plastomes per proplastid were found to be lower than those of chloroplasts (Sakai *et al.*, 1998). Thus, inducing a callus phase and culture with selection should increase selection stringency and the chances of reaching homoplasmy. The development of media to extend the callus phase prior to shoot regeneration in strawberry may also offer an improvement to the plastid transformation procedures developed within this study.

7.3 Future work and applications

The characterisation of the strong near root-specific *FavRB7* gene promoter described in this study provides a basis from which transgenes may be expressed almost exclusively in the roots of strawberry. There are several benefits to localising transgene expression in the roots of crop species. Restricting transgene expression to the roots of the plant may go some way to satisfying regulations regarding food safety and to alleviate public concerns over the consumption of genetically modified food. Additionally, as discussed there are many insect and fungal pathogens posing agronomically important threats which specifically target the roots in strawberry. The use of the *FavRB7* promoter to confer expression of, for example, Bt toxins, proteinase inhibitors or plant lectins may impart resistance to some of these insect predators. Expression of an endochitinase, antimicrobial peptide or ribosome-inactivating protein under control of the *FavRB7* promoter has the potential to confer resistance to some of the many fungal pathogens of strawberry.

A further application of the *FavRB7* promoter may be that of rhizosecretion, that is the secretion of molecules from roots into the rhizosphere. Rhizosecretion can be used as a method for phytoremediation. For example expression of mercury ion reductase (*merA*) and organomercurial lyase (*merB*) under control of the *FavRB7* promoter could improve the ability of roots to detoxify mercury in the soil. Rhizosecretion may also be used to produce recombinant proteins using hydroponics. Root-specific transgene expression using the *FavRB7* promoter may reduce the metabolic drain of producing compounds in tissues where they are not required and may also increase recombinant protein yields.

Further studies could be undertaken to further characterise the *cis*-acting regulatory elements responsible for the root-specific activity of the *FavRB7* promoter. This could be achieved through the construction of a series of transformation vectors, containing a succession of *FavRB7* promoter truncations to enable further elucidation of the *cis*-acting regulatory elements present in this promoter. Electrophoretic mobility

shift assays could also be used to establish the binding affinity between specific regions of the promoter and nuclear extracts from different tissues of both tobacco and strawberry. The expression pattern conferred by the *FavRB7* promoter should also be established in other heterologous species, particularly within the *Rosaceae*. Many important fruits such as apples, pears, plums, peaches, raspberries as well as strawberries belong to the *Rosaceae*. The *FavRB7* promoter may have significant broad applications if activity was found to be root-specific in other *Rosaceous* crops.

Further work also needs to be carried to quantitatively establish the levels of *gusA* expression in a range of tissues of the pSCVFavRB7 tobacco lines. It also needs to be established that the *FavRB7* promoter is active in regenerating tobacco callus tissues, thus enabling selectable marker gene expression during the transformation process. However, the histochemical data presented here suggests that the *FavRB7* promoter may confer strong constitutive expression in tobacco and thus provide a viable alternative to the CaMV 35S promoter. This may be desirable to allay fears over the use of a viral promoter when constitutive transgene expression is required.

The development of plastid transformation in strawberry described in this thesis is in its infancy. Whilst the work reported here represents a significant advance, the three confirmed plastid transformed lines presented remain heteroplasmic and further optimisation of the tissue culture regimes applied to these lines is required to attain homoplasmy. The series of plastid transformation vectors created during this study may have a generic application in *Rosaceous* crops. The conservation of plastome gene sequence and gene order in higher plants means that the strawberry plastome sequence surrounding the transcription units and the strawberry plastome regulatory elements therein are likely to function in other higher plant species, particularly within the *Rosaceae*. Thus, the pFav series of vectors could be utilised to enable plastid transformation in fruit crops such as apple, pear, plum, peach and raspberry.

In the future the effect of targeting several different regions of the strawberry plastome may increase transformation efficiencies (Ruf *et al.*, 2001). Another potential improvement may be to use a binding-type selectable marker gene. This selection

method has the potential to produce homoplasmic lines within a single regeneration cycle (Dix and Kavanagh, 1995). In the absence of spontaneous mutation acquired spectinomycin resistance in strawberry and genetic and physiological factors specifically influencing the selection of transformed plastome copies in strawberry this method may prove more applicable than the use of a dominant selectable marker gene such as *aadA*.

