Genetic Analysis of *At4CL* Gene Regulation and *AtMyb* subfamily 14 Functional Characterization in *Arabidopsis thaliana*

by

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ABSTRACT

Lignin is an important biopolymer that is developmentally deposited in secondary cell walls of specialized plant cells (e.g. tracheary elements and fiber cells) and also in response to stresses such as wounding. Lignin biosynthesis occurs via the phenylpropanoid pathway, in which the enzyme 4-coumarate:CoA ligase (4CL) plays a key role by catalyzing the formation of hydroxycinnamoyl-CoA esters. These esters are subsequently reduced to the corresponding monolignols. Four *At4CL* genes have been identified in *Arabidopsis thaliana* (*At4CL1-At4CL4*). *At4CL1* and *At4CL2* genes are developmentally up regulated and co-expressed with other genes involved in lignin biosynthesis. Also, they are co-expressed in response to stresses such as wounding. This co-expression is probably through the engagement of common regulatory elements and cognate transcription factors such as Mybs and their recognition sites. In this thesis, I undertook three projects with the goals to identify components of the signaling pathway(s) regulating developmental expression and wound responsiveness of the *At4CL* genes, to localize cis regulatory elements controlling developmental and wound responsiveness of *At4CL1* and *At4CL2* genes, and to investigate the functions of a subfamily of *Arabidopsis* Myb transcription factors.

First, *Arabidopsis* transgenic lines containing *At4CL1*::GUS or *At4CL2*::GUS transgenes were mutagenized in order to find and map At4CL signaling pathway mutants. Several lines with reproducible patterns of reduced GUS-expression were identified. However, the GUS-expression phenotype segregated in a non-Mendelian manner in all of the identified lines. Also, GUS expression was restored by 5-azacytidine treatment suggesting DNA methylation of the transgene. Southern analysis confirmed DNA methylation of the proximal promoter sequences of the transgene only in the mutant lines. In addition, retransformation of *At4CL::GUS* lines with further *At4CL* promoter constructs resulted in a comparable GUS-silencing phenotype with higher frequency. Taken together, these results suggest that the isolated mutants are epimutants. Apparently, two specific modes of silencing were engaged in *At4CL1::GUS* and *At4CL2::GUS* (trans)genes silencing. While silencing in the seedlings of the *At4CL1::GUS* line was root-specific, it was global in the *At4CL2::GUS* line. Also,

At4CL1::GUS transgene silencing was confined to the transgene but *At4CL2::GUS* silencing was extended to the endogenous *At4CL2* gene.

In the second project, we generated a series of transgenic *Arabidopsis* plants containing promoter fragments and parts of the transcribed region of the *At4CL2* gene fused to the *GUS* reporter gene, in order to localize *cis* regulatory elements which are involved in developmental and wound responsiveness of this gene. We found that positive and negative regulatory elements effective in modulating developmental expression or wound responsiveness of the gene are located both in the promoter and transcribed regions of the *At4CL2* gene. Also, histochemical GUS assays and molecular studies indicated a biphasic wounding response of the *At4CL2* gene, attributing early or late response to distinct *cis*-regulatory elements involved in the response, suggesting that different signaling pathways may be involved in these different responses.

In the third project, I initiated strategies to knock down/out multiple members of *AtMyb* subfamily #14 genes in *Arabidopsis* in an attempt to find phenotypes related to loss of function of these genes, since functional redundancy within the subfamily appears to have hampered previous studies. Single *AtMyb* gene knock down or knock out lines did not reveal any mutant phenotypes but RNAi generated *AtMyb84* knockdown lines in the *AtMyb68* knock out background showed small rosettes and a delay in shoot development.

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LIST OF ABBREVIATIONS

ACC synthase	Aminocyclopropane-1-carboxylate synthase
Ago4	*Argonaute4*
AP2	APETALA2
ARF	Auxin response factors
At	Arabidopsis thaliana
AtPTR3	peptide transporter
bHLH	helix-loop-helix
BCAT5	branched-chain amino acid transaminase 5
CAD	Cinnamyl alcohol dehydrogenase
CaMV3	Cauliflower mosaic virus
CCOMT	Caffeoyl-CoA 3-O-methyltransferase
C4H	Cinnamate 4-hydroxylase
CHS	*Chalcone synthase*
CMT3-DMTase	Chromo-domain containing methyltransferase)
Col	Arabidopsis Columbia ecotype
DCE	1,2-dicholoroethane
Dcl3	*Dicer-like3*
DDM1	Deficient in DNA Methylation 1
*dhl*A	Dehalogenase A
DMT	DNA methyltransferase
DRM-DMTases	Domains Rearranged Methyltransferases
dsRNA	Double stranded RNA
EMS	Ethyl-Methyl- Sulfonate
EREBP	Ethylene responsive binding element protein
ERF1	*Ethylene Response Factor1*
5-aza	5-azzacytidine
4CL	4-coumarate::CoA ligase
5mC	Cytosine methylation
GFP	Green Florescence Protein

GUS	B-glucuronidase
HDGS	homology dependent gene silencing
HB	Homeobox
JAs	Jasmonic acids
Kan	Kanamycin
Kb,	Kilo base
LB	Luria Bertani Broth
Ler	Landsberg *erecta Arabidopsis* ecotype
LWRE	Late Wound Response Element
MAPK	Mitogen Activated Protein Kinase
MET1-DMTs	DNA Methylase Met1
MOM1	*Morpheus' Molecule 1*
MOPS	3-(N-Morpholino)propanesulfonic acid
MPSS	Massive Parallel Signature Sequencing
MS	Murashige and Skoog
NAPS	Nucleic acid and protein Service Unit at UBC
NPT	Neomycin Phosphotransferase
ORF	Open Reading Frame
PAL	Phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PLACE	Plant cis-acting regulatory DNA elements
PTGS	Post transcriptional gene silencing
RdDM	RNA directed DNA Methylation
RdRp	RNA Dependent RNA Polymerase
rdr2	RNA-dependent RNA polymerase2
RNAi	RNA interference
RT	Reverse transcription
SAIL collection	Syngenta *Arabidopsis* Insertion Library
SDS	Sodium Dodecyl Sulfate
siRNAs	Short inhibitory RNAs
STP4	Sugar transporter

SSC	Standard Saline Citrate Buffer
SUP	*SUPERMAN* gene
TBE	Tris, Boric Acid, EDTA
T-DNA	transferred -DNA
TGS	Transcriptional gene silencing
3LE	4CL3 like element
UTR	Un-translated Region
WPK1	Phytochrome-Regulated kinase1
X-gluc	5-bromo-4-chloro-3-indolyl-B-D-glucuronide
Zm	ZEA mays

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CHAPTER 1
GENERAL INTRODUCTION

1.1 PLANT SIGNAL TRANSDUCTION

Plants respond to a large number of developmental and environmental signals through changes in gene expression. This requires that signals reach the nucleus in order to regulate the expression of target genes. First, perception of the signal by an appropriate receptor molecule is necessary and then the signal will be transduced to the nucleus via intermediates, often targeting transcription factors. The cell membrane separates the protoplasm of the cell from its surrounding environment and presents a barrier to many signals that may alter gene expression. Only small lipophilic molecules such as steroid hormones are able to diffuse into the cytoplasm but the cell membrane is impermeable to large water-soluble molecules. Therefore, responses of the cell to extra-cellular hydrophilic signaling molecules (ligands) is often through the specific interaction of these ligands and the extra-cellular domain of plasma membrane receptor proteins. Receptors may physically transport the ligand inside the cell or binding of the ligands may convert the receptor from an inactive to an active form or vice versa and activate or inactivate its cytosolic domain. The process, from signal perception to target gene activation, is called signal transduction (Lewin 2004).

After signal perception by an extra-cellular or intracellular receptor, the signal may be propagated through a number of different signal transduction pathways. A common pathway relies on the activation of serial protein kinases of the Mitogen Activated Protein Kinase (MAPK) class (Buchanan *et al.*, 2000). The signal leads to the activation of effectors, some of which may act in the cytosol (for example to affect the cytoskeleton), and some may carry the signal into the nucleus and affect activity of the transcription factors. Ultimately, a given transcription factor interacts with a specific DNA sequence in the promoter of target gene and increases or decreases the expression level of the target gene (Lewin 2004).

To elucidate the molecular mechanisms underlying several signaling pathways in plants, researchers have utilized the genetically facile plant *Arabidopsis thaliana* to isolate mutants that confer altered responses to various stimuli. An example of a well - characterized signal transduction pathway in *Arabidopsis* analyzed through mutant

analysis is the ethylene-signaling pathway. Ethylene is a gaseous plant hormone that affects many developmental and stress processes such as germination, senescence, fruit ripening, and pathogen response (Bleecker 2000). Ethylene (the ligand) interacts with a specific membrane-associated receptor resulting in activation of ethylene responses by inhibiting a negative regulator of the response. In the absence of ethylene this receptor is functionally active and constitutively activates a serine/threonine (Ser/Thr) kinase, which in turn is a negative regulator of ethylene responses (Wolanin *et al.*, 2002: Potuschak *et al.*, 2003). Downstream of this negative regulatory kinase are several positive regulators of ethylene responses, which are not all fully characterized. One of these positive regulators is a transcription factor that controls the expression of its immediate target genes such as *Ethylene Response Factor1* (*ERF1*) (Solano *et al.*, 1998). ERF1 is itself a transcription factor that binds to a GCC-box present in the promoters of many ethylene-inducible, defense-related genes (Guo *et al.*, 2004). Thus, in the presence of ethylene, a signal transduction pathway is activated that relies on the repression of the negative regulator of the pathway, allowing ethylene induced gene expression to occur.

1.2 PLANT TRANSCRIPTION FACTORS

The RNA polymerase II complex alone is only able to catalyze transcription at a very low (basal) level. Transcription at higher rates requires that other proteins such as transcription factors bind to the *cis*-regulatory elements in the DNA around the gene. Plant transcription factors are modular proteins typically composed of DNA binding domain and effector domains that regulate the frequency of transcription of target gene(s) (Pabo *et al.*, 1992: Liu *et al.*, 1999). In eukaryotes, multi-protein complexes that mediate gene expression are commonly formed through the combinatorial action of transcription factors and co-activators. Complexes are bound to the conserved promoter elements in precise spatial orientations and on the basis of both specific protein-DNA and protein-protein interactions (Griffiths *et al.*, 2004). A fraction of all genes in the sequenced eukaryotic genomes encode transcription factors. According to the sequence of their DNA binding domain, the majority of transcription factors can be assigned to specific families (Pabo *et al.*, 1992, Ülker *et al.*, 2004) (Table 1-1).

Table 1.1 Sizes of major *Arabidopsis* transcription factor gene families in comparison to other species (according to Riechmann *et al.*, 2000)

	Predicted Gene Number			
Gene Family	*Arabidopsis*	*Drosophila*	*C. elegans*	*S.cerevisiae*
Myb	~130	35	16	19
AP2/EREBP	150	0	0	0
NAC	105	0	0	0
bHLH/MYC	100	61	38	8
bZIP	100	24	18	15
HD	90	113	88	10
Z-C_2H_2	85	352	138	47
MADS	80	2	2	4
WRKY	75	0	0	0
ARF	42	0	0	0
Dof	41	0	0	0

More than 1,600 putative transcription factor genes have been identified in the *Arabidopsis thaliana* genome. This represents about 6% of the total ~26,000 *Arabidopsis* genes (Riechmann *et al.*, 2000). Some of the transcription factor families (e.g Mybs, bZIPs and Homeodomain) are found in plants, animals and fungi. Transcription factor families such as ARF (auxin response factors) are plant specific (Rubin *et al.*, 2000).

1.2.1 Plant Myb transcription factors

The first Myb transcription factor gene was identified as a chicken oncogene derived from the avian myeloblastoma virus and was called *v-Myb* (Klempnauer *et al.*, 1982). *V-Myb* is a truncated version of *c-Myb*, which is well-conserved gene in all vertebrates examined to date (Weston *et al.*, 1998). The plant Myb transcription factors were first discovered by similarity of their DNA binding domain to v-Myb. The presence of a Myb binding domain is the common feature of all Myb proteins that is conserved amongst animals, plants and yeasts (Lipsick *et al.*, 1996). The Myb domain typically consist of one to three imperfect repeats called R1, R2 and R3. However, R2 and R3 repeats alone are necessary and sufficient for sequence-specific DNA binding (Howe *et al.*, 1990; Saikumar *et al.*, 1990; Gabrielsen *et al.*, 1991). Each repeat is about 53 amino acids long and contains three helices which form a helix helix-turn-helix structure with the second and third helix forming the helix-turn-helix (HTH) DNA-binding structure (Frampton *et al.*, 1991). Although Myb proteins are common to all the characterized eukaryotes, in higher plants this protein family is extraordinarily amplified. In contrast to animals, most plant *Myb* genes belong to the *R2R3-Myb* category and about 126 putative R2R3-type *Myb* genes in the *Arabidopsis* genome have been identified, which are classified in 22 phylogenetic subfamilies (Stracke *et al.*, 2001).

Plant Mybs are involved in a variety of cellular processes such as phenylpropanoid (Sablowski *et al.*, 1994) or tryptophan biosynthesis (Stracke *et al.*, 2001) and regulation of the phosphate starvation response (Rubio *et al.*, 2001). Also they are involved in control of cell fate determination, regulation of the cell cycle and circadian clock-regulated gene expression (Meissner *et al.*, 1999; Martin *et al.*, 1997: Jin *et al.*, 1999: Zimmermann *et al.*, 2004). There are also reports of Mybs that function with other transcription factors such as BHLH and BZIP by protein-protein interaction in controlling flavonoid biosynthesis (Hartmann *et al.*, 2005). Thus, Mybs form a relatively large family of plant transcription factors with potential roles for controlling gene expression, some of which have been shown to be important in regulating the phenylpropanoid biosynthetic pathway (Jin *et al.*, 2000).

1.2.2 Methods of studying plant transcription factors

After sequencing of the *Arabidopsis thaliana* genome, a variety of reverse genetic approaches have been used to analyze the function of genes with unknown specific functions. Creation of large populations of mutants by either T-DNA insertion or transposon tagging is a well-known method (Maes *et al.*, 1999; Walbot *et al.*, 2000; Krysan *et al.*, 1999). Originally, these populations were used to screen for insertion mutations in a particular gene using a PCR-based strategy with a gene specific primer in combination with T-DNA (or transposon) border primers. Today, transposon and T-DNA collections such as the collections generated at the Salk Institution (http://signal.salk.edu/tabout.html) are compiled in databases with bordering plant DNA sequences, which provide individual mutant lines with T-DNA insertions in genes of interest to researchers.

RNA interference (RNAi) naturally occurs in almost all the eukaryotes examined to date, including plants in which its biochemistry has been characterized (Tang *et al.*, 2003; Matthew 2004). RNAi in plants has multiple consequences such as transcriptional and post-transcriptional gene silencing through RNA degradation and/or DNA methylation respectively (Bender 2004). RNAi has been frequently used as an alternative method to generate loss-of-function mutants for specific plant genes (Abbott *et al.*, 2002; Zamore *et al.*, 2002; Tomari *et al.*, 2005). For example, RNAi has been used to down regulate the tomato *Blind* gene that encodes a Myb transcription factor. In the *blind* mutant of tomato, initiation of lateral meristems during the shoot and inflorescence development is blocked leading to reduction of the number of lateral axes, which is manifested in reduction of shoot and inflorescence branching. Using positional cloning, the *Blind* gene was isolated and it was shown that encodes an R2R3 class *Myb* gene. RNAi induced *blind* phenocopies confirmed the identity of the isolated gene. In this experiment 17 out of 19 independent RNAi transgenic plants showed reduction in the number of lateral shoots and the number of flower per inflorescence (Schmitz *et al.*, 2002).

In order to generate RNAi silenced lines, a DNA construct containing complementary 150-500 bp sense and antisense inserts which are separated by an artificial intron is

typically made, and introduced into the plant genome (Helliwell *et al.*, 2003; Mattew *et al.*, 2004). Transcription of this construct results in self-complementary double stranded RNA (dsRNA). Presence of the dsRNA activates the RNAi silencing system in which dsRNA is recognized and specifically cleaved by the Dicer enzyme to give small siRNAs of 21–26 nucleotides. These small siRNAs then generate short single stranded RNAs that hybridize to the target RNAs or DNA by sequence complementarity and direct a protein complex to cleave target mRNAs or methylate DNA (Bender 2004; Gendrel *et al.*, 2005). For some genes without inverted sequences it is accepted that increased transcript copy number may trigger dsRNA formation through the function of known RNA dependent RNA polymerases and activate RNAi machinery (Shubert *et al.*, 2004).

RNAi transformants usually exhibit mutant phenotypes at a much higher frequency than the plants transformed with either the antisense or the sense gene alone (Chuang *et al.*, 2000). However, as with the use of other loss-of-function approaches, a gene that shares function with related genes is difficult to functionally characterize unless potential redundant genes are knocked -out or -down at the same time (Zhang 2003). Overall, interruption of gene expression by RNAi has been often utilized in plant genetics. This method provides the flexibility necessary for the characterization of genes of diverse function and complements the T-DNA insertion reverse genetics approach.

1.3 EPIGENETIC CONTROL OF GENE EXPRESSION

The process of transcription is affected by a number of different factors including pre-initiation and RNA polymerase II complex formation, transcription factor binding and by factors required for chromatin remodeling (Griffiths *et al.*, 2004). As a result of histone modification and/or DNA methylation chromatin structure may change and result in gene silencing (Fagard *et al.*, 2000). Also, in many organisms including plants, different kinds of RNAs are involved in chromatin alteration and gene silencing (Gendrel *et al.*, 2005; Bender 2004). The study of chromatin change and its effect on inheritance is generally equated with epigenetics (Henikoff *et al.*, 2004; Gendrel *et al.*, 2005), which is defined as the study of mitotically and/or meiotically heritable changes of gene expression without any change of DNA sequence (Wu *et al.*, 2001). Also, epigenetics is defined as the memory of transcriptional activity, which is regulated through the binding of specific

chromosomal proteins and the covalent modification of chromatin (Lippman *et al.*, 2005).

Heterochromatin is the condensed area of chromatin and is generally considered as transcriptionally inactive or silenced, whereas euchromatin represents the transcriptionally active form of chromatin. As well as DNA methylation, a variety of post-translational modifications of histones such as acetylation, phosphorylation and ubiquitylation have been reported during chromatin modification. However, only methylation of DNA has been reported so far to directly result in silencing (Jenuwein *et al.*, 2001). The level of DNA methylation, diverse covalent modifications of histones, histone variants and specific associations with non-histone proteins are different between hetero- and euchromatin (Richards *et al.*, 2002). The functional relationship between plant DNA-methylation and histone modifications that lead to chromatin compaction and gene silencing is still under investigation (Tariq *et al.*, 2004). There are also reports indicating that RNAi silencing machinery is involved in chromatin remodeling. Three pathways of RNAi silencing have been considered in recent reports; two pathways target mRNAs for either degradation or translational repression and the third pathway is chromatin-based in several organisms including *Arabidopsis*, ending in DNA methylation and histone modifications (Lippman 2004, Gendrel 2005). In summary, in many organisms heterochromatin is silenced by conserved mechanisms of epigenetic modification of histones and DNA. This epigenetic silencing, as well as higher order of packing of chromatin into heterochromatin is believed to prevent illegitimate and harmful recombination and transposition and deleterious over-expression of genes (Lippman 2004).

1.3.1 DNA methylation in plants

DNA methylation is widespread among plants and vertebrates and is widely considered as the mechanism for defending genomes against selfish DNA sequences such as transposable elements and retroviruses (Bird 2002; Martienssen *et al.*, 2001; Chan *et al.*, 2005). It has been shown that transposons are mobilized as a result of reduction in the level of DNA methylation in *Arabidopsis* genome (Miura *et al.*, 2001). Plants also use DNA methylation to modulate the expression of repeated gene families (Lawrence *et al.*, 2004). DNA methylation regulates gene expression through inhibition of transcription

initiation or arresting transcription elongation, acts as an imprinting signal and suppresses recombination between homologous DNA molecules (Colot *et al.*, 1999; Chan *et al.*, 2005).

Cytosine methylation (5mC) is always present in the transcriptionally silent chromatin in plants (Bird 2002). Cytosine methylation in symmetrical CG dinucleotide sites is an evolutionarily conserved DNA modification in vertebrates, plants and some fungi (Bird 2002; Finnegan *et al.*, 2000). As well as preferred symmetrical CG sites (Bender 2004), plants show significant levels of cytosine methylation in non-CG sequences, which include symmetrical CNG and asymmetric CNN sequences (where N = A, T or G) (Finnegan *et al.*, 1994; Gendrel *et al.*, 2005; Chan *et al.*, 2005). DNA methyltransferase (DMT) enzymes are responsible for DNA methylation and are conserved among protists, plants, fungi and animals (Colot *et al.*, 1999). In the *Arabidopsis* genome at least 10 of these genes have been found and some of them are conserved with animal genes (Gendrel 2005). These genes are categorized into three types of DMTases, two of which are plant specific (Bender 2004). The plant specific DRM-DMTases (Domains Rearranged Methyltransferases) family are involved in methylation of asymmetric cytosines. The plant specific CMT3-DMTase (Chromo-domain containing methyltransferase) maintains methylation of CNG cytosines, and MET1-DMTase is related to its mammalian counterpart and maintains methylation of CG sites (Wada *et al.*, 2003; Cao *et al.*, 2002; Cao *et al.*, 2003).

Many of these DMTases are engaged in RNA directed DNA Methylation (RdDM) (Bender 2004; Gendrel *et al.*, 2005). In this RNAi system, dsRNA, from different sources such as viroids, cytoplasmic viruses, transcribed inverted repeats, and overabundant or aberrant RNAs from genes or transgenes will be cleaved into 21-26 nucleotide small interfering RNAs (siRNA). These siRNA molecules may guide DNA methylation to homologous sequences. In other words, DNA methylation is one of several downstream mechanisms that siRNA can use to down-regulate gene expression during transcriptional gene silencing (Chan *et al.*, 2005; Bender 2004; Gendrel *et al.*, 2005).

In some cases of transposon and transgene silencing in plants, a connection between RNAi and DNA plus histone methylation has been established based on several kinds of evidence. First, it was reported that RNA-induced silencing in plants often

results in DNA methylation (Wasseneger *et al.*, 1994). Then, in *Arabidopsis* and tobacco, it was shown that dsRNAs targeting the promoter of transgenes induce DNA methylation and transcriptional silencing (Mette *et al.*, 2000). Finally, it has been shown that, members of conserved gene families such as *Argonaute4* (ago4), *dicer-like3* (dcl3), and RNA-dependent RNA polymerase2 (rdr2), are responsible for the initiation of silencing on the transcriptional level in plants (Zilberman *et al.*, 2003; Chan *et al.*, 2004; Xie *et al.*, 2004).

In conclusion, different cytosine residues in the DNA are methylated by different DMTases. Also, the RNAi system apparently specifies methylation of certain parts of the genome by sequence complementarity and engaging/guiding DMTases.

1.3.2 (Trans) gene silencing in plants

It seems that there is a common theme for the silencing of developmental genes, viroids and viral DNAs, transgenes and transposons. Silencing of developmental genes plays an important role during development. For example the *SUPERMAN* (*SUP*) gene of *Arabidopsis* encodes a transcription factor and is expressed during flower development (Sakai *et al.*, 2000). Epigenetic mutant alleles of *SUP* have been described, in which the *SUP* transcribed region becomes heavily methylated (Ito *et al.*, 2003). The flower of wild type *Arabidopsis* contains six stamens (male reproductive organs) and two fused central carpels (female reproductive structure). Mutations in the *SUPERMAN* gene (*sup-5* mutants for example) increase the number of stamens to 12 and carpels to 3 on average (Jacobsen *et al.*, 1997: Schultz *et al.*, 1991; Bowman *et al.*, 1992). Several heritable but unstable *sup* epi-alleles have also been recognized in which the level of *SUP* RNA is reduced. In these epimutants, cytosines in a specific area within the *SUP* gene are hyper methylated. Epimutants show a similar phenotype to, but weaker than, that of sup mutants with an average of 8 stamens and 3 carpels. Demethylation of the *SUP* gene is associated with the reversion of epialleles and restores the levels of *SUP* RNA (Jacobsen *et al.*, 1997).

Silencing of transposons is important for the protection of genome integrity. For example, the sequence of maize (*Zea mays*) is over 30 times of the size of *Arabidopsis*, and 83% of it consists of transposable elements. The much smaller *Arabidopsis* genome

has a limited number of transposable elements (Bevan *et al.,* 1998). To control the activity of transposable elements, *Arabidopsis* and maize have silenced transcription of the majority of these elements via methylation (Martienssen 1998). Plants also use gene silencing as a mechanism to defend their genomes against viruses (Ratcliff *et al.,* 1997, Covey *et al.,* 1997). It is known that plant recovery from viral disease involves viral RNA silencing through viral dsRNA degradation (Baulcombe 2004).

Often, the expression level of the same transgene construct varies in between independent lines, and this variation can sometimes be attributed to gene silencing, especially, when the transgene has been active for a few generations and then is epigenetically inactivated (Fagard 2000). This suggests that transgenes can be perceived as plant genome invaders and that plant defensive methylation systems are activated in response to the introduction of transgenes (Matzke *et al.,* 1998).

Two possible molecular mechanisms have been considered for gene and transgene silencing: Transcriptional gene silencing (TGS) which blocks transcription of the (trans) gene and post transcriptional gene silencing (PTGS) which involves degradation of the RNA product of the (trans) gene (De Carvalho *et al.,* 1992; Meyer *et al.,* 1993; Fagard 2000). PTGS, which is initiated during the development, may result in systemic silencing of the transgene but after meiosis, it is reset. TGS on the other hand, is both meiotically and mitotically heritable although, reversion of transgene expression is possible (Schubert *et al.,* 2004). In *Arabidopsis*, TGS is mainly correlated with the methylation of the promoter (Kumpatla *et al.,* 1997; Chandler *et al.,* 2001) whereas in PTGS, mainly coding sequences are methylated (Vaucheret 1998; Stam *et al.,* 1998; Chandler *et al.,* 2001; Fagard 2000; Schubert *et al.,* 2004).

It is not clear whether methylation is a cause or a consequence of silencing (Fagard *et al.,* 2000). Results gathered from the impairment of two nuclear proteins, which are involved in TGS suggests that TGS could operate in methylation-dependent or – independent pathways (Chandler *et al.,* 2001). Impairment of DDM1, a chromatin remodeling protein, results in the release of both TGS and methylation of transgenes and silent retrotransposons (Jeddeloh *et al.,* 1999), while the impairment of another novel protein called MOM1 only releases TGS but not the methylation of transgenes (Amedeo *et al.,* 2000).

