How the Pigment Stripes Form in Snapdragon (*Antirrhinum majus*) Flowers: a study of the molecular mechanism of venation pigmentation patterning in flowers

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Plant Molecular Biology

at Massey University, Palmerston North, New Zealand

Yongjin Shang

2006

Abstract

Floral stripes are a common pigmentation pattern in plants. Defining the molecular mechanisms of the striped pattern formation will aid understanding of how a gene can be differentially regulated across a population of similar cells. In the venation phenotype of *Antirrhinum majus*, the anthocyanin pigment is typically confined to the adaxial epidermal cells overlaying the petal veins.

To explore how this pattern forms this study focused on the expression and regulation of *Venosa*, a *Myb* regulator of anthocyanin biosynthesis. Pigment complementation experiments demonstrated that the lack of a MYB factor caused the lack of pigment in the cells outside the venation pigmentation domain. An allele of *Venosa* was isolated and identified. It was a mutant version of functional *Venosa* due to the central part being replaced by a transposon. Phenotype / genotype analysis indicated that the venation pigmentation patterning was due to the functional *Venosa*. *In situ* mRNA hybridisation showed that *Venosa* was expressed from the xylem to the adaxial epidermis, and was controlled spatially and quantitatively by a signal associated with the petal veins. *Venosa* expression provided the longitudinal axis for venation pigmentation stripes, and determined the location and intensity of the pigmented cells. Because another factor required for pigmentation, a bHLH factor, is specifically expressed in epidermal cells and it provides the transverse axis. The pigmented stripes are the cross expression domain of these two kinds of factors.

The transcriptional controlling property of a 2.4 kb (relative to the ATG) promoter region of the *Venosa* gene was analysed. The -900 bp fragment was characterised in detail using 5′-end deletion mutagenesis. A heterologous host, tobacco, was used for analysis in stable transgenics. The homologous host, *Antirrhinum*, was used for transient assays. The efficacy and efficiency of different reporter genes (intron-containing GUS, GFP, *Venosa* cDNA and genomic *Venosa*) and enhancement systems (transcriptional enhancer, translational enhancer, inhibitor of post transcriptional gene silencing and a two-step signaling amplification system) for the detection of low-level reporter gene expression were also tested. The strength of expression correlated to the length of the promoter fragment, and expression was detected using deletions down to

-500 bp, although only weak expression was found. This expression was flower specific but not vein related in both plant hosts. No expression was detected in petals of either host with fragments shorter than -500 bp. The results suggest that the fragment from -380 bp to -900 bp positively affected *Venosa* expression at the transcriptional level, but might not be sufficient to define venation. A possibility is that the venation controlling property is negatively controlled at the epigenetic level, such as DNA methylation status and / or chromatin structure.

The role of gibberellin and sugar in the pigment and venation patterning formation of *Antirrhinum* was studied. The results suggest that gibberellin is not required for pigmentation or venation patterning. Convincing evidence on the role of sugar signaling could not be obtained from the experiments, due to the difficulty in separating the impact on pigmentation from other functions of sugars in petal development.

In addition, the *in situ* analysis detected the expression of a gene probably related to aurone biosynthesis that may be a regulatory gene of this biosynthetic pathway.

Acknowledgements

This acknowledgement is to the following people who have assisted me with my PhD study:

To my supervisors Kathy Schwinn, Kevin Davies and Paula Jameson, for giving me the opportunity to be involved in an interesting and challenging research project; for training me in many aspects, especially in lab techniques, research strategies, presentation skills and writing abilities; for supporting and encouraging me to finish this study, an important step in my life. Your assistance that has been built into my ability and confidence in scientific research is so precious and valuable, that I always feel so lucky and cannot thank you enough.

To Cathie Martin (John Innes Centre), for giving me valuable advice during the research. Cathie initiated the study of venation pigmentation patterning and provided the opportunity to further explore this phenomenon.

To Huaibi Zhang, for frequently giving me assistance in solving various problems; to Erin O'Donohue, for patiently teaching me *in situ* hybridisation technique; to Liz Nickless, for providing assistance in confocol microscopy; to Simon Deroles, for excellent support in computing; to Ian King, for carefully culturing so many transgenic plants; to Ray Rains, for providing quality plant material; to Steve Arathoon and Jan Manson, for providing countless chemical solutions; to Tony Corbett, for excellent photography and poster-design; to Andrew Mullan, Beverley Hoffmann and Margaret Young, for providing quality media.

To Donald Hunter, David Lewis, Murry Boase, Dave Brummell, Julian Heyes, Marian Mckenzie, Ross Lill, Jocelyn Eason, Ranjith Pathirana, Keren Neilsen, Lyn Watson, Sheryl Somerfield, Michael Bennett, Lei Wang, Dacey Ryan, Tatyana Pinkney, John Javallana, Nady Pathirana, Nicholas Albert, Toni Waugh, Camela Lee, Margaret Burling, Nigel Gapper, Vern Collette, John Harris, Deepa Patel, Yvonne Dommels, Philip West, for your supporting and helping in various ways.

Table of Contents

List of Figures

List of Tables

Abbreviations

A_{260}	absorbance at 260 nm
A_{600}	absorbance at 600 nm
A	adenine
ANS	anthocyanidin synthase
AS	acetosyringone
ATP	adenosine triphosphate
6-BAP	6-benzylamino purine
bp	base-pairs
^{o}C	degrees Celsius
C	cytosine
CaMV 35S	cauliflower mosaic virus 35S promoter
cDNA	complementary DNA
CHS	chalcone synthase
cm	centimetre
cv	cultivar
ATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DFR	dihydroflavonol 4-reductase
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetracetic acid
EtBr	ethidium bromide
F3H	flavanone 3-hydroxylase
g	gram
G	guanine
GA	gibberellin
GA_3	gibberellic acid
GBV	genomic big venosa

GFP	green fluorescent protein
GMO	genetically modified organism
GSV	genomic small venosa
GUS	ß-glucuronidase
gVenosa	genomic *Venosa*
h	hour
IGUS	intron GUS
IPTG	isopropyl-ß-D-thiogalactoside
Kan	kanamycin
kb	kilo base-pairs
KV	kilo volts
L	litre
LB	Luria-Bertani (media or broth)
M	molar, moles per litre
min	minute
µg	micro gram
mg	milligram
mL	millilitre
µM	micro molar, micro moles per litre
MOPS	3-[N-morpholino] propanesulphonic acid
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog Basal Medium
NaHAc	sodium acetate
ng	nanogram
NOS	nopaline synthase
nptII	neomycin phosphotransferase gene
OCS	octopine synthase
PCR	polymerase chain reaction
pmol	pico-molar, pico moles per litre
rATP	riboxyadenosine triphosphate
rCTP	riboxycytidine triphosphate
rGTP	riboxyguanosine triphosphate
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
rUTP	riboxyuradine triphosphate
SDS	sodium dodecyl suphate
SSC	saline sodium citrate buffer
T	thymine
TBE	tris borate EDTA buffer
TBS	tris-buffered saline solution
TE	tris-EDTA buffer
TFs	transcription factors
Tris	tris(hydroxymethyl)aminomethane
Tween20	polyoxyethylenesorbitan monolaurate
U	uracil
V	volts
VEN	*Venosa* promoter deletions
Vv	*Venosa/venosa* heterozygous
vv	*venosa/venosa* homozygous
v/v	volume per volume
w/v	weight per volume
X-Gluc	5'-bromo-4-chloro-3-indoyl-ß-D-glucuronide

Chapter 1

Introduction

1.1   Overview

Pigmentation patterns in petals are prominent features in many angiosperm plants. Various pigmentation patterns such as stripes, spots, circles and irregular patches occur naturally. These patterns, when integrated with different colour and pigment intensity, can become very complex (Figure 1.1). To achieve such patterns, the plant must control pigment production not only in a tissue-specific manner, but also to the level of the individual cell. The anthocyanin biosynthesis pathway has been elucidated at the level of the structural gene, and is even partly understood at the level of the regulatory gene. However, little is known about how such complex petal pigmentation patterns form. Defining the molecular mechanisms of pigment pattern formation will aid understanding of how a gene can be differentially regulated across a population of similar cells.

Among the various pigmentation patterns that occur, stripes are common in nature (Figure 1.2), and they occur in the model species *Antirrhinum majus* (antirrhinum). In the venation phenotype of antirrhinum, anthocyanin pigment is confined to the adaxial epidermal cells overlaying the petal veins (Figure 1.3). Three *myb* genes regulate anthocyanin biosynthesis in this model species (Schwinn *et al.*, 2006). One of the *myb* genes, *Venosa*, was isolated from a venation phenotype in a *Rosea1* mutant (*rosea^{dorsea}*) background. It has been hypothesised that the venation pigmentation patterning is due to the localised expression of *Venosa*.

Based on this, the purpose of this PhD project, which is a component of a Marsden Fund of New Zealand project, was to investigate the molecular mechanisms controlling venation pigmentation patterning in the petals of antirrhinum by functionally analysing *Venosa* and exploring its upstream regulatory mechanism.

1

Figure 1.1 Photographs illustrating complexity of floral pigmentation patterning in orchid. (A, B and C) *Paphiopedilum* hybrids;(D) *Phalaenopsis* hybrid. Photos courtesy of Dr Kevin Davies.
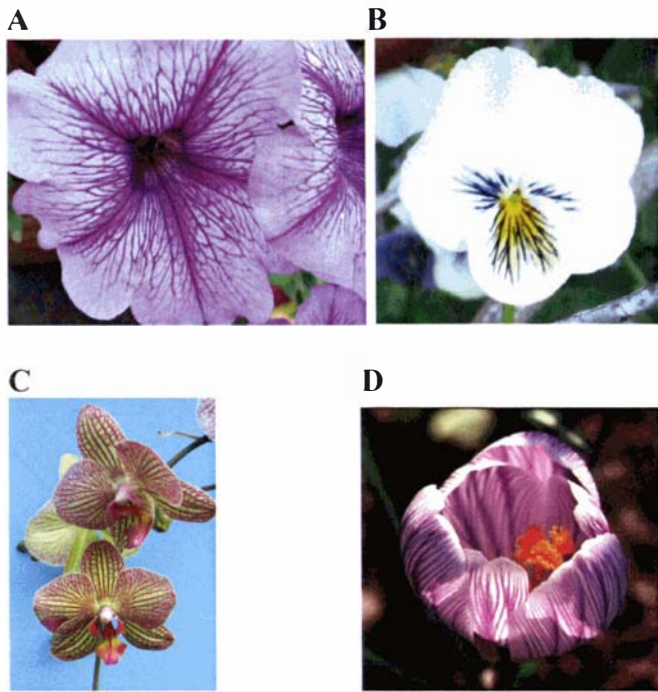
Figure 1.2 Floral venation pigmentation patterning is common in nature. (A) *Petunia hybrida* (common name Petunia); (B) *Viola hybrid* (common name Pansy); (C) *Phalaenopsis hybrid*; (D) *Crocus vernus*. Photos courtesy of Drs Kevin Davies and Guojun Sun.
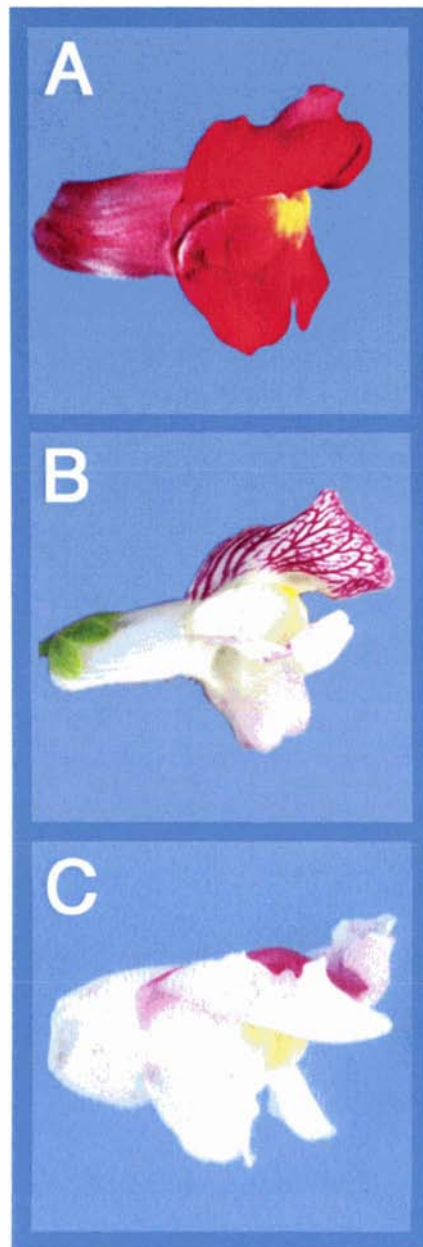
Figure 1.3   Three selected phenotypes of *A. majus*. (A) Fully red flower, which is controlled by *Rosea1* and accepted as the wildtype due to its widespread occurrence in nature; (B) Venation phenotype, which is hypothetically controlled by *Venosa*, and was experimentally characterised its genotype, *Venosa/venosa*, in this study; (C) *rosea*^{dorsea} phenotype, a mutant of *Rosea1* (Its genotype is *rosea1/rosea1* in *Rosea* locus). It was also experimentally characterised as the genotype *venosa/venosa* in *Venosa* locus in this study. *Rosea1* and *Venosa* are different loci and not alleles. The phenotype of *Venosa* can't be seen unless *Rosea1* is mutated such as in *rosea*^{dorsea} background. Photos courtesy of Dr Kathy Schwinn.

1.2   Anthocyanin pigments in plants

1.2.1   Anthocyanins as plant pigments and their biological functions in flowers

The common plant pigments can be grouped into four classes according to their chemical structures and biosynthetic pathway: chlorophylls, betalains, carotenoids and flavonoids. (Davies, 2004). Flavonoids represent a large class of secondary plant metabolites and play many key functions in plant development (Gould and Lister, 2006). According to chemical structure, they can be sub-grouped into chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins (or proanthocyanidins) (Winkel-Shirley, 2001).

Anthocyanins are the most significant flavonoid plant pigments. They are synthesised in the cytosol, and then transported into acidic vacuoles by specific transporters for biological functions (Kitamura, 2006). Robert Boyle (1664, cited from Onslow, 1925) provided the idea of anthocyanin as an indicator in acid and alkali reactions. A wide range of colours result from their synthesis. These include blue, purple, violet, mauve, magenta, and nearly all the red shades. The colour of anthocyanins is determined by the structure of molecules, pH value in vacuoles, the concentration of metal ions and (flavonoid) co-pigment, and the way anthocyanins are packaged (Quattrocchio *et al.*, 2006). They accumulate in all plant organs including flowers, fruits, leaves, stems, seeds, tubers and roots, but are especially dominant in petals of flowers. Kay *et al.* (1981) examined 201 species from 60 angiosperm families and gave a detailed description of pigment distribution and cell structure in petals. In flowers, anthocyanins are mainly located in the vacuoles of petal epidermal cells, where they can most effectively contribute to flower colour. Unusually, anthocyanins were found in the mesophyll cells in most members of the *Boraginaceae* and a few species of *Liliaceae*. In these instances, their localisation was correlated with morphological differences in the shape of the epidermal cells (Kay *et al.*, 1981).

Anthocyanins play multiple roles as flower pigments: in signalling between plants and insects; in response related to nutrient availability; in male fertility of some species; in

defense as microbial agents and feeding deterrents; in the modulation of auxin transport and in UV protection (Winkel-Shirley, 2001).

1.2.2   Anthocyanin biosynthesis

The isolation of the structural genes involved in anthocyanin biosynthesis has been achieved by a combination of genetic, biochemical and molecular approaches. The anthocyanin biosynthetic pathway is well established (Holton and Cornish, 1995; Davies and Schwinn, 2005). It is summarised in Figure 1.4. The precursors for the synthesis of virtually all flavonoids, including anthocyanins, are malonyl-CoA and 4-coumaroyl-CoA. Chalcone synthase (CHS) catalyses the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaroyl-CoA to yield naringenin chalcone. Chalcone isomerase (CHI) then catalyses the stereospecific isomerisation of the yellow-coloured naringenin chalcone to the colourless naringenin. Naringenin is converted to dihydrokaempferol (DHK) through a hydroxylation by flavanone 3-hydroxylase (F3H). Dihydroflavonols (DHFs) are then converted to flavan-3, 4-*cis*-diols (leucoanthocyanidins) through reduction by dihydroflavonol 4-reductase (DFR). Leucoanthocyanidins are colourless and unstable precursors of coloured anthocyanidins. Their conversion to anthocyanidins is catalysed by anthocyanidin synthase (ANS).

The anthocyanidins can be further modified by glucosylation. This reaction is most commonly carried out by the enzyme UDP-glucose:flavonoid 3-*O*-glucosyltransferase (A3GT). In antirrhinum after anthocyanin 3-*O*-glucoside formation, a rhamnose moiety may be added to the glucose residue by the UDP-rhamnose:anthocyanidin-3-*O*-glucoside rhamnosyltransferase (A3RT). Anthocyanidin 3-*O*-glucosides may be modified by further glycosylation, methylation and acylation. The anthocyanin precursors, such as flavanones and DHFs, are commonly modified by 3' or 3',5' hydroxylation, carried out by the flavonoid 3'-hydroxylase or flavonoid 3',5'-hydroxylase. In *Antirrhinum*, the anthocyanins are either pelargonidin (4'-OH) or cyanidin (3',4'-OH) based.
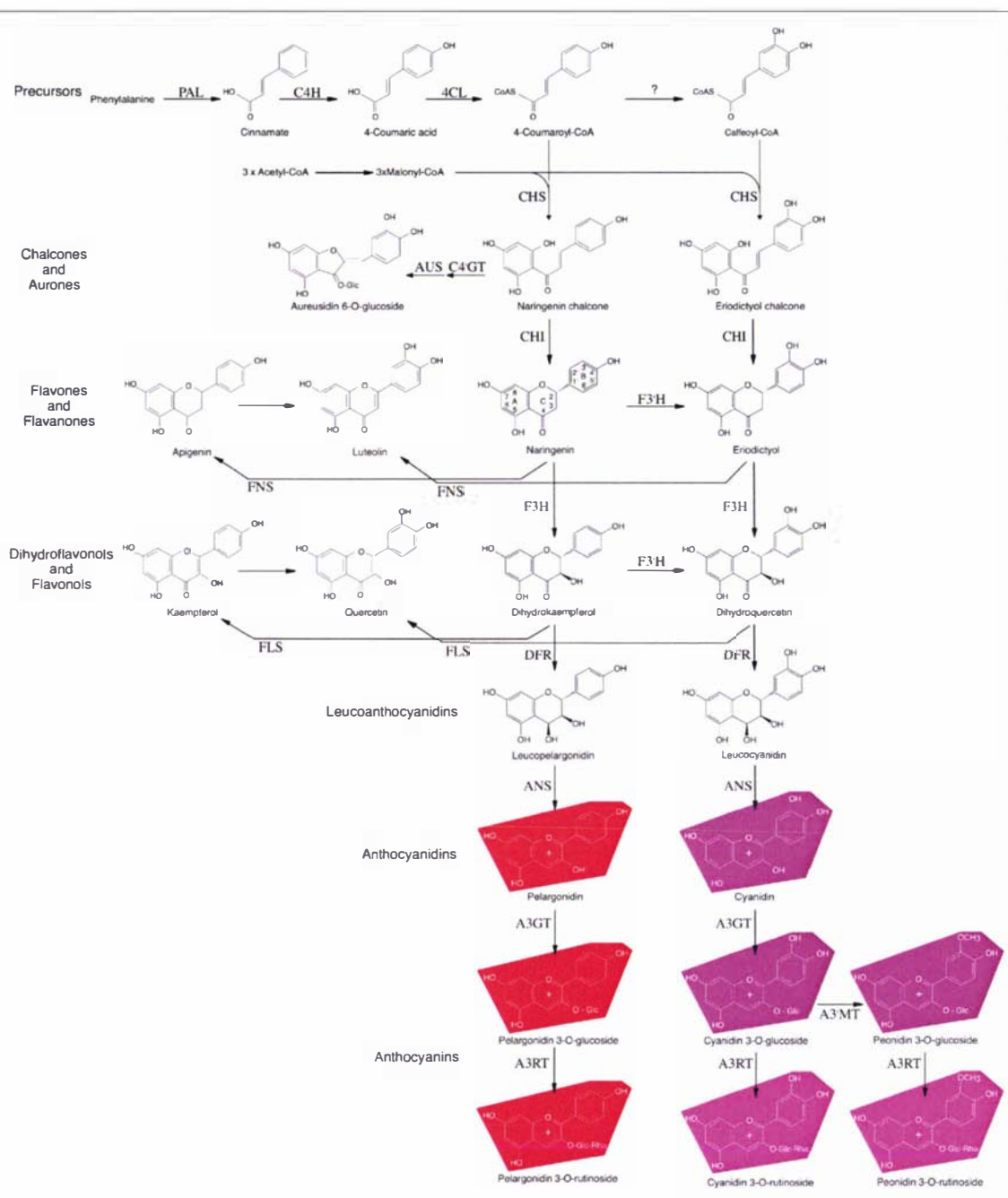
Figure 1.4   Anthocyanin biosynthesis pathway in *Antirrhinum*. Names in lower case represent pathway intermediates and products. Upper case letters represent enzymes. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; C4'GT, chalcone 4' glucosyltransferase; AUS, aureusidin synthase; CHI, chalcone isomerase; F3H, flavonone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; A3GT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase; A3RT, UDP-rhamnose:anthocyanidin-3-*O*-glucoside rhamnosyltransferase; A3M'T, UDP-rhamnose:anthocyanidin-3-*O*-glucoside methyltransferase; FLS, flavonol synthase; FNS, flavone synthases. The diagram was drawn by Dr Kevin Davies and reproduced with permission.

7

1.3   Gene regulation of anthocyanin biosynthesis in model plant species

1.3.1   Regulation of anthocyanin biosynthesis in maize

The regulatory mechanism of anthocyanin biosynthesis was originally studied in maize (*Zea mays*), in which it was found that two families of transcription factors control anthocyanin biosynthesis. The first is the *C1/Pl* family, which is comprised of two homologues R2R3-MYB factors (Paz-Ares *et al.*, 1986, 1987; Cone *et al.*, 1993). The second is the *R/B* gene family, which includes *R, B, Lc, Sn* and *Hopi* regulating pigmentation in different tissues of the plant (Chandler *et al.*, 1989; Ludwig *et al.*, 1989; Tonelli *et al.*, 1991; Consonni *et al.*, 1992, 1993; Petroni *et al.*, 2000). The *R/B* gene family encodes bHLH proteins, which are highly homologues to each other (Ludwig and Wessler, 1990).

Genetic studies and transient expression assays revealed that individual family members alone were not sufficient to induce anthocyanin biosynthetic gene expression. Activation requires the presence of a member from each family in the cell. The R2R3-MYB and bHLH factors are believed to act in partnership. They interact directly through the amino terminus of the bHLH and the R3 repeat of the MYB domain within the transcription activation complex (Goff *et al.*, 1992; Sainz *et al.*, 1997). The interaction between MYB and bHLH seems not to be required to increase the DNA-binding specificity of MYB factors, since *C1* alone is able to bind to a specific site within the promoter of the *A1* (DFR) gene (Sainz *et al.*, 1997). In addition, *B*, a bHLH partner lacking the bHLH domain can still bind *C1* and stimulate anthocyanin synthesis suggesting that the role of the bHLH partner is by interaction with the MYB partner (Goff et al., 1992; Hernandez *et al.*, 2004).

In maize, combinatorial interactions between differentially expressed members of the two distinct classes of factors define developmentally regulated anthocyanin production profiles, and all of the anthocyanin biosynthetic genes are regulated in a single group. The combination of *C1* and *R* induces pigmentation in the kernels, while *P1* and *B* together are responsible for pigmentation in mature tissues. The *R/B* family members

are functionally equivalent and highly similar in sequence, so their tissue specificities appear to be due to differences in their promoters (Ludwig *et al.*, 1989, Ludwig and Wessler 1990; Goff *et al.*, 1992; Consonni *et al.*, 1993). The *C/Pl* family shows less allelic diversity than *R/B* family (Cone *et al.*, 1993). The regulation of pigmentation in maize also involves inhibitors (Coe, 1985; Styles and Coe, 1986; Franken *et al.*, 1994), which may decrease the structural gene transcription by heteromerising with activators (Franken *et al.*, 1994; Goff *et al.*, 1991). The inhibitory regulators also show tissue specificity of expression (Coe, 1985; Styles and Coe, 1986).

It is not clear how the anthocyanin biosynthetic pathway is controlled at upstream regulation in maize. *Viviparous1 (Vp1)* is a regulator of *C1*. The *vp1* mutant, which has an ABA insensitive phenotype (Robichaud and Sussex, 1986), encodes a transcription factor (McCarty *et al.*, 1991; McCarty, 1995). *Vp1* is seed specific and required for seed maturation (in which seed pigmentation is one component) and germination. There are four prominent conserved domains in *VP1*, designated A1, B1, B2 and B3 (Giraudat *et al.*, 1992). *VP1* is a direct activator of the *C1* gene (Hattori *et al.*, 1992; Kao *et al.*, 1996; Suzuki *et al.*, 1997). Activation of *C1* is mediated by the binding of B3 to the Sph element in the *C1* promoter (Hattori *et al.*, 1992; Kao *et al.*, 1996; Suzuki *et al.*, 1997). Maize *Vp1* complements *Arabidopsis thaliana* (arabidopsis) *abi3* and confers a novel ABA / auxin interaction in the roots of arabidopsis, but it remains unknown whether *VP1 / ABI3* mediated interaction between auxin and ABA signalling also occurs during normal seed development (Suzuki *et al.*, 2001). In addition, a novel regulatory gene, *PAC1* (pale aleurone colour1), affecting seed pigmentation via *B* and *C1* (Selinger and Chandler, 1999), is a WD-repeat (WDR) protein (Carey *et al.*, 2004).

It was found that epigenetic control could also be involved in anthocyanin regulatory gene expression in maize. The tissue-specific patterns of a maize *myb* transcription factor, *pericarp colour1 (p1)*, were epigenetically regulated (Cocciolone *et al.* 2001). Developmental patterns of chromatin structure and DNA methylation were responsible for its epigenetic expression (Hoekenga *et al.* 2000). The maize *unstable factor for orange1* is a dominant epigenetic modifier of a tissue-specific silent allele of *p1* (Chopra *et al.*, 2003). In addition to the *myb* factors, the bHLH factors such as *b1* have been shown to be epigenetically controlled (Stam *et al.*, 2002).

1.3.2   Regulation of anthocyanin biosynthesis in petunia and *Arabidopsis*

In petunia (a range of studied species, including *Petunia hybrida*), the regulation of pigmentation is well characterised in petals. MYB and bHLH factors are involved in controlling pigmentation. *An1* encodes a bHLH factor that activates the transcription of structural genes, including *DFR*, and a regulatory gene, *MYB27* (Spelt *et al.*, 2000). The expression of *An1* is regulated by *An2* and *An4* (Spelt *et al.*, 2000). *An2* and *An4* are *myb* genes with different spatial domains of expression. *An2* is expressed only in the petal limb (Quattrocchio *et al.*, 1999), while *An4* is expressed in anthers (Spelt *et al.*, 2002). The activity of one or more of these transcription factors seems to be regulated post-transcriptionally by *An11*, which encodes a cytosolic WDR protein, because *An11* is required for anthocyanin production and expressed independently from *An1* and *An2* throughout plant development. In addition to regulation of pigmentation, *An1*, *An2* and *An11* control the vacuolar pH in petal cells and the morphology of the seed coat epidermis (Quattrocchio, 1994; de Vetten *et al.*, 1997; Spelt *et al*, 2002). A gene yet to be molecularly characterized, *An12* increases pigmentation in regions of the petal outside the veins. Mutation at the *An12* locus result in a flower with a venation pattern: dark pigmentation in the veins and paler pigmentation throughout the rest of the petal limb (Gerats *et al.*, 1989).

In arabidopsis, in addition to a MYB factor (encoded by *TRANSPARENT TESTA 2*) (*TT2*) (Nesi *et al.*, 2001), a bHLH factor (encoded by *TRANSPARENT TESTA 8*) (*TT8*) (Nesi *et al.*, 2000) and a WDR protein (encoded by *TRANSPARENT TESTA GLABROUS 1*) *(TTG1)* (Walker *et al.*, 1999; Western *et al.* 2001, Baudry *et al.*, 2004), two other transcription factors, the homeodomain protein *ANTHOCYANINLESS2 (ANL2)* (Kubo *et al.*, 1999) or *TRANSPARENT TESTA 16 (TT16)* (Nesi *et al.*, 2002) and the zinc finger protein *TRANSPARENT TESTA 1 (TT1)* (Sagasser *et al.*, 2002), are involved in the production of proanthocyanidin polymers in the seed coat. Probably, these factors regulate seed coat development by acting upstream of the MYB and bHLH regulators such as *TT2* (Debeaujon *et al.*, 2003; Nesi *et al.*, 2002; Sagasser *et al.*, 2002), a *myb* gene controlling proanthocyanidin accumulation in developing seed (Nesi *et al.*, 2001). Overexpression of the *myb* genes *Production of Anthocyanin Pigment 1* and *2 (PAP1* and *PAP2)* up-regulates the accumulation of anthocyanin in the whole plant

(Borevitz *et al.*, 2000). The arabidopsis transcription factor *MYB12* transcriptionally up-regulated CHS and FLS (Mehrtens *et al.*, 2005).

Hartmann *et al.* (2005) studied how three factors, MYB, bZIP and bHLH interacted with *cis*-acting elements of the promoters of phenylpropanoid biosynthesis genes in controlling light and tissue-specific activation. In their results, a bHLH and a MYB factor cooperate to determine the tissue-specific production of flavonoids, and a bZIP factor and a MYB factor cooperate to determine the light responsiveness. The bZIP, MYB and bHLH factors can bind to the relevant *cis*-acting elements of flavonoid biosynthesis gene promoters respectively (Hartmann *et al.*, 2005), and the MYB can form into a homodimer or interact with the bHLH to form into a heterodimer. In addition, a WDR factor interacts with only a bHLH factor or the bHLH / MYB complex (Baudry *et al.*, 2004).

A model of the controlling mechanism of expression of anthocyanin biosynthesis genes is presented schematically in Figure 1.5 based on these studies.

1.3.3 Regulation of anthocyanin biosynthesis in antirrhinum

1.3.3.1 Spatial and temporal control of anthocyanin production in antirrhinum

As described in the previous section, regulatory genes that control expression of the structural genes of the anthocyanin biosynthetic pathway have been identified in several plants. These genes influence the intensity and pattern of anthocyanin produced and generally control expression of many different structural genes. Study of co-regulation of structural genes can be carried out by either enzyme assays or mRNA expression assays.

The anthocyanins accumulate during petal development in antirrhinum primarily as a result of increased biosynthesis of the biosynthetic enzymes. The temporal increase of these enzymes is most likely due to increased transcription of the structural genes (Jackson *et al.*, 1992). In wild type flowers of antirrhinum, anthocyanin biosynthesis is restricted to the epidermal cells. The expression of structural genes can be tightly spatially coordinated with respect to cellular position within the corolla (Jackson *et al.*,
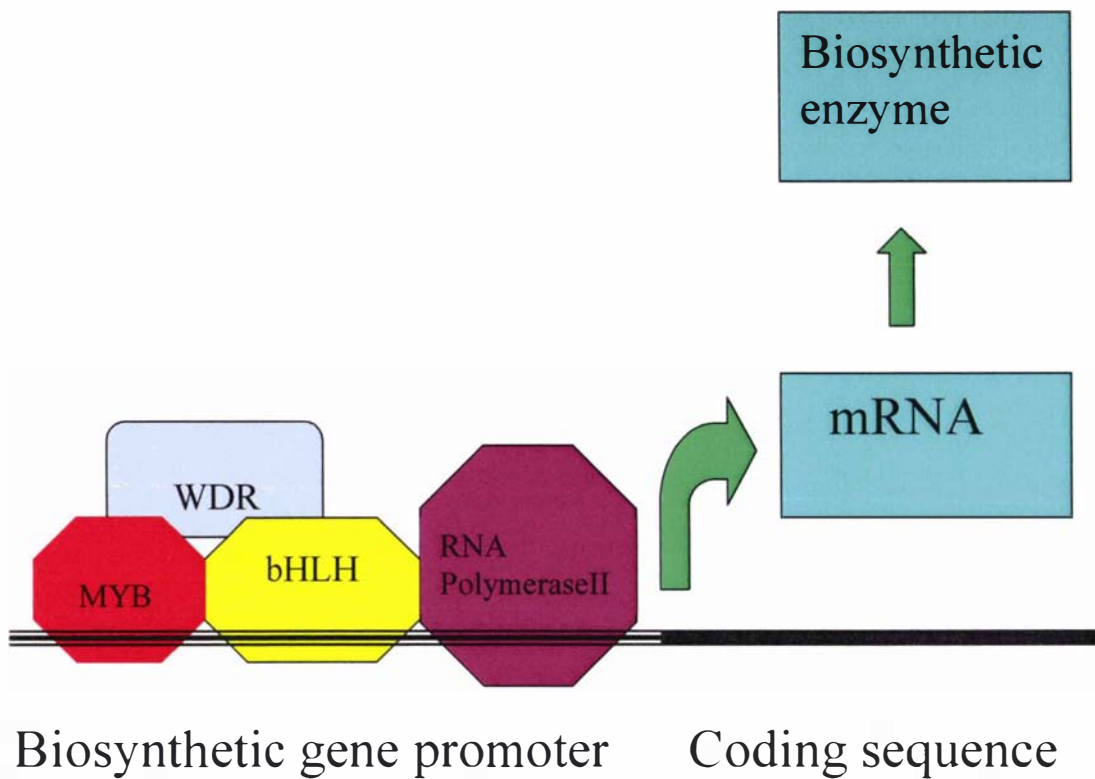
Figure 1.5   The current model of gene regulation of anthocyanin biosynthesis. Three factors, MYB, bHLH and WDR form into a complex, which binds to the structural gene promoter region with the DNA binding domains in MYB and bHLH, and interacts with the RNA polymerase II holoenzyme via general transcription factors to activate the transcription of the anthocyanin biosynthesis genes.

1992; Martin and Gerats, 1993). The expression of all the tested biosynthetic genes including CHS, F3H, DFR, *Candi* and F3GT varies across flowers, but shows the same pattern over the epidermis and is spatially co-ordinated; it is highest in the cells of the inner epidermis of the lobes and the cells of both inner and outer epidermis at the base of the flower tube; it is lowest in the upper region of the tube. This pattern of expression correlates with the pattern and intensity of anthocyanin pigmentation (Jackson *et al.*, 1992).

Expression of the anthocyanin biosynthetic genes is also temporally coordinated in antirrhinum flowers. A detailed time-course analysis of steady state transcript levels for six of the genes required for anthocyanin production (Jackson *et al.*, 1992) showed that the expression of CHS and CHI peaked slightly earlier than the other gene products, and CHI transcript alone was very high in young buds (0-5 mm). Based on the different expression profiles and their response to regulatory genes (Almeida *et al.*, 1989; Bartlett, 1989; Martin *et al.*, 1991; Jackson *et al.*, 1992) the biosynthetic genes were divided into two groups: early biosynthetic genes encoding CHS and CHI, and late biosynthetic genes encoding F3H, DFR, ANS and F3GT (Martin *et al.*, 1991; Jackson *et al.*, 1992).

In petunia petals, there is also a regulatory division between early biosynthetic genes and late biosynthetic genes, but this division occurs after F3H (Beld *et al.*, 1989; Quattrocchio *et al.*, 1993; Weiss *et al.*, 1993). In arabidopsis, as in petunia, genes for DFR and ANS are regulated differently to those for CHS, CHI and F3H (Kubasek *et al.*, 1992; Shirley *et al.*, 1995; Pelletier and Shirley, 1996; Pelletier *et al.*, 1997; Pelletier *et al.*, 1999). However, in maize, all the biosynthetic genes are simultaneously induced in the aleurone cell layer in the kernels (Dooner and Nelson, 1977; Dooner, 1983; Cone *et al.*, 1986; Paz-Ares *et al.*, 1986; Chandler *et al.*, 1989; Ludwig *et al.*, 1989; Ludwig and Wessler, 1990).

1.3.3.2 Transcription factors controlling anthocyanin biosynthesis in antirrhinum flowers

A bHLH gene, *Delila* (*Del*), and three *myb* genes, *Rosea1*, *Rosea2* and *Venosa* have been cloned and characterised from antirrhinum (Goodrich *et al.*, 1992; Schwinn *et al.*,

2006). Together with data for another bHLH factor, *Mutabilis (Mut)* (P.Piazza, C. Tonilli, and C. Martin, unpublished data, cited from Schwinn *et al.*, 2006), the study of Schwinn *et al.* (2006) shows that there are multiple spatial pigmentation domains for control of anthocyanin pigmentation within the flower, and the controlling mechanism is complex. All three *myb* genes are expressed in the tube and lobes of the flower, but they have different spatial patterns of expression within the flower. *Rosea1* functions in the tube and lobes, and controls anthocyanin pigmentation through the positive regulation of late biosynthetic genes (Schwinn *et al.*, 2006). *Rosea2* has little if any effect in the central regions of the lobe and no effect in the other organs of the flower, and produces a far weaker pigmentation phenotype than *Rosea1* due, at least in part, to a lower expression level. It was hypothesised that *Venosa* had a more restricted spatial expression domain and may only express in the inner epidermis overlaying the petal veins. The expression domain is different between the two bHLH factors. *Del* expresses in the corolla lobe and tube, while *Mut* functions only in the lobe. Recently, a cDNA for the antirrhinum WDR factor has also been identified (Kathy Schwinn, personal communication).

The partnership between the three MYB factors and the two bHLH factors was tested by making double and triple mutants. All three MYB factors can interact with DEL in the tube. In the lobe, ROSEA1 and VENOSA can interact with both MUTABILIS and DELILA, whereas ROSEA2 can interact only with DELILA (Schwinn *et al.*, 2006).

There are other genes identified by mutations and involved in the controlling network of antirrhinum anthocyanin pigmentation. These genes include *Eluta, Mutabilis, Picturata, Diluta* and *Vitrix*. They may encode bHLH, MYB, WDR, homeodomain protein, or zinc finger proteins respectively (Martin and Gerats, 1993). The phenotypes with variant anthocyanin patterning reflect the operating domains of anthocyanin regulatory genes, and some patterns are also due to the mutation of certain regulators such as *rosea^{dorsea}*, which results from the mutation of the *myb* gene *Rosea* (Schwinn *et al.*, 2006).

1.4   Signals that might regulate anthocyanin biosynthesis in flowers

1.4.1   Action of gibberellins during petal development

Anthocyanin accumulation is an integral part of flower development (Martin and Gerats, 1993). Activation of the anthocyanin pathway during petal development requires a complex interaction between environmental and developmental signals (Weiss, 2000). One of the plant hormones, the gibberellins (GAs), is produced in the developing anthers (Pharis and King, 1985; Weiss *et al.*, 1995; Itoh *et al.*, 1999; Rebers *et al.*, 1999; Kaneko *et al.*, 2003) and plays an important role in petal development (Weiss and Halevy, 1989; Jacobsen and Olszewski, 1991; Goto and Pharis, 1999). The role of GAs in petal pigmentation in petunia has been well studied (Weiss and Halevy, 1989; Weiss *et al.*, 1992, 1995). Removal of anthers from young green flower buds, inhibited corolla growth and pigmentation, and application of GA_3 replaced the anthers in their effect on both processes (Weiss and Halevy, 1989). At later stages, after the transition to the phase of rapid elongation, the corolla was no longer dependent on the anthers or exogenous GA for growth and pigmentation (Weiss *et al.*, 1989). GA may be required only for the initiation of these processes but not for their maintenance. It was concluded that GAs are produced in the developing anthers and transported to the corollas, where they induce growth and pigmentation (Weiss *et al.*, 1995). In the study of the "Red Star" phenotype of petunia, treatment with GA_3 can completely suppress the formation of the star pattern (van der Krol *et al.*, 1989). GAs control anthocyanin accumulation through the induction of the expression of both late biosynthetic genes and early biosynthetic genes (Weiss *et al.*, 1995). This control is indirect, in which GA may first induce the synthesis of trans-acting factors which, in turn, activate the entire anthocyanin pathway (Weiss *et al.*, 1992).

In addition, GA_3 has been shown to promote other processes in corolla development, including respiration and expression of genes from primary metabolic pathways and the induction of *gibberellin-induced gene1* (*gip1*) expression (Ben-Nissan and Weiss, 1995). GA may induce an entire developmental program via the activation of master regulatory genes. *Myb92*, induced by GA in a primary fashion, is such a candidate (Weiss, 2000).

GA$_3$ also promotes anthocyanin accumulation in detached *Hyacinthus orientalis* (Hosokawa *et al.*, 1996) and Baccara rose flowers (Zieslin *et al.*, 1974), and pigmentation in expanding cucumber petals (Vainstein *et al.*, 1994). However, in *Phlox* flowers GA inhibits anthocyanin synthesis in the petals (Weiss, 2000). In *Phlox*, unlike in petunia, anthocyanins accumulate at the early stages of flower development, before the rapid expansion of the petals. It is possible that GA-controlled pigmentation only occurs when pigmentation accumulation is directly tied to cell expansion of the petal (Weiss, 2000). The dwarf varieties in pea are due to impaired gibberellin production (Stoddart, 1987), and they can have coloured flowers (Mendel, 1865, cited by Martin and Gerats, 1993). Gibberellins are probably not required for floral pigmentation in pea, or gibberellin production in pea anthers differs from that in internodes (Martin and Gerats, 1993).

It is not known whether GA$_3$ acts as a signal in petal development and pigmentation of antirrhinum.

1.4.2 The role of sugar signalling in flower pigmentation

The flower is a major sink for assimilates, and a shortage of carbohydrates often inhibits flower development (Halevy, 1987). The role of sugars in flower development may be multifunctional: they can act as an energy source (Moalem-Beno *et al.*, 1997), as osmotic regulators (Ho and Nichols, 1977; Bieleski, 1993), as precursors for metabolic processes and as signalling molecules (Neta-Sharir *et al.*, 2000). Pulsing the cut flower with sugar improves flower development and pigmentation in commercial practice. Increased sucrose concentration enhanced petal growth and pigmentation in detached flowers of rose (Kuiper *et al.*, 1991), *Liatris spicata* (Han, 1992), *Eustoma grandiflorum* (Kawabata *et al.*, 1995) and *Hyacinthus orientalis* (Hosokawa *et al.*, 1996).

In *in vitro* conditions, detached petunia corollas elongated and pigmented only in the presence of sucrose and GA$_3$ in the light (Weiss and Halevy, 1989). Sucrose was required for the transcription of the *chs* gene and it enhanced the effect of the GA$_3$ (Weiss *et al.*, 1992; Moalem-Beno *et al.*, 1997). Other metabolic sugars such as glucose

and fructose had the same effect as sucrose (Weiss *et al.*, 1992). In arabidopsis and soybean leaves, sugars regulated *chs* expression directly (Tsukaya *et al.*, 1991; Sadka *et al.*, 1994). To act as signal molecules sugar phosphorylation by hexokinase is required to initiate signal transduction in most cases (Jang and Sheen, 1997). Sugars do not act as signalling molecules via modification of the osmotic potential of petal cells (Moalem-Beno *et al.*, 1997), nor are they dependent on their metabolism in glycolysis or on changes in phosphate level (Neta-Sharir *et al.*, 2000). It has been suggested that sugars may promote the GA-signal-transduction pathway or induce a specific *trans*-acting factor, which in turn, induces various GA-induced genes (Weiss, 2000). Recently, it is reported that the anthocyanin biosynthetic pathway could be specifically induced by sucrose in arabidopsis (Teng *et al.*, 2005; Solfanelli *et al.*, 2006). *MYB75/PAP1* gene was required in this induction. Sucrose, in a concentration-dependent way, induces *MYB75/PAP1* mRNA accumulation (Teng *et al.*, 2005).

It is not known whether sugars act as signals in petal pigmentation and patterning of antirrhinum.

1.4.3   The role of light controlling anthocyanin biosynthesis

Light is one of the most important environmental stimuli regulating anthocyanin accumulation, and acts both as an essential stimulus and as a factor that modulates the intensity of pigmentation (Piazza *et al.*, 2002). Under low light intensity plants develop pale flowers with low levels of anthocyanin (Biran and Halevy, 1974; Griesbach, 1992). When detached petunia flowers were cultured in the dark, corolla growth, anthocyanin accumulation and *chs* expression were strongly inhibited (Weiss and Halevy, 1991; Moscovici *et al.*, 1996). However, covering the attached young flowers on illuminated plants of petunia (Weiss and Halevy, 1991; Moscovici *et al.*, 1996) and rose (Biran and Halevy, 1974) did not inhibit petal growth and pigmentation. It appears that the green leaves perceived the light and transmitted a signal to the corolla (Moscovici *et al.*, 1996).

UV-B is required for normal flower pigmentation in apple (Dong *et al.*, 1998), and UV radiation promotes anthocyanin accumulation in kangaroo paw flowers (Ben-Tal and King, 1997). However, UV radiation has no effect on anthocyanin biosynthesis in the

flowers of petunia, *Geranium, Phlox, Impatiens, Lobelia* (Klein, 1990) and antirrhinum (Weiss, 2000).

In maize, the Myb and bHLH genes regulating pigmentation are light responsive (Scheffler *et al.*, 1994; Procissi *et al.*, 1997; Tonelli *et al.*, 1991, 1994; Petroni *et al.*, 2000; Piazza *et al.*, 2002). Different light qualities are differentially effective in the modulation of anthocyanin synthesis and accumulation in maize (Mereghetti *et al.*, 1991; Galbiati *et al.*, 1994; Piazza *et al.*, 2002).

In the "Red Star" phenotype of petunia, high light and low temperature can lead to larger uncoloured areas. However, the period during which pattern formation can be influenced is very short and takes place early in flower development (Marheineke, 1936, cited from Martin and Gerats, 1993).

There are no reports on the role of light in anthocyanin biosynthesis in antirrhinum.

1.4.4   Other factors affecting anthocyanin biosynthesis

In addition to GAs, other plant hormones can affect anthocyanin biosynthesis during plant development. Abscisic acid modulates anthocyanin accumulation in maize seeds by its ability to regulate *C1* gene expression (Kao *et al.*, 1996). Cytokinin treatment also stimulated anthocyanin accumulation in some plant organs (Deikman and Hammer, 1995; Piazza *et al.*, 2002).

Other factors affecting anthocyanin biosynthesis include temperature (Dela *et al.*, 2003; Wang *et al.*, 2005), the circadian clock (Deikman and Hammer, 1995), wounding (Pacholati *et al.*, 1985) and infection (Nicholson *et al.*, 1987). However, they may not be of particular relevance to flowers.

1.5   Pigmentation patterns in flowers

1.5.1   Unstable and stable patterns in flowers

There are many different pigmentation patterns that occur naturally in flowers. Pattern generally implies the localisation of pigment in specific areas. Flower pigmentation patterns can generally divided into unstable patterns and programmed 'stable' patterns. Onslow (1925) reviewed evidence of the diversity and complexity of pigmentation patterning in flowers. Early inheritance experiments of pigmentation patterning, which are based on Mendelian genetics, revealed multiple mechanisms of variant pattern formation. Onslow (1925) pointed out that of the patterns (that included spots, lines and streaks), some (*Papaver*, *Tropaeolum*) were inherited independently of the background colour of the flower, whereas the factors for others were intimately associated in various ways with the background colour factors (*Digitalis*, *Primula*). Some patterns were thought likely due to mutations.

Unstable mutations may present as unusual mosaic patterns through periclinal or mericlinal chimeras. The unstable anthocyanin expression is caused by the mobility of transposable elements (Coen *et al.*, 1986; Kho *et al.*, 1977). In cells in variegated areas, a transposable element is excised from a gene encoding an enzyme involved in a pigment biosynthetic pathway or from a gene involved in the transcriptional regulation of these enzymes. For example, in antirrhinum a mutant with ivory flowers with red spots, *pallida recurrens*, is unstable because of the insertion of the *Tam 3* transposable element near the promoter of the *pallida* (DFR) gene (Coen *et al*, 1986). In petunia, a white flowered mutant of the *an1* (bHLH) locus contained red spots due to an apparent back mutation in the epidermal cells of the young corolla (Kho, *et al.*, 1977).

The pigmented patterns in petals result primarily from the differential expression of the pigment biosynthetic genes, and this expression is controlled by regulatory genes (Martin and Gerats, 1993; Schwinn *et al.*, 2006). In antirrhinum, the *Del* gene product is required for anthocyanin biosynthesis in the flower tube (see Section 1.3.3.2) and lack of this transcriptional regulator results in the lack of pigment in the tube of this mutant (Almeida *et al.*, 1989; Martin *et al.*, 1991), but the flower lobes are fully pigmented.

This pattern is produced by the loss of function of *Del* in conjunction with *Mut* having limited activity in petals compared to the lobes.

In the study of the "Red Star" phenotype of petunia, run-on transcription study shows that the *chs* gene transcript does not accumulate in the acyanic areas. This fact suggests that the star pattern is the result of post-transcriptional control of *chs* expression (van der Meer, 1991, cited by Martin and Gerats, 1993).

1.5.2 Pigmentation patterning during petal development

Some patterns of pigmentation can be related to petal structures, such as in antirrhinum and tobacco where the corolla consists of two distinct regions, the tube and the lobes or limb (Figure 1.6). The epidermal cells in the tubes and lobes or limb are structurally distinct (Drews *et al.*, 1992). In some phenotypes, the region of differentiation is accompanied by a different pigmentation pattern (Martin and Gerats, 1993). The inner epidermal cells of the limb or lobes develop a specialised conical form compared to the normal flattened shape of epidermal cells such as in the tube (Kay *et al.*, 1981). These conical cells contain the highest concentrations of anthocyanins, and it is believed that they reflect incident light to enhance the impact of their pigment as a signal (Kay *et al.*, 1981). Other patterns do not follow clear structural boundaries between pigmented and unpigmented cells such as in wild type antirrhinum (Stubbe, 1966) and the "Red Star" phenotype of petunia (Martin and Gerats, 1993). Interestingly, the star pattern of petunia is variable and depends on environmental conditions (especially light) that do not affect flower morphology (Martin and Gerats, 1993). In antirrhinum, the shape of the pigmented conical cells is under control of the *myb* gene *Mixta* (Noda *et al.*, 1994). In *mixta* recessive lines the cells are flattened and the flower colour is dulled and less intense (Noda *et al.*, 1994).

Considering the relationship between pigmentation and petal morphogenesis, homeotic genes may play a role in the regulation of pigmentation, because their expression continues late into organ morphogenesis (Martin and Gerats, 1993). Although there is no strong evidence for direct transcriptional control of the anthocyanin biosynthetic genes in flowers by homeotic genes *in vivo*, it is possible that *deficiens (Def)* and *globosa (Glo)* (which determine petal and stamen identity) (Coen and Meyerowitz,

1991) activate genes such as *Del*, which in turn activate parts of the anthocyanin biosynthetic pathway (Martin and Gerats, 1993). This would be analogous to the genes identified for seed coat formation in arabidopsis (e.g. TT16) that also regulate the proanthocyanin regulatory factors (Nesi *et al.*, 2002).

Figure 1.6   Tobacco (*Nicotiana tabacum*) flower consists of two distinct regions, the tube and limb, and its pigmentation patterning is related to the petal structure, in which pigmentation only occurs in the limb.

1.5.3   Striped pigmentation patterning in flowers

Striping is a common form of pigment variation in many flowers and has been studied since the early 20th century (Onslow, 1925). Striped pigmentation patterning is the arrangement of colour in bands, stripes, or elongated patches. Onslow (1925) provided an early description of this pigmentation patterning:

> "these markings may vary in thickness from the narrowest hair-like streaks to broad bands, or elongated patches, which may then occupy almost the whole of the flower. It is difficult to define the limits of striping for, on the one hand, among striped varieties we frequently find sectorial variations in which colour is definitely and symmetrically confined to a half, a third, or some other fraction of the flower. On the other hand, striping may pass into spotting or blotching, and it is questionable whether spotted and blotched flowers should be placed in the same category, though their genetical behaviour may be similar".

Despite these early studies, there is surprisingly little information on the determinates and inheritance of flower colour patterning. Apparently, striping is a phenomenon that occurs in healthy plants. It can also occur when a plant is in an unhealthy condition, but this is rare (Hildebrand, 1893, 1896, cited by Onslow, 1925). The striping is most usual

in connection with anthocyanin, and the common form of striping is that of anthocyanin on an albino background, either white or yellow (Onslow, 1925). Among striped patterning plants many are venation patterning ones.

With exception of recent research on transposons, most studies on the inheritance of striping, in antirrhinum and other striped flowers, are still in the older literature (Correns, 1910; de Vries, 1911: Emerson, 1914; Gregory, 1911, cited by Onslow, 1925). Researchers attempted to explain this phenomenon using Mendelian principles and bud sports. However, convincing explanation for the inheritance of striping in genera such as *Antirrhinum* and *Mirabilis* from a classical genetics point of view was not achieved at that time, perhaps due to unknown transposon activities. However, in *Primula sinensis* striping behaves as a simple recessive to a self-coloured form (Gregory, 1911, cited by Onslow, 1925). It was reported more recently that the corolla striping in *Salpiglossis sinuate* was controlled by a recessive gene (*st*) (Conner and Erickson, 1991).