Once homoplasmy is achieved in strawberry, plastid transformation may be used to confer a number of important agronomic traits to this crop. High levels of insecticidal or antimicrobial protein expression in the leaves and roots of strawberry could provide a means of protection against the multitude of pathogens that affect this crop. The expression of osmo-protectants such as trehalose to confer drought tolerance to strawberry could further extend the range and season of strawberry crops. As a means of commodity enhancement plastid transformation would also enable the production of polymers, biopharmaceuticals, antibodies and edible vaccines in strawberry.

One of the drawbacks of plastid transformation is that plastids are present in most plant tissues in specialised forms. Hence, relatively high levels of transgene expression are commonly achieved throughout the plant, which is not always desirable. The two technologies developed here could be combined to enable root-specific plastid-localised transgene expression. The use of the bacterial T7 RNA promoter to drive plastid encoded transgenes in strawberry in conjunction with a nuclear encoded T7 RNA polymerase, linked to chloroplast targeting sequences under control of the *FavRB7* promoter has the potential to restrict plastid transgene expression to the roots.

As a rule, *Agrobacterium*-mediated nuclear transformation using constitutive promoters to confer transgene expression has thus far been employed in strawberry genetic modification. The findings presented in this thesis represent a significant advancement and refinement in the methods available for the genetic modification of strawberry, enabling targeted, regulated and contained transgene expression in this species.

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Appendix 1

Plant culture media

Abbreviations

24-D: 2,4-dichlorophenoxyacetic acid   **BAP**: 6-Benzyl-amino purine

IBA: Indole-3-butyric acid   **MS**: Murashige and skoog salts

NAA: 1-napthylacetic acid   **TDZ**: thiadiazuron

GA₃: Gibberellin 20-oxidase

Ingredients stated are amounts per litre,

Strawberry proliferation media			Strawberry rooting media	
	SC5	**SMI**	**R 13**	**R 37**
MS	4.4 g	4.4 g	4.4 g	2.2 g
Sucrose	30 g	30 g	30 g	30 g
BAP	0.5 mg	0.224 mg	-	-
GA₃	0.25 mg	0.1 mg	-	-
IBA	0.1 mg	0.104 mg	3 mg	-
pH	5.7	5.7	5.7	5.7
Agar	7.5 g	7.5 g	7.5 g	7.5 g

Strawberry regeneration media

	ZN102	**Z1**	**ZN105**	**ZD102**	**ZD103**	**ZD202**	**BN202**	**BN205**
MS	4.4 g	4.4 g	4.4g	4.4 g	4.4 g	4.4 g	4.4 g	4.4 g
Glucose	30 g	30 g	30g	30 g	30 g	30 g	30 g	30 g
TDZ	1 mg	1 mg	1 mg	1 mg	1 mg	2 mg	-	-
NAA	0.2 mg	-	0.5 mg	-	-	-	0.2 mg	0.5 mg
24D	-	-	-	0.2 mg	0.3 mg	0.2 mg	-	-
BAP	-	-	-	-	-	-	2 mg	2 mg
pH	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7
Gerite	2.5 g	2.5 g	2.5 g	2.5 g	2.5 g	2.5 g	2.5 g	2.5 g

D-xylose substitution media

In strawberry regeneration media varying proportions of the 30 g/ L glucose normally supplied were substituted with D-xylose during the non-antibiotic selection system studies. All other components were as previously described.

D-xylose as percentage of the total carbohydrate supplied

/ l	100	98.3	96.6	91.6	83.3	75	66	50	33	16	0
glucose	-	0.5 g	1 g	2.5 g	5 g	7.5 g	10 g	15 g	20 g	25 g	30 g
xylose	30 g	29.5 g	29 g	27.5 g	25 g	22.5 g	20 g	15 g	10 g	5 g	-

Tobacco proliferation media	Tobacco regeneration media
RM	**RMOP**
MS 4.4 g	4.4 g
Glucose 30 g	30 g
Thiamine 0.9 mg	0.9 mg
BAP -	1 mg
24D -	0.1 mg
pH 5.8	5.8
Agar 6 g	6 g

Appendix 2

Primers used during *FavRB7* gene and promoter studies

TobRB71A 5' TTGGYAGCRTYGGTGAYTCTTT 3'

TobRB71B 5' ATGGTKCCWAGTGAGCCCTT 3'

TobRB72A 5' AGCMAAYATHTCRGGTGGCCAT 3'

TobRB72B 5' AATGATCGMGCNGGRTTCATTGA 3'

FavRB7SS 5' TTATGCATGCAGTCGACGGCCCGG 3'

FavRB7N 5' TCTTCACCATGGTTTGATCACTGGAAAAT 3'