Researchers have suggested several models to explain TGS and PTGS. In one model, TGS occurs when there are identical sequences to the newly introduced promoter of the transgene in the genome, while PTGS occurs when the transcribed region of the transgene shows sequence identity with another sequence in the genome (Dudley 2003). In a favored RNA–mediated silencing model, the common theme is that silencing is triggered by the formation of dsRNA molecules. If dsRNA is homologous to the promoter sequence of the transgene, TGS may occur and if it is homologous to the transcribed region, PTGS may occur (Chandler *et al.,* 2001). In support of this model, siRNA molecules matched with transcribed region of the transgene have been found which are considered as hallmark of PTGS (Hamilton *et al.,* 1999). Also, discovery of siRNA specific for the promoter sequence of the transgene correlates TGS with RNAi systems (Mette *et al.,* 2000; Sijen *et al.,* 2001). Double stranded RNAs, which are required in RNAi based silencing system, could be formed through the transcription from the inverted repeat sequences (Waterhouse *et al.,* 1998) or through the function of RNA Dependent RNA Polymerase (RdRp) enzymes on aberrant or over abundant RNAs (Gendrel *et al.,* 2005). DsRNA molecules in turn, trigger the RNAi system and finally methylation of the transgene (Chandler *et al.,* 2001; Gendrel *et al.,* 2005).

If TGS only affects the transgene itself and not the unlinked homologous endogenous sequences, it is called *cis*-silencing and if silencing affects both transgene and homologous DNA sequences simultaneously, it is called *cis* and *trans* silencing. Also, if TGS affects the homologous DNA but not the transgene it is called *trans* silencing (Fagard *et al.,* 2000).

1.3.3 Factors leading to TGS

Several factors are known to be involved in TGS in eukaryotes. The expression of an active gene in the euchromatin may be affected by the presence of heterochromatin in the vicinity. This is manifested as mosaic or variegated expression of an active gene as seen in *Drosophila melanogaster* (Wakimoto 1998). In plants it has been proposed that the variability of the level of transgene expression among the transformants could be attributed to the integration position of transgenes in different lines (Jones *et al.,* 1985; Peach and Velten 1991; Day *et al.,* 2000). That means that proximity of the transgene to

heterochromatin (Pr¨ols *et al.,* 1992) or methylated repetitive elements (Lohuis M, *et al.,* 1995) may lead to transgene silencing simply by the position of integration. Also, integration of multiple copies of a transgene in a particular spatial arrangement may lead to methylation and TGS (Davies *et al.,* 1997; Mittelsten *et al.,* 1998). Based on these reports, a chromatin-level silencing model has been suggested in which repressive chromatin may spread from adjacent sequences into the transgene. Then, the silenced transgene may pass the silencing state to another homologous transgene or to endogenous genes, which are linked to or independent from original silenced transgene (Chandler *et al.,* 2001).

Consistent with this model it has been documented in *Arabidopsis* and tobacco transgenic plants harboring CaMV35S promoter::GUS constructs that different transgenic lines show several hundred fold GUS expression level differences (Hobbs *et al.,* 1990; Holtorf *et al.,*1995). In spite of these notions, recent research in *Arabidopsis* has assessed the influence of the integration position of 35S::GUS transgene and no significant transgene expression differences between these lines in different positions of integration was reported. Also in the same report, stable expression was observed for all the lines in which the weaker nopaline synthase promoter was fused to NPT (Neomycin Phospho- Transferase) that confers kanamycin resistance (Schubert *et al.,* 2004).

Silencing has frequently been associated with repetitive T-DNA insertions in the transgenic plants (Muskens *et al.,* 2000). On the other hand, it is documented that tandem arrangement of T-DNA inserts and / or inverted T-DNA repeat configurations, were not sufficient to trigger transgene silencing (Lechtenberg *et al.,* 2003, Schubert *et al.,* 2004).

There has been a favored idea that silencing is triggered by a threshold concentration of a specific transgene transcript (Lindbo *et al.,* 1993). In agreement with this idea, the frequency of silencing has been positively correlated with the promoter strength of transgenes (Que *et al.,* 1997). Also, homozygous transgenic plant lines show stronger silencing than hemizygous plants (Vaucheret *et al.,* 1998). In the most recent report, for less than a certain number of transgene copies in the genome, a positive correlation between copy number and gene expression was observed while more than a certain number of identical transgene copies triggered gene silencing (Schubert *et al.,* 2004). Overall, position effects, repetitive elements and repeat arrangements of T-DNAs do not

account for all the variability of transgene expression observed in *Arabidopsis* and other plants, and a key trigger for silencing appears to be the surpassing of a threshold level of gene expression. The nature of the transgene coding sequence apparently determines the gene specific threshold for silencing (Schubert *et al.*, 2004).

1.3.4 Modifiers of TGS

Transgenic *Arabidopsis* plants containing extra copies of the *Chalcone synthase* (*CHS*) gene have shown homology dependent *CHS* silencing (Davies *et al.*, 1997). Mutagenesis of these transgenic plants and screening for modifiers of *CHS* (trans) gene silencing resulted in the isolation of mutants such as *hog1* and *sil1*. These silencing modifiers were able to restore the expression of silenced *CHS* (trans) gene plus kanamycin (npt) and hygromycin (hpt) resistant genes on the same T-DNA (Furner *et al.*, 1998). Hog1 is a partial loss of function mutation in the S-adenosyl homocysteine hydrolase enzyme that generally inhibits trans-methylation metabolism (Rocha *et al.*, 2005) In the *hog1-1* mutant, *CHS*, npt and hpt transgenes are reactivated and hypomethylated. The sil1 mutation is an allele of HDA6 (histone deacetylase), which has been found to confer partial demethylation of CG sequences at specific regions of the genome (Probst *et al.*, 2004). In the *sil1-1* mutant, the functions of transgenes are also restored but transgenes are not hypomethylated, very similar to a nonallelic mutant called *mom1*, which is involved in silencing, probably through chromatin remodeling (Amedeo *et al.*, 2000). Introduction of such mutant alleles into transgenic plants with silenced transgenes may reveal the nature of transgene silencing in those plants.

1.4 THE PHENYLPROPANOID PATHWAY

The phenylpropanoid pathway is required for the biosynthesis of a series of natural products based on a phenylpropane skeleton derived from L-phenylalanine. The flow of carbon from primary metabolism into an array of secondary phenylpropanoid products is through the general phenylpropanoid pathway (Figure 1-1). Specific branch pathways derive their basic phenylpropanoid unit from this core series of reactions (Hahlbrock and Scheel 1989; Douglas *et al.*, 1996). The functional significance of phenylpropanoid compounds in plants has been further corroborated through genetic and molecular analysis of mutants that are defective in phenylpropanoid regulatory or structural genes (e.g., Jin *et al.*, 2000; Landry *et al.*, 1995), analysis of phenotypes of transgenic plants with altered expression of key phenylpropanoid structural or regulatory genes (e.g., Elkind *et al.*, 1990; Tamagnone *et al.*, 1998), and through generation of transgenic plants engineered for novel phenolic pathways (Hain *et al.*, 1993; Mayer *et al.*, 2001). The general part of this pathway is composed of three enzymes, phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate::CoA ligase (4CL). PAL is a tetrameric enzyme that catalyzes the first step, the de-amination of L-phenylalanine to produce cinnamic acid (Bolwell *et al.*, 1986). Cinnamic acid is then hydroxylated at the para-position by C4H, a cytochrome P450 mono-oxygenase (Teutsch *et al.*, 1990). The product of C4H is *p*-coumarate, which is further modified by other hydroxylases and O-methyltransferase to generate other derivatives of cinnamic acid such as caffeic acid, ferulic acid and sinapic acid. The enzyme 4CL catalyzes the formation of CoA esters of hydroxy-cinnamic acids, and these activated intermediates serve as the substrates for specific branch pathways, such as those leading to the synthesis of flavonoids and lignin (Noel *et al.*, 2005).

In the lignin biosynthetic pathway, the *p*-coumaryl-CoA substrate is converted to three types of lignin monomers; 4-coumaryl, coniferyl, and sinapyl alcohol which constitute *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) moieties of lignin, respectively (Fig 1-2). In this pathway, enzymes such as coumaryl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) and caffeoyl-CoA 3-O-methyltransferase (CCOMT) are involved (Peter *et al.*, 2004). The composition of three lignin monomers in

lignin varies between species, at different developmental stages or in response to stresses (Lewis and Yamamoto, 1990). Lignin provides mechanical strength and hydrophobicity to the cell wall. Biosynthesis and deposition of lignin is developmentally regulated, and occurs in tracheary elements, vessels and fibers during the xylem differentiation (Peter *et al.*, 2004). Lignin synthesis is also induced in response to environmental stresses such as wounding and pathogenic infection.

Deposition of flavonoid pigments in plant organs also occurs in tissue and cell type specific manner, such as in the epidermal cells of petals and in the aleurone layer of maize kernels. Free phenolics acids, or their conjugates and esters, are other phenylpropanoid products that in combination with flavonoid pigments act as efficient sunscreens and antioxidant chemicals in epidermal cell layers of leaves. These compounds protect plants against damaging UV light (Landry *et al.*, 1995; Li *et al.*, 1993).

The biosynthesis of phenylpropanoid compounds is also induced upon environmental stimuli such as wounding, pathogen infection, and UV irradiation (Hahlbrock and Scheel, 1989; Douglas, *et al.*, 1992; Dixon and Paiva, 1995). After wounding, the accumulation of lignin may seal off the wound sites to protect the plants from the loss of water and from pathogenic infection. Evolution of lignin biosynthesis is thought to have been a major adaptation that allowed vascular plants to successfully colonize the terrestrial environment (Whetten and Sederoff 1995).

Genes encoding the enzymes in the general phenylpropanoid pathway and several branch pathways have been isolated from many plant species (Dixon and Harrison, 1990 Raes *et al.*, 2003). In *Arabidopsis thaliana*, four genes have been annotated as members of the At*PAL* gene family and At*PAL1*, 2 and 4 have demonstrated de-amination activity (Cochrane *et al.*, 2004). Only one At*C4H* gene is present in the *Arabidopsis* genome (Mizutani *et al.*, 1997; Urban *et al.*, 1997; Raes *et al.*, 2003), although multiple family members have been detected in other plants (Betz *et al.*, 2001).

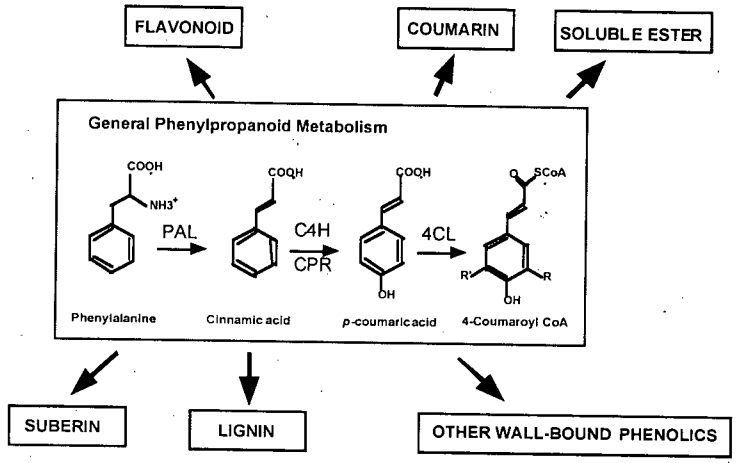


Figure 1-1 General Phenylpropanoid Metabolism (according to Hahlbrock *et al.*, 1989). Thicker arrows indicate branch pathways emanating from the general pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase.

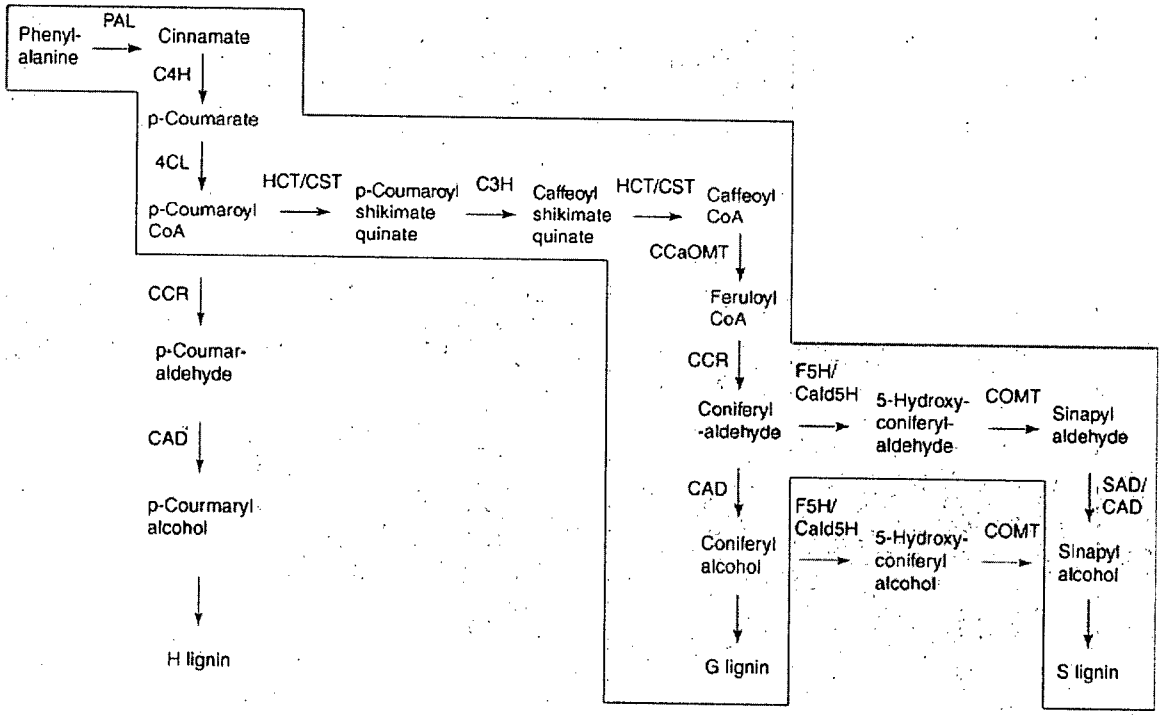


Figure 1-2 Lignin biosynthetic pathway according to Peter *et al.*, (2004).

1.4.1 *At4CL* genes

In all of the plant species studied so far, small gene families encode for 4CL enzymes and corresponding *4CL* genes have been cloned from several plants (for example, Douglas *et al.*, 1987; Ehlting *et al.*, 1999; Raes *et al.*, 2003; Hamberger *et al.*, 2004). *In vitro*, 4CL enzyme can catalyze CoA-esterification of multiple substrates, including *p*-coumaric acid, caffeic acid, ferulic acid 5-hydroxyferulic acid and sinapic acid (Lee *et al.*, 1997; Hu *et al.*, 1998, Ehlting *et al.*, 1999). In *Arabidopsis*, *At4CL1*, *At4CL2*, *At4CL3* and *At4CL4* genes have been cloned. It is known from the published *Arabidopsis* genome sequence that these are the only four *4CL* genes in the *Arabidopsis 4CL* gene family. At4CL1 and At4CL2 share 83% amino acid identity while At4CL3 and At4CL4 share 61% and 66% identity with At4CL1, respectively (Hamberger *et al.*, 2004). *At4CL1*, *At4CL2* and *At4CL4* are considered as more closely related class I genes, while *At4CL3* is in the less closely related class II clad in the *4CL* phylogenetic tree (Ehlting *et al.*, 1999). Class I isoenzymes have been associated with the biosynthesis of lignin and structurally related soluble or cell wall-bound phenylpropanoid derivatives, whereas class II isoenzymes have been associated with flavonoid biosynthesis (Ehlting *et al.*, 1999) and are in a single copy in plants studied so far.

Different *4CL* genes often show different expression patterns throughout plant development. In the mature plant, *At4CL1* and *At4CL2* are strongly expressed in bolting stems and root respectively. Also, these two genes are expressed throughout the inflorescence stem development and their expression increases during the later stages of development (Lee *et al.*, 1995; Mizutani *et al.*, 1997; Ehlting *et al.*, 1999). *At4CL3* is most highly expressed in non-vascular tissue in leaves and flowers (Ehlting *et al.*, 1999). After wounding or infection by *Peronospora parasitica*, the expression of At*4CL3* is not affected but the other At*4CL* genes (class I) are expressed in response to these stresses (Ehlting *et al.*, 1999).

1.4.2 Control of gene expression in phenylpropanoid pathways

It has been shown that transcripts of genes encoding enzymes in the phenylpropanoid pathway coordinately accumulate in a tissue/cell type specific manner during plant development. Coordinate expression is also activated in response to different environmental stimuli (Hahlbrock and Scheel, 1989; Douglas *et al.*, 1996; Dixon *et al.*, 1995). In *Arabidopsis*, expression of *PAL*, *C4H* and *4CL*, is coordinately activated during development and also in response to the wounding (Lee *et al.*, 1997, Bell-Lelong et all., 1997; Mizutani *et al.*, 1997, Koopmann *et al.*, 1999). It is believed that the phenylpropanoid pathway is regulated primarily through control of transcription of the corresponding genes, possibly by a common signaling pathway and transcription factors. According to this model, the presence or activity of common transcription factors is modulated by developmental and environmental signals, and these factors in turn regulate the expression of sets of genes encoding enzymes in the phenylpropanoid pathway, and thus the activity of the pathway (Hahlbrock and Scheel 1989; Douglas *et al.*, 1996).

In support of this model, coordinated expression of genes encoding phenylpropanoid pathway enzymes requires common transcription factors and also common *cis*-regulatory elements. The P and L promoter boxes are characteristic of all genes involved in general phenylpropanoid metabolism investigated so far and have been described for a wide range of plant species. These boxes contain a putative Myb transcription factor binding-site. At least one copy of P and L promoter boxes has been found in the promoter proximal region of all four *At4CL* genes and also parsley 4CL genes (Lois *et al.*, 1989; Logemann *et al.*, 1995, Ehlting *et al.*, 1999, Hamberger *et al.*, 2004). The presence of three perfect W-box sequences (Eulgem *et al.*, 2000) only in the *At4CL4* gene promoter has been reported, two of which fulfill the operational W-box criterion of WRKY transcription factor binding (Hahlbrock *et al.*, 2003). These common *cis*-elements provide a basis for the coordinate regulation of the transcription of structural genes in the phenylpropanoid pathway in response to developmental and environmental stresses such as wounding.

1.5 WOUNDING RESPONSE IN PLANTS

1.5.1 Plant stress responses are interrelated

Compared to mobile animals, non-mobile plants cannot escape biotic and abiotic stresses and are in constant danger of wounding by environmental stresses like wind, sand, and herbivores. Open mechanical wounds are potentially a passage for infectious pathogens to enter the plant tissues. Plants have developed defensive responses against wounding to combat this danger. For example, lignin and phenolic compounds, which originate from the phenylpropanoid pathway, rapidly accumulate at the sites of attack by pathogen or after wounding (Bowles 1990). It is suggested that stresses such as mechanical wounding, drought, freeze and osmotic stress are interrelated by using partially overlapping signaling pathways (Cheong *et al.*, 2002). For example, wounded tissues may induce local osmotic stress responses (Reymond *et al.*, 2000; Denekamp *et al.*, 2003). Also, it has been shown that wound and pathogen induced signaling pathways share common intermediates (Romeis *et al.*, 1999).

1.5.2 Role of plant hormones in the wounding response

Plant hormones are involved in the activation of wounding responses. For example, jasmonic acid and related compounds (JAs) have a central role in the early wound response (Farmer and Ryan 1990; Mc Conn *et al.*, 1997; Schaller 2001, Turner *et al.*, 2002). Transcription of JA biosynthetic enzymes is up regulated, active JAs level is increased and defense-related genes are activated shortly after wounding (He *et al.*, 2005). Like the systemin peptide (Pearce *et al.*, 1991) and its receptor (Scheer and Ryan 2002) that only found in Solanaceae plants, JA may also serve as a systemic signal in the wound-induced systemic responses of the other plants (Li *et al.*, 2002).

There are also reports that ethylene (O'Donnell *et al.*, 1996) and abscisic acid (Pena-Cortés *et al.*, 1989) contribute to changes in gene expression during wounding responses. JA and ethylene-responsive elements and S boxes, all responsive to elicitor, wounding, and pathogen stimuli, have been found in the genes involved in lignin biosynthesis (Rushton *et al.*, 2002). JA induces *4CL* gene expression in tobacco but the mechanisms and the signal transduction pathways leading to wound-induced

phenylpropanoid gene activation are still not clear (Ellard and Douglas 1996).

1.5.3 Duration of activated plant gene expression in the wounding response

It has been reported that steady-state mRNA levels of ~8% of the 8,200 genes present on an *Arabidopsis* microarray are altered in response to wounding, and many of these genes are also osmotic stress- and heat shock-regulated (Cheong *et al.*, 2002). The expression levels of many defensive genes transiently increase to the maximum levels within 90-120 min post wounding, followed by decreases towards the baseline (Reymond *et al.*, 2000), while other genes are activated much later. For example, the Wound-Responsive and phytochrome-Regulated kinase1 (*WPK1*) gene in maize (*Zea mays* L.) is transiently activated immediately after wounding. This gene is also up-regulated rapidly and transiently after jasmonic acid treatment. The *AtPTR3* peptide transporter gene on the other hand is activated 4-h post wounding and gradually increases the response up to 24 h (Karim *et al.*, 2005). Sugar transporter (*STP4* and *AtSUC*) genes are also activated within 3 h post wounding (Truernit *et al.*, 1996; Meyer *et al.*, 2004). Transgenic poplar plants containing the *Eucalyptus gunnii CAD2* gene promoter fused to GUS showed wound responsiveness of the transgene only after 24h post wounding and no response was detected 0 or 48 h post wounding (Lauvergeat *et al.*, 2002). Wounding also induces the expression of a large number of transcription factor genes including members of the AP2, WRKY, and Myb transcription factor families (Cheong *et al.*, 2002). For example, *AtMyb32* (Preston *et al.*, 2004) and *AtMyb4* (Jin *et al.*, 2000) are known to be involved in the wounding response.

1.5.4 Biphasic phenylpropanoid gene expression in the wounding response

Wounding or infection may induce deposition of lignin to protect plant tissues against invading pathogens. To do so, wounding coordinately induces many phenylpropanoid pathway genes such as *PAL, C4H, 4CL, CCR, COMT* (Dixon & paiva 1995; Lee *et al.*, 1995; Bell-Lelong *et al.*, 1997; Meyer *et al.*, 1998; Mizutani *et al.*, 1997; Ehlting *et al.*, 1999; Reymond *et al.*, 2000).

Several reports show that *At4CL1* mRNA accumulates rapidly but transiently in the

wounded *Arabidopsis* leaves 1-2 h post wounding (Lee *et al.*, 1995; Ehlting *et al.*, 1999). The wound-induced expression pattern of *At4CL1* and *At4CL2* are similar although the maximum amount of *At4CL2* mRNA is considerably lower than that of *At4CL1* mRNA. *At4CL3* mRNA levels do not change after the wounding (Ehlting *et al.*, 1999) and the wound responsiveness of *At4CL4* has not been tested.

A report has shown two phases of coordinated wounding response by *PAL*, *4CL*, and *C4H* in parsley within 24 hours. In this report, *PAL*, *4CL*, and *C4H* transcript levels reach a maximum by about 10 h post wounding of leaves, followed by a decrease in RNA levels before rising to a second peak around 24 hours, after which transcript levels declined (Logemann *et al.*, 1995). Such a biphasic wounding response has also been reported by others and suggests that multiple signaling routes may exist for phenylpropanoid gene transcriptional activation in response to wounding (Batard *et al.*, 2000).

1.6 RESEARCH GOALS

1.6.1 Research Objectives for three research projects

Objective # 1- Screen mutagenized populations of *Arabidopsis* lines expressing 4CL-reporter gene fusions for mutants defective in developmental activation of *4CL* expression

In an attempt to identify genes required for activation of *4CL* expression, transgenic *Arabidopsis thaliana* lines containing *4CL:GUS* transgenes were mutagenized and putative mutants affected in *4CL* expression were sought. Mutant phenotypes were not inherited in a Mendelian fashion, suggesting that gene silencing had been activated. The objective of this work shifted to understanding the mechanisms of *4CL* (trans)gene silencing in these lines.

Objective # 2- To search for *cis*-regulatory elements involved in developmental and stress activation of *4CL2* gene expression in *Arabidopsis*

At4CL2 gene expression is developmentally regulated and also is regulated in response to wounding. In an attempt to identify *cis*-regulatory elements responsible for developmental expression and wounding response of the gene, transgenic plants containing fusions of different parts of the *At4CL2* gene to *GUS* were prepared and *At4CL2:GUS* expression monitored in transgenic plants relative to the expression of the endogenous gene.

Objective # 3- To determine the potentially redundant functions of *AtMyb* sub-family #14 transcription factors by reverse genetics

Attempts to find a phenotype in an *AtMyb68* knock out line had previously failed, possibly due to the presence of related, partially redundant *AtMyb* genes in AtMyb subfamily # 14. I identified *AtMyb84* as the most likely gene that could have redundant function to *AtMyb68*, and used reverse genetics (RNAi and T-DNA knockouts) to investigate the functions of *AtMyb68* and *AtMyb84*.

CHAPTER 2
MATERIALS AND METHODS

2.1 PLANT GROWTH CONDITIONS

2.1.1 General

In all of the experiments, *Arabidopsis thaliana* seeds were cold treated for 1-2 day at 4°C on germination medium. Then, seeds were germinated at 20 ° C either on soil (Suns*Hine* mix 5 plug, Sungrow Horticulture, Saba Beach, AB) or in Petri dishes containing ½x MS (Murashige and Skoog) salts (Sigma-Aldrich), supplemented with 1% sucrose and 0.6% agar medium. At the first time of watering, soil in pots was saturated with distilled water containing Miracle Gro powder (4 g/l) (Scotts Canada Ltd. Mississauga, ON), subsequently tap water was added to the base of pots. To help seedling establishment, pots were covered with plastic wrap (Resinite, AFP Canada Inc., West hill, ON) after sowing seeds on saturated soil or soon after transplantation of seedlings from plats to the pots. One week after germination of seeds on the soil or 3 days after transplantation of seedlings from the plate to the pots, the plastic wrap was cut with a razor blade, and 2-3 days later was completely removed. Plants were maintained in long day conditions (18h light) at 20° C from germination to senescence.

2.1.2 Plant material

At4CL1::GUS, *At4CL2::GUS*, *At4CL3::GUS*, and all of the *At4CL::dhlA+At4CL::GUS* transgenic lines were provided by Dr. Ehlting (Max Planck Institute, Germany) in *Arabidopsis thaliana* ecotype Landsberg *erecta* (Ler). For *At4CL1::GUS* and *At4CL2::GUS* transgenic plants, lines with stable *GUS* expression over several generations were chosen for further work. *At4CL4::GUS* lines were provided by Dr. Hamberger in the Columbia background. Two homozygous silencing modifier mutant lines (hog1-1 (0/0), and Sil 1-1 (0/0), (Furner *et al*, 1998) were obtained from the Nottingham stock center (http://arabidopsis.info/).

Wild-type *Arabidopsis* Columbia-1 (Col) was used in the *At4CL2* wounding response and *AtMyb* knock down/out projects.

A Basta resistant *AtMyb68* T-DNA knock out line from the SAIL collection (http://www.tmri.org/en/partnership/sail_collection.aspx) in the Columbia background (Wang 2003) was transformed with *AtMyb84* RNAi construct containing kanamycin resistance gene. An *AtMyb84* T-DNA insertion line (Salk #, http://signal.salk.edu) was obtained from the SALK population (Alonso *et al.*, 2003).

T-DNA insertion locations and genotypes were determined by PCR using genomic DNA from each line. Table 2.1 shows the primers used to confirm the T-DNA insertion in the *AtMyb84* knock out line. Genomic DNA was obtained from 1-2 leaves of plants using the miniprep method of Edwards *et al.* (1991). Five µl of DNA was used in PCR reactions.