1.6 *Antirrhinum majus* as a model species for the study of pigmentation patterning

Antirrhinum is a member of the dicotyledonous family *Scrophulariaceae*. It is perennial, simple to cultivate, with a relatively short life cycle, self-fertile and easy to outcross. These features are advantageous in genetic analysis. The inflorescence is at the end of the shoot. Flowers are set on a very short stem and point in all directions. The flower shows bilateral symmetry. Bracts are much shorter than the petals, and sepals are egg shaped and about 5 mm in length. Petals are 2 to 3 cm in length when mature, fused to form a corolla with a tube and five lobes. The flower is relatively large, which makes it easy to collect a particular tissue or organ for molecular analysis. Furthermore, a series of transposable elements has been cloned and characterised (Coen and Carpenter, 1986; Sommer *et al.*, 1988), contributing to the usefulness of antirrhinum as a model species for plant development studies.

The bud finishes cell division when about 10 mm long and extends to the full mature length by cell expansion, which takes around 12 days in total (Jackson, 1991).

Anthocyanins are present at a very early stage of flower development and accumulation increases as flowers develop from the bud to the mature flower (Bartlett, 1989). Anthocyanins accumulate during the petal elongation stage, which is after the establishment of many of the boundaries in petal form (Coen *et al.*, 1986). They are restricted to the inner and outer epidermal cells of the petals, and are more abundant in the cells of the inner epidermis of the lobes and the cells of both inner and outer epidermis at the base of the flower tube (Martin and Gerats, 1993). In wildtype lines the throat and face of the corolla are pigmented with the yellow aurones. The aurones are also epidermally located (Asen *et al.*, 1972) and increase in abundance when the flower develops (Geissman *et al.*, 1954). Aurone is a type of flavonoid. Its biosynthesis pathway is shown in Figure 1.4.

As an important subject of scientific research, the floral pigmentation in this species has been studied for several decades in diverse aspects from genetics and biochemistry to molecular biology ([Basteson, 1902; Wheldale, 1907, 1909; Baur, 1910; Wheldale, 1913, 1914], cited by Onslow, 1925; [Scott-Moncrieff, 1930, 1936; Geissman *et al*, 1954; Jorgensen and Geissman, 1954, 1955; Geissman and Harborne, 1955; Dayton, 1956; Harborne, 1963], cited by Schwinn, 1999; Harrison and Stickland, 1974, 1977, 1978; Stickland and Harrison, 1974; Stickland *et al.*, 1976; Forkmann and Stotz, 1981). As a result, the anthocyanin biosynthetic pathway has been elucidated. The antirrhinum anthocyanin biosynthetic genes which have been isolated and characterised, include those for CHS, CHl, F3H, DFR, ANS and A3GT (Wienand *et al.*, 1982; Martin *et al.*, 1985, 1991; Coen *et al.*, 1986; Sommer and Saedler, 1986; Beld *et al.*, 1989) (Figure 1.4). Such information provides the basis for further study on the spatial and temporal control of anthocyanin biosynthesis in antirrhinum flowers. Some regulatory genes controlling this biosynthetic pathway have also been cloned and characterised (see Section 1.3.3.2). This makes it possible to further study the mechanism of pigmentation patterning.

As a traditional ornamental plant, in addition to the original species phenotypes of the *Antirrhinum* genus, many more phenotypes have been developed by breeders. Obviously, the occurrence of various pigmentation patterns can make these ideal materials for the study of floral pigmentation patterning. One striking phenotype identified in a range of *Antirrhinum* species is venation pigmentation patterning, in

23

which the epidermis over the veins is intensely pigmented with anthocyanins. The hypothesis behind the studies reported in this thesis is that the striped pattern is due to the spatial expression of *Venosa*; that this spatial property is transcriptionally controlled; and that a high level signal such as gibberellin or sugar directs this patterning. As will be apparent from this introductory chapter, this hypothesis was formed from knowledge on the activity of other regulatory factors in antirrhinum, as little was known of the control of patterning, and from results on GA and sugar actions in other species, as their role in antirrhinum flowers was also unknown.

1.7   The aims and objectives of the project

The overall objective of this project was to determine, at the gene and molecular level, the regulatory mechanism of venation anthocyanin pigmentation patterning in the flowers of the model species *Antirrhinum majus.*

Based on the isolated *myb* regulatory gene *Venosa*, the specific research aims of this project were:

- To determine the role of *Venosa* in the formation of pigment venation patterning in antirrhinum flowers by genetic complementation, mRNA *in situ* hybridisation and RNAi inhibition experiments.

- To determine the controlling mechanism of the *Venosa* promoter by promoter deletion assays using both transient expression and stable transgenic plants.

- To determine whether gibberellin and/or sugar play a role in the venation pigmentation patterning of the petals in antirrhinum by emasculation, girdling and *in vitro* supplementation experiments.

Chapter 2

Methods and Materials

2.1   Plant material

Seeds of *Antirrhinum majus* were germinated in pots, and the plants grown in a glasshouse at Crop & Food Research, Palmerston North. Environmental conditions in this glasshouse were variable: it was partially temperature controlled (vents opened and electric fans switched on when the temperature exceeded 21°C); lighting was not controlled. The GMO glasshouse conditions were similar. Plants of *A. majus* showing the venation phenotype were usually selected for study. Figure 2.1 A and B show the full developmental sequence of flowers on an *Antirrhinum* inflorescence.

The following lines were used in this study: line CT128 showed the venation phenotyp; lines CC112 and CL144 showed the *rosea*^{dorsea} phenotype; line CC112P showed fully pigmented except for the inner tube area of the flower; line 522 was fully pigmented with venation patterning in the inner tube area of the flower. The corresponding phenotypes are shown in Figure 1.3.

The *Antirrhinum* species used in this study also included *A. graniticum, A. mollissimum, A. meonanthemum, A. barrelieri, A. latifolium* and *A. molle.*

Tobacco transgenic lines from Professor Cathie Martin were also analysed. These lines harboured three *Venosa* promoter deletion constructs, in which three fragments, -760 bp, -1.6 kbp and -2.4 kbp relevant to ATG start site, drive GUS. They were provided as F2 seeds.

A

B

50,   40,   30,   25,   20, 12, 9, 7, 5, 3, 2, Bud length (mm)
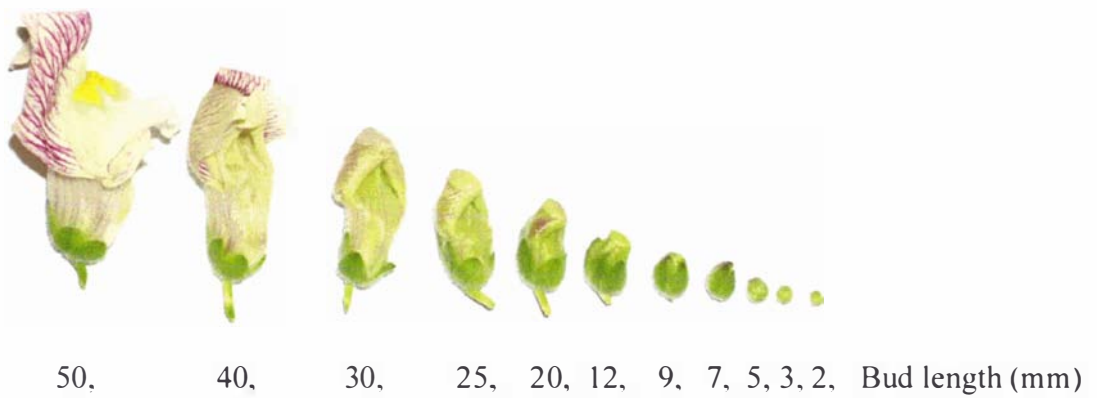
Figure 2.1   Flower developmental stages of *A. majus* venation phenotype (in *rosea*[dorsea] background) in inflorescence (A) and determined in bud length (B).

2.2 General bacterial growth and plasmid purification methods

The general plasmid isolation and bacteria growth methods were used primarily to prepare plasmid DNA for cloning experiments, DNA amplifications, particle bombardment experiments and making stable transgenic tobacco plants. All glassware used with plasmid manipulations and for growth of bacteria cultures was autoclaved.

2.2.1 Growth of bacterial cultures

Media used:
- LB - 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, and 1% NaCl pH 7.0; for solid media 1.5% (w/v) bactoagar was added.
- YEB - 0.1% (w/v) yeast extract, 0.5% (w/v) beef extract (Bovril), 0.05% (w/v) bacto-peptone, 0.05% (w/v) sucrose, 0.05 (w/v) $MgSO_4$, pH 7.0; for solid media 1.5% (w/v) bactoagar was added.

Liquid *E.coli* cultures were grown in LB media at 37°C with vigorous shaking at 250 rpm. Appropriate antibiotics were added after autoclaving the media. Liquid *A. tumefaciens* cultures were grown in YEB or LB media at 28°C with vigorous shaking at 250 rpm.

2.2.2 Plasmid DNA preparation

Plasmids were purified from bacteria (*E.coli*) grown in liquid culture containing the appropriate antibiotics. Two methods (I and II) were used for DNA preparation:

1. A quick miniprep of plasmid DNA was used for the selection of positive colonies. Solutions used in this method:

- Solution I – 50 mM glucose, 25 mM Tris-HCL, 10 mM EDTA pH 8.0.
- Solution II - 0.2 M NaOH, 1% SDS (w/v).
- Solution III - 3 M potassium acetate, 11.5 % (v/v) glacial acetic acid.

A 1.5 ml LB culture grown overnight with appropriate antibiotics was harvested by centrifugation at 14 000 rpm for 5 min at room temperature in a benchtop microfuge. The pellet was resuspended in 100 µl of Solution I. 200 µl of freshly prepared Solution II was added and mixed by gently inverting the tube 5 times. 150 µl of Solution III was added and mixed by gently inverting the tube 5 times. The tube was centrifuged at 14000 rpm for 5 min at room temperature. The supernatant was removed into a new tube (care was taken to avoid including any white precipitate with the supernatant). The DNA was precipitated with 2 volumes of ice-cold absolute ethanol and was recovered by centrifugation at 14 000 rpm for 10 min at room temperature. The pellet was washed with 70% (v/v) ethanol and air-dried, then resuspended in 50 µl autoclaved distilled water containing 1 µl of 10mg/ml RNase A.

II. For preparation of plasmid DNA for sequencing and particle bombardment experiments commercial kits (SIGMA GenElute™ Plasmid Miniprep Kit, QIAGEN QIAprep® spin miniprep kit, QIAGEN Plasmid Midi Kit or Maxi Kit) were used. The protocols were based on the manufacturers' recommendations. The bacteria were lysed under alkaline condition by adding a given buffer solution, buffer P1. The lysate was subsequently neutralised by adding buffer P2. The bacterial lysate was then purified by centrifugation and the clear supernatant was passed through a membrane column to bind the DNA. The bound DNA was washed with ethanol solution, and eluted with sterile water. Purified DNA was stored at -20°C.

2.2.3   Transformation of *E. coli* by heat shock

Frozen competent NovaBlue *E.coli* cells were thawed on ice. An aliquot (50 µl) was dispensed into a sterile 1.5 ml microfuge tube. Plasmid DNA (10 ng) or ligation reaction mix (about 50 – 100 ng in total) was added and gently mixed with the cells, which were then incubated on ice for 10 min. Cell-DNA mixture was heat shocked at 37°C for 1 min then incubated on ice for 10 min for recovery. 300 µl of LB was then added. If SCS 110 strain cells were used an incubation at 37°C for 30 min was done after adding the LB. 50 µl was plated onto one LB plate containing the appropriate antibiotic and 300 µl was plated onto another, then grown overnight at 37°C.

2.2.4 Transformation of *A. tumefaciens* by electroporation

Cell-porator (Life Technologies, INC) was used for electroporation. Aliquots (20 µl) of frozen *A. tumefaciens* strain LBA4404 competent cells (Invitrogen, Life technologies) were thawed on ice and 1 µl (100 - 500 ng) plasmid DNA added, mixed, then the sample was pipetted into the chilled electroporation cells between the two raised points. The units were switched on and the Booster was set to the following parameters: LOW = Ω, CAPACITANCE = 330, CHARGE RATE = Fast, KΩ reading = 4. The cells were placed into the electroporation chamber, which was then closed tightly and connected up to the machine. Whilst on charge mode the 'up' button was pressed until the desired 300V was obtained. Then the unit was switched to ARM mode and the trigger button was pressed for one second (the booster unit read about 1.8V). The cells were taken out and pipetted into a 50 ml tube containing 1 ml LB medium. The cells were cultured in a shaking incubator at 28°C for 2 h. 50 µl was taken out, spread onto the appropriate plate and cultured in a 28°C incubator for several days. Colonies were picked for downstream research.

2.2.5 Storage of bacteria

Bead stocks were prepared using Microbank^{TM} microbial storage (PRO-Lab, Diagnostics) according to the protocol manual and stored at - 80°C until required.

2.3 General DNA methods

2.3.1 Quantification of DNA

DNA was quantified either by spectrophotometry, or by agarose gel electrophoresis and staining with ethidium bromide. For spectrophotometric quantification, a UV-160A UV-visible Recording Spectrophotometer was used (Shimadzu). DNA was diluted and dispensed into quartz cuvettes. Absorbance readings at 260 and 280 nm wavelength light were recorded. The quantity of DNA was calculated according to the formula C = OD_{260} x 50 x D for double stranded DNA. D is the dilution factor. 260/280 ratios were

used as an indication of purity with a reading of about 1.8 indicating relatively pure DNA samples.

To quantify DNA by agarose gel eletrophoresis, DNA was run on 0.8-2.0% (w/v) agarose gels along with a known concentration of specific molecular weight markers (High DNA mass ladder or Low DNA mass ladder, Invitrogen). Gels were stained with ethidium bromide and the intensity of fluorescence of stained DNA was compared to the known masses of the molecular weight markers.

2.3.2   Electrophoresis of DNA

DNA separation by electrophoresis was carried out by loading DNA in 0.8-2.0% agarose gels in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) or 1 x TBE (90 mM Tris-borate, 1mM EDTA) buffers. Appropriate DNA molecular weight markers were used, either 1 kb plus DNA ladder or 100 bp DNA ladder (Invitrogen). Ethidium bromide staining was done either by incorporating ethidium bromide in the gel or by staining the gel in ethidium bromide solution. Stained DNA was visualised with a digital imaging system (MultiImage^{TM} light cabinet, Alphainnotech Corporation).

2.3.3   Amplification of DNA by PCR

Oligonucleotide primers were synthesised by Sigma Genosys and resuspended to a stock concentration of 1 mM. The stocks were further diluted to a final concentration of 10 μM for PCR stocks. The PCR reactions were performed in 50 μl final volume containing 5 μL 10x PCR buffer, 1.5 μL each primer (from 10 μM stock solution), 1.5 μL each deoxynucleotide triphosphate (dNTPs) (from 10 mM stock solution) (Roche), and 2.5 units of polymerase, either Taq (5 u/μL) (Roche, QIAGEN), Platinum *Pfx* (5 u/μL) (Invitrogen, Life technologies) or PWO (5 u/μL) (Roche). The remaining volume was made up of water, Mg^{2+} (final concentration 1.5 μM) and template DNA (1-5 ng plasmid DNA, or 50-350 ng genomic DNA). For colony PCR, colonies were picked into 20 μL water, then 5 μL of the mixture were used as template. The following standard PCR cycle conditions were used in this study: one cycle of 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min/kb; then one cycle of 72°C for 7 min. The PCR program varied slightly depending on the primer set

used. The annealing temperature, for example, depended on the melting temperature of the primers and the elongation time related to the size of the amplification product. PCR reactions were performed in a Eppendorf Mastercycler gradient or Techne PHC-3 thermal cycler.

2.3.4   DNA purification, digestion and ligation

DNA was digested with restriction enzymes (Roche). Double digests or partial digests were used according to experimental requirements. To purify digested DNA or PCR products MinElute Gel Extraction Kit (QIAGEN) or High Pure PCR Product Purification Kit (Roche) were used. DNA ligation was carried out using the Rapid DNA ligation kit (Roche). When necessary DNA fragments were dephosphorylated prior to ligation by treating with Shrimp Alkaline Phosphatase (Roche).

2.3.5   Sequencing

All sequencing was carried out at either Waikato DNA Sequencing Facility at the University of Waikato, or the Massey University DNA Sequencing Facility. DNA sequences were analysed using the DNA star software package (LASERGENE, Madison Wisconsin). The sequence data of newly identified genes is supplied in appendix including the attached CD in sequencher format.

2.4   Plant RNA and DNA extraction protocol

The extraction of plant RNA and DNA was based on the following protocol developed by Dr Huaibi Zhang (Crop and Food Research, Palmerston North, New Zealand). A CTAB (Cetyltrimethylammonium bromide) extraction buffer was prepared in 50-mL Nunc tubes containing:

> 0.3g CTAB power (2% w/v)
> 0.3g SDS or 1.5 mL 20% SDS (2% w/v final)
> 0.15g PVP (soluble)
> 1.5 mL 1 M Tris-HCl (pH 8)
> 0.75 mL 0.5 M EDTA (pH 8)

6.0 mL 5 M NaCl

The components were mixed and the solution was made up to the 15 ml marker and autoclaved. The extraction buffer was warmed to 55°C in a water bath. 0.3 mL ß-mercaptoethanol was added to 15 mL extraction buffer before 1-3 g ground tissue was added. The material was mixed completely by inverting the tube and then vortexing for 1 min. An equal volume of chloroform:isoamylalcohol (24:1) was added, then the tube was vortexed for 1 min and incubated at 55°C for 15 min. The sample was spun at 6500 rpm (ss-34 rotor) at 4°C for 15 min to separate phases, then the supernatant was removed to a 50-mL Nunc tube and kept on ice. An equal volume of chloroform:isoamylalcohol was added to the supernatant, then the sample was vortexed for 30 seconds and spun at 6500 rpm for 15 min. The aqueous supernatant was removed to a new plastic tube. The volume of supernatant was measured accurately and 1/3 volume of 8 M LiCl (-20°C) was added to the supernatant and mixed. RNA was precipitated overnight at 4°C and harvested by centrifugation at 10000 rpm (ss-34 rotor) for 20 min at 4°C. The supernatant was transferred carefully into a new Nunc tube for DNA extraction. The RNA pellet was dissolved in 400 µl 3 M sodium acetate and transferred into a 1.5 mL tube, then reprecipitated by adding 2.5 volume 100% ethanol. The sample was spun at 13000 rpm for 20 min, the ethanol was discarded and 70% ethanol was used to wash the RNA pellet. The RNA pellet was then air-dried and dissolved in 200 µL water. For DNA isolation, 1 volume of room temperature isopropanol was added to the supernatant contained in a Nunc tube, then DNA was spun down at 1000 rpm for 20 min. The DNA pellet was washed with 70% ethanol and then dried. The DNA was dissolved in 200 µL water.

A commercial kit, Nucleon Extraction & Purification (Amersham, Life Science) was used as an alternative way for DNA extraction. DNA extraction was carried out according to the manufacturer's manual. The plant material (1.0 g fresh weight) was disrupted by grinding in liquid nitrogen, then cells were lysed with potassium / SDS. The cell debris and salt precipitate were removed by Nucleon PhytoPure resin and chloroform. DNA was then precipitated and washed.

2.5   Construct generation

2.5.1   construct generation for genetic complementation

The p7GSV plasmid was made as follows. A PCR product of the genomic *Venosa* gene was cloned into the KpnI/SmaI digested pART7 vector (Gleave, 1992; Figure 2.2) to generate 35S:genomic *Venosa* construct. A 35S:*Lc* cDNA construct (lab stock pLc349)
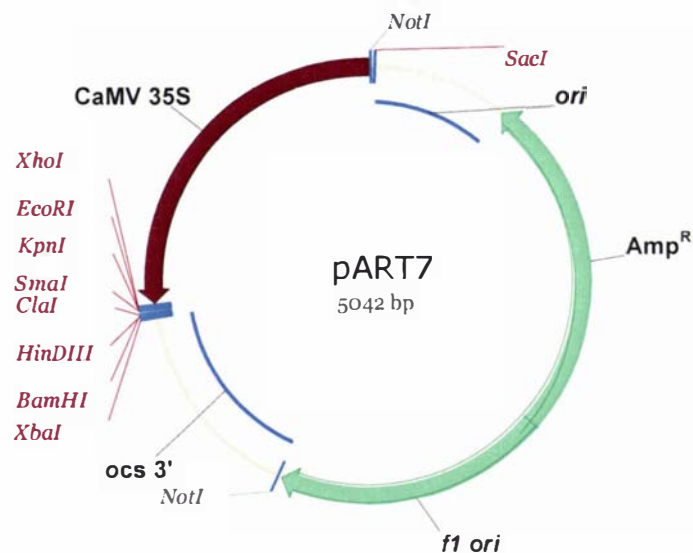
Figure 2.2   The map of the vector used for promoter deletion constructs, pART7. A PCR product of genomic *Venosa* gene was cloned into the KpnI/SmaI digested vector to generate a 35S:genomic *Venosa* construct.

served as control in this experiment. *Lc* (*Leaf colour*) is a bHLH gene controlling anthocyanin biosynthesis in maize (Goff *et al.*, *1992*; Sainz *et al.*, *1997*).

2.5.2 Preparation of *Venosa* promoter 5' deletion constructs

The strategy for making the required deletion constructs is schematically presented in Figure 2.3. *mgfp* (Haseloff *et al.*, 1997) was amplified with PCR using Y05/Y06 (Appendix I) as primers and plasmid pPN92 (a construct of 35S:GFP) as template, then cloned downstream of the 35S promoter and into KpnI/SmaI digested pART7. The insert was confirmed with sequencing. It was designated as p7GFP.

Cis-acting elements are typically short sequences. To help retain potential functional *cis* elements for the promoter assay of 5' deletion mutagenesis, the 900 bp fragment of the *Venosa* promoter was analysed with the PLACE database (http://www.dna.affrc.go.jp/PLACE/), a database of plant *cis*-acting regulatory DNA elements (Higo *et al.*, 1999), so that any predicted elements could be left intact. The PLACE map is shown in Appendix VI. Seven regions without any element in the PLACE map were determined to make 5' deletions. Accordingly, six forward primers were designed (see Appendix I). A series of 5' deletions were made at intervals of about 100 bp (Figure 2.4) with PCR using construct pJAM1371 (*-2.4 kVEN:GUS*, made by our UK collaborator) as template, and cloned into SacI/KpnI digested p7GFP, replacing the 35S promoter. The insert was confirmed by sequencing. The deletion constructs using other reporters such as *Rosea* cDNA, IGUS and genomic *Venosa* were made in a similar manner. How many deletion constructs were made using a given reporter gene depended on the necessity of the experiments. All the constructs are summarized in Table 2.2.

The constructs were transformed into *E.coli* strains Novablue or SCS110. Midiprep plasmids prepared using commercial kits were used for transient assay by particle bombardment.
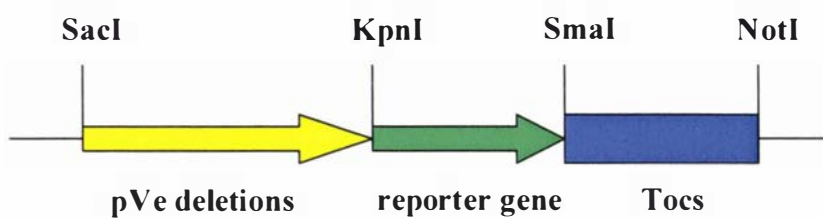
Figure 2.3 Schematic representation of the structure of deletion constructs (not to scale). The gene encoding GFP, IGUS or Myb Anthocyanin Regulator (MAR) was cloned into KpnI / SmaI digested pART7 (see Figure 2.2) first, then the *Venosa* promoter deletion fragments were cloned into SacI/KpnI sites to produce a series of *Venosa* promoter deletion constructs. pVe deletions, *Venosa* promoter 5' end deletion fragments; reporter gene, the gene encoding GFP, IGUS or MAR; Tocs, ocs terminator.

A

AAAATAAGGTAATATTTGTATATCGAGCATTTTAACACTTTGCTGACATTACTAACTTTTGTTGGCATTTAAAC

GGTGGGACTGGTCCCTGGTGCATGCAGCGACAAACTTCAGGGAATTCATATTATTAATATCTTGGAGATGGGG

TATTTAAGACAAATCTTAATTAAGGCGTTACAAATTGTATCCCTAAATATTAAATGTTTCAGtATAATATATAAG

CAAATTGAGAAGAGGTCATGTATTCCAATATATCATTATTTATCTATACTTATGCAGCACGACTATTTAGTGCT

TAAAAGGAGCCTACTGGATCCATCGACTTTCAGTTAAAACCCGTCGTTACAAATCAATTATTTAACATAACTGC

TAATGGAGGTTTCTTTGTTAATTAATATCTGTATAAAACAAGTAATAAATACAAACAAGTAAAAATCAGTATAA

AATGAACATAAAACTTAgAACAACAATCAAAAGATTGTGATCCGGCTGGAAGCTAACCAATTAGTAAAGGAAAT

AAATAAACGATTTGCAGAAACGGGAAATGATAGATTAAAAGAAATCATCGAGTATGTCTTCAATGGGGCATGA

TAGGTGTTGCACTGAATATAGATATTTCTACTTTATGGTCGTTAAATATGCACCGTCATAATCCAGATTGAGTG

AATTGAAGGCAAAAAGATACGGAAATGGCATACATAAACTGGTGAAGGCTTCCATATTCAAGAATCATTATTA

GTAAAGGAAGGTGCAGTTAACTAGAGATTCAATTAATATTCTTATTTCTCCACACAATGTGCATATATGTAACA

TAATATAGATGTAGGTTTTAAGCTTGCACAGAACGAAATAGAGCAATAGGCAAATCAAGTGAACACCCATG

B

Figure 2.4 The main 5' end *Venosa* promoter deletions. (A)The positions of forward primers are highlighted in red and indicated by arrow. The position of reverse primer was highlighted in blue before the start coden TAG. (B) The graphic showing the corresponding deletions. The pink bar represents 5' end *Venosa* promoter deletion fragments; the black bar represents reporter genes; the green bar represents ocs terminator.

Table 2.1   List of *Venosa* promoter deletion constructs

The template and primers of the reporter gene	Construct name	construct
pPN92 (a construct of 35S:mGFP) as template, Y05/Y06 as primers	p7GFP	*35S:GFP*
	pVE01	*-746VEN:GFP*
	pVE04	*-630VEN:GFP*
	pVE05	*-500VEN:GFP*
	pVE06	*-380VEN:GFP*
	pVE07	*-260VEN:GFP*
	pVE08	*-150VEN:GFP*
pJAM1468 (a construct of 35S:*Rosea* cDNA) as template, Y03/Y04 as primers	p7Rosea cDNA	*35S:Rosea*
	pVE11	*-746VEN:Rosea*
	pVE14	*-630VEN:Rosea*
	pVE15	*-500VEN:Rosea*
	pVE16	*-380VEN:Rosea*
	pVE17	*-260VEN:Rosea*
	pVE18	*-150VEN:Rosea*
pMOG410 (Vancanneyt *et al.*, 1990) as template, Y25/Y26 as primers	p7IGUS	*35S:IGUS*
	pVE40	*-900VEN:IGUS*
	pVE41	*-746VEN:IGUS*
	pVE44	*-630VEN:IGUS*
	pVE45	*-500VEN:IGUS*
	pVE46	*-380VEN:IGUS*
	pVE47	*-260VEN:IGUS*
	pVE48	*-150VEN:IGUS*
	pVE71	*-120VEN:IGUS*
	pVE72	*-100VEN:IGUS*
	pVE73	*-90VEN:IGUS*
	pVE40-2	*promoterless:IGUS*
Venation phenotype genomic DNA as template, Y44/Y45 as primers	p7GSV	*35:gVenosa*
	pVE900GSV	*-900VEN:gVenosa*
	pVE500GSV	*-500VEN:gVenosa*
	pVE200GSV	*-150VEN:gVenosa*
	pVE120GSV	*-120VEN:gVenosa*
	pVE100GSV	*-100VEN:gVenosa*
	pVE90GSV	*-100VEN:gVenosa*
	pVE0GSV	*promoterless:gVenosa*
Constructs made by UK collaborator	pJAM1334[a]	*-760VEN:GUS*
	pJAM1367[a]	*-1.6kVEN:GUS*
	pJAM1371[a]	*-2.4kVEN:GUS*

a: structure of constructs is based on information from Professor Cathie Martin (John Innes Centre), and has not been confirmed by sequencing.

2.5.3 Arabidopsis ubiquitin promoter transcriptional enhancer and Omega translational enhancer constructs

A 1.4 kb fragment of *Arabidopsis* ubiquitin promoter upstream of the TATA box was amplified by PCR using *Arabidopsis* genomic DNA as template and Y28/Y29 as primers, and cloned into SacI digested p7IGUS. The sequence of the insert was confirmed by sequencing. This 1.4 kb fragment was cut off and cloned into SacI digested p27IGUS, p27VEN900IGUS and p27VEN700IGUS in both directions (pART27 is a binary vector, which is used to make stable transgenic plants. Its map is shown in Figure 2.6. All the binary constructs including p27IGUS, p27VEN900IGUS and p27VEN700IGUS were described in Section 2.5.6). The schematic structure is shown in Figure 2.5 A. All the constructs are summarized in Table 2.2. They were designated as p27UFIGUS (forward ubiquitin-35S:IGUS in pART27), p27URIGUS (reverse ubiquitin-35S:IGUS in pART27), p27UF900IGUS (forward ubiquitin-900 bp *Venosa* promoter:IGUS in pART27), p27UR900IGUS (reverse ubiquitin-900 bp *Venosa* promoter:IGUS in pART27), p27UF700IGUS (forward ubiquitin-700 bp *Venosa* promoter:IGUS in pART27) and p27UR700IGUS (reverse ubiquitin-700 bp *Venosa* promoter:IGUS in pART27) respectively.

Ω is a 68 bp translational enhancer (Gallie *et al.*, 1987). It was made from two oligonucleotides, positive and negative strands (Y41/Y30), and cloned into KpnI/HindIII digested pART7, then IGUS was inserted into HindIII. This construct was designated as p7ΩIGUS. The fragment of -900 bp *Venosa* promoter was cut off from *-900VEN:IGUS*, then cloned into SacI/KpnI digested p7ΩIGUS. It was designated as pVe900ΩIGUS. The physical map is shown in Figure 2.5 B.

2.5.4 Gal4 two-step transcriptional amplification system constructs

Method I

A 0.8 kb fragment upstream of the TATA box of the *Venosa* promoter was amplified with PCR using pJAM1371 as template and Y39/Y13 as primers. This fragment was designated as ΔpVe and cloned into the SacI/KpnI digested pART7. The corresponding construct was designated as pΔVe. Plasmid pC-4956:ET15 (Johnson *et al.*, 2005) was

digested with KpnI and the small fragment containing the Gal4 trapper was purified. It was cloned into the KpnI digested pΔVe. The plasmid with correct orientation of the insert was checked using restriction fragment analysis and confirmed by sequencing. The structure is shown in Figure 2.5 C. This construct is designated as pΔVetrapper.

The adaxial area of venation phenotype antirrhinum petals was bombarded with pΔVetrapper or pC-4956:ET15.

Method II

pC-4956:ET15 was digested with KpnI. The small fragment was the enhancer trapper (Figure 2.5 C). It was then digested into two fragments with HindIII. One fragment was gene for Gal4 protein, in which GAL4 was driven by -40 bp of 35S core promoter. Another was reporter system, in which *mgfp* was driven by -90 bp of 35S core promoter combining five tandem repeats of GAL4 binding site (Figure 2.5 C). The petals of venation phenotype antirrhinum were bombarded with each fragment or a mixture of both.

2.5.5   Construction of constructs to use PTGS inhibitor p19 in transient assay

To test the efficacy and efficiency of p19 (Voinnet *et al.*, 2003) in transient assays (Section 2.6) of *Venosa* promoter activity, four different methods were attempted.

Method I   A *35S:p19* construct (supplied by Dr Kathy Schwinn and designated pPN 308) and each *VEN:IGUS* construct were co-precipitated on gold particles then bombarded into the adaxial epidermal cells of *rosea*^{dorsea} petals, or leaf epidermal cells of tobacco (*Nicotiana tabacum* cv. Samsun). The petals were also bombarded with a mixture of *-700VEN:GUS* and pART7.

Method II   *35S:p19* and *VEN:IGUS* were constructed into one vector (Figure 2.5 D), then bombarded into epidermal cells. The constructs were made using the following strategy: the fragment containing 35S:p19:terminator was digested from pBIN61-p19 (from Roger Hellens, HortResearch), and the fragment containing

VEN:IGUS:terminator was cut off from pVeIGUS, then both fragments were cloned into pDAHCROP3E (from Don Hunter, Crop & Food Research).

Method III   The petal tissue was bombarded with *35S:p19* construct, then with *VEN:IGUS*. To test if two different constructs can be introduced into one cell with separate bombardments, the adaxial area of *rosea^dorsea* petals was bombarded with *35S:GFP* then *35S:Venosa*.

Method IV   A *-900VEN:p19* construct was made. The fragment of -900 bp *Venosa* promoter was amplified with PCR using pJAM1367 as template and Y13/Y46 as primers (see Appendix I), then cloned into SacI/XhoI digested pPN308, so that it replaced the 35S promoter. It was designated as pVe900p19. This plasmid was co-precipitated with *VEN:IGUS* plasmid onto gold particles, then bombarded into petal epidermal cells.

Table 2.2 **The constructs used for enhancement of the expression of *Venosa* promoter deletions in particle bombardment**

Enhancement strategy	Construct name	Structure
1.4 kbp fragment upstream TATA box of Arabidopsis ubiquitin promoter as an enhancer	p27UFIGUS	*UF35S:IGUS*
	p27URIGUS	*UR35S:IGUS*
	p27UF900IGUS	*UF-VEN900:IGUS*
	p27UR900IGUS	*UR-VEN900:IGUS*
	p27UF700IGUS	*UF-VEN700:IGUS*
	p27UR700IGUS	*UR-VEN700:IGUS*
Omega fragment as an enhancer	p7 ΩIGUS	*35S:ΩIGUS*
	pVe900ΩIGUS	*-900VEN:ΩIGUS*
Gal4 enhancement system	pΔVetrapper	*ΔVe-40bp35Score:Gal4 trapper*
	Gal4 trapper	*pC-4956:ET15*
P19 as an inhibitor of PTGS	pVe900p19	*-900VEN:p19*
	pVe2.4p19	*-2.4kVEN:IGUS* and *35S:p19* in one vector
	pVe700p19	*-746VEN:IGUS* and *35S:p19* in one vector
	pVe200p19	*-150VEN:IGUS* and *35S:p19* in one vector
	pVe0p19	*Promoterless:IGUS* and *35S:p19* in one vector
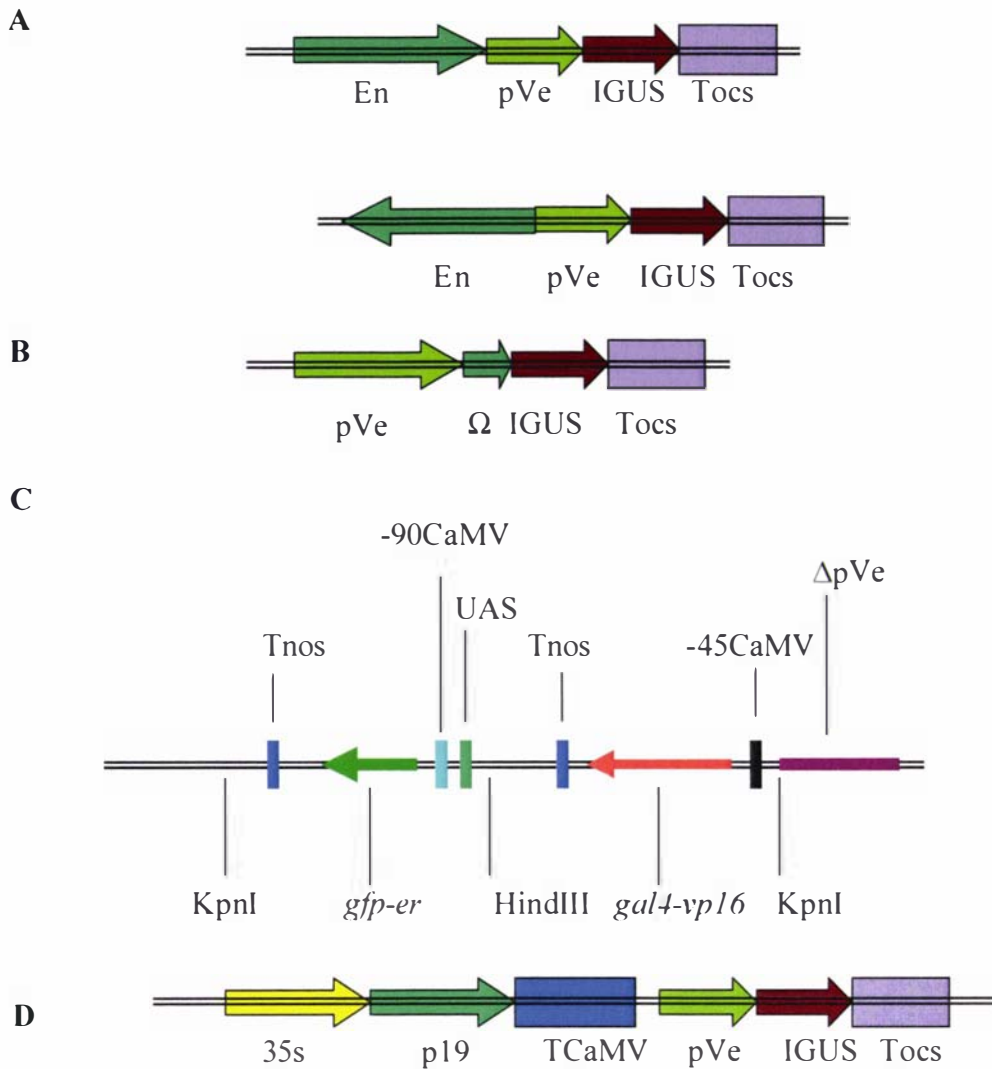
Figure 2.5   The constructs developed for strategies to enhance the expression of weak promoter deletions. The schematic structures are not to scale.

(A) Fusion of the transcriptional enhancer of Arabidopsis ubiquitin promoter upstream of *Venosa* promoter deletions.

(B) Cloning of the omega translational enhancer upstream of IGUS.

(C) Fusion of ΔpVe upstream of the -45CaMV fragment of the Gal4 two-step transcriptional amplification system.

(D) Fusion of *35S:p19* and *VEN:IGUS* into one expression vector.

Abbreviations are 35S, CaMV 35S promoter; p19, the sequence encoding the p19 protein of tomato bushy stunt virus; IGUS, intron GUS; Tocs, bacterial octopine synthase gene terminator; En, transcriptional enhancer; Ω, 68 bp sequence of 5'-leader of tobacco mosaic virus; ΔpVe, a 700 bp fragment upstream of *Venosa* core promoter; -45CaMV, -45CaMV 35S minimal promoter; gal4-vp16, gal4-vp16 transcriptional activator fusion gene; Tnos, bacterial nopaline synthase gene terminator sequence; UAS, five tandem repeats of the upstream activation sequence element GAL4 binding site; -90CaMV, -90CaMV 35S minimal promoter TATA; gfp-er, sequence encoding green fluorescent protein (mgfp5) with a signal to target the protein to the endoplasmic reticulum.

2.5.6 Construction of binary vectors

Binary vector pART27 (Gleave, 1992) was used for making the constructs for stable transformation described in this section. The cloning strategy used is shown schematically in Figure 2.6. The *VEN:REPORTER* deletion fusions were cut out with SacI / NotI from the corresponding cloning vector pART7, then cloned into the SacI / NotI sites of pART27. All binary constructs are listed in Table 2.3. Empty vector pART27 was used as a negative control, and *35S:IGUS* and *35S:GFP* constructs were the positive controls.

The construct plasmid was purified from *E. coli* strain Novablue using the procedure outlined in Section 2.3.4, then used to transform *A. tumefaciens* strain LBA4404 by electroporation. A positive colony harboring the correct construct was identified by checking the DNA using back transformation into *E. coli*. Plasmid DNA rearrangement can occur during *A.tumefaciens* transformation, and back transformation into *E. coli* with plasmids purified from *A.tumefaciens* colonies allowed the construct to be checked by restriction digestion.

A
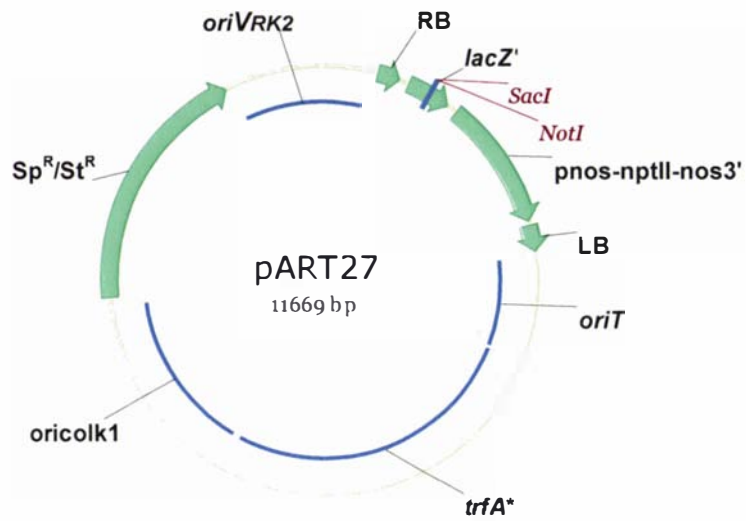
B
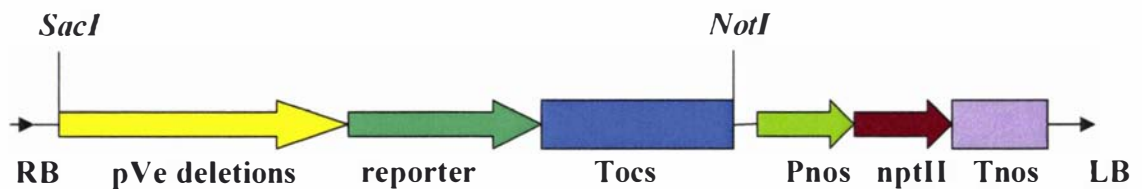
Figure 2.6 The strategy for making binary constructs for stable tobacco transgenics. (A) Binary vector pART27 used for making binary constructs. (B) Schematic representation of the T-DNA region (not to scale) of the binary vector constructs. The pVe promoter fragment:reporter:terminator cassette (see Figure 2.3) were digested with SacI/NotI, then cloned into the SacI/NotI sites of pART27 to produce a series of pVe27 binary constructs. RB, right border; pVe deletions, venosa promoter deletions; reporter, GFP, IGUS or MYB regulator of anthocyanin biosynthesis; Tocs, ocs terminator; Pnos, nos promoter; nptII, neomycin phosphotransferase II gene for kanamycin resistance; Tnos, nos terminator; LB, left border.

Table 2.3   Binary constructs

Construct Name	Construct Description
pVE21	-746VEN:mgfp
pVE24	-630VEN:mgfp
pVE25	-500VEN:mgfp
pVE26	-380VEN:mgfp
pVE27	-260VEN:mgfp
pVE28	-150VEN:mgfp
pVE31	-746VEN:Roseal
pVE34	-630VEN:Roseal
pVE35	-500VEN:Roseal
pVE36	-380VEN:Roseal
pVE37	-260VEN:Roseal
pVE38	-150VEN:Roseal
pVE50	-900VEN:IGUS
pVE51	-746VEN:IGUS
pVE54	-630VEN:IGUS
pVE55	-500VEN:IGUS
pVE56	-380VEN:IGUS
pVE58	-150VEN:IGUS
pPN92	CaMV35S:mGFP
p27IGUS	CaMV35S:IGUS
pART27	empty vector

2.6   Particle bombardment for transient gene expression

2.6.1   Preparation of gold particles

Gold particles (1.0 micron) (BIO-RAD LABORATORIES PTY LTD) were weighed out (100 mg) in 1.5 ml microfuge tube and washed briefly in 1 ml isopropanol. They were then washed three times with sterile water. On the last wash the gold particles were suspended in the water with sonication, then while pippetting up and down frequently, 50 µl portions were aliquoted out into Eppendorf tubes. They were stored at 4°C for later use.

2.6.2 Precipitation of DNA onto gold particles

Construct DNA (usually 10 µg, but sometimes as little as 1 µg depending on the experiments and the amount of DNA available) was added to the gold particle aliquot (50 µL) to a total volume of 60 µL (water was added to reach the final volume) and mixed briefly. Then 20 µL of 0.1 M spermidine was added with brief mixing. 50 µL of 2.5 M $CaCl_2$ was added and the mixture vortexed thoroughly. It was left for at least 3 min, during which it was vortexed three times. The gold was spun down briefly and 90 µL of supernatant was discarded. The gold particles were resuspended by pipetting up and down. A 5 µL aliquot was used for each firing.

2.6.3 Particle bombardment

A modified Helium gene gun (Finer *et al.*, 1992) was used for particle bombardment (Figure 2.7). The gun was cleaned and the filters which carry the DNA-coated gold particles were changed for different DNA samples. The shooting parameters were as follows: 300 kPa helium pressure; 30 micro second solenoid release time; 13 cm shooting distance; -14 kPa vacuum pressure. The material to be shot was petal or young leaf, sterilised (surface sterilised for 10-15 min in 10% (v/v) bleach containing 1-2 drops of 100m/L TWEEN, and then rinsed thoroughly in sterile water) or unsterilised. Tissue was bombarded once or twice, depending on the experiment. The plant materials were then cultured on ½ MS medium under light (16 hours photoperiod) at 25°C.
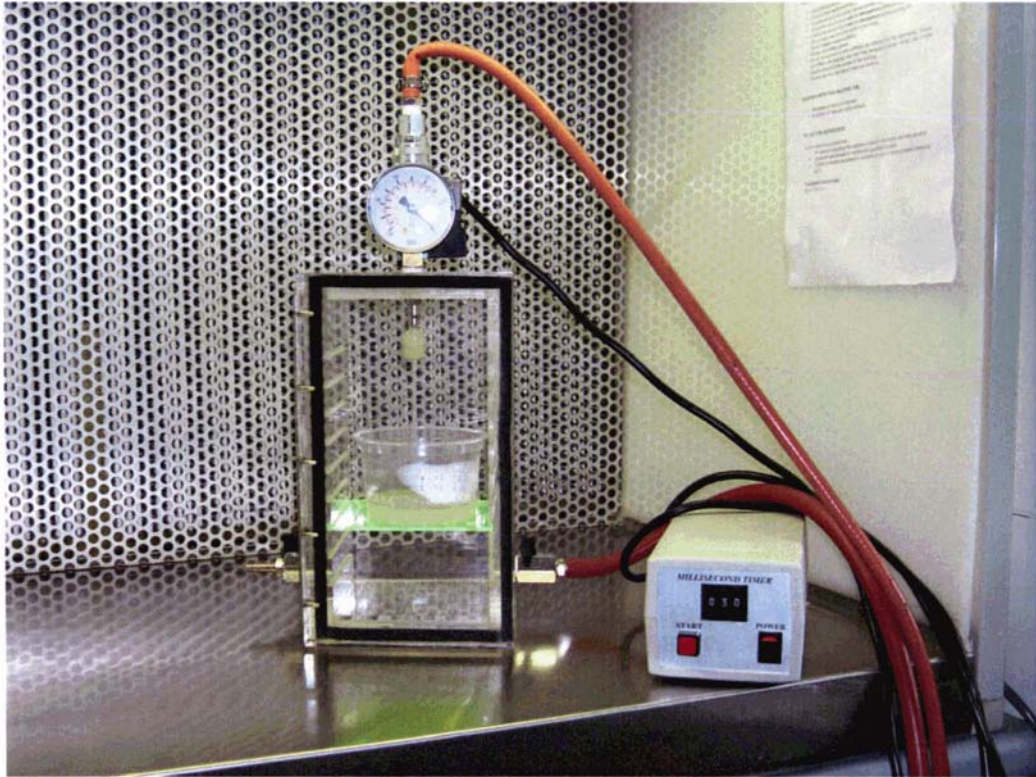
Figure 2.7   Helium particle inflow gun used in the biolistic bombardments in this study.

2.6.4   Plant material

The dorsal petals of nearly fully opened flowers of either the venation phenotype of antirrhinum or of *rosea*^{dorsea} were used unless stated otherwise. Both adaxial and abaxial sides were bombarded depending on the reporter used.

When the petals of *rosea*^{dorsea} were to be used with constructs containing a MYB anthocyanin regulator (*Rosea* or *Venosa*), they were first checked under the microscope and those without individual pigmented cells in the adaxial area were selected.

Two flowers were tested for one construct (tested gene or control) each time, and each experiment was repeated at least twice. The response of the tissue to the bombardment was observed using light microscopy over the following five days.

2.7   Stable tobacco transgenics

2.7.1   Preparation of *A. tumefaciens* culture

Bead stocks of *A. tumefaciens* strain LBA4404 containing the binary vectors (Table 2.4) were inoculated into 40 mL LB broth containing antibiotic(s). They were cultured in a shaking incubator at 28°C until the bacteria growth reached an $A_{600} > 0.5$.

2.7.2   Transformation

The following media were used in the experiments:
- Base medium (MS salts + B5 vitamins +30 g/L sucrose + 0.8% agar);
- Shooting medium (Base media + 0.1 µg/L NAA + 1.0 µg/L BAP);
- Shooting selection medium (Shooting media + 150 mg/L Kanamycin + 500 mg/L Ticarcillin);
- Rooting selection medium (Base media + 150 mg/L Kanamycin + 250 mg/L Ticarcillin).

Young healthy leaves from stock *in vitro Nicotiana tabacum* (cv. Samsun) plants or surface sterilised leaves from young plants in the glasshouse were cut into approximately 5 mm^2 discs to a total of 50 pieces for each construct, avoiding the central midrib. 30 discs were transferred into a beaker containing the *A.tumefaciens* culture and became thoroughly wetted. The discs were put onto sterilised Whatman paper until most of the excess liquid culture had been absorbed. They were then placed adaxial (top) side down on each plate with shooting medium. The discs were co-cultivated with *A.tumefaciens* in a 24°C culture room under low light for 3 days, then transferred to shooting selection medium. Twenty discs that had not been in contact with *A.tumefaciens* were used as controls: ten were transferred to shooting selection medium to ensure that the selection agent used prevented development of calli or shoots. The other ten leaf discs were kept on shooting medium to make sure that callus tissue did develop under the hormone regime being used. After 2-4 weeks, shoots began to emerge from the callus tissue. The explants were transferred to fresh media regularly. Once shoots were obviously elongated they were excised and transferred into a fresh

individually labeled media tub containing rooting selection medium. 20-50 independent lines were harvested for each construct. When the independent plants had proliferated adequately, a stem was cut off for each independent plant and transferred to individually labeled medium tubs containing rooting selection medium for backup purposes. One plant was planted into soil in the glasshouse for each independent line. The number of independent lines for each construct is listed in Table 2.4. Only plants with normal phenotypes were analysed.

The seeds for transgenic plant lines containing three other constructs (*-760VEN:GUS*, *-1.6kVEN:GUS* and *-2.4kVEN:GUS*) were obtained from Prof. Cathie Martin (UK). The seeds were sterilised and screened for rooting in media supplemented with kanamycin. The rooted plants were grown to flowering in the GMO greenhouse.

Table 2.4   The number of independent tobacco transgenic lines produced for each construct

Construct Name	Construct Description	Number of Lines
pVE21	*-746VEN:mgfp*	21
pVE24	*-630VEN:mgfp*	17
pVE25	*-500VEN:mgfp*	23
pVE26	*-380VEN:mgfp*	16
pVE27	*-260VEN:mgfp*	13
pVE28	*-150VEN:mgfp*	22
pVE31	*-746VEN:Roseal*	24
pVE34	*-630VEN:Roseal*	29
pVE35	*-500VEN:Roseal*	23
pVE36	*-380VEN:Roseal*	24
pVE37	*-260VEN:Roseal*	24
pVE38	*-150VEN:Roseal*	22
pVE50	*-900VEN:IGUS*	18
pVE51	*-746VEN:IGUS*	29
pVE54	*-630VEN:IGUS*	18
pVE55	*-500VEN:IGUS*	27
pVE56	*-380VEN:IGUS*	30
pVE58	*-150VEN:IGUS*	57
pPN92	*CaMV35S:mGFP*	15
p27IGUS	*CaMV35S:IGUS*	9
pART27	empty vector	15

2.8   Reporter gene assays

Light microscopy (Olympus BM2) was used for the observation of red pigment in the lines harboring *Rosea* cDNA constructs. To observe GUS reporter activity, the tissue was stained with X-gluc first. Fluorescent microscopy (Olympus SZX) and confocal microscopy (Leica) was used for the observation of GFP in the lines harboring GFP constructs. Images were captured and examined using a Leica DC 50 digital camera attached to a microscope.

Two methods were used for GUS staining:

Method 1. Histochemical assay for GUS expression was performed as described by Jefferson *et al.* (1987). Flower samples were immersed in staining buffer, then infiltrated for about one minute, during which time the petal tissue became translucent. They were then incubated 12 – 48 h at 37°C in the original staining buffer. The X-gluc buffer was then replaced with 70% (v/v) ethanol to remove the chlorophyll and preserve the sample. Sometimes a brown colour occurred due to tissue necrosis. To remove this brown colour the samples were transferred into 100% (v/v) ethanol + 5% (v/v) acetic acid solution and bathed at 70°C for 30 minutes.