3'FavRB71 5' GAACCCGGCTGTCACTT 3'

3'FavRB72 5' CTAACCGGCATTTTCTACTGGA 3'

3'FavRB73 5' GCTCTGTTTGTGGGGGTCTC 3'

FavRB71 5' GTTTTGGCAGCGTTGGTGACTCT 3'

FavRB72 5' AAATGCCGGTTAGGATGGTGATGT 3'

FavRB73 5' GTGCCTAGTGAGCCCTTCTTG 3'

FavRB74 5' TCATCGCCACCCTTCTTTTC 3'

Ubq1 5' TGACCAGCAGCGACTTATTTTT 3'

Ubq2 5' GGGTACGTCCATCCTCCAACT 3'

FRB7GSP1 5' GCGACGGAGACCCCCACAAACAGAGCAAAT 3'

FRB7GSP2 5' AAGGGTGGCGATGAACTCAGCTAGGTAAGA 3'

M13 forward 5' GTAAAACGACGGCCAG 3'

M13 reverse 5' CAGGAAACAGCTATGAC 3'

FavPGUS1 5' CCGTCCTTGGGTCCATCCTCTAT 3'

FavPGUS2 5' ATCCGCATCACGCAGTTCAA 3'

NptIIA 5' GAGGCTATTCGGCTATGA

NptII B 5' ATCGGGAGCGGCGATACC 3'

GUS27 5' CCTGTAGAAACCCCAACCCGTG 3'

GUS392 5' CCCGGCAATAACATACGGCGTG 3'

Appendix 3

Primers used during plastid transformation studies.

RRN16SSP1 5'AAATTAATATTCGCCTGGAGTTCGCT 3'

RRN16 KPNSGF 5'ATTATGGTACCGCGATCGCTTTCCATTTTTATTTGC 3'

ATPB1 5'CGRCCYTKAACTACCAGAGCRTT 3'

ATPB2 5'CTGTATAAACGCDGGCGCAGATC 3'

RBCL1 5'GGTTGAGGAGTTACTCGGAATGC 3'

RBCL2 5'CGGTGGATGTGAAGAAGTAGACC 3'

PSBA1 5'CAAACTAGTCCCTTTGGTGTCTTT 3'

PSBA2 5'TRGCWCGATTRGAACCATACCTTG 3'

PSBA3 5'CAYGGTTATTTTGGYCGATTGAT 3'

PSBA4 5'GCGGYTCCCTATTYAGTGCTATG 3'

RPL2 1 5'CRGGTGCTGTAGCGAAACTGATT 3'

RPL2 2 5'AACAGTCGGACAAGTRGGDAATG 3'

PSBABGL 5'ACTGCAGATCTCCATCTATCAATGGATAAGGCTT 3'

PSBAECOSGF 5'TATAGAATTCGCGATCGATTACTTGTAAAAAAGAATACAACAT 3'

AADAPAG1 5'AAAATTCATGAGGGAAGCGGTGATCGCCG 3'

AADAXHO1 5'TTACTCGAGTTTCATTATTTGCCGACTA 3'

XYLAPAG1 5'TTAATCATGAATAAATATTTTGAGAACG 3'

XYLAXHO1 5'ATTATCTCGAGTTATTCTGCAAACAAAT 3'

SMGFPBGLAPA 5'TATAGGGCCCAGATCTTATTTGTATAGTTCATCCATG 3'

aadAF 5' TCCGCGCTGTAGAAGTCACCATTG 3'

aadAR 5' CCGGCAGGCGCTCCATTG 3'

GA1 5' ATTCCCTTAAGCTCGATCCTGTTGA 3'

GA2 5' GCTTACGTTGTCCCGCATTTG 3'

Sf1 5' TCCGCCTACACGTCTTGCTTTTACTGCT 3'

Sf2 5' CCCTGGGATTTGACGGCGGACTT 3'

aadA1 5' AGTGATATTGATTTGCTGGTTACG 3'

aadA2 5' GCGCGATTTTGCCGGTTACTGC 3'

gfp281 5' TACGTGCAGGAGAGAGGACCAT 3'

gfp715 5' TAGGGCCCAGATCTTATTTGTAT 3'

Plast1 5' CGTAGCTCGCTTCTTTTTGGGTGT 3'

Plast2 5' TATGAGTCTCTTCCTTTTTGCGTATG 3'

xylA860 5' ACGCCAGAATAAACGGTGTA 3'

xylA1241 5' AATCTGGCTGCGCTCTAATG 3'