2.1.3 *Agrobacterium* and plant transformation

We used several different binary vectors including pBAR (Becker *et al.*, 1992), pART (Gleave 1992), and pCAMBIA (Hajdukiewicz *et al.*, 1994) for the transformation of *Agrobacterium tumefaciens* strain GV3303 in different projects. After heat shock transformation of competent *Agrobacterium* using binary vectors carrying the gene of interest, single antibiotic-resistant colonies were selected on LB plates containing kanamycin (100mg/L), gentamycin (25mg/L), and rifampicilin (25mg/L). Then a few colonies were inoculated into 5 ml of LB broth and grown at 28°C overnight. These bacterial cultures were tested for the presence of the appropriate transgene using PCR followed by sequencing of PCR products. This 5-ml culture was also used to inoculate 500 ml of LB broth supplemented with gentamycin, rifampicilin, and kanamycin in 1 L flasks. These cultures were incubated with shaking at 28°C, 24 h or longer to OD_{600}~0.8. *Agrobacterium* cultures were precipitated by centrifugation for 15 min at 4°C and 5000 rpm. The pellets were resuspended in a solution of 5% (W/v) sucrose, 0.05% (V/V) Silwet L-77 (Lehle Seeds, Round Rock TX). Plants containing about 10-cm tall flowering stems with many young buds in the inflorescences were used for transformation. The inflorescences of the plants were dipped for 1 second into the *Agrobacterium* suspension (Clough and Bent, 1998). The pots with dipped plants were kept dark overnight, after which they were returned to normal growth conditions and to complete their seed development. Seeds harvested from transformed plants were germinated on MS-agar

plates containing kanamycin (50 μg/L) or hygromycin (50 μg/L) plus Vancomycin (40 μg/L) (all antibiotics from Sigma) (Katavic *et al.*, 1994). Kanamycin or hygromycin resistant seedlings (T1 generation) were transferred to soil for selfing to produce T2 populations, which would contain individuals homozygous for the transgenes. The ratio of antibiotic sensitive seedlings/ resistant seedlings showed the number of transgene loci and T3 generation individuals with 100% resistance were used as homozygous lines for further studies.

2.2 GENE EXPRESSION ANALYSIS

2.2.1 RNA gel blot analysis

Arabidopsis 10-cm tall stems, 10 day-old seedlings, roots from plants grown in liquid media (supplemented with 3% sucrose), and wounded leaves from 35 day-old plants were harvested and immediately frozen in liquid nitrogen to provide plant material for RNA or DNA analysis experiments. Using TRIZOL^{R} Reagent, total RNA was extracted and purified as described by manufacturer (Invitrogen Life Technology). The RNA quality samples was assessed by running 1 μg of RNA on a 1% agarose gel and visualizing rRNA bands. About 12-15 μg of high quality total RNA per sample was separated by electrophoresis on 1.2% agarose gels containing 10 % MOPS and 5% formaldehyde. Blots were prepared as described by Koetsier *et al.* (1993) onto Hybond-N^{+} membranes (Amersham Bioscience) using 20X SSC as a transfer solution followed by baking at 80°C for 2h to fix the RNA on the membrane. Pre-hybridization was carried out in (1M NaCl, 5%SDS, and 10% g/v Dextran sulfate) at 65°C overnight. PCR-generated or midi prep plasmid DNA preparations were used to prepare labeled probes. To purify DNA templates and labeled probes, PCR Purification or Gel Extraction kits were used (Qiagen). DNA probes were prepared with ^{32}P-ATP (Amersham) and the Random Primers DNA Labeling System (Invitrogen) using > 50ng template DNA per 50 μl reaction. Blots were washed in 2X SSC, 0.5% SDS, 65°C for 30 min and then in 0.2 SSC, 0.1% SDS. 65°C, 3X for 15 min. Membranes were exposed overnight at room temperature using phospho-screens then scanned with a STORM 860 Phospho-imager (Amersham Pharmacia Biotech). Most of the blots were sequentially hybridized, starting with probes corresponding to the gene of interest followed by actin probe as a loading

control. The intensity of the bands detected by the phospho-imager was quantified using the Imagequant software.

2.2.2 Genomic Southern blots

Total genomic DNA was extracted from 10 day-old *4CL1::GUS*, *4CL2::GUS*, wild type *Arabidopsis* (Ler), and epimutant line (1-A and 2-8) seedlings grown on MS media using the DNAeasy Plant Mini kit (Qiagen) and Nucleon PHYTO pure Plant DNA extraction (Amersham Biosciences) kit according to the manufacture's protocol. Total genomic DNA samples (15 μg) were thoroughly digested by appropriate restriction enzymes, and DNA fragments were separated on 20-cm long 1.0-1.5% agarose-TBE gels at 25 volts. The DNA fragments in the gel were denatured using 1.5 M NaCl, 0.5 N NaOH, for 45 min with gentle agitation, followed by gel neutralization using three times (15-min each) washes of (1M Tris–HCl, pH 7.4, and 1.5M NaCl), and gels were blotted onto Hybound- N^+ membrane (Amersham) using 10 X SSC as the transfer buffer based on a standard protocol (Sambrook *et al.*, 1989). The membrane was baked at 80°C for 2h to fix the DNA fragments, and pre-hybridized and hybridized to the probes as described with RNA gel blot analysis. Table 2.1 shows the primers used to generate probes for northern and Southern analysis.

Primer name	Primer sequence
-1kb4CL1-Forward	5'- GGTCTCCAAAGTTGAATTAAATGGTTGTAG
GUS-Reverse	5'-TCGCGATCCAGACTGAATGCCCAC
4CL1/ORF-Forward	5'-CTCTTGTAAAACACAACCTGTTTCGA
4CL1/ORF-Reverse	5'-GTTTCCTAACGCCAAGCTTGGTCAGGGC
-2504CL2-Forward	5'-ATGTCGACAGCATGGATAATGATAGTAGAGTAGC
84Eco-F	5'-AAGAATTCGGATCCAAGATCGAAGATCAAGAACTGG
84 Rev primers	5'-ATGGTACCAATCGATTTGAATCAGAATAAACAAGAGAGC
LBb1	5'-GCGTGGACCGCTTGCTGCAACT
4CL1/RT-F	5'-CTAATGCCAAACTCGGTCAGGGATAC

Table 2.1 Sequences of PCR primers used to generate DNA hybridization probes and investigate the T-DNA insertion in the *Myb84* Salk knock out line.

2.2.3 Quantitative RT-PCR

Total RNA samples were isolated, qualified, and quantified as described for RNA gel blot analysis. RNA samples (2 µg RNA/ 20 µl reaction), were used to generate first strand cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technology) following the manufacturer's protocol. Gene-specific and intron-spanning primers (Table 2.2) were used in PCR reactions to amplify corresponding cDNA sequences. Template (cDNA) amounts and PCR cycle numbers were optimized using actin cDNA-specific primers in serial PCR reactions. General PCR conditions were 95°C for 2 min, followed by 25-31 cycles of (94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min), followed by 72°C for 5 min, using Taq-polymerase/ 25µl total reaction. PCR products were separated on 1% agarose gels, stained with ethidium bromide, visualized, and photographed under UV transluminator and a digital camera using AlphaImager 1220.

Primer name	Primer sequence
4CL1/RT-F	5'-CTAATGCCAAACTCGGTCAGGGATAC
4CL1/RT-R	5'-CTCTTGTAAAACACAACCTGTTTCGAC
4CL2/RT-F	5'-CCTAACGCCAAGCTTGGTCAGGGCTATGGG
4CL2/RT-R	5'-CACCAGCATCTTCTTCCTTCATGGCGACG
4CL3/RT-F	5'-CTCAATTCACTCCGATCCGGCGC
4CL3/RT-R	5'-CTGGATCGTTCAAGTACTCTTTCATGAT
4CL4/RT-F	5'-CGAGGTCATCAGCTCATGAAAGGTTAT
4CL4/RT-R	5'-CGCTTGTAGTGAACCACCTGTTTGTTTAC
Actin-F	5'-GCGACAATGGAACTGGAAT
Actin-R	5'-GGATAGCATGTGGAAGTGCATACC

Table 2.2 Sequences of primers used in semi-quantitative PCR reactions.
(Primer sequences were adapted from Hamberger *et al.*, 2004)

In order to study changes in *At4CL* gene expression in response to wounding, four week-old leaves of two *Arabidopsis* (Col-01) rosettes grown in separate pots under long day conditions (16 h day at 20°C, 90% humidity), and were each punched 40 times using

pipette tips. At 0-72 hours after *in planta* wounding, leaves were harvested and frozen in liquid nitrogen and 200 mg leaf material was used for total RNA extraction. Leaves from parallel control plants grown under the same conditions were harvested without wounding.

2.2.4 Histochemical GUS assays

Seedlings or organs of transgenic plants expressing GUS, in parallel with positive (35S::GUS) and negative (wild-type) controls were incubated in GUS assay buffer containing 100mM phosphate buffer pH 7.0, 0.1% Triton-X-100, 1mM potassium ferricyanide, 1mM potassium ferrocyanide, and 1mg/ml 5-bromo-4-chloro-3-indolyl-B-D-glucuronide (X-gluc; Jefferson, 1987) at 37C for 4 h (or longer if noted). The assay buffer was removed and 95% ethanol was added to stop the reaction and remove the chlorophyll. Plant materials were kept in water 15 min before photography.

2.3 DNA SEQUENCE ANALYSIS

For restriction enzyme mapping and sequence manipulation, Seqpup software (http://iubio.bio.indiana.edu/soft/molbio/seqpup/java/seqpup-doc.html) was used. *At4CL* gene sequences were found at (http://www.arabidopsis.org/info/genefamily/Raes.html), and *AtMyb* gene sequences were found at (http://www.arabidopsis.org/info/genefamily/Myb.html). For homology searches and alignments, the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) was used. The PLACE software program (http://www.dna.affrc.go.jp/PLACE) was used to search for putative *cis* regulatory elements. DNA sequencing was performed using the di-deoxy chain termination method with BIG dye 3.0 (Applied Biosystems) and a PRISM 377 automated sequencer (Applied Biosystems; NAPS unit, NAPS Unit, UBC). Primers used for sequencing are listed in table 2.3.

Primer name	Primer sequence
2Pr-R	5'-TTGTGTCG**CCATGG**ATCAGAAGTTAATATC
- 110 F	5'-ATGTCGACATATGGAAACACTGATCATCATGC
- 250 F	5'-ATGTCGACAGCATGGATAATGATAGTAGAGTAGC
- 420 F	5'-ATGTCGACCTACTCATAATGACCAATGAATG
- 750 F	5'-ATGTCGACGTCTTGGAAGAGTACTGTTAAAGAG
-1.6kF	5'-ATCCCGGGGAATTCCATCATTTCAGTAGAGAGGATC
2Cod-R	5'-AT**CCATGG**GGTTCATTAATCCATTTGCTAGTCTTGCTCTTAG
2ORF-F	5'-GAT**CCATGG**CGA CACAAGATGTGATAGTCAATG
+ 500 cod- F	5'-CACCCATGGACTCAATACCGGAGAAGATTTCG
+ 1000 cod-F	5'-TACCATGGTTAAGTCTGGAGCAGCTCCTC
+ 1500 cod-F	5'-TGACCATGGACTGTTTTAACTTTTAGAGCGTCTAC
+ 2000 cod-F	5'-GGGCCATGGGAATGACAGAAGCAGGTCCGG
-1kb4CL1-For	5'- GGTCTCCAAAGTTGAATTAAATGGTTGTAG
GUS-Reverse	5'-TCGCGATCCAGACTGAATGCCCAC
4CL1/ORF-Re	5'-GTTTCCTAACGCCAAGCTTGGTCAGGGC
pHannibal-F	5-CCCACTATCCTTCGCAAGACCC
pHanibal-R	5-CAACGTGCACAACAGAATTGAAAGC
Hanni-intron reverse	5'-CATACTAATTAACATCACTTAAC
Hanni-intron forward	5-CATGTCATTGTGTTATCATTGTC

Table 2-3 Sequences of primers used to prepare plasmid constructs and also for sequencing reactions.

2.4 PLASMID CONSTRUCTS

2.4.1 Strategies to generate *At4CL::GUS* plasmid constructs

Various *At4CL2* promoter fragments were PCR amplified using (-1.6 kF), (-750F), (-420F), (-110F), (2Pr-R) (Table 2.3) and fused to GUS in the pBT10 vector (Figure 2.1). Inclusion of the *NcoI* restriction site in the (2Pr-R) reverse primer enabled fusion of amplified promoter fragments to the *GUS* gene. The 1.6-kb construct was digested with *XbaI* to eliminate 600 bp of the upstream promoter sequences to prepare the 950bp construct.

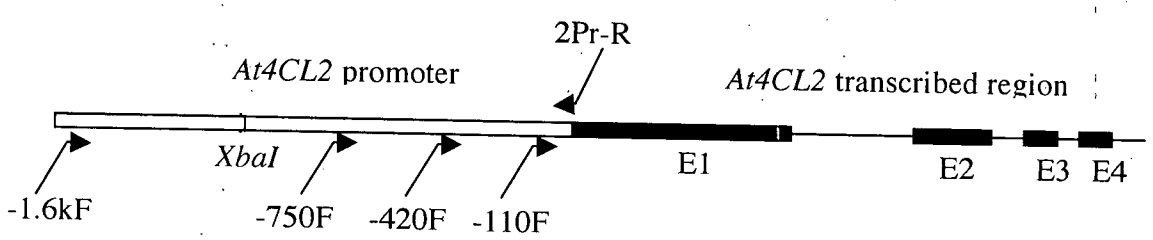


Figure 2-1 Relative position of the primers used to amplify *At4CL2* promoter fragments. *At4CL2* promoter fragments of different lengths were amplified using the indicated primers and were fused with GUS in PBT10. Empty bars represent promoter and solid black bars represent exon (E1-E4).

The Expand Long Template PCR system (Roche) was used to amplify the full length *At4CL2* gene from *Arabidopsis thaliana* (Col) genomic DNA using (1.6k-F) and (2Cod-R) primers and 4.3kb full-length PCR products were generated (Figure 2.2-A). These PCR products were cut by *XbaI* and *NcoI* restriction enzymes and the resulting 3.7-kb fragment (containing the 950-bp promoter plus transcribed region) was fused in-frame with the GUS-ORF that was already installed in pBluescript. This clone was designated the full-length *4CL2::GUS* construct (Figure 2.2-A & -B). Using *Xba1* and *Sac*1 restriction enzymes, the full-length *4CL2::GUS* construct was transferred to the pBAR binary vector.

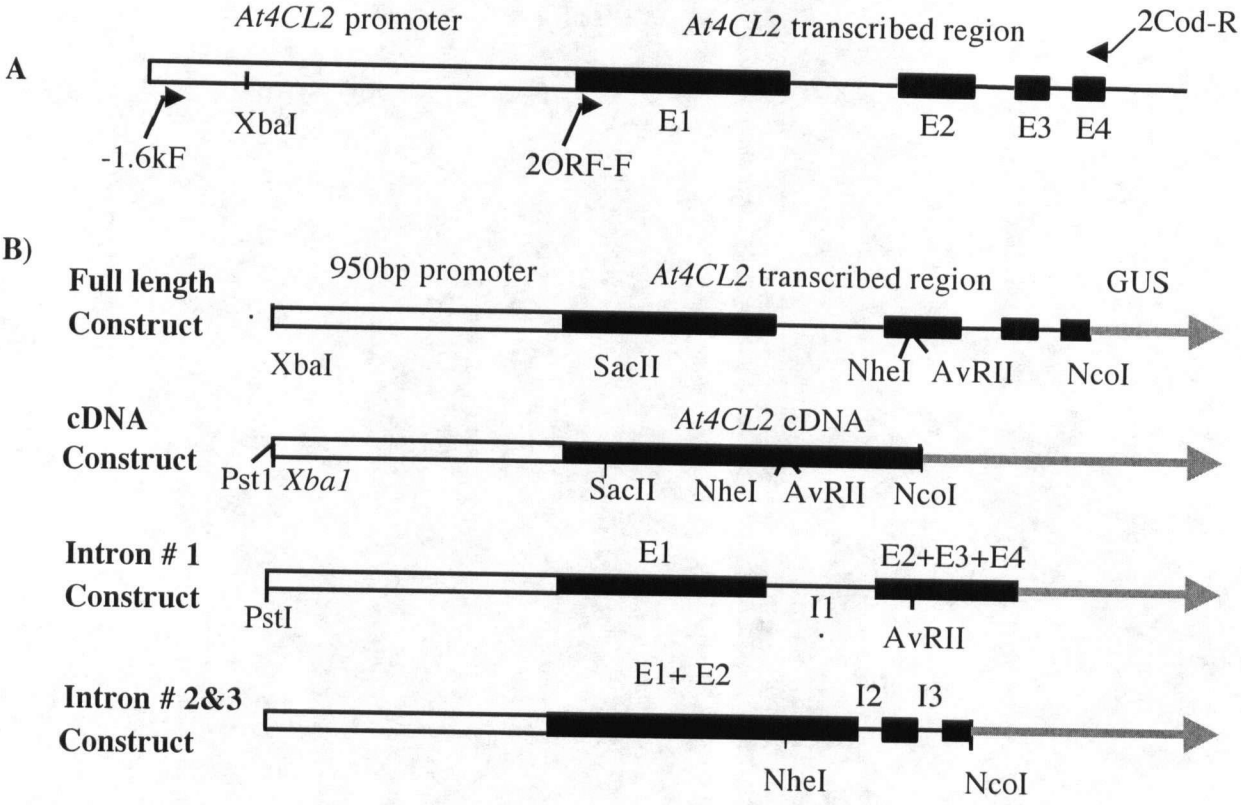


Figure 2-2 Schematic representation of *At4CL2* promoter and transcribed regions fused to GUS. **A)** Locations of primers used to amplify *At4CL2* promoter plus transcribed regions. **B)** Structure of *At4CL2*::*GUS* constructs in pBluescript. Empty bars represent promoter fragments and solid bars represent exon (E1-E4). The name of each clone referred to in the text is indicated at the left.

In order to delete the introns from the full-length *4CL2::GUS* construct and generate a *At4CL2* promoter::At*4CL2*-cDNA::GUS construct, the *At4CL2*-cDNA was amplified using 2ORF-F and 2Cod-R primers (Figure 2.2-A), both containing *Nco*1 sites (Table 2.3). Using *NcoI* and *Sac*II restriction enzymes, a 2-4 kb DNA fragment containing all three introns was removed from the full-length *4CL2::GUS* construct in pBluescript and substituted by 1.3 kb of the *4CL2-cDNA* (Figure 2.2B). Using *Sac*1 and *XbaI* restriction enzymes, the 950 bp promoter::*4CL2cDNA::GUS* construct in

pBluescript was partially digested and the 4.6 kb fragment was sub-cloned into pCambia 1300 (http://www.cambia.au) binary vector.

To add introns # 2 and 3 to the *4CL2-cDNA::GUS* construct in pBluescript, an 850-bp DNA fragment containing introns 2 and 3 was cut out from full length *4CL2::GUS* construct in pBluescript clone using *NcoI* and *Nhe*I. This fragment was used to substitute the corresponding area (625 bp) in the *4CL2-cDNA::GUS* construct in pBluescript that had been cut with the same enzymes (Figure 2.2-B). An *Nco*I-*XbaI* restriction fragment containing this construct was transferred to the pCambia1300 binary vector. To prepare the intron 1 construct, a 2950-bp *Avr*II-*Pst*1 restriction fragment containing intron 1 from the full-length construct was substituted for corresponding fragment in the cDNA construct (Figure 2.2-B).

As described above, based on the presence of convenient restriction enzyme sites, DNA constructs were transferred to different binary vectors for transformation of *Arabidopsis* plants. The pCambia 1300 was used for intron 1, intron # 2 & 3, and cDNA constructs. The pBAR binary vector was used for the full length *4CL2::GUS* construct and the 125 bp, 500 bp, and 750 bp constructs and pCambia 1305 was used for the 950 bp construct. The sequence integrity of all constructs was confirmed by sequencing (UBC, NAPS unit).

Agrobacterium strain GV3101, *Arabidopsis thaliana* plants were transformed with the constructs as described above. At least 10 transgenic lines for each construct were selected for subsequent analysis.

2.4.2 Strategies for RNAi construct generation

2.4.2.1 *AtMyb68, AtMyb84, and GUS* RNAi constructs

As a sense arm for the RNAi construct, 400 bp of *AtMyb84* coding sequences were PCR amplified using (M84-Ec-Ba-F) and (M84-Kp-Cl-R) primers (Table 2.3). These products were cloned into pHannibal (Wesley *et al.*, 2001) obtained from (CSIRO Plant Industry, Canberra, Australia) using *Bam*H1 and *Kpn*I restriction enzymes whose sites were engineered in the primers. A 315 bp fragment as antisense arm was PCR amplified using (M84-Xba-F) and (M84-kp-Cl-R) primers, was cut by *XbaI* and *Cla*I, and cloned into pHannibal which already had the 400-bp sense arm (Figure 2.3-B).

Primer name	Primer sequence
M68-EcXbH-F	5-TTGAATTCTAGAAGCTTCAGCACAAACTCACCATTACCAAAC
M68-*Bam*-F	5-AAGGATCCTTTGCTATCATGAGCAGCAGCAC
M68-Kp-CL R	5-ATGGTACCACATCGATTTGGCGCATTGAAGTAACTTGC
M84-Ec-Ba-F	5-AAGAATTCGGATCCAAGATCGAAGATCAAGAACTGG
M84-Xba-F	5-GATCTAGAACTGGAGAAAACAAACCTCATC
M84-Kp-Cl-R	5-ATGGTACCAATCGATTTGAATCAGAATAAACAAGAGAGC
Gus-Xb-Ec-F	5-ACTCTAGAATTCAAAAAACTCGACGGCCTGTG
Gus-Xho-F	5-AACTCGAGGGCCTGTGGGCATTCAGTCTG
Gus-Kp-Cl-R	5-TCGGTACCTATCGATTATTGACCCACACTTTGCCG
M84-R-Kpn	5'-TACGGTACCCCCAATTAATAATAATGATGTACG

Table 2-4 Sequences of PCR primers used to prepare RNAi constructs.

In similar steps, 355-bp sense and 350-bp antisense arms for *AtMyb68* were PCR amplified using (M68-EcXbaH-F) or (M68-Bam-F) and (M68-Kp-CLR) primers and cloned in pHannibal using *Hin*dIII and *Kpn*I restriction sites for cloning sense arm and *Bam*HI and *Cla*I sites for cloning the antisense arm (Figure 2-4, A). I chose ~ 350 bp of the down stream region of these two genes in order to provide DNA for the RNAi constructs. Two genes in these areas did not show any identity except for a domain of 50bp (with 83% identity) but even in this domain no more than 12 bp were identical in each sub domain. The amplified area is the least similar between *AtMyb68* and *AtMyb84*.

In order to prepare the GUS RNAi construct, 270 bp of the GUS-ORF was amplified using (Gus-Xho-F) and (Gus-Kp-Cl-R) primers (Table 2-4) and cut by *Xho*I and *Kpn*I restriction enzymes and cloned into pHannibal as the sense arm. The 312-bp antisense arm was also amplified using (Gus-XbaI -Eco-F) and (Gus-Kp-Cl-R) primer. PCR products were cut by *XbaI* and *Cla*I and joined them to the pHannibal vector already containing the sense arm (Figure 2-3 C).

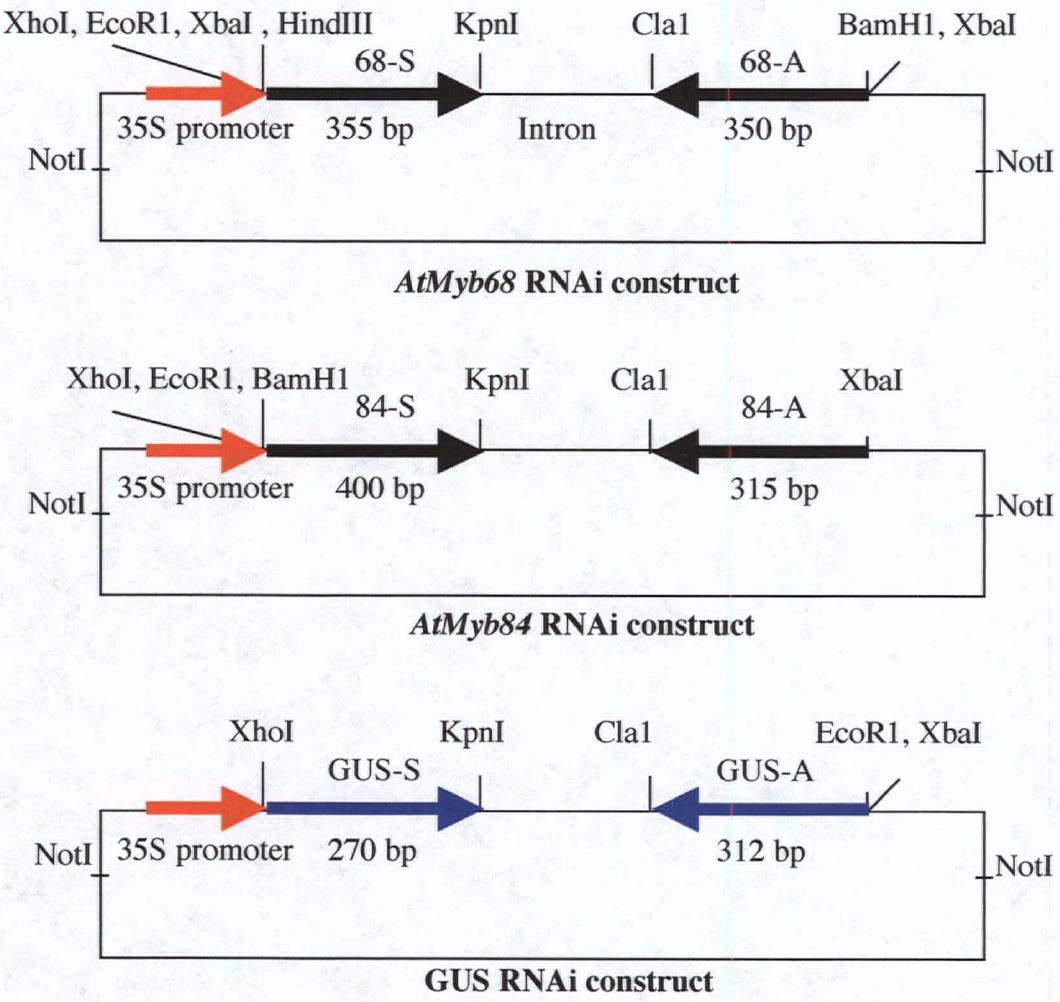


Figure 2-3 RNAi constructs for *AtMyb68, AtMyb84* and GUS in pHannibal

2.4.2.2 Cloning of double RNAi constructs

Using *Xho*I and *Eco*RI restriction enzymes, *GUS* RNAi and *AtMyb84* RNAi constructs in pHannibal were cut and *GUS* RNAi was ligated upstream of the *AtMyb84* RNAi construct in pHannibal (Figure 2-4, B). Using a similar strategy, *AtMyb68* and *GUS* RNAi constructs were fused (Figure 2-4, A). Also, using *Eco*RI and *Bam*HI restriction sites, *AtMyb68* and *AtMyb84* RNAi constructs were fused to each other to make a single construct containing both RNAi constructs (Figure 2-4, C).