X-Gluc buffer: X-Gluc (5-bromo-4-chloro-3-indoyl-ß-Dgluconide) was first dissolved in a small amount of dimethyl formamide first, then made up to 0.5 mg/L X-gluc in 50 mM phosphate buffer pH 7.0, 1% (v/v) Triton.

Method 2. The procedure was the same as the method described by Hemerly *et al.* (1993). Flowers were prefixed with cold 90% (v/v) acetone for 1 h at -20°C, washed twice with 100 mM sodium phosphate buffer, pH 7.4, and immersed in the enzymatic reaction mixture. In this method the staining buffer was 100 mM phosphate buffer pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10 mM EDTA, 1 mM X-Gluc.

GUS stained tissue was observed under a light microscope to determine the pattern of staining. For some GUS positive lines the GUS staining patterning was further examined using sectioning. Thin sections were made by hand using a razor blade.

2.9   Agro-infiltration

Agro-infiltration was also used for the transient assay of *Venosa* promoter deletions. The *Venosa* promoter deletion constructs were made using binary vector pART27, then transformed into Agro strain LBA 4404 (see Table 2.3 for the binary construct details). The main analysis of the deletion constructs with Agro-infiltration used *N. tabacum* (cv. Samsun) flowers.

Infiltration of attached tobacco flowers with *A. tumefaciens* (*Agrobacterium*) strain LBA 4404 harboring *35S:IGUS* or *35S:GFP* constructs was carried out using the following protocol:

Agrobacterium cells were cultured in 10 ml LB + selection antibiotic overnight. The cells were centrifuged and re-suspended in medium 1003 (AB media salts + NaH_2PO_4 240 mg / L+ glucose 10 g / L + MES 14.693 g / L) containing 100 µM acetosyringone (AS) and cultured for 4 h. The cells were then centrifuged and re-suspended in 1% (w/v) glucose solution supplemented with AS. The solution was adjusted pH 5.3 beforehand. The density of Agrobacterium cells was adjusted to $A_{600}=0.5$ with the same solution. Flower buds or opened flowers in the GMO glasshouse were pierced with a needle and infiltrated with the Agro culture using a syringe. It was found that the buds could be treated from about 15 mm in length to fully opened flowers. After 2.5 to 3 days the buds were stained for GUS activity with the standard protocol or observed for GFP under the fluorescent microscope.

Tobacco flowers developed normally after *in vivo* Agro-infiltration. Staining was apparent in epidermal cells within 1.5 days when the IGUS reporter was used. Detailed observation demonstrated that all kinds of tissue cells could be transformed.

The above protocol was also used to test the efficacy of Agro-infiltration of petunia and *Antirrhinum* flowers.

2.10 *In situ* hybridisation of *Venosa* mRNA

2.10.1 Plant material

Seven different species of *Antirrhinum* with or without venal pigmentation patterning were used (Table 2.5). Tissue from the *rosea*^{dorsea} phenotype (no venation) was used as a negative control. The plant tissue fixation and embedding was carried out by our UK collaborator Professor Cathie Martin (John Innes Centre). The blocks were mailed to our lab and all subsequent procedures carried out in Palmerston North.

Table 2.5 **Species and phenotype of plant material used for *in situ* mRNA hybridisation of *Venosa***

Species	Phenotype	Material code	Bud stage (mm)
A. majus	*rosea*^{dorsea}/ No Venation	AA 104	15
A. majus	*rosea*^{dorsea}/ Venation	AA 114	15
A. graniticum	No venation	AA 123	10
A. mollissimum	Venation	AA 124	5
A. meonanthemum	Venation	AA 125	10
A. barrelieri	Venation	AA 126	10
A. latifolium	Venation	AA 127	15
A. molle	Venation	AA 128	5

2.10.2 Precautions to avoid RNase contamination

General precautions to avoid RNAse contamination were observed throughout the course of the experiment. Gloves were changed when gloved hands touched RNase contaminated working surfaces. Glassware used for RNA work was autoclaved and baked at 180°C for at least 2 h. Plasticware was either new (plastic pipettes) or treated overnight in 3% (v/v) decon 90 (Decon Laboratories Limited) and rinsed with sterile

water. Solutions for RNA work were prepared with sterile distilled water, then autoclaved.

2.10.3 Sectioning

Tissues were sectioned at 8 μm. Sections were cut using a microtome (Porter-Blum Microtome, JB-4, Sorvall), floated on water at 42°C and captured with precoated microscope slides (Fischer, Scientific, US and / or Esco Superfrost Plus coated microscope slides, Biolab Scientific). Microscope slides were baked at 42°C in an incubator overnight and stored at room temperature for 3-4 weeks prior to *in situ* mRNA hybridisation.

2.10.4 Probe synthesis

When designing the probe for the detection of *Venosa* transcription, the aim was to get a gene specific probe. The binding domain of *myb* factors is highly conserved, and several *myb* genes are known to express in the antirrhinum flowers (Schwinn *et al.*, 2006). DNA alignment showed that *Venosa* was highly similar to *Rosea1* and *Rosea2* in the binding domain, but differed in the C-terminal domain. So a portion of the C-terminal domain was used as the probe for gene specific hybridisation. There is an XhoI in the middle of the *Venosa* C-terminal domain. A plasmid containing *Venosa* cDNA was provided by Cathie Martin (John Innes Centre). The DNA fragments for transcription of probes were derived from PCR-amplified cDNA using this plasmid as template, *Venosa* cDNA was cloned into KpnI/SmaI digested pART7, then the XhoI/SmaI fragment from p7Venosa-cDNA plasmid was cloned into XhoI/SmaI digested pBluescript KS+ (Figure 2.8).

A plasmid containing antirrhinum CHS was provided by Dr Kevin Davies. The DNA fragments for transcription of probes were derived from PCR-amplified cDNA using this plasmid as template. CHS cDNA was cloned into the XbaI/ClaI digested pBluescript K+. The presence of the complete gene was confirmed by sequencing. This construct is recorded as pBSKS+CHS.

A
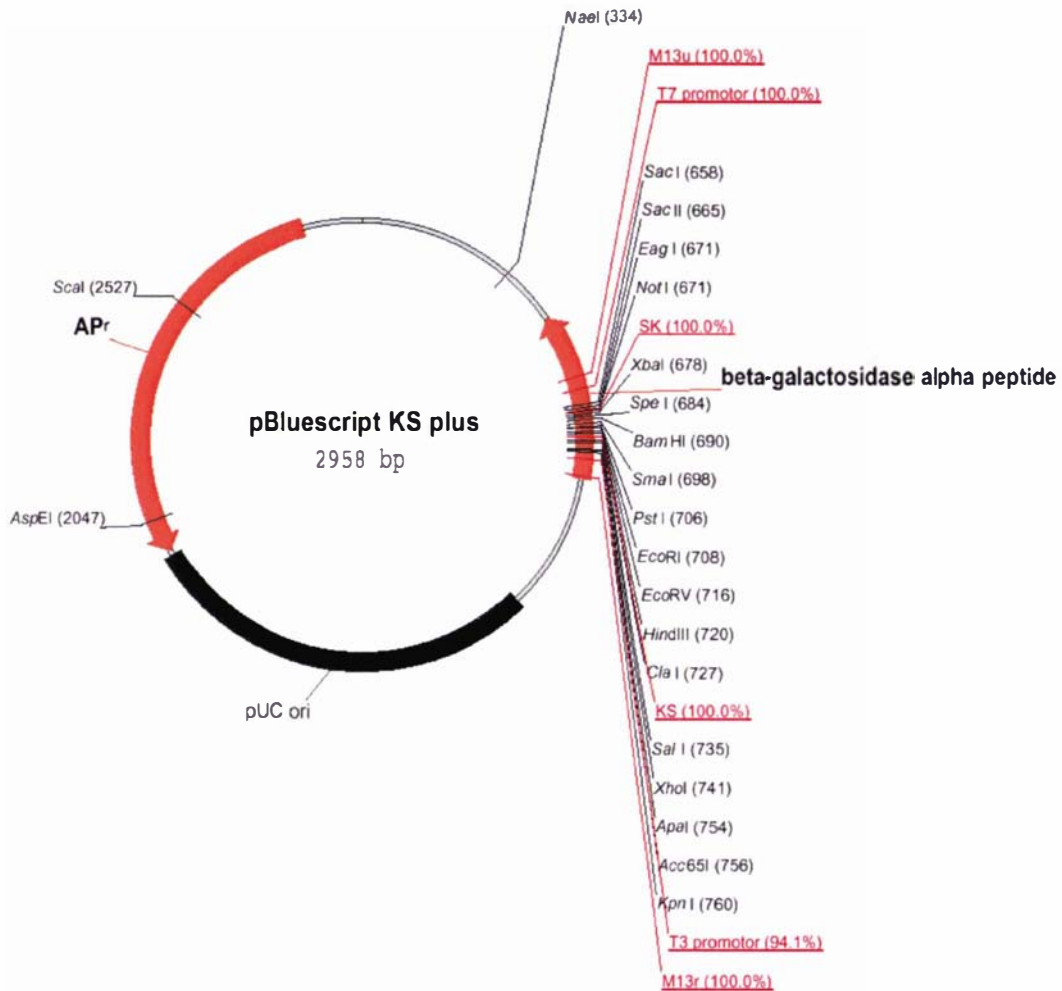
B

Figure 2.8 The probe for *Venosa* mRNA *in situ* hybridisation. (A) The map of pBluescript KS+ vector used for making the probe. Cited from Internet: www.rzpd.de/.../ pBluescript_KS_plus_pic.shtml. (B) Schematic presentation of genomic *Venosa* (not to scale), in which part of the C-terminal domain, XhoI downstream fragment, is used as probe. *Venosa* cDNA was cloned into KpnI/SmaI digested pART7 (see Figure 2.2), then the XhoI/SmaI fragment from p7Venosa-cDNA plasmid was cloned into XhoI/SmaI digested pBluescript KS+ vector. Line, intron; box, exon; pink bar, probe region from XhoI to stop coden, TAA.

The anti-sense probe and sense probe of CHS and *Venosa* were synthesised with a kit (Roche Applied Science) according to the manufacturer's instructions. They were cloned into pBluescript KS+ vector. DNA templates were prepared from plasmid DNA and linearised at restriction sites downstream of the cloned insert. After restriction digest, the DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation. Digoxigenin-labeled RNA probes were synthesised with *in vitro* transcription using T3 and T7 RNA polymerase according to the manufacturer's instructions (Roche Applied Science). A negative control was set using sense probes. The transcription reaction was carried out in microfuge tubes consisting of the following: 1 μg purified template DNA and sterile distilled water to a final volume of 13 μl, 10x NTP labeling mixture (2 μl), 10x transcription buffer (2 μl), RNase inhibitor (1 μl), and RNA polymerase T3 or T7 (2 μl).

The reaction solution was mixed gently and centrifuged briefly, then the mixture was incubated at 37°C for 3 h. RNA-free DNase 2 μl was added to the transcription mixture, and the mixture incubated for a further 15 min to remove template DNA. Transcription was stopped by adding 2 μl 0.2 M EDTA (pH 8.0), and the RNA transcript was precipitated by 2.5 μl 4 M LiCl and 75 μl ethanol at -80°C for 1 h. After centrifuging for 20 min at 4°C, the supernatant was removed and the pellet was washed, centrifuged, air-dried and dissolved in 50 μl sterile distilled water. Reaction mixture (2 μl) was loaded onto a 1.5% formaldehyde gel to determine if the expected RNA product was present. 1 μl reaction mixture was used for quantification with spectrophotometry:

C (μg / ml, concentration) = R (the read number under A_{260}) x D (dilution factor) x 40 (μg/ml)

A (μg, amount) = C x V (ml, sample volume)

Because CHS was 1.3 kb, the RNA probes were hydrolysed to reduce probe size and allow better penetration of probes into the tissue. Transcripts for both antisense and sense probes were hydrolysed into about 0.2 kb long fragments. These were chemically degraded to a mean length of 150-250 bp by the addition of sodium carbonate buffer (carbonate buffer 50 μl was added to the 50 μl of transcript solution), pH 10.2 at 60°C using the formula below to calculate the incubation time for each probe:

$$t = (Li-Lf) / (K) (Li) (Lf)$$

Where   t   hydrolysis time in minutes

                   Li   initial length in kb

                   Lf   final length in kb = 0.2 kb

                   K   rate constant for hydrolysis = 0.11 kb / min

The hydrolysis reaction was stopped by adding 5 µl of 10% acetic acid and the transcripts were precipitated by 10 µl of 3 M NaHAc and 260 µl ethanol and left at -80°C for 1 h. After centrifugation for 20 min at 4°C and washing / spinning with ethanol, the pellets were air-dried and taken up again in 50 µl of sterile distilled water. Hydrolysed transcripts (2 µl) were run through a 1.5% formaldehyde gel to confirm they were of the expected size. Transcripts were stored in aliquots at -80°C.

The transcripts for the *Venosa* probe did not need to be hydrolysed due to their inherently suitable length of 0.2 kb (Figure 2.8 B).

2.10.5 Pretreatments

The microscope slides with the mounted tissue were put into a slide rack and passed through the following series of solutions for section pretreatments. The slides were dewaxed firstly by passing through two separate histoclear (National Diagnostics) with ten minutes per change, then through sequential rehydration steps, one and half minutes in 100% ethanol, a half minute in each solution of 95% ethanol, 75% ethanol + 0.85% NaCl, 50% ethanol + 0.85% NaCl, 30% ethanol + 0.85% NaCl, two minutes in 0.85% NaCl and PBS (phosphate buffered saline solution). The next step was pronase treatment (0.124 mg/ml) in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA) for 10 minutes. This step degrades proteinaceous material and allows cell permeability for probe penetration. The tissues were washed with 1 x PBS solution added 2% glycine for two minutes before post fixation with 4% formaldehyde in PBS for ten minute. The tissues were washed by passing through two separate PBS solution with two minutes per wash. This wash was followed by acetylation reaction (3 ml acetic anhydride in 0.1 M triethanolamine), which reduced background through acetylation of exposed charges. After washes in 1 x PBS the tissues were dehydrated back through the ethanol solution

with fresh ethanol solution at the end. The slides were air-dried before probe application.

2.10.6 RNA *in situ* hybridisation and immunological detection

RNA probes were diluted at the required hybridisation concentrations with hybridisation buffer (50% formamide, 300 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 10% dextran sulphate, 1% denhardts, 100 μg/ml tRNA), which was made fresh on the day. The quantity of DIG-labeled probe required for adequate colour development was determined empirically. In an attempt to find the optimal probe concentration, four concentrations of the anti-sense and sense probes for each gene were tried with 0.25 μl, 0.5 μl, 1.0 μl and 2.0 μl. The cross sections of tube and lobe of AA114 (*Venos /rosea^{dorsea}* phenotype) were used as plant material. When the optimal concentration of probes were determined, all species or phenotypes were tested, in which one slide for a tube section and one slide for a lobe section for each species or phenotype were selected according to their position in the flower for maximum likely *Venosa* expression.

The volume of probe was diluted with 10 volumes of 0.5 x TE, heated for 2 min at 80°C, then cooled on ice and hybridisation solution added. A 200 μl aliquot of solution containing the probe was applied to a slide and a second slide was lowered onto it like a cover slip. Thus, a set of two microscope slides facing each other with the probe between them like a sandwich. The slides were placed in a covered plastic container where the slides were laid down on an elevated level and the bottom of the container was line with wet paper towel to maintain a humid atmosphere inside the box. The paper was wet with 2 x SCC in 50% formamide. Hybridisations were set up overnight at 50°C.

After an overnight incubation, low and high stringency washes were done at 50 °C. The microscope slide pairs were separated carefully and incubated in 2 X SSC (SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7) in 50% formamide at 50°C for twice washes. Per wash was one hour. The tissues were washed in NTE (0.1 mM N$_a$CL + 0.002 mM Tris-HCL + 0.0002 mM EDTA (pH 8.0) autoclaved) for 5 minutes. It was followed by RNase treatment (20 μg/ml RNase in NTE buffer) for 30 minutes. After twice passing

through (5 minutes each time) the NTE buffer, the tissues were incubated in 0.2 x SSC at 50°C for one hour. A final rinse was done using 1 x PBS at room temperature for 1 min. Immunological detection was done by blocking treatment first. The microscope slides were put into a glass dish containing blocking solution (0.1% BM reagent in buffer 1). The dish was put on shaker for gentle shaking for 45 min. After wash with buffer 1 for 45 min, antibody reaction was done using 1:2500 dilution of DIG-AP conjugate in buffer 1. The slides were placed in boxes wetted with paper towel and incubated at room temperature for 90 minutes. The slides were separated carefully and antibody was washed off with buffer 1 for four times. This was followed by further three times washes with detection buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$).

The colour reaction was done with Color Reagent solution (40 µl colour reagent + 2 ml detection buffer). Slide sandwiches were made by adding Colour Reagent solution 200 µl, then put into a water moistered box and incubated at room temperature in the dark overnight. The progress of the colour reaction was monitored. Slides were separated, rinsed with water, air dried and mounted. Images were captured and examined using a Leica DC 50 digital camera attached to a microscope.

2.11   Isolation and identification of a mutant *Venosa* allele

2.11.1 Isolation and characterisation of two *Venosa* genomic clones

Based on the *Venosa* cDNA sequence, the primers Y44/Y45 (see Appendix I) were designed for isolating *Venosa* (from ATG to stop codon). Genomic DNA was extracted from leaf tissue of the venation phenotype line CT128 using the method given in section 2.2.3. Gradient PCR was performed, with the annealing temperature rising from 54°C to 66°C. Two fragments were amplified and cloned into KpnI/SmaI digested pART7. Representative clones for each were sequenced three times over their entire length from both directions (see Appendix I for the primers used in sequencing, Appendix II and III for the sequence data). The function of these genes was assayed by transient expression, in which the petals of *rosea*^{dorsea} were bombarded with the standard biolistic protocol.

2.11.2 Comparison of the promoter structure of two *Venosa* genomic clones

To compare the promoter regions of the two fragments the following primers were used for PCR. Y45 was used as reverse primer, while Y01, Y14, Y15, Y16, Y17, Y18 and Y44 were used as forward primers respectively (see Appendix I for primer details). The set of primers used was the same as for the *Venosa* promoter deletion experiments.

2.11.3 Genotype determination for different phenotypes using PCR

Genomic DNA was extracted from lines with different flower phenotypes, including CT128 (venation phenotype), CC112 and CL144 (both show the *rosea*^{dorsea} phenotype), CC112P (fully red flower without pigment in its inner tube area) and 522 (fully red flower with venation patterning in its inner tube area). The extracted DNA was used as template for PCR with primers Y44/Y45 (see Appendix I), which generated fragments of different sizes characteristic of each allele.

2.12   Inhibition of *Venosa* expression using RNAi

2.12.1 *Venosa* RNAi construct

A vector to generate hairpin mRNA from pRNA69 (contact Dr Kevin Davies for this vector), was used to make an RNAi construct. *Venosa* cDNA was PCR amplified using p7Venosa template and Y50/Y53 primers (see Appendix I). The fragment has four embedded restriction sites as 5'--XbaI/EcoRI -- *Venosa* cDNA -- BclI/BamHI--3', so that the sense was cloned into EcoRI/BclI and the anti-sense into XbaI/BamHI digested pRNA69 vector containing a 35S promoter of cauliflower mosaic virus upstream of the sense and antisense multi-cloning site separated by a *YABBY5* intron. Novablue was the host strain. The structure of the construct was confirmed by sequencing. It is recorded as pVenosa-RNAi. The structure is schematically shown in Figure 2.9.

EcoRI   BclI   BamHI   XbaI

35S promoter   *Venosa* cDNA   intron (Y5)   *Venosa* cDNA   ocs terminator
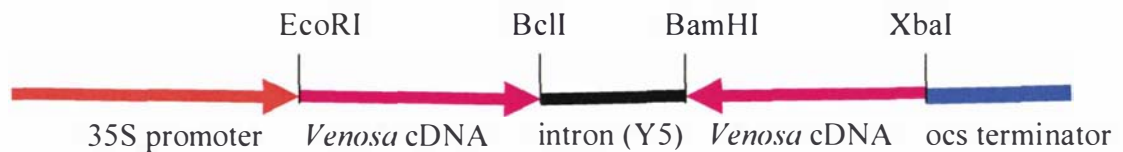
Figure 2.9   Schematic representation of the structure of pVenosa-RNAi construct (not to scale).

2.12.2 Plant material

Venation phenotype *A.majus* flower buds of different developmental stages (7 mm – 15 mm in bud length) (Figure 2.3 B) were used as plant material in this experiment. The standard particle bombardment protocol (see Section 2.6) was used. The bud was placed erect in the medium, and then shot from its front. The shot buds were cultured in medium #2 (1/2 MS medium) in culture room conditions. Their response was observed over the following days.

2.13   Investigation of the role of gibberellin and sugar signaling in controlling venation pigmentation patterning

2.13.1 Emasculation experiments

Aantirrhinum plants were grown as described in Section 2.1. Among the population of antirrhinum plants showing the venation phenotype, the intensity of the pigmentation was somewhat variable between different plants. To obtain consistent plant material, 15 plants were propagated in the glasshouse through cuttings from one plant (CT128, 1/5), which had a good venation phenotype in its flowers.

Flowers of varying stages of development could be seen on any individual inflorescence (Figure 2.1). Hence, flowers at different developmental stages could be emasculated on one inflorescence. Emasculation was performed on buds ranging from 4 mm length to fully opened flowers. The flowers or young buds were cut longitudinally along the middle of the lower petals. The anthers were removed carefully with forceps. All the flowers on three inflorescences were emasculated. For the controls, the flowers and young buds were cut in the same way, but the anthers were not disturbed. The control

61

sample contained two inflorescences. Observations were carried out over ten days following emasculation, although other flowers would fully senescence within this time frame.

2.13.2 Experiments testing detached petal response to GA_3 supplementation *in vitro*

Young buds were harvested for feeding experiments. The buds were measured with a ruler, and grouped according to bud length: 3-4 mm, 4-5 mm, 5-6 mm, 6-7 mm and 7-8 mm (measured from the base of the sepal and excluding the pedicel). The buds were sterilised with 15% (v/v) Janola + a few drops of Tween 20, placed under vacuum for 2 min and shaken for 15 min. Buds were then rinsed three times with sterile water. The buds were dissected under the microscope. The lobes of the two dorsal petals were cultured face (adaxial side) up on different solid media (0.7% (w/v) agar, pH 7.0). 12-50 samples were cultured on each plate, depending on the media and experiments, and maintained under lights (16 h photoperiod) at 25°C. The appropriate concentration of applied GA_3 was first determined by testing a series of concentrations. The media used for this purpose were adjusted to pH 7.0 and solidified with 0.7% (w/v) agar. They were:

1. MS without sucrose
2. MS + 3% (w/v) sucrose
3. MS + 3% (w/v) sucrose + 0.03 mg/L GA_3
4. MS + 3% (w/v) sucrose + 0.1 mg/L GA_3
5. MS + 3% (w/v) sucrose + 0.3 mg/L GA_3
6. MS + 3% (w/v) sucrose + 1.0 mg/L GA_3
7. MS + 3% (w/v) sucrose + 1.5 mg/L GA_3
8. MS + 3% (w/v) sucrose + 3.0 mg/L GA_3
9. MS + 3% (w/v) sucrose + 15 mg/L GA_3

A combined supplementation for sugar supplementation protocol was established after the determination of the appropriate GA_3 concentration in conjunction with other preliminary experiments. This protocol is shown in Figure 2.10 A. In this combined supplementation approach, the excised petals were pre-cultured in MS media supplemented with GA_3 in the dark at 25°C for three days. The petals were then

transferred onto media without GA_3 and cultured in the dark for another two days (the manipulation of the tissue was rapid and done under weak light). Petals of similar size and shape were then selected and transferred onto media with GA_3 (tested samples) or without GA_3 (control samples), and cultured under light (16 h photoperiod) at 25°C. The response of the samples was observed and recorded in the following five days. The media used in the GA_3 experiments were adjusted to pH 7.0 and solidified with 0.7% (w/v) agar. They were:

1. MS without sucrose
2. MS + 3% (w/v) sucrose
3. MS + 3% (w/v) sucrose + 0.1 mg/L GA_3

2.13.3 Experiments testing detached petal response to different sugar supplements *in vitro*

The experimental procedure was similar to the one described for the GA_3 experiments above, but the media used were different. The protocol is shown in Figure 7.2 B. The media used in this experiment were:

1. MS + 0.1 mg/L GA_3 (no sucrose)
2. MS + 0.1 mg/L GA_3 + 3% (w/v) sucrose
3. MS + 0.1 mg/L GA_3 + 3% (w/v) glucose
4. MS + 0.1 mg/L GA_3 + 3% (w/v) mannose
5. MS + 0.1 mg/L GA_3 + 3% (w/v) xylose
6. MS + 0.1 mg/L GA_3 + 3% (w/v) manitol
7. MS + 0.1 mg/L GA_3 + 3% (w/v) 3-O-methylglucose
8. MS + 0.1 mg/L GA_3 + 3% (w/v) sucrose + 50 mM mannoheptulose
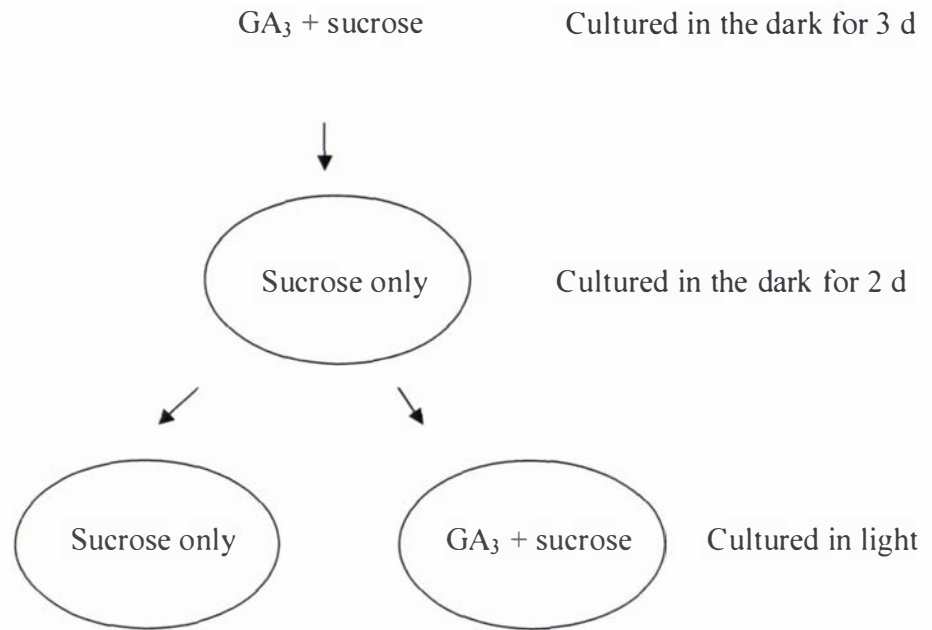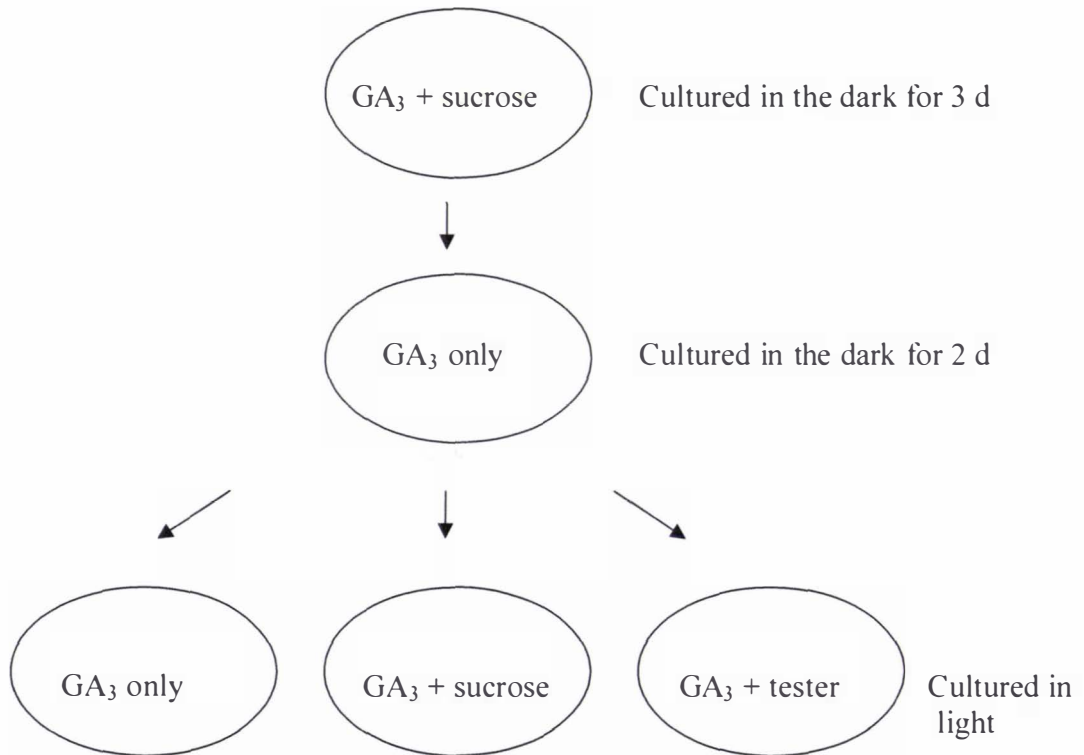
Figure 2.10   Protocol to test the response of detached petals to (A) gibberellin and (B) sugar *in vitro*. 'Tester' stands for other sugars or sugar analogues.

2.13.4 Girdling experiments

The stem of the inflorescence was girdled, rather then the pedicel, because the pedicel was too short to be girdled. The top younger buds and the lower older buds were removed as were the leaves above the girdling area, so that the focus was on one or two bud stages on one individual inflorescence. The stems were girdled for 1 cm in the lignified stem area by removing the outer layer (phloem) using forceps. Buds with the same treatment but without girdling served as control. Because the buds ≥ 8 mm in length were already pigmented at the base of the tube, the effect of girdling on buds 4-7 mm in length was tested.

Chapter 3

Venosa controls the venation pigmentation patterning in the petals of antirrhinum

3.1   Introduction

Pigment patterning in the flowers of venation phenotype is shown in Figure 3.1. One of the *myb* genes, *Venosa*, was identified by the venation phenotype in a *Rosea1* mutant (*rosea* [dorsea]) background (Figure 1.3). Given that *Venosa* could not be seen in the wildtype due to the epistatic expression of *Rosea* and was seen only in a *Rosea* mutant (*rosea* [dorsea]) background (Figure 1.3), it has been suggested that the expression of the MYB transcription factor, VENOSA, controls the venation phenotype (Schwinn *et al.*, 2006). Hence, it has been hypothesised that the venation pigmentation patterning may be due to the localised expression of *Venosa*. The purpose of the work reported in this chapter was to investigate the function of *Venosa* in controlling venation pigmentation patterning.

Three experiments were carried out in this chapter: (1) Pigment complementation in the venation phenotype of antirrhinum was assessed using particle bombardment; (2) the expression pattern of *Venosa* was determined by mRNA *in situ* hybridisation and, to confirm the role of *Venosa* in controlling the venation pigment patterning in the *Antirrhinum* genus, other species were also subjected to mRNA *in situ* hybridisation; and (3) inhibition of *Venosa* expression, in which an RNAi construct was introduced into the potential pigment cells with particle bombardment.
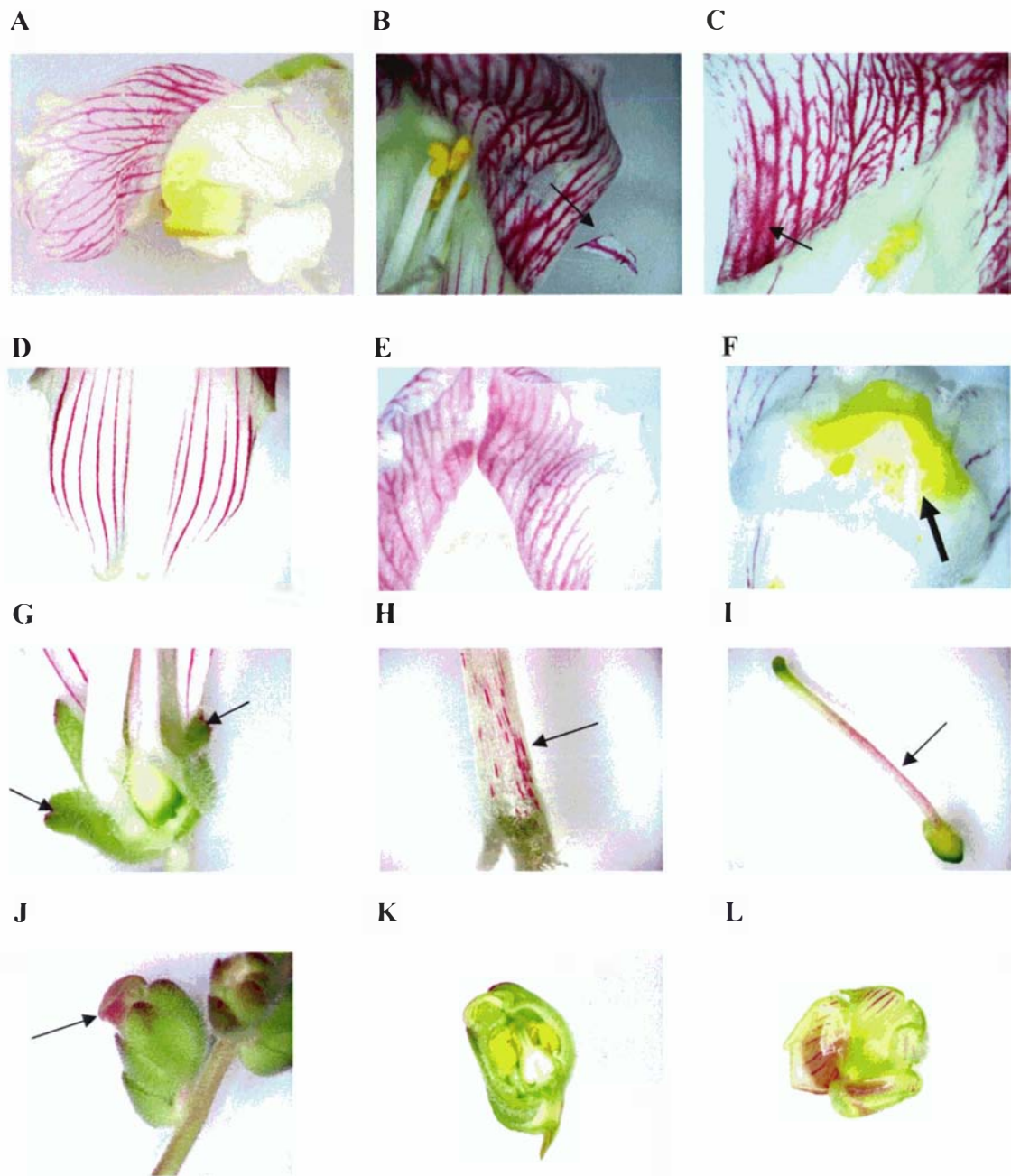
Figure 3.1 Anthocyanin pigment patterning in venation phenotype. (A) Front view of mature flower; (B) Pigment only produced in adaxial epidermal cells; (C) Pigment venation patterning is not clear at petal edge; (D) Pigmentation patterning in adaxial tube; (E) No pigment in abaxial epidermal cells; (F) Anthocyanin pigment patterning in abaxial lower petals. The yellow pigment, aurone is indicated with arrow; Pigment also occurs in sepal (G), filament (H) and style (I); (J) Pigment occurs in abaxial epidermal cells when bud is in very early developmental stages, and disappears in late development stages; (K) Longitudinal cut of a bud, and pigmentation patterning occurs in adaxial epidermal cells in this early development stage (L).

3.2 Results

3.2.1 Floral development and pigmentation

The flowers in antirrhinum are generated in inflorescences. Flowers of all developmental stages, from very early to fully opened, are found on one inflorescence. Flower developmental stages determined by bud length are shown in Figure 2.1. The occurrence of venation pigmentation patterning corresponds to the development of petals. Dissection revealed that pigment venation patterning was present at the tube base by the time the bud had reached 8 mm in length. From this stage the pigment venation pattern expands and intensifies accompanying the cell division and petal expansion.

3.2.2 Non-pigmented cells in the petal epidermis can be pigmented by *Venosa* expression

The results of complementation experiments are shown in Figure 3.2. In the petal tissue expressing the venation phenotype, cells in the non-pigmented domain could be complemented by transient expression of *Venosa* driven by the CaMV 35S promoter, and the foci were not correlated with veins. No foci were observed in the control samples containing 35S:Lc. The non-pigmented tissue includes both adaxial and abaxial epidermis of tube and lobe.

In the abaxial epidermal cells, the pigmented cells induced by particle bombardment could be observed clearly. However, in the adaxial area, in most cases if not all, it was difficult to distinguish the newly generated pigmented cells from the original ones. Many originally pigmented cells were damaged by particle bombardment, and the venation pattern was disrupted in the area that had been bombarded. The damage of cells by bombardment was demonstrated via pigment as a monitor from this experiment. Although the cells complemented for pigmentation could not always be distinguished from the background resulting from the shooting damage in the central area of the petal, cells complemented into pigmentation could be recognized at the edge area, where the less damage occurred.

3.2.3   *Venosa* expression detected by *in situ* hybridisation is in a vein-specific manner

The structure of flower tissue is revealed in the cross sections. In petals, the vascular tissue is arranged with xylem on the adaxial and phloem on the abaxial side.

No significant signal was found from *in situ* hybridisation with CHS sense probe. In contrast to this negative control, *in situ* hybridisation with CHS anti-sense probe showed an obvious CHS expression pattern in the epidermis of the both adaxial and abaxial sides of the floral organs, as expected (Figure 3.3).

In the tube area of antirrhinum flowers *Venosa* mRNA expression was localised to the adaxial side of petal tissue in a vein-specific manner (Figure 3.4 A, B and C). A similar hybridisation signal was also detected in the lobes (Figure 3.4 D). The hybridisation signal varied in strength and location depending on the location of veins. It could occur in the xylem cells, sub-epidermal cells and epidermal cells adjacent to the xylem (Figure 3.5). A similar expression pattern was detected in the tube area of a range of other *Antirrhinum* species (Figure 3.6 and Table 3.1), while no expression was detected in the lobe area at the developmental stage used (data not shown).

Table 3.1   Plant material and result of *in situ* mRNA of *Venosa*

Species	Phenotype	Material code	Bud stage (mm)	*In situ* mRNA hybridisation detected
A. majus	*rosea*^{dorsea}/ No Venation	AA 104	15	-
A. majus	*rosea*^{dorsea}/ Venation	AA 114	15	+
A. graniticum	No venation	AA 123	10	-
A. mollissimum	Venation	AA 124	5	+
A. meonanthemum	Venation	AA 125	10	+
A. barrelieri	Venation	AA 126	10	+
A. latifolium	Venation	AA 127	15	+
A. molle	Venation	AA 128	5	+

No similar venation patterning of expression was detected in tissue with the non-venation *rosea^{dorsea}* phenotype (AA104), which served as a negative control (Figure 3.7 A and B). Nor was it observed in *A. graniticum* (AA123), which does not have venation pigment patterning either, and was another possible negative control (Figure 3.7 C).

In all samples the pattern of *in situ* hybridisation of the *Venosa* sense probe was similar to the one for the anti-sense probe (data was not shown).

An unexpected patterning of *in situ* signal was detected in both the flower tube (Figure 3.7 A and C) and throat area (Figure 3.8 A and B) of flowers. Strong expression was detected in a limited area of the adaxial side in epidermal cells, sub-epidermal cells and inner tissue (Figure 3.9). Clearly this pattern was not vein-specific, and it occurred in both the *rosea^{dorsea}* venation and non-venation phenotypes and in *A. graniticum*. Surprisingly, this pattern matches the localisation of the yellow aurone pigment in the tube and lobe (Figure 3.7 B and Figure 3.8 C, D and E).

3.2.4 *Venosa* RNAi

To test the efficacy of particle bombardment for the *Venosa* RNAi experiment, buds of length 7 mm, 10 mm, 13 mm and 15 mm were bombarded with RNAi construct and cultured under culture room conditions. The gold particle alone was the negative control. No difference occurred in the pigment development between the tested RNAi samples and negative controls. However, there were problems associated with culture of the flowers. The buds at length 7 mm and 10 mm did not develop normally. The buds at length 13 mm developed to fully opened, but with poor pigmentation. The buds at length 15 mm developed normally and had normal pigmentation.

A adaxial lobe / adaxial tube / abaxial lobe / abaxial tube

B adaxial lobe / adaxial tube / abaxial lobe / abaxial tube
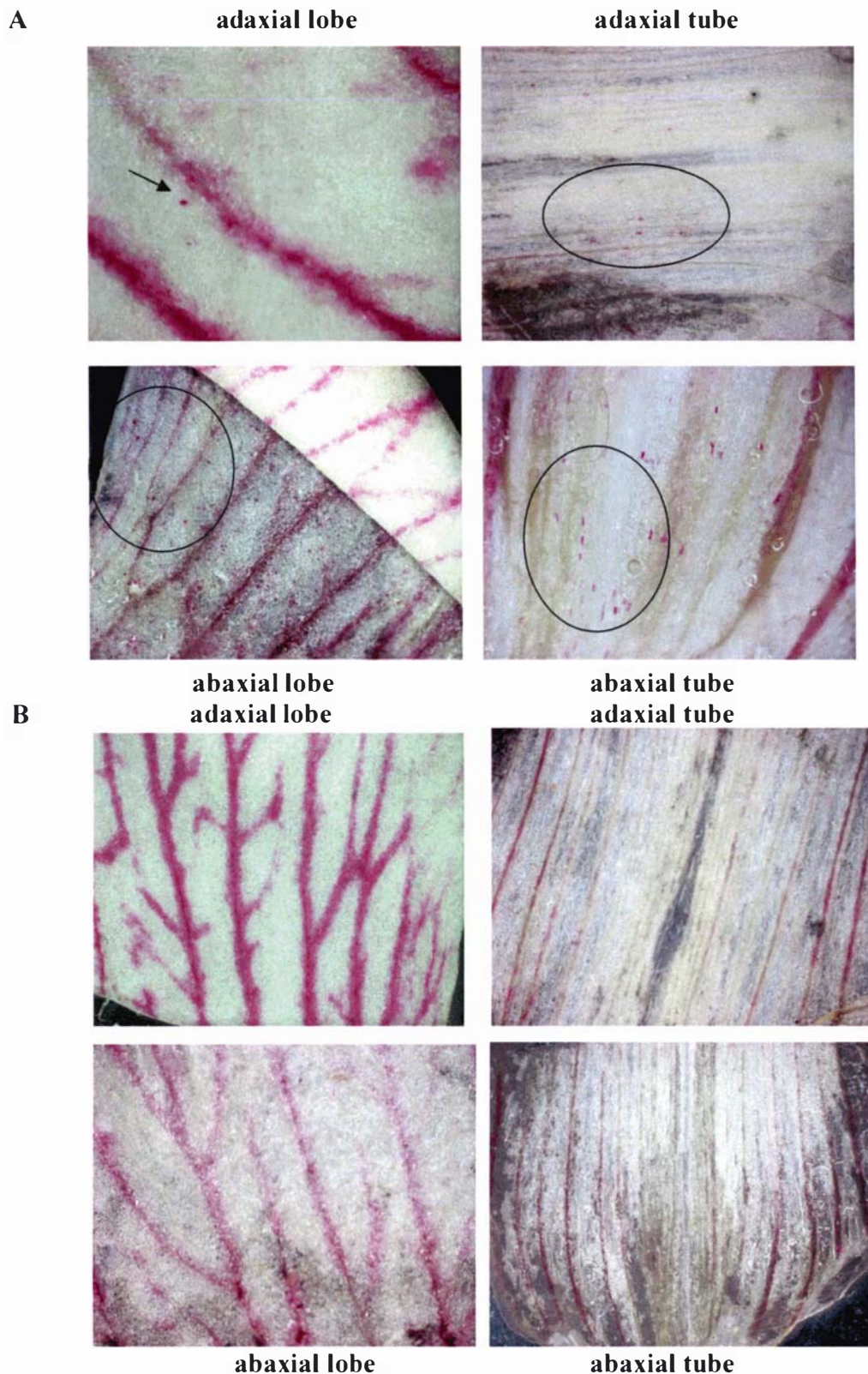
Figure 3.2 Pigmentation complementation. Non-pigmented cells in the petal epidermis were pigmented following transient expression of *Venosa*. Representative regions of bombarded *A. majus Venosa* flowers are shown. (A) 35S:genomic *Venosa* (construct p7GSV) induced pigmentation outside the vein areas when biolistically introduced. The foci are indicated with the arrow and circles. (B) No foci occurred with the 35S:*Lc* (construct pLc349) negative control.

Antisense probe   **Sense probe**
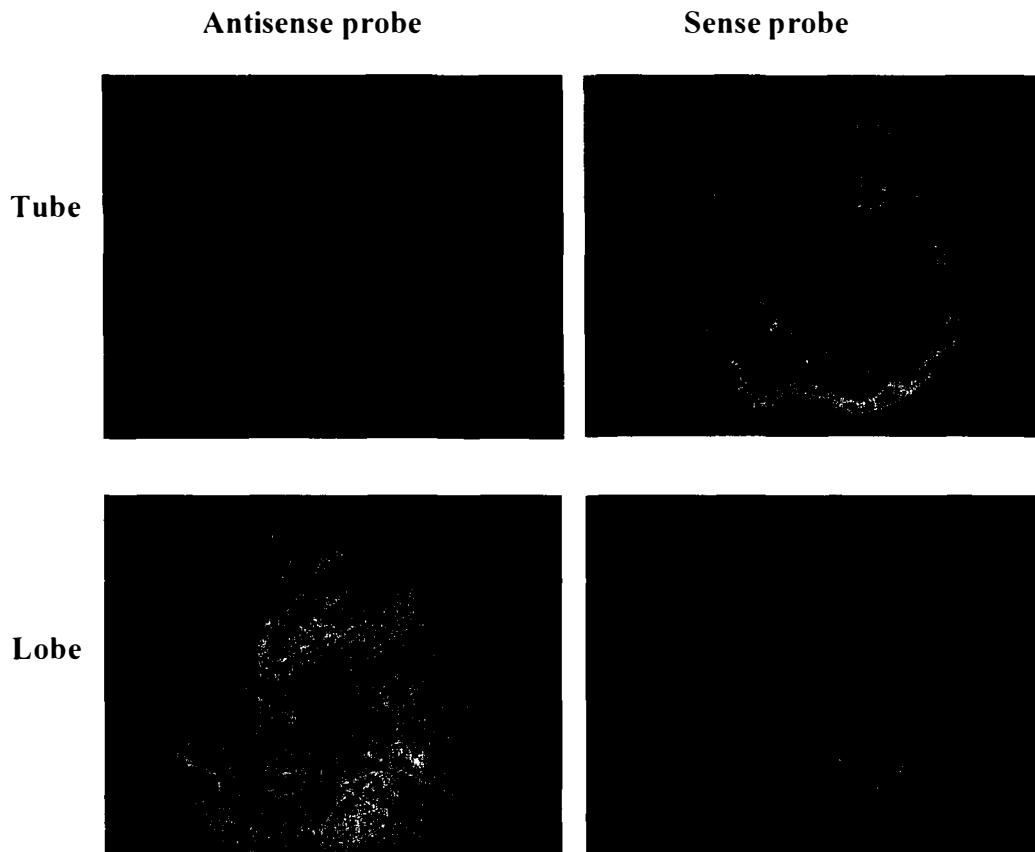
Tube

Lobe

Figure 3.3 CHS *in situ* mRNA expression pattern in sections of flowers of *A. majus*. Transverse sections through the tube or lobe were probed with RNA antisense or sense (control) probes and hybridisation detected using DIG-labeled mRNA generated in *in vitro* from pBluescript + containing CHS from *A. majus*.

72

A

B

C

D

E
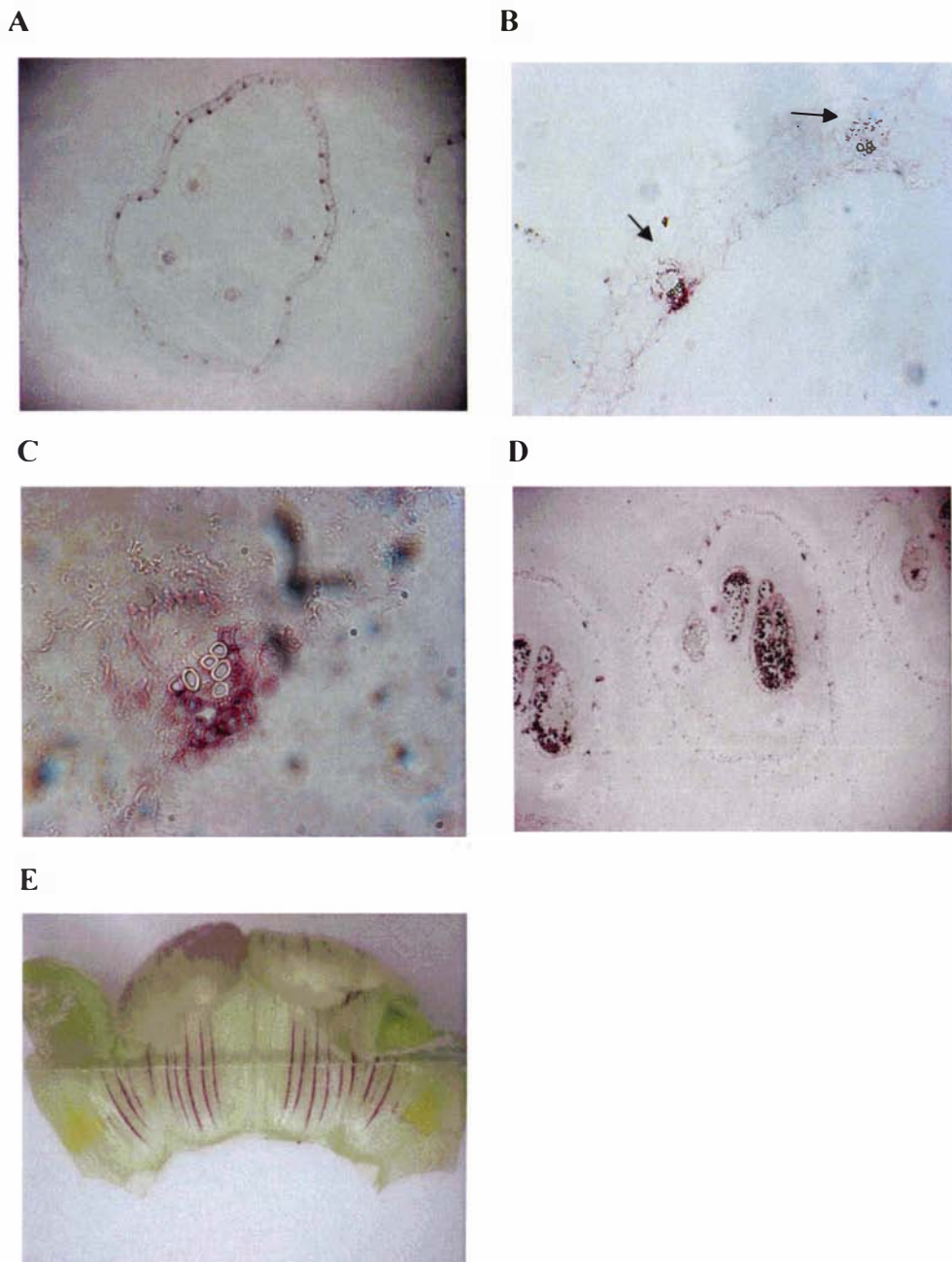
Figure 3.4   *Venosa in situ* mRNA expression pattern in the petal of *A. majus. Venosa in situ* mRNA expression pattern in tube area (A, B 400x and C 1000x) and in the lobe area (D). The expression signal can be seen in the adaxial epidermis above the veins and the cells immediately below these towards the xylem vessels. All examples are for the *Venosa* antisense RNA probe detected using DIG-labeled mRNA generated in *in vitro* from pBluescript+ containing a C-terminal region of *Venosa* from *A .majus*. Arrows indicate veins: (E) Venation pigmentation patterning in the adaxial tube of a 10 mm length bud.