GX1 5' TCCCGTCGTCCTTGAAAGAGATGGTC 3'

GX2 5' GCATTAGAGCGCAGCCAGATTGTC 3'

263

Appendix 4

Sequences isolated during construction of plastid transformation vectors

SEQ 1: 3179 bp excised from plasmid pStraw4.5. *Stu* I (5') and *Psi* I (3') are highlighted, bases removed during cloning are in lowercase. The internal *Bst1107*I site is also highlighted.

```
aggCCTTCTACCTCCACGCGGCATTGCTCCGTCAGGCTTTCGCCCATTGC      50
GGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT      100
CCCAGTGTGGCTGATCATCCTCTCGGACCAGCTACTGATCATCGCCTTGG      150
TAAGCTATTGCCTCACCAACTAGCTAATCAGACGCGAGCCCCTCCTCGGG      200
CGGATTCCTCCTTTTGCTCCTCAGCCTACGGGGTATTAGCAGCCGTTTCC      250
AGCTGTTGTTCCCCTCCCAAGGGCAGGTTCTTACGCGTTACTCACCCGTC      300
CGCCACTGGAAACACCACTTCCCGTCCGACTTGCATGTGTTAAGCATGCC      350
GCCAGCGTTCATCCTGAGCCAGGATCGAACTCTCCATGAGATTCATAGTT      400
GCATTACTTATAGCTTCCTTGTTCGTAGACAAAGCGGATTCGGAATTGTC      450
TTTCATTCCAAGGCATAACTTGTATCCATGCGCTTCATATTCGCCTGGAG      500
TTCGCTCCCAGAAATATAGCCATCCCTACCCCCTCACGTCAATCCCACGA      550
GCCTCTTATCCATTATCATTCGATGACGGCGGGGAAGCAAATAAAAATGG      600
AAAAACTCACATTGGGTTTAGGGATAATCAGGCTCGAACTGATGACTTCC      650
ACCACGTCAAGGTGACACTCTACCGCTGAGTTATATCCCTTCCCTTGCCT      700
TGCCCCCCATCGAGAAATAGAACTGACTAATCCTAAGGCAAAGGGTCGAG      750
AAACTCAACGCCATTATTCTTGAACAACTGAACAACTTGGAGCCGGGCCT      800
TCTTTTCGCACTATTACAGATACGAAAATAATGGGAAAAATTGGATTCAA      850
TTGTCAACTGTTCCTATCGGAAATAGGATTGACTACGGATTCGAGCCATA      900
GCACATGGTTTCAGAAAACCGTACGATTTTCCCGATCTAAATTCTAAATA      950
AAGTAGGTTTTACATGAAGAAGATTTGGCTCAGCATGTTCTCGGGTAGGA      1000
GAAGAGCCCGACTCAGTATTCTTAAAAAAAAAAAATAGAGAAATCAGAACC      1050
AAGTCAAAATGATACGGATCAACTCCTTCTTCTTGCGCCAAAGATCTTAT      1100
CATTTCCGAAGAAACAGGAGTTACATCTCTTTTCCATTTTCATTCAAGAG      1150
TTCTTATGTGTGTCCACGCCCCTTCGAGACCCCGAAAAGTGGACAAATTC      1200
CTTTTCTTAGGAACACATACAAGATTCGTCACTACAAAAAGGATAATGGT      1250
AACCCCACCATTAACTACTTCATTTATGATTTATGAATTTCATAGTAATA      1300
GAAATACATGTCCTACCGAGACAGAATTTGGAACTTGCTATCCCCTTGCC      1350
TAGCAGGCAAAGATTTACCTCCGTGGAAAGGATAATTCATTCGGATCAAC      1400
ATGAGAGTCCAACTACATTGCATTGCCATAATCCATGTTGTATATTTGAA      1450
AGAGGTTGATCCCCTTGCTTCTCTCATGTTACAATCCTCGTCCCGCTGAG      1500
CCCCCCTTTATCCTCGGTCCACAGAGACAAAATGTAGGACTGGTGCCAAC      1550
AGTTCATCACGGAAGAAAGGACTCACTGAGCCGAGATCACTAACTAATAC      1600
```