Sequences of the all of these RNAi constructs were verified, transferred to pART binary vector using *Not*I and transformed into *Agrobacterium* for *Arabidopsis* (Col) transformation as described above.

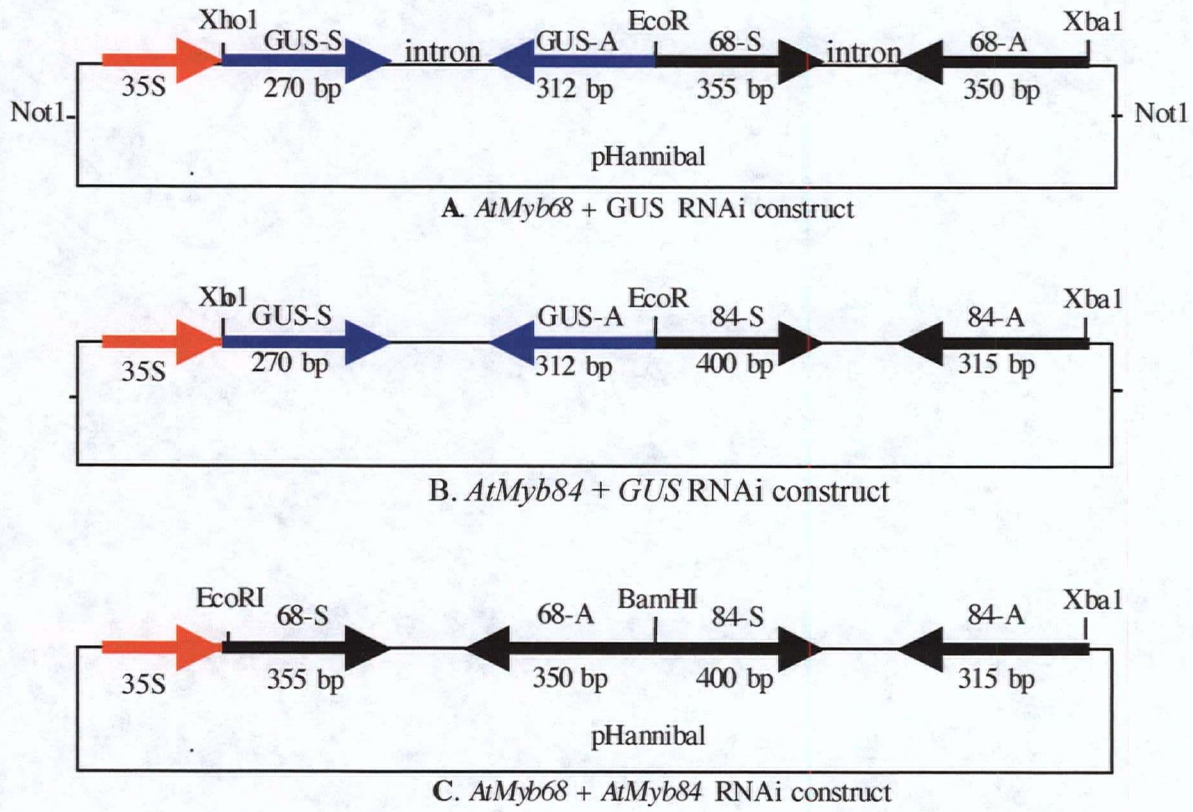


Figure 2-4 Double RNAi constructs for *AtMyb68, AtMyb84* and GUS in pHannibal

2.5 MUTANT ANALYSIS

2.5.1 Screening for GUS expression mutants

Five thousand seeds from the selected homozygous *At4CL1::GUS* line were mutagenized by EMS (0.25%, 8 h) and were grown in 100 pots to set their seeds and collect these seeds as 100 pools containing M2 generation. These M2 seeds were grown in liquid media containing MS salts. Ten day-old seedlings were screened for changes of developmental GUS expression in the root and shoot.

2.5.2 Screening for *dhl*A expression mutants

Basta resistant T1 generation plants containing double transgenes (*At4CL1::GUS* plus *At4CL1::dhlA*), (*At4CL2::GUS* plus *At4CL2::dhlA*), or (*At4CL3::GUS* plus *At4CL3::dhlA*) were selected by Basta and ~15 independent homozygous lines were identified in the T3 generation for all three cases.

The *Xanthobacter autotrophicus* GJ10 Dehalogenase A (*dhl*A) gene (Janssen *et al.*, 1989) works as a negative selection marker in *Arabidopsis* (Naested *et al.*, 1999) when *dhl*A-expressing plants are treated with a haloalkane pro-toxin substrate, such as 1,2-dicholoroethane (DCE) (Janssen *et al.*, 1994). In order to optimize the selection system for DCE treatment, 0-6 day-old seedlings of wild type *Arabidopsis* (Ler) plants grown in MS media were treated with 5- 40 µl of DCE (1.256 g/ml; Sigma-Aldrich). Plants were treated once/day for three days. I found that treatment of 3 day-old *Arabidopsis* seedlings on solid MS medium with 10 µl of DCE for 3 days showed the least amount of toxicity in wild type plants, but was toxic to a *35S::dhlA* control line.

2.5.3 Phenotypic and genetic analysis of putative mutants

In order to analyze the phenotype of putative *At4CL1::GUS* expression mutants, overnight GUS assays were performed on 10 day-old seedlings of putative mutants grown on MS media. The morphology of putative mutants grown in pots was compared with wild type plants.

In order to genetically analyze the inheritance of the mutant phenotype, reciprocal crosses were made between three selected putative *At4CL1::GUS* expression mutants and the T5 *4CL1::GUS* non-mutated progenitor line. In order to avoid pollen contamination, the top flowers of each mutant and non-mutant plant were emasculated and cross-

pollinated after 2 days. The GUS assays were performed on the F1 and F2 generation of these crosses.

2.5.4 Azacytidine treatment of mutant plants

The demethylation agent 5-azacytidine (5-aza), a cytosine analog, was used to prevent DNA methylation (Prakash and Kumar, 1997). Seedlings from putative mutant lines were grown on solid media supplemented with 40 mg/L of 5-aza (Sigma-Aldrich) and 50 µg /ml kanamycin. Ten day-old seedlings treated in this way were stained for GUS expression in parallel with control lines.

CHAPTER 3

GENETIC ANALYSIS OF *At4CL* EXPRESSION AND EPIGENETIC SILENCING OF *4CL* EXPRESSION

3.1 INTRODUCTION

4-coumarate::CoA ligase (4CL) (EC 6.2.1.12) enzymes catalyze the formation of CoA esters from cinnamic acids in the plant general phenylpropanoid pathway. These esters are intermediate substrates for specific branch pathways, such as those leading to the synthesis of flavonoids and lignin (Hahlbrock and Scheel, 1989; Dixon and Paiva 1995; Douglas 1996).

4CL genes are recognized as small gene families in all the plant species studied so far and the genes encoding for 4CLs have been cloned from several plants (reviewed by Raes *et al.*, 2003). *At4CL1*, *At4CL2, At4CL3* (Lee *et al.*, 1995; Ehlting *et al.*, 1999 and *At4CL4* (Hamberger *et al.*, 2004) encode the complete *4CL* gene family in *Arabidopsis*. At4CL1 and At4CL2 share 83% amino acid sequence identity and 74% cDNA identity in cDNA coding regions (Ehlting *et al.*, 1999; Hamberger *et al.*, 2004).

At4CL1 and At4CL2 isoenzymes both are associated with biosynthesis of lignin and structurally related soluble or cell wall-bound phenylpropanoid derivatives in the xylem tissues of the plant vasculature (Lee *et al.*, 1995, Mizutani *et al.*, 1997, Ehlting *et al.*, 1999), similar to the parsley (*Petroselinum crispum*) *4CL1* gene, the first *4CL* gene cloned (Hauffe *et al.*, 1991). From the analysis of organ-specific expression of *At4CL1* and *At4CL2* genes in *Arabidopsis* it is known that both *At4CL1* and *At4CL2* have high expression levels in seedling roots. In adult plants, *At4CL1* is the only gene family member strongly expressed in bolting stems (Ehlting *et al.*, 1999).

At4CL1 and *At4CL2* promoters contain multiple common *cis* regulatory elements that are also found in other genes encoding enzymes in the general phenylpropanoid and lignin-specific biosynthetic pathways (Ehlting *et al.*, 1999; Hamberger *et al.*, 2004). Considering the high identity of At4CL1 and At4CL2 plus common *cis* regulatory elements, a common mode of transcriptional regulation and partial functional redundancy is a strong possibility.

Epigenetic mechanisms can alter the transcription patterns of endogenous genes as well as transgenes, and control tissue specific gene expression (Fagard and Vaucheret 2000). DNA methylation causes gene inactivation in eukaryotes (Bird 1992; Martienssen and Richards, 1995). Also, it is widely considered as a mechanism for defending genomes against the effects of transposable element and virus proliferation (Ratcliff, *et al.,* 1997; Martiensen, 2001; Miura *et al.,* 2001). It is also a controlling factor for developmental gene expression (Sakai *et al.,* 2000; Ito *et al.,* 2003), a modulator of the expression of duplicated gene family members (Lawrence 2004), and a silencer of transgenes (Kilby *et al.,* 1992; Stam *et al.,* 1997; Fagard, 2000; Chandler *et al.,* 2001).

Transgene expression in plants is controlled by incompletely understood gene silencing mechanisms. There are examples of single copy transgene silencing but often, homology between the interacting multiple (trans) genes is critical in silencing (Meyer and Saedler 1996). Transgenes can be silenced after a few generations of activity and may also silence the homologous endogenous gene (Vaucheret and Fagard, 2001).

Two molecular mechanisms have been proposed to mediate HDGS that are not necessarily exclusive. In transcriptional gene silencing (TGS) promoters of two (trans) genes are homologous. This form of silencing blocks transcription of the (trans) gene and is both meiotically and mitotically heritable (Fagard and Vaucheret, 2000). TGS is mainly associated with heavy methylation of the promoter sequences (Meyer *et al.,* 1993; Neuhuber *et al.,* 1994; Park *et al.,* 1996).

Post-transcriptional gene silencing (PTGS) involves degradation of the cytosolic mRNA product of the (trans) gene (Meyer *et al.,* 1993; de Carvalho *et al.,* 1992), based on homology that is confined to the coding regions (Muskens *et al.,* 2000). Double stranded RNA (Waterhouse *et al.,* 2001), high RNA expression levels (Napoli *et al.,* 1990), and otherwise aberrant RNAs may trigger PTGS (Balcombe, 2004; Gendrel *et al* 2005). In PTGS, mainly coding sequences are methylated (Vaucheret *et al.,* 1998, Stam *et.al.,*1998, Chandler *et al.,* 2001) and this methylation is triggered by dsRNA with an identical sequence (Bender, 2004). PTGS can also be initiated by a single transgene (Muskens *et al.,* 2000).

Here, we show that *At4CL1* and *At4CL2* promoters drive GUS reporter gene in overlapping expression patterns in the *Arabidopsis* vascular system. I used mutagenized

populations of *At4CL::GUS* transgenic lines to isolate multiple mutant lines with reduced *GUS* expression. All mutant lines showed non-Mendelian inheritance of the *GUS* expression phenotypes and the majority were sensitive to treatment by the demethylating agent, 5-aza that restored the original *At4CL::GUS* transgene expression in the roots, implicating alterations in DNA methylation in the loss of GUS expression. Southern analysis confirmed DNA methylation was confined to the promoters of the transgenes in the mutant lines, supporting methylation-mediated TGS. Increasing the number of transgenes in the non-mutated lines resulted in similar phenotypes at a high frequency. Taken together our data suggest that epigenetically silenced *At4CL* epimutants had been isolated. *At4CL1::GUS* epimutants differed from *At4CL2::GUS* epimutants with respect to organ-specific gene silencing, suggesting two different silencing modes for two closely related family members.

3.2 RESULTS

3.2.1 NEGATIVE SELECTION OF *AT4CL* SIGNALING PATHWAY MUTANTS

3.2.1.1 Generation of transgenic *Arabidopsis* lines containing *4CL::Reporter* transgenes

I, intended to screen for mutants in genes required for activation of developmentally regulated *At4CL* expression in mutagenized transgenic lines containing *At4CL::GUS* or *At4CL::dhlA* transgenes. Transgenic *Arabidopsis thaliana* (Ler) lines containing 1.0-kb *At4CL1*, 1.6-kb *At4CL2* and 0.6-kb *At4CL3* promoter fused to *GUS* transgenes, were generated by Dr. J. Ehlting. The *At4CL1::GUS* and *At4CL2::GUS* transgenic lines showed GUS expression throughout the plant vasculature while *At4CL3::GUS* line showed the expression of GUS in areas other than the vasculature (Figure 3-1). Both representative *At4CL1::GUS* and *At4CL2::GUS* transgenic lines (T5 generation) were crossed to wild type (Ler) plants and segregation of kanamycin resistance (encoded on the T-DNA) in the F2 generations indicated a single transgene locus in both cases. These *At4CL1::GUS* and *At4CL2::GUS* transgenic lines were used in

two parallel genetic screens aimed at identifying mutants affected in developmental regulation of *At4CL1* and *At4CL2*, using *GUS* expression as a visual phenotype.

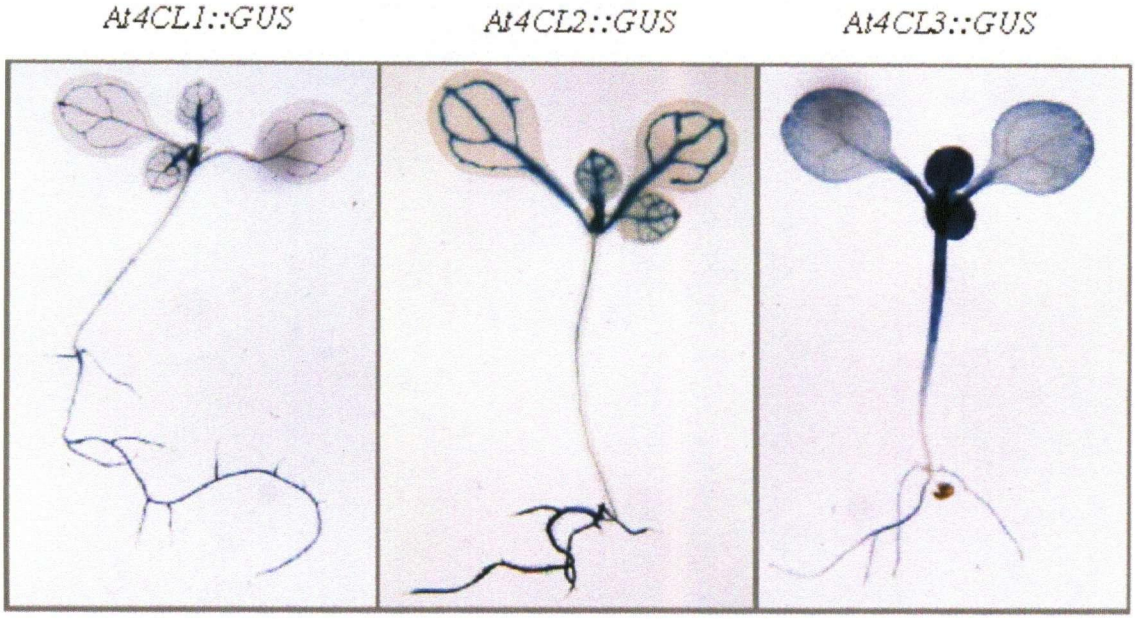


Figure 3-1 Developmentally regulated GUS expression in *At4Cl1::GUS, At4CL2::GUS* and *At4CL3::GUS* lines (data from Dr. J. Ehlting, unpublished).

The dehalogenase-A (*dhlA*) gene, when driven by the CaMV35S promoter, has been shown to be an effective negative selectable marker in *Arabidopsis* plants fed with the pro-toxin substrate 1,2-dichloroethane (DCE) (Janssen *et al.,* 1994; Næsted *et al.,* 1999). In collaboration with Dr. Ehlting, I explored the use of *dhlA* to select for mutants affected in developmental regulation of *At4CL* promoter activity. To establish a negative selection system for *At4CL* promoter activity, Dr. Ehlting fused the *dhlA* gene to *At4CL1*, *At4CL2* and *At4CL3* promoters, and introduced the *At4CL1::dhlA, At4CL2::dhlA* and *At4CL3::dhlA* fusions into *At4CL1::GUS, At4CL2::GUS* and *At4CL3::GUS* transgenic plants, respectively, to generate lines harboring both *At4CL::GUS* and *At4CL::dhlA* transgenes. This would allow dual screening for mutants impaired in *At4CL* promoter activity by negative selection upon feeding with DCE, and histochemical assay of GUS expression in mutagenized populations derived from such lines.

From the primary transformants, I identified ~15 independent homozygous lines for each of *At4CL1::dhlA, At4CL2::dhlA,* and *At4CL3::dhlA* transgenic lines. The

majority of these lines showed monohybrid ratios for the *At4CL::dhlA* transgenes inheritance. Using *At4CL* promoter specific and dhlA/ORF specific reverse primers, the presence of *At4CL::dhlA* transgenes in the plants was confirmed.

3.2.1.2 Negative selection system optimizing for *At4CL::dhlA* lines

I first optimized the system to establish the highest amount of DCE that was not toxic for wild type *Arabidopsis* plants after 3 days of treatment. Treatment of wild type plants (10 µl of DCE/ day) three times over three days, was not toxic to wild type plants. To test this system, multiple (at least 15) homozygous *At4CL::dhlA* lines in the appropriate *At4CL::GUS* background were treated with DCE under conditions optimized for negative selection of a control *35S::dhlA* line and wild type plants.

In multiple experiments, DCE treatment caused delay of wild type seed germination and growth, but was lethal for the *35S::dhlA* seeds or seedlings. Germination and growth of *At4CL::dhlA* lines in the *At4CL::GUS* background, were also delayed but DCE treatment was not lethal as their growth resumed after treatment. In all of the *At4CL::dhlA* lines with *At4CL::GUS* background, plants responded similarly to wild type plants (data not shown), and negative selection did not work regardless of whether plants were grown in MS media or soil (Figure 3-2).

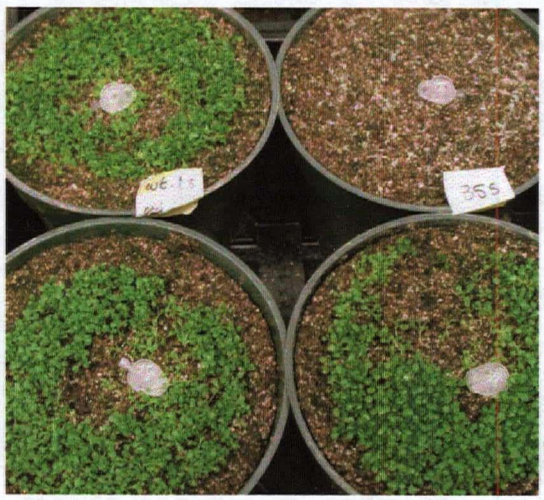


Figure 3-2 Treatment of *At4CL::dhlA* lines with DCE and comparison of their sensitivity to the 35S::dhlA line.

Northern blot analysis performed on all *At4CL::dhlA* lines failed to detect *dhlA* transcripts (data not shown) although semi-quantitative RT-PCR did detect low levels of *dhlA* mRNA in a few tested lines (Figure 3-3). This suggested that, unexpectedly, the *At4CL* promoters were ineffective in multiple lines to drive sufficient *dhlA* expression for negative selection to be effective.

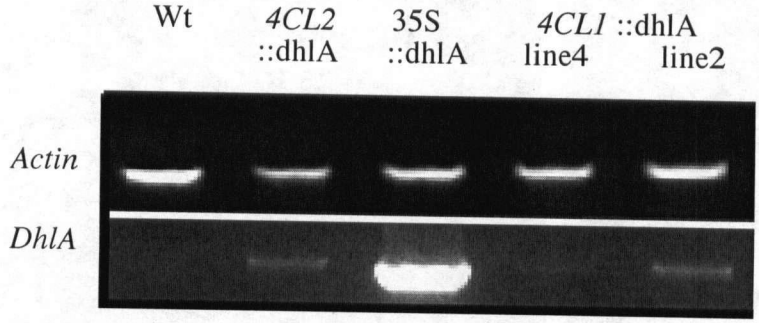


Figure 3-3 Semi quantitative RT-PCR in order to compare of *dhlA* expression levels in *At4CL::dhlA* lines and the *35S::dhlA* line. Actin was amplified as a control for RNA amount.

3.2.1.3 *At4CL::GUS* transgenes are silenced in *At4CL::dhlA* lines

I examined *At4CL::GUS* transgene expression in the *At4CL::dhlA/At4CL::GUS* lines and found that more than 50% of the lines showed down regulation of *At4CL::GUS* transgenes (Figure 3-4).

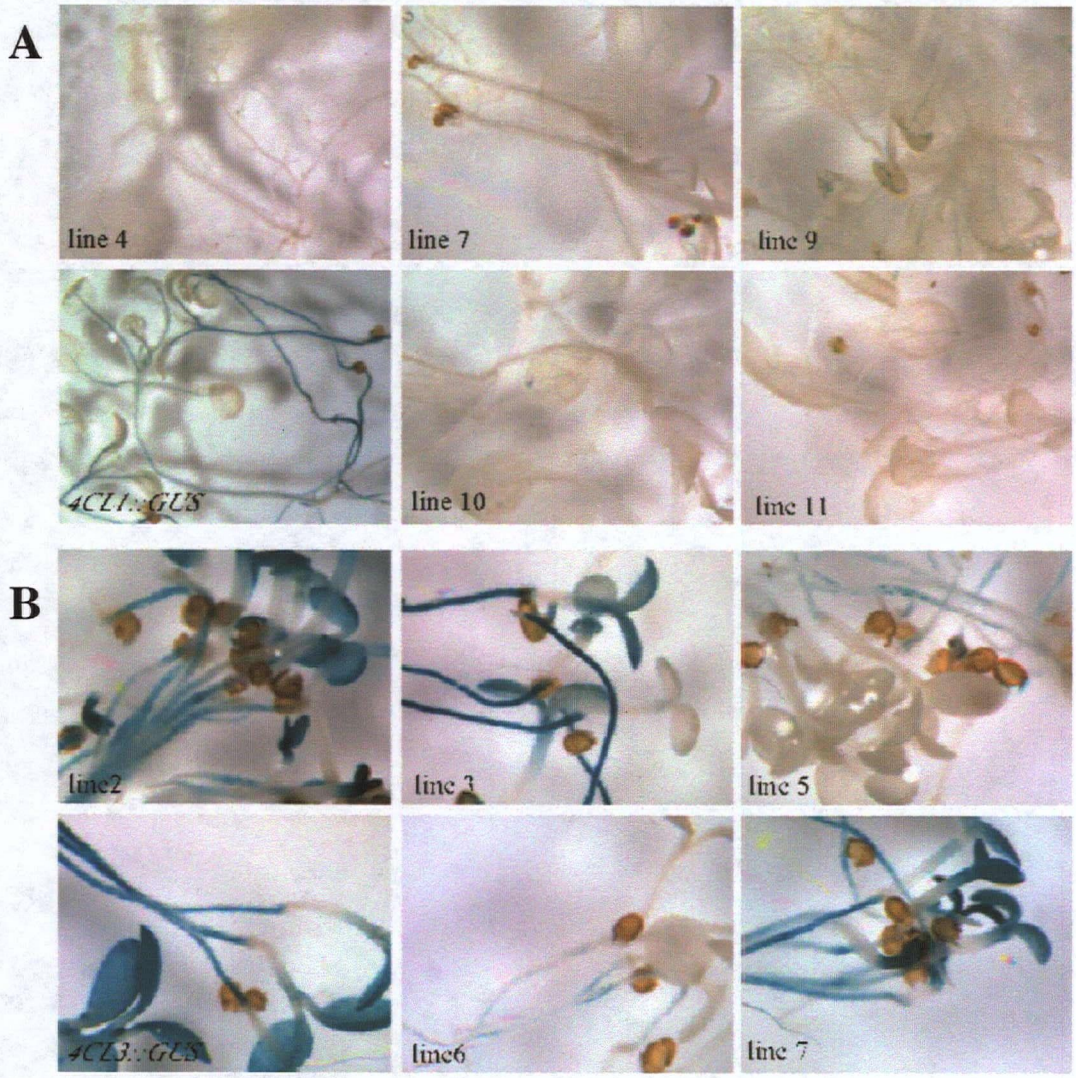


Figure 3-4 GUS expression in the several lines of *At4CL::dhlA/At4CL::GUS* transgenic plants. **A)** *At4CL1::dhlA* lines in the *At4CL1::GUS* background. **B)** *At4CL3::dhlA* lines in the *At4CL3::GUS* background.

To test if DNA methylation could play a role in the unexpected loss of *GUS* expression, selected *At4CL::dhlA* lines showing down regulated *GUS* expression were treated with 5-aza to inhibit DNA methylation. This treatment restored developmentally regulated *GUS* expression to the visible levels (Figure 3-5).

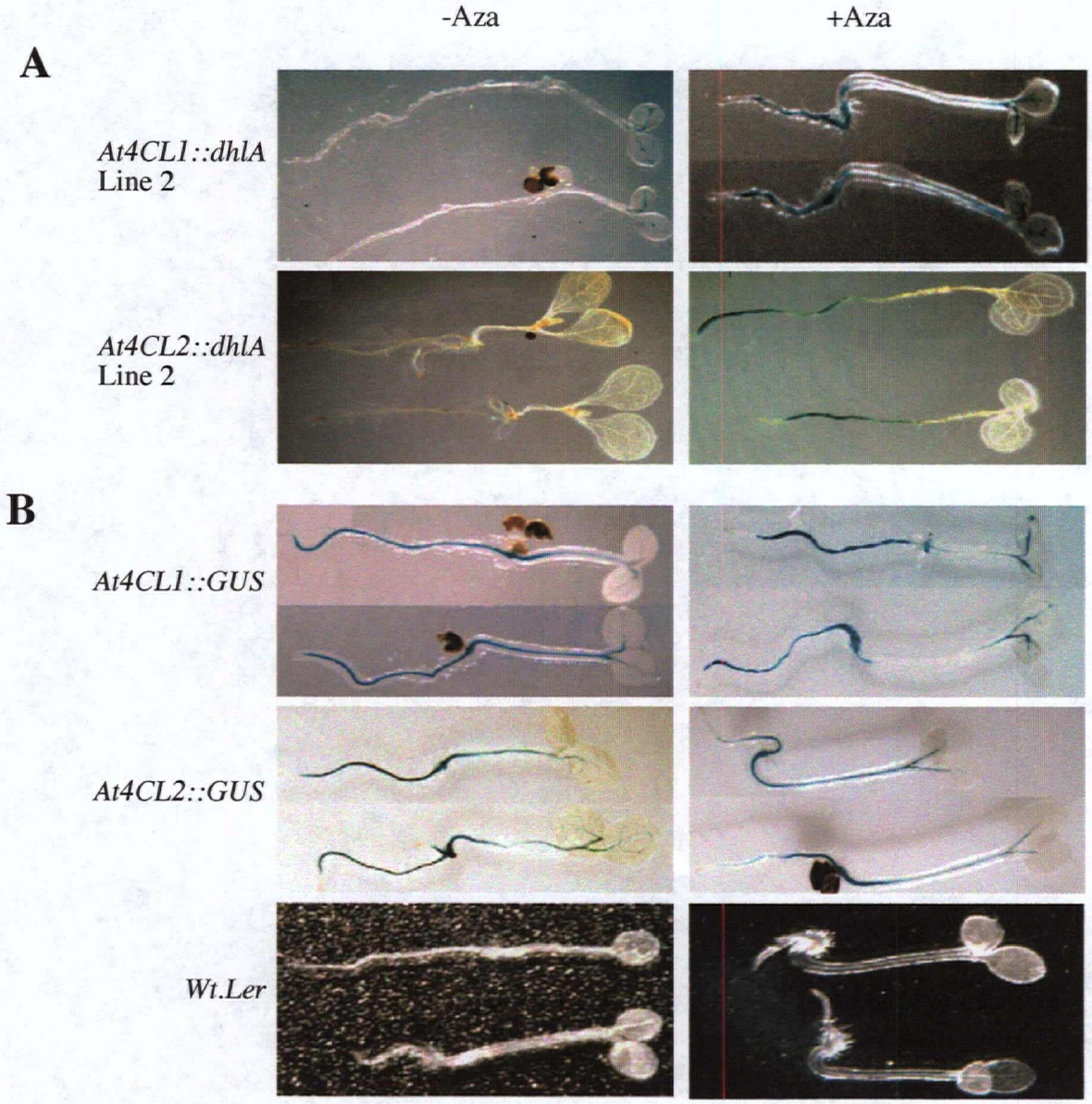


Figure 3-5 GUS-activity in *At4CL::reporter* transgenic lines and wild type controls. **A)** GUS activity in *At4CL::dhlA/At4CL::GUS* lines with or without treatment with 5-aza. **B)** GUS activity in *At4CL::GUS* progenitor lines and wild type plants.