A
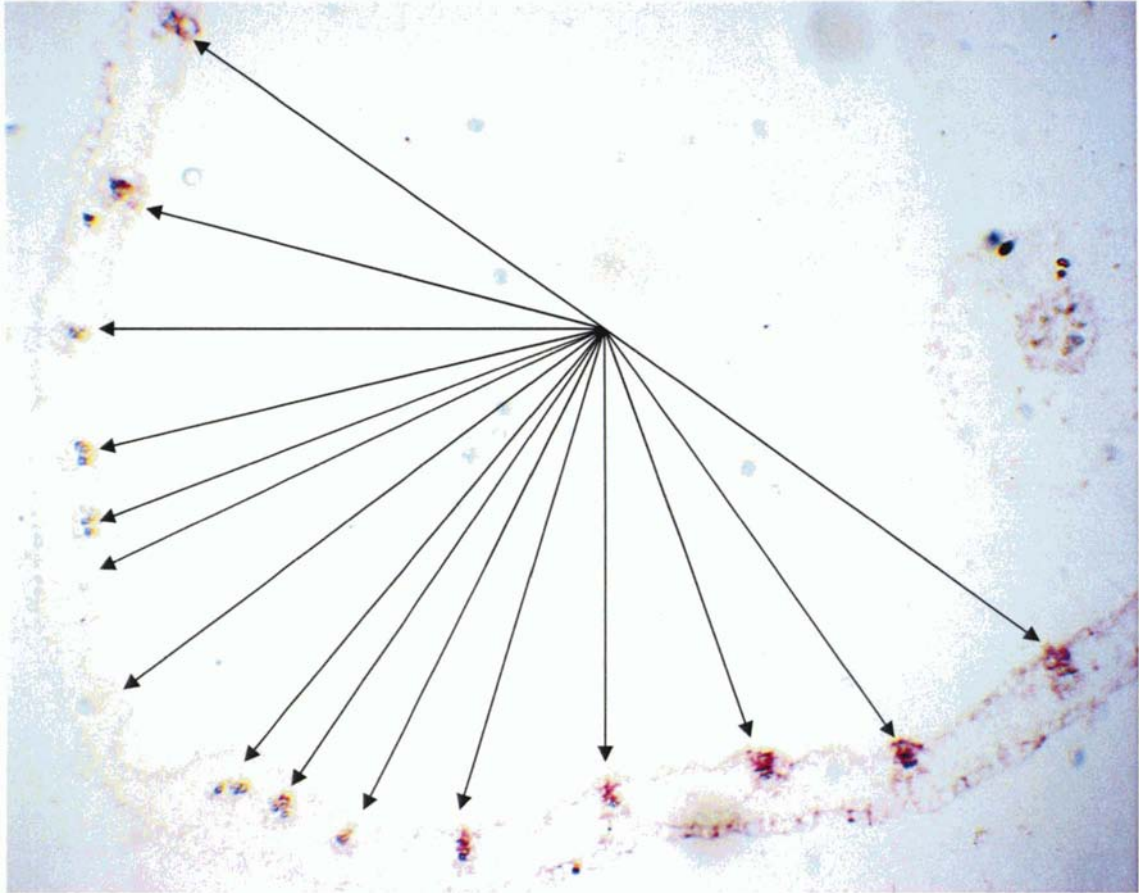
Figure 3.5 Variation in the expression pattern of *Venosa* in the tube region of *A. majus*. The arrows indicate the location of veins. This pattern was detected using DIG-labeled *Venosa* antisense mRNA probe generated in *in vitro* from pBluescript+ containing a C-terminal region of *Venosa* from *A. majus*. Arrows indicate veins and positive signal.
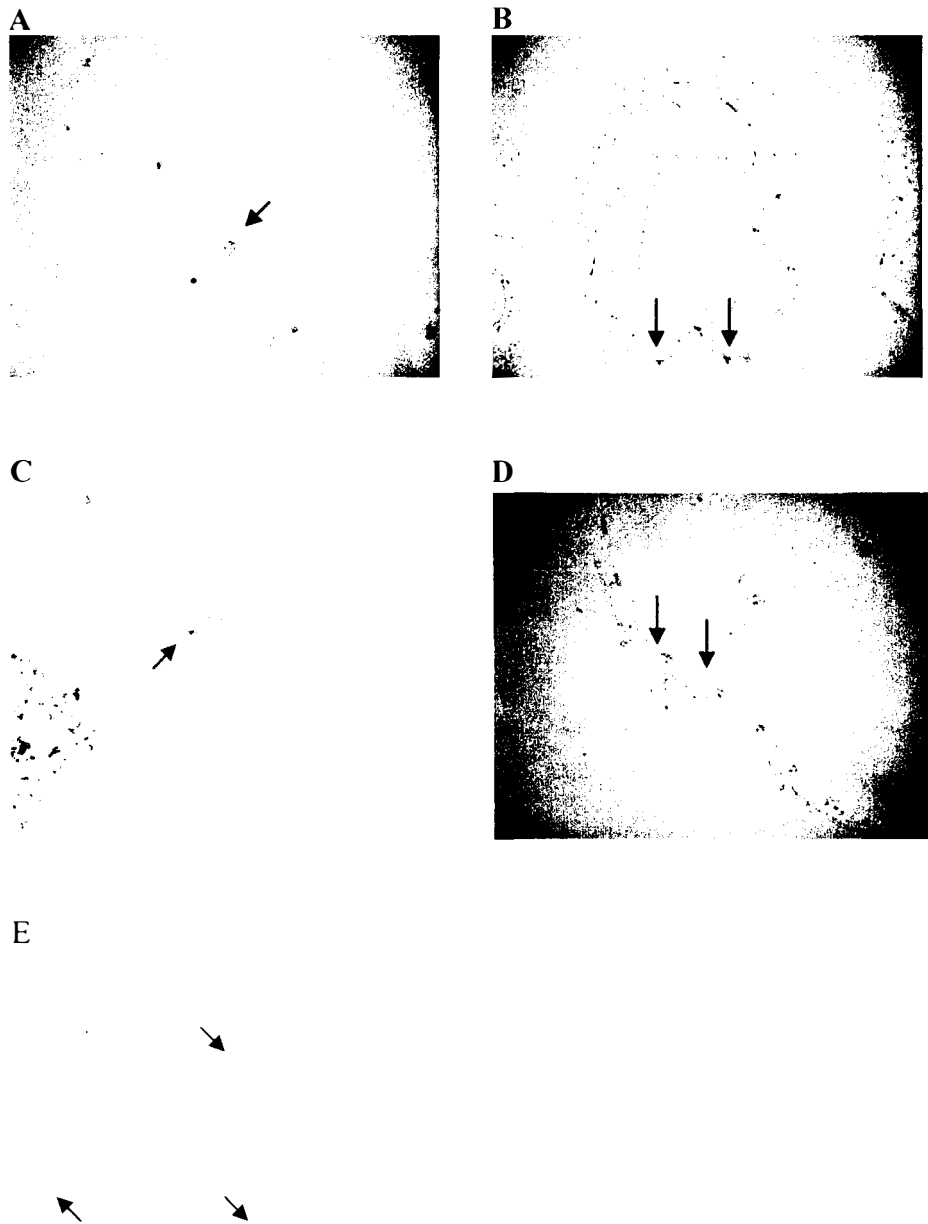
Figure 3.6 *Venosa in situ* mRNA expression pattern in the petal tubes of four *Antirrhinum* species. (A) *A. meonanthemum* (AA125); (B) *A. mollissimum* (AA124); (C) *A. latifolium* (AA127); (D) *A. barrelieri* (AA126); (E) *A. molle* (AA128). All examples are for the *Venosa* antisense probe detected with DIG-labeled mRNA generated in *in vitro* from pBluescript+ containing a C-terminal region of *Venosa* from *A. majus*. Arrows indicate examples of positive signals.
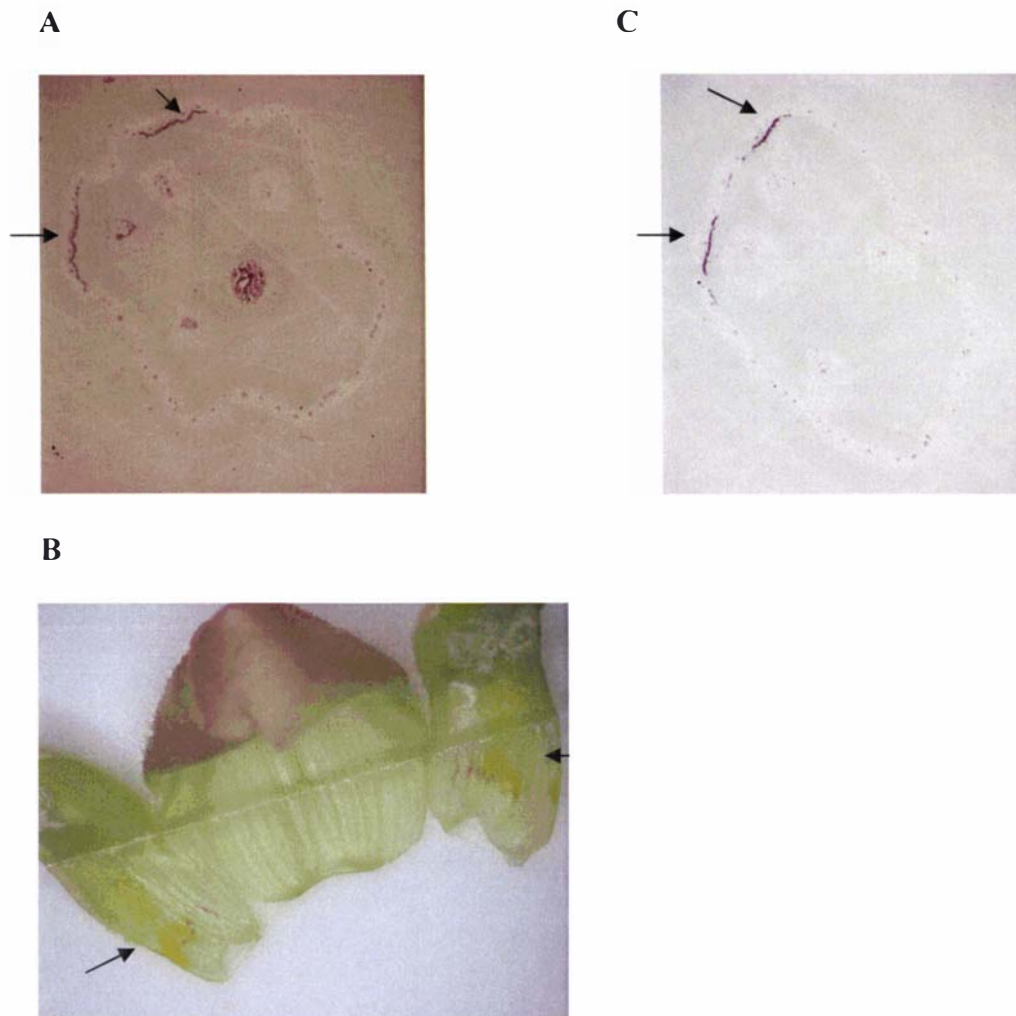
Figure 3.7 *Venosa in situ* mRNA expression pattern in the petal tubes of two *Antirrhinum* samples which lack venation pigmentation. (A) *rosea* ^{dorsea} *A. majus* (AA104); (B) The pigmentation patterning in the inner tube of *rosea* ^{dorsea} *A. majus*. Bud length is 10 mm. No anthocyanin pigment is seen in the adaxial epidermis. Arrows indicate the sites of occurrence of aurone pigmentation; (C) *A. graniticum* (AA123). Arrows show the location of *in situ* positive signals. All examples are for the *Venosa* antisense probe detected with DIG-labeled mRNA generated in *in vitro* from pBluescript+ containing a C-terminal region of *Venosa* from *A.majus*.
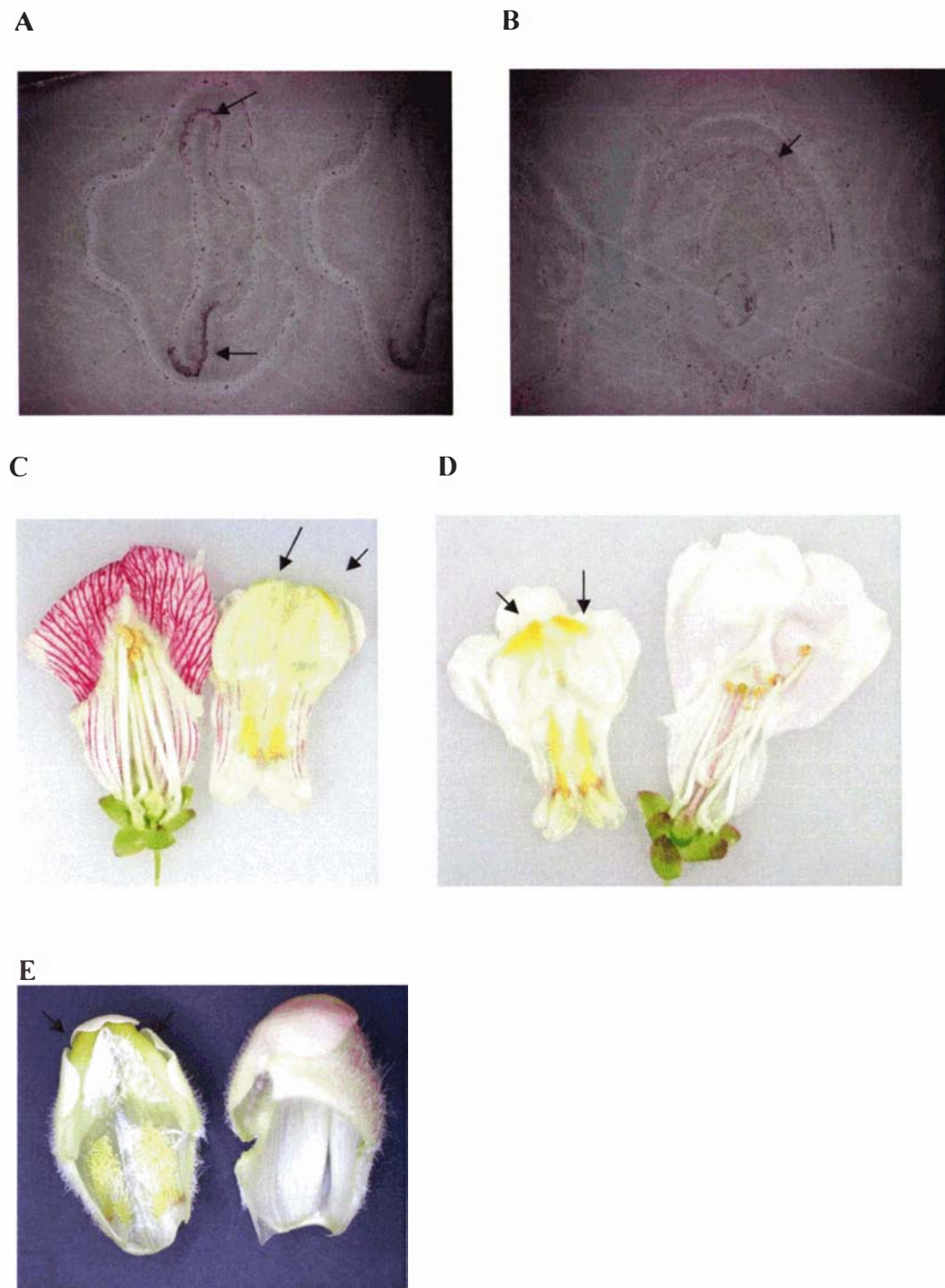
Figure 3.8   Probing of the aurone pattern in the lobe area of *Antirrhinum* flowers.
The *in situ* pattern of hybridisation to the *Venosa* antisense probe in the sections of
flower lobes of (A) *A. latifolium* (AA127) and (B) *A. meonanthemum* (AA125). The
probe is DIG-labeled *Venosa* antisense mRNA generated in *in vitro* from pBluescript+
containing a C-terminal region of *Venosa* from *A. majus*. Arrows indicate the *in situ*
positive signal; (C) The aurone pigment patterning in the venation phenotype of *A.
majus* mature flower. The aurone pigment patterning in the *rosea*[dorsea] phenotype of *A.
majus* mature flower (D) and in 10 mm bud length (E). Arrows indicate the aurone
pattern in the adaxial epidermis of the lobe.
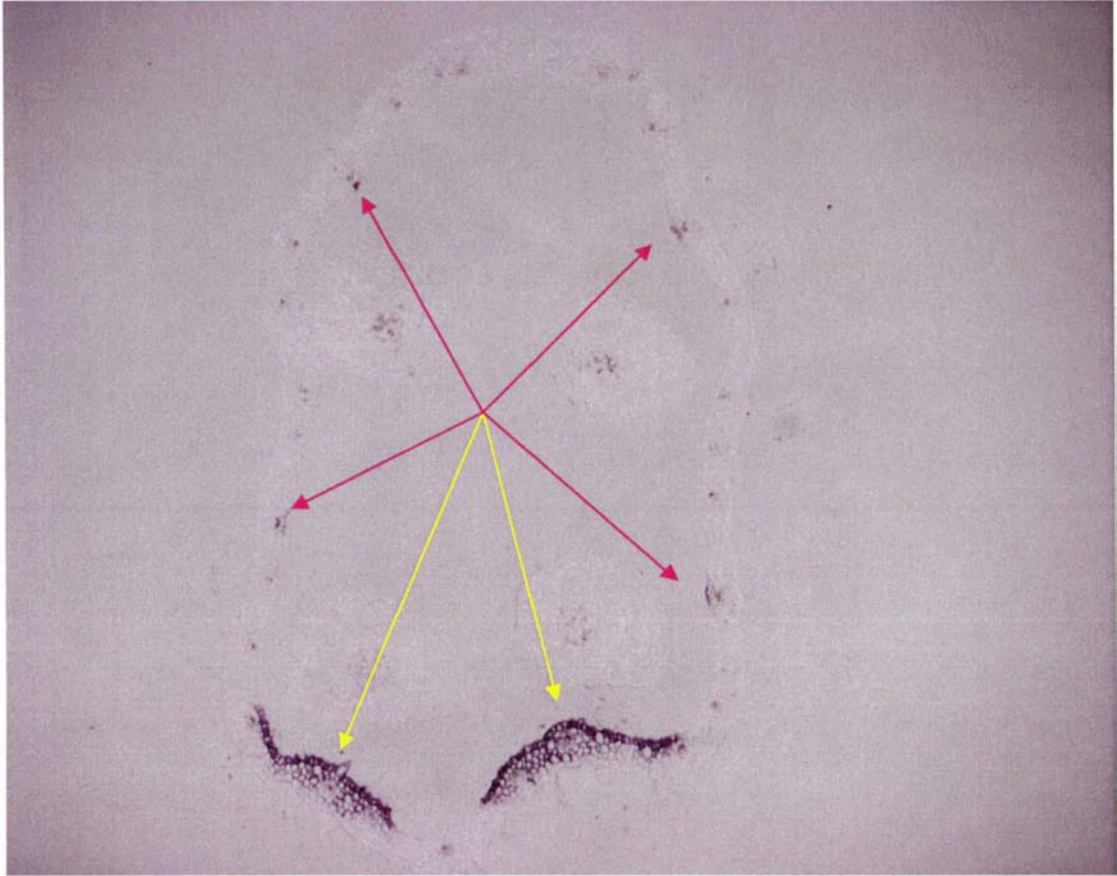
Figure 3.9 *Venosa* antisense probe signal matches with the two pigmentation patterns, the anthocyanin venation pattern and the aurone patch pattern in *A. molle* (AA128). The probe is DIG-labeled *Venosa* antisense mRNA generated in *in vitro* from pBluescript+ containing a C-terminal region of *Venosa* from *A. majus*. Pink arrows indicate the anthocyanin venation pattern, and the yellow arrows indicate the aurone patch pattern.

3.3   Discussion

3.3.1   Cells lacking anthocyanin pigment in the non-pigment domain is due to lack of MYB protein, suggesting that the venation pigmentation patterning is due to the localised expression controlled by a *myb* gene promoter

To activate the anthocyanin biosynthesis pathway, several factors, such as MYB, bHLH and WDR, are required (see section 1.3). Pigment could be induced by the 35S:VENOSA construct in the cells of the non-pigment region of the petal. This included the whole dorsal area and the areas between the venal pigment 'lines' in the facial area (Figure 3.2). This complementation experiment demonstrated that the non-pigmentation of the epidermal cells in the venation phenotype of antirrhinum was due to the lack of a MYB factor, while other factors such as the bHLH and WDR proteins were produced endogenously or induced by the MYB protein. The 35S promoter driving *Venosa* activated pigment production in cells is not associated with veins, thereby suggesting that the *Venosa* promoter is important to the pattern. In addition, the fact that *Venosa* tissue was used in these experiments indicates that there is nothing in the tissue itself that inhibits *Venosa* from functioning in cells away from veins. Thus, venation pigmentation patterning is likely due to localized expression of a *myb* gene.

3.3.2   Venation pigmentation patterning was due to *Venosa*

When designing the probe for the detection of *Venosa* transcription, the aim was to get a gene specific probe. The binding domain of *myb* factors is highly conserved, and several *myb* genes are known to express in the antirrhinum flowers (Schwinn *et al.*, 2006). DNA alignment showed that *Venosa* was highly similar to *Rosea1* and *Rosea2* in the binding domain, but differed in the C-terminal domain. So a portion of the C-terminal domain was used as the probe for gene specific hybridisation. The location and intensity of the hybridisation signal reflected the spatial pattern and strength of the *Venosa* expression, respectively. In the tube area, *in situ* mRNA hybridisation showed that *Venosa* expression and strength is selective to the location of the veins. It also appears related to the maturity of the veins. Although its expression was always related to the veins with the exception of the aurone region, not all the veins induced or

promoted *Venosa* expression, perhaps due to these being less developed veins. For example, no *Venosa* expression occurred in the veins situated in the front of the tube (Figure 3.4 A and B). In the lobe area of flowers *Venosa* mRNA expression also showed similar venation patterning, which matched the visual marker, the pigment venation patterning (Figure 3.4 D).

In summary, as the expression pattern and strength of *Venosa* (Figure 3.4 A and B) closely matched the pigment venation patterning and strength (Figure 3.4 E). It suggests that not only venation pigment patterning, but also probably pigment strength, is controlled by *Venosa* strictly.

Figure 3.5 shows the variation of *Venosa* mRNA expression. It seems that the expression of *Venosa* is induced by a signal related to the veins. This signal may be translocated from the xylem to the adaxial epidermis in a polar manner, and may then accumulate in the epidermal cells. Possibly, this signal is produced accompanying xylem development and transported in an active and polar manner forming the longitudinal axis of pigmentation. Epidermal specific expression of a bHLH is likely to define the transverse axis, and the pigmented stripes are the cross expression domain of these two kinds of factors. This conclusion is supported by work showing DELILA or MUTABILIS are required for venation pigmentation patterning with VENOSA (Schwinn *et al.*, 2006), and that DELILA is expressed specifically in the petal epidermal cells (Jackson *et al.*, 1992).

In situ hybridisation experiments were carried out with six other *Antirrhinum* species, of which five have pigment venation and one does not (Table 2.6). Vein-specific *in situ* mRNA expression was detected in the tube area in all the five venation species, and no expression was detected in the non-venation species (Figure 3.7). This supports the conclusion that the pigment venation patterning in the *Antirrhinum* genus is controlled by *Venosa*.

At the stages sampled (Table 2.6), *Venosa* expression was detected in both the tube and lobe area in *A.majus*, but only in the tube area of other species. Either no *Venosa* expression occurred in the sampled lobe cross sections, or sampling was too early in development for *Venosa* to express in the sections of other species. Indeed, in the

flower of *A.majus* the pigment occurs at the base of the tube at an early developmental stage, and expands up to the lobe area during flower development. In some other species the pigment venation patterning is weak and limited in petals even in fully opened flowers (Schwinn *et al.*, 2006).

3.3.3 The performance of the controls in the *in situ* mRNA experiments

Venosa gene transcript level is thought to be low. If strong background occurs in *in situ* hybridisation, this low expression may result in difficulty in the detection of the mRNA expression pattern. A good positive control was necessary to be sure of the experimental procedure and patterning. CHS expressed specifically in the epidermal cells of petals of antirrhinum has been detected in *in situ* mRNA hybridisation (Jackson *et al.*, 1992), and therefore, was a suitable control. CHS was selected, based on previous work (Kevin Davies, personal communication) as a control not only for the experiment procedure but also the expression patterning. The results showed, as expected, that CHS is expressed specifically in the epidermal cells of both tube and lobe area of *A. majus*. No vein-specific patterning occurred.

The negative control, the sense CHS probe, generated a pattern similar to the anti-sense probe in the *in situ Venosa* mRNA expression experiments. A much higher concentration of sense probe than anti-sense probe can result in strong background in *in situ* hybridisation (Erin O'Donoghue, personal communication). In this case, it was not the concentration that resulted in the background because, to avoid this background, similar concentrations were used for both kinds of probes. To answer why the sense probe performed similarly to the anti-sense probe, alignment was carried out with Clustal W. The result suggested that the sense and anti-sense fragments used as probes had 48% identity. However, when the sense or anti-sense *Venosa* probe was aligned with CHS cDNA with Clustal W, over 40% identity occurred in some area of the CHS sequence. Clearly, the high sequence similarity between the sense and anti-sense probes also cannot explain the background presented.

In most cases, if not all, the sense probe can result in a specific background (Cathie Martin, personal communication). Indeed, a mutant plant line lacking expression of the target gene is a better negative control than a sense probe in *in situ* hybridisation

experiments. Fortunately, the *rosea^dorsea* phenotype (AA104) serves as such a negative control. As outlined in Chapter 4, the genotype corresponding to this phenotype is homologous for a mutant *Venosa* allele (GBV) in genotype. AA123 also serves as another negative control, due to the lack of venation phenotype in this species. As expected, no venation patterning of expression was found for either of these two negative controls.

3.3.4 A possible link with a regulator of aurone biosynthesis

In addition to the vein-specific expression pattern of *Venosa*, a pattern matching the yellow colour in the flowers occurred in the *in situ* mRNA expression experiments (Figure 3.7, 3.8 and 3.9). It is possible that another regulator controlling the yellow pigmentation was detected by cross-hybridisation due to the probing of similar C-terminal domains.

The yellow colour is due to aurone production, which distributes in the epidermal cells of limited regions in the petals of the *Antirrhinum* genus (Kevin Davies, personal communication). The aurone biosynthetic pathway has been only partially elucidated (see Figure 1.4). A cDNA has been isolated from *A. majus* corresponding to a polyphenol oxidase (PPO) variant (Nakayama *et al.*, 2000, 2001). It is thought to be the aureusidin synthase (AUS), which converts chalcones to aurones (Figure 1.4). It is not known if other enzymatic steps and regulatory factors are involved in aurone production (Schwinn and Davies, 2004). No similarity was found in the alignment of the *Venosa* probe and the AUS sequence. AUS is only expressed in the epidermal cells (Davies *et al.*, 2006). Compared with this, the pattern tested with the *Venosa* probe was expressed in epidermal cells, sub-epidermal cells and inner tissue (Figure 3.8 A and Figure 3.9). However, as with venation, the epidermal specificity of aurone biosynthesis could be provided by a co-regulator. *Venosa* itself is unlikely to be the regulator, as only anthocyanin pigment was induced by the 35S:genomic *Venosa* construct following particle bombardment (Section 3.2.2) and aurones are still produced in the homozygous line of the mutant *venosa* allele (see Chapter 4 for detail). Thus, the possibility remains that a regulatory gene for aurone biosynthesis, which shares high similarity to *Venosa*, was being detected by *Venosa* probe used.

Aurones are plant flavonoids that provide bright yellow colours in the flowers of species such as antirrhinum, coreopsis and cosmos (Nakayama *et al.*, 2000; Schwinn and Davies, 2004). Recent advances have been made on the therapeutical potential of aurones such as anticancer, antihormonal activity and antidiabetes compounds, and for treating parasitic and microbial infections (Boumendjel, 2003). Undoubtedly, isolation of the gene being detected, whether it functions as a structural or a regulatory gene, would advance the elucidation of this important pathway.

3.3.5 Particle bombardment was not effective for *Venosa* RNAi

RNAi is a method widely used to study gene function, and so was attempted in this study. Particle bombardment was tested as a method to deliver the RNAi owing to the difficulty of antirrhinum transformation mediated with *Agrobacterium tumefaciens*, and previous success with transient RNAi inhibition of *Rosea1* (Kathy Schwinn, personal communication). However, the *Venosa* RNAi results showed that buds at early stages of development (≤ 10 mm) might be too young to develop under the culture conditions used, but buds ≥ 15 mm might be too advanced for the RNAi experiment, because the *Venosa* gene has already been expressed and the pigment induced at this stage. Possibly, the optimal bud developmental stage at which the bud would be bombarded might be 13 mm. However, the pigment and patterning developed poorly with buds of this length, and presented discontinuous pigment venation 'lines'. Any non-pigmented cells resulting from RNAi, if RNAi did occur, were difficult to distinguish from the undeveloped pigment cells due to use of young, cultured buds. Thus, RNAi proved an unsuitable approach in this case.

3.4 Conclusion

The non-pigmented domain in the flowers of venation phenotype *A. majus* was due to lack of the expression of a MYB factor, while other required factors for pigmentation were accessible endogenously. Notably, there was nothing in the *Venosa* tissue used that inhibited *Venosa* from functioning in cells away from veins. This suggested that venation pigmentation patterning was due to the spatial expression of a *myb* gene and the *Venosa* promoter is important to the pattern. *In situ* mRNA hybridisation showed

that *Venosa* transcriptional expression was in a vein-specific manner. The spatiality and strength of *Venosa* expression determines the location and intensity of anthocyanin pigment in the *Antirrhinum* genus. In the cross section, *Venosa* expressed from xylem cells to adaxial epidermal cells in a band. It would appears *Venosa* expression may be controlled by a signal that diffuses from the xylem to the adaxial epidermis. Hybridisation matching the pattern of the yellow colour in the flowers of *Antirrhinum* genus was also detected. This is possibly detected a regulator of the aurone biosynthesis pathway.

Chapter 4

Isolation and identification of a mutant *Venosa* allele

4.1 Introduction

Gene function can be characterised by genotype – phenotype comparison. Isolation and identification of *Venosa* alleles improves understanding of venation pigmentation patterning. Two fragments were amplified when specific primers were used in PCR for isolating genomic *Venosa* DNA to use as a reporter gene. Sequencing and functional analysis demonstrated that they were alleles. One fragment represented the functional *Venosa* allele, while the other fragment was non-functional due to mutation. This chapter describes the isolation and identification of this mutant allele of *Venosa*. The work has been published in The Plant Cell (Schwinn *et al.*, 2006).

4.2 Results

4.2.1 Isolation and characterisation of two different *Venosa* genomic clones

As was mentioned in Chapter 3, a genomic *Venosa* clone was used for the pigment complementation experiment. Based on the *Venosa* cDNA sequence the primers Y44/Y45 (see Appendix I) were designed for isolating *Venosa* (from ATG to stop codon). Genomic DNA was extracted from leaf tissue of the venation phenotype line CT128. Gradient PCR was performed, with the annealing temperature rising from 54°C to 66°C. Two bands of DNA were amplified clearly with gel analysis of *Venosa* PCR products (Figure 4.1). One band, at approximately 2.4 kbp, was designated as GSV (Genomic Small *Venosa*) and the other, at about 3.2 kbp, was designated as GBV (Genomic Big *Venosa*). The products were cloned into pART7, and the two constructs were designated as p7GSV and p7GBV.

The GSV sequence alignment with the *Venosa* cDNA sequence showed that GSV was likely the functional *Venosa* allele. The GSV PCR product was 2427 bp with three

exons and two introns (Figure 4.2, Appendix II), which is the typical structure of plant *myb* genes.

Comparison of GBV sequence (Appendix III) to the GSV and *Venosa* cDNA sequences showed that GBV was a mutated version of GSV. The GBV PCR product was 3158 bp

Figure 4.1   Two specific DNA fragments were amplified using venation phenotype genomic DNA as template in gradient PCR. Lane 1: 1 kb+ ladder; lane 2 to lane 7 showing PCR products using annealing temperatures of 56, 58, 60, 62, 64, 66°C respectively in gradient PCR. 1% (w/v) TBE agarose gel was stained with EtBr.

A
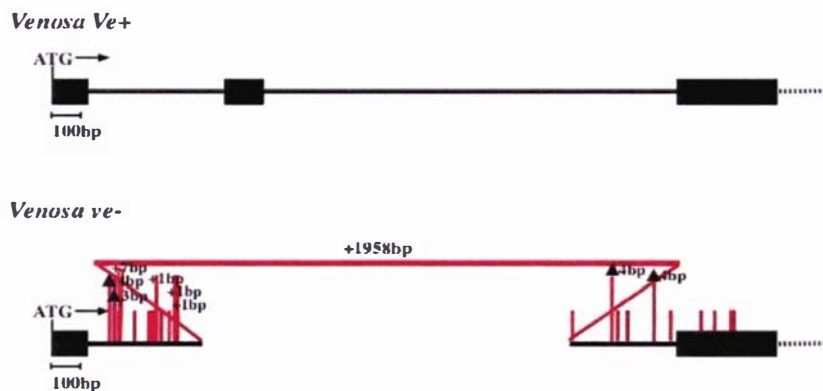
Figure 4.2   Structural comparison of two *Venosa* alleles. The black boxes indicate the exons. The verticle red bars indicate the locations of small differences of nucleotide. The +1958bp horizontal red bar indicates the replacement fragment, which seems to be a transposon due to direct repeats at its ends. This figure is taken from Schwinn *et al.*, (2006).

long. A 1958-base fragment in GBV replaces the central part of GSV from the middle of intron 1 to the middle of intron 2 (from +510 bp to +1731 bp), which includes the second exon (Figure 4.2.A). A direct repeat flanks this fragment. This direct repeat is 40 bp with two nucleotides unmatched (Appendix IV). A BLAST search with the insert sequence found no significant matches in the GenBank database. Other differences between GBV and GSV are minor and comprise 23 mostly single-nucleotide changes over the 3158 bp GBV sequence.

To test whether GSV was a functional allele and GBV was not, transient assays were conducted. Many pigment foci occurred when p7GSV was used for transient assay (Figure 4.3.A), while no foci occurred in the p7GBV particle bombardment experiments (Figure 4.3.B). The GFP fluorescent foci occurred from the internal control p7GFP (*35S:GFP*), which was co-precipitated with the tested construct on the gold particle. This suggested that no pigment foci were due to the non-expression of p7GBV.

A   B
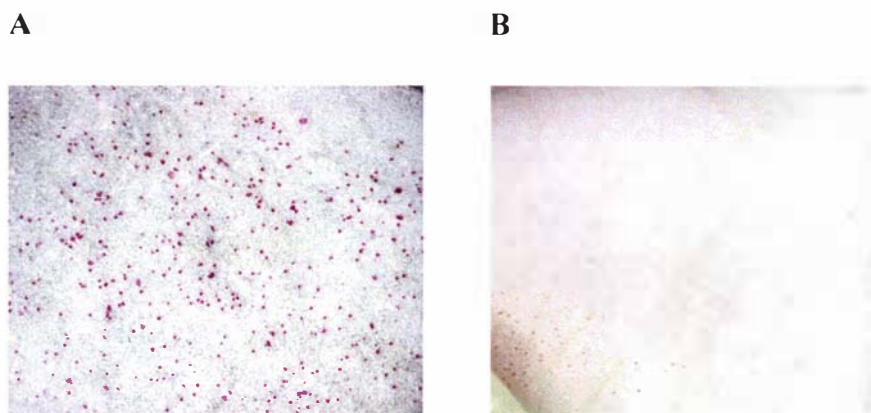
Figure 4.3 Functional analysis of two *Venosa* alleles. (A) Pigment was induced in the epidermal cells of *rosea^dorsea* phenotype by the 35S:GSV construct introduced with particle bombardment. (B) No pigment was induced by the 35S:GBV construct introduced with particle bombardment. The foci are the traces of gold particle clusters, not pigment cells. To cover bigger field lower magnification was used in this photo.

4.2.2 Structural similarity between GBV promoter and GSV promoter

The PCR amplified products analysed by agarose gel electrophoresis showed that two bands always occurred with the primer combinations Y16/Y45, Y17/Y45, Y18/Y45 and Y44/Y45. The pattern of fragments was in a gradient manner, which corresponded to the primers Y16, Y17, Y18 and Y44 (Figure 4.4). This result suggested that in the

fragment -380 bp relative to the ATG the GBV promoter was likely to be similar in structure to the GSV promoter.
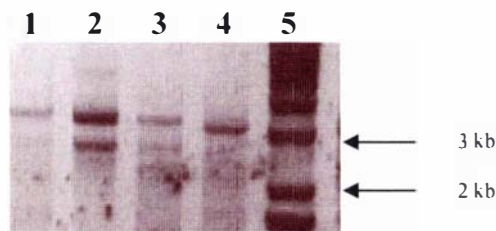
Figure 4.4 GBV and GSV possibly share same or similar promoter region. Genomic DNA of venation phenotype was used as template, Y45 was used as reverse primer and the primers for different 5' end promoter deletions were used as forward ones. Lane 1: Y16 (-380 bp); lane 2: Y17 (-260 bp); lane 3: Y18 (-150 bp); lane 4: Y44 (0 bp); lane 5: 1 kb+ ladder. PCR products were run in 1% (w/v) TBE agarose gel, and the gel was stained with EtBr.

4.2.3 Genotypes of the tested lines

PCR results showed that lines CC112, CL144 (both show the $rosea^{dorsea}$ phenotype) and CC112P gave only the GBV fragment and are likely homozygous for the GBV allele (Figure 4.5). As expected, venation phenotype line CT128 gave both GSV and GBV fragments, so is likely to be heterozygous for the two alleles. Strikingly, no band occurred for line 522.
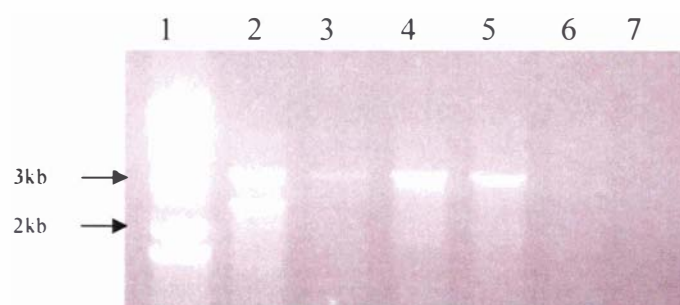
Figure 4.5 Using *Venosa* specific primers in PCR to determine the *Venosa* genotypes in different phenotype lines. Lane 1: 1 kb+ ladder; lane 2: venation phenotype from CT128; lane 3: $rosea^{dorsea}$ phenotype from CC112; lane 4: $rosea^{dorsea}$ phenotype from CL144; lane 5: fully red phenotype line CC112p; lane 6: fully red phenotype line 522 Full Red; lane 7: negative control of no template. PCR products were run in 1% (w/v) TBE agarose gel, and the gel was stained with EtBr.

4.3 Discussion

4.3.1 GSV and GBV are alleles

As will be introduced in Chapter 5, the intention was to use the *Venosa* gene as a reporter for the transient assay of promoter deletions. To improve the amplification specificity of PCR, long primers were designed and a high annealing temperature (66°C) was used in amplifying *Venosa* from genomic DNA. Genomic DNA extracted from the leaf tissue of venation phenotype line CT128 was used as template. This line was thought to be heterozygous at the *Venosa* locus (Cathie Martin, personal communication), and its venation phenotype indicated that there was a functional *Venosa* allele present. Two specific fragments were isolated using PCR, and cloned downstream of the 35S promoter in pART7. Sequencing these two fragments demonstrated that the *Venosa* cDNA corresponded to the sequence of GSV, and GBV is a mutant version of GSV. A 1958-base fragment replacing the central part of GSV has generated the mutant. This fragment is thought a possible transposon because it contains a 789 bp (263 amino acids) open reading frame and imperfect 40 bp direct repeat termini (see Appendix IV), which is a typical structure in some transposons. For example, the yeast Ty elements (Hauber *et al.*, 1985). In addition to the transposon replacement, there were 23 small differences in the remaining areas between GBV and GSV. This high level of similarity indicates GBV is an allele of GSV. Further PCR analysis using a set of primers for the promoter region showed that as far as -380 bp relative to the ATG, the GBV promoter shared high similarity with the GSV promoter (Figure 4.4). Thus, there is strong evidence that GBV is an allele of GSV, in support of the genetics that suggest only one *Venosa* locus (Cathie Martin, personal communication). The GBV promoter was not isolated due to its non-founction.

Transposable elements are a heterogeneous class of genetic elements that can insert at new locations on chromosomes. They vary in structure, mechanism of transposition, the fate of the donor element, and their choice of target sites. Some transposable elements can insert into the genome as intronic sequences, a mechanism that has been proposed for the insertion of introns (Menssen *et al.*, 1992). For example, a 743 bp fragment was found in intron 2 of maize *P1-rr* and *P1-wr*, but not in *P1-rw* and *p2* alleles. A 10 bp

direct repeat (5`-TGATTTTGAC-3`) flanks this fragment (Athma *et al.*, 1992). Different to the insertion of a genomic locus, the transposon in GBV replaced the fragment between the two introns. Thereafter, exon 2 and parts of intron 1 and intron 2 were removed. It is usual that a transposon inserts at a new location, but unusual that it replaces a fragment when it moves to this new location. Although several transposons have been identified from antirrhinum (Coen *et al.*, 1986; Sommer *et al.*, 1988), the sequence in GBV had no significant similarity to any transposon sequence in the GenBank database. It may be a new transposon, or have lost activity during gene evolution.

Because exon 1 and exon 3 were not altered at the DNA level, if GBV is expressed and splicing occurs in a similar manner as GSV, exon 1 + exon 2 could provide a reading frame and produce a truncated protein. Transposon replacement of an intronic fragment may be a mechanism of gene development and evolution, by which new proteins could be produced.

4.3.2   GSV represents a functional *Venosa* allele and GBV a non-functional allele

Venosa transcript was detected in the venation phenotype line but not detected in the *rosea*^*dorsea* lines (Schwinn *et al.*, 2006). This result is consistent with the experiment of *Venosa* mRNA *in situ* hybridization (Chapter 3). Because the probe used in the experiment of *Venosa* mRNA *in situ* hybridization was also suitable for the detection of expression of the mutant *venosa* allele, the results suggest that *venosa* is not transcribed or its transcripts are degraded quickly. The transient particle bombardment assay further showed that GSV was functional and GBV was not functional (Figure 4.3), thereby indicating that GSV is the functional, transcribed *Venosa* allele.

4.3.3   *Venosa / venosa* genotypes were consistent with venal / non-venal phenotype

Identification of the mutant *venosa* allele helps to explain the polymorphisms observed for the *Venosa* gene and contributes to the understanding of the evolution of pigment venation patterning. Genotype – phenotype comparisons showed that the venation phenotype of line CT128 was heterozygous (*Vv*), while the non-venation phenotype (*rosea*^*dorsea* phenotype) of lines CC112 and CL144 were GBV homozygous (vv). This

provides evidence not only for the non-functionality of GBV, but also for the function of *Venosa*, *i.e.* the venation phenotype is due to *Venosa* (see Chapter 3).

It is also consistent with the genotype – phenotype comparison in line CC112P, in which the corolla is fully pigmented except for the inner tube. Because in *A. majus* flowers the fully pigmented phenotype controlled by *Rosea* masks the venation patterning controlled by *Venosa*, the venation phenotype could not be seen in fully red flowers. The genotype of CC112P (vv genotype) is consistent with its phenotype, in which there is no pigment venation patterning in the inner tube area.

No fragment was amplified using line 522. Because there is a clear venation patterning in the inner tube area of this fully red line, it is not known whether the failure of amplification could be due to the quality of genomic DNA template, or due to a different locus controlling this patterning, or to other alleles of *Venosa*.

4.4   Conclusion

A mutant allele of *Venosa* was isolated and identified. Its central part including the second exon was replaced by a transposon. Probably, its non-functionality was due to the lack of the second exon. Lines showing the non-venation phenotype are homozygous for this mutant allele, while lines showing the venation phenotype are due to the presence of a functional *Venosa* allele.

Chapter 5

Transient assay of *Venosa* promoter 5' deletion constructs

5.1   Introduction

Transient assay is a common method used to analyse gene expression. Compared with making stable transgenic plants the prominent advantage of this method is that it is much quicker to get results. As one of the main transient assay methods, particle bombardment was considered for this project. Furthermore, an efficient *Agrobacterium* mediated transformation system has not been developed to make stable transgenic antirrhinum plants, while particle bombardment can be a practical way to transform antirrhinum for transient assay. As the zones where *Venosa* is expressed include the petal epidermis and the epidermal cells, the target cells should be transformed efficiently by particle bombardment.

Our UK collaborater (Prof. Cathie Martin) conducted a preliminary analysis of a 2.4 kbp fragment (Appendix V) of the *Venosa* promoter using tobacco transgenics. A -760 bp deletion presented very weak expression, but probably had a venal expression pattern (Cathie Martin, personal communication). The proximal region of a gene usually plays an important role in transcriptional control. Given this, the -900 bp fragment was targeted to be analysed in detail. The aim of this part of the project was to analyse how *Venosa* is regulated by *cis*-elements in this fragment using transient assay. Unexpectedly, several technical questions were raised during the work. For example, when the bombardment is foci negative, what is the reason for undetectable expression. Is it due to low sensitivity of the detection method? Is gene expression too weak or is there no expression? When the bombardment is foci positive, how to determine if it is vein-related for its cell specificity? Obtaining convincing evidence and answering such questions proved challenging. To address these issues, GFP, intron-containing GUS (IGUS) and MYB anthocyanin regulators (*Rosea* cDNA and genomic *Venosa*) were used. The reporting mechanism of these three types differs. GFP indication is a simple physical procedure, in which blue light or UV is used to excite the GFP to emit green

fluorescent light. This fluorescence signal cannot be amplified. GUS indication is a chemical procedure, in which blue colour results from reaction of β-Glucuronidase to the substrate, X-Gluc. The signal can be strengthened by the reaction time and the substrate concentration. The anthocyanin regulator indicates expression via up-regulation of the anthocyanin biosynthetic pathway, which may allow promoter expression signal to be significantly amplified by signaling pathway efficiency. GFP and GUS are common reporters used in molecular biology studies. Their expression images closely reflect their spatial transcription due to lack of post-transcriptional and post-translational modification. The anthocyanin regulator can be used as reporter only in appropriate tissues in which the anthocyanin biosynthetic pathway can be induced. Its expression domain should be considered carefully, because it may be affected by unknown post transcriptional mechanisms.

Transcription factors are usually present at low levels, due to their signaling role, likely reflecting weak promoter activity. Weaker expression than the endogenous gene may then occur in promoter assays, due to promoter deletions. This may result in difficulty in detecting the reporter gene signal. In order to overcome this difficulty two ways could be used. One way is to improve the detection method, and another is to quantitatively improve the expression of the tested promoter.

It has been reported that *cis*-element strength of the ROL6 promoter was enhanced by placing the maize *Ubi1* flanking region upstream of the ROL6-SynCore promoter, and that the enhanced ROL6 promoter retained the root-preferred activity (Lu and Bruce, 2000). This suggested that the flanking region of the ubiquitin promoter might enhance expression without seriously affecting the expression domain. To improve the expression of the tested *Venosa* promoter fragments, a fragment upstream of the TATA box of the *Arabidopsis* ubiquitin promoter was tested as an enhancer.

Plant viral 5'-UTR region can influence expression of reporter genes in plants (Gallie *et al.*, 1987; Dowson Day *et al.*, 1993; Qin *et al.*, 2000). Omega (Ω) was chosen to test from among the available leaders. It is a 68 bp sequence of the 5'-leader of tobacco mosaic virus (TMV) that has been shown to act as a translational enhancer in transgenic plants (Gallie *et al.*, 1987; Dowson Day *et al.*, 1993; Qin *et al.*, 2000), increasing expression 3-7 fold over the native leader (Dowson Day *et al.*, 1993).

Two-step transcriptional amplification systems have been reported for enabling tissue-specific over-expression of genes. Bacteriophage T7 RNA polymerase (T7 system) directed inducible and tissue-specific over-expression of foreign genes in transgenic tobacco and rice. The recombinant protein was expressed at a 3-10 fold higher level when compared with transgenes expressed directly under the control of unmodified tissue-specific promoters (Nguyen *et al.*, 2004). Another two-step transcriptional amplification system is the Gal4 system, in which the target promoter drives an artificial transcriptional activator, Gal4-VP16 fusion protein, and it in turn activates the target transgene expression under the control of Gal4-responsive elements. The level of transgene expression could be augmented greatly in animal cells using this system (Fang *et al.*, 1998). It has also been used as a method for enhancing transgene expression using weak promoters in animals (Segawa *et al.*, 1998; Koster and Fraser, 2001; Lyer *et al.*, 2001), and for enhancer or tissue specific promoter trapping in stable transgenic plants (Wu *et al.*, 2003; Yang *et al.*, 2004; Johnson *et al.*, 2005; Nakayama *et al.*, 2005). Thus it had promise to enable use of GUS or GFP reporter genes with the *Venosa* promoter. GFP is a particularly attractive reporter gene for this study, as the GFP foci can be readily visualised in relation to the vein, due to the distinct vein background produced by red fluorescence of lignin under blue or UV light.

The transient assay using particle bombardment is based on the detection of transient gene expression. It has recently been shown that post-transcriptional gene silencing (PTGS) can stop the ectopic protein expression after 2-3 days, limiting the efficiency of this method (Voinnet *et al.*, 2003). Viral suppressors of PTGS can alleviate the host silencing response, hence enhancing the transient expression of a broad range of proteins. The p19 protein encoded by tomato bushy stunt virus (TBSV) is an effective suppressor of PTGS, and has been shown to improve protein production by 50-fold in some instances (Voinnet *et al.*, 2003). Use of p19 may be an approach to enhance expression of the VEN:IGUS (*Venosa* promoter deletions drive IGUS) constructs.

To amplify expression from the promoter the above four measures - transcriptional enhancement (*Arabidopsis* ubiquitin enhancer), translational enhancement (omega element), inhibition of gene silencing (P19), and two-step transcriptional amplification system (Gal4 system) - were tested for their efficacy and efficiency. Combined with

such experimental modification, the promoter fragment of -2.4 kb was tested, with a detailed analysis of the -900 bp region.

Agro-infiltration is a transient assay mediated by *Agrobacterium*-infection, which had been shown to be effective for gene expression (Yang *et al.*, 2000). This approach avoids the need for cell culture and growth of stable transgenics generated from calli, so provides a rapid method for assaying the function of some types of gene; transgenes can be assayed within a few days of Agro-infiltration. This method has been used for transgenic complementation (Bendahmane *et al.*, 2000; Van der Hoorn *et al.*, 2000; Johansen and Carrington, 2001; Shao *et al.*, 2003), promoter analysis (Yang *et al.*, 2000) and protein production (Vaquero *et al.*, 1999, 2002). *Venosa* expresses in petals, so if petal tissue could be transformed by Agro-infiltration, the expression pattern could be observed much quicker than flowers from stable transgenics. Agro-infiltration of the petal tissue of tobacco, petunia and antirrhinum was tested for the *Venosa* promoter 5' deletions in work reported in this chapter.

5.2   Results

5.2.1   *-700 VEN:GFP* and *-700 VEN:IGUS* constructs fail to give foci in transient assays with particle bombardment

The petals from buds from 1 cm length to just fully opened flowers of both venation and *rosea*^{dorsea} phenotypes were bombarded with *-700VEN:GFP* and *-700VEN:IGUS*. No foci were observed over five days of examination. In contrast, after 2-4 days many foci occurred in the petals bombarded with the *35S:GFP* or *35S:IGUS* constructs used as positive controls (Figure 5.1).

The petals from just fully opened flowers of *rosea*^{dorsea} were bombarded with pJAM1371 (*-2.4kVEN:GUS*), pJAM1367 (*-1.6VEN:GUS*) or pJAM1334 (*-760VEN:GUS*), which were constructed by our UK collaborator. No GUS foci were seen from these constructs.
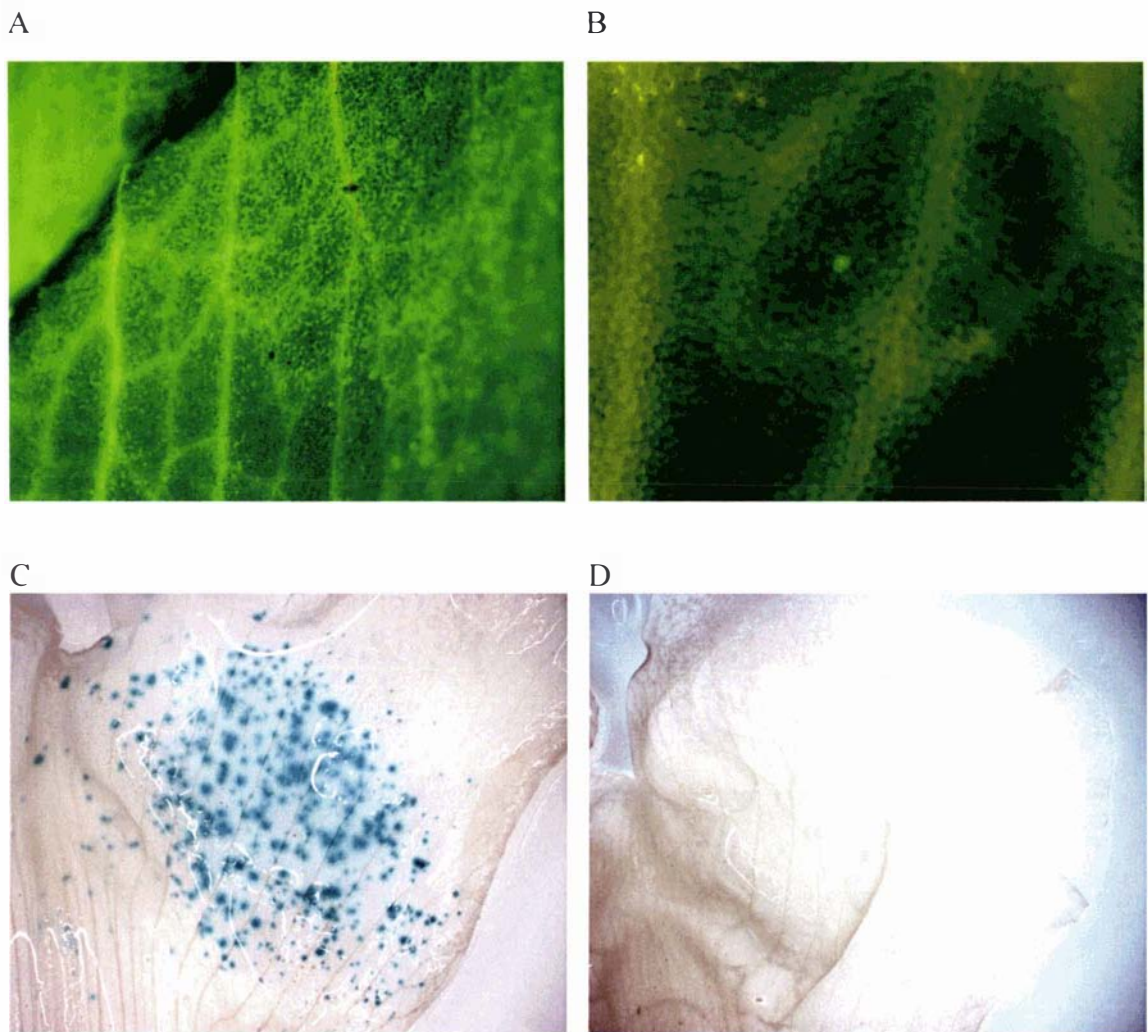
Figure 5.1   Transient assay using particle bombardment transformation of antirrhinum petals of the *rosea^dorsea* line. (A) GFP foci of the *35S:GFP* control construct. (B) Transformation with *VEN:GFP* constructs. No GFP foci were apparent. (C) GUS foci of the *35S:IGUS* control construct. (D) Transformation with *VEN:IGUS* constructs. No GUS foci were apparent.