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TAATGAAAATGCTAATATAATAGAAAAGAACTGTCGTTTCTGTATACTTT        1650
CCCCGGTTCCGTTGCTACCGCGGGCTTTACGCAATCGATCGGATCATATA        1700
GATATCCCTTCAACACAACATAGGTCATTGAAAGGATCTCGGAGACCCAC        1750
CAAAGCACGAAAGCCAGGATCTTTCAGAAAATGGATTCCTATTCGAAGAG        1800
TGCATAACCGCATGGATAAGCTCACACTAACCCGTCAATTTGAGATACAA        1850
TTCGGGATTTTCCTTGGGAGGTATCGGGAAGGAATTGGAATGTAATAATA        1900
TCGATTTATATAGATACATTTATACAGATACAGAAAAAAAGGTTATCTAT        1950
TGATTCAAACGTGTACCTACGGGATAGGGATAGAGAAAGAGGAAAAACCG        2000
AAGATTTCACATAGTATTTTTGATCGAAAAATCAATTTTATTTATTTCGT        2050
ACCTTTCGCTTAATGAGAAAATGGGTCAGATTATACAGGATCAAATCTAT        2100
GGGACTTAAGGAATGATGGAAGGGAATAAAATAAAAAAAGAAAAGAGAAT        2150
AAGTAAATAAAAATGAAATAGAAGAGCCCAGATTCAAAATGAATGAAAAC        2200
GTGACTGAATTGGTCCTGTCTTCGGAAGGGAGTGGAAGACGGGAAGAGAT        2250
TCTCGAACAAGGAAAAGGATCCAATGACTTCAAAAGAATTGAACGAGGAG        2300
CCGTATGAGGTGAAAATCTCATGTACGGTTCTGTAGAGTGGCAGTAAGGG        2350
TAACTTATCTGTCAACTTTTCCACTATCACCCCCAAAAAACCAAACTCTG        2400
CCTTACGTAAAGTTGCCAGAGTACGCTTAACCTCTGGATTTGAAATCACT        2450
GCTTATATACCTGGTATTGGCCATAATTTACAAGAACATTCTGTAGTCTT        2500
AGTAAGAGGGGGGAGGGTTAAAGATTTACCCGGTGTGAGATATCACATTG        2550
TTCGAGGAACCCTAGATGCTGTCGGAGTAAAGGATCGTCAACAAGGGCGT        2600
TCTAGTGCGTTGTAGATTCTTATCCAAGACTTGTATCATTTGATGATGCC        2650
ATGTGAATCGCTAGAAACATGTAAAGTGTATGGCTAACCCAATAACGAAA        2700
GTTTCGTAAGGGAACCGGAGCAGGCTACCACGAGACAAAAGATCTTCTTT        2750
CTAAAGAGATTCTATTCGGAACTATTATATGTCCAAGGTCCAATATTGAA        2800
ATAATTTCAGAGGTTTTCCTTGACTTTGTCCGTGTCAACAAACAATTCGA        2850
AATGCCTCGACTTTTTTAGAACAGGTCCGAGTCAAATAGCAATGATTCGA        2900
AGCACTTCTTTTTACACTATTTCGGAAACCCAAGGACTCAATCGTATGGA        2950
TATGTAAAATACAGGATTTCCAATCCTAGCAGGAAAAGGAGGGAAACGGA        3000
TACTCAATTTAAAGTGAGTAAACAGAATTCGATACTCGATCTCATAGATA        3050
CATATATAATTCTGTGGAAAGCCGTATTCGATGAAAGTCGTATGTACGGC        3100
TTGGAGGGAGATCTTTCCTATCTTTCGAGATCCACCCTACAATATGGGGT        3150
CAAAAAGCCAAAATAAATTATTTTAGCCCTTAtaa                       3185
```

SEQ 2: 3351 bp excised from plasmid pTob4.4. *Mbi* I (5') and *Psi* I (3') are highlighted, bases removed during cloning are in lowercase. The internal *Bst1107*I site is also highlighted.