Selected *At4CL::dhlA* lines were also co-treated with DCE and 5-aza to investigate potential methylation of *At4CL::dhlA* transgenes. Although both of these chemicals delayed the growth of wild type plants, treatment of *At4CL::dhlA* lines with 5-aza made them more sensitive to DCE treatment, as shown by chlorotic leaves of the seedlings treated with both DCE and 5-aza (Figure 3-6). Overall, these data suggest that in *At4CL::dhlA/At4CL::GUS* lines, both *At4CL::GUS* and *At4CL::dhlA* transgenes had been epigenetically silenced by DNA methylation and that as a result of transgene silencing, or in combination with other unknown reasons, the negative selection system using *At4CL::dhlA* was not functional in the *Arabidopsis*.

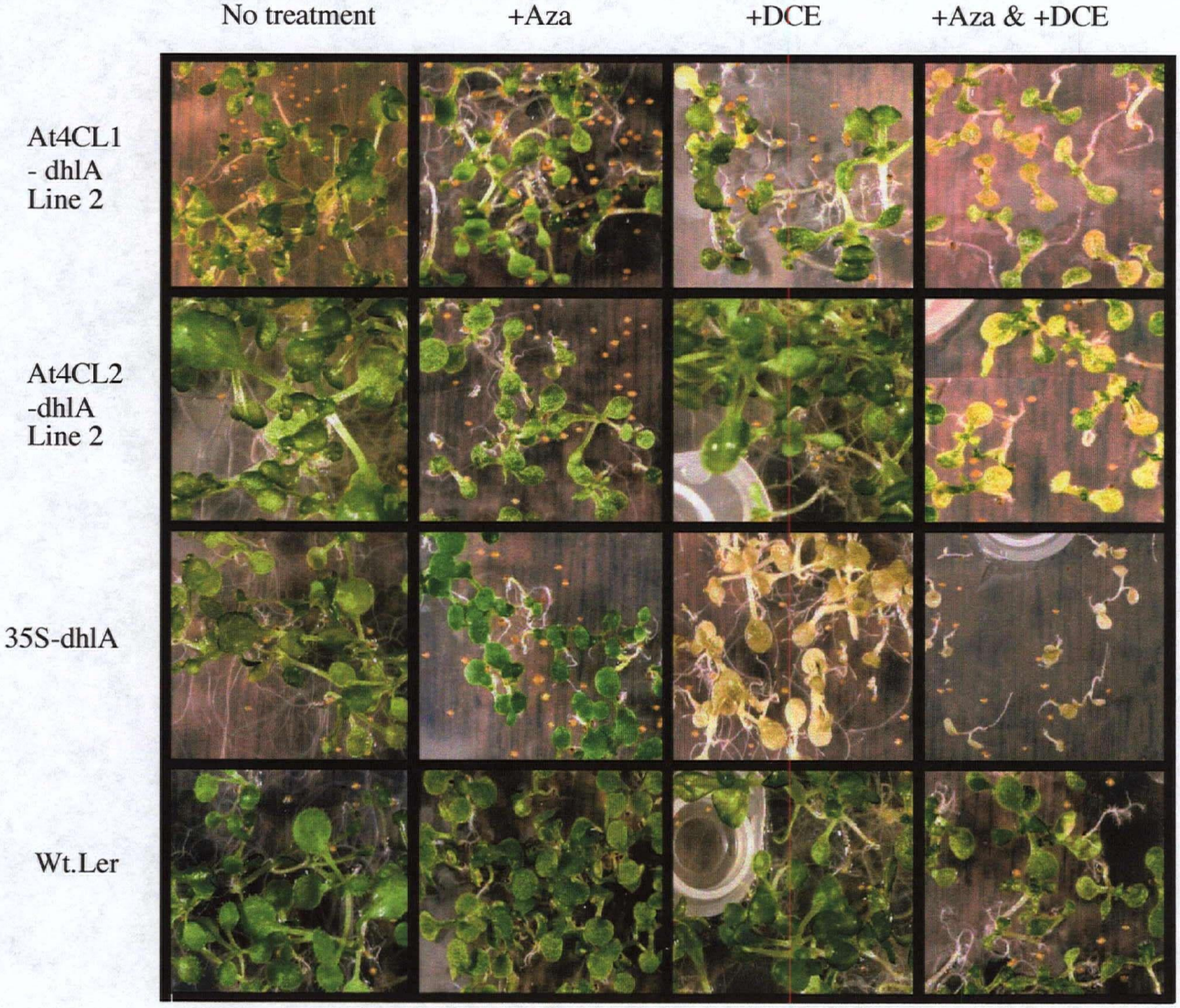


Figure 3-6 DCE sensitivity of *At4CL::dhlA* lines with or without treatment by 5-aza compared with positive (35S::dhlA line) and negative (Wt.Ler) controls.

3.2.2 ALTERNATIVE SCREEN FOR At4CL SIGNALING PATHWAY MUTANTS

3.2.2.1 Screen for mutants with reduced At4CL1-driven GUS expression

As an alternative to negative selection for mutants affected in *At4CL* promoter activation, I used *GUS* expression as a screen for loss of *At4CL* promoter activation. 5000 seeds of transgenic *Arabidopsis* (Ler) plants containing a *At4CL1::GUS* transgene were mutagenized and grown in soil (50 seeds in each pot) and selfed to make the M2 generation. M2 seeds were harvested as 100 M2 pools by Dr. Ehlting and I screened >100 seeds from each pool (total of 8000 M2 seeds from 80 pools), looking for ectopic or reduced developmental *GUS* expression in the leaves of two-week old plants. Overall, I found 14 putative mutants. In parallel, 8000 M2 seeds of mutagenized *At4CL2::GUS* line were screened by Dr. Ehlting and >10 putative mutants were identified.

3.2.2.2 Phenotype analysis of putative mutants lines

Representative phenotypes of mutants affected in *At4CL1::GUS* expression showing weak, patchy, or complete lack of *GUS* expression in the vasculature are shown in Figure 3-7.

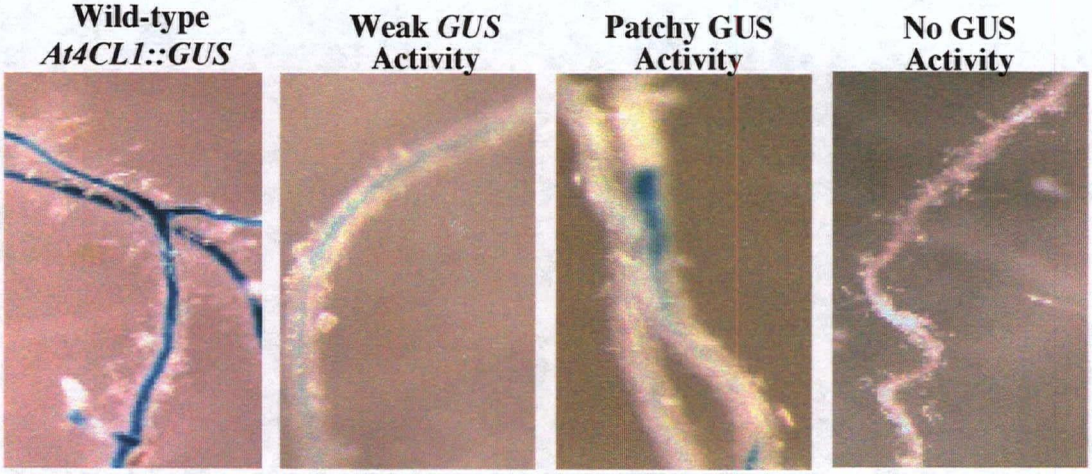


Figure 3-7 Histochemical analysis of GUS activity in the roots of representative putative *At4CL::GUS* expression mutants.

All of the putative mutants in the *4CL1::GUS* background (e.g. mutant 1-A) were severely affected in *GUS* expression in the root, with slight or no reduction of *GUS* expression in the veins of the cotyledons. At maturity, these mutant plants showed global loss of *GUS* expression in all organs (Figure 3-8). The strongest mutants in the *4CL2::GUS* background (e.g. mutant 2-8) showed global loss of developmental *GUS* expression in both seedlings and mature plants (Figure 3-9).

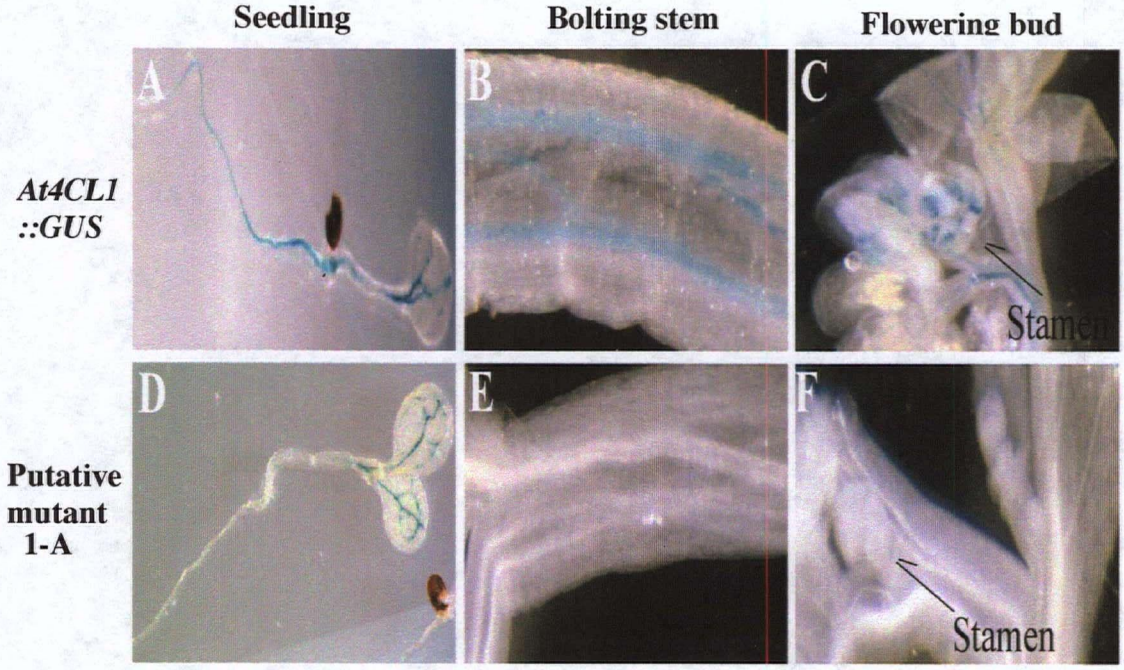


Figure 3-8 Histochemical analysis of GUS activity in the 1-A putative mutant is compared with GUS activity in the progenitor background *At4CL1::GUS* line.

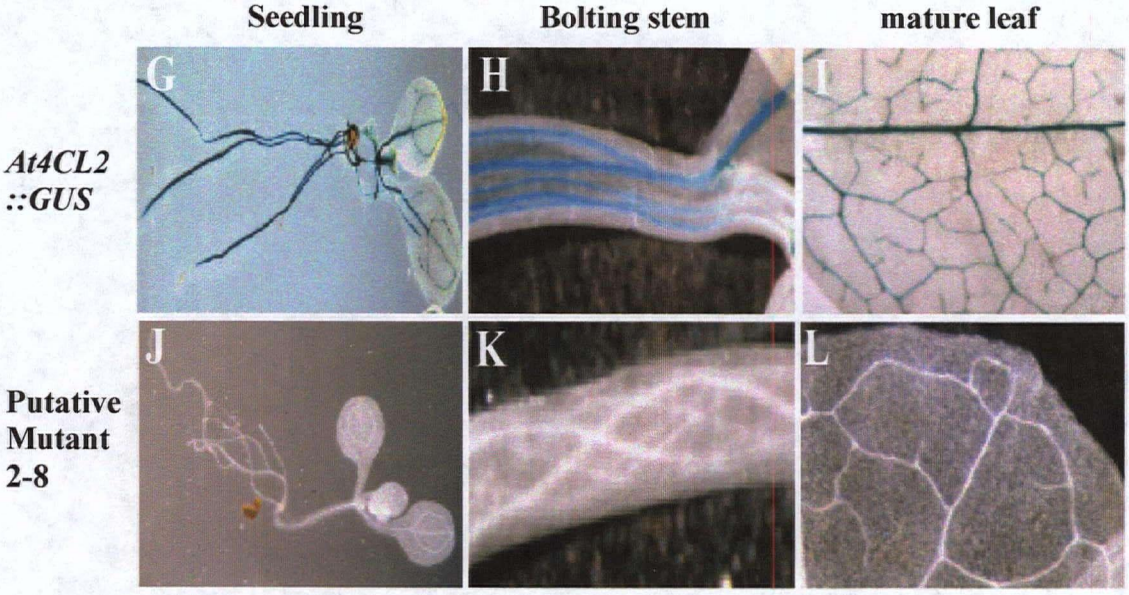


Figure 3-9 Histochemical analysis of GUS activity in the putative mutant 2-8 is compared with GUS activity in the progenitor background *At4CL2::GUS* line. Mutant (2-8), was originally identified by Dr. Juergen Ehlting.

No morphological differences were detected in putative mutants (1-A, 1-B, & 1-C) relative to the progenitor line (*At4CL1::GUS*) and wild-type plants (Figure 3-10 left). Only one of the mutants (1-C) showed a longer hypocotyl compared to non-mutant plants (Figure 3-10 right) but further genetic analysis showed that the long hypocotyl phenotype was not linked to the GUS down regulation phenotype.

Figure 3-10 Growth and development of putative mutants. Left, Five-week old mutants and control plants. Right, long hypocotyl phenotype in mutant 1-C

3.2.2.3 Genetic analysis of putative mutants lines

Individuals of all 14 putative *4CL1::GUS* mutant lines were crossed to the non-mutant *4CL1::GUS* transgenic parental line both as pollen donors or recipients. Inheritance of the mutant phenotype (loss of histochemically detectable GUS activity) was analyzed in the F1 and F2 generations. In all the cases, the phenotype was inherited in a non-Mendelian manner as shown in Table 3-1 for three of these mutants (1-A, 1-B and 1-C).

Table 3-1 Genetic analysis of *4CL1::GUS* mutants

Male parent	Female parent	F1 phenotype		F2 phenotype	
		#GUS+	#GUS-	#GUS+	#GUS-
WT[1]	1-A-1[2]	30	70	0	80
1-A-1	WT	25	10	0	50
WT	1-A-2	10	30	0	45
1-A-2	WT	25	35	0	50
WT	1-B-1	0	70	0	42
1-B-1	WT	25	35	0	47
WT	1-B-2	10	20	0	45
1-B-2	WT	0	40	0	40
WT	1-C-1	0	10	0	55
1-C-1	WT	1	115	0	35
WT	1-C-2	0	20	0	50
1-C-2	WT	0	3	0	45

[1] The wild-type line used was the *4CL1::GUS* progenitor line

[2] Two separate individuals from each of the mutant lines 1-A, 1-B, and 1-C were crossed in a reciprocal manner to WT (*4CL1::GUS)* plants

Similar results were observed by Dr. Ehlting in crosses of more than 10 putative *4CL2::GUS* expression mutants (such as mutant 2-8). To exclude the possibility of mutations in the *At4CL::GUS* transgenes, we sequenced the *4CL1::GUS* and *4CL2::GUS* transgenes in the 1-A and 2-8 mutant lines and confirmed their integrity.

Crosses of non-mutagenized *At4CL2::GUS* lines from more advanced generations to wild type plants showed Mendelian segregation of both kanamycin resistance and *GUS* expression phenotypes, in leaf veins, as expected. However, in spite of the Mendelian inheritance of the kanamycin resistance phenotype, 83% of analyzed F2 plants derived

from the *At4CL1::GUS* line (T14) crossed to wild type showed root specific lack of *GUS* expression. These results suggest that the putative mutants affected in *At4CL::GUS* expression may be epigenetic mutants affected in transgene expression through transgene silencing, and that such silencing can occur without EMS mutagenesis in these lines.

3.2.2.4 Treatment of mutant lines with 5-aza

To examine the possibility of *At4CL::GUS* transgene DNA methylation in the mutant lines, I grew three mutant lines in the *At4CL1::GUS* back ground (1-A, 1-B, 1-C) and one mutant in the *At4CL2::GUS* background (mutant 2-8) in MS media supplemented with 5-aza and kanamycin. Histochemical analysis of GUS expression in 10 day old seedlings in each case showed restoration of *GUS* expression in > 90% of seedling roots treated with 5-aza while non-treated control seedlings showed the mutant phenotype. Representative results for 1-A and 2-8 mutants are shown in Figure 3-11.

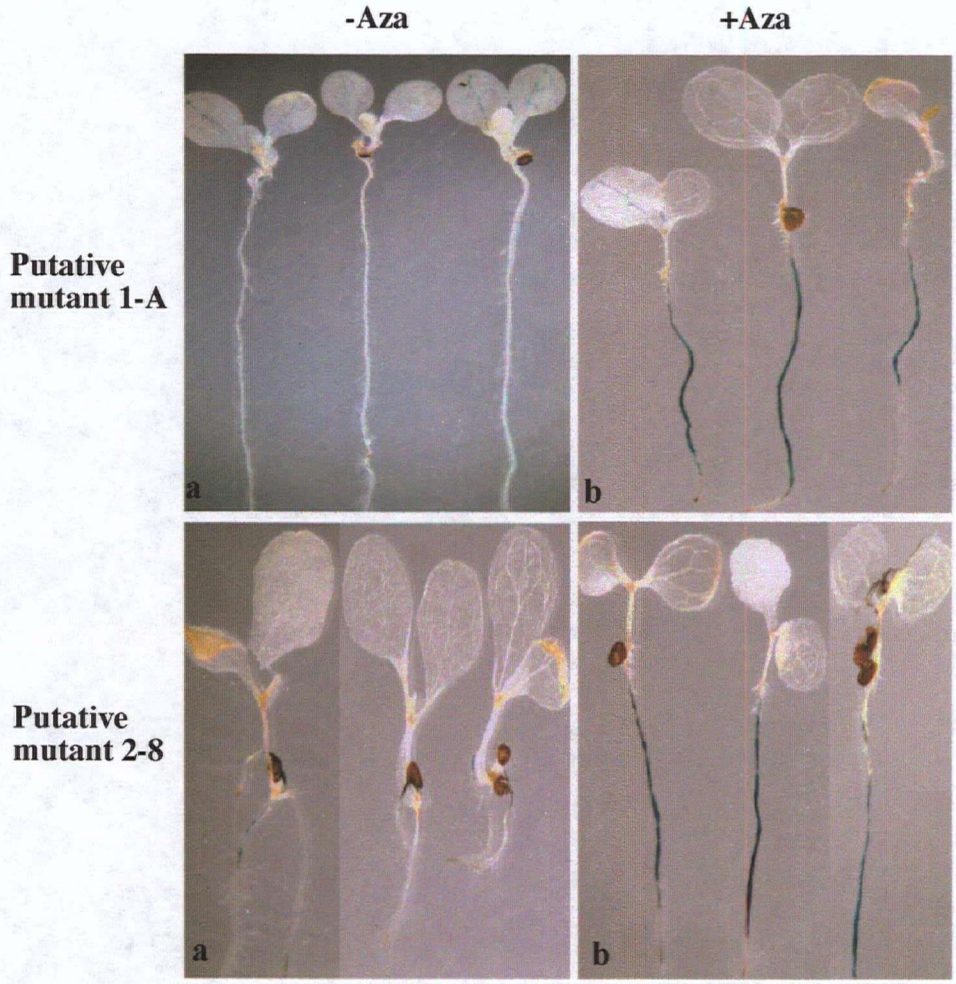


Figure 3-11 *GUS* expression in *At4CL::GUS* mutants in the presence or absence of 5-aza. Representative phenotypes from treatment of 10 ten-day old seedlings from each line are shown. Wild type and non-mutagenized controls are shown in Figure 3-5 B.

These results strongly suggest that transgene DNA methylation plays a role in the loss of *GUS* expression observed in mutants, and suggests that the mutants are in fact epimutant in which the transgene has been epigenetically silenced.

During the course of mutant screening, the non-mutated *At4CL1::GUS* line was also selfed and used as positive controls for the GUS assays. I observed an increasing frequency of GUS down regulation in this non-mutated line after the T7 generation. This spontaneously occurring GUS down-regulation phenotype of the *At4CL1::GUS* line was

similar to that of mutant 1-A shown. Such variation was not observed in the *At4CL2::GUS* line. When I grew seeds from the original T5 generation of the *At4CL1::GUS* line, 10 day-old seedlings also showed variation of *GUS* expression which was removed by 5-aza treatment (Figure 3-12). This is also supportive of my suggestion that the *At4CL1::GUS* mutants we identified are epimutants that arose in the population by epigenetic silencing of the transgene.

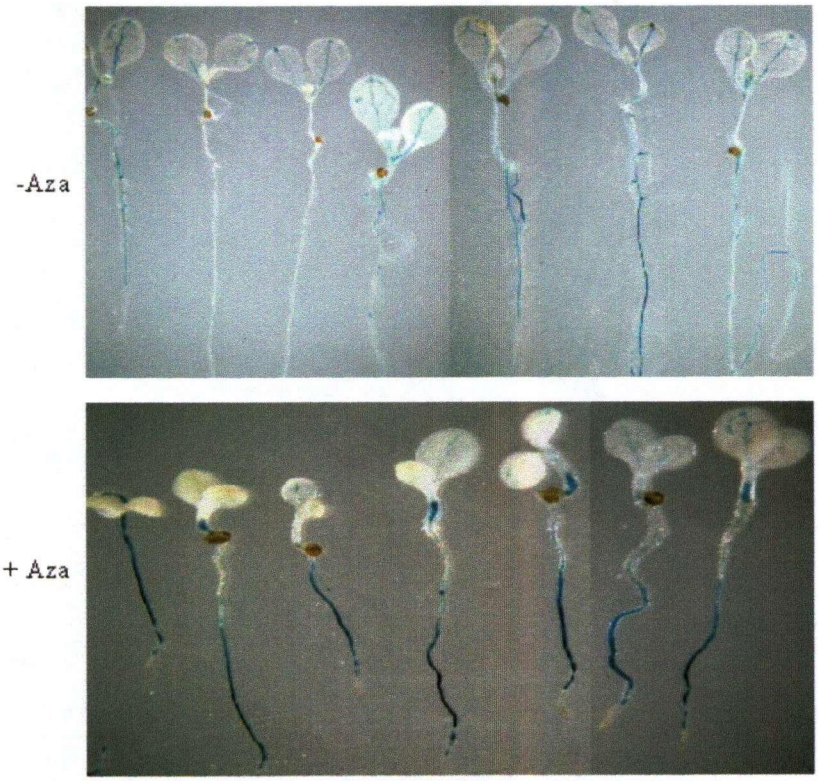


Figure 3-12 *GUS* expression in T5 generation seedlings of the *At4CL1::GUS* line in the presence and absence of 5-aza.

3.2.2.5 Southern Blot Analysis of Mutant Lines

To investigate the methylation status of the transgene promoters in the mutant lines, I used methylation sensitive isoschizomer restriction enzymes such as *Msp*I/*Hpa*II and *Mbo*I/*Sau*3A1 to restrict the genomic DNA from mutants 1-A and 2-8 respectively. *Msp*I is only inhibited when the outermost cytosine in its recognition site (**C**CGG) is methylated, allowing detection of methylation in a non-CG context, and is otherwise is

able to cut at the recognition site. However, the *Hpa*II is inhibited by methylation of either cytosine of this recognition site allowing detection of CpG and CpNpG methylation (Cao and Jacobsen 2002). As shown in Figure 3-13, there is a single *Msp*I/*Hpa*II (T**CCGG**T) restriction site in the 1 kb *At4CL1::GUS* promoter, whose cleavage in combination with cleavage in the T-DNA and *GUS Msp*I/*Hpa*II sites would result in generation of 266-bp and 870-bp promoter fragments. Cleavage of the promoter of endogenous *At4CL1* gene is predicted to yield 1200-bp and 228-bp promoter fragments. Several other recognition sites in the transcribed regions of *GUS* and *At4CL1* result in ~ 88-245 bp fragments.

I hybridized Southern blots of *Msp*I or *Hpa*II restricted genomic DNA from mutant and control lines with two different probes spanning different areas of the *4CL1::GUS* transgene. As shown in Figure 3-13, probe #1 hybridized only to 1200-bp fragments in wild-type DNA cut with either enzyme, while the probe hybridized to 1200-bp and 870-bp fragments in the *At4CL1::GUS* control line after both *Hpa*II and *Msp*I digestion, showing that the *Msp*I/*Hpa*II restriction site was not methylated in this line. In the line 1-A also, probe #1 detected a 1200-bp band but this fragment would co-migrate with a smaller 1136-bp band, resulting from the fusion of 266 and 870 bp bands due to lack of cleavage at the methylated *MspI/HpaII* site. The ~1136-bp band in *Hpa*II restricted DNA of line 1-A is clearly thicker and more intense than the band of similar size of the same DNA restricted by *Msp*I, suggesting the presence of the predicted doublet band. This indicates CG methylation of the recognition site and partial CNG methylation of the same site.

Similarly, probe #2 hybridized strongly to the expected 870-bp and 260-bp fragments in the DNA from *At4CL1::GUS* line and weakly to the 1200 bp fragments of the endogenous gene generated by both *Hpa*II and *Msp*I digestion of *At4CL1::GUS* control DNA. In contrast, hybridization of #2 to digested genomic DNA isolated from mutant 1-A roots and seedlings revealed a 1136-bp band that was stronger in the *Hpa*II restricted DNA and also a band bigger than 1200 bp that was unique to the *Hpa*II restricted DNA (bands marked with asterisks, Figure 3-13). The sizes of these fragments suggest that they arose as a result of the inability of *Hpa*II to cut its recognition site within the *At4CL1::GUS* and/or endogenous *At4CL1* promoters. Methylation of other

*Msp*I sites upstream of the *At4CL1::GUS* transgene in the T-DNA may created a >1200-bp band.