5.2.2 Efficacy of *Venosa* as reporter gene in particle bombardment

20-30 weak foci occurred in both the adaxial and abaxial areas of *rosea*^{dorsea} petals bombarded with -*900VEN:gVenosa* (Figure 5.2 A and B) or -*100VEN:gVenosa* (Figure 5.2 C). However, 20-30 weak foci also occurred in the adaxial area of *rosea*^{dorsea} phenotype when bombarded with *promoterless:gVenosa* construct (Figure 5.2 D). In comparison, over 100 strong foci occurred in the petals bombarded with the positive control construct, *35S:gVenosa* (data not shown). No foci occurred in the petals bombarded with the negative control construct, pART7 empty vector (Figure 5.2 E).

5.2.3 Efficacy of the *Arabidopsis* ubiquitin transcriptional enhancer and omega translational enhancer

Many GUS foci occurred in the petals bombarded with p27UF900IGUS and p27UF700IGUS, but fewer when bombarded with p27UR900IGUS and p27UR700IGUS. In contrast, similar amounts of foci occurred between p27UFIGUS and p27URIGUS (data not shown).

No GUS foci were observed in the petals bombarded with pVe900ΩGUSI. Over one hundred GUS foci occurred in the petals bombarded with either *35S:ΩIGUS* or *35S:IGUS*, but no difference in foci number was apparent between these two constructs.

5.2.4 Particle bombardment assays using the Gal4 enhancement system

Many fluorescent foci occurred in the petals bombarded with pΔVetrapper. They were not vein-related (Figure 5.3 B and C). Similarly, many foci occurred in a non-vein-related pattern when using the pC-4956:ET15 control (Figure 5.3 A). A similar number of foci and distribution pattern occurred with the bombardment of the mixture of the Gal4 producing fragment and GFP reporter fragment (Figure 5.3 D). Several weak GFP foci occurred from the bombardment of the GFP reporter fragment alone (Figure 5.3 E).
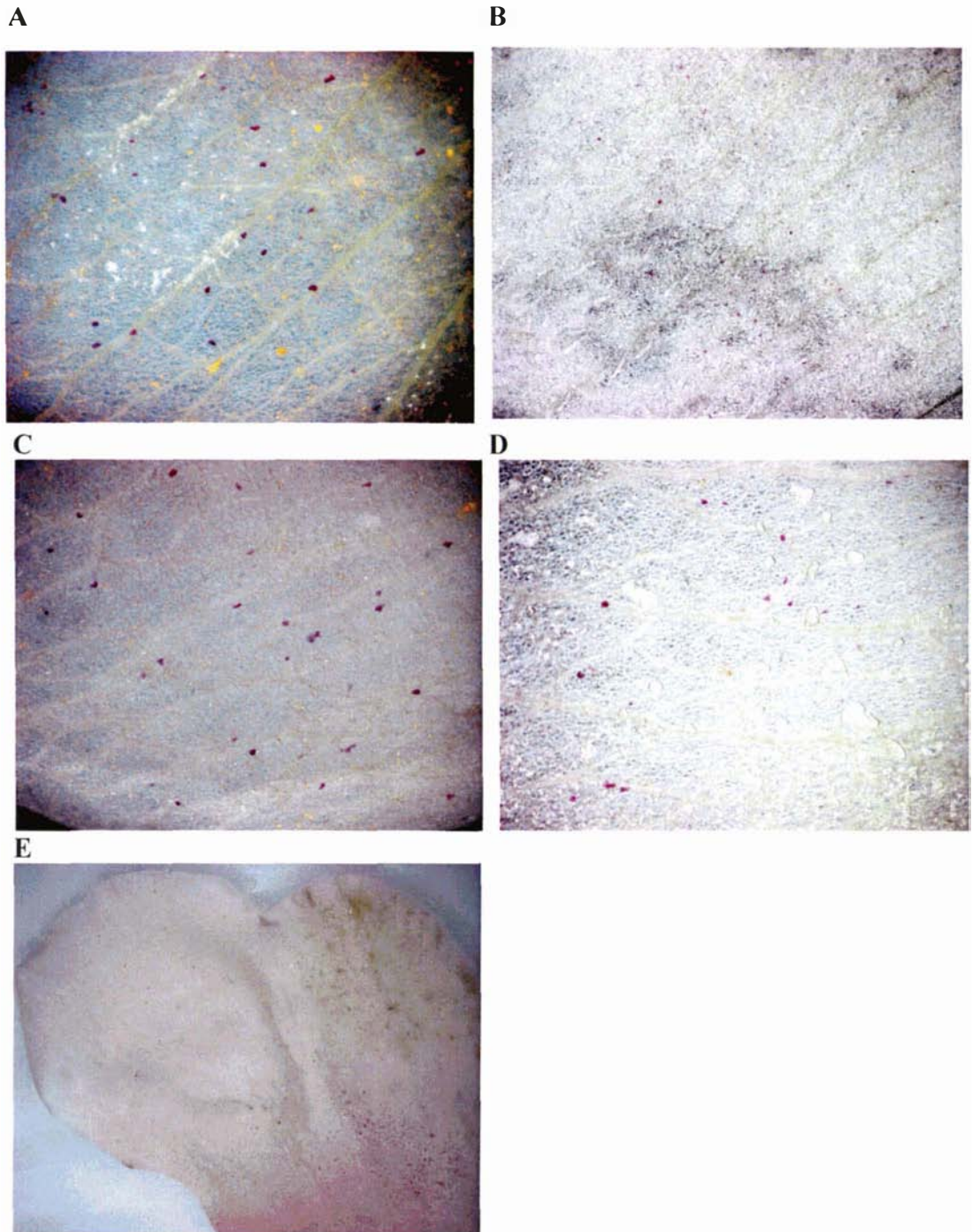
Figure 5.2 *Venosa* promoter transient assay using particle bombardment with genomic *Venosa*. The petals of *rosea^dorsea* were bombarded. Positive expression of the constructs is observed as single pigmented cells (pigmented foci). Expression pattern of *-900VEN:gVenosa* construct in adaxial area (A) and abaxial area (B); (C) Expression pattern of *-100VEN:gVenosa* construct in adaxial area; (D) Expression of promoterless:genomic *Venosa* construct in adaxial area; (E) Expression of the pART7 empty vector control.
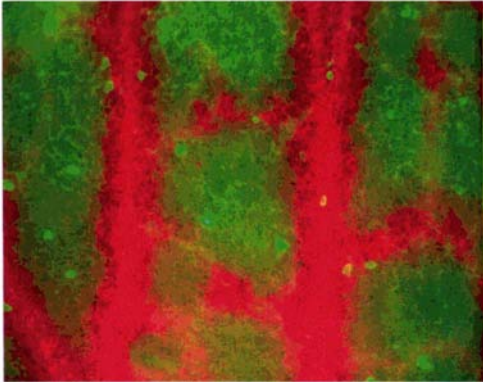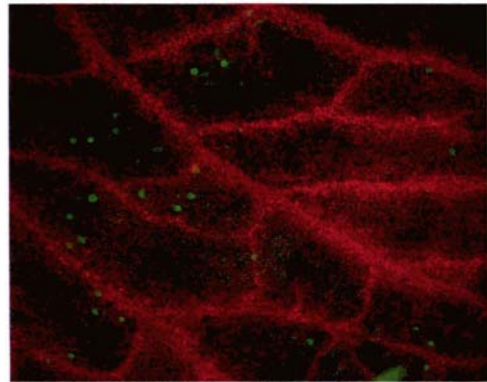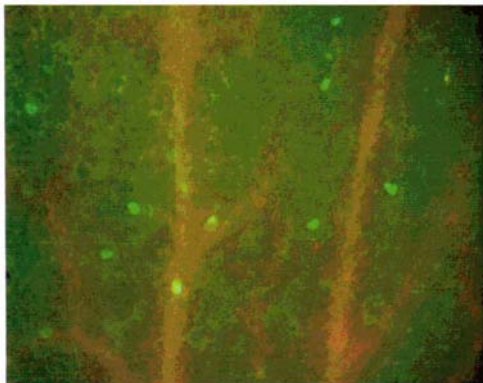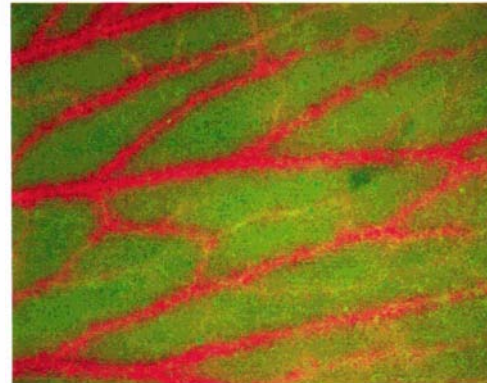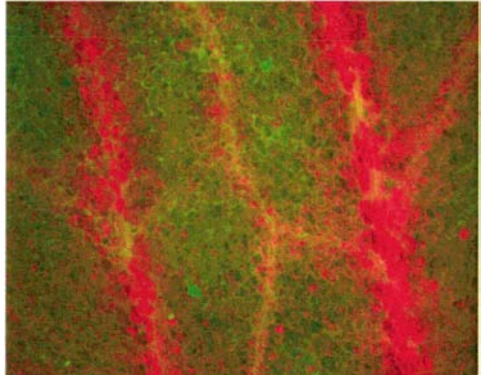
Figure 5.3 Transient assay with particle bombardment using Gal4 two-step transcriptional amplification system. The GFP reporter gene was used in all cases, and the adaxial region of petals from venation phenotype *A. majus* was the target tissue. (A) Expression pattern of the plasmid pC-4956:ET15 (Gal4 trapper); (B) Expression pattern of pΔVe trapper construct. The tissue was water-infiltrated to aid photography; (C) Expression pattern of pΔVe trapper construct; (D) Expression pattern of a mixture of -90CaMV:GFP fragment and -40CaMV:Gal4 fragment. (E) Expression pattern of -90CaMV:GFP.

5.2.5 p19 as a suppressor of gene silencing

Method I: When the petals of venation phenotype (Figure 5.4 C) or tobacco leaves (Figure 5.5 C) were bombarded with the mixture of *35S:p19* and *VEN:IGUS* constructs, GUS foci occurred in all combinations, even in the *35S:p19 + promoterless:IGUS* combination, although in this case only a few foci were observed (Figure 5.5 A). No foci occurred in the petals bombarded with *VEN:IGUS* only (Figure 5.5 B and D, and 5.1 D). The number and strength of foci decreased as the promoter fragment became shorter, based on observation with the naked eye. The foci location was not vein related. Foci also occurred in the petals bombarded with a mixture of *-700VEN:IGUS* and pART7 (Figure 5.4 A).

Method II: Many foci were obtained with *35:p19 + VEN:IGUS* in the same vector, and using the standard bombardment protocol it was hard to determine if they were vein-related, due to the high number of foci. A lower amount of plasmid, 400 ng, was tested to reduce foci number and it could then be seen that the foci were not vein-related.

Method III: Very few foci were ever observed when using sequential bombardment of the *35S:p19* construct and *VEN:IGUS*. Of the foci that did occur, some were seen with the -150 bp promoter, and they looked vein-related (Figure 5.6 D), but this result could not be repeated. No foci occurred using shorter promoter fragments. It was observed that both pigment and GFP fluorescence could occur in the same cells when the petals were bombarded with *35S:GFP* first, then subsequently with *35S:Rosea* (Figure 5.5 E and F).

Method IV: When the petals of venation phenotype of antirrhinum were bombarded with the mixture of *-900VEN:p19* and *-900VEN:IGUS*, GUS foci occurred (Figure 5.6 A). Further bombardment experiments showed that deletion constructs as short as *-500VEN:GUS* were positive for GUS staining. The number of foci decreased as the length of the promoter fragment decreased. The foci occurrence appeared unrelated to the veins (Figure 5.6 B).

5.2.6   Agro-infiltration assay

When *35S:GUS* was used for Agro-infiltration, attached (Figure 5.7 A) or detached (Figure 5.7 C) tobacco flowers showed clear GUS staining in epidermal cells (Figure 5.7 B). Similar results were achieved when using *35S:GFP* (Figure 5.8 A and C). The GFP fluorescence intensity was similar to that of stable *35S:GFP* transgenics (chapter 6). No GUS staining (Figure 5.8 D) or GFP (Figure 5.8 B) fluorescence occurred in the respective negative controls.

Different developmental stages were tested with *-900VEN:IGUS* and *-700VEN:IGUS* using Agro-infiltration. No GUS staining occurred in the Agro-infiltrated attached or detached tobacco flowers with these constructs (data not shown).

Infiltrating detached petunia flowers with *35S:IGUS* and *35S:GFP* constructs showed similar results as for tobacco (Figure 5.7 E and F), with positive signal for samples ranging from 15 mm length buds to fully opened flowers.

Infiltrating detached petals of venation antirrhinum with *35S:IGUS* or *35S:GFP* constructs gave no observed GUS staining or GFP fluorescence (data not shown).

5.3   Discussion

5.3.1   The expression of *Venosa* promoter deletions was too weak to be detected using IGUS or GFP as reporters for transient assays

In the transient assays no foci occurred using the *VEN:GUS*, *VEN:IGUS* or *VEN:GFP* constructs, suggesting that activity of the *Venosa* promoter is too weak to give adequate reporter protein levels. Thus, analysis of the deletion constructs was not possible using the standard reporter gene approach used with most promoter analyses.

Foci occurred in the bombardment experiment of *VEN:gVenosa*. However, they occurred also with the *promoterless:gVenosa*. That the induced pigmentation was due to *Venosa* was demonstrated by the lack of colour foci when using controls such as gold

A
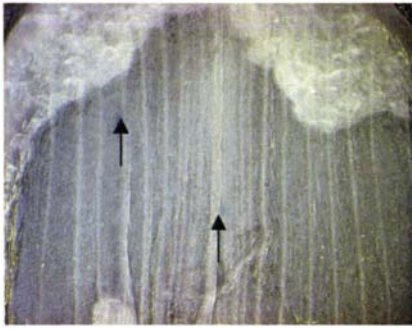
B

C
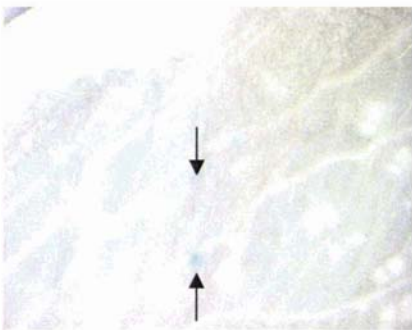
Figure 5.4   Transient expression using p19 as inhibitor of PTGS. Petals from just fully opened venation phenotype flowers were used as materials. (A) Foci occurred in the petals bombarded with a mix of the constructs -*700VEN:GUS* and pART7. (B) Foci occurred in the petals bombarded with a mix of -*150VEN:lGUS* and *35S:p19*. (C) Foci occurred in the petals bombarded with -*150 VEN:lGUS* and *35S:p19* separately.
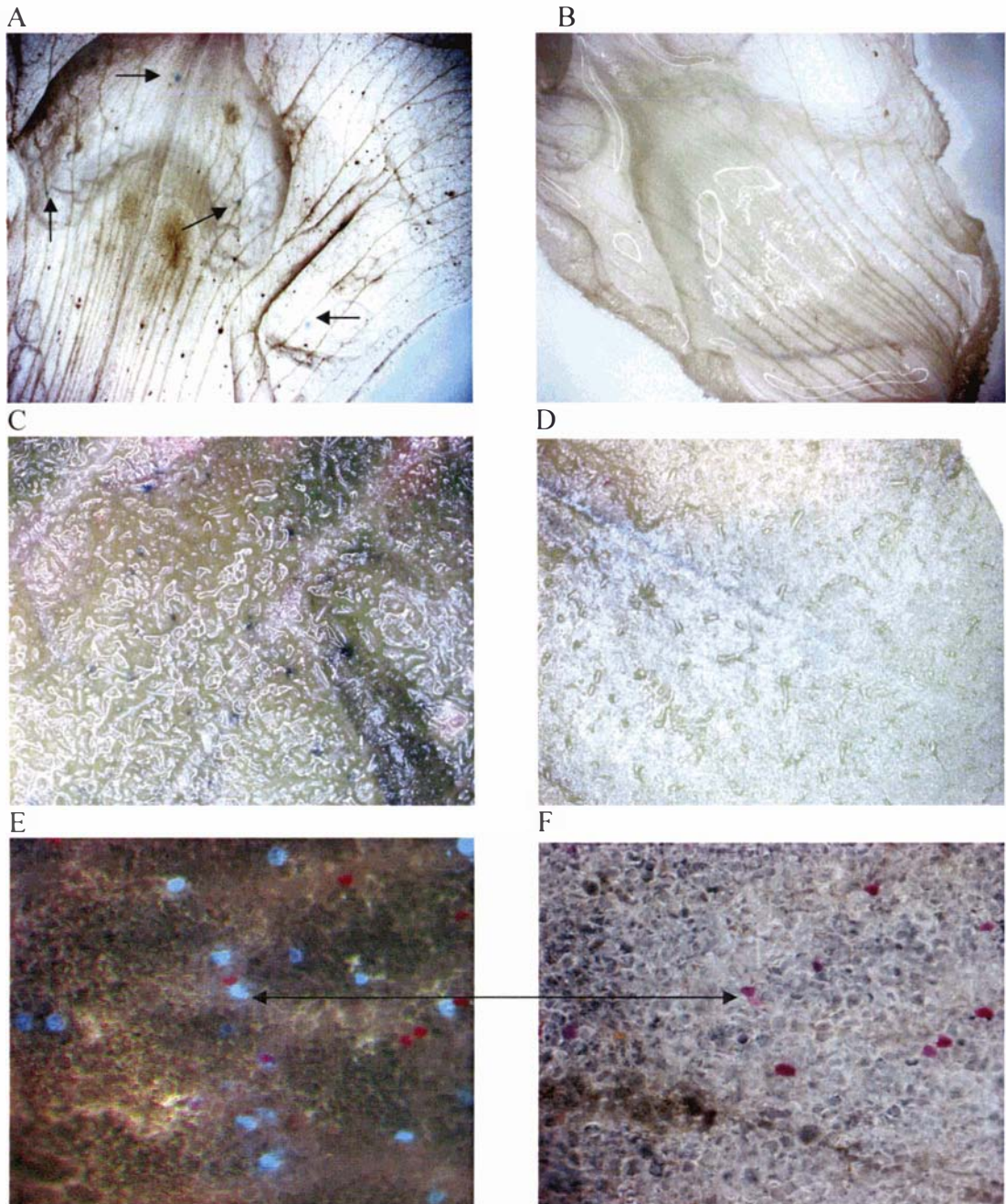
Figure 5.5 Transient assay using *35S:p19* construct. (A) Foci occurred in the petals bombarded with a mix of pVe40-2 (*promoterless:lGUS* construct) and *35S:p19*. (B) No foci occurred in the petals bombarded with the pVe40-2 construct only. (C) Foci occurred in tobacco leaves bombarded with the mixture of *-700VEN:lGUS* and *35S:p19*. (D) No foci occurred in the tobacco leaves bombarded with *-700VEN:lGUS* construct only. (E and F) Two constructs could be introduced into same cells separately. GFP (E) and pigment (F) occurred in the same cell (arrow) when first *35S:GFP* and then subsequently *35S:Venosa* were separately introduced into the epidermal cells of *rosea^dorsea* phenotype petals by particle bombardment.
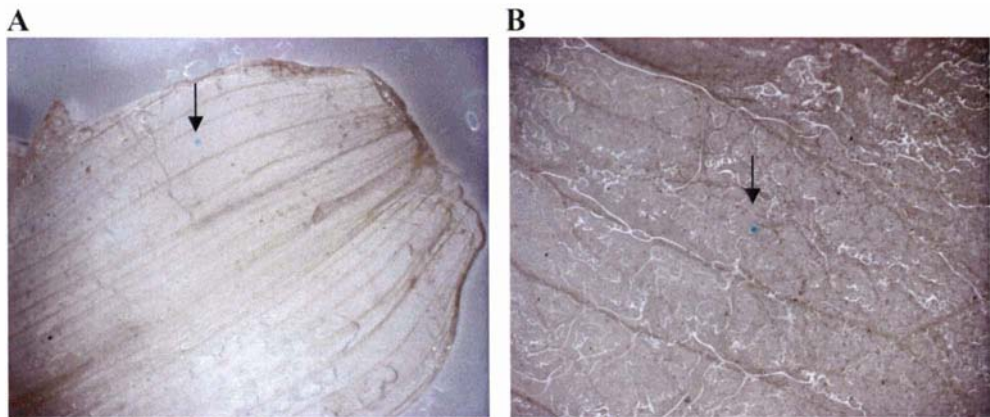
Figure 5.6   *-900VEN:p19* can enhance *VEN:IGUS* activity, resulting in a low number of GUS-staining foci when co-introduced into the epidermal cells of venation phenotype petals with particle bombardment. (A) Foci occurred in the petals bombarded with a mix of *-900VEN:p19* and *-900VEN:IGUS*. (B) Foci occurred in the petals bombarded with a mix of *-900ProVEN:p19* and *-500VEN:IGUS*.
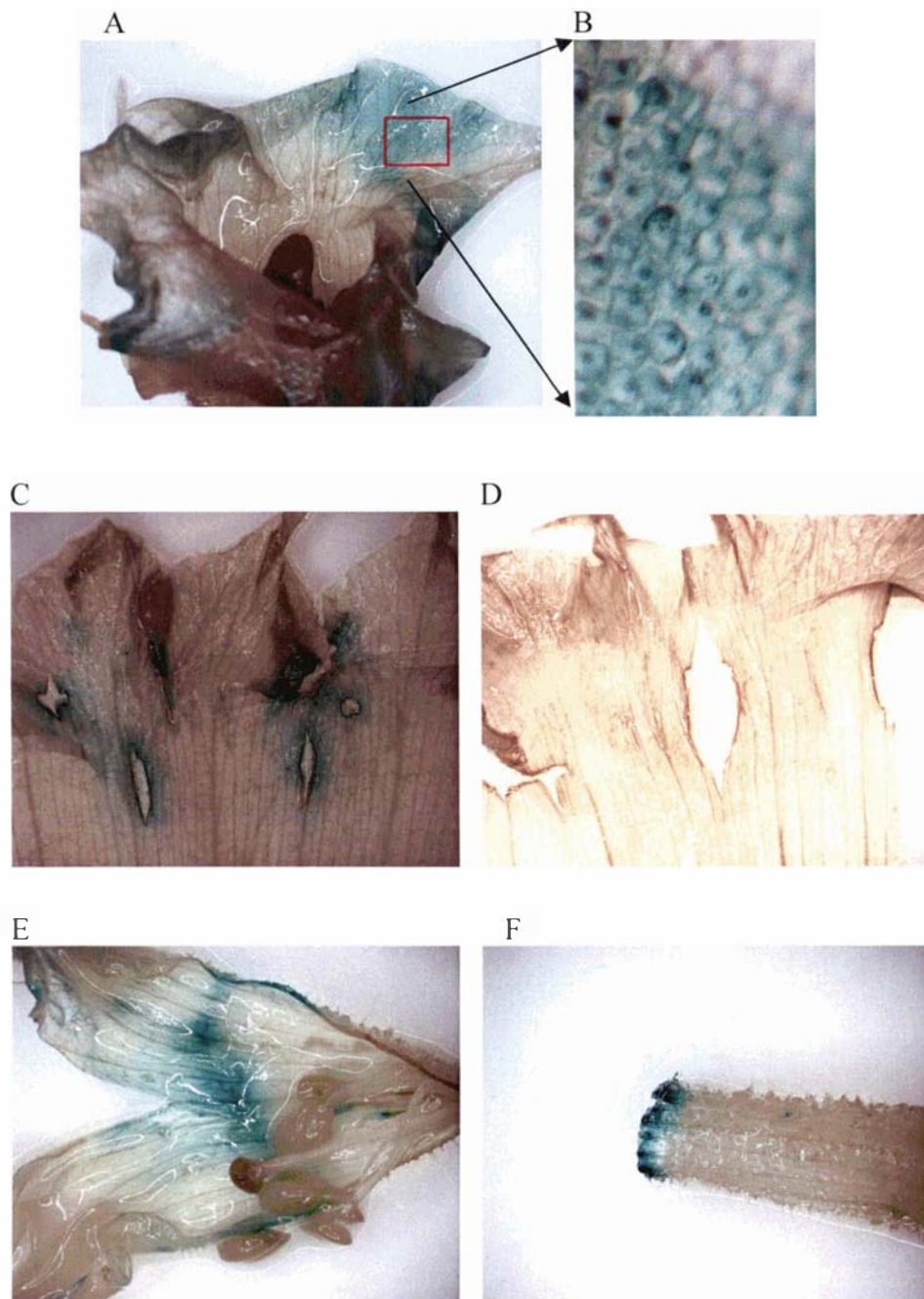
Figure 5.7 A transient expression system for flowers using Agro-infiltration and *35S:IGUS*. GUS staining occurred in mature tobacco limb epidermal cells after either detached (A and B) or attached (C) tobacco flowers were Agro-infiltrated. (D) No GUS staining occurred in the negative control using pART27. GUS staining also occurred in petunia limb (E) and tube (F) after the detached flower tissue was Agro-infitrated with *35S:IGUS*.

alone or pART7. These results showed that anthocyanin biosynthesis was regulated by the MYB anthocyanin regulator very sensitively and efficiently. They also suggested that low level of MYB protein could be produced in the promoterless construct under particle bombardment conditions. Perhaps the backbone of the vector or the 'contamination' (see Section 5.3.4 for detail) provided the transcription machinery for this low level production. Therefore, the MYB anthocyanin regulator was also not appropriate for the promoter analysis using particle bombardment-mediated transient assays.

5.3.2 Arabidopsis ubiquitin promoter transcriptional enhancer and Omega translational enhancer were not effective for *Venosa* promoter analysis

Based on the *Ubi1* flanking region reported to be a gene enhancer, arabidopsis ubiquitin promoter enhancer was fused upstream of *Venosa* promoter deletion fragments in two directions. The shooting results showed that many more foci occurred using the forward direction construct than the reverse direction, which suggested that forward direction was much stronger than reverse direction in transcription enhancement. This does not fit the definition of an enhancer, which should not show directional preferance. In addition, the enhancement fragment may have broad promoter activity, which may mask the specificity of the assayed *Venosa* promoter deletions.

Sandhu *et al.* (1998) reported that an A/T-rich sequence of the pea plastocyanin gene (*PetE*) promoter acted as a quantitative enhancer of gene expression in transgenic tobacco and potato plants. This was considered for my study, but it was also shown that this enhancer element required a chromatin context to increase transcription and failed to increase reporter gene expression when the same constructs were introduced transiently into plant cells (Chua *et al.*, 2001).

The Ω enhancer was also tested for improving translational efficiency. It was fused between the promoter and reporter gene. Particle bombardment results showed that no foci occurred using the *-900VEN:ΩIGUS* construct, and no obvious difference could be recognized in strength between *35S:ΩIGUS* and *35S:IGUS*. This suggested that the enhancement effect of Ω might be limited for transient expression.

5.3.3   The Gal4 system was not effective for *Venosa* promoter analysis

The GAL4 system was thought to be very promising for use in this project and, based on the plasmid of pC-4956:ET15 (Figure 5.9 A), pΔVetrapper was made. However, particle bombardment results showed that pΔVetrapper expressed in a non-vein related manner similar to the expression pattern of the control construct, pC-4956:ET15 (Figure 5.3 A, B, and C). To clarify which fragment affected the expression, the purified GFP reporter fragment was used for bombardment. A few foci occurred (Figure 5.3 E). This suggested that the -90 bp 35S core promoter could express at detectable levels under particle bombardment conditions, although it was only at low levels. Probably these occasional foci were due to the high copy number of the construct in these cells or the interaction between the molecules precipitated on the gold particles. When the petals were bombarded with the mixture of GFP reporter fragment and the Gal4 producing fragment, many foci occurred (Figure 5.3 D). This clearly showed that Gal4 protein could be produced somehow under particle bombardment conditions. It may result from the basal transcription of -40 bp 35S core promoter, or the accumulation of the basal transcription due to high copy number or the "contamination" (see Section 5.3.4 for detail) resulting from the interaction between the molecules. Gal4 protein might be very low in amount but still able to amplify the signal by its specific binding. Due to this strong background, the GAL4 system is not effective for the transient assay of promoter deletions under particle bombardment condition. An optimal construct for this assay is suggested in Figure 5.9 B. It is optimal due to the promoter deletion containing its own core promoter region. However, this has yet to be tested due to the time limitation of this study.

5.3.4   p19 is not appropriate for the transient assay of *Venosa* promoter activity

35S:p19 plasmids were co-precipitated with *Venosa* promoter deletion constructs plasmids onto gold particles and introduced into the petal epidermal cells. The results showed that foci occurred when using all constructs, even the promoterless construct. However, the number of the foci did decrease in accordance with the amount of promoter deleted. This suggests that *35S:p19* did improve the expression of the deletion constructs but that 'contamination' of expression occurred concurrently. This altered
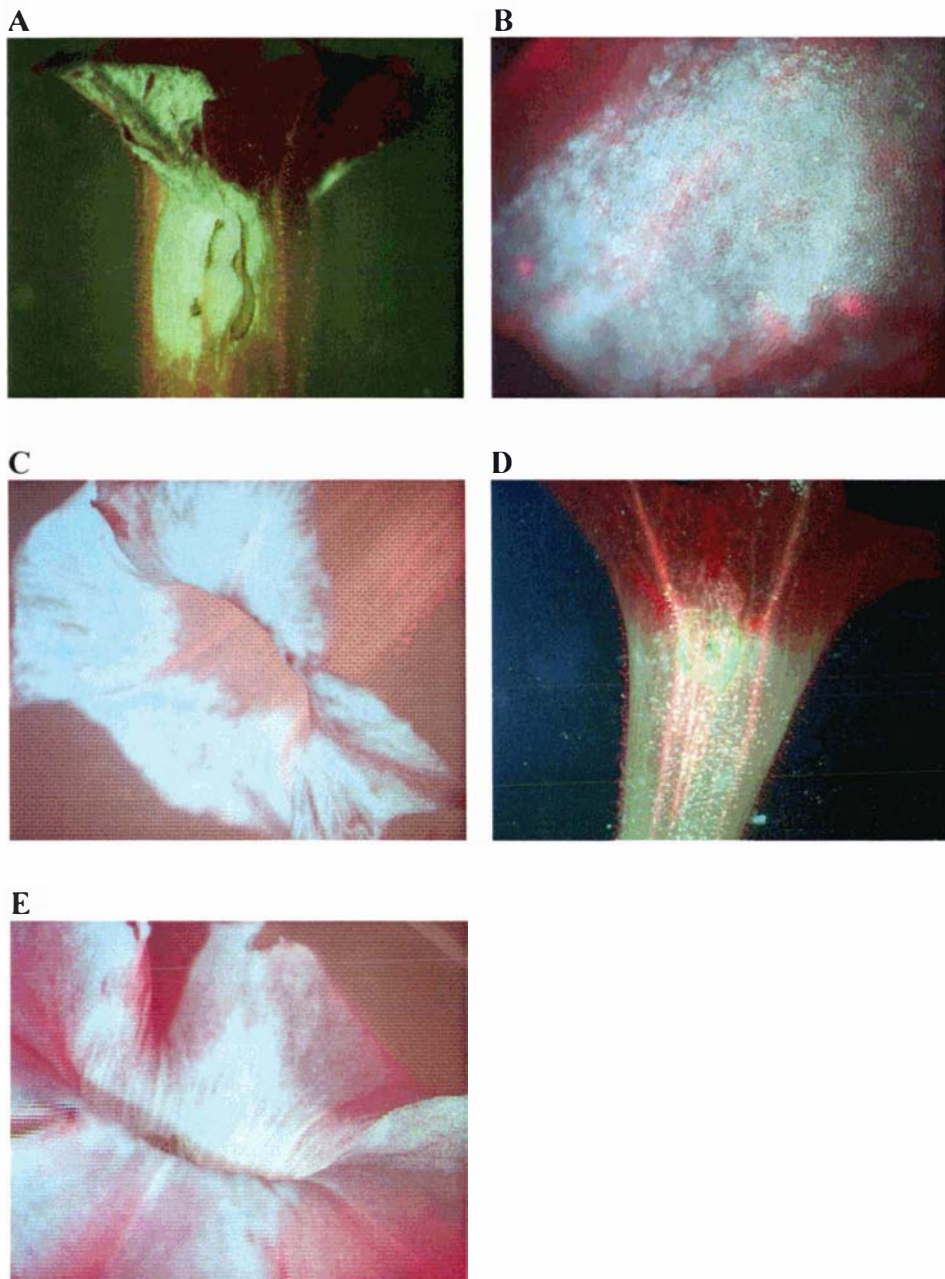
Figure 5. 8 A transient expression system for tobacco flowers using Agro-infiltration and *35S:GFP*. (A) GFP fluorescence occurred in the epidermal cells of the limb of attached (A and B) or detached (C) tobacco flowers Agro-infiltrated with *35S:GFP*. (D) No GFP fluorescence occurred in the flowers Agro-infiltrated with pART27. (E) GFP fluorescence in the limb of tobacco stably transformed with *35S:GFP*.
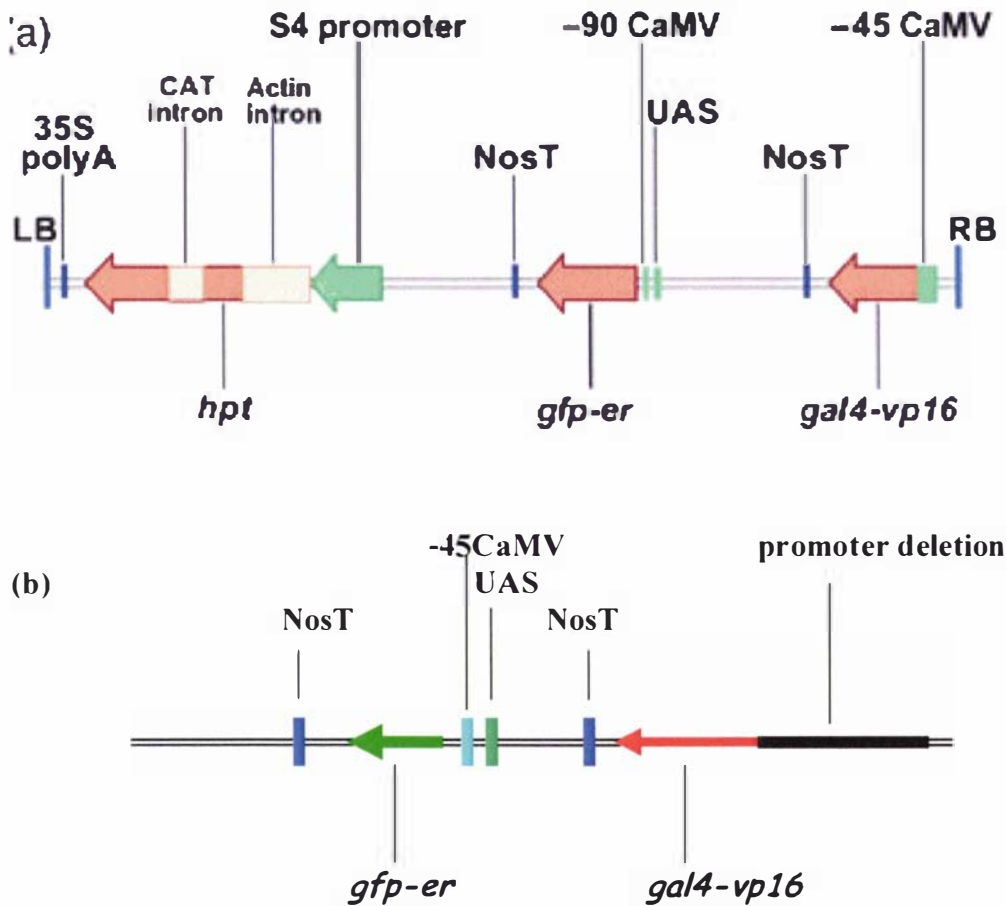
Figure 5.9 Structure of Gal4 two-step transcriptional amplification system.

(a) The GAL4-based enhancer trap pC-4956:ET15. Cited from Alexander *et al*. (2005). From the right border (RB): -45CaMV, -45CaMV 35S minimal promoter; *gal4-vp16*, *gal4-vp16* transcriptional activator fusion gene; NosT, bacterial nopaline synthase terminator sequence; UAS, five tandem repeats of the upstream activation sequence element GAL4 binding site; -90CaMV, -90CaMV 35S minimal promoter TATA; *gfp-er*, green fluorescent protein (mgfp5) targeted to the endoplasmic reticulum; S4, subterranean clover mosaic virus promoter; actin intron, rice actin intron 1; CAT intron, castor bean catalase intron 1; hpt, hygromycin phosphotransferase gene; 35SpolyA, CaMV 35S 3'UTR polyA signal; LB, left border.

(b) A proposed Gal4-based promoter assay system, YJ-815. Its design is based on the particle bombardment transient expression assay of pC-4956:ET15. It may maintain the specificity of promoter deletions more than pC-4956:ET15.

expression pattern possibly resulted from the interaction between the 35S promoter of the p19 construct and the *Venosa* promoter deletions during co-precipitation and co-transformation. To test this possibility, tobacco leaves were bombarded with a mixture of *-700VEN:IGUS* and *35S:p19*. The positive GUS staining foci (Figure 5.5C) confirmed the effect on expression pattern, because no GUS staining occurred in the stable transgenic tobacco leaves harboring *VEN:GUS* or *VEN:IGUS* (Chapter 6), or in the leaves bombarded with *VEN:IGUS* alone (Figure 5.5 D).

Compared with the bombardment of *-700VEN:IGUS* / pART7 (Figure 5.4 A), many more GUS foci occurred in the bombardment of *VEN:IGUS* / *35S:p19*, suggesting that p19 indeed improved transgene expression by inhibition of PTGS in particle bombardment. That GUS foci did also occur in petals bombarded with a mixture of *-700VEN:GUS* and pART7 (Figure 5.4 A) suggests that the 35S promoter is also a factor, not just p19 itself.

This effect of the 35S promoter can also be found in the published literature. For example, Figure 5 C in Verdonk *et al.* (2005), in which the authors quantify the transient gene expression after bombardment with the mixture of tested constructs, and the error resulting from this effect was decreased with internal and external controls as references.

Cross-talk between a strong promoter on one gene and expression of another gene on the same construct is certainly possible, and the results suggest that cross-talk effects can occur. The separate bombardments of two different constructs may avoid this problem. To test this, the petals were bombarded with *35S:Rosea* and *35S:GFP* separately. As a few cells had both pigment and GFP fluorescence, it seems possible for two constructs to be successfully introduced and expressed in the same cells (Figure 5.5 E and F), but that the frequency is too low for practical purposes.

To avoid effects of the 35S promoter, *-900VEN:p19* was made and tested. This approach was reasonably successful. When the petals of venation phenotype of antirrhinum were bombarded with the *-900VEN:p19* and the test *VEN:IGUS* constructs, GUS foci occurred for constructs down to *-500VEN:IGUS*. The foci appear non-vein

related. Thus, the promoter region of -380 bp to -900 bp positively affected *Venosa* expression at the transcription level, but may not be solely responsible for venation.

That the foci occurred only in the presence of p19 suggested that the -900 bp fragment has only weak activity, and detection was normally prevented by PTGS. Given the wounding involved in particle bombardment, the high plasmid copy number introduced, and the need to suppress PTGS, it is also possible that the expression pattern found by this method does not accurately reflect that of the native *Venosa* gene.

5.3.5 The efficacy and efficiency of Agro-infiltration as a method for promoter analysis

The use of floral tissue for transient assays with Agro-infiltration has not been reported prior to these studies. The petals of three species were tested with Agro-infiltration using *35S:IGUS* and *35S:GFP* as reporter constructs. The petals of tobacco and petunia could be transformed efficiently. Based on sectioning, transformed tissue included all cell layers. *Venosa* promoter deletion constructs were tested using this established protocol. but no staining occurred. probably due to the weak promoter activity.

Antirrhinum petals could not be transformed by Agro-infiltration. This negative result was not a result of failure of the infiltration process, as the *Agrobacterium* solution could be infiltrated into the petal tissue effectively. The failure more likely resulted from a lack of successful *Agrobacterium*-plant cell interaction. Only the *Agrobacterium* strain LBA4404 was used. It may be necessary to test other Agrobacterium strains for antirrhinum Agro-infiltration.

5.4   Conclusion

Compared with making stable transgenics, the transient assay with particle bombardment is much quicker, and allows large numbers of constructs to be tested quickly. However, as illustrated by these studies, it has a number of disadvantages. While it works well for some analyses, its efficacy and efficiency for promoter analysis

is very dependent on the target promoter. Despite attempting many techniques, only some of the issues could be overcome for the *Venosa* promoter.

Determining whether the foci that were obtained were vein-related was another challenge. It was difficult to set an objective standard due to the disappearance of the natural marker, the pigmentation pattern, during the experimental procedure. In the adaxial epidermis, foci located exactly between the middle of veins were considered non-vein related. In the abaxial epidermis any foci present were considered non-vein related, as no pigment normally occurs in this area in intact flowers.

The expression of the -900 bp fragment could only be detected as GUS foci in the presence of p19. These results suggested that the -900 bp fragment could function for transcription, but weakly. The promoter deletion assay showed that the expression decreased in accordance to the deletion and stopped at -380 bp. This suggests that there are important *cis* elements in the fragment of -380 bp to -900 bp. If these data with p19 are valid, it also suggests that the *Venosa* promoter region tested is not the prime determinant of the vein-related gene expression. However, the low number of foci obtained, and the need to use p19, means the results are not as robust as those from stable transgenics.

Chapter 6

Venosa promoter analysis in stable transgenics of tobacco

6.1   Introduction

Making stable transgenics is an alternative to transient assays for promoter analysis. It has advantages which include the stable expression and low copy number of the transgene. In addition, transgene expression and copy number can be assayed quantitatively and, unlike many biolistic transient assays in which the transformed cells are mainly, or exclusively, epidermal cells, all cells are transgenic. Overall, stable transgenics should show an expression pattern closer to that of the natural gene.

To explore how venation patterning forms in antirrhinum and how the *Venosa* gene is controlled, making stable transgenic antirrhinum was initially considered, because the homologous host is obviously preferable. However, generation of stable transgenic antirrhinum is difficult, and an efficient *Agrobacterium*-mediated transformation system has not been established in our lab. Tobacco can be effectively transformed via *Agrobacterium*-mediated transformation. Tobacco has an active anthocyanin biosynthetic pathway in the flowers, in which weak venation patterning can be recognised (Figure 6.1), and has been used previously for studying function of anthocyanin genes. Thus, it was thought it might be suitable for *Venosa* promoter analysis as a heterologous host.

As a transcriptional regulatory gene, *Venosa* expression is very weak (Cathie Martin, personal communication). Consequently, its promoter deletions may give even weaker expression. This low level expression may result in difficulty in detecting transgene expression. Choosing a suitable reporter gene was, therefore, critical. The three reporter genes (*gfp*, *Rosea* and *gus*) each have advantages and disadvantages (see Chapter 5 for details), thus, all of them were used to improve the possibility of transgene detection and the accuracy of observation. In addition, in consideration of the long timeframe needed for stable transgenic plants, a series of stable transgenic tobacco plants
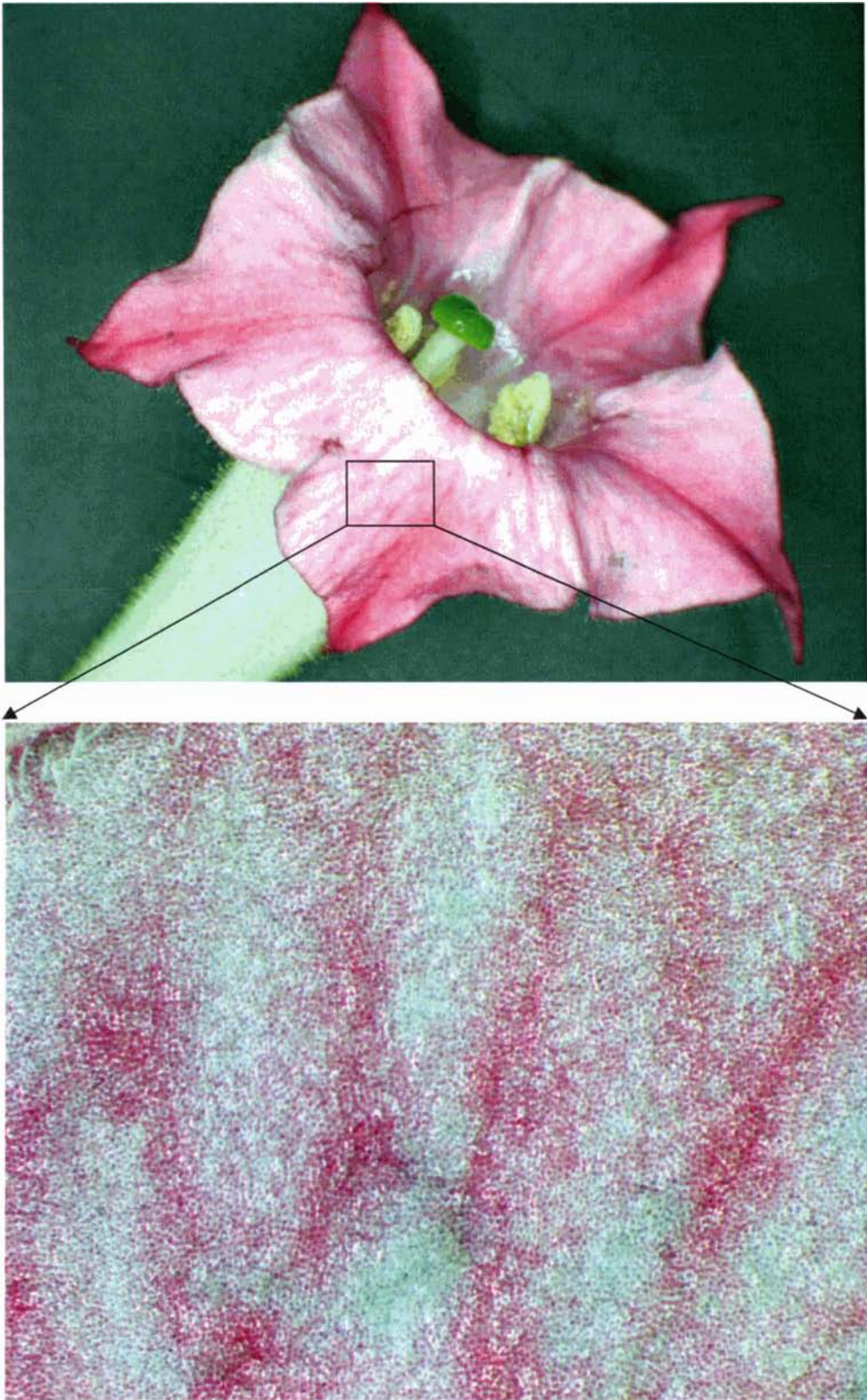
Figure 6.1 Weak venation pigmentation patterning is visible in the limb of *Nicotiana tabacum* flowers.

containing promoter deletions driving the three respective reporter genes were generated concurrently.

This chapter introduces the strategies of *Venosa* promoter analyses using stable transgenic tobacco plants and the expression patterns of promoter deletion constructs.

6.2 Results

6.2.1 Transgenic lines harboring *Rosea* cDNA or GFP constructs

All the transgenic plants harboring *VEN:Rosea* deletion constructs were compared with tobacco wildtype plants and negative control plants harboring an empty pART27 vector. No difference was observed in the strength and patterning of flower pigmentation with either the naked eye or light microscopy.

For GFP observation, the positive control (lines harboring *35S:GFP* construct) and negative control (non-transgenic wildtype lines and lines harboring empty vector) were observed first. Non-transgenic flower tissue emitted green fluorescence which varied depending on the developmental stage. This auto-fluorescence is likely due to lignin, which is one of the components of the cell wall. Venation patterning was observed, probably reflecting the higher lignin content in vascular bundles. The auto-fluorescence decreased when the flower developed to maturity. No auto-fluorescence was observed in the pigmented limb of fully opened flowers, and only faint auto-fluorescence was observed in the tube area. Variation in the strength of the GFP signal in the positive control plants was observed, and in plants with weak GFP fluorescence the signal was possibly masked by pigment in the limb, and by auto-fluorescence in the tube. Fifteen 35S:GFP plants were categorized into four groups according to their strength and pattern of emitting GFP (Table 6.1).

Two different developmental stages of flower were examined for the main data set. One stage was when pigmentation of the flower tip occurred, and the other stage was when flowers were fully opened. All the transgenic plants harboring *VEN:GFP* deletion constructs were observed with fluorescent microscopy. No difference of strength and

patterning of green fluorescence was observed between the tested samples and the negative controls.

Table 6.1   **Fluorescence microscopy observation of stable tobacco transgenic plants harboring *35S:GFP***

Lines	Description
1,2,5,6,14	GFP emission was weak and could be recognised only in the tip of the flower. Anthocyanin masked the GFP in most areas.
3,9,15	GFP emission could be just recognised from the anthocyanin background in the limb.
4,7,8,11,12,13	GFP emission masked the anthocyanin. It could be seen in the whole petal, and was more obvious in the limb than in the tube.
10	GFP emitted in all petal cells, but was stronger in the vascular system.

The *VEN:GFP* constructs may express only weakly in stages prior to flower pigmentation. Such weak GFP may be masked by auto-fluorescence at these stages. If the constructs expressed in a vein-specific manner as expected, the venation pattern of auto-fluorescence may impact significantly on the GFP observation. To try to avoid this, observation was carried out with confocal fluorescence microscopy, which would allow measurement of the epidermal cells alone. All the lines harboring *VEN:GFP* constructs were analysed. No difference of strength and patterning of green fluorescence was observed between the tested samples and the negative controls.

6.2.2   Transgenic lines harboring IGUS constructs

Independent lines were made for each construct after screening for rooting on kanamycin (Figure 6.2 A), and transferred into the GMO green house for flowering (Figure 6.2 B). Flowers of four different stages (Figure 6.2 C) were stained with GUS staining solution for all the lines harboring the *VEN:IGUS* deletion constructs. The staining pattern is shown in Figure 6.3. The results are recorded in Appendix VII and summarized as following:

A
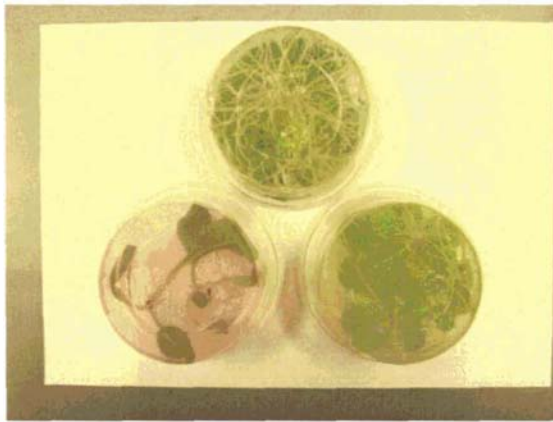
Non-transgenics on Kan   Transgenics on Kan

B

C
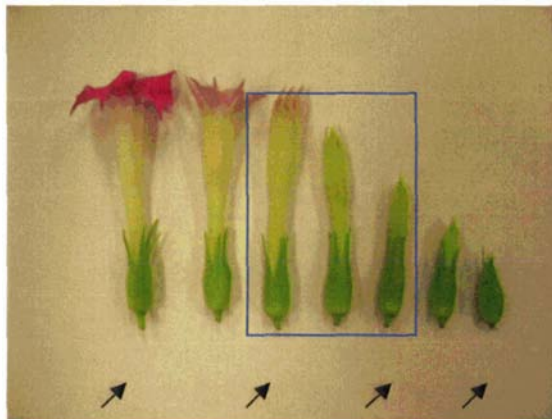
Figure 6.2   Transgenic plants containing *Venosa* promoter deletion constructs. (A) The rooting screen for the transgenics, which generated roots on media supplemented with kanamycin. MS: MS culture media. Kan: kanamycin. (B) Transgenic plants in the GMO greenhouse. (C) The developmental stages of the tobacco flower. The four stages tested for GUS staining are indicated with arrows. The stages presenting GUS staining are highlighted in the rectangle.