```
ccgCTCCACCGGAAATTCCCTCTGCCCCTACCGTACTCCAGCTTGGTAGT        50
TTCCACCGCCTGTCCAGGGTTGAGCCCTGGGATTTGACGGCGGACTTAAA        100
AAGCCACCTACAGACGCTTTACGCCCAATCATTCCGGATAACGCTTGCAT        150
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```
CCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGATGCTTATTCCC      200
CAGATACCGTCATTGCTTCTTCTCCGGGAAAAGAAGTTCACGACCCGTGG      250
GCCTTCTACCTCCACGCGGCATTGCTCCGTCAGCTTTCGCCCATTGCGGA      300
AAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCC      350
AGTGTGGCTGATCATCCTCTCGGACCAGCTACTGATCATCGCCTTGGTAA      400
GCTATTGCCTCACCAACTAGCTAATCAGACGCGAGCCCCTCCTCGGGCGG      450
ATTCCTCCTTTTGCTCCTCAGCCTACGGGGTATTAGCAGCCGTTTCCAGC      500
TGTTGTTCCCCTCCCAAGGGCAGGTTCTTACGCGTTACTCACCCGTCCGC      550
CACTGGAAACACCACTTCCCGTCCGACTTGCATGTGTTAAGCATGCCGCC      600
AGCGTTCATCCTGAGCCAGGATCGAACTCTCCATGAGATTCATAGTTGCA      650
TTACTTATAGCTTCCTTGTTCGTAGACAAAGCGGATTCGGAATTGTCTTT      700
CATTCCAAGGCATAACTTGTATCCATGCGCTTCATATTCGCCCGGAGTTC      750
GCTCCCAGAAATATAGCCATCCCTGCCCCCTCACGTCAATCCCACGAGCC      800
TCTTATCCATTCTCATTGAACGACGGCGGGGGAGCAAATCCAACTAGAAA      850
AACTCACATTGGGCTTAGGGATAATCAGGCTCGAACTGATGACTTCCACC      900
ACGTCAAGGTGACACTCTACCGCTGAGTTATATCCCTTCCCCGCCCCATC      950
GAGAAATAGAACTGACTAATCCTAAGTCAAAGGGTCGAGAAACTCAACGC      1000
CACTATTCTTGAACAACTTGGAGCCGGGCCTTCTTTTCGCACTATTACGG      1050
ATATGAAAATAATGGTCAAAATCGGATTCAATTGTCAACTGCCCCTATCG      1100
GAAATAGGATTGACTACCGATTCCGAAGGAACTGGAGTTACATCTCTTTT      1150
CCATTCAAGAGTTCTTATGCGTTTCCACGCCCCTTTGAGACCCCGAAAAA      1200
TGGACAAATTCCTTTTCTTAGGAACACATACAAGATTCGTCACTACAAAA      1250
AGGATAATGGTAACCCTACCATTAACTACTTCATTTATGAATTTCATAGT      1300
AATAGAAATACATGTCCTACCGAGACAGAATTTGGAACTTGCTATCCTCT      1350
TGCCTAGCAGGCAAAGATTTACCTCCGTGGAAAGGATGATTCATTCGGAT      1400
CGACATGAGAGTCCAACTACATTGCCAGAATCCATGTTGTATATTTGAAA      1450
GAGGTTGACCTCCTTGCTTCTCTCATGGTACACTCCTCTTCCCGCCGAGC      1500
CCCTTTTCTCCTCGGTCCACAGAGACAAAATGTAGGACTGGTGCCAACAA      1550
TTCATCAGACTCACTAAGTCGGGATCACTAACTAATACTAATCTAATATA      1600
ATAGTCTAATATATCTAATATAATAGAAAATACTAATATAATAGAAAAGA      1650
ACTGTCTTTTCT<mark>GTATAC</mark>TTTCCCCGGTTCCGTTGCTACCGCGGGCTTTA      1700
CGCAATCGATCGGATTAGATAGATATCCCTTCAACATAGGTCATCGAAAG      1750
GATCTCGGAGACCCACCAAAGTACGAAAGCCAGGATCTTTCAGAAAACGG      1800
ATTCCTATTCAAAGAGTGCATAACCGCATGGATAAGCTCACACTAACCCG      1850
TCAATTTGGGATCCAAATTCGAGATTTTCCTTGGGAGGTATCGGGAAGGA      1900
TTTGGAATGGAATAATATCGATTCATACAGAAGAAAAGGTTCTCTATTGA      1950
TTCAAACACTGTACCTAACCTATGGGATAGGGATCGAGGAAGGGGAAAAA      2000
CCGAAGATTTCACATGGTACTTTTATCAATCTGATTTATTTCGTACCTTT      2050
CGTTCAATGAGAAAATGGGTCAAATTCTACAGGATCAAACCTATGGGACT      2100
TAAGGAATGATATAAAAAAAAGAGAGGGAAAATATTCATATTAAATAAAT      2150
ATGAAGTAGAAGAACCCAGATTCCAAATGAACAAATTCAAACTTGAAAAG      2200
GATCTTCCTTATTCTTGAAGAATGAGGGGCAAAGGGATTGATCAAGAAAG      2250
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```
ATCTTTTGTTCTTCTTATATATAAGATCGTGATTGGATCCGCATATGTTT      2300
GGTAAAGAGAATAATCTTATCCTTTGAGAATAATCAAAAATGGACAGTGT       2350
TCAATTGGAACATGAAAACGTGACTAAATTGGTCCTAGTTACTCTTCGGG       2400
GCGGAGTGGAAGAAGGGGGGGGATTCTCGAACGCGGAAAGGATCCAATGAA     2450
TTCGAAAGAATTGAACGAGGAGCCGTATGAGGTGAAAATCTCATGTACGG       2500
TTCTGTAGAGTGGCAGTAAGGGTGACTTATCTGTCAACTTTTCCACTATC       2550
ACCCCAAAAAAACCAAACTCTGCCTTACGTAAAGTTGCCAGAGTACGATT       2600
AACCTCTGGATTTGAAATCACTGCTTATATACCCGGTATTGGCCATAATT       2650
TACAAGAACATTCTGTAGTCTTAGTAAGAGGGGGAAGGGTTAAGGATTTA       2700
CCCGGTGTGAGATATCACATTGTTCGAGGAACCCTAGATGCTGTCGGAGT       2750
AAAGGATCGTCAACAAGGGCGTTCTAGTGCGTTGTAGATTCTTATCCAAG      2800
ACTTGTATCATTTGATGATGCCATGTGAATCGCTAGAAACATGTGAAGTG       2850
TATGGCTAACCCAATAACGAAAGTTTCGTAAGGGGACTGGAGCAGGCTAC      2900
CATGAGACAAAAGATCTTCTTTCTAAAGAGATTCGATTCGGAACTCTTAT       2950
ATGTCCAAGGTTCAATATTGAAATAATTTCAGAGGTTTTCCCTGACTTTG      3000
TCCGTGTCAACAAACAATTCGAAATACCTCGACTTTTTTAGAACAGGTCC      3050
GAGTCAAATAGCAATGATTCGAAGCACTTCTTTTTACACTATTTCGGAAA      3100
CCCAAGGACTCAATCGTATGGATATGTAAAATACAGGATTTCCAATCCTA      3150
GCAGGAAAGGGAGGGAAACGGATACTCAATTTAAAGTGAGTAAACAGAAT      3200
TCCATACTCGATCTCATAGATACATATAGAATTCTGCGGAAAGCCGTATT      3250
CGATGAAAGTCGTATGTACGGCTTGGAGGGAGATCTTTCATATCTTTCGA      3300
GATCCACCCTACAATATGGGGTAAAAAAGCCAAAATAAGTGATTTTAGCC      3350
CTTATaa                                                 3357
```

SEQ 3: *rrn16* promoter isolated from strawberry. *Kpn* I / *Sgf* I (5') and *Ssp* I recognition sites are highlighted, bases removed during cloning are in lowercase.