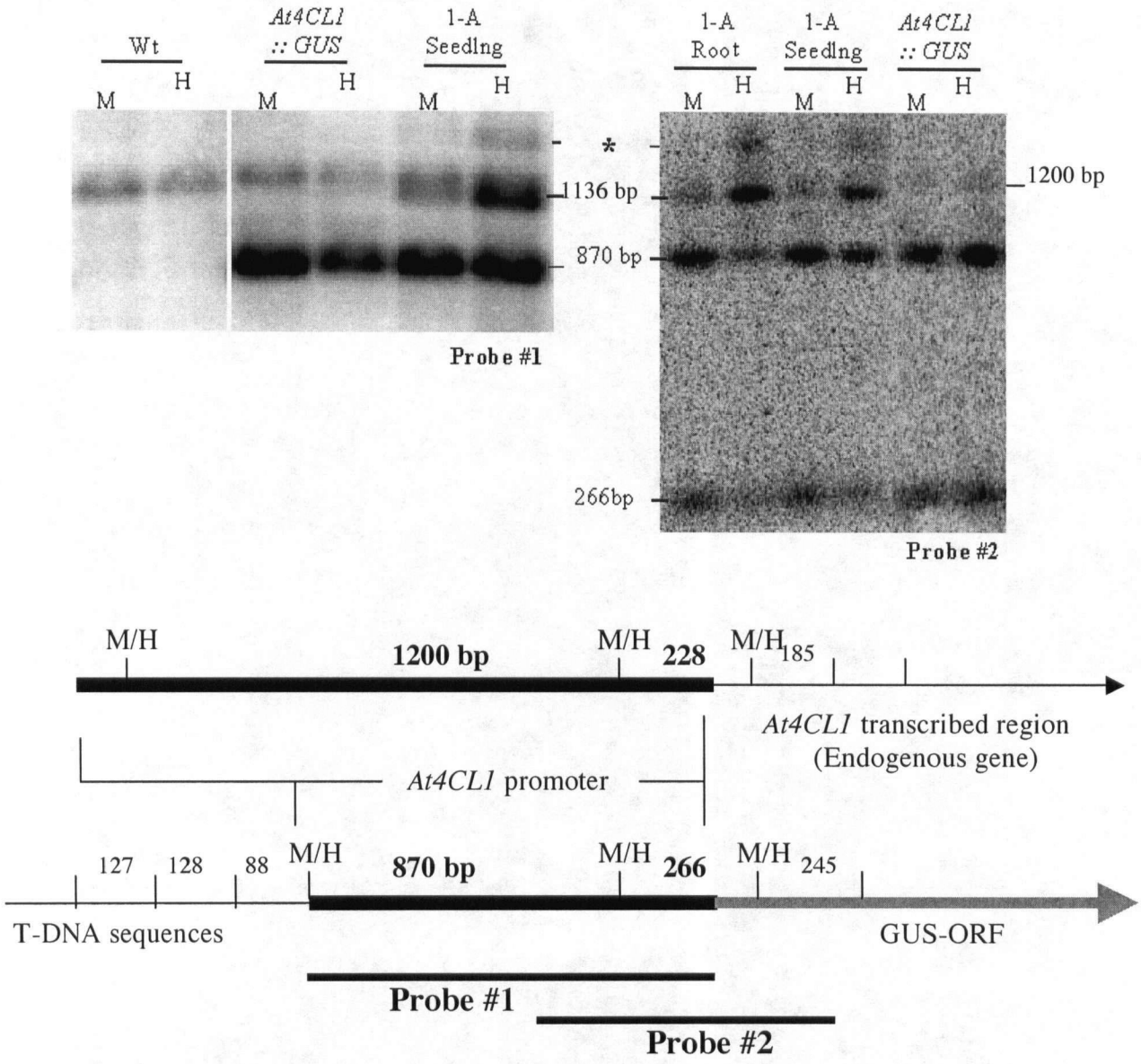


Figure 3-13 Southern blot analysis of methylation status of the *At4CL1* promoter in mutant 1-A seedlings. The positions of radiolabeled probes #1 and #2 are shown below restriction maps of the endogenous *At4CL1* gene and the *At4CL1::GUS* transgene. M/H

indicates *Msp*I and *Hpa*II respectively. Sizes of predicted restriction fragments are given in bp. Probe #2 was more biased towards the transgene sequences including the *GUS* gene.

I conducted a similar experiment to investigate the methylation states of the *At4CL2* promoter in the mutant 2-8. In this case, I used the *MboI*/*Sau*3AI isoschizomer pair, with *Sau*3A being methylation sensitive and its specificity determined by bases flanking the GATC core recognition site. As shown in Figure 3-14, there is a single GATC *MboI*/*Sau*3AI recognition site in the *At4CL2* promoter. This analysis showed that digestion of mutant 2-8 genomic DNA with *Sau*3A generated an *At4CL2* promoter fragment unique to the mutant. The size of this fragment (1165bp, Figure 3-14) is consistent with failure of *Sau*3A to cut at the *Mbo*/*Sau*3AI recognition site in *At4CL2* promoter, resulting of the fusion of 979 bp and 186 bp fragments to yield a 1165 bp *Sau*3A fragment in the mutant. This fragment was not observed in *Sau*3A digested DNA from the *A4CL2::GUS* control line. These data are consistent with increased methylation of the *At4CL1* and *At4CL2* promoters in epigenetic mutant lines in which *GUS* expression is silenced.

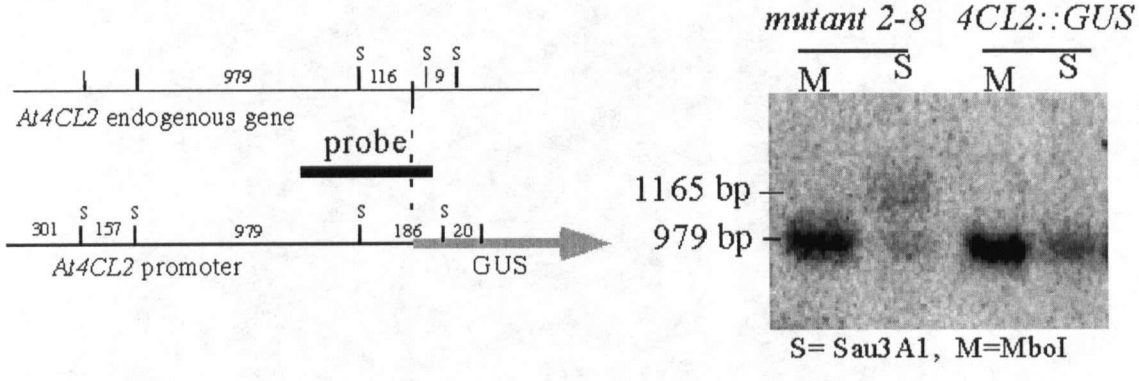


Figure 3-14 Southern blot analysis of methylation status of the A*t4CL2* promoter in the mutant 2-8 seedlings. The position of the radiolabeled probe is shown between restriction maps of the endogenous *At4CL2* gene and the *At4CL2::GUS* transgene. S, *MboI*/*Sau*3AI restriction sites. Sized of predicted restriction fragments are given in bp.

3.2.2.6 Southern Blot Analysis of Transgene Copy Number

In order to determine the copy number of transgenes in the *At4CL::GUS* transgenic lines, Southern blot analysis was conducted. Restriction enzymes indicated in Figure 3-15 do not cut in the *At4CL* promoter::*GUS* transgene contained in the T-DNA, ensuring only one band to be detected per T-DNA molecule using GUS sequences as probe. Multiple bands present in each digest suggest the presence of ~ six *4CL1::GUS* and two *4CL2:: GUS* transgenes

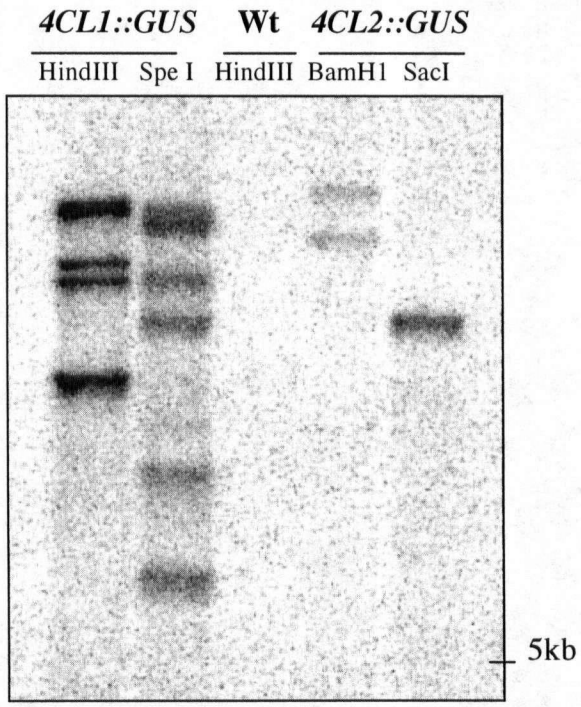


Figure 3-15

Investigation of *At4CL::GUS* transgene copy number in the transgenic lines. Southern analysis was performed using the enzymes indicated. Bands corresponding to the *At4CL1::GUS* transgene should be larger than 6 kb and those corresponding to the *At4CL1::GUS* transgene should be larger than 5.5 kb.

3.2.2.7 Northern blot analysis of mutant lines

To investigate the expression status of the endogenous *At4CL1* gene in the mutant 1-A and a representative *4CL1::dhlA/At4CL1::GUS* line, both of which showed transgene silencing, northern blot analyses were conducted using RNA extracted from 10 day old seedlings and a *At4CL1*-specific probe. The results, consistent among several northern blot experiments, showed that the endogenous *At4CL1* gene was not down regulated (lanes 1 and 4, Figure 3-16) relative to expression in control plants.

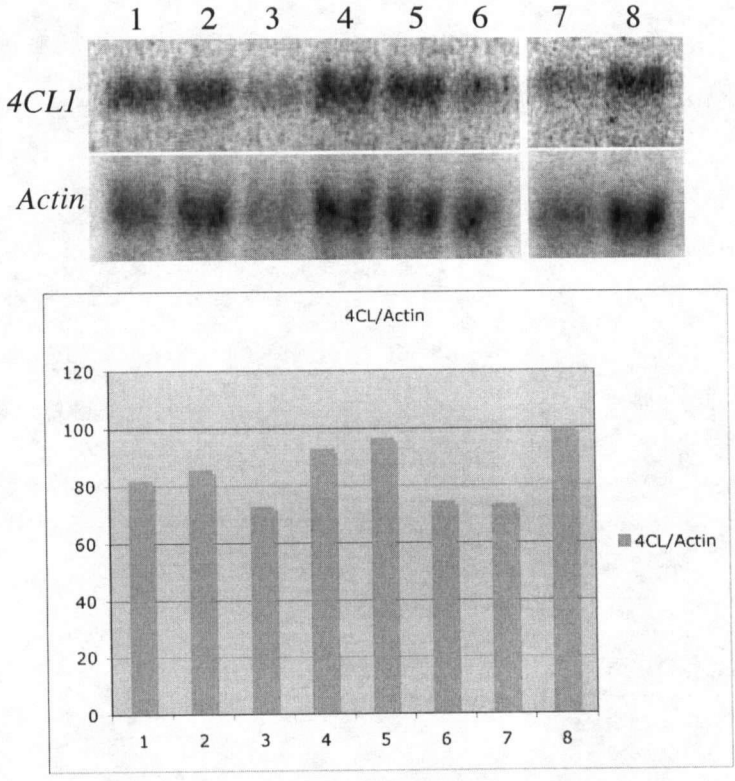


Figure 3-16 Northern analysis of *At4CL1* gene expression in *At4CL1::GUS* lines. Total RNA was extracted from 1) mutant 1-A, 2) Wild-type, 3) *At4CL1::GUS*, 4) *At4CL1::dhlA*, 5) mutant sil1-1, 6) mutant hog1-1, 7) Mutant 2-8, 8) Wild-type. The blot was hybridized to an *At4CL1*-specific probe, then stripped and hybridized to an actin probe to control for RNA loading. Intensities of *At4CL1* signals relative to actin signals are shown at the bottom. ~20% difference differences between the samples were not

significant in repeated tests as indicated by variability in two wild type samples #2 and # 8.

I also investigated the expression status of the endogenous *At4CL2* gene in the mutant 2-8 and a representative *4CL2::dhlA/At4CL2::GUS* line, using an *At4CL2*-specfic probe. Again, the results were consistent among multiple experiments, but in contrast to the *At4CL1* lines, showed that the endogenous *At4CL2* gene was significantly down regulated in the silenced lines (lanes 1 and 4, Figure 3-17). Silencing of the endogenous gene was specific to mutant 2-8, since *At4CL2* expression was not affected in mutant 1-A, affected in *At4CL1::GUS* expression (Figure 3-17, lane 5). This suggests that *cis*-silencing (affecting the transgenes alone) occurred in the mutant 1-A and the *4CL1::dhlA/At4CL1::GUS* line, while both *cis*- and *trans*- silencing occurred in the mutant 2-8 and the *At4CL2::dhlA/At4CL2::GUS* line.

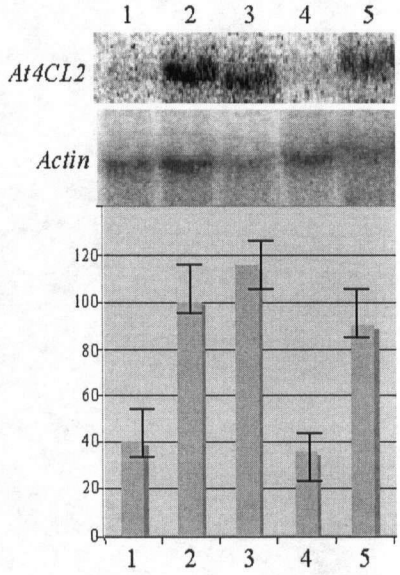


Figure 3-17 Northern analysis of *At4CL2* gene expression in *At4CL2::GUS* lines, Total RNA was extracted from 1) mutant 2-8, 2) wild-type, 3) *At4CL2::GUS,* 4) *At4CL2::dhlA*, 5) mutant 1-A lines. The blot was hybridized to an *At4CL2*-specific probe, then stripped and hybridized to an actin probe to control for RNA loading. Intensities of *At4CL2* signals relative to actin signals are shown at the bottom. Band intensities were measured

in this experiment, and two replicates. The average intensities ± standard error are shown at the bottom

3.2.2.8 Effect of two silencing modifier mutants on *4CL::GUS* transgene silencing

To better understand the silencing mechanisms of the *At4CL::GUS* transgenic lines, I crossed the mutants 1-A and 2-8 to the *Arabidopsis hog1 and sil1* mutants that are defective in homology-dependent gene silencing of the *Arabidopsis CHS* gene (Furner *et al.*, 1998). The hog1 (homology dependent gene silencing 1) is a partial loss of function mutation in the S-adenosyl-L- homocytosine hydrolase enzyme that generally inhibits trans-methylation metabolism. In the *hog1-1* mutant the activity of this hydrolase enzyme is reduced (Rocha et al. 2005). The sil1 mutant (modifiers of silencing 1, Furner et al., 1998) is a new allele of histone deacetylase (*AtHDA6)* and mutations in the this gene influence histone acetylation levels and reactivate silent and methylated transgenes and endogenous repeats and confer partial demethylation of CG sequences at specific regions of the genome (Probst *et al.*, 2004). Crossing of mutants 1-A and 2-8 as male parents into the *hog1-1* and *sil1-1*, did not restore the expression of *At4CL1::GUS* or *At4CL2::GUS* transgenes in the kanamycin resistant F2 generation. These results suggest the existence of distinct gene silencing pathways for homology dependent *CHS* and *At4CL* gene silencing pathways.

3.3 DISCUSSION

3.3.1 *At4CL1* and *At4CL2* promoters direct similar GUS expression patterns in the vascular tissues.

Fusion of *At4CL1* and *At4CL2* promoters to the *GUS* reporter gene and generation of transgenic *Arabidopsis* plants showed that the promoters direct overlapping patterns of vascular-specific *GUS* expression in different organs (Figure 3-1), similar to the pattern described for *Arabidopsis* plants expressing a parsley *4CL1*promoter-*GUS* fusion (Lee *et al.*, 1995). The overlapping patterns of vascular-specific expression directed by *At4CL1* and *At4CL2* promoters were predictable considering the presence of multiple common conserved *cis* regulatory elements, and similar expression patterns characterized by northern analysis (Ehlting *et al.*, 1999). Consistent with previous northern analysis, I

found the strongest *GUS* expression of *At4CL1::GUS* and *At4CL2::GUS* transgenes in the roots of the seedlings, and strong *GUS* expression in the vasculature of bolting stems. Taken together with the high identities of the *At4CL1* and *At4CL2* coding sequences and the similar substrate specificities of the recombinant enzymes (Ehlting *et al.*, 1999), the similar expression patterns dictated by their promoters suggest functional redundancy of these two genes. Since 4CL plays an essential role in lignin biosynthesis, loss of endogenous *4CL* expression would be predicted to lead to a severe phenotype. However, functional redundancy is a likely explanation for the lack of an obvious phenotype in mutant 2-8, in which the endogenous *At4CL2* gene was down regulated (Figure 3-17) together with the *At4CL2::GUS* transgene (Figure 3-9).

3.3.2 Evidence that mutants affected in *At4CL::GUS* expression are epigenetically silenced epimutants.

We screened EMS mutagenized M2 populations and found multiple putative mutants impaired in *4CL1::GUS* or *4CL2::GUS* transgene expression. These mutants showed different intensities and patterns of reduced developmental *GUS* expression (Figure 3-7), initially suggesting potential lesions in genes required to activate developmentally regulated *GUS* expression. Selected mutants were crossed to the progenitor wild-type transgenic lines, and the inheritance of the *GUS* expression phenotype was analyzed in F1 and F2 generations. The results of the analysis of three mutants in the *At4CL1::GUS* background (summarized in Table 3-1) showed non-Mendelian inheritance of the phenotypes. I focused on the mutant 1-A as a representative mutant of the *At4CL1::GUS* line showing root-specific lack of *GUS* expression in seedlings, but global loss of *GUS* expression in the mature plants (Figure 3-8) and mutant 2-8 as a representative mutant in the *4CL2::GUS* transgenic background showing apparent complete loss of developmental *GUS* expression (Figure 3-9). Genetic analysis of this mutant also showed non-Mendelian inheritance (Dr. J. Ehlting, personal communication). Based on the non-Mendelian inheritance of the mutant phenotypes we hypothesized the involvement of transgene silencing in generating the *GUS* expression phenotypes, rather than mutations in second genes required for *4CL* promoter activation.

Sequencing of transgenes in the mutant plants confirmed sequence integrity, and after several generations in which the lack of *GUS* expression was stably inherited, treatment of mutant lines with 5-aza efficiently restored *GUS* expression, suggesting reversible silencing of the transgenes, associated with changes in methylation status of DNA (Figure 3-11), since this reagent is a well-characterized inhibitor of cytosine methylation (Fieldes 1994). Consistent with this interpretation, Southern blot analysis of mutants confirmed increased methylation of the respective *4CL* promoters in the 1-A and 2-8 mutants (Figure 3-13 and 3-14). Taken together, these data strongly suggest that the mutants we isolated are epigenetic mutants (epimutants) of the *At4CL::GUS* transgenes, and that epigenetic silencing is mediated by methylation of promoter DNA.

3.3.3 *At4CL::GUS* transgene silencing is specific and occurs naturally.

GUS expression was stable in the *At4CL2::GUS* line from the T1-T16 generation, but introduction of an *At4CL2*::*dhlA* construct into *At4CL2*::*GUS* transgenic plants in the T5 generation resulted in *GUS* down regulation in >50% of the lines similar to what we saw for *At4CL1::dhlA* lines (Figure 3-4). Also, none of the lines were sensitive to DCE treatment (Figure 3-2), which would be predicted if the *At4CL2* promoters were inactive in driving *dhlA* expression. I hypothesized that both of the *At4CL2*::*dhlA* and *At4CL2*::*GUS* transgenes were silenced in these lines, triggered by introduction of the *At4CL2*::*dhlA* transgene. Treatment of these lines with 5-aza made them more sensitive to DCE treatment (Figure 3-6) and also restored *GUS* expression (Figure 3-5). Similar results were observed for *At4CL1::dhlA* lines. Also, the occurrence of *GUS* down regulation similar to the mutant 1-A phenotype in the non-mutated original T5 generation of *At4CL1*::*GUS* transgenic plants became apparent after three years reservation of seeds, and this phenotype was reversed by 5-aza treatment (Figure 3-12). These data suggest that silencing of *4CL::GUS* transgenes occurred in *At4CL1*::*GUS* and *At4CL::dhlA* lines in the absence of EMS mutagenesis, and that mutants such as 1-A and 2-8 represent pre-existing epigenetically silenced variants.

Epimutants were isolated from seedlings grown on kanamycin containing media. This probably selected against epimutants in which the entire T-DNA was methylated. On the other hand, the occurrence of kanamycin resistant epimutants indicates that the

region of the T-DNA containing transgene had been specifically silenced. Apparently, the silencing machinery specifically targeted the *At4CL::GUS* transgene. Southern analysis showed that methylation of the transgene was restricted to their promoters (Figures 3-13 and 3-14).

HDGS related mechanisms have been reported to be involved in the regulation of gene expression of gene family members in non-transgenic plants (Martienssen, 1998; Luff *et al.,* 1999; Chandler, 2000). The down regulation of endogenous *At4CL2* gene expression in the mutant 2-8 was specific to *At4CL2* and *At4CL1* was not affected (Figure 3-16 line 7). Conversely, *At4CL2* gene expression was not affected in the mutant 1-A, which affected *At4CL1::GUS* expression (Figure 3-17 line 5). To confirm the specificity of *At4CL::GUS* silencing, I crossed mutant 1-A to the *At4CL2::GUS* line and mutant 2-8 to the *At4CL1::GUS* line to see if silencing of the *At4CL1::GUS* transgene could trigger silencing of *At4CL2::GUS* and vice versa. F1 seedlings showed *GUS* expression in both crosses suggesting that silencing of *At4CL1::GUS* may not affect the expression of the *At4CL2::GUS* transgene and vice versa (data not shown). However, an alternative explanation for these results cannot be ruled out. In summary, these data suggest that transgene-triggered gene silencing occurred in the lines investigated, and that this silencing is gene family member specific.

3.3.4 Silencing of *At4CL11* and *At4CL2* (trans) genes is best explained through a 5'-UTR threshold mechanism.

Although TGS and PTGS are mechanistically related methods of silencing (Fagard and Vaucheret 2000; Sijen *et al.,* 2002), specific methylation of multiple identical promoters of (trans) genes and 5-aza-restorable expression point to TGS versus PTGS of *At4CL::GUS* transgenes. To evaluate the critical characteristics of the transgene loci that could have directed TGS of *At4CL::GUS* and *At4CL::dhlA* transgenes we considered variables such as transgene locus structure, chromosomal environment of transgene and transgene expression level that have been shown to affect TGS (Fagard and Vaucheret 2000).

The presence of inverted repeat sequences in transgenes (Wang and Waterhouse, 2000, Chuang and Meyerowitz, 2000; Mette *et al.,* 2000), and direct or inverted repeat

arrangements of multiple T-DNAs (Hobbs *et al.,* 1990, Assaad *et al.,* 1993, Jorgensen *et al.,* 1996) have been associated with silencing. However, tandem T-DNA configurations and arrangements may not be sufficient to trigger TGS (Lechtenberg *et al.,* 2003, Shubert *et al.,* 2004). Since TGS of *At4CL1::GUS* line became apparent spontaneously in the T7 generation, the arrangement of multiple T-DNAs carrying the *At4CL1::GUS* transgene could play a role. However, regardless of transgene copy number and arrangement, TGS of the *At4CL2::GUS* transgene was detectable only after introduction of an independent *At4CL2::dhlA* transgene into this line. Therefore, it is hard to consider locus arrangement of the transgenes to be the primary cause of *At4CL2::GUS* transgene silencing.

TGS has also been attributed to the chromosomal environment of transgenes (Jones *et al.,* 1985; Peach and Velten 1991; Lohuis *et al.,* 1995; Jakowitsch *et al.,* 1999; Day *et al.,* 2000). Also, abrupt change in GC content of flanking sequences is considered to be a factor affecting silencing (Meyer *et al.,* 1993; Matzke and Matzke 1998). However, similar to other reports (Jorgensen *et al.,* 1996; Hobbs *et al.,* 1993; Schubert *et al.,* 2004) it seems that transgene chromosomal position may not have played a primary role in triggering *At4CL2::GUS* or *At4CL2::dhlA* silencing. If chromosomal sequences flanking the *At4CL2::GUS* transgene locus, for example, had a role in its silencing, this should have been effective before the insertion of the second transgene (*At4CL2::dhlA*) or during the 14 generations of stable transgene expression in this line. However, we may attribute the low rate (0.125%) of silencing in the *At4CL2::GUS* line (represented by mutant 2-8) to the spontaneous TGS (Prols and Meyer, 1992). Then, increased frequency of transgene silencing *At4CL2::dhlA* line could be attributed to the trans-silencing through DNA pairing interaction (Matzke *et al.,* 1994; Bender *et al.,* 1995; Park *et al.,* 1996). For the *At4CL1::GUS* line, we cannot rule out the involvement of transgene locus environment in TGS, since transgene expression was less stable in this line.

Some reports (Lindbo *et al.,* 1993; Jorgenson *et al.,* 1996; Wassenegger *et al.,* 1998) suggest that surpassing a threshold level of transgene expression may result in silencing. This hypothesis is supported by a correlation between frequency of silencing and transgene promoter strength (Que *et al.,* 1997) and also the report of stronger silencing in homozygous transgenic plants compared with hemizygous siblings (Vaucheret *et al.,* 1998). In a recent report it is suggested that if transgene expression

levels exceed a specific threshold, PTGS will be triggered, but at the sub-threshold expression levels, the expression from multi-copy transgenes is additive (Schubert *et al.*, 2004).

Only after retransformation of the *At4CL2::GUS* line with the additional *At4CL2::dhlA* construct, the *At4CL2::GUS* transgene was silenced, suggesting trans-inactivation phenomena (Matzke 1993) has happened. Based on the threshold hypothesis, this could be explained on the basis that the transgene transcript copy number may not have surpassed the hypothetical threshold for silencing in *At4CL2::GUS* plants until the introduction of the second transgene (*At4CL2::dhlA*) with a common *At4CL2* promoter and 5'-UTR sequence. We suggest that 5'-UTR common to both transcripts was detected by a *At4CL2* gene-specific threshold sensing system, causing *trans*-silencing of *At4CL2::reporter* transgenes and the endogenous *At4CL2* gene in the *At4C2::dhlA* and mutant 2-8 lines (Figure 3-17). Sporadic up regulated *At4CL2::GUS* transgene expression in the vicinity of enhancers may have elevated transgene expression above the threshold and resulted in epimutants like mutant 2-8. Based on this hypothesis we predict that the endogenous *At4CL2* promoter as well as the *At4CL2::GUS* transgene promoter should be hyper-methylated, but the analysis shown in Figure 3-14 could not distinguish between the endogenous and transgene promoters. This hypothesis could also be further tested by introduction of *At4CL2* 5'-UTR sequences in *At4CL2::GUS* plants under the control of a strong promoter other than *At4CL2* promoter. We would predict this to result in a co-suppression like phenomenon (Napoli *et al.*, 1990).