- No GUS signal was detected for the constructs *-1.6kVEN:GUS* and *-2.4kVEN:GUS*. Positive GUS signal was detected for all the other constructs. In particular, the ovule was stained in some samples of all constructs.

- For each construct there were both positive and negative lines. The staining results were not consistent for GUS signal strength and patterning across all independent lines from each construct.

- The number of positive lines decreased as the promoter length decreased.

- All the flower organs could be stained positive in -900 bp and -700 bp deletions (Figure 6.3 A, B, C, D, E, F, G and H). The staining pattern was variable in lines harbouring different deletions. For example, the style was stained in one line while it was not stained in another line. Petals could be stained in lines of the -500 bp deletion construct. No staining occurred in the petals of shorter deletion lines.

- In GUS positive petals the GUS staining was not observed in the patterns expected, such as the venation patterning seen for *Venosa* mRNA *in situ* hybridisation. It occurred in both adaxial and abaxial epidermal cells (Figure 6.3 H, I and J). Stronger staining usually occurred in vascular bundles rather than other tissues, giving a venation staining pattern in this situation (Figure 6.3 J, K and L).

- No staining was observed in the leaves of any deletion lines, including those in which the flowers were GUS positive.

- No staining occurred in the flowers or leaves of negative control lines. The flowers and leaves of nine positive control lines were stained. Six lines were GUS positive, with strong staining in all flower organs and the leaves.
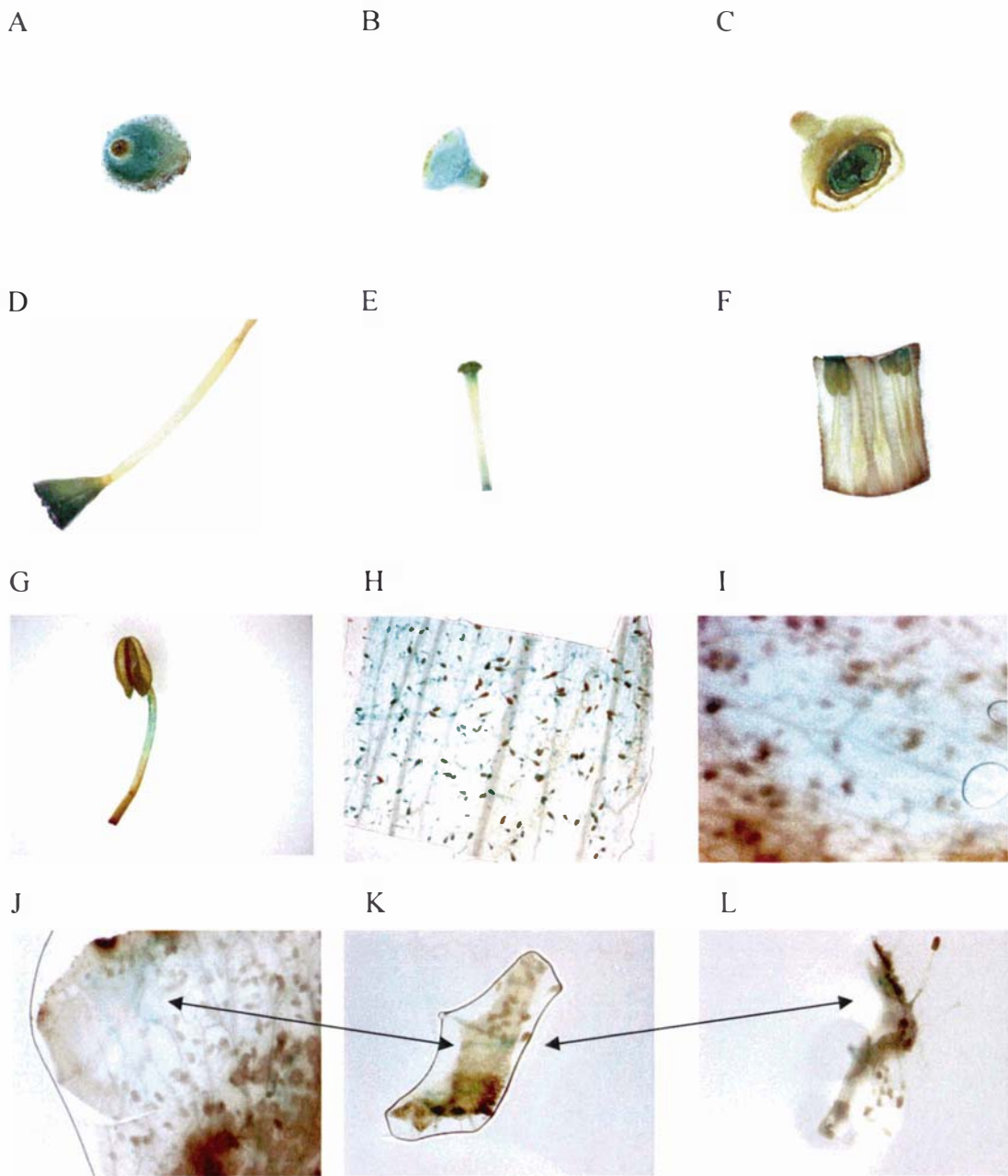
Figure 6.3 The GUS staining pattern of *VEN:IGUS* deletion constructs in transgenic tobacco flowers. -900:IGUS construct staining in floral organs: the base of sepal (A), receptacle (B), ovule (C), ovary and style (D), stigma (E), anther (F), filament (G) and outer area of petal tube (H). (I and J) −500:IGUS staining in petal. (K and L) The stronger staining occurred within the vascular bundle system. Photos were taken from two directions of one hand section.

6.3 Discussion

6.3.1 Transgenic tobacco plants harboring *VEN:Rosea* or *VEN:GFP* deletion constructs

Anthocyanin regulators have been used successfully as reporters in plant transgenic studies. For example, cell autonomous anthocyanin pigmentation, produced by the anthocyanin regulatory genes *B* and *C1* controlled by the constitutive CaMV35S promoter, was used to optimize biolistic gene delivery into embryogenic wheat scutellum cultures (Chawla *et al.*, 1999). In addition, tobacco transgenics harboring *35S:Rosea* have increased pigmentation of flowers (Cathie Martin, personal communication), suggesting ROSEA could be a suitable visual marker for this study. However, compared with the negative control, no difference was observed in the patterning and strength of anthocyanin pigmentation in transgenic lines harboring *VEN:Rosea* deletion constructs. Probably the amount of ROSEA protein produced was too low to affect the strength of pigment sufficiently to be observed, due to the weakness of the promoter deletions.

GFP has been used extensively as a visible marker for promoter analysis. However, it may not be suitable if it is expressed too weakly, especially in plant tissues that have a high background auto-fluorescence. Lignin fluoresces at 580 nm, which is the wavelength used for GFP observation. GFP could not be detected using fluorescence microscopy or confocal fluorescence microscopy in any of the transgenic lines harboring these deletion constructs. Probably the GFP reporter was expressed too weakly, and the fluorescence emission from low levels of GFP protein was masked by the background auto-fluorescence or by anthocyanin pigment.

6.3.2 Transgenic lines harbouring *VEN:IGUS* constructs

GUS is the most frequently used reporter for promoter analysis in transgenics. The advantage of GUS as a reporter is that the staining signal for the detection of gene expression can be amplified by the chemical reaction (Mantis and Tague, 2000). However, in addition to the need for destruction of the sample in the GUS assay,

another disadvantage is that staining may not reflect the real expression pattern of the tested promoter due to diffusion or poor penetration of substrate (Taylor, 1997). Diffusion may over-report promoter activity, while poor penetration may under-report promoter activity. To improve the staining quality some additional staining protocols have been developed. In this study, the salts and acetone pre-treatment (see section 2.3.6.3 method II) were used to inhibit diffusion of the stain and to improve chemical penetration (Hemerly *et al.*, 1993; Sessions *et al.*, 1999; Regan *et al.*, 1999).

Sectioning was used to observe the expression pattern of the deletion constructs. The staining occurred in various tissues of the petal. It was stronger in vein tissue than in any other tissues (Figure 6.3 J, K and L). It is unlikely that this patterning resulted from diffusion from the epidermis, or from the uneven penetration of the staining solution. If that was the case, the staining of epidermis should be stronger than that of the veins.

Although the expression of the deletion constructs was floral specific, unexpectedly it was not the pattern shown by *in situ* mRNA analysis of antirrhinum petals. The cause of this difference in patterns is not known. It may be due to the heterologus plant host, tobacco, not having the same signaling pathway to control venation patterning as in antirrhinum, or it may be that the promoter deletions do not include sequences that control venation expression.

When staining strength of the GUS positive lines was compared visually, expression from the -900 and -746 bp promoter fragments was markedly stronger than that from the -630 bp and -500 bp fragments. This suggests that an enhancer element is located between -746 bp and -630 bp. A petal ubiquitous transcription factor(s) may interact with this *cis* element(s).

Among 27 lines harboring -*500VEN:1GUS*, only two lines stained weakly in the tip area of petals. This may reflect the weak expression from this promoter fragment. No staining occurred in the petals of the lines harboring the deletions -*380VEN:1GUS*, suggesting that no *cis* elements capable of driving significant petal gene expression were present in this fragment.

There were some transgenic lines for all deletions with positive staining in ovule tissue. This occurred even for the -150 bp deletion, but was not seen in the negative control, so is not endogenous GUS activity. It may reflect basal transcription in undifferentiated cells, or there may be an ovule specific element in the promoter fragment of -150 bp.

In the plants generated from the seeds harboring GUS constructs, only two lines harboring *-760VEN:GUS* presented weak GUS staining, and none of the lines harboring *-1.6kVEN:GUS* or *-2.4kVEN:GUS* showed any staining. The reason for this is unknown, but it should be noted that only limited molecular analysis of these plasmid constructs has been carried out, and no Southern analysis of the transgenics.

Southern blotting to determine transgene copy number was not done for the plants generated in this study. The reasons for this are, firstly, the large number of independent lines for each construct was thought sufficient to cover for variations due to copy number differences. Secondly, analysis of stable tobacco *Rosea* promoter transgenics produced by the same method suggested the strength of GUS staining was not positively correlated to transgene copy number (Nilangani Pathirana, personal communication).

6.3.3   Detection efficiency of GFP and GUS as reporters in stable transgenics

Selecting a suitable reporter and an efficient detection approach is critical in promoter analysis, especially for weak promoters such as transcription factors. GFP and GUS are two important marker proteins used in plant gene expression studies. The transient assay and stable transgenic analyses showed that *Venosa* promoter activity detection was weak when using IGUS constructs, and not detectable when using GFP constructs. This difference may be due to the different efficiency of the reporters. The utility of GFP and GUS was also compared in the analysis of a relatively weak *Arabidopsis* promoter (Mantis and Tague, 2000). They found that, as with the tobacco results, the background fluorescence of *Arabidopsis* tissues and organs makes it difficult to detect weak promoter activity driving GFP, a problem due to the lack of amplification of the GFP signal. The low or no GUS background, and the enzymatic amplification of the signal makes GUS more easily and more accurately detected for weak promoter

activity. These authors comment that GUS rather than GFP is the choice for preliminary studies of promoter activity, particular those that have weak activity.

A GUS-GFP fused reporter may be a useful alternative for such studies. To combine the advantage of GUS, its high sensitivity in histochemical staining, with the advantages of GFP as a visual marker, a bifunctional reporter producing GUS-GFP fused protein was tested in *Arabidopsis* and *Lotus japonicus* (Quaedvlieg *et al.*, 1998). The fused protein could function as a reporter. Using this bifunctional reporter may improve the possibility of detection of weak promoter activity.

Alternative techniques have been developed to detect weak expression. It is reported that vacuole GFP could be easily degraded by light, especially blue light. This light dependent degradation resulted in the failure to observe GFP fluorescence in the vacuoles of plant organs (Tamura *et al.*, 2003). Transferring the plants from the light into the dark improved the observation of GFP-fluorescent vacuoles (Tamura *et al.*, 2003). Luby-phelps *et al.* (2003) developed a procedure for visualizing GFP expression in fixed tissue after embedding. The antigenicity of the GFP is retained after treatment so that the GFP localisation can be visualised after labeling with anti-GFP antibodies. Rech *et al.* (2003) used fluorescence microscopy to detect weak GUS activity in phenol-rich tissues.

6.4   Conclusion

To analyse the *Venosa* promoter, a series of promoter deletion binary constructs were made. Expression of *Rosea* and GFP could not be detected in the transgenic lines. This was probably due to the weak *Venosa* promoter and low expression of the reporter genes used. Hence, they were not suitable in the *Venosa* promoter analysis. However, GUS stained positively in transgenics. GUS staining was floral specific, but did not show venation patterning. The fragment of -380 bp to -900 bp could drive IGUS for detectable expression. There is the possibility of a *cis*-element enhancing expression located between -630 bp and -746 bp upstream of the *Venosa*.

Chapter 7

Investigation of the role of gibberellin and sugar signaling in controlling venation pigmentation patterning

7.1 Introduction

A number of signals might regulate anthocyanin biosynthesis in flowers (Section 1.4). Of these signals, gibberellin and sugar were considered of particular interest for this study. Gibberellins are required for the development of pigment in petunia flowers (Section 1.4.1), and sugar plays a signaling role in pigmentation in some species (Section 1.4.2). A hierarchy of signals must be involved in determining the venation pigmentation patterning in flowers. This chapter focuses on the possible role of gibberellin and sugar signaling in the formation of the anthocyanin venation patterning in *Antirrhinum* flowers. Experiments involving emasculation and gibberellin acid (GA$_3$) supplementation of petals *in vitro* were conducted to assess whether GA signaling plays a role in venation patterning. Girdling and sugar supplementation of petals *in vitro* were tested to assess whether a signaling role could be ascribed to sugars.

Emasculation refers to the removal of anthers. As summarised in the Introduction (Section 1.4.1), at anthesis gibberellin is produced in the developing anthers (Pharis and King, 1985; Weiss *et al.*, 1995; Itoh *et al.*, 1999; Rebers *et al.*, 1999; Kaneko *et al.*, 2003) and is required for petal development (Weiss and Halevy, 1989; Jacobsen and Olszewski, 1991; Goto and Pharis, 1999). Removing anthers could inhibit gibberellin production and, hence, its effect on the pigmentation and patterning in petals may be tested.

An alternative method to test gibberellin signaling is GA$_3$ supplementation of petals cultured *in vitro*. GA$_3$ is a biologically active gibberellin often used in plant tissue culture and physiological studies. The response of *in vitro* cultured petals to different media supplemented or not supplemented with GA$_3$ may indicate the signaling role of GA$_3$ in pigmentation and patterning.

Girdling removes the phloem in a ring around the inflorescence stem. Mason and Maskell (1928) observed that sugar transport in the stem was blocked by girdling, and since then girdling has been used as a method to study the translocation of substances in plants. For example, it was used to determine whether or not various substances were equally accumulated in stems of tomato plants in which transport had been interrupted by girdling (Bonner, 1944), or to determine stem xylem as a possible pathway for mineral retranslocation from senescing leaves to the ear in wheat (Martin, 1982). At anthesis, sugar translocates mainly from the source (leaves) to the sink (flowers) via the stem and pedicel. The pedicels of antirrhinum flowers are too short and thin to be girdled, while the stems of inflorescences can be girdled. Hence, girdling may be an approach to assess the importance of carbohydrate on development of pigmentation patterning.

Similar to GA_3 supplementation of petals cultured *in vitro*, sugar supplementation is an alternative approach to test its signaling role. The cultured petals may respond differentially to the media supplemented with different sugars, such as metabolic sugars or non-metabolic sugars. This may indicate not only the importance of sugar but also the signaling pathway, because only metabolisable sugar can act as a signal molecule via phosphorylation by hexokinase (Section 1.4.2). Different sugar or substitute material or sugar metabolic inhibitors were tested: sucrose, glucose and mannose are metabolic sugars; xylose and manitol are non-metabolic sugars; 3-o-methylglucose is a nearly non-metabolisable glucose analogue; mannoheptulose is a sugar metabolic inhibitor.

7.2 Results

7.2.1 Emasculation experiments

When the flower bud was between 4 and 7 mm in length, it failed to develop after emasculation, and the petals gradually senesced (Figure 7.1 A). When the bud length was between 7 and 10 mm, the flower developed slowly and was smaller, but it was pigmented and patterned as normal (Figure 7.1 B). When the bud length was greater

125

than 10 mm, emasculation did not affect the development of the flower, its pigmentation or its patterning (data not shown).

7.2.2 Spontaneously arising variant flowers

Several aberrant flowers developed on plants in the glasshouse during hot weather (January and February in New Zealand). The flowers were all positioned near the top of the inflorescence and were at the earliest developmental stages. Only the two dorsal petals developed while other organs including pistil, stamen and lateral and ventral petals failed to develop. The 'mutant' flowers were noticeably smaller than the normal ones, and had normal venation pigmentation patterning (Figure 7.1 C and D).

7.2.3 Response of detached petals to gibberellin supplementation *in vitro*

The experiments initially focused on determining the developmental stage at which the excised petal was sufficiently mature to become pigmented in response to a signal. The concentration of GA_3 and length of exposure to GA_3 needed to induce pigmentation were also tested. Preliminary experiments were carried out by culturing petals at different developmental stages on different media (Figure 7.2 A). The observations showed that 0.1-1.0 mg/l GA_3 was sufficient for petal development and pigmentation (Figure 7.2 B). However, it was difficult to conclude from these preliminary experiments whether GA was playing a role as a signal in patterning, because the petals of controls (MS without GA_3) and test samples (MS with GA_3) did not develop equally. The latter developed faster and were larger than the former (Figure 7.2 B). Accompanying petal development, which absolutely needed the GA_3 supplement to occur, venation pigmentation patterning also occurred.

Preliminary experiments showed that cultured petals from buds 2-8 mm in length expanded but did not develop pigmentation in the dark (data not shown), and that light was required for venation pigmentation. Further, petals at different developmental stages were shown to be differentially sensitive to light. Based on the above two preliminary experiments, a combined supplementation procedure was developed (Figure 2.10 A), and a series of petals of varying developmental stage were tested. For petals from buds 4-5 mm, 5-6 mm, 6-7 mm and 7-8 mm in length, equivalent

126
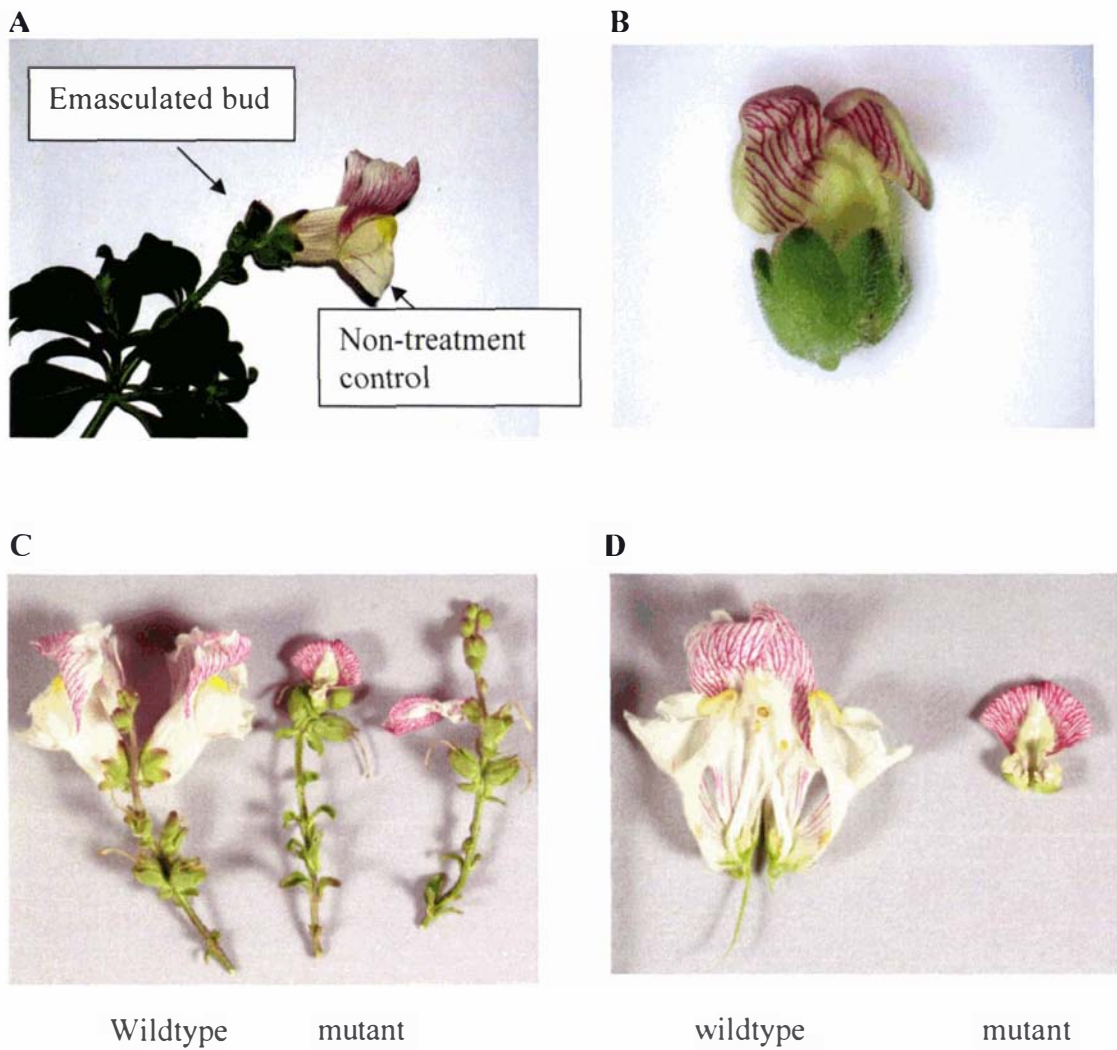
Figure 7.1 Development of emasculated flowers and mutant flowers. (A) Emasculated flower bud shorter than 7 mm in length did not develop. (B) Emasculated flower bud 7 mm in length developed normal pigmentation and patterning, but was slower in petal formation development and was smaller in petal size. Aberrant flowers lacking sexual organs and showing pigmentation patterning: intact (C) and dissected (D).

development occurred between control samples (MS + sucrose) and GA_3 supplemented samples (Figure 7.2 C). None of the petals from buds 4-5 mm in length became pigmented, whereas some petals from other later developmental stages developed pigment in culture whether or not supplemented with GA_3. Control (without GA_3) petals from buds 5-6 mm, 6-7 mm and 7-8 mm in length showed similar pigmentation patterning as samples supplemented with GA_3. However, for the petals pigmented in the culture either with or without GA_3, the venation pigmentation pattern appeared only in very limited areas, and did not occur in the whole sample (Figure 7.2 D and E).

7.2.4 Response of detached petals to different sugar supplements *in vitro*

Petals from buds 5 – 8 mm in length developed equally in the media supplemented with GA_3 and either sucrose, glucose, mannose or sucrose plus mannoheptulose. Compared with the test samples, the control samples (without sugar supplementation at the last culture period) were somewhat smaller. No pigmentation occurred in either test samples or control. In the media supplemented with xylose, mannitol or 3-O-methylglucose, the petals were wrinkled and senescent (example shown for mannitol in Figure 7.3).

Pigmentation occurred in several petals excised from buds 4-5 mm in length when the media was supplemented with GA_3 and either sucrose or glucose. However, the pigmentation was not venation patterning (Figure 7.4).

7.2.5 Girdling experiments

Buds $\leq$ 5 mm in length did not develop following girdling (Figure 7.5 A). Following girdling the dorsal petals of buds 5-7 mm in length expanded and developed a weak venation pattern (Figure 7.5 B). The petals developed more slowly than those of control buds and were of a smaller final size (Figure 7.5 C). They opened in 7 d and gradually senesced by 10 d. One flower developed differently, acquiring a weak but complete pigmentation pattern occurring on the inner lobes of the upper petals (Figure 7.5 C).

A

3-4 4-5 5-6 6-7 7-8
Bud length (mm)

B

MS   MS +sucrose

MS+0.05 mg/L   MS+0.1 mg/L   MS+1.0 mg/L
GA₃+sucrose   GA₃+sucrose   GA₃+sucrose

C

	MS	MS+ sucrose	MS	MS+ sucrose	MS+GA₃ +sucrose
Petals from buds 5-6 mm in length					
Petals from buds 4-5 mm in length					

D

E

MS + sucrose

MS+0.1mg/L GA₃ + sucrose
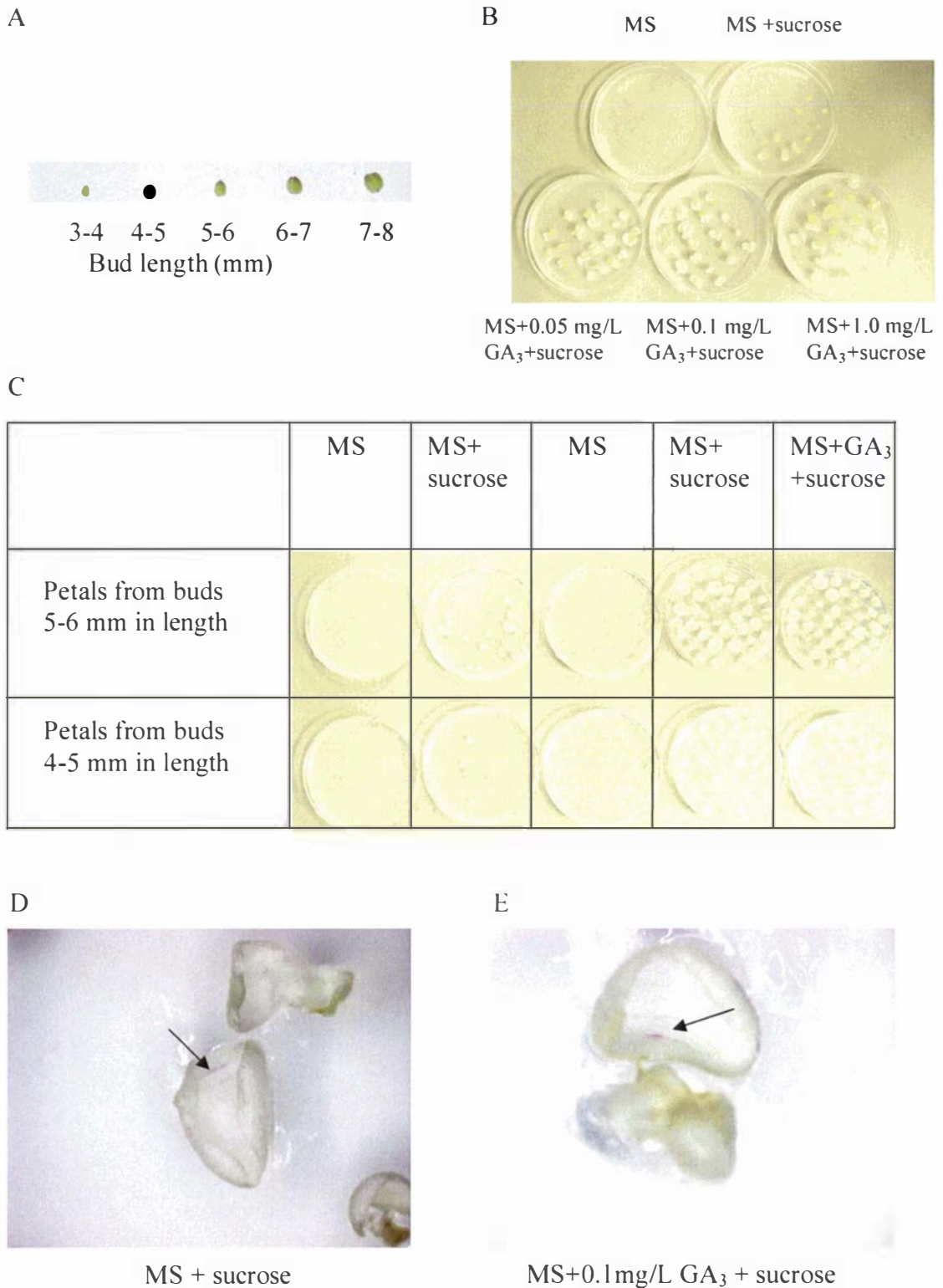
Figure 7.2 GA₃ supplementation of detached petals *in vitro*. (A) Bud development stages for petal samples. (B) Petals developed unequally in GA₃-supplemented and GA₃ –free media. (C) Comparison of petal development in the combined supplementation protocol. Petals developed similarly in GA₃-supplemented and GA₃-free media. Venation pigmentation patterning occurred in both GA₃-supplemented (D) and GA₃-free (E) media.
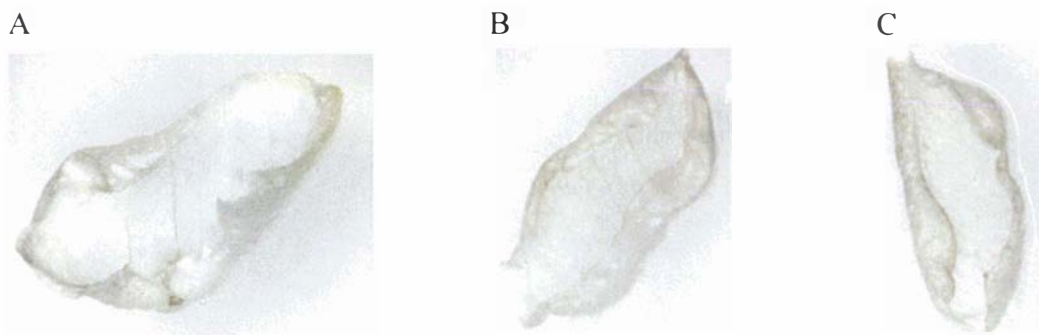
Figure 7.3 Sugar supplementation of detached petals *in vitro*. The response of petals excised from 7-8 mm buds after culture in (A) MS + sucrose, (B) MS and (C) MS + manitol. All media were supplemented with GA_3.
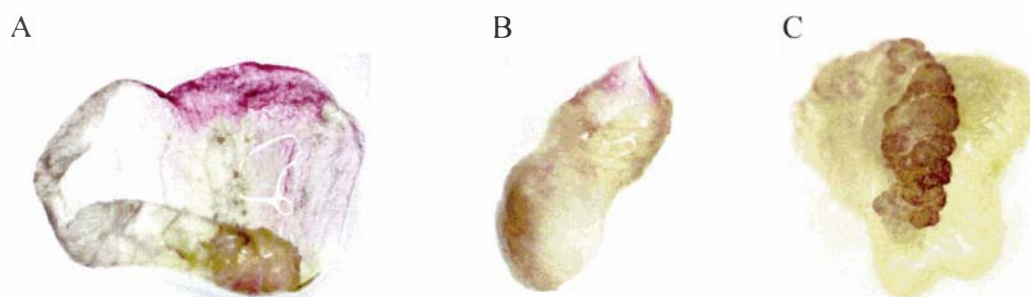
Figure 7.4 Pigmentation and abnormal patterning could be induced by culturing petals, removed from buds <5 mm in bud length, in MS media + sucrose + GA_3. Pigmentation occurred in non-vein-specific patterning in some petals excised from the buds 4-5 mm in length and cultured in MS + mannose (A) and in MS + sucrose (B). (C) Pigmentation occurred in non-vein-specific patterning in some petals excised from the buds 2-3 mm in length and cultured in MS + sucrose + GA_3.

A

B

C
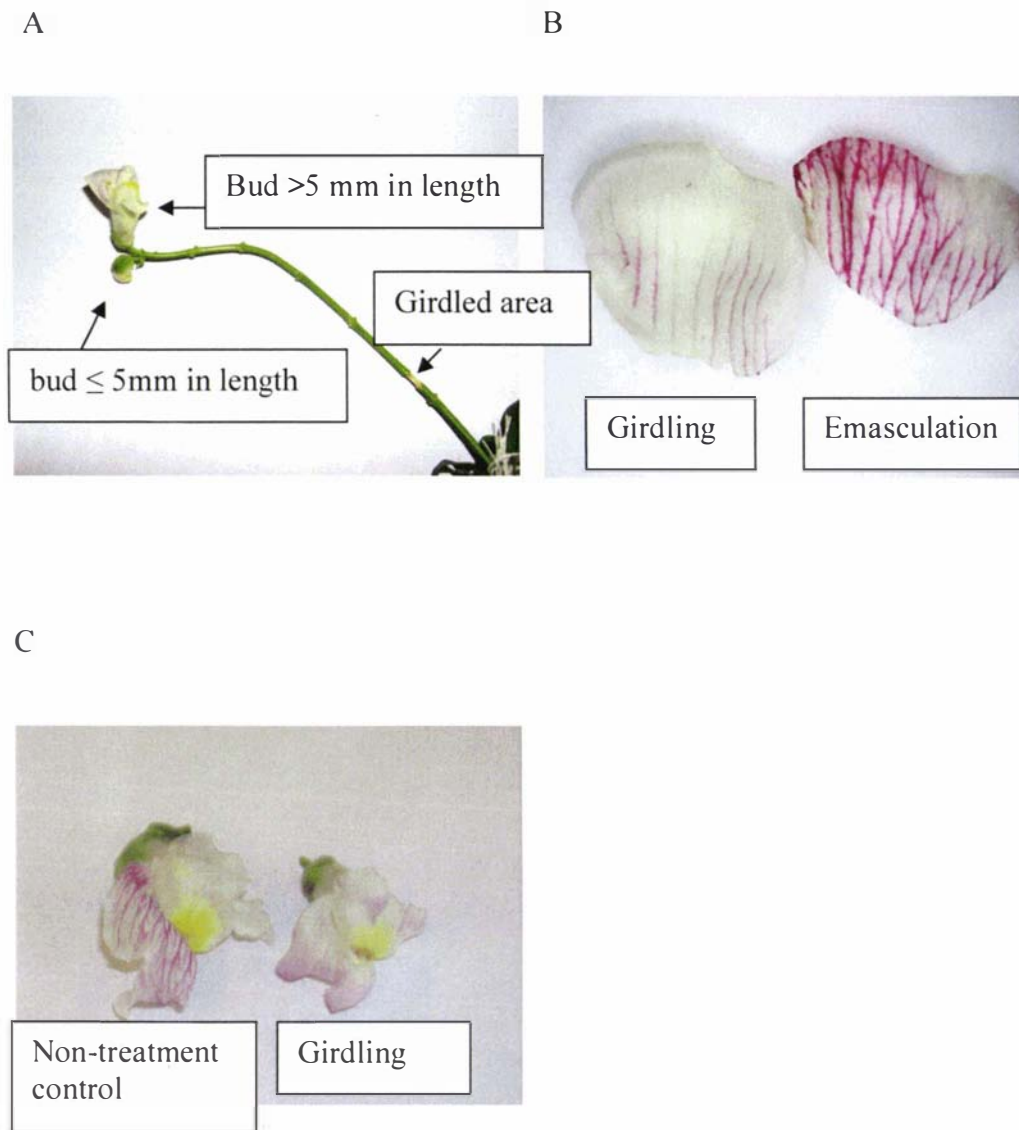
Figure 7.5 Response of flower buds to girdling of the inflorescence stem. (A) Girdling stopped flower development when the bud was 5 mm long, and decreased flower development when the bud was longer than 5 mm. (B) Girdling inhibited pigmentation patterning in petals. (C) Girdling could induce pigment and abnormal patterning occasionally.

7.3 Discussion

7.3.1 Emasculation experiments and naturally mutated individual flowers

In antirrhinum flowers, cell division within the bud is complete when the bud is approximately 10 mm in length, and cell expansion accounts for the rest of the growth of the bud during development (Jackson, 1992). The venation pigmentation pattern first appears in the adaxial epidermis of the tube base once the buds reach 8 mm in length, and subsequently spreads to other areas. Emasculating buds 7-10 mm in length delayed their maturity and caused the developed flowers to be smaller, but did not affect their pigmentation and patterning which developed normally. These results suggest that GA is not required for the production of pigment, the formation of the patterning or for the maintenance of the patterning. Taken together with the fact that the emasculated buds > 10 mm developed normally, it appears that the patterning is defined during the cell division stage, at which time the anthers are required for petal growth. After the transition to the phase of rapid elongation, the corolla is no longer dependent on the anthers (or GA) for growth and pigmentation.

Emasculating buds 4-7 mm in length inhibited their development. The failure to develop probably resulted from removal of the anthers, although physical damage may be another factor, but which could not be avoided in emasculating such small buds. From these experiments it is not clear what role if any the anthers play in patterning at this early stage of flower development, because patterning had occurred by the time buds were 8 mm in length, and smaller emasculated flowers (≤ 7 mm) did not develop.

The aberrant flower phenotype detected in the glasshouse may be related to high temperature. The temperature of the glasshouse was partially controlled with vents and electrical fans, but occasionally in January and February the temperature was over 35°C, even though the vents were set to open at 21°C. The developmental phenomenon may have resulted in somatic mutation related to transposon activity, as several transposons have been identified in antirrhinum (Coen *et al.*, 1986; Sommer *et al.*, 1988). It was unlikely that GA could be produced from the malformed flowers, as they lacked anthers. Probably as a consequence, petal development was reduced. Given that normal

venation pigment patterning occurred in these mutant flowers, this indicates that GA was not required for the initiation and maintenance of anthocyanin pigmentation, or for its patterning, agreeing with the findings from emasculation. The aberrant flowers also overcome one of the short-comings of emasculation - the physical damage of the bud.

Emasculating *Rosea1* wildtype flowers (the fully red phenotype) or flower buds was also carried out (data not shown). However, as pigment could be recognized in the petals of buds 4 mm in length, at a very early stage of petal development, it was impossible to emasculate without disturbing petal development.

7.3.2 Response of detached petals to gibberellin supplementation *in vitro*

The relationship between gibberellin and venation pigmentation patterning was tested by culturing detached petals in media supplemented with GA_3. The initial experiments indicated that GA_3 was required for petal expansion, during which pigment patterning occurred. To test GA_3 signaling in pigment patterning, by comparing the response of the cultured petals to GA_3, it was critical to have petals developing equally. Therefore, a combined supplementation approach was developed, in which the test petals were initially cultured in MS + sucrose + GA_3 in the dark for three days. This step provided a set of uniform petals developed to such a stage that they would synthesise pigment when all necessary factors including light were provided. The petals were then transferred onto media without GA_3 and cultured in the dark for another two days. The purpose of this step was to reduce the carry-over of GA_3 to the next culture step. The similarly developed petals were transferred onto media with GA_3 (test samples) or without GA_3 (control samples), and cultured in light. This step was to supply light, a required factor for pigment development of antirrhinum flowers, and compare the petal response to the different media. This system was successful for allowing +/- GA_3 treatment of well developed petals. However, similar pigmentation patterning developed with or without GA_3, supporting the conclusion that GA_3 is not required for the initiation or maintenance of venation pigmentation patterning in antirrhinum.

It was found that light was required for pigment development in antirrhinum flowers, because no pigmentation occurred or accumulated in petals cultured in the dark, even if

the culture period was extended. Light is also required for the accumulation of anthocyanin in petunia flowers (Weiss and Halevy, 1991; Moscovici *et al.*, 1996).

Interestingly, the venation pigmentation pattern appeared only in very limited areas of cultured petals and did not occur across the whole petal (Figure 7.2). This may have been a consequence of the relatively weak light levels in the culture rooms or, alternatively, the veins of the detached petals may not have fully developed.

Pigment in *Roseal* wildtype (the fully red phenotype) appeared at an even earlier stage than the venation phenotype. Unfortunately, this made it technically difficult to test the GA$_3$ signaling in *Roseal* wildtype using *in vitro* culture.

7.3.3   Response of detached petals to different sugar supplements *in vitro*

Preliminary experiments showed that young petals from buds 4-8 mm in length cultured in media without sucrose did not grow. This suggested that sugar was necessary for petal development at this stage. Sugar was required for petal expansion, during which the pigment patterning also occurred. To test sugar signaling in pigment patterning, by comparing the response of the cultured petals to sugar, it was critical to have petals developing equally as for the GA$_3$ experiment. The same system was used as for GA$_3$ treatment, but lacking sugar to reduce the sugar carry-over between step 1 and step 3.

After the culture procedure, the samples in the media supplemented with GA$_3$ and with the non-metabolic sugars appeared senescent. Possibly sugars or substitute material in these media could not be assimilated effectively due to their non-metabolic property. Although the samples in the media supplemented with metabolic sugars looked 'healthy', no pigment was observed. However, it was not known whether the petals were physiologically normal and able to sense the signal for pigmentation given the extensive *in vitro* treatments.

Sugar is required in petals both as a carbon source for the anthocyanin and as an energy resource. In *in vitro* conditions, detached petals were cultured in the medium without sugar for two days to decrease the carry-over of sucrose to the following culture step. Possibly, petal development was very sensitive to sugar assimilation at these stages.

Shortage of sugar could affect multiple pathways, which may not have recovered following re-supplementation with sugar. In conclusion, it is technically difficult to test the role of sugar in signaling to venation patterning separately from general flower development using *in vitro* supplementation.

Pigmentation did occur in some *in vitro* cultured petals that were excised from the buds 4-5 mm in length (Figure 7.4). This suggested that pigment could be induced during the *in vitro* culture procedure at this early developmental stage, but that the programmed venation property was disturbed. As the *Venosa* line lacks a functional copy of *Rosea1*, this suggests that *Venosa* gene expression can be induced in cells away from the veins, at least in these 'extreme' conditions.

7.3.4   Girdling experiments

One of the negative factors in the girdling experiments was that sugar assimilation could not be avoided completely. Although the photosynthetic organs such as leaves and bracts were removed in the girdled samples, photosynthetic activity by stem and flower buds could produce some assimilates. If the buds remaining on the girdled inflorescence were 5-7 mm in length, the petals in the girdled samples still expanded, possibly because of a weak supply of photosynthates. However, compared with the non-girdled controls, the petals in the girdled treatment developed more slowly, and were smaller and only weakly pigmented. This suggested that girdling did affect petal development and pigmentation but not enough to cause a difference in pigmentation patterning at this stage.

These experiments demonstrated that girdling inhibited markedly petal expansion of buds ≤ 5 mm at the time of girdling. Because pigment and venation patterning accompany petal expansion, the influence of carbohydrate signaling on the pigment venation patterning could not be determined. In addition, girdling not only precluded translocation of photosynthates, but possibly also perturbed translocation of other important signalling molecules.

Pigmentation was also induced in the domain between the veins in one sample (Figure 7.5 C). Because it occurred in a *Venosa/rosea^{dorsea}* phenotype, probably *Venosa* or

135

rosea^{dorsea} was induced. The *rosea^{dorsea}* line has a mutated but functional *rosea1* allele that is normally expressed in the abaxial surface of dorsal petals. As with the *in vitro* result, this observation suggested that the pigmentation patterning could be disturbed by treatments that severely disrupted the normal developmental and environmental signals.

Girdling experiments were also attempted with *Rosea1* phenotype plants (full red wild type). However, it was more technically difficult to achieve information about the role of sugar signaling to pigmentation due to pigment biosynthesis occurring at a very early stage (4 mm bud length).

7.4   Conclusion

A protocol for GA_3 and / or sugar supplementation of detached petals cultured *in vitro* was developed. The results suggest that GA is not required for venation. The emasculation results together with the naturally occurring flower mutants confirmed that GA was not required for the initiation and maintenance of pigmentation and venation patterning in antirrhinum flowers. Sugar supplementation of detached petals cultured *in vitro* and girdling suggested that sugar is required for petal development and pigmentation. However, conclusive results for a signaling role for sugar could not be achieved, because it was technically difficult to separate the signaling role, if it has one, from the general impact on flower development.

Chapter 8

General discussion

8.1 Summary of the aims and results of the study

The overall aim of this thesis was to extend our understanding of how a plant can differentially regulate gene activity in similar cells during development by exploring the mechanisms controlling venation pigmentation patterning in antirrhinum flowers. This naturally occurring striped vein-associated patterning provided a visual marker for investigating gene expression and regulation during development. Using the *Venosa* gene from the venation phenotype of antirrhinum, a model plant used for the study of plant pigment development, the study focused on three questions: Does *Venosa* control pigment venation? If so, how is *Venosa* gene transcription controlled for the vein property? Are gibberellin or sugar high level signals controlling the venation patterning?

8.1.1 Venation pigmentation patterning is due to *Venosa* gene activity

To answer the first question three different experimental approaches were taken: complementation of the non-pigmented cells, *in situ* mRNA analysis and *Venosa* expression inhibition using RNAi. Except for the *Venosa* RNAi experiment, in which convincing information could not be achieved due to the efficacy of the method itself, the experiments were successful in demonstrating that *Venosa* controls pigment venation patterning.

The ability of 35S:VENOSA to induce pigment in cells away from the veins demonstrated that it is the lack of the MYB factor that causes the acyanic regions, while other necessary components such as a bHLH factor or WDR repeat protein are present in a non-vein related expression pattern. Also, the data indicate that there is nothing in tissue that stops *Venosa* from functioning in cells away from veins. This suggests that the spatial specific expression of *Venosa*, or a similar MYB, is determining the venation pigmentation due to a vein-associated mechanism.

Another line, *rosea^dorsea*, was also used as a target in the complementation experiments. The cells in its non-pigment regions were also pigmented by 35S:VENOSA. Because this line is homozygous for the mutant *venosa* allele (GBV), and lacks *Rosea* expression in the non-pigmented regions, it suggests that it was functional *Venosa* (GSV) that controlled the pigment venation patterning in the *rosea^dorsea* background.

In the venation phenotype, the pigment occurs specifically in the inner petal epidermal cells overlying the veins, and the anthocyanin regulatory gene might be expected to have a similar pattern of expression. To determine the expression domain of *Venosa*, *in situ* hibridisation of *Venosa* mRNA expression was carried out. A vein-specific expression pattern was found corresponding to the pigment pattern in all samples with venation phenotype, but not in the control samples such as *rosea^dorsea* of *A. majus* and *A. graniticum*, which lack venation pigment. These results further suggest that pigment venation is controlled by the cell specific expression of *Venosa*, not only in *A. majus* but also in other species of *Antirrhinum*. In addition to the evidence from these experimental approaches, a non-functional allele of *venosa* (GBV) was identified, whose presence as the homozygote correlated with lack of pigment venation.

The *in situ* mRNA hybridisation showed that the expression of *Venosa* was not only in the adaxial epidermal cells overlying the petal veins, but also in the sub-epidermal cells and xylem cells in a band towards the inner epidermis. This suggests a polar signal diffusing from xylem to the adaxial epidermis is inducing the expression of *Venosa*. It also suggested that *Venosa* expression provided the longitudinal axis for venation pigmentation stripes, while another required factor for pigmentation, a bHLH factor, is specifically expressed in epidermal cells, therefore providing the transversal axis. The pigmented stripes are the cross expression domain of these two kinds of factors. Such an idea is shown schematically in Figure 8.1.

In addition, based on the 35S:*Venosa* complementation experiments and the *Venosa* mRNA *in situ* hybridisation results, it can be determined that pigment venation is at least partially determined at the level of control of *Venosa* transcript abundance, probably through transcriptional control of *Venosa* gene expression.
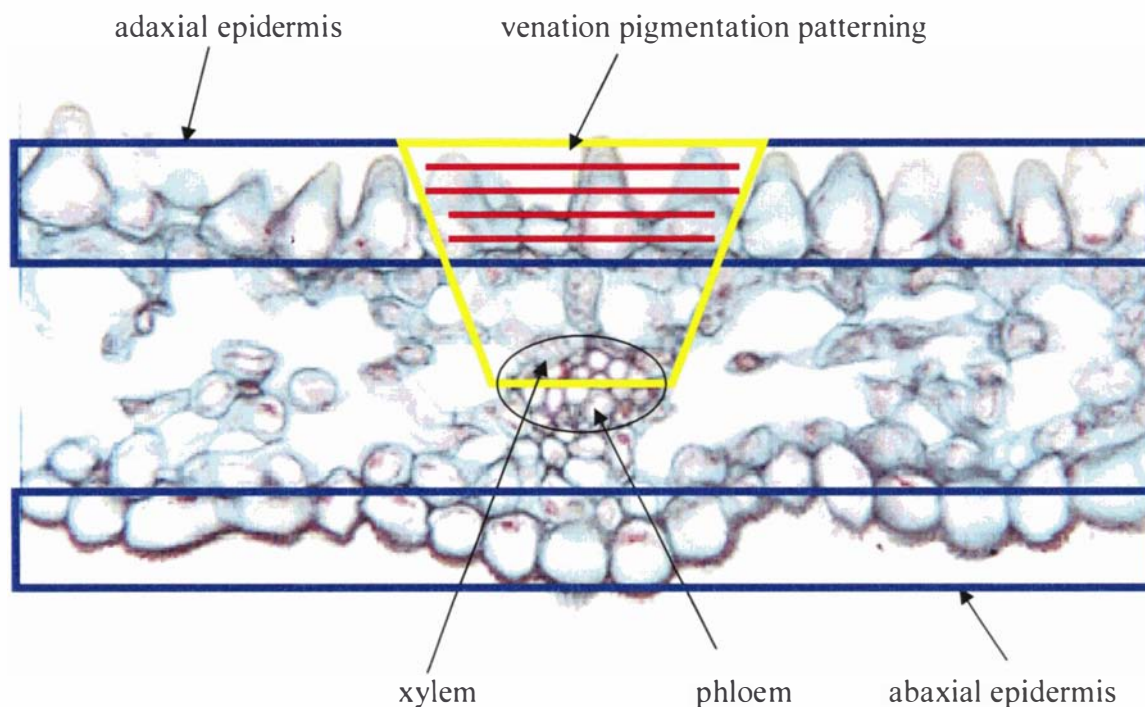
Figure 8.1 The mechanism of venation pigmentation patterning formation in antirrhinum is schematically shown in cross section. The two blue rectangles indicate the expression domain of a bHLH factor, which is DELILA in the tube, and DELILA or MUTABILIS in the lobe; the yellow trapezoid indicates the expression domain of *Venosa*, which is induced by a polar signal diffusing from xylem to adaxial epidermis; the red lines indicate the venation pigment patterning, which is the cross expression domain of the two kinds of factors. The photo of petal cross section was obtained from the internet to aid this explanation (www.uri.edu/artsci/bio/plant).

8.1.2 Analysis of the *Venosa* promoter transcriptional activity

The particle bombardment complementation and *in situ* analysis suggested transcriptional regulation of *Venosa* is likely to determine pigment venation. To identify some of the signals controlling transcription of the *Venosa* promoter, -2.4 kb (relative to the ATG) of the promoter region was analysed. The proximal 900 bp of this was characterised in detail using 5´-end deletion mutagenesis. The homologous host, *A. majus*, was used in transient assays with particle bombardment. The results showed that the expression of this 900 bp fragment was too weak to be detected when IGUS or GFP was used as the reporter gene. Constructs with VENOSA as the reporter could induce pigment in the dorsal epidermal cells. However, further testing showed that even the

promoterless construct could induce pigment. This suggested that the promoterless construct could be expressed weakly under particle bombardment conditions, and VENOSA was too sensitive to be a suitable reporter for promoter analysis in this case. It also demonstrated that *Venosa*, through its role as a transcription factor, required only very low expression to perform its regulatory function.

Because of the weak promoter activity, the efficacy and efficiency of different signaling enhancement systems was also tested. A PTGS suppressor, p19, was effective in enhancing the transient assays. Using a p19 system, it was shown that the number of positive GUS foci decreased with decreasing promoter length, with foci occurring down to -500 bp. However, the pattern of foci occurrence was non-vein related.

The assay results could be affected by biolistic parameters, the physiological status of plant material and the copy number of transgene in the transformed individual cells. The consistency of shooting results could be increased by optimizing biolistic parameters and using consistant plant material, but it is impossible to control and determine the transgene copy number in the bombarded cells. Therefore, it was not possible to determine the impact of copy number on promoter expression. In stable transgenics generated via particle bombardment, the integrated transgene copy number can be as high as 20 (Powlowski and Somers, 1996), and the copy number may be much higher in transient expression. Although the copy number effect could not be determined accurately, the presence of GUS foci only when p19 was co-expressed with *VEN:IGUS* constructs suggests that high copy number could not overcome the weak *Venosa* promoter activity.

A heterologous host, tobacco, was used for *Venosa* promoter analysis in stable transgenic plants. As with transient assays, the level of GUS reporter activity decreased as the length of the promoter decreased, and expression was detected down to -500 bp. Expression was flower specific but not vein related.

A weak venation patterning occurs in wildtype tobacco flowers. However, it is not known if this shares a common control mechanism with antirrhinum. Because expression of the *Venosa* promoter deletion constructs was flower specific in tobacco transgenics, the 900 bp promoter fragment kept at least some, if not all, of its expression

properties from antirrhinum. This suggests that tobacco may share a similar mechanism of pigment venation formation with antirrhinum.

Based on the transient assay and stable transgenic plants, it is concluded that the fragment of -380 bp to -900 bp of the *Venosa* promoter is required to promote *Venosa* expression at the transcription level, but may not be sufficient for the vein-related occurrence of *Venosa* transcript.

8.1.3 The possible role of GA₃ and sugar in the formation of the venation pigmentation patterning of antirrhinum flowers

Two approaches were taken to study the influence of GA: GA₃ supplementation of *in vitro* cultured flowers, and removal of the anthers of the flowers, as the predicted organ for GA production. The combined results suggested that GA was not required for the formation of venation pigmentation patterning. These findings were supported by the occurrence of normal venation patterning in aberrant flowers lacking the sexual organs.