```
ggtACCGCGATCGCTTTCCATTTTTATTTGCTTCCCCGCCGTCATCGAAT       50
GATAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGGGATGGCT       100
ATATTTCTGGGAGCGAACTCCAGGCGAATatt                         132
```

SEQ 4: *psbA* 3'TR isolated from strawberry. *Bgl* II (5') and *Eco* RI / *Sgf* I (3') recognition sites are highlighted, bases removed during cloning are in lowercase.

```
agaTCTCCATCTATCAATGGATAAGGCTTTGATATTAGTATATATGAGTT       50
CGTGAAACTTTTGAAAGTAAAGGAGCAATACTAAATTTCTTGTTATATCA       100
AGAGGTTTTGTGTTGCTCCTTTACTCTAATTAAATTAGTATTTTTTAGTA       150
CTATTTTTTTTTAGATTAAAATAAAAAGGTTTTTATTTATGTTGTATTCT       200
TTTTTACAAGTAATGCGATCGCGAAttc                             228
```

SEQ 5: *aadA* gene. *Pag* I (5') and *Xho* I (3') recognition sites are highlighted, bases removed during cloning are in lowercase.

```
tCATGAGGGAAGCGGCGATCGCCGAAGTATCGACTCAACTATCAGAGGTA    50
GTTGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTT    100
GTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAGTGATATTGATT    150
TGCTGGTTACGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTG    200
ATCAACGACCTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGCGAGATTCT    250
CCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGC    300
GTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGAC    350
ATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTAT    400
CTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGG    450
CGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTA    500
AATGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGA    550
GCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCG    600
GCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATGGAGCGCCTG    650
CCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGG    700
ACAAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTG    750
TCCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGAAAC    800
TCGAg                                                 805
```

SEQ 6: *xylA* gene. *Pag* I (5') and *Xho* I (3') recognition sites are highlighted, bases removed during cloning are in lowercase.