Contributions of aberrant promoter transcripts to *trans*-TGS of un-linked transgene has also been reported (Mette *et al.*, 1999, and 2000; Sijen *et al.*, 2001) and it is widely accepted that aberrant RNA species direct some, if not all of the heterochromatin formation in the plants (Bender 2004). The 5'-UTRs of *At4CL1* and *At4CL2* genes are not predicted to form dsRNA structures, but we cannot rule out the involvement of chimeric transgene transcripts in the silencing. Taken together, our data are not consistent with the involvement of transgene structure or chromosomal environment in the TGS we observed. Rather it seems *At4CL2::GUS* and *At4CL2::dhlA* TGS is mostly consistent with a transcript threshold mechanism, which is able to detect common 5'-UTRs in transcripts derived from different genes.

3.3.5 Unanswered aspects of *At4CL11* and *At4CL2* (trans) gene silencing

An alternative explanation for the observed silencing is that *At4CL2* 5'-UTR RNA sequences may have surpassed the threshold and/or were involved in the formation of an aberrant RNA, resulting in dsRNA triggered activation of the PTGS system and simultaneous promoter sequence methylation. This would explain the silencing of the endogenous *At4CL2* gene. This scenario may not apply for the silencing of the *At4CL1::GUS* line, as the endogenous *At4CL1* gene was shown to be unaffected (Figure 3-16).

There is no clear explanation for the lack of endogenous *At4CL1* gene down regulation in lines silenced for *At4CL1::GUS* expression. The 1-kb *At4CL1* promoter sequence used to generate *At4CL1::GUS* transgene has 27% G.C pairs content including 15 CG dimers and 18 CNG trimers that could be substrates for methylation. In the 1.55-kb *At4CL2* promoter, there are 37% G.C pairs including 19 CG dimers and 54 CNG trimers. If promoter methylation has been triggered by dsRNA produced from the transgenes transcripts, methylation of the target *At4CL1* promoter would not be as dense as methylation of the target *At4CL2* promoter simply because of lower number of CG and CNG sequences. Therefore, less *At4CL1* promoter methylation may have resulted in weaker TGS manifested by unaffected endogenous *At4CL1* gene expression.

The root-specific silencing of *At4CL1::GUS* in seedlings is also puzzling. However, the *At4CL1* gene has been shown to have the highest expression level in the root (Ehlting *et al.*, 1999). This may have made it easier for *At4CL1::GUS* transgene expression to surpass the threshold level of 5'-UTR containing RNA in the root, resulting in preferential *At4CL1::GUS* silencing of the transgene in that organ. Elevated *4CL1* gene expression in the bolting stem and elsewhere later in development (Elthing *et al.*, 1999) may have resulted the threshold of expression being surpassed, resulting in TGS (Figure 3-8). Therefore lack of *At4CL1::GUS* line silencing in the veins of cotyledons may be attributed to the lower expression level of *At4CL1* gene in this area. However, it is not clear why silencing *At4CL1::GUS* transgenic in the mature plants is not meiotically heritable to the cotyledons of the next generations.

3.3.6 Conclusion

At4CL1 and *At4CL2* genes are closely related and show overlapping expression patterns. Although there are reports of silencing between alleles (paramutation; Brink 1973, Matzke and Matzke, 1993) and duplicated members of gene families (Matzke 1996), cross silencing between these two genes did not occur, suggesting that *At4CL* transgene silencing mechanisms are able to distinguish between related family members possibly through non homologous promoter sequences.

Mechanisms underlying transgene silencing still are not fully known. The *hog1* and *sil1* mutants, that affect the pathway used for *CHS* silencing, did not affect *At4CL::GUS* transgene silencing and *At4CL* gene expression (Figure 3-16), suggesting that that distinct mechanisms underlie silencing of these genes, both involved the phenylpropanoid pathway. It is now clear that dsRNA acts as a sequence specific signal for TGS by promoting promoter methylation (Mathieu and Bender, 2004). If such a hypothetical dsRNA were GUS specific, we should have seen signs of GUS down regulation in F1 plants derived from crossing mutant 1-A to *At4CL2::GUS* plants or mutant 2-8 to *At4CL1::GUS* plants. The lack of such down regulation, and other clues lead us to favor a role of gene-specific *5-UTR*s in triggering an RNAi system and subsequent TGS of *At4CL* genes. However, regardless of the mechanism of silencing, *At4CL1::GUS* and *At4CL2::GUS* lines illustrate the diversity of silencing phenomena that may be encountered upon transgenes insertion and expression.

CHAPTER 4

DEVELOPMENTAL AND WOUNDING RESPONSE *CIS* ELEMENTS IN *AT4CL2 GENE*

4.1 INTRODUCTION

The general phenylpropanoid pathway in plants channels carbon flow to different branch pathways via sequential actions of the phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL) enzymes. Genes that encode these enzymes are coordinately activated in response to developmental cues and to non-developmental signals such as wounding or irradiation with UV light (Dixon and Paiva 1995). Members of 4-coumarate:CoA ligase (*4CL*) (EC 6.2.1.12) gene families encode isoenzymes that catalyze the formation of CoA esters of cinnamic acids. These esters may be used as substrates in specific branch pathways, such as those leading to the synthesis of flavonoids and lignin (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995; Douglas, 1996).

Four members of the *At4CL* gene family, *At4CL1*, *At4CL2, At4CL3* and *At4CL4* have been identified and cloned in *Arabidopsis* (Lee *et al.,* 1995; Ehlting *et al.,* 1999; Hamberger *et al.,* 2004). At4CL1 and At4CL2 are the closest family members with 83% identity at the amino acid level (Hamberger *et al.,* 2004). *At4CL* genes appear to have specialized developmental and biochemical functions. *At4CL1* and *At4CL2* and the isoenzymes encoded by these genes are associated with the biosynthesis of lignin and structurally related soluble or cell wall-bound phenylpropanoid derivatives in the xylem tissues of the plant vascular system (Lee *et al.,* 1995, Mizutani *et al.,* 1997, Ehlting *et al.,* 1999). The At4CL3 enzyme may participate in the biosynthetic pathway leading to flavonoids and the *At4CL3* gene is mostly expressed in aerial organs like flowers and mature leaves (Ehlting *et al.,* 1999) where flavonoids play a major role in UV protection. *At4CL4* is expressed in low level in aerial parts of the plants (B. Hamberger, unpublished). In addition to developmental expression, expression of *4CL* genes in different plants is also activated by external stimuli such as wounding, pathogen infection, and UV irradiation (Douglas *et al.,* 1987; Schmelzer *et al.,* 1989). In *Arabidopsis*, *At4CL1* and *At4CL2* transcripts accumulate rapidly but transiently in

response to wounding while *At4CL3* mRNA levels are not affected (Ehlting *et al.*, 1999). The response of *At4CL4* to wounding is yet unknown.

A typical approach used to identify *cis*-regulatory elements controlling plant gene expression is to fuse target gene promoter sequences to a reporter gene, and assay reporter gene expression in transgenic plant lines. Commonly, enhancers and core promoter elements are located in the promoter proximal region. When promoters containing these elements are fused to the reporter gene, they typically drive reporter gene expression in specific tissues, organs and cells and/or drive expression in response to external stimuli. There are also examples of the presence of regulatory elements within exons, introns or other areas (Douglas *et al.*, 1991; Zhang *et al.*, 1994; Gidekel *et al.*, 1996; de Boer *et al.*, 1999; Ito *et al.*, 2003).

Using this approach, the locations of *cis*-regulatory elements in the parsley (*Petroselinum crispum*) *4CL1* promoter that direct developmental expression have been defined (Hauffe *et al.*, 1991; Hauffe *et al.*, 1993; Neustaedter *et al.*, 1999). Some of these 4CL elements, termed AC elements or P and L boxes are highly conserved in phenylpropanoid gene promoters (Logemann *et al.*, 1995; Douglas, 1996; Raes *et al.*, 2003), are binding sites for MYB transcription factors (Feldbrügge *et al.*, 1997), and are also found in the promoters of *At4CL* genes (Ehlting *et al.*, 1999; Hamberger and Hahlbrock, 2004).

Here, I show that *Arabidopsis thaliana* (Ler) *At4CL1* and *At4CL2* promoter::GUS fusions direct developmental GUS expression to the vasculature of leaves, stems, and roots, whereas the *At4CL4* promoter does not direct detectable developmentally regulated GUS expression. I report the locations of *cis*-regulatory elements controlling developmental and wound-induced expression of the *At4CL2* gene. Using promoter deletions, I identified the locations of *cis*-elements directing developmental expression in upstream promoter regions where they were expected. RNA analysis has shown rapid wound responsiveness of the *At4CL2* gene (Ehlting *et al.*, 1999), and data presented here show that *At4CL2* expression is activated in a biphasic manner, with early and late responses. However, *At4CL2* promoter::*GUS* expression in wounded transgenic plants did not mimic this response. In contrast, fusions including both the promoter and transcribed region of the *At4CL2* gene to GUS directed wound-induced expression, but

only the late wound response was detected. Through elimination of different introns I found multiple domains within the transcribed region containing positive and negative regulatory sequences involved in wound responsiveness of the *At4CL2* gene. We also report lack of developmental GUS expression driven by the *At4CL4* promoter but show its strong early and persistent wounding response.

4.2 RESULTS

4.2.1 Developmental and wound-induced expression patterns directed by *At4CL* Promoter::GUS fusions

Transgenic plants containing *At4CL1, At4CL2, At4CL3*, and *At4CL4* promoter::GUS constructs (Figure 4-1, C) were generated and introduced into *Arabidopsis* plants by Drs. J. Ehlting and B. Hamberger (unpublished), and I performed histochemical GUS assays on multiple transgenic homozygous lines for each construct. Ten-day old seedlings containing *At4CL1*::GUS and *At4CL2*::GUS constructs showed similar patterns of developmentally regulated GUS expression throughout the plant, and largely restricted to the vasculature (Figure 4-2, f & g; Figure 3-1). Expression of *At4CL3::GUS* was not restricted to the vascular system, but instead appeared to be associated with leaf and stem surfaces, and was especially high in young leaves and upper part of hypocotyls (Figure 3-1) consistent with a function hypothesized for 4CL3 in flavonoid biosynthesis (Ehlting *et al.*, 1999). Developmental GUS expression in the mature leaves of *At4CL4::GUS* line was not detectable in multiple lines.

Seedlings and leaves from three to four-week old mature plants of representative transgenic lines for the constructs in Figure 4-1, as well as a 35S::GUS control line, were examined in parallel for developmentally regulated and wound-induced GUS expression. Leaves were wounded *in planta* for 2 to 72 hours before assays were carried out for GUS expression. The 35S control line showed predicted constitutive developmental GUS expression and lack of wound-induced expression (Figure 4-2, a). The *At4CL4::GUS* line responded to wounding by less than 2 h post-wounding, but did not show any developmentally regulated expression (Figure 4-2, h). The *At4CL3::GUS* line showed no wound response while the *At4CL1::GUS* line showed developmental expression and responded to wounding only after 72 h, with no wound-induced expression detectable after 2 h (Figure 4-2, g).

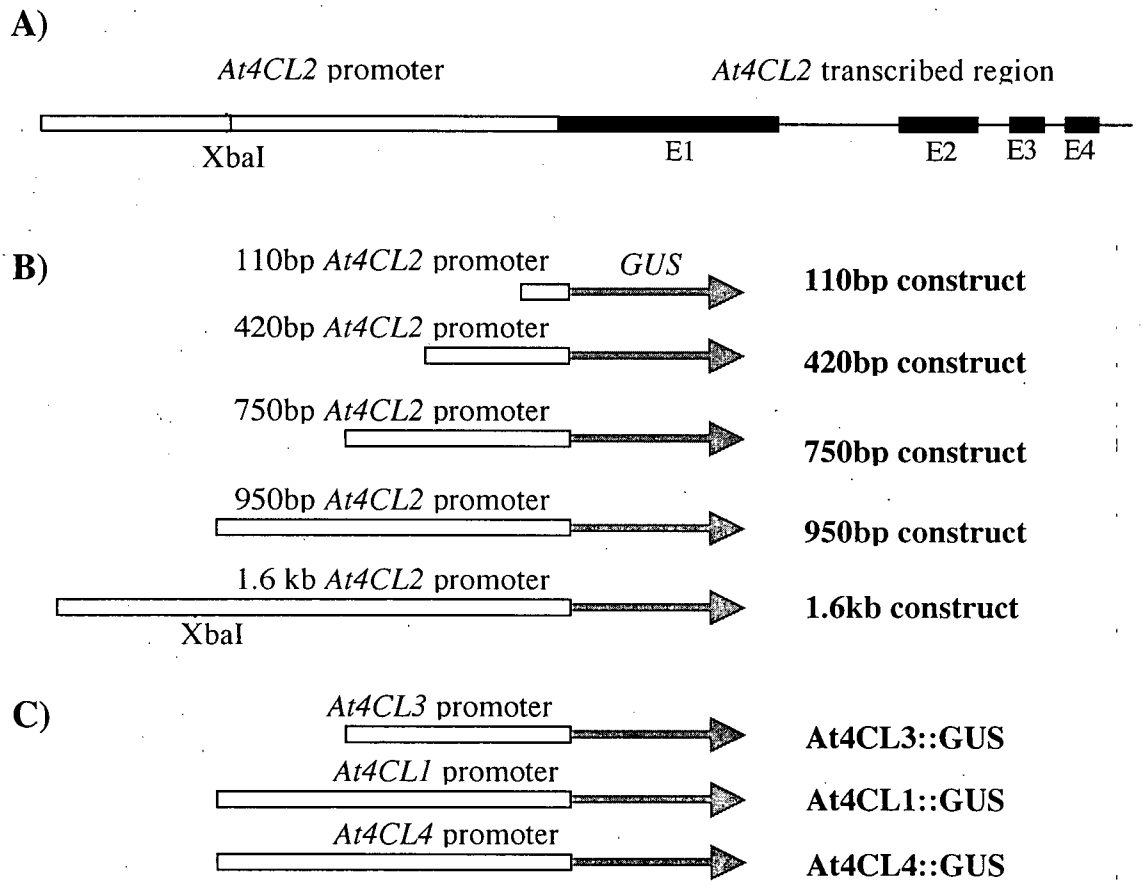


Figure 4-1 Schematic representation of *At4CL* promoter-*GUS* fusion constructs, **A)** *At4CL2* gene **B)** *At4CL2* promoter-*GUS* fusion constructs and **C)** Structure of *At4CL1*, *At4CL3* and *At4CL4* promoter-*GUS* fusions in transgenic plants provided by Drs. J. Ehlting and B. Hamberger. Empty bars represent promoter fragments and solid bars represent exons (E1-E4). The name of each clone that is referred to in the text, is indicated at the right.

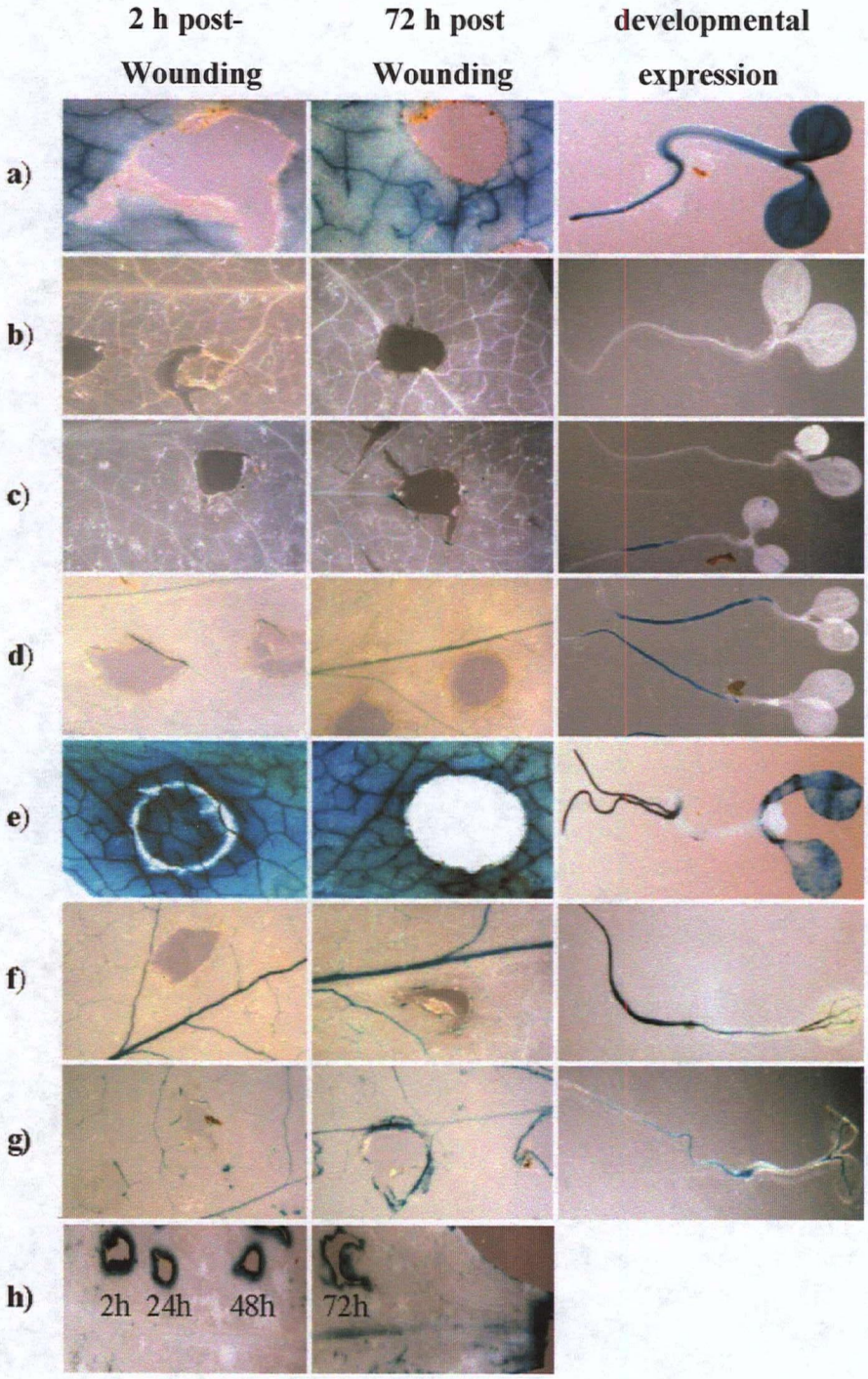


Figure 4-2 Wounding response and developmental expression of *At4Cl* promoters. Histochemical assays for GUS expression were performed on representative lines with the following transgenes: **a)** 35S::GUS line **b)** 110bp of *At4CL2*promoter:: *GUS* line , **c)**

420bp of *At4CL2* promoter::*GUS* line, **d)** 750bp of *At4CL2* promoter::*GUS* line, **e)** 950bp of *At4CL2* promoter::*GUS* line, **f)** 1.6kb of *At4CL2* promoter::*GUS* line, **g)** 1 kb of *At4CL1* promoter::*GUS* line. **h)** 1kb of *At4CL4* promoter::*GUS* line.

Finally, while the 1.6kb *At4CL2* promoter directed developmental GUS expression as expected in this line, I did not see any GUS expression in response to wounding by 2 or 72 h directed by this promoter (Figure 4-2, f). These results indicate that, while *At4CL1* and *At4CL2* promoters direct developmentally regulated expression as predicted from previous experiments, wound induced expression directed by the promoters is distinct from patterns of endogenous wound-induced expression (rapid up-regulation of transcript levels by 1h post-wounding) previously observed (Ehlting *et al.*, 1999).

4.2.2 Evidence for positive developmental and negative wound inducible *cis*-regulatory elements in the *At4CL2* promoter

I generated a series of truncated *At4CL2* promoter fragments, fused them to GUS (Figure 4-1, B) and 10 day-old seedlings of 8-12 transgenic lines made from each construct were assayed for GUS expression. Developmental expression and wound-induced GUS expression in seedlings and leaves from three to four week-old mature plants of representative lines for each construct are shown in Figure 4-2, b-e.

My data showed that the 110-bp *At4CL2* promoter (Figure 4-1, B) is not sufficient to drive GUS expression on a visible level (Figure 4-2, b). The 420-bp promoter fragment (Figure 4-1, B) directed weak GUS expression only in some of the lines (Figure 4-2, c). However, lines containing the 750bp construct (Figure 4-1, B) showed clear visible developmental GUS expression in the vascular tissues of the seedlings and mature leaves (Figure 4-2, d) indicating the presence of strong positive regulatory element(s) in the -750 to -420 bp interval of the *At4CL2* promoter region.

All the lines containing the 950bp construct (Figure 4-1, B) showed strong developmental GUS expression in the root but apparent ectopic expression in the cotyledons and mature leaves outside of the vascular tissues (Figure 4-2, e) in a pattern reminiscent of *At4CL3::GUS* expression (Figure 3-1). This expression was distinguishable from 35S::GUS expression, especially in seedlings (compare Figures 4-2, a and e). Thus, -950/-750 bp regions of the *At4CL2* promoter appear to harbor one or

more positive regulatory element(s) that drive expression very similarly to the *At4CL3* gene and was called *At4CL3*-like positive *cis* -element (3LE).

As shown above, transgenic lines carrying a 1.6 kb *At4CL2* promoter::GUS fusion (Figure 4-2, f) showed strong vascular-specific expression in both seedlings and mature leaves. The GUS expression intensity of this construct seemed stronger than that of the -750 bp construct and was clearly specific to the vasculature system (Figure 4-2, d and f). It seems, elements that enhance developmental expression or repress the ectopic expression are located between -950 bp and -1.6 kb sequences.

Wounding treatment of these lines showed that only the 950-bp *At4CL2* promoter::GUS construct directed wound-induced GUS expression (Figure 4-2, e). Wound-induced GUS expression was evident at 2 h post wounding, but appeared transient and had disappeared by 72 h after wounding (Figure 4-2, e). Other promoter::GUS lines only showed stronger expression in some cases, but this was restricted to the veins in the wounded area (Figure 4-2, c, d, f). To examine the dynamics of wound-induced expression driven by the 950-bp *At4CL2* promoter::GUS construct in more detail, single leaves were wounded for different times over a period of 72 h, followed by staining for GUS expression. As shown in Figure 4-3 for a representative leaf, wound-induced GUS expression peaked by 2-4 h, and was no longer detectable by 48 h. Taken together, these data suggest the presence of a *cis* element(s) in the -950/-750 bp region of the *At4CL2* promoter responsible for an early wound response while the region between -950 bp and -1.6 kb apparently contains element(s) that negatively affect the wound response.

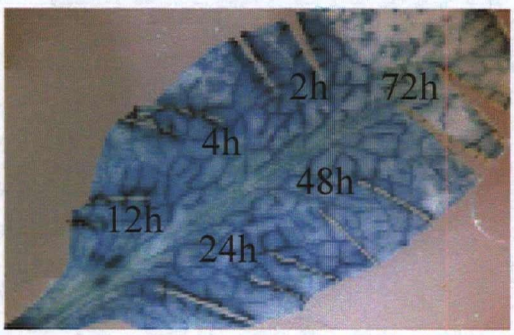


Figure 4-3 Developmental and wounding response expression of 950bp *At4CL2* promoter fused to GUS. The same leaf was wounded for the times shown before

histochemical assay of GUS expression.

4.2.3 *Cis*-elements that specifying developmental and wound-induced expression in the *At4CL2* transcribed regions

To test whether elements directing developmental and/or wound responsiveness of *At4CL2* are located within the transcribed regions of the gene, the *At4CL2* gene including 950-bp of promoter and the entire transcribed region (except the 3'-UTR) were fused in-frame to GUS (Full length construct; Figure 4-4).

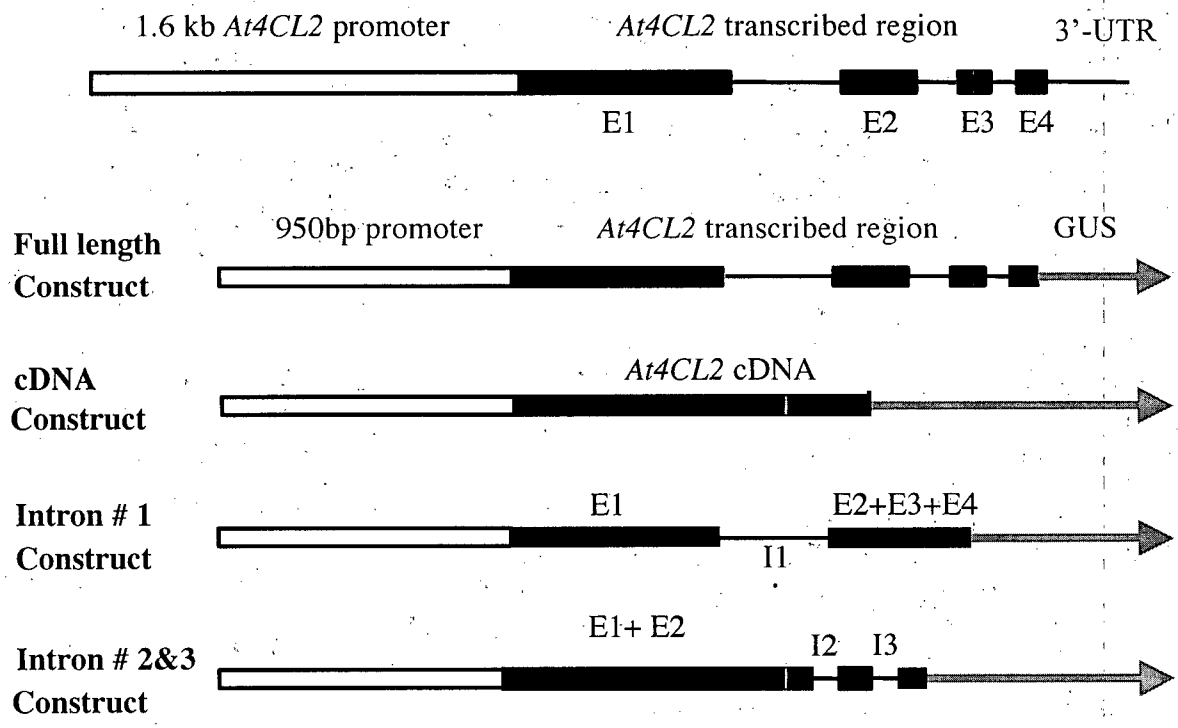


Figure 4-4 *At4CL2* promoter (950 pb) plus *At4CL2* transcribed region fragments fused to GUS. Empty bars represent promoter and solid bars represent exon (E1-E4). The name of each clone is indicated in front of it and is used in the text.

Transgenic *Arabidopsis* lines containing this construct showed developmental GUS expression that was very similar to the 1.6-kb *At4CL2* promoter::GUS line (Figures 4-5, a).