This conclusion is different to the results for petunia, but has similarities with those for Phlox. In petunia, pigmentation occurs at a late stage of flower development and GA is required for the production of anthocyanin (Weiss and Halevy, 1989; Weiss *et al*, 1992, 1995). In Phlox, pigmentation occurs at an early stage of flower development, before the rapid expansion of the petals, and GA application inhibits anthocyanin biosynthesis in the petals (Weiss, 2000). In antirrhinum, pigmentation occurs at an early stage of the flower development, before the cell division period is complete. This study of antirrhinum supports the viewpoint that GA is not required for pigment initiation in flowers that synthesise pigment early in development, but is required in flowers that pigment later. A wider study of more species would help to confirm whether this is a consistent pattern of regulation.

Removing the supply of sugar affected the intensity of pigment, suggesting that sugar is required for pigmentation. However, it was technically difficult to test the role of sugar as signal for venation patterning separately from its impact on general flower development. Hence, it was difficult to get a convincing conclusion due to the multiple roles of sugar in plant development. Because the venation patterning is due to *Venosa*,

the research should be confined to test if the sugar signaling targets *Venosa*. It is reported that sucrose can specifically induce the anthocyanin biosynthetic pathway in arabidopsis (Teng *et al.,* 2005; Solfanelli *et al.,* 2006). This induction requires the *MYB75/PAP1* gene. Sucrose, in a concentration-dependent way, induces *MYB75/PAP1* mRNA accumulation (Teng *et al.,* 2005). *PAP1* is a *myb* gene that up-regulates the anthocyanin biosynthesis pathway. It would be worth testing further whether *Venosa* is induced by sucrose, whose transport is dependent on vein development.

In addition, the results from girdling and sugar supplementation experiments show that non-vein-specific pigmentation patterning can occur in the early stages of petal development. This suggests that the pigment venation patterning is controlled strictly by developmental and environmental factors. Possibly, other components such as auxin, due to its polarity property, should be tested as a high level signal controlling venation pigmentation patterning.

8.2 A hypothesis for the control of *Venosa* gene expression and venation patterning

This study showed that the pigment venation patterning is controlled by the spatial expression of *Venosa*. Based on promoter analysis, the 900 bp proximal region of the *Venosa* promoter is necessary but may not be sufficient for the expression domain of venation patterning. Thus, it is worth considering alternative hypotheses for the venation control mechanism. Two hypotheses are provided and compared below:

Vein-related suppression of post-transcriptional down-regulation of Venosa

MicroRNAs (miRNAs) are small RNA molecules encoded in the genomes of plants and animals. Typically about 21-mer in length, these RNAs regulate the expression of genes by binding to specific target mRNAs. In plants, miRNAs generally display near-perfect complementarity to a single site within the target message and direct the cleavage at this site. The target site is often, but not always, the 3'-untranslated regions (3'-UTR) of mRNAs. MiRNAs have been shown to regulate diverse developmental processes, including organ separation, polarity and identity, and to modulate their own biogenesis

and function (for a review see Dugas and Bartel, 2004). MiRNAs can restrict gene expression to a specific organ or tissue by post-transcriptional down-regulation resulting from a miRNA-induced mRNA degradation pathway. For example, in arabidopsis, the expression of *MYB33/MYB65* is restricted to anthers by miR159 posttranscriptional down-regulation. Mutation of the miR159 target sequence results in increased expression of *MYB33* in tissues outside the anther and dramatic pleiotropic developmental defects (Millar and Gubler, 2005).

Based on this mechanism, a hypothesis involving miRNA is provided. In this hypothesis *Venosa* expresses in a flower specific but non-vein related manner at the transcriptional level, but is down-regulated in the non-vein area at the post-transcriptional level. If this is the case, the vein-specific regulatory mechanism would act on miRNA action rather than *Venosa* transcription.

Epigenetic control of vein-related expression of Venosa

The term 'epigenetic' refers to heritable gene expression patterns determined by how the DNA of a gene is 'packaged' or further modified (*eg.* by methylation) rather than its primary DNA sequence. Within tightly packed DNA, genes are often maintained in an 'off' state by repressor protein, and hence are not readily available to the transcription machinery until signal transduction alleviates the repression. Nucleosomal histones are the target of a variety of post-translational modification signals, including acetylation, phosphorylation, ubiquitination and methylation. (Eberharter and Becker, 2002; Schubeler and Elgin, 2005; Verdone *et al.*, 2005).

In this model the venation-controlling property of *Venosa* is negatively controlled, and a vein-related molecule quenches this inhibition. The vein-related signaling molecule might induce the remodeling of chromatin structure and/or DNA methylation status, to allow the remodeled DNA to be accessible to flower-specific TFs to activate transcription. When the *Venosa* promoter is introduced via particle bombardment or into a heterologous host it may not be influenced by these epigenetic signals, and hence not drive vein-related reporter gene expression.

There is some evidence supporting this second hypothesis over that of miRNA. In particular, the venation phenotype does not segregate in a Mendelian manner in some experiments (Schwinn *et al.*, 2006).

There is some precedent for control of anthocyanin pigmentation via chromatin modification. It is reported that the tissue-specific patterns of a maize MYB transcription factor, *pericarp colour (p1)*, are epigenetically regulated (Cocciolone *et al.*, 2001). *P1-wr* (white pericarp / red cob) and *p1-rr* (red pericarp / red cob) are two alleles determining two different pigmentation patterns. However the *p1-wr* promoter / cDNA directs transgenic plants with a red pericarp / red cob pattern, not the predicted white / red pattern. It was suggested that the regulatory elements required for tissue-specific regulation of *P1-wr* may either be missing from the transgene construct or were not reproduced in the transgenic experiment. Further analyses found that *p1-wr* allele differs considerably from *p1-rr* with respect to gene structure and the level of DNA methylation formed. The *p1-wr* allele contains six *p1* gene copies present in a hypermethylated and multicopy tandem array. The maize *Unstable factor for orange1(Ufo1)* modifies *p1-wr* expression to confer pigmentation in kernal pericarp by reducing the methylation of *p1-wr* DNA sequence (Chopra *et al.*, 2003). The study of two other *p1* alleles, *p1-Blotched* and *p1-Rhoades*, demonstrated that developmental patterns of chromatin structure and DNA methylation was responsible for epigenetic expression of *p1*, and chromatin structure rather than DNA methylation was the primary epigenetic determinant for the phenotypic difference between these alleles (Hoekenga *et al.*, 2000).

In situ hybridisation showed that *Venosa* mRNA expression patterning matched the pigment patterning in the epidermis in the vein-specific manner, but was beyond the epidermis and extended into the inner tissue. This suggests a signal that diffuses from xylem to the adaxial epidermis turns on the expression of *Venosa*. What is this signal? Is it transported into the petals via the vascular system or is it established during xylem formation? Is it transported in a controlled system from xylem to adaxial epidermal cells or does it diffuse passively? Does it target *Venosa* directly or via other *trans*-acting elements? One candidate signal molecule is auxin, due to its role in vascular development and the scope for polar translocation. I hypothesise that auxin enters the cell by active transport through spatial auxin transporter molecules in the plasma

membrane, then activates an enzyme molecule by binding to it. This enzyme molecule releases the 'off' state of *Venosa* by remodeling the chromatin structure or changing the methylation status of its promoter region, then a floral-specific transcription factor activates the transcription of *Venosa*.

8.3   Limitations of the study and future experimental directions

The need to rely on transient assays and heterologous systems may have been a major negative factor in the research on the *Venosa* promoter. Related to this, the lack of an efficient *A. tumefaciens*-mediated transformation system is a big limitation for antirrhinum as an important model species for the investigation of not only floral pigmentation, but also other morphological features of floral development. Although a number of genes related to floral pigmentation and morphogenesis have been isolated from antirrhinum, most of these genes have not been characterised by analysis of their expression in transgenic antirrhinum plants. *A. tumefaciens*-mediated transformation of antirrhinum has been reported (Heidmann *et al.* 1998; Cui, *et al.* 2000, 2001, 2003). In particular, Cui *et al.* (2003) reported that efficient *A. tumefaciens*-mediated transformation of antirrhinum was achieved via indirect shoot organogenesis from hypocotyl explants of seedlings. In their protocol, 8-9% transformation efficiency was reached based on the recovery of transformed plants after 4-5 months of culture. However, it has not been reported that this protocol has been used for any gene function study or promoter analyses to date. This protocol was not attempted in this study due to time limitations, but would be an appropriate future research aim.

The possible role of epigenetic or post-transcriptional mechanisms in defining *Venosa* gene expression could be explored further. *Cis*-acting binding sites for transcription activators may not explain the patterned expression of genes observed during development, nor a large range of phenomena that result from gene transplacement (review, Gasser *et al.*, 1998). Current data suggest that epigenetics play an important role in regulation of some genes (reviews, Courey and Jia, 2001; Eberharter and Becker, 2002). However, little is known about how epigenetic modifications are targeted to specific domains or loci of the genome. There are studies on epigenetic regulation in maize, as mentioned earlier for the *pl* gene. Also it has been reported that histone acetylation affects expression of cellular patterning genes in the arabidopsis root

epidermis (Xu *et al.*, 2005). An appropriate approach to study the controlling mechanism of *Venosa* might be to examine the epigenetics further.

In addition to the exploration of an epigenetic control mechanism of *Venosa*, another experiment should examine whether a regulator of aurone biosynthesis in antirrhinum has been detected. Because the pattern of yellow colour in antirrhinum was probed exactly in the *in situ* mRNA hybridisation experiment, this opens up the possibility of identifying a gene related to the aurone biosynthetic pathway.

References

Almeida, J., Carpenter, R., Robins, T.P., Martin, C. and Coen, E.S. (1989) Genetic interactions underlying flower color patterns in *Antirrhinum majus*. Genes and Development, 3, 1758-1767.

Asen, S., Norris, K. H. and Stewart, R.N. (1972) Copigmentation of aurone and flavone from petals of *Antirrhinum majus*. Phytochemistry, 11, 2739-2741.

Athma, P., Grotewold, F. and Peterson, T. (1992) Insertional mutagenesis of the maize *P* gene by intragenic transposition of *Ac*. Genetics, 131, 199-209.

Bartlett, N.J.R. (1989) The genetic control of anthocyanin biosynthesis in *Antirrhinum majus*. PhD Thesis, University of East Anglia.

Basteson, W. (1902) Note on the resolution of compound characters by cross-breeding. Proceedings American Philosophical Society, Cambridge, XII, pp. 50-54.

Baudry, A., Heim, M. A., Dubreucq, B., Caboche, M., Weisshaar, B. and Lepiniec, L. (2004) TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. The Plant Journal, 39, 366–380.

Baur, E. (1910) Vererbungs- und Bastardierungsversuche mit Antirrhinum. Zs. Indukt. Abstammungslehre, Berlin, III, pp. 34-89.

Beld, M., Martin, C., Huits, H., Stuitje, A. R. and Gerats, A.G.M. (1989) Flavonoid synthesis in *Petunia hybrida*: partial characterisation of dihydroflavonol 4-reductase genes. Plant Molecular Biology, 13, 491-502.

Bendahmane, A., Querci, M., Kanyuka, K. and Baulcombe, D.C. (2000) *Agrobacterium* transient expression system as a tool for the isolation of disease resistance genes: application to the *Rx2* locus in potato. The Plant Journal, 21, 73–81.

Ben-Nissan, G. and Weiss, D. (1995) Developmental and hormonal regulation of a triosephosphate isomerase gene in petunia corollas. Journal of Plant Physiology, 147, 28-62.

Ben-Tal, Y. and King, R.W. (1997) Environmental factors involved in coloration of flowers of Kangaroo Paw. Scientia Horticulturae, 72, 35-48.

Bieleski. R.L. (1993) Fructan hydrolysis drives petal expansion in the ephemeral daylily flower. Plant Physiology, 103, 213-219.

Biran, I. and Halevy. A.H. (1974) Effect of varying light intensities and temperature treatments applied to whole plants, or locally to leaves or flower buds, on growth and pigmentation of 'Baccara' roses. Physiologia Plantarum, 31, 175-179.

Bonner, J. (1944) Accumulation of various substances in girdled stem of tomato plants. American Journal of Botany, 31, 551-555.

Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A. and Lamb, C. (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. The Plant Cell, 12, 2383-2394.

Boumendjel, A. (2003) Aurones: A subclass of flavones with promising biological potential. Current Medicinal Chemistry, 10, 2621-2630.

Boyle, R. (1664) Experiments and considerations touching colours, London.

Britton, G. (1988) Biosynthesis of carotenoids. Plant pigments, edited by Goodwin, T. W. Academic Press. pp. 133-177.

Bruce, W.B., Christensen, A.H., Klein, T., Fromm, M. and Quail, P.H. (1989) Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. Proceedings of the National Academy of Sciences USA, 86, 9692-9696.

Carey, C.C., Strahle, J.T., Selinger, D.A. and Chandler, V.L. (2004) Mutations in the *pale aleurone color1* regulatory gene of the *Zea mays* anthocyanin pathway have distinct phenotypes relative to the functionally similar TRANSPARENT TESTA GLABRA1 gene in *Arabidopsis thaliana.* The Plant Cell, 16, 450-464.

Chandler, V.L., Radicella, J.P., Robbins, T.P., Chen, J and Turks, D. (1989) Two regulatory genes of the maize anthocyanin pathway are homologous: Isolation of *B* utilizing *R* genomic sequences. The Plant Cell, 1, 1175-1183.

Chawla, S.H., Cass, A.L. and Simmonds, A.J. (1999) Developmental and environmental regulation of anthocyanin pigmentation in wheat tissues transformed with anthocyanin regulatory genes. In Vitro Cellular & Developmental Biology – Plant, 35, 403-408.

Chopra, S., Cocciolone, S. M., Bushman, S., Sangar, V., McMullen, M. D. and Peterson, T. (2003) The maize *unstable factor for orange1* is a dominant epigenetic modifier of a tissue specifically silent allele of *pericarp color1*. Genetics, 163, 1135-1146.

Christensen, A.H., Sharrock, R.A., Quail, P.H. (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoteractivity following transfer to peotoplasts by electroporation. Plant Molecular Biology, 18, 675-689.

Chua, Y.L., Brown, C.A.P. and Gray, J.C. (2001) Targeted histone acetylation and altered nuclease accessibility over short regions of the pea plastocyanin gene. The Plant Cell, 13, 599-612.

Cocciolone, S.M., Chopra, S., Flint-Garcia, S.A., Mcmullen, M.D. and Peterson, T. (2001) Tissue-specific patterns of a maize *Myb* transcription factor are epigenetically regulated. The Plant Journal, 27, 467-478.

Coe, E.H.Jr. (1985) Phenotypes in corn: control of pathways by alleles, time and place. Plant Genetics, edited by Freeling, M., pressed by Alan R. Liss, Inc., New York. pp. 509-521.

Coen, E.S., and Carpenter, R. (1986) Transposable elements in *Antirrhinum majus*: Generators of genetic diversity, Trends in Genetics, 20, 292-296.

Coen, E.S. and Meyerowitz, E.M. (1991) The war of the whorls: Genetic interactions controlling flower development. Nature, 353, 31-37.

Coen, E.S., Carpenter, R. and Martin, C. (1986) Transposable elements generate novel spatial patterns of gene expression in *Antirrhinum majus*. Cell, 47, 285-296.

Cogdell, R. (1988) The function of pigments in chloroplasts. Plant pigments, edited by Goodwin, T. W. Acabemic Press. pp. 183-225.

Cone, C.K., Cocciolone, M.S., Burr, A.F., and Burr, B. (1993) Maize anthocyanin regulatory gene *pl* is a duplicate of *cl* that functions in the plant. The Plant Cell, 5, 1795-1805.

Cone, K.C., Burr, F.A. and Burr, B. (1986) Molecular analysis of the maize regulatory locus *Cl*. Proceedings of the National Academy of Sciences USA, 83, 9631-9635.

Conner, P. and Erickson, H.T. (1991) Inheritance of corolla striping and flower colors in *Salpiglossis sinuata*. HortScience, 26, 1549-1550.

Consonni, G., Geuna, F., Gavazzi, G. and Tonelli, C. (1993) Molecular homology among members of the *R* gene family in maize. The Plant Journal, 3, 335-346.

Consonni, G., Viotti, A., Dellaporta, S. L. and Tonelli, C. (1992) cDNA nucleotide sequence of *Sn*, a regulatory gene in maize. Nucleic Acids Research, 20, 373.

Courey, A.J. and Jia, S. (2001) Transcriptional repression: the long and the short of it. Genes and Development, 15, 2786-2796.

Cui, M., Handa, T. and Ezura, H. (2003) An improved protocol for Agrobacterium-mediated transformation of *Antirrhinum majus* L. Molecular Genetics and Genomics, 270, 296-302.

Cui, M., Takayanagi, K., Kamada, H., Nishimura, S. and Handa, T. (2000) Transformation of *Antirrhinum majus* L. by a *rol* –type multi-autotransformation (MAT) vector system. Plant Science, 159, 273-280.

Cui, M., Takayanagi, K., Kamada, H., Nishimura, S. and Handa, T. (2001) Efficient shoot regeneration from hairy roots of *Antirrhinum majus* L. transformed by the *rol* type MAT vector system. Plant Cell Reports, 20, 55-59.

Davies,K.M., Marshall, G.B., Marie Bradley, J., Schwinn, K.E., Bloor, S.J., Winefield, C.S. and Martin, C.R. (2006) Characterisation of aurone biosynthesis in *Antirrhinum majus*. Physiologia Plantarum, 128, 593-603.

Davies, K.M and Schwinn, K.E. (2003) Transcriptional regulation of secondary metabolism. Funtional Plant Biology, 30, 913-925.

Davies, K.M. (2004) Chapter 1, An introduction to plant pigments in biology and commerce. Plant pigments and their manipulation, annual Plant Reviews, Volume 14, edited by Davies, K.M., CRC Press, pp. 1-18.

Davies, K.M., Spiller, G.B., Bradley, J.M., Winefield, C.S., Schwinn, K.E., Martin, C.R. and Bloor, S.J. (2001) Genetic engineering of yellow flower colours. Acta Horticulturae., 560, 39-44.

Dayton, T.O. (1956) The inheritance of flower colour pigments. 1. The genus Antirrhinum. Journal of Genetics, 54, 249-260.

de Vetten, N., Quattrocchio, F., Mol, J. and Koes, R. (1997) The *an11* locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals. Genes and Development, 11, 1422-1434.

Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M. and Lepiniec, L. (2003) Proanthocyanidin-accumulating cells in Arabidopsis testa: Regulation of differentiation and role in seed development. Plant Cell, 15, 2514-2531.

Deikman, J. and Hammer, P.E. (1995) Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana.* Plant Physiology, 108, 47-57.

Dela, G., Or, E., Ovadia, R., Nissim-Levi, A., Weiss, D. and Oren-Shamir, M. (2003) Changes in anthocyanin concentration and composition in 'Jaguar' rose flowers due to transient high-temperature conditions. Plant Science, 164, 333–340.

Dong, Y.H., Beuning, L., Davies, K., Mitra, D., Morris, B. and Kootstra, A. (1998) Expression of pigmentation genes and photo-regulation of anthocyanin biosynthesis in developing Royal Gala apple flowers. Australian Journal of Plant Physiology, 25, 245-252.

Dooner, H.K. (1983) Co-ordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Molecular and General Genetics, 189, 136-141.

Dooner, H.K. and Nelson, O.E. (1977) Genetic control of UDP-glucose: flavonol-*O*-glucosyltransferase in the endosperm of maize. Biochemical Genetics, 15, 509-515.

Dowson Day, J.M., Ashurst, L.J., Mathias, F.S., Watts, W.J., Wilson, A.T.M. and Dixon, A.R. (1993) Plant viral leaders influence expression of a reporter gene in tobacco. Plant Molecular Biology, 23, 97-109.

Dowson Day, M.J., Ashurst, J.L. and Dixon, R.A. (1994) Plant expression cassettes for enhanced translational efficiency. Plant Molecular Biology Reporter, 12, 347-357.

Drews, G N., Beals, T.P., Bui, A.Q. and Goldberg, R.B. (1992) Regional and cell-specific expression patterns during petal development. The Plant Cell, 4, 1383-1404.

Dugas, V.D. and Bartel, B. (2004) MicroRNA regulation of gene expression in plants. Current Opinion in Plant Biology, 7, 512-520.

Eberharter, A. and Becker, P.B. (2002) Histone acetylation: a switch between repressive and permissive chromatin. EMBO Journal, 3, 224-229.

Emerson, R.A. (1914) The inheritance of a recurring somatic variation in variegated ears of maize. American Naturalist, New York, XLVIII, 87-115.

Fang, B., Ji, L., Bouvet, M. and Roth, A.J. (1998) Evaluation of GAL4/TATA *in vivo*. The Journal of Biological Chemistry, 273, 4972-4975.

Finer, J.J., Vain, P., Jones, M.W. and McMullen, M.D. (1992) Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Reports, 11, 323-328.

Forkmann, G. and Stotz, G. (1981) Genetic control of flavanone 3-hydroxylase activity and flavonoid 3'-hydroxylase activity in *Antirrhinum majus* (Snapdragon). Zeitschrift fuer Naturforschung, 35c, 411-416.

Formann, G. (1991) Flavonoids as flower pigments: The formation of the natural spectrum and its extension by genetic engineering. Plant Breeding, 106, 1-26.

Franken, P., Schrell, S., Peterson, P.A., Saedler, H. and Wienand, U. (1994) Molecular analysis of protein domain function encoded by the myb-homologous maize genes *C1. Zm1 and Zm 38.* The Plant Journal, 6, 21-30.

Galbiati, M., Chiusi, A., Peterlongo, P., Mancinelli, A. and Gavazzi, G. (1994) hotoinduction of anthocyanin in maize: a genetic approach. Maydica, 39, 89-95.

Gallie, R.D., Sleat, E.D., Watts, W.J., Turner, C.P and Wilson, A.T.M. (1987) A comparison of eukaryotic viral 5'-leader sequences as enhancers of mRNA expression *in vivo*. Nucleic Acids Research, 15, 8693-8711.

Gasser, S.M., Paro, R., Stewart, F. and Aasland, R. (1998) Epigenetic control of transcription introduction: the genetics of epigenetics. CMLS Cellular and Molecular Life Sciences, 54, 1-5.

Geissman, T.A. and Harborne, J.B. (1955) The chemistry of flower pigmentation in *Antirrhinum majus*. IV. The albino (-mm-nn) form. Archives of Biochemistry and Biophysics, 55, 447-454.

Geissman, T.A., Jorgensen, E.C. and Johnson, B.L. (1954) The chemistry of flower pigmentation in *Antirrhinum majus*. Color genotypes. I. The flavonoid components of the homozygous P, M, Y color types. Archives of Biochemistry and Biophysics, 49, 368-388.

Gerats, A.G.M., Beld, M., Huits, H. and Prescott, A. (1989) Gene tagging using homologous and heterologous transposable elements. Developmental Genetics, 10, 561-568.

Giraudat, J., Hauge, B.M., Valon, C., Amalle, J., Parcy, F. and Goodman, H.M. (1992) Isolation of the *Arabidopsis* ABI3 gene by positional cloning. The Plant Cell, 4, 1251-1261.

Gleave, P.A. (1992) A versatile binary vector system with a T-DNA organisational structure conductive to efficient integration of cloned DNA into the plant genome. Plant Molecular Biology, 20, 1203-1207.

Goff, S.A., Cone, K.C. and Chandler, V.L. (1992) Functional analysis of the transcriptional activator encoded by the maize B gene: evidence for a direct functional interaction between two classes of regulatory proteins. Genes and Development, 6, 864-875.

Goff, S.A., Cone, K.C. and Fromm, M.E. (1991) Functional analysis of the maize C1 transcriptional activator: Comparison of wild-tpye and dominant inhibitor proteins. Genes and Development, 5, 298-309.

Goodrich, J., Carpenter, R. and Coen, E. (1992) A common gene regulates pigmentation pattern in diverse plant species. Cell, 68, 955-964.

Goodwin, T. and Britton, G. (1988) Distribution and analysis of carotenoids. Plant pigments, edited by Goodwin, T. W. Acabemic Press. pp. 61-127.

Gorton, H.L. and Vogelmann, T.C. (1996) Effects of epidermal cell shape and pigmentation on optical properties of *Antirrhinum* petals at visible and ultraviolet wavelengths. Plant Physiology, 112, 879-888.

Goto, N. and Pharis, R.P. (1999) Role of gibberllins in the development of floral organs of the giberellin-deficient mutant, *ga1-1*, of *Arabidopsis thaliana*. Canadian Journal of Botany, 77, 944-954.

Gould, K.S. and Lister, C. (2006) Flavonoid functions in plants. Flavonoids chemistry, biochemistry and applications, editted by Andersen, Ø.M. and Markham, K.R. Published by CRC Press. pp. 367-442.

Gregory, R.P. (1911) Experiments with *Primula sinensis*. Journal of Genetics, Cambridge, I, pp. 72-132.

Griesbach, R.J. (1992) Correlation of pH and light intensity on flower color in potted *Eustoma grandiflorum* Grise. HortScience, 27, 817-818.

Halevy, A.H. (1987) Assimilate allocation and flower development. Manipulation of flowering, edited by Atherton J.G., pressed by Butterworth, London, pp. 363-378.

Halevy, A.H. and Mayak, S. (1981) Senescence and postharvest physiology of cut flowers part II. Horticultural Reviews, 3, 59-143.

Han, S.S. (1992) Role of sucrose in bud development and vase life of cut *Liatris spicata* (L.) Wild. HortScience, 27, 1198-1200.

Harborne, J.B. (1963) Plant polyphenols. X. Flavone and aurone glycosides of *Antirrhinum*. Phytochemistry, 2, 327-334.

Harborne, J.B. (1988) The flavonoids: recent advances. Plant pigments, edited by Goodwin, T.W. Acabemic Press. pp. 299-345.

Harrison, B.J. and Stickland, R.G. (1974) Precursors and genetic control of pigmentation. 2. Genotype analysis of pigment controlling genes in ayanic phenotypes in *Antirrhinum majus*. Heredity, 33, 112-115.

Harrison, B.J. and Stickland, R.G. (1977) Point of action of the Eosinea gene in *Antirrhinum majus*. John Innes Annual Report, pp. 40.

Harrison, B.J. and Stickland, R.G. (1978) Precursors and genetic control of pigmentation. 4. Hydroxylation and methoxylation stages in anthocyanidin biosynthesis. Heredity, 40, 127-132.

Hartmann, U., Sagasser, M., Mehrtens, F., Stracke, R. and Weisshaar, B. (2005) Differential combinatorial interactions of *cis*-acting elements recognized by R2R3-MYB, bZIP, and bHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. Plant Molecular Biology, 57, 155-171.

Haseloff, J., Siemering, R.K., Prasher, C.D. and Hodge, S. (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proceedings of the National Academy of Science USA, 94, 2122-2127.

Hattori, T., Vasil, V., Rosenkrans, L., Hannah, L.C., McCarty, D.R. and Vasil, I.K. (1992) The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory for anthocyanin biosynthesis during seed maturation in maize. Genes and Development, 6, 609-618.

Hauber, J., Nelbock-Hochstetter, P. and Feldmann, H. (1985) Nucleotide sequence and characteristics of a Ty element from yeast. Nucleic Acids Research, 13, 2745-2758.

Heidmann, I., Efremova, N., Saedler, H. and Schwarz-sommer, Z. (1998) A protocol for transformation and regeneration of *Antirrhinum majus*. The Plant Journal, 13, 723-728.

Hemerly, A. S., Ferreira, P., de Almeida Engler, J., Van Montagu, M., Engler, G. and Inze, D. (1993) *Cdc2a* expression in *Arabidopsis* is linked with competence for cell division. The Plant Cell, 5, 1711-1723.

Hernandez, J.M., Heine, G.F., Irani, N., Feller, A., Kim, M., Matulnik, T., Chandler, V.L. and Grotewold, E. (2004) Different mechanisms participate in the R-dependent activity of

the R2R3 MYB transcription factor G1. The Journal of Biological Chemistry, 279, 48205-48213.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant cis-acting regulatory DNA elements (PLACE) database:1999. Nucleic Acids Research, 27, 297-300.

Ho, L.C. and Nichols, R. (1977) Translocation of C-sucrose in relation to changes in carbohydrate content in corollas cut at different stages of development. Annals of Botany, 41, 227-242.

Hoekenga, O.A., Muszynski, M.C. and Cone, K.C. (2000) Developmental patterns of chromatin structure and DNA methylation responsible for epigenetic expression of a maize regulatory gene. Genetics, 155, 1889-1902.

Holton, T.A. and Cornish, E.C. (1995) Genetics and biochemistry of anthocyanin biosynthesis. The Plant Cell, 7, 1071-1083.

Hosokawa, K., Fukunaga, Y., Fukushi, E. and Kawabata, J. (1996) Production of acylated anthocyanins by blue flowers of *Hyacinthus orientalis* regenerated in vitro. Phytochemistry, 41, 1531-1533.

Itoh, H., Tanaka-ueguchi, M., Kawaide, H., Xinbo, C., Kamiya, Y. and Matsuoka, M. (1999) The gene encoding tobacco gibberellin 3-hydroxylase is expressed as the site of GA action during stem elongation and flower organ development. The Plant Journal, 20, 15-24.

Jackson, D., Roberts, K. and Martin, C. (1992) Temporal and spatial control of expression of anthocyanin biosynthetic genes in developing flowers of *Antirrhinum majus*. The Plant Journal, 2, 425-434.

Jacobsen, S.E. and Olszewski, N.E. (1991) Characterization of the arrest in anther development associated with gibberellin deficiency of the *gib-1* mutant of tomato. Plant Physiology, 97, 409-414.

Jang, J.C. and Sheen, J. (1997) Sugar sensing in higher plants. Trends in Plant Science, 2, 208-213.

Jefferson, R.A,. Kavanagh, T.A and. Bevan, M.W (1987). Gus fusion: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal. 6, 3901-3907.

Johansen, L.K. and Carrington, J.C. (2001) Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*mediated transient expression system. Plant Physiology. 126, 930–938.

Johnson, A.A.T., Hibberd, J.M., Gay, C., Essah, P.A., Haseloff, J., Tester, M. and Guiderdoni, E. (2005) Spatial control of transgene expression in rice (*Oryza sativa* L.) using the GAL4 enhancer trapping system. The Plant Journal, 41, 779-789.

Jorgensen, E.C. and Geissman, T.A. (1954) The chemistry of flower pigmentation in *Antirrhinum majus*. II. Glycosides of *PPmmYY, PPMMYY, ppmmYY* and *ppMMYY* color genotypes. Archives of Biochemistry and Biophysics, 54, 72-82.

Jorgensen, E.C. and Geissman, T.A. (1955) The chemistry of flower pigmentation in *Antirrhinum majus* color genotypes. III. Relative anthocyanin and aurone concentrations. Archives of Biochemistry and Biophysics, 55, 389-402.

Kaneko, M., Hironori, I., Yoshiaki, I., Tomoaki, S., Miyako, U.T., Motoyuki, A. and Makoto, M. (2003) Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? The Plant Journal, 35, 104-115.

Kao, C.Y., Cocciolone, S.M., Vasil, I.K. and McCarty, D.R. (1996) Localization and interaction of the *cis*-acting elements for abscisic acid, VIVIPAROUS1, and light activation of the C1 gene of maize. The Plant Cell, 8, 1171-1179.

Kawabata, S., Ohta, S.M., Kusuhara, Y. and Sakiyama, R. (1995) Influences of low light intensities on the pigmentation of *Eustoma grandiflorum* flowers. Acta Horticulturae, 405, 173-178.

Kay, Q.O.N., Daoud, H.S. and Stirton, C.H. (1981) Pigment distribution, light reflection and cell structure in petals. Botanical Journal of the Linnean Society, 83, 57-84.

Kho, K.F.F., Bolsman-Louwen, A.C., Vuik and Bennink, G.J.H. (1977) Anthocyanin synthesis in a white flowering mutant of *Petunia hybrida*. Planta, 135, 109-

Klein, R.M. (1990) Failure of supplementary ultraviolet radiation to enhance flower color under greenhouse conditions. HortScience, 25, 307-308.

Kitamura, S. (2006) Transport of flavonoids: from cytosolic synthesis to vacuolar accumulation. The Science of Flavonoids, edited by Grotewold, E, pressed by Springer Science+Business Media, Inc. pp. 123-146.

Koster, R.W. and Fraser, S.E. (2001) Tracing transgene expression in living zebrafish embryos. Developmental Biology, 233, 329-346.

Kubasek, W.L., Shirley, B.W., McKillop, A., Goodman, H.M., Briggs, W. and Ausubel, F.M. (1992) Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedlings. The Plant Cell, 4, 1229-1236.

Kubo, H., Peeters, A.J., Aarts, M.G., Pereira, A. and Koornneef, M. (1999) *ANTHOCYANINLESS2*, a homeobox gene affecting anthocyanin distribution and root development in Arabidopsis. The Plant Cell, 11, 1217-1226.

Kuiper, D., van Reenen, H.S. and Ribot, S.A. (1991) Effect of gibberellic acid on sugar transport into petals of 'Madelon' rose flowers during bud opening. Acta Horticulturae, 298, 93-95.

Lauvergeat, V., Rech, P., Jauneau, A., Guez, C., Coutos-Thevenot, P. and Grima-Pettenati, J. (2002) The vascular expression pattern directed by the *Eucalyptus gunnii* cinnamyl alcohol dehydrogenase *EgCAD2* promoter is conserved among woody and herbaceous plant species. Plant Molecular Biology. 50, 497-509.

Lu, C.A., Lim, E.K. and Yu, S.M. (1998) Sugar response sequence in the promoter of a rice Alpha-amylase gene serves as a transcriptional enhancer. Journal of Biology and Chemistry, 273, 10120-10131.

Lu, G. and Bruce, W.B. (2000) A novel *cis*-acting element conferring root-preferred gene expression in maize. Journal of Plant Physiology, 156, 277-283.

Luby-phelps, K., Ning, G., Fogerty, J. and Besharse, J.C. (2003) Visualization of identified GFP-expressing cells by light and electron microscopy. The Journal of Histochemistry & Cytochemistry, 51(3), 271-274.

Ludwig, S.R. and Wessler, S.R. (1990) Maize *R* gene family: Tissue-specific helix-loop-helix proteins. Cell, 62, 849-851.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L. and Wessler, S.R. (1989) *Lc,* a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the *myc* homology region. Proceedings of the National Academy of Science USA, 86, 849-851.

Lyer, M., Wu, L., Carey, M., Wang, Y., Smallwood, A. and Gambhir, S.S. (2001) Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. Proceedings of the National Academy of Science USA, 98, 14595-14600.

Mantis, J. and Tauge, B.W. (2000) Comparing the utility of ß-glucuronidase and green fluorescent protein for detection of weak promoter activity in *Arabidopsis thaliana*. Plant Molecular Biology Reports. 18, 319-330.

Marheineke, J. (1936) Ueber den Einfluss van Aussenfaktoren auf das Blⵀtenfarbmuster von *Petunia hybrida grandiflora, Calliopsis bicolor, Mimulus tigrinus* und *Primula malacoides.* Jahrb. F. Wiss. Botanik, 83, 593-656.

Martin, C. and Gerats, T. (1993) Control of pigment biosynthesis genes during petal development. The Plant Cell, 5, 1253-1264.

Martin, C., Carpenter, R., Sommer, H., Saedler, H. and Coen, E.S. (1985) Molecular analysis of instability in flower pigmentation in Antirrhinum majus, following isolation of the *pallida* locus by transposon tagging. EMBO Journal, 4, 1625-1630.

Martin, C., Prescott, A., Mackay, S., Bartlett, J. and Vrijlandt, E. (1991) Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. The Plant Journal, 1, 37-49.

Martin, P. (1982) Stem xylem as a possible pathway for mineral retranslocation from senescing leaves to the ear in wheat. Australia Journal of Plant Physiology, 9, 197-207.

Mason, T.G. and Maskell, E.J. (1928) Studies on the transport of carbohydrates in the cotton plant. 11. The factors determining the rate and the direction of movement of sugars. Annual Botany (London) 42, 571-636.

McCarty, D.R. (1995) Gnetic control and intergration of maturation and germination pathways in seed development. Annual Review of Plant Physiology and Plant Molecular Biology, 46, 71-93.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M. and Vasil, I.K. (1991) The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. Cell, 66, 895-905.

McElroy, D., Zhang, W., Cao, J. and Wu, R. (1990) Isolation of an efficient actin promoter for use in rice transformation. The Plant Cell. 2, 163-171.

Mehrtens, F., Kranz, H., Bednarek, P. and Weisshaar, B. (2005) The Arabidopsis transcription factor MYB12 is a flavonal-specific regulator of phenylpropanoid biosynthesis. Plant Physiology. 138, 1083-1096.

Mendel, G. (1865). Versuche über Pflanzen-Hybriden. Verhandlungen des Naturforschenden Vereins in Brünn, 4, 3-47.

Menssen, A., Hohmann, S., Martin, W., Schnable, P.S., Peterson, P.A., Saedler, H. and Gierl, A. (1992)The En/Spm transposable element of *Zea mays* contains splice sites at the

termini generating a novel intron from a *dSpm* element in the *A2* gene. EMBO Journal, 9, 3051-3057.

Mereghetti, M., Tonelli, C. and Gavazzi, G. (1991) Developmental expression of light inducible genes of the *R* family in immature seeds of maize. Maydica, 36, 337-342.

Millar, A.A. and Gubler, F. (2005) The Arabidopsis *GAMYB-Like* genes, *MYB33* and *MYB65*, are microRNA-regulated genes that redundantly facilitate anther development. The Plant Cell, 17, 705-721.

Mitsuhara, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., Gotoh, Y., Katayose, Y., Nakamura, S., Honkura, R., Nishimiya, S., Ueno, K., Mochizuki, A., Tanimoto, H., Tsugawa, H., Otsuki, Y. and Ohashi, Y. (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant and Cell Physiology. 37, 49-59.

Moalem-Beno, D., Tamari, G., Leitner-Dagan, Y., Borochov, A. and Weiss, D. (1997) Sugar-dependent gibberellin-induced chalcone synthase gene expression in petunia corollas. Plant Physiology, 113, 419-424.

Mol, J., Stuitje, A., Gerats, A., van der Krol, A. and Jorgensen, R. (1989) Saying it with genes: Molecular flower breeding. Trends in Biotechnology. 7, 148-135.

Moscovici, S., Moalem-Beno, D. and Weiss, D. (1996) Leaf-mediated light responses in petunia flowers. Plant Physiology, 110, 1275-1282.

Nakama, T., Yonekura-Sakakibara, K., Sato, T., Kikuchi, S., Fukui, Y., Fukuchi-Mizutani, M., Ueda, T., Nakao, M., Tanaka, Y., Kusumi, T. and Nishino, T. (2000) Aureusidin synthase: a polyphenol oxidase homolog responsible for flower coloration. Science, 290, 1163-1166.

Nakayama, N., Arroyo, M.J., Simorowski, J., May, B., Martienssen, R. and Irish, F.V. (2005) Gene trap lines define domains of gene regulation in Arabidopsis petals and stamens. The Plant Cell, 17, 2486-2506.

Nakayama, T., Sato, T., Fukui, Y., Yonekura-Sakakibara, K., Hayashi, H., Tanaka, Y., Kusumi, T. and Nishino, T. (2001) Specificity analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration. FEBS Letter, 499, 107-111.

Nesi, N., Debeaujon, I., Jond, C., Pelletier, G., Caboche, M. and Lepiniec, L. (2000) The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in *Arabidopsis* siliques. The Plant Cell, 12, 1863-1878.

Nesi, N., Debeaujon, I., Jond, C., Stewart, A.J., Jenkins, G.I., Caboche, M. and Lepiniec, L. (2002) The TRANSPARENT TESTA16 locus encodes the ARABIDOPSIS BSISTER MADS domain protein and is required for proper development and pigmentation of the seed coat. The Plant Cell, 14, 2463-2479.

Nesi, N., Jond, C., Debeaujon, I., Caboche, M. and Lepiniec, L. (2001) The arabidopsis *TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. The Plant Cell, 13, 2099-2114.

Neta-Sharir, I., Shoseyov, O. and Weiss, D. (2000) Sugars enhance the expression of gibberellin-induced genes in developing petunia flowers. Physiologia Plantarum, 109, 196-202.

Nguyen, H.T., Leelavathi, S. and Reddy, V.S. (2004) Bacteriophage T7 RNA polymerase-directed, inducible and tissue-specific over-expression of foreign genes in transgenic plants. Plant Biotechnology Journal, 2, 301-310.

Nicholson, R.L., Kollipara, S.S., Vincent, J.R., Lyons, P.C. and Caden-Gomez, G. (1987) Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. Proceedings of the National Academy of Science USA, 84, 5520.

Noda, K.L., Glover, B., Linstead, P. and Martin, C. (1994) Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor. Nature, 369, 661-664.

Noscovici, S., Moalem-Beno, D. and Weiss, D. (1996) Leaf-mediated light responses in petunia flowers. Plant Physiology, 110, 1275-1282.

Onslow, M.W. (1925) The Anthocyanin Pigments of Plants. Second edition. Cambridge at the university press.

Pacholati, S.F., Nicholson, R.L. and Butler, L.G. (1986) Phenylalanine ammonia-lyase activity and anthocyanin accumulation in wounded maize mesocotyls, Journal of Phytopathology, UAR, 115-165.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P.A. and Saedler, H. (1987) The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. EMBO Journal, 6, 3553-3558.

Paz-Ares, J., Peterson, P.A. and Saedler, H. (1990) Molecular analysis of the *C1-I* allele from *Zea mays*: a dominant mutant of the regulatory *C1* locus. EMBO Journal, 9, 315-321.

Paz-Ares, J., Wienand, U., Peterson, P.A. and Saedler, H. (1986) Molecular cloning of the *C* locus of *Zea mays*. A locus regulating the anthocyanin pathway. EMBO Journal, 5, 829-833.

Pelletier, M.K. and Shirley, B.W. (1996) Analysis of flavanone-3-hydroxylase in Arabidopsis seedlings. Coordinate regulation with chalcone synthase and chalcone isomerase. Plant Physiology, 111, 339-345.

Pelletier, M.K., Burbulis, I.E. and Winkel-Shirley, B. (1999) Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in *Arabidopsis* seedlings. Plant Molecular Biology, 40, 45-54.

Pelletier, M.K., Murrell, J.R. and Shirley, B.W. (1997) Characterization of flavonol synthase and leucoanthocyanidin dioxygenase genes in Arabidopsis. Plant Physiology, 113, 1437-1445.

Petroni, K., Cominelli, E., Consonni, G., Gusmaroli, G., Gavazzi, G. and Tonelli, C. (2000) The tissue specific expression of the maize regularory gene *Hopi* determines germination-dependent anthocyanin accumulation. Genetics, 155, 323-336.

Pharis, R.P. and King, R.W. (1985) Gibberellins and reproductive development in seed plants. Annual Review of Plant Physiology, 36, 517-568.

Piazza, P., Antonia, P., Gareth, I.J. and Chiara, T. (2002) Members of the *c1/pl1* regulatory gene family mediate the response of maize aleurone and mesocotyl to different light qualities and cytokinins. Plant Physiology, 128, 1077-1086.

Powlowski, W.P. and Somers, D.A. (1996). Transgene inheritance in plants genetically engineered by microprojectile bombardment. Molecular Biotechnology, 6, 17-30.

Procissi, A., Dolfini, S., Ronchi, A. and Tonelli, C. (1997) Light-dependent spatial and temporal expression of pigment regulatory genes in developing maize seed. The Plant Cell, 9, 1547-1557.

Qin, H., Guo, H., Jia, Y., Li, L. and Tian, Y. (2000) Effect of UTRs from TMV-RNA on the expression of foreign gene in transgenic plants. Chinese Science Bulletin, 45, 1681-1685.

Quaedvlieg, N.E.M., Schlaman, H.R., Admiraal, P.C., Wijting, S.E., Stougaard, J. and Spaink, H. (1998) Fusions between green fluorescent protein and ß-glucuronidase as sensitive and vital bifunctional reporters in plants. Plant Molecular Biology, 38, 861-873.

Quattrocchio, F., Baudry, A., Lepiniec, L. and Grotewold, E. (2006) The regulation of flavonoid biosynthesis. The Science of Flavonoids, eddited by Grotewold, E, pressed by Springer Science+Business Media, Inc. pp. 97-122.

Quattrocchio, F. (1994) Regulatory genes controlling flower pigmentation in *Petunia hybrida*. PhD thesis (Amsterdam: Vrije Universiteit).

Quattrocchio, F., Wing, J.F., Leppen, H.T.C., Mol, J.N.M and Koes, R.E. (1993) Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. The Plant Cell, 5, 1497-1512.

Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J. and Koes, R. (1999) Molecular analysis of the *anthocyanin2* gene of *Petunia* and its role in the evolution of flower color. The Plant Cell, 11, 1433-1444.

Rau, W. (1988) Functions of carotenoids other than in photosynthesis. Plant Pigments, edited by Goodwin, T. W. Acabemic Press. pp. 231-252.

Rebers, M., Kaneta, T., Kawaide, H., Yamaguchi, S., Yang, Y.Y., Imai, R., Sekimoto, H. and Kamiya, Y. (1999) Regulation of gibberellin biosynthesis genes during flower and fruit development of tomato. The Plant Journal, 17, 241-250.

Rech, P., Grima-Pettenati, J. and Jauneau, A. (2003) Fluorescence microscopy: a powerful technique to detect low GUS activity in vascular tissues. The Plant Journal, 33, 205-209.

Regan, S., Bourquin, V., Tuominen, H. and Sundberg, B. (1999) Accurate and high resolution *in situ* hibridization analysis of gene expression in secondary stem tissues. The Plant Journal, 19, 363-369.

Robichaud, C.S. and Sussex, I.M. (1986) The response of *viviparous1* and wild-type embryos of *Zea mays* to culture in the presence of abscisic acid. Journal of Plant Physiology, 126, 235-242.

Rudiger, W. and Schoch, S. (1988) Chlorophylls. Plant Pigments, edited by Goodwin, T. W. Acabemic Press. pp. 1-50.

Sadka, A., Dewald, D., May, G.D., Park, W.D. and Mullet, J.E. (1994) Phosphate modulates transcription of soybean *vspB* and other sugar-inducible genes. The Plant Cell, 6, 737-749.

Sagasser, M., Lu, G.H., Hahlbrock, K. and Weisshaar, B. (2002) *A. thaliana* TRANSPARENT TESTA 1 is involved in seed coat development and defines the WIP subfamily of plant zinc finger proteins. Genes and Devevelopment, 16, 138-149.

Sagi, L., Panis, B., Remy, S., Schoofs, H., De Smet, K., Swennen, R. and Cammue, B.P.A. (1995) Genetic transformation of banana and plantain (Musa spp) via particle bombardment. Bio/Technology. 13, 481-485.

Sainz, M.B., Grotewold, E. and Chandler, V.L. (1997) Evidence for direct activation of an anthocyanin promoter by the maize C1 protein and comparison of DNA binding by related Myb domain proteins. The Plant Cell, 9, 611-625.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, a Laboratory Manual. 2nd Edition. Cold Spring Harbour Laboratory Press.

Sandhu, J.S., Webster, C.l. and Gray, J.C. (1998) A/T-rich sequences act as quantitative enhancers of gene expression in transgenic tobacco and potato plants. Plant Molecular Biology, 37, 885-896.

Scheffler, B., Franken, P., Schutt, E., Schrell, A., Saedler, H., and Wienand, U. (1994) Molecular analysis of *C1* alleles in *Zea mays* defines regions involved in the expression of this regulatory gene. Molecular and General Genetics. , 242, 40-48.

Schenk, P.M., Elliott, A.R. and Manners, J.M. (1998) Assessment of transient gene expression in plant tissue using the green fluorescent protein as a reference. Plant Molecular Biology Reports, 16, 313-322.

Schubeler, D and Elgin, S.C.R. (2005) Defining epigenetic states through chromatin and RNA. Nature Genetics, 37, 917-918.

Schwinn, K. (1999) Regulation of anthocyanin biosynthesis in *Antirrhinum majus.* Ph.D Thesis, University of East Anglia.

Schwinn, K.E. and Davies, K.M. (2004) Chapter 4, Flavonoids, In: Plant pigments and their manipulation, Annual Plant Reviews, Volume 14, edited by Kevin Davies, CRC Press, pp. 92-136.

Schwinn, K., Venail, J., Shang, Y., Mackay, S., Alm, V., Butelli, E., Oyama, R., Bailey, P., Davies, K. and Martin, C. (2006) A small family of *MYB*-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. The Plant Cell, 18, 831-851.

Scott-Montcrieff, R. (1930) Natural anthocyanin pigments. II. The magenta flower pigment of *Antirrhinum majus*. Biochemical Journal, 24, 753.

Scott-Montcrieff, R. (1936) A biochemical survey of some mendelian factors for flower colour. Journal of Genetics, 32, 117-170.

Segawa, T., Takebayashi, H., Kakehi, Y., Yoshida, O., Narumiya, S. and Kakizuka, A. (1998) Prostate-specific amplification of expanded polyglutamine expression: a novel approach for cancer gene therapy. Cancer Research, 58, 2282-2287.

Selinger, D.A. and Chandler, V.L. (1999) A mutation in the *pale aleurone color 1* gene idenfifies a novel regulator of the maize anthocyanin pathway. The Plant Cell, 11, 5-14.

Sessions, A., Weigel, D. and Yanofsky, M.F. (1999) The *Arabidopsis thaliana MERISTEM LAYER 1* promoter specifies epidermal expression in meristems and young primordial. The Plant Journal, 20, 259-263.

Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E. and Innes, R.W. (2003) Cleavage of *Arabidopsis* PBS1 by Bacterial Type III Effector. Science, 301, 1230–1233.

Shirley, B.W., Kubasek, W.L., Storz, G., Bruggenmann, E., Koornneef, M., Ausubel, F.M. and Goodman, H.M. (1995) Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. The Plant Journal, 8, 659-671.

Solfanelli, C., Poggi, A., Loreti, E., Alpi, A. and Perata, P. (2006) Sucrose-Specific Induction of the Anthocyanin Biosynthetic Pathway in Arabidopsis. Plant Physiology, 140, 637-646.

Sommer, H. and Saedler, H. (1986) Structure of the chalcone synthase gene of *Antirrhinum majus*. Molecular and General Genetics, 202, 429-434.

Sommer, H., Carpenter, R., Harrison, B. J. and Saedler, H. (1985) The transposable element *Tam3* of *Antirrhinum majus* generates a novel type of sequence alteration upon excision. Molecular and General Genetics, 199, 225-231.

Sommer, H., Hehl, R., Krebbers, E., Piotrowiak, R., Lnnig, W.E. and Saedler, H. (1988) In Nelson, O. (ed), Proc. International Symposium Plant Transposable Elements. Plenum, New York, pp. 227-236.

Spelt, C., Quattrocchio, F., Mol, J. and Koes, R. (2000) *anthocyanin1* of petunia encodes a basic-helix loop helix protein that directly activates structural anthocyanin genes. The Plant Cell, 12, 1619-1631.

Spelt, C., Quattrocchio, F., Mol, J. and Koes, R. (2002) ANTHOCYANIN1 of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. The Plant Cell, 14, 2121-2135.

Stam, M., Belele, C., Dorweiler, J.E., and Chandler, V.L. (2002) Differential chromatin structure within a tandem array 100 kb upstream of the maize *b1* locus is associated with paramutation. Genes and Development, 16, 1906-1918.

Stickland, R.G. and Harrison, B.J. (1974) Precursors and genetic control of pigmentation. 1. Induced biosynthesis of pelargonidin, cyanidin and delphinidin in *Antirrhinum majus*. Heredity, 33, 108-112.

Stickland, R.G., Harrison, B.J. and Barnes, C. (1976) Guantitative effects of partial dominance and optical activity of precursors on anthocyanidin production. John Innes Institute Annual Report, pp. 56-57.

Stoddat, J.L. (1987) Genetic and hormonal regulation of stature. In Developmental Mutants, edited by H. Thomas and D. Grierson. Cambridge University Press, pp. 155-180.

Stubbe, H. (1966) Genetik und Zytologie von *Antitthinum* L. sect. *Antirrhinum*. (Jena, Germany: Veb. Gustav Fischer, Verlag).

Styles, E.D. and Coe, E.H., Jr. (1986) Unstable expression of an *R* allele with *a3* in maize. Journal of Heredity, 77, 389-393.

Suzuki, M., Kao, C.Y. and McCarty, D.R. (1997) The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. The Plant Cell, 12, 35-52.

Suzuki, M., Kao, C.Y., Cocciolone, S. and McCarty, D.R. (2001) Maize VP1 complements *Arabidopsis abi3* and confers a novel ABA/auxin interaction in roots. The Plant Journal, 28, 409-418.

Swcheffler, B., Franken, P., Schutt, E., Schrell, A., Saedler, H. and Wienand, U. (1994) Molecular analysis of the *C1* alleles in *zea mays* defines regions involved in the expression of this regulatory gene. Molecular and General Genetics, 242, 40-48.

Takumi, S., Otani, M. and Shimada, T. (1994) Effect of six promoter-intron combinations on transient reporter gene expression in einkorn, emmer and common wheat cells by particle bombardment. Plant Science, 103, 161-166.