```
tCATGAATAAATATTTTGAGAACGTATCTAAAATAAAATATGAAGGACCA    50
AAATCAAACAATCCTTATTCTTTTAAATTTTACAATCCTGAGGAAGTAAT    100
CGATGGTAAGACGATGGAGGAGCATCTTCGCTTTTCTATAGCTTATTGGC    150
ACACTTTTACTGCTGATGGAACAGATCAATTTGGCAAAGCTACCATGCAA    200
AGGCCATGGAATCACTATACAGATCCTATGGACATAGCTAAAGCAAGGGT    250
AGAGGCAGCATTTGAGTTTTTTGATAAGATAAATGCACCGTATTTCTGCT    300
TCCATGATAGAGATATTGCCCCTGAAGGAGACACTCTTAGAGAGACGAAC    350
AAAAATTTAGATACAATAGTTGCTATGATAAAGGATTACTTGAAGACCAG    400
CAAGACGAAAGTTTTGTGGGGTACTGCGAATCTTTTCTCCAATCCAAGAT    450
TTGTGCATGGTGCATCAACGTCTTGCAATGCCGATGTTTTCGCATATTCT    500
GCAGCGCAAGTCAAAAAAGCACTTGAGATTACTAAGGAGCTTGGTGGCGA    550
AAACTACGTATTCTGGGGTGGAAGAGAAGGATATGAGACACTTCTCAATA    600
CAGATATGGAGTTTGAGCTTGATAATTTTGCAAGATTTTTGCACATGGCT    650
GTTGATTATGCAAAGGAAATCGGCTTTGAAGGCCAGTTCTTGATTGAGCC    700
GAAGCCAAAGGAGCCTACAAAGCATCAATACGACTTTGACGTGGCAAATG    750
TATTGGCATTCTTGAGAAAATACGATCTTGACAAATATTTCAAAGTTAAT    800
ATCGAAGCAAATCATGCAACATTAGCATTCCATGATTTCCAGCATGAGCT    850
AAGATACGCCAGAATAAACGGTGTATTAGGATCGATTGACGCAAATACGG    900
```

GTGATATGCTATTAGGATGGGATACAGATCAGTTCCCTACAGATATACGC 950

ATGACAACACTTGCTATGTATGAAGTTATAAAGATGGGTGGATTTGACAA 1000

AGGCGGACTCAACTTCGATGCGAAAGTAAGACGTGCTTCATTTGAGCCAG 1050

AAGATCTTTTCTTGGGTCACATAGCAGGAATGGATGCTTTTGCAAAAGGC 1100

TTCAAAGTGGCTTACAAGCTTGTAAAAGATAGGGTATTTGACAAGTTCAT 1150

CGAAGAAAGATATGCAAGCTACAAAGATGGCATAGGTGCAGATATTGTAA 1200

GTGGGAAAGCTGATTTTAGAAGTCTTGAAAAGTATGCATTAGAGCGCAGC 1250

CAGATTGTCAACAAATCAGGAAGACAAGAGCTATTAGAGTCAATCCTAAA 1300

TCAGTATTTGTTTGCAGAATAA<mark>CTCGA</mark>g 1328

SEQ 7: *smGFP* gene incorporating *xylA/ aadA* adapter. *Pst* I and *Xho* I (5')
recognition sites are highlighted as are *Bgl* II and *Sma* I (3'), bases removed during
cloning are in lowercase and adapter sequence is underlined.

at<u>ctgcagctcg**AG**</u>ATGACTAATTAGAAGGGAGGGAATCATGAGTAAAGG 50

<u>AGAAGAACTTTTCACT</u>GGAGTTGTCCCAATTCTTGTTGAATTAGATGGTG 100

ATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCA 150

ACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACC 200

TGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCT 250

TTTCAAGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAGAGCGCC 300

ATGCCTGAGGGATACGTGCAGGAGAGGACCATCTCTTTCAAGGACGACGG 350

GAACTACAAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCA 400

ACAGGATCGAGCTTAAGGGAATCGATTTCAAGGAGGACGGAAACATCCTC 450

GGCCACAAGTTGGAATACAACTACAACTCCCACAACGTATACATCACGGC 500

AGACAAACAAAGAATGGAATCAAAGCTAACTTCAAAATTAGACACAACA 550

TTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCA 600

ATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACA 650

ATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTC 700

TTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATAC 750

AAATA<mark>Agatctgggccc</mark>tata 771