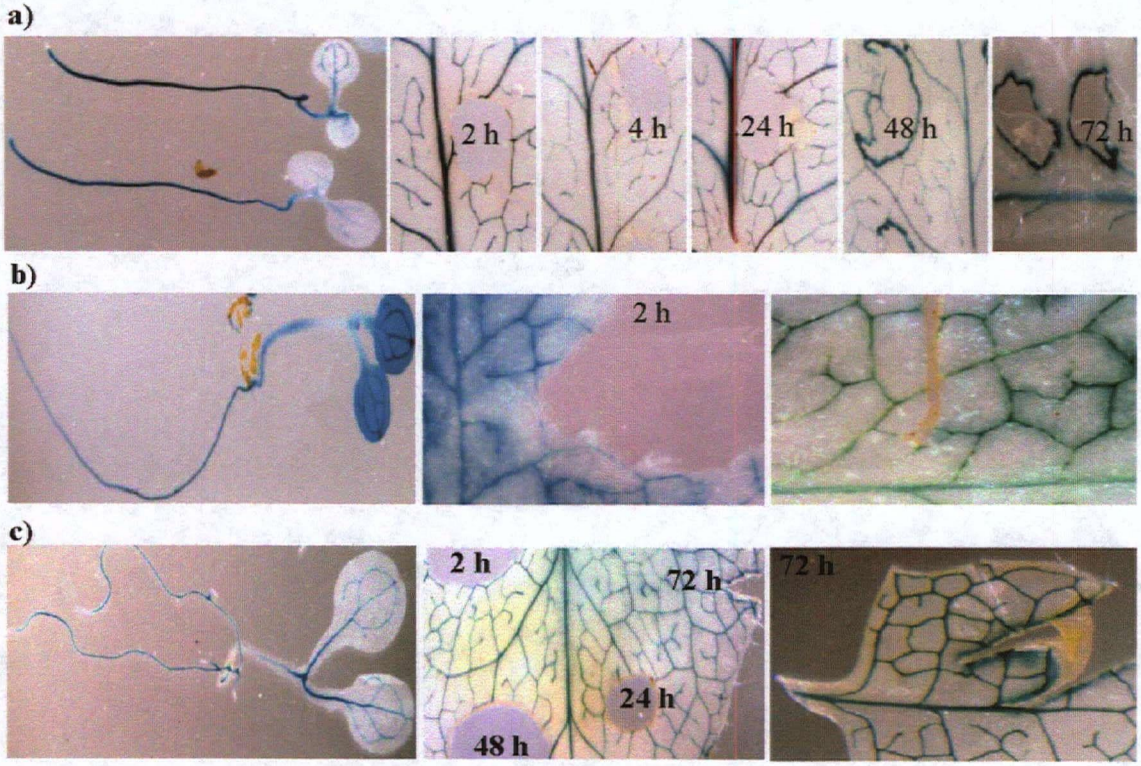


Figure 4-5 Developmental and wound-induced expression in transgenic lines containing 950-pb *At4CL2* promoter and different transcribed regions fused to *GUS*. **a)** Full length *At4CL2::GUS*, **b)** *At4CL2* cDNA::*GUS*, and **c)** *At4CL2* intron #2&3::*GUS* lines. Developmental expression in seedlings is shown at the left. Four-week old leaves were wounded and GUS assays performed 2-72 h post-wounding, right-hand panels

In contrast to the 1.6-kb promoter, however, the full-length construct directed wound-induced GUS expression that was detectable after 48-72 h, but no early wound response such as that observed for the -950-bp *At4CL2* promoter GUS fusion was observed in these lines (Figures 4-5, a). Based on these results, we postulate the presence of a Late-Wound Response Element in the transcribed portion of the *At4CL2* gene. To locate the area where the LWRE resides, I removed all of the introns from the construct by substituting the transcribed region of the *At4CL2* full-length construct with the

At4CL2 cDNA sequence (Figure 4-4). All of the transgenic lines containing the *At4CL2*-cDNA::*GUS* construct showed strong developmental *GUS* expression, mostly restricted to vascular tissue, but showed ectopic GUS expression in surrounding tissues as well (Figures 4-5, b). This developmental expression pattern was highly reproducible, being observed in most of the *At4CL2*-cDNA::*GUS* lines (Figure 4-6). However, wounding did not induce GUS expression in any of these lines (Figures 4-5, b).

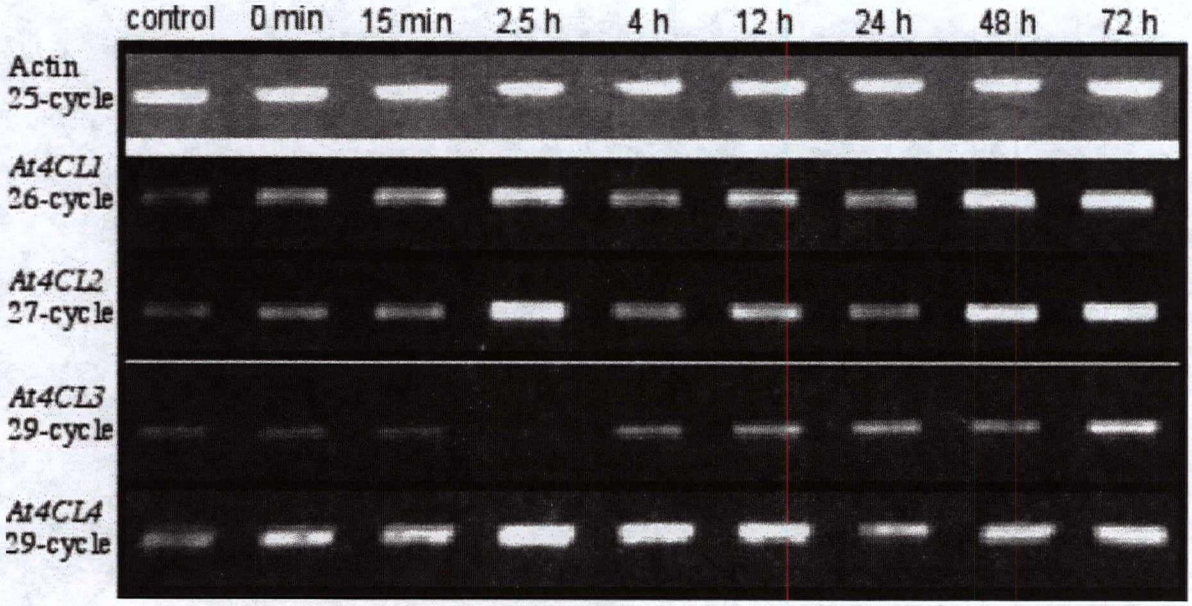
Figure 4-6 Developmental GUS expression in mature leaves of nine independent *At4CL2* cDNA:: *GUS* lines (2-10), All lines showed ectopic GUS expression outside of veins except line 1 with no GUS expression.

These data suggest that a LWRE might be located in one of the *At4CL2* introns. To test this, intron # 1 of the *At4CL2* gene was inserted into *At4CL2*-cDNA::*GUS* construct (Figure 4-4) but none of the T1 transgenic lines showed developmental or wound induced *GUS* expression. RT-PCR analysis showed expression of the transgene and sequence analysis did not reveal any mutation in the transgene transcript or promoter sequence. Introns # 2 and # 3 were also inserted into the *At4CL2*-cDNA::*GUS* construct (Figure 4-4) and transgenic plants containing this construct were assayed for developmental and wound-induced GUS expression. These lines showed strong vascular specific developmental *GUS* expression and activation of *GUS* expression in response to wounding at 72 h, although this response was weaker than the response seen in lines harboring the full-length line construct (Figures 4-5, c). We conclude that a LWRE resides in intron # 2 or # 3.

4.2.4 Biphasic wound induction of *At4CL2* Expression

Our results suggested that the kinetics of wound induced *At4CL* expression might be more complex than it was previously realized (Ehlting *et al.,* 1999). I used semi-quantitative RT-PCR to test the expression of the endogenous *At4CL* gene family

members in response to wounding in leaves. *At4CL1* and *At4CL2* mRNA levels transiently increased 2.5 h post-wounding and then returned to a basal level, before again increasing to a maximum 48-72 h after wounding (Figure 4-7). *At4CL4* transcript levels also increased by 2.5 h post-wounding but remained elevated for at least 12 h. *At4CL3* expression was rapidly down regulated in response to wounding, but recovered to basal levels by 4 h post wounding. Thus, *At4CL* gene family members display a diverse set of responses to wounding.



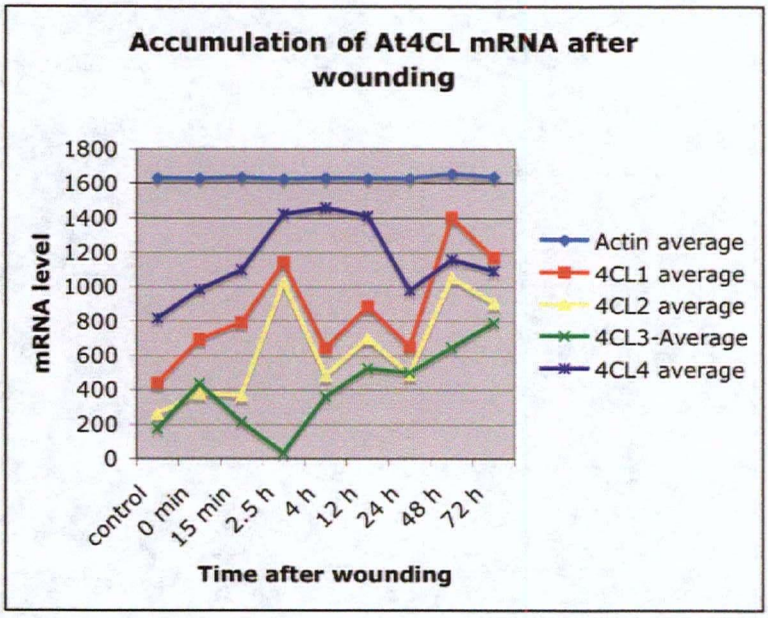


Figure 4-7 Wounding response of *At4CL* genes analyzed by semi-quantitative RT-PCR. Wounding was performed on 3-4 week old *Arabidopsis* leaves, and RNA isolated at the given times after wounding. Cycle numbers were adjusted empirically for each gene so that amplification was in the linear range, and amplification of actin was used to control for variations in RNA amounts between samples. The experiment was repeated three times using leaves of independent plants and the averages of band intensities (pixel number as measured by Alpha Imager software) over the course of the experiment are shown at the bottom as mRNA level.

4.3 DISCUSSION

4.3.1 Differential and biphasic wound responsiveness of *4CL* gene family members in *Arabidopsis*

Induced expression of phenylpropanoid genes plays a key role in plant responses to many environmental stresses by activating the biosynthesis of defensive compounds (Dixon and Paiva 1995). Wounding coordinately induces many phenylpropanoid pathway genes such as *PAL*, *C4H*, and *4CL* (Dixon and Paiva 1995; Ohi *et al.*, 1990; Lee *et al.*, 1995; Bell-Lelong *et al.*, 1997; Meyer *et al.*, 1998; Mizutani *et al.*, 1997). *Arabidopsis* offers a system to study mechanisms underlying stress activated phenylpropanoid gene expression, and the *At4CL* gene family provides an opportunity to test whether different gene family members with potentially different biochemical functions (Ehlting *et al.*, 1999) respond differentially to environmental stimuli. *At4CL1* and *At4CL2* mRNAs have been reported to rapidly but transiently accumulate in an hour post-wounding in the detached *Arabidopsis* leaves while *At4CL3* gene expression is reported to be not affected (Ehlting *et al.*, 1999). In this study, I focused my attention on wound-induced *At4CL* gene expression, with an emphasis on *At4CL2*.

Wound-induced gene expression may be activated by signaling pathways that partially overlap with those activated by other stresses such as drought, freeze and osmotic stress (Reymond *et al.*, 2000; Denekamp *et al.*, 2003) and this overlap has been shown for pathogen-induced signaling pathways as well (Romeis *et al.*, 1999). Plant hormones such as jasmonic acid (JA) and related compounds play a central role in rapid localized and systemic wound responses in plants (Farmer and Ryan 1992; Schaller 2001,

Turner *et al.* 2002; Li *et al.* 2002; He *et al.,* 2005). JA- and ethylene-responsive elements and the S box, all of which confer responsiveness to elicitation, wounding, and pathogen infection have been found in the genes that are involved in lignin biosynthesis (Rushton *et al.,* 2002). It has been shown that parsley *4CL1* gene expression is activated by JA treatment (Ellard and Douglas, 1996) and stresses such as wound, UV, and pathogen infection activate *At4CL* gene expression in *Arabidopsis* (Ehlting *et al.,* 1999).

The activation of *At4CL* gene expression by wounding is part of a complex global response. Wounding induced up-regulated expression of 8% of the 8000 genes on an *Arabidopsis* microarray (Cheong *et al.,* 2002), and wound-induced genes and proteins exhibit different induction kinetics in many plants. For example, a GFP-Nitrilase 1 fusion protein aggregates in the cells directly abutting the wounded area in 30-60 min post mechanical wounding (Cutler and Somerville, 2005). In wounded tomato plants, Phospholipase A activity has increased systemically in a biphasic manner, peaking at 15 min and again at 60 min post wounding (Narváez-Vásquez *et al.,* 1999). The peptide transporter (*AtPTR3*) gene on the other hand is activated in 4 h and gradually increases in response up to 24 h (Karim *et al.,* 2005). The sugar transporter (*AtSTP4)* gene is also activated in 3 h post-wounding (Truernit *et al.,* 1996; Meyer *et al.,* 2004). Wounding also induces the expression of transcription factors that have recognition sites on the phenylpropanoid pathway genes (Hara *et al.,* 2000, Cheong *et al.,* 2002). It has been shown that the expression of *AtMyb32* (Preston *et al.,* 2004) and *AtMyb4* (Jin *et al.,* 2000), regulators of phenylpropanoid gene expression, was altered by wounding and may play roles in regulating wound-induced expression.

I used RT-PCR to assay changes in mRNA abundance of *At4CL* gene family members up to 72 h after *in planta* wounding of the leaves. This analysis indicated the presence of a biphasic wounding response for *At4CL1* and *At4CL2* genes (Figure 4-7). Similar to a previous report (Ehlting *et al.,* 1999) *At4CL1* and *At4CL2* expression was rapidly, coordinately, and transiently activated (by 2.5 h) by wounding. Our analysis shows that *At4CL4* expression is also rapidly and coordinately activated, but is not as transient as *At4CL1* and *At4CL2*, perhaps reflecting the distinct biochemical function of At4CL4, which specifically activates sinapic acid (Hamberger and Hahlbrock, 2004). In contrast to these genes, our results show that *At4CL3* expression was rapidly down

regulated, possibly again reflecting a specialized biochemical function for At4CL3, which appears to be primarily involved in flavonoid biosynthesis (Ehlting *et al.*, 1999).

The second phase of wound activated *At4CL1* and *At4CL2* expression started between 24 and 48 h post-wounding and lasted up to 72 h after the onset of wounding (Figure 4-7). The biphasic response of the *At4CL2* gene is consistent with late *GUS* expression directed by the full-length and intron 2&3 lines (Figures 4-5, a and c), and the early response directed by the 950-bp promoter::GUS construct (Figure 4-3). A biphasic wounding response for phenylpropanoid pathway genes has been reported in parsley and also in Jerusalem artichoke (*Helianthus tuberosus*) tubers (Logemann *et al.*, 1995; Batard *et al.*, 2000). However, both responses occurred within 24 h after wounding. Based on their results, those authors suggested multiple signaling routes might exist for phenylpropanoid gene transcriptional activation (Batard *et al.*, 2000). Similarly, in *Arabidopsis*, it appears that multiple wound-generated signals may affect different *At4CL* gene family members differently, and that a single gene such as *At4CL2* is regulated by multiple wound-generated signals, leading to a complex pattern of wound-induced transcriptional regulation that could be reflected in a complex array of wound responsive *cis*-regulatory elements associated with the gene.

4.3.2 Multiple *cis*-regulatory elements are involved in developmental regulation of *At4CL2* gene expression

The *At4CL1* and *At4CL2* promoters (Figure 4-1) directed developmentally regulated GUS expression in the vasculature (Figure 3-1 or 4-2), as reported for other *4CL* genes (Hauffe *et al.*, 1991; Lee *et al.*, 1995). In contrast, the *At4CL4* promoter fused to GUS (Figure 4-1) did not direct detectable developmental expression in the mature leaves (Figure 4-2, h). Coupled with the rapid wound-induced expression of *At4CL4* (Figure 4-2, h, and 4-7) this suggests that At4CL4 may play a primarily defensive role. Consistent with a hypothesized role for At4CL3 specific to flavonoid biosynthesis (Ehlting *et al.*, 1999), *At4CL3::GUS* was expressed most highly in young organs and tissues, possibly in epidermal cells (Figure 3-1).

In order to map the locations of elements specifying *At4CL* expression patterns, several different *At4CL2* promoter fragments (Figure 4-1, B) were fused to *GUS* and developmental and wounding response expression of transgenes was assayed (Figure 4-2,

b-f). Expression results showed that a minimum of 750 bp of the *At4CL2* promoter is required for strong vascular- specific developmental GUS expression (Figure 4-2, d), although a few lines with the 420-bp promoter::GUS construct were able to drive weak developmental GUS expression (Figure 4-2, c). Accordingly, critical *cis*-regulatory element(s) are likely to reside in the -750/-420 bp fragment of the *At4CL2* promoter, in addition to the P and L boxes located more proximally (Ehlting *et al.*, 1999) (Figure 4-8). Using a Database of Plant Cis-acting Regulatory DNA Elements (PLACE, Higo *et al.*, 1999) (http://www.dna.affrc.go.jp/PLACE) several putative *cis*-regulatory elements were found in this fragment including potential Myb, WRKY, Myc, and GATA elements.

Developmental expression directed by the 950-bp construct (Figure 4-2, e) was distinct from that directed by both shorter and longer promoter fragments (750-bp and 1.6-kb constructs; Figure 4-1, B), and the observed pattern was more similar to that of *At4CL3::GUS* expression (Figure 3-1) in which expression extends to cell types outside of the vascular system in leaves. I used the same 950-bp promoter and fused it with *At4CL2* transcribed regions to generate *At4CL2*-cDNA::*GUS* and *At4CL2*-intron #2&3::*GUS* constructs (Figure 4-4). The above described GUS expression pattern for the 950 construct, was not observed in *At4CL2*-intron # 2&3::*GUS* lines (Figures 4-5, c), but was evident in *At4CL2*-cDNA::*GUS* lines (Figures 4-5, b). Thus, this pattern is likely intrinsic to the truncated 950-bp promoter by itself or in combination with the cDNA. It is possible that the junction between the flanking vector sequences and the -950 bp promoter created novel regulatory sequences responsible for the observed phenotype. To avoid this potential problem, future experiments could substitute deleted promoter sequences with other sequences. Regardless of this possibility, we suggest that the -950/-750 bp promoter fragment may have positive regulatory element(s) (3LE; Figure 4-8) and this element(s) direct gene expression in a very similar manner to the gene expression directed by the 635-bp *At4CL3* promoter (Figure 3-1). If so, there appear to be other negative elements in the -1.6-kb/-950 fragment and in introns 2 or 3 that negatively affect the 3LE function and restrict expression to the vascular-specific patterns directed by the 1.6kb promoter *At4CL2*::*GUS* (Figure 4-2, f), *At4CL2*-intron #2&3::*GUS* (Figure 4-5, c), and *At4CL2*::*GUS* full length constructs (Figure 4-5, a). Figure 4-8 represents all the deduced developmental cis elements and their relationship in the *At4CL2* gene. Searching

the -950/-750 sequence for potential cis-elements using PLACE revealed multiple tandem ARR1 *cis*-elements (involved in positive transcriptional regulation; Sakai *et al.,* 2001) and also multiple GT-1 motif and GT1 consensus sequences, which play roles in pathogen and salt- induced gene expression (Park *et al.,* 2004).

Compared with shorter promoter fragments lines, the 1.6 kb *At4CL2* promoter (Figure 4-1, B) directed the strongest xylem specific GUS expression (Figure 4-2, f). Therefore, we suggest another positive regulatory element is located between the -1.6-kb /-950 bp sequences (Figure 4-8). Several potential *cis*-elements including an I-box that was shown to be a recognition site for the LeMyb1 transcriptional activator in tomato (Rose *et al.,* 1999) were found in this region using the PLACE program.

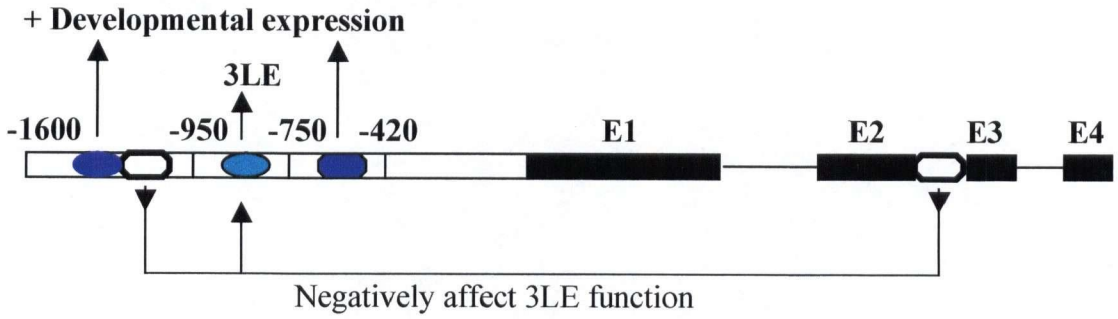


Figure 4-8 Summary of deduced locations of positive and negative regulatory elements in the *At4CL2* gene affecting developmentally regulated expression. Positive elements are indicated by blue ovals, negative elements by open ovals, and the 3LE element specifying ectopic expression by a turquoise oval, Solid boxes labeled E1 to E4 represent exons, open boxes represent 5' promoter sequences upstream of the ATG start codon. Base pair coordinates upstream of the ATG are given.

Addition of introns #2 and #3 to the cDNA construct (Figure 4-4), restricted 950-bp promoter-driven GUS expression to the vascular system (Figures 4-5, c) and a negative regulatory element in either of these two small introns was deduced to be responsible for the inactivation of 3LE element function in the -950/-750 bp fragment (Figure 4-8). Introns #2 and #3 both are about 110 bp in length, and at +30 and +40 positions of the

intron #2 and the +80 position of intron #3 a tetra-nucleotide (CACT) *cis* element was found that was shown to be a *cis*-regulatory element for mesophyll-specific gene expression in *Flaveria trinervia* (Gowik *et al.*, 2004). Mutagenesis of these putative elements could be used to test their roles in tissue specific expression of the *At4CL2* gene.

When added to the *At4CL2*-cDNA::*GUS* construct (Figure 4-4), intron #1 had the apparent and unexpected effect of blocking GUS expression in all of the 20 tested lines. RT-PCR confirmed the expression of the transgene, at least at low levels, and sequencing of RT-PCR product confirmed the integrity of the sequence. Even though a yet un-detected mutation may have been generated during the cloning process, it is possible that intron #1 contains a negative regulatory element with a repressive effect on developmental *At4CL2* mRNA accumulation or on translation.

4.3.3 Multiple *cis*-regulatory elements are involved in modulating *At4CL2* early wound responsiveness

The 950-bp *At4CL2* promoter fused to *GUS* was the only one of several *At4CL2::GUS* constructs that directed the early and transient wound response (Figures 4-2, e and 4-3) characteristic of the endogenous *At4CL2* gene (Ehlting *et al.*, 1999). GUS expression in response to wounding directed by smaller promoter fragments such as 750-bp *At4CL2* promoter, was restricted to some of the veins in the wounded area (Figure 4-2, d). This GUS expression could be attributed to the greater accessibility of the X-Gluc substrate from the wound site to these veins. This suggests the presence of a positive regulator of the early wound response in the -950/-750 region of the *At4CL2* promoter (Figure 4-9).

Other transgenic lines with the same promoter, such as those harboring *At4CL2*-cDNA::*GUS*, *At4CL2*-intron # 2&3::*GUS*, and full-length constructs (Figure 4-4), did not show such an early wound response (Figure 4-5). Furthermore, such an early response was not observed in lines harboring the 1.6-kb *At4CL2* promoter fused to GUS. Figure 4-9 represents the deduced cis elements involved in the early wound response and their relationship. Based on the results, elements that negatively regulate the early wound response appear to exist in the promoter and transcribed regions of the gene. One negative regulatory element may reside in the -1.6kb/-950 fragment, while another

appears to be in an exon, since early wound responsiveness was absent in both *At4CL2*-cDNA::*GUS* and *At4CL2*-intron 2&3::*GUS* lines (Figures 4-2 and 4-5).

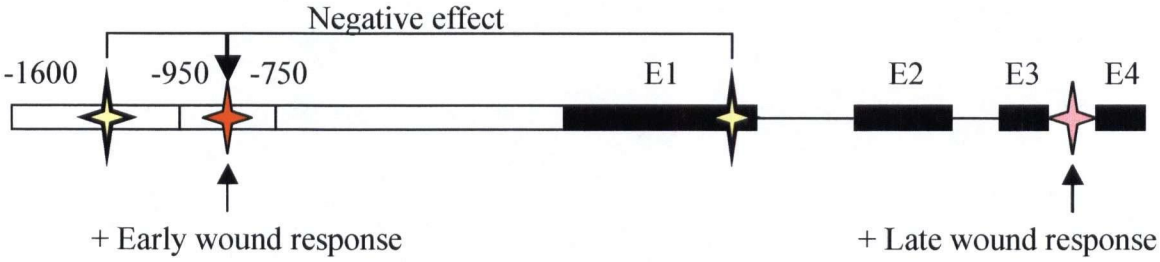


Figure 4-9 Summary of deduced positions of positive and negative regulatory elements in the *At4CL2* gene affecting wound inducible expression. Approximate positions for the positive regulatory elements, are indicated by red and purple stars and position of negative elements are indicate by yellow stars. Solid boxes labeled E1 to E4 represent exons, open boxes represent 5' promoter sequences 5' to the ATG start codon. Base pair coordinates upstream of the ATG are given.

In spite of non-detectable developmental GUS expression, *At4CL4::GUS* transgenic lines (Figure 4-1, C) showed a strong early wounding response, which was sustained for 72 h (Figure 4-2, h). This is consistent with the kinetics of wound induced *At4CL4* mRNA accumulation in wounded leaves (Figure 4-7). In both experiments levels of wounding induced *At4CL4* mRNA accumulation and *At4CL4*-driven GUS expression were distinguishable from the control even after 10 min post wounding (data not shown). This characteristic distinguishes *At4CL4* from the other *At4CL* genes, and suggests the presence of strong wound responsive *cis*-regulatory elements in the 1-kb *AtCL4* promoter used in these experiments.

4.3.4 Intronic *cis*-regulatory elements are involved in the late wounding response of *At4CL2* gene expression.

The line expressing the 1-kb promoter *At4CL1::GUS* construct (Figure 4-1, C) showed wound-activated GUS expression by 72 h post-wounding (Figure 4-2, g), a response characteristic of the endogenous gene clearly evident by RT-PCR (Figure 4-7

). On the other hand, none of the *At4CL2* promoter fragments directed the *At4CL2* late wounding response (Figure 4-2, b-f). These data suggest the absence of positively and negatively acting *cis*-elements for the late wound response in the *At4CL2