Tamura, K., Shimada, T., Ono, E., Tanaka, Y., Nagatani, A., Higashi, S., Watanabe, M., Nishimura, M. and Hara-Nishimura, I. (2003) Why green fluorescent fusion proteins have not been observed in the vacuoles of higher plants. The Plant Journal, 35, 545-555.

Taylor, C.B. (1997) Promoter fusion analysis: an insufficient measure of gene expression. The Plant Cell, 9, 273-275.

Teng, S., Keurentjes, J., Bentsink, L., Koornneef, M. and Smeekens, S. (2005) Sucrose-specific induction of anthocyanin biosynthesis in arabidopsis requires the *MYB75/PAP1* gene. Plant Physiology, 139, 1840-1852.

Tonelli, C., Consonni, G., Faccio Dolfini, S., Dellaporta, S.L., Viotti, A. and Gavazzi, G. (1991) Genetic and molecular analysis of *Sn*, a light-inducible, tissue specific regulatory gene in maize. Molecular and General Genetics, 225, 401-410.

Tonelli, C., Faccio, D.S., Ronchi, A.Q., Consonni, G. and Gavazzi, G. (1994) Light inducibility and tissue specificity of the *R* gene family in maize. Genetica, 94, 225-234.

Tsukaya, H., Ohshima, T., Naito, S., Chino, M. and Komeda, Y. (1991) Sugar-dependent expression of the *CHS-A* gene for chalcone synthase from petunia in transgenic *Arabidopsis*. Plant Physiology, 97, 1414-1421.

Vainstein, A., Halevy, A. H., Smirra, I. And Visgnevetsky, M. (1994) Chromoplast b iogenesis in *Cucumis sativus* corollas: Rapid effect of giberellin A_3 on the accumulation of a chromoplast-specific carotenoid-associated protein. Plant Physiology, 104, 321-326.

Van der Hoorn, J.A.L., Laurent, F., Roth, R. and De Wit, P.J.G.M. (2000) Agroinfiltration is a versatile tool that facilitates comparative analyses of *Avr9/cf-9*-induced and *Avr4/Cf-4*-induced necrosis. Molecular Plant–Microbe Interaction, 13, 439–446.

Van der Krol, A. R., de Lange, P., Gerats, A. G. M., Mol, J. N. M. and Stuitje, A. R. (1989) Antisense chalcone synthase genes in *Petunia*: Visualisation of variable transgene expression, Molecular and General Genetics, 220, 204-212.

Van der Meer, I. (1991) Regulation of flavonoid gene expression in *Petunia hybrida*. Ph.D. Thesis, Vrije University of Amsterdam.

Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, L. and Rocha-Sosa, M. (1990) Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Molecular Gemonics and Genetics, 220, 245-250.

Vaquero, C., Sack, M., Chandler, J., Drossard, J., Schuster, F., Monecke, M., Schillberg, S. and Fischer, R. (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. Proceedings of the National Academy of science

USA, 96, 11128–11133.

Vaquero, C., Sack, M., Schuster, F., Finnern, R., Drossard, J., Schumann, A. and Fischer, R. (2002) A carcinoembryonic antigenspecific diabody produced in tobacco. FASEB Journal, 16, 408–410.

Verdone, L., Caserta, M. and Mauro, E.D. (2005) Role of histone acetylation in the control of gene expression. Biochemical Cell Biology, 83, 344-353.

Verdonk, J.C., Haring, M.A., van Tunen, A.J. and Schuurink, R.C. (2005) *ODORANT1* regualtes frangrance biosynthesis in petunia flowers. The Plant Cell, 17, 1612-1624.

Voinnet, O., Rivas, S., Mestre, P. and Baulcombe, D. (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. The Plant Journal, 33, 949-956.

Vries, H. de (1911) The mutation theory, Translated by J. B. farmer and A. D. Darbishire. (Die mutations theories, Leipzig, 1901.).

Walker, A.R., Davison, P.A., Bolognesi-Winfield, A.C., James, C.M., Srinivasan, N., Blundell, T.L., Esch, J.J., Marks, M.D. and Gray, J.C. (1999) The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. The Plant Cell, 11, 1337-1350.

Wang, C.Q., Zhang, Y.F. and Tao, L. (2005) Activity changes of calmodulin and Ca^{2+}-ATPase during low-temperature-induced anthocyanin accumulation in *Alternanthera bettzickiana*. Physiologia Plantarum, 124, 260-266.

Weiss, D. (2000) Regulation of flower pigmentation and growth: Multip;le signaling pathways control anthocyanin aynthesis in expanding petals. Physiologia Plantarum, 110, pp. 152.

Weiss, D. and Halevy, A.H. (1989) Stamens and gibberellin in the regulation of corolla pigmentation and growth in *Petunia hybrida*. Planta, 179, 89-96.

Weiss, D. and Halevy, A.H. (1991) The role of light reaction in the regulation of anthocyanin synthesis in petunia corollas. Physiologia Plantarum, 81, 127-133.

Weiss, D., van Blokland, R., Kooter, J.M., Mol, J.N.M. and van Tunen, A.J. (1992) Gibberellic acid regulates chalcone synthase gene transcription in the corolla of *Petunia hybrida.* Plant Physiology, 98, 191-197.

Weiss, D., van der Huit, A.H., Kroon, J.T.M., Mol, J.N.M. and Kooter, J.M. (1993) The *Petunia* homologue of the *Antirrhinum candi* and *Zea mays A2* flavonoid genes; homology to flavanone-3-hydroxylase and ethylene forming enzyme. Plant Molecular Biology, 22, 893-897.

Weiss, D., van der Luit, A., Knegt, E., Vermeer, E., Mol, J.N.M. and Kooter, J.M. (1995) Identification of endogenous gibberellins in petunia flower, induction of anthocyanin biosynthetic gene expression and the antagonistic effect of abscisic acid. Plant Physiology, 107, 695-702.

Western, T.L., Burn, J., Tan, W.L., Skinner, D.J., Martin-McCaffrey, L., Moffatt, B.A. and Haughn, G.W. (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis.* Plant Physiology 127, 998-1011.

Wheldal;e, M. (1913) The flower pigments of *Antirrhinum majus.* 1. Method of preparation. Biochemical Journal, Cambridge, 7, 87-91.

Wheldale, M. (1910) Further observations upon the inheritance of flower-colour in antirrhinum majus. Report to the Evolution Committee. Royal Society of London, Rpt. V, 1-26.

Wheldale, M. and Bassett, H.Ll. (1914) The chemical interpretation of some mendelian factors for flower colour. Proceedings of Royal Society of London, LXXXVII B, 300-311.

Wienand, U., Sommer, H., Schwarz, Zs., Shepherd, N., Saedler, H., Kreuzaler, F., Ragg, H., Fautz, E., Hahlbrock, K., Harrison, B.J. and Peterson, P.A. (1982) A general method to

identify plant structural genes among genomic DNA clones using transposable element induced mutations. Molecular and General Genetics, 187, 195-201.

Winkel-Shirley, B. (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology, 126:485-493.

Wu, C., Li, X., Yuan, W., Chen, G., Kilian, A., Li, J., Xu, C., Li, X., Zhou, D., Wang, S. and Zhang Q. (2003) Development of enhancer trap lines for functional analysis of the rice genome. The Plant Journal, 35: 418-427.

Xu, C.R., Liu, C., Wang, Y.L., Li, L.C., Chen, W.Q., Xu, Z.H. and Bai, S.N. (2005) Histone acetylation affects expression of cellular patterning genes in the Arabidopsis root epidermis. Proceedings of the National Academy of Science USA, 102, 14469-14474.

Yang, Y., Li, R. and Qi, M. (2000) *In vivo* analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. The Plant Journal, 22, 543-551.

Yang, Y., Peng, H., Huang, H., Wu, J., Jia, S., Huang, D and Lu, T. (2004) Large-scale production of enhancer trapping lines for rice functional genomics. Plant Science, 167, 281-288.

Zieslin, N., Biran, I. and Halevy, A. H. (1974) The effect of growth regulators on the growth and pigmentation of 'Baccara' rose flowers. Plant Cell Physiology, 15, 341-349.

Appendix I

List of primers used

Primers for *Venosa* promoter deletions. All primers are 5'--3' direction and restriction enzyme sites are highlighted.

Forward primers for promoter deletions. Highlighted is SacI.

Y01 (-746 bp)   cga gag ctc gga gat ggg gta ttt aag ac

Y14 (-630 bp)   cgc gag ctc gta ttc caa tat atc att

Y15 (-500 bp)   cgc gag ctc tgg agg ttt ctt tgt taa

Y16 (-380 bp)   cgc gag ctc gga agc taa cca att agt

Y17 (-260 bp)   cgc gag ctc tgg tcg tta aat atg cac

Y18 (-150 bp)   cgc gag ctc caa gaa tca tta tta gta

Y32 (-120 bp)   this primer change mybcore and palindrome cagttaact into gctctaact.
                     cgc gag ctc taa cta gag att caa tta

Y33 (-100 bp)   this primer starts from 5 bp upstream TATA box.
                     cgc gag ctc tat tct tat ttc tcc aca

Y34 (-90 bp)   this primer starts from 6 bp downstream of TATA box.
                     cgc gag ctc aca atg tgc ata tat gta

Y39   3' end deletion 1 (from TATA box) (from 3' to 5' Kpnl)

ccg gta ccg cga ata tta att gaa tct cta

Y40   3' end deletion 2 (-19 bp relevant to TATA box) (from 3' to 5' KpnI)

ccg gta cct agt taa ctg cac ctt cct

Reverse primers for *venosa* promoter deletions. Highlighted is KpnI.

Y02 (0 bp)   ccg gta ccg ggt gtt cac ttg att tgc cta

5' - KpnI – *Rosea1* - SmaI - 3'

Y03   Forward (5' - KpnI – *Rosea1* - 3')

ctg gta cca tgg aaa aga att gtc gtg gag

Y04   Reverse (3' - SmaI – *Rosea1* - 5')

cta ccc ggg tta att tcc aat ttg ttg g

5' - KpnI - *mgfp* - SmaI - 3'

Y05   Forward (5' - KpnI - mgfp - 3')

gcc ggt acc aag gagata taa caa tg

Y06   Reverse (3' - SmaI - *mgfp* - 5')

gat ccc ggg tta aag ctc atc atg tt

Y07, Y08, OCS and K149 are for sequencing:

Y07   For pART7 SacI/MCS cassette forward (from 5' to 3') sequencing (upstream about 30 bp of T7 and upstream about 70 bp of SacI)

cac aca gga aac agc tat ga

Y08   For 5'-*mgfp* reverse (from 3' to 5') sequencing

gtg aca agt gtt ggc cat gg

OCS   For pART7 SacI / MCS cassette reverse (from 3' to 5') sequencing (sequence is omitted)

K149   For *Rose a1* 5' reverse sequencing (sequence is omitted)

Y25   forward primer for lGUS with KpnI embedded

cgc <u>ggt acc</u> atg tta cgt cct gta gaa acc cca

Y26   reverse primer for lGUS with SmaI embedded

<u>ggg</u> tca ttg ttt gcc tcc ctg ctg

omega oligo (KpnI / HindIII)

Y30   Omega positive strand:

cgt att ttt aca aca att acc aac aac aac aaa caa caa aca aca tta caa tta cta ttt aca att aca

Y41   Omega negative strand:

agc ttg taa ttg taa ata gta att gta atg ttg ttt gtt gtt tgt tgt tgt tgg taa ttg ttg taa aaa tac ggt ac

Y27   Forward primer lGUS with HindIII

g ccg <u>aag ctt</u> atg tta cgt cct gta gaa acc cca

Y28   Arabidopsis ubi promoter fragment upstream TATA box, forward primer. SacI is embedded

cta <u>gag ctc</u> aag ctt cgg att tgg agc caa gtc tca taa acg

Y29   Reverse primer. SacI is embedded

cgc gag ctc cca cat aac aaa aat tgg aga tca gat acg

Y44   genomic *Venosa* forward primer. Kpnl is embedded

gcc ggt acc atg gga aat aat cct ctt gga gta aga aaa ggc

Y45   genomic venosa reverse primer. Smal is embedded

ggg tta atc tac gta gtc cgc aaa atc gag caa ttc

Y46   pVe reverse primer with Xhol

tcg act cga ggg gtg ttc act tga ttt gcc ta

Primers for making *Venosa* RNAi construct

The vector pRNA69 is used. To get PCR product as Xbal- EcoRI---*Venosa*---Bcll-BamHI, the Y50 and Y53 primers are used. Sence *Venosa* is achieved by EcoRI / Bcll digest and antisence *Venosa* is achieved by Xbal / BamHI digest

Y53   Forward primer:

gct cta gag aat tca tgg gaa ata atc ctc ttg gag

Y50   Reverse primer:

cgg gat cct gat cat taa tct acg tag tcc gca aaa tc

Y54   pVe2.4 kb Sacl forward primer

cgc gag ctc cga aag aag aaa gtt taa agt tta ttt agt tat acc ac

Y55   middle GBV reverse primer, just behind the point different with GSV

cgt atg gct ctg gtg cca aat tga tag aga tcg gga

Y56   middle GSV reverse primer, just behind the point different with GBV

gca tgt cat cga cct cag aga tag aaa gta gtc aag

Sequencing primers for GBV and GSV

Y47　Sequencing for both GBV and GSV about forward 420bp relevant start codon
gaa agg tca ttt ttt agt agt tct tgc g

Y48　Sequencing for both GBV and GSV about reverse 600bp relevant stop codon
cca gaa acg gtc atc aac att ttc caa ag

Y51　The second forward primer for GBV about 900bp relevant start coden
ttg cag cac cgc cca agc tgt tc

Y52　The second reverse primer for GBV about 1200bp relevant stop coden
cat gaa ctt cct tca aga tca gca g

Y57　GBV forward primer, just before 1440 bp
gga act tag atc aca aga aag tga g

Y58　GBV reverse primer, just before 1440 bp
ctc act ttc ttg tga tct aag ttc c

Y59　GBV forward primer, just after 1920 bp
ctg ctg atc ttg aag gaa gtt cat g

Y60　GBV and GSV forward primer, the middle of 2580 bp
ctt tggaaa atg ttg atg acc gtt tct gg

Y61　GBV reverse primer just after 900 bp
gaa cag ctt ggg cgg tgc tgc aa

Y62　GBV and GSV reverse primer, in 420 bp

cgc aag aac tac taa aaa atg acc ttt c

Y63   GSV forward primer in 900 bp

aga gcc tct tac att gat ggt

Y64   GSV reverse primer in 900 bp

acc atc aat gta aga ggc tct

Y65   GSV forward primer behind 1320 bp

gag tca aag cta gca aca aaa tcc

Y66   GSV reverse primer behind 1320 bp

gga ttt tgt tgc tag ctt tga ctc

Appendix II

The hard copy of the nucleotide sequence of genomic *Venosa* allele 1 (GSV). Upper

case indicates exon; lowercase indicates intron; Start codon and stop codon are highlighted

in red. The sequence data is also supplied on the attached CD in text file and sequencher

format (*.SPF file).

```
ATGGGAAATAATCCTCTTGGAGTAAGAAAAGGCAC
ATGGACCAAAGAAGAAGATATTCTCTTGAAGCAAT  70
GCATAGAGAAGTATGGGGAAGGTAAGTGGCATCAA
GTTCCTATTAGAGCAGGtaatccttcttctccgga  140
Gatctaaatattatataataacattttttaaataa
atactttttttttttttttataaagtttggactt  210
ttaaagtagtcaaaagtatatatgtggtgttagca
aatgcttattagaagggttagttggcttttgcacc  280
tacataagcacatttttactgtttgttaacttttg
cttctgtttgtcttaaaaaaagttagaaaaagcac  350
aaataaaagaacatgtgcttttttcaactgcttttg
aaaatatggtattttaaatttttttttaaagaaag  420
gtcattttttagtagttcttgcgaacacctaatta
atactttttagtagcttttttaaaaagatttgcaaat  490
gctattaacttttttgtcaaaattcacttaaaatga
ctttaaaattagtaaattctcttgactactttcta  560
tctctgaggtcgatgacatgcaggGCTAAATAGGT
GCAGGAAGAGTTGCAGGATGAGATGGTTAAATTAT  630
CTAAGTCCCAATATTAAGAGAGGTTCCTTCACAAG
AGATGAAGTGGATCTCATTGTGAGGCTTCATAAGC  700
```

TTTTGGGGAACAGgtaagcttggagtcgccattat

tattgggattcaaatttataattcatacatatcca 770

agaattattttattaaattataaaagcttacaaat

tcaatttcgagtaataactagcaacaaaattccgt 840

gctacaaaaaaccaacttttcttcaacactaacag

ccaaactcatcaaaagagcctcttacattgatggt 910

taaattttaaagagatttgaagttctaataatata

accaaggcataaaagtactcaaatagtgtgtagaa 980

atttaatatatataaattcaaattttttaattcttt

tattgtatagttaagagataatgttatatttacca 1050

tatagtattttcaaccttttcagttttttggtcatc

gtttaattttgtttttgtgtcatatgatcacttta 1120

cattattatgtattatttattttataatattttttt

ttcttgttgggcagtattttcacttaatcttttta 1190

tcaagcataaagtattttttttttcatagtcttcca

aaaacaatcgtcgggtcaactttatgcagataaca 1260

gattgaatttctgaatacactcttattgcacgggt

tggagatttcttcaaccatttgaaaattcaatttt 1330

gagtcaaagctagcaacaaaatcctgtgctacaaa

caatcaaactcatcaaaagagttgctggttaaatt 1400

ttaaagagatttgaagttctaataatataaccaag

gaacaaaagtactcaaataatgtgttgaaaattta 1470

atctatataaattcatattttaaatcttttattg

tatagttaagagataatattatatttactatatag 1540

tattttcatcttttttcagttttttggtcatcattta

aatttgttttcgtgtcgtatcgtatcatcacttttt 1610

atattattatgaatttttatttatattatattt

ttttcttggttgggatataatttcacttattcttt 1680

ttatcaagcttaaagttctctttttcgtagtcttc

caagaacaatcatcgggcaactcaatgcatattga 1750

atttctgaatacacacctattgcacgggctggaga

tttcttttcagattaaattttcagccatttaaata 1820

tttagttaaattctttggaaaatgttgatgaccgt

ttctggccgagttaaaggctcggatcgatccgggg 1890

ttttgttaccgagtgttcgttacaccgaattctca

acaatctgtctaacttttttaccctctaaactgaa 1960

ttaaagagaacaaacaggactaattaagtgtgtaa

acaaccatgcggctaacatgtgcacgcttataaga 2030

agctaatgcatgcccacagataccacgaacattaa

ataattatcctgtctacttttgatgcagATGGTCG 2100

TTGATTGCCGGAAGACTTCCCGGAAGAACGGGTAA

CGATGTGAAAAACTTCTGGAATACCCACTTCGAGA 2170

AGAAGTCCGGAGAACGAGAGAATACGGAAAATATA

AACCCGAAACTCATCAACTCGAGCAATATAATAAA 2240

ACCCCAACCTCGTACCTTCTTGAAACTGCGTCCCA

AGGAAACAAAGAAACAGAAAAATATACGGAACGTT 2310

TGTACAGCAAATGATGACAAACAGCAGCCGTTGTC

CACGTCCGGACAGTTAGAAGAAGTGAATGAACGCA 2380

TTCGGTGGTGGAGTGAATTGCTCGATTTTGCGGAC

TACGTAGATTAA 2427

Appendix III

The hard copy of the nucleotide sequence of genomic *venosa* allele 2 (GBV). The start and stop codons are highlighted. The sequence data is also supplied on the attached CD in text file and sequencher format (*.SPF file). Its alignment with GSV is also supplied on the attached CD in the format with apr extension (*.apr file).

ATGGGAAATAATCCTCTTGGAGTAAGAAAAGGCAC

ATGGACCAAAGAAGAAGATATTCTCTTGAAGCAAT 70

GCATAGAGAAGTATGGGGAAGGTAAGTGGCATCAA

GTTCCTATTAGAGCAGGTAATCCTTCTTCTCCGGA 140

GATCTAAATATTATATAATAACATTTTTTAAATAA

ATACTTTTTTTTTTTTATAAAGTTTGGACTTAAGT 210

AGTCAAAACTCAAAAGTATATATGTGGTGTTAGCA

AATGCTTATTAGAAGGGTTAGTTGGCTTTTGCATC 280

TACATAAGCACATTTTTACTGTTTGTTAACTTTTG

CTTCTGTTTGTCATAAAAAAATGTAGAAAAAAGCA 350

CAAATCAAAGAACATGTGCTTTTTCAACTGCTTTT

GAAAATATGGTATTTTAATTTTTTTTTTTAAAAGA 420

AAGGTCATTTTTTAGTAGTTCTTGCGAACACCTAA

TTAATACTTTTAGTAGCTTTTTAAAAAGATTTGCA 490

AATGCTATTAACTTTTTGTCAAGATTCTCCAATCC

ATTTCTTCTAATTTCCCGATCTCTATCAATTTGGC 560

ACCAGAGCCATACGTATCCTTTATTTTCCGCTGCA

TTATTTTGTTTTACTTTACAGTTTTTTTGTGTTCA 630

ATTCGTTTTTTGAAAAAAAAAAAAAAAAATTTCAAA

AATCCAGAAAATACAGAAAATCTGCCGAGAAAAAA 700

AAAAAAAAAAAATCTGAAAACAAAGGAGCATGCGT

CCCACGCGCCGTCACCAAGCAACGGCAGAAGTCGC 770

AACACGCGACCTCCGTCTTCCAGATCCGGATCCGT

CGCTCCTCCGCAGCTCTCCACCGAATTCGTCGTCT 840

TCTAGATCCAGATCCGTCGCTCCTCTGCAGATCCA

CCACCATCGCCTATTCTCTCCGACGGTGACGCGCC 910

TTGCAGCACCGCCCAAGCTGTTCGCCGCCGTCACA

ACCCCTTGATCGCGAATTTTTTTTGCTGTTCCGCT 980

GCCACACCGCCGCACACTGCACTGCAGCCGCCACA

CCGCCGTATCAGTCACGTTCCAGTCGGGTCTCCGC 1050

CGCCGCTCTCATCTGCTTAGTCACCACCGACCGCC

ATCAACGCCTCACATACTGTTCTCCCGTATTCCTG 1120

CCGAGGAAAAAAATCGCCCAGCCTTGGGTTTGGGC

CGAGAAAAGGAAAAAAAAAAAGAATTGGGCCTTG 1190

TTCTCTTTGCATTTGGGCCGAAATTGGACAAGAAA

TGGGCCGCCAACATTCAAACTTGGGCTACTGAAAT 1260

TGTTTGGTAAAGTTATTCTTTTTTATTGACTTTTG

GACTTTAATTATTTGTGGGCAAGTTTAAACATCAT 1330

TATGCCTTGGTCACGTGCAATAGATCAATCATTTG

ATAGAATCCTCAGTTTACTGGACTTACTGAAAGAG 1400

GAATTTCTTAAGGAACTTAGATCACAAGAAAGTGA

GTTAGAAAATCTTGAGAATAGTTCCATACTTACGC 1470

ATGATCTTCAAAGCACTATACATTTATCTGAAAAT

AATACTTGTGAGATTACTGAAAATATTAAGAGTGA 1540

TGTCCCTGATTTTGATAGCACCCATGCGTTGCCTC

ACACTGAAGAACGCATTATGCTACCTTCTGTTCTA 1610

CCACCCACTTGTCCTCCTAAGCCTGACATGAGTAG

CAATCAAAATTTTCTATCAGCTACCCCTGGTGAGA 1680

ATTTGTTAGCTTCTAGAATTGCTTGTAAGCTGCAA

GTTAAATCTTGTCTATTATTGAGAGCACCATTTTT 1750

TGAAGTTAAGGATAGCTTGTTGGACTTGCCTCCAA

ATTTATTTGAGAGTCCTTACTTCAATAAGAAAGAT 1820

TTAACTTTTTATCAAATTATTATTGGGCATATTGA

ATTTTCTGCTAGTGGTTCAGGAGGTTTTGCAACTT 1890

CAAAGATTTCAGTGCATAAGCTACTTCCTATCCAA

CTGCTGATCTTGAAGGAAGTTCATGTTCGTTTCAA 1960

CACCAAAGATGGACAATTTCTATATTTGGTTCGAT

GGAAAGGTCGTCCAGATTTGGCTGATTTTGGAAGT 2030

TCGATTGTTGCATTCTCTCAATTTCAACACTTCTT

GACGGGTATCATTTTGCAAACTCTCCGGGGGCGAG 2100

TTTTTTCAAGCCGGGGAGAATGATGGAAATCAAGA

ATATTATTTTAGAATTATAGTTTAATTAGAGTTTA 2170

TTTAGATTATGAGTCTTATTTATTTCCTAGTTATT

TATTTCATAGTTATCTTGTTAAGAGTTTTAGTATG 2240

GTAGGTTTCTTTTTTAAAGCCTATAAATAAAGGGC

TAGAGTCTTATTGTTTAGTACGCTTTGATGAATAT 2310

GAATTATTGATTGTTGTTTGCAATTTACTCCCCTA

ATTTATGTCTTCTTTCCCCTAAATTATCTTCTTAT 2380

TCTTCTGAATTCTTCCTCAATTCCCTCTAAATTCT

GATTCTTCTATTCTAATTCTCCAATCCCTTTCTTC 2450

TAATTTCCCAATCTCTATCAGCAACTCAATGCATA

TTGAATTTATGAATACACACCTATTGCACGGGCTG 2520

```
GAGATTTCTTTTCAGATTAAATTTTCAGCCATTTA
AATATTTAGTTAAATTCTTTGGAAAATGTTGATGA  2590
CCGTTTCTGGCCGAGTTAAAGGCTCCGATCCGGGG
TTTTGTAACCGAGTGTTCGTTACACCGAATTCTCA  2660
ACAGTCTGTCTAACTTTTTTACCCTCTAAACTGAA
TTAAAGAGAACAAACAGGACTAATTAAGTGTGTAA  2730
ACAACCATGCGGCTAACATGTGCACGCTTATAAGA
AGCTAATGCCCACAGATACCACGAACATTAAATAA  2800
TTATCCTCTCTACTTTTGATGCAGATGGTCGTTGA
TTGCCGGAAGACTTCCCGGAAGAACGGGTAACGAT  2870
GTGAAAAACTTCTGGAATACCCACTTCGAGAAGAA
GTCTGGAGAACGAGAGAATACGGAAAATATAAACC  2940
CGAAACTCATCAACTCCAGCAATATAATAAAACCC
CAACCTCGTACCTTCTTGAAACTGCGTCCTAAGGG  3010
AACAAAGAAACAGAAAAATATACGGAACGTTTGTA
CAGCAAATGATGACAAACAGCAGCCGTTGTCCACG  3080
TCCGGACAGTTAGAAGAAGTGAATGAACGCATTCG
GTGGTGGAGTGAATTgCTCGATTTTGCGGACTACG  3150
TAGATTAA  3158
```

Appendix IV

The hard copy of the sequence of the possible transposon located in the central part of *venosa* allele 2 (GBV). The direct repeat at both ends is marked in red colour. Two non-matched nucleotides in this repeat are underlined. A longest ORF (open reading frame) is marked in green colour. The sequence data is also supplied on the attached CD in text file.

```
ATTCTCCAATCCATTTCTTCTAATTTCCCGATCT

CTATCAATTTGGCACCAGAGCCATACGTATCCTTT  70

ATTTTCCGCTGCATTATTTTGTTTTACTTTACAGT

TTTTTTGTGTTCAATTCGTTTTTTTGAAAAAAAAAA  140

AAAAAATTTCAAAAATCCAGAAAATACAGAAAATC

TGCCGAGAAAAAAAAAAAAAAAAAAAATCTGAAAACA  210

AAGGAGCATGCGTCCCACGCGCCGTCACCAAGCAA

CGGCAGAAGTCGCAACACGCGACCTCCGTCTTCCA  280

GATCCGGATCCGTCGCTCCTCCGCAGCTCTCCACC

GAATTCGTCGTCTTCTAGATCCAGATCCGTCGCTC  350

CTCTGCAGATCCACCACCATCGCCTATTCTCTCCG

ACGGTGACGCGCCTTGCAGCACCGCCCAAGCTGTT  420

CGCCGCCGTCACAACCCCTTGATCGCGAATTTTTT

TTGCTGTTCCGCTGCCACACCGCCGCACACTGCAC  490

TGCAGCCGCCACACCGCCGTATCAGTCACGTTCCA

GTCGGGTCTCCGCCGCCGCTCTCATCTGCTTAGTC  560

ACCACCGACCGCCATCAACGCCTCACATACTGTTC

TCCCGTATTCCTGCCGAGGAAAAAAATCGCCCAGC  630
```

CTTGGGTTTGGGCCGAGAAAAGGAAAAAAAAAAA

GAATTGGGCCTTGTTCTCTTTGCATTTGGGCCGAA 700

ATTGGACAAGAAATGGGCCGCCAACATTCAAACTT

GGGCTACTGAAATTGTTTGGTAAAGTTATTCTTTT 770

TTATTGACTTTTGGACTTTAATTATTTGTGGGCAA

GTTTAAACATCATTATGCCTTGGTCACGTGCAATA 840

GATCAATCATTTGATAGAATCCTCAGTTTACTGGA

CTTACTGAAAGAGGAATTTCTTAAGGAACTTAGAT 910

CACAAGAAAGTGAGTTAGAAAATCTTGAGAATAGT

TCCATACTTACGCATGATCTTCAAAGCACTATACA 980

TTTATCTGAAAATAATACTTGTGAGATTACTGAAA

ATATTAAGAGTGATGTCCCTGATTTTGATAGCACC 1050

CATGCGTTGCCTCACACTGAAGAACGCATTATGCT

ACCTTCTGTTCTACCACCCACTTGTCCTCCTAAGC 1120

CTGACATGAGTAGCAATCAAAATTTTCTATCAGCT

ACCCCTGGTGAGAATTTGTTAGCTTCTAGAATTGC 1190

TTGTAAGCTGCAAGTTAAATCTTGTCTATTATTGA

GAGCACCATTTTTTGAAGTTAAGGATAGCTTGTTG 1260

GACTTGCCTCCAAATTTATTTGAGAGTCCTTACTT

CAATAAGAAAGATTTAACTTTTTATCAAATTATTA 1330

TTGGGCATATTGAATTTTCTGCTAGTGGTTCAGGA

GGTTTTGCAACTTCAAAGATTTCAGTGCATAAGCT 1400

ACTTCCTATCCAACTGCTGATCTTGAAGGAAGTTC

ATGTTCGTTTCAACACCAAAGATGGACAATTTCTA 1470

TATTTGGTTCGATGGAAAGGTCGTCCAGATTTGGC

TGATTTTGGAAGTTCGATTGTTGCATTCTCTCAAT 1540

TTCAACACTTCTTGACGGGTATCATTTTGCAAACT
CTCCGGGGGCGAGTTTTTTCAAGCCGGGGAGAATG 1610
ATGGAAATCAAGAATATTATTTTAGAATTATAGTT
TAATTAGAGTTTATTTAGATTATGAGTCTTATTTA 1680
TTTCCTAGTTATTTATTTCATAGTTATCTTGTTAA
GAGTTTTAGTATGGTAGGTTTCTTTTTTAAAGCCT 1750
ATAAATAAAGGGCTAGAGTCTTATTGTTTAGTACG
CTTTGATGAATATGAATTATTGATTGTTGTTTGCA 1820
ATTTACTCCCTAATTTATGTCTTCTTTCCCCTAA
ATTATCTTCTTATTCTTCTGAATTCTTCCTCAATT 1890
CCCTCTAAATTCTGATTCTTCTATTCTAATTCTCC
AATCCCTTTCTTCTAATTTCCCAATCTCTATCA 1958

Appendix V

The sequence of 2.4 kb fragment upstream of ATG of *Venosa*. This sequence comes from Prof. Cathie Martin (UK). The sequence data is also supplied on the attached CD in text file.

GTTAACATCGAAAGAAGAAAGTTTAAAGTTTATTTAGTTATACCACAACGCCA

GTTAGATTAACATGATATAAACAAATCTGtTATATTCAACTTTGTACCATATTAG

CATCCCTGTAAAAATATTCATCACGTTTCTTTCTTCCTCAATGGCCTGGTGTGCA

GTACAATTAACATAGTTTCTATTGCAATTCAACATCTTTTGTAAAAGTAATTCA

GTGAGAAACAGAAAGTGTGCATTTTTAGACGGCTCTTGAAGTTATTCCAGGAA

CCATAAAATTAACCCATTACCATTGGTATTCTTACATATCATTTACTTCAAAGA

AATATATGTTGCGGAAGGTTTTTCATCACCAAAGTACATGAACTAATCAGACAC

ATTACAGGAAGCGAAACATTCACACAACTGCAAGGTTATTATGAGTTGAACAT

CATTATTAAACAACTAATACCATTAAACCTATAGTAAACATCCTCATTTTGGTA

GTTGGAACAAACCCAATGCCATCGAGTTTTCTGGGTTGTTGAATGAAATGCATG

GACATAATTTGAAGGATAAATCAGGAAGATGACAAAAATGGTAAGGGCATTGA

AGTAATTCATTAGAATTGGGTGTGCCATATTCAAATGCGTATTACGAATCCCGG

GGTCCTTGGGGAGATTGATTCAggAAAATATTCCCGGGTCACATGGATTGACCC

TGGAATCAATGGGAGTAACCATACGTGACATGGGCCCAGCTTAGCTCATATTA

TTTCCCATAATAAGGGCCTAATATGGGCAACCATACAAttcTTATGTACATGGGT

TCTTCAAGATCTATTACCAAGGAAGTTCATGAGTATTAGAAAACTTGAACTCCT

AATGAATTACTTTCTTGATGGATTGGATCTTTTTATCTCTTCAACTCCTTCTTGA

AATAGGATTTATCAAAGTATATATTATTTCCAAAATAGATTGGATCTAATGATC

TCTTTAAAGCCATTTTGGAATAAGTTTCTATCCAAATATATAGTTTTCAAAACG

TAGGTAATCTCGTAGAGAATGTCAATCACATTAGGAATACATCTTCCGTTGGAC

TCATAAAACCCAAATCCCACTTGTGCTGGGATTAATGGGCCTTCTCGGCAGGGG

TGTATGTACCTTAAGGCTGGGGAGGGAGCCTTTGGGCCCCCTTTATTAATACCT

TTTAAGTTTAATTTAGATTATTAATGCCTTTTAAGTTTAATTTAGΛΛGAAGGAA

AATATATGGCTCAAGTAAATATAAGATGAATTACATCTACATAAATATATTTTA

ACACTTTCTGATAAATTATCATGATACTTCAATTAGTTTATTGTCTTTCATAACC

ACTTCTTGTTAAATGATGTGCATTACTTGCTTTGTAAAAATTATTAGTTGATGGA

AACATAAAACTTTTACTAAGCAATTGATGTTACTTTATGTAAGATTACATTATT

TATATAGACTTTTTAATTTCTATTTTGTGATGGAATGCTAAGAAAATAAGGTAA

TATTTGTATATCGAGCATTTTAACACTTTGCTGACATTACTAACTTTTGTTGGCA

TTTAAACGGTGGGACTGGTCCCTGGTGCATGCAGCGACAAACTTCAGGGAATT

CATATTATTAATATCTTGGAGATGGGGTATTTAAGACAAATCTTAATTAAGGCG

TTACAAATTGTATCCCTAAATATTAAATGTTTCAGtATAATATATAAGCAAATTG

AGAAGAGGTCATGTATTCCAATATATCATTATTTATCTATACTTATGCAGCACG

ACTATTTAGTGCTTAAAAGGAGCCTACTGGATCCATCGACTTTCAGTTAAAACC

CGTCGTTACAAATCAATTATTTAACATAACTGCTAATGGAGGTTTCTTTGTTAA

TTAATATCTGTATAAAACAAGTAATAAATACAAACAAGTAAAAATCAGTATAA

AATGAACATAAACTTAgAACAACAATCAAAGATTGTGATCCGGCTGGAAGC

TAACCAATTAGTAAAGGAAATAAATAAACGATTTGCAGAAACGGGAAATGATA

GATTAAAAGAAATCATCGAGTATGTCTTCAATGGGGCATGATAGGTGTTGCAC

TGAATATAGATATTTCTACTTTATGGTCGTTAAATATGCACCGTCATAATCCAG

ATTGAGTGAATTGAAGGCAAAAAGATACGGAAATGGCATACATAAACTGGTGA

AGGCTTCCATATTCAAGAATCATTATTAGTAAAGGAAGGTGCAGTTAACTAGA

GATTCAATTAATATTCTTATTTCTCCACACAATGTGCATATATGTAACATAATAT

AGATGTAGGTTTTAAGCTTGCACAGAACGAAATAGAGCAATAGGCAAATCAAG

TGAACACCCATG

Appendix VI

Identification of potential *cis*-elements in *Venosa* promoter using the Web Signal Scan Program
Database searched: PLACE

This is the sequence you submitted
>../../tmp/sigscan//signalseq.7120 [Unknown form], 881 bases, 25700246 checksum.
GAAAATAAGGTAATATTTGTATATCGAGCATTTTAACACTTTGCTGACATTACT
AACTTTTGTTGGCATTTAAACGGTGGGACTGGTCCCTGGTGCATGCAGCGACAA
ACTTCAGGGAATTCATATTATTAATATCTTGGAGATGGGGTATTTAAGACAAAT
CTTAATTAAGGCGTTACAAATTGTATCCCTAAATATTAAATGTTTCAGtATAATA
TATAAGCAAATTGAGAAGAGGTCATGTATTCCAATATATCATTATTTATCTATA
CTTATGCAGCACGACTATTTAGTGCTTAAAAGGAGCCTACTGGATCCATCGACT
TTCAGTTAAAACCCGTCGTTACAAATCAATTATTTAACATAACTGCTAATGGAG
GTTTCTTTGTTAATTAATATCTGTATAAAACAAGTAATAAATACAAACAAGTAA
AAATCAGTATAAAATGAACATAAAACTTAgAACAACAATCAAAAGATTGTGAT
CCGGCTGGAAGCTAACCAATTAGTAAAGGAAATAAATAAACGATTTGCAGAAA
CGGGAAATGATAGATTAAAAGAAATCATCGAGTATGTCTTCAATGGGGCATGA
TAGGTGTTGCACTGAATATAGATATTTCTACTTTATGGTCGTTAAATATGCACC
GTCATAATCCAGATTGAGTGAATTGAAGGCAAAAAGATACGGAAATGGCATAC
ATAAACTGGTGAAGGCTTCCATATTCAAGAATCATTATTAGTAAAGGAAGGTG
CAGTTAACTAGAGATTCAATTAATATTCTTATTTCTCCACACAATGTGCATATA
TGTAACATAATATAGATGTAGGTTTTAAGCTTGCACAGAACGAAATAGAGCAA
TAGGCAAATCAAGTGAACACCC

RESULTS OF YOUR SIGNAL SCAN SEARCH REQUEST

../../tmp/sigscan//signalseqdone.7120: 881 base pairs
Signal database file: user.dat

Factor or Site Name	Loc.(Str.)		Signal Sequence	SITE #
GT1CONSENSUS	site	1 (+)	GRWAAW	S000198
TATABOX5	site	3 (-)	TTATTT	S000203
GT1CONSENSUS	site	9 (+)	GRWAAW	S000198
ROOTMOTIFTAPOX1	site	12 (-)	ATATT	S000098
ROOTMOTIFTAPOX1	site	13 (+)	ATATT	S000098
GATABOX	site	22 (-)	GATA	S000039
CACTFTPPCA1	site	37 (+)	YACT	S000449
TBOXATGAPB	site	38 (+)	ACTTTG	S000383
DOFCOREZM	site	39 (-)	AAAG	S000265
WRKY71OS	site	45 (+)	TGAC	S000447
CACTFTPPCA1	site	51 (+)	YACT	S000449

DOFCOREZM	site	57 (-) AAAG	S000265
RAV1AAT	site	61 (-) CAACA	S000314
RYREPEATLEGUMINBOX	site	93 (-) CATGCAY	S000100
RYREPEATBNNAPA	site	94 (-) CATGCA	S000264
RYREPEATBNNAPA	site	96 (+) CATGCA	S000264
ROOTMOTIFTAPOX1	site	123 (+) ATATT	S000098
POLASIG3	site	125 (-) AATAAT	S000088
TATABOX3	site	127 (+) TATTAAT	S000110
TATABOX3	site	128 (-) TATTAAT	S000110
ROOTMOTIFTAPOX1	site	131 (-) ATATT	S000098
GATABOX	site	133 (-) GATA	S000039
TATABOXOSPAL	site	149 (+) TATTTAA	S000400
ARR1AT	site	160 (-) NGATT	S000454
CARGCW8GAT	site	163 (+) CWWWWWWWWG	S000431
CARGCW8GAT	site	163 (-) CWWWWWWWWG	S000431
CAATBOX1	site	182 (-) CAAT	S000028
GATABOX	site	186 (-) GATA	S000039
MYBST1	site	186 (-) GGATA	S000180
ROOTMOTIFTAPOX1	site	194 (-) ATATT	S000098
ROOTMOTIFTAPOX1	site	195 (+) ATATT	S000098
GATABOX	site	211 (+) GATA	S000039
GT1CONSENSUS	site	211 (+) GRWAAW	S000198
IBOXCORE	site	211 (+) GATAA	S000199
ROOTMOTIFTAPOX1	site	214 (-) ATATT	S000098
TATABOX4	site	216 (+) TATATAA	S000111
INRNTPSADB	site	225 (-) YTCANTYY	S000395
CAATBOX1	site	227 (-) CAAT	S000028
NODCON2GM	site	233 (-) CTCTT	S000462
OSE2ROOTNODULE	site	233 (-) CTCTT	S000468
QELEMENTZMZM13	site	236 (+) AGGTCA	S000254
WBOXNTERF3	site	237 (-) TGACY	S000457
WRKY71OS	site	238 (-) TGAC	S000447
CCAATBOX1	site	248 (+) CCAAT	S000030
CAATBOX1	site	249 (+) CAAT	S000028
ROOTMOTIFTAPOX1	site	250 (-) ATATT	S000098
GATABOX	site	254 (-) GATA	S000039
POLASIG3	site	258 (-) AATAAT	S000088
TATABOX5	site	259 (+) TTATTT	S000203
GT1CONSENSUS	site	262 (-) GRWAAW	S000198
IBOXCORE	site	263 (-) GATAA	S000199
GATABOX	site	264 (-) GATA	S000039
CACTFTPPCA1	site	270 (+) YACT	S000449
CACTFTPPCA1	site	292 (-) YACT	S000449
PYRIMIDINEBOXOSRAMY1A	site	299 (-) CCTTTT	S000259
DOFCOREZM	site	300 (+) AAAG	S000265
CACTFTPPCA1	site	309 (+) YACT	S000449
DOFCOREZM	site	324 (-) AAAG	S000265

MYBCORE	site	328 (+) CNGTTR	S000176
MYB2AT	site	328 (-) TAACTG	S000177
MYB2CONSENSUSAT	site	328 (-) YAACKG	S000409
HEXAMERATH4	site	338 (+) CCGTCG	S000146
CGACGOSAMY3	site	339 (-) CGACG	S000205
ARR1AT	site	349 (-) NGATT	S000454
CAATBOX1	site	352 (+) CAAT	S000028
POLASIG3	site	354 (-) AATAAT	S000088
TATABOX5	site	355 (+) TTATTT	S000203
TATABOXOSPAL	site	356 (+) TATTTAA	S000400
MYB2AT	site	365 (+) TAACTG	S000177
MYB2CONSENSUSAT	site	365 (+) YAACKG	S000409
MYBCORE	site	365 (-) CNGTTR	S000176
POLLEN1LELAT52	site	381 (-) AGAAA	S000245
DOFCOREZM	site	384 (-) AAAG	S000265
AMYBOX1	site	385 (-) TAACARA	S000020
GAREAT	site	385 (-) TAACAAR	S000439
MYBGAHV	site	385 (-) TAACAAA	S000181
TATABOX3	site	392 (-) TATTAAT	S000110
ROOTMOTIFTAPOX1	site	395 (-) ATATT	S000098
GATABOX	site	397 (-) GATA	S000039
CACTFTPPCA1	site	412 (-) YACT	S000449
POLASIG1	site	415 (+) AATAAA	S000080
CACTFTPPCA1	site	429 (-) YACT	S000449
SEF4MOTIFGM7S	site	431 (-) RTTTTR	S000103
ARR1AT	site	435 (-) NGATT	S000454
CACTFTPPCA1	site	439 (-) YACT	S000449
INRNTPSADB	site	444 (-) YTCANTYY	S000395
RAV1AAT	site	466 (+) CAACA	S000314
CAATBOX1	site	469 (+) CAAT	S000028
ARR1AT	site	470 (-) NGATT	S000454
DOFCOREZM	site	475 (+) AAAG	S000265
NODCON1GM	site	475 (+) AAAGAT	S000461
OSE1ROOTNODULE	site	475 (+) AAAGAT	S000467
ARR1AT	site	477 (+) NGATT	S000454
CAATBOX1	site	479 (-) CAAT	S000028
GTGANTG10	site	482 (+) GTGA	S000378
MYBATRD22	site	498 (+) CTAACCA	S000175
MYB1AT	site	499 (+) WAACCA	S000408
REALPHALGLHCB21	site	500 (+) AACCAA	S000362
CCAATBOX1	site	502 (+) CCAAT	S000030
CAATBOX1	site	503 (+) CAAT	S000028
CACTFTPPCA1	site	508 (-) YACT	S000449
TAAAGSTKST1	site	510 (+) TAAAG	S000387
DOFCOREZM	site	511 (+) AAAG	S000265
GT1CONSENSUS	site	514 (+) GRWAAW	S000198
TATABOX5	site	516 (-) TTATTT	S000203

MARABOX1	site	517 (+) AATAAAYAAA	S000063
POLASIG1	site	517 (+) AATAAA	S000080
TATABOX5	site	520 (-) TTATTT	S000203
POLASIG1	site	521 (+) AATAAA	S000080
ARR1AT	site	527 (+) NGATT	S000454
POLLEN1LELAT52	site	535 (+) AGAAA	S000245
GT1CONSENSUS	site	542 (+) GRWAAW	S000198
GATABOX	site	548 (+) GATA	S000039
ARR1AT	site	551 (+) NGATT	S000454
DOFCOREZM	site	557 (+) AAAG	S000265
POLLEN1LELAT52	site	559 (+) AGAAA	S000245
ARR1AT	site	562 (-) NGATT	S000454
CACTFTPPCA1	site	570 (-) YACT	S000449
CAATBOX1	site	580 (+) CAAT	S000028
GATABOX	site	591 (+) GATA	S000039
RAV1AAT	site	597 (-) CAACA	S000314
CACTFTPPCA1	site	602 (+) YACT	S000449
ROOTMOTIFTAPOX1	site	607 (-) ATATT	S000098
GATABOX	site	613 (+) GATA	S000039
ROOTMOTIFTAPOX1	site	614 (+) ATATT	S000098
POLLEN1LELAT52	site	617 (-) AGAAA	S000245
CACTFTPPCA1	site	621 (+) YACT	S000449
NTBBF1ARROLB	site	622 (+) ACTTTA	S000273
DOFCOREZM	site	623 (-) AAAG	S000265
TAAAGSTKST1	site	623 (-) TAAAG	S000387
TATABOXOSPAL	site	634 (-) TATTTAA	S000400
ROOTMOTIFTAPOX1	site	637 (-) ATATT	S000098
ASF1MOTIFCAMV	site	646 (-) TGACG	S000024
WRKY71OS	site	647 (-) TGAC	S000447
ARR1AT	site	652 (-) NGATT	S000454
ARR1AT	site	657 (+) NGATT	S000454
INRNTPSADB	site	657 (-) YTCANTYY	S000395
CAATBOX1	site	659 (-) CAAT	S000028
CACTFTPPCA1	site	663 (-) YACT	S000449
GTGANTG10	site	664 (+) GTGA	S000378
INRNTPSADB	site	666 (-) YTCANTYY	S000395
CAATBOX1	site	668 (-) CAAT	S000028
DOFCOREZM	site	679 (+) AAAG	S000265
NODCON1GM	site	679 (+) AAAGAT	S000461
OSE1ROOTNODULE	site	679 (+) AAAGAT	S000467
GATABOX	site	682 (+) GATA	S000039
GT1CONSENSUS	site	687 (+) GRWAAW	S000198
GTGANTG10	site	708 (+) GTGA	S000378
ROOTMOTIFTAPOX1	site	720 (+) ATATT	S000098
ARR1AT	site	729 (-) NGATT	S000454
POLASIG3	site	733 (-) AATAAT	S000088
CPBCSPOR	site	735 (+) TATTAG	S000491

CACTFTPPCA1	site	739 (-) YACT	S000449
TAAAGSTKST1	site	741 (+) TAAAG	S000387
DOFCOREZM	site	742 (+) AAAG	S000265
MYBCORE	site	753 (+) CNGTTR	S000176
MYB2AT	site	753 (-) TAACTG	S000177
MYB2CONSENSUSAT	site	753 (-) YAACKG	S000409
ARR1AT	site	764 (+) NGATT	S000454
CAATBOX1	site	769 (+) CAAT	S000028
TATABOX3	site	771 (-) TATTAAT	S000110
ROOTMOTIFTAPOX1	site	774 (-) ATATT	S000098
ROOTMOTIFTAPOX1	site	775 (+) ATATT	S000098
-10PEHVPSBD	site	776 (+) TATTCT	S000392
TATABOX5	site	781 (+) TTATTT	S000203
POLLEN1LELAT52	site	784 (-) AGAAA	S000245
CAATBOX1	site	794 (+) CAAT	S000028
CARGCW8GAT	site	812 (+) CWWWWWWWWG	S000431
CARGCW8GAT	site	812 (-) CWWWWWWWWG	S000431
ROOTMOTIFTAPOX1	site	815 (-) ATATT	S000098
CAATBOX1	site	857 (+) CAAT	S000028
ARR1AT	site	866 (-) NGATT	S000454
EBOXBNNAPA	site	869 (+) CANNTG	S000144
MYCCONSENSUSAT	site	869 (+) CANNTG	S000407
EBOXBNNAPA	site	869 (-) CANNTG	S000144
MYCCONSENSUSAT	site	869 (-) CANNTG	S000407
CACTFTPPCA1	site	871 (-) YACT	S000449
GTGANTG10	site	872 (+) GTGA	S000378

Appendix VII   GUS staining results for the stable tobacco transgenics

independent line	GUS staining in floral organs	GUS staining in petal
pVE50		
1	+	+
2	+	+
3	+	+
4	−	−
5		
6	+	+
7	+	+
8		
9	−	
10	−	−
11		
12	−	+
13	−	
17	−	−
18	−	−
19	+	+
21	+	−
22		−
pVE51		
1	−	
2	+	+
3		
4	+	+
5	−	−
6	+	+
7	−	
8	+	+
9	+	
10	+	−
11	+	+
12	−	
13	+	+
14	+	+
15		
16	+	−
17	+	−

18	–	–
19	+	+
20	+	–
21	–	–
22	–	–
23	+	–
24	+	+
25	–	–
26	–	–
27	–	–
28	+	–
29	+	+
pVE54		
1	–	–
2	+	–
3	–	–
4	+	+
5	–	–
6	+	+
7	–	–
8	–	–
10	+	+
11	–	–
12	–	–
13	–	–
14	–	–
15	–	–
16	+	–
17	–	–
18	+	–
pVE55		
1	+	+
2	+	–
3	–	–
4	–	–
5	–	–
6	–	–
7	–	–
8	+	–
9	–	–
10	+	–
11	–	–
12	–	–
13	–	–

14		−	−
15		−	−
16		−	−
17		−	−
18		−	
19		+	−
20		−	
21		−	−
22		−	−
23		−	−
24		−	
25			
26		+	+
27		−	
pVE56			
1			
2		−	
3		−	−
4		−	−
5		−	−
6		−	−
7		−	
8		+	−
9		−	
10		−	
11		+	−
12		−	−
13		−	−
14		−	−
16		−	−
17		+	−
18		−	−
19		−	
20		−	
21		+	−
22		−	−
23			
24		+	−
25		−	−
27		−	−
28		−	−
29		−	
30		−	−
pVE58			

201

	1		
	2		
	3		
	4		
	5		
	6		
	7		
	8		
	9		
	10		
	11		
	12		
	13		
	14		
	15		
	16		
	17		
	18		
	19		
	20		
	21		
	22		
	23		
	24		
	25		
	26		
	27		
	28		
	29		
	30		
	31	+	
	32		
	33		
	34		
	35		
	36		
	37		
	38		
	39		
	40		
	41		
	42		
	43	+	
	44		

202

45		+	–
46		–	–
47		–	–
48			
49		–	–
50		–	
51		–	–
52		–	–
53			
54		–	–
55			
56		–	
57		–	–
58		–	–
59		–	
p27IGUS			
1			
2		–	–
3		+	+
4		+	+
5		+	+
6		+	+
7		+	+
8		+	+
9		+	+
-760ProVEN:GUS			
1	No resistance to kanamycin		
3	No resistance to kanamycin		
4		+	–
5		+	+
6		+	–
9		–	
10		–	–
12		+	
15		+	
16		+	+
-1.6kVEN:GUS			
1			
2		–	–
4		–	–
5		–	–
6			
8		–	–
9		–	

203

10		—
12		—
13		—
14		—
16		—
17		—
18		—
-2.4kVEN:GUS		
3		—
7		—
8		—
9	No resistance to kanamycin	
11		—
12		—
13		—
14		—
15		
16		—
19		—
20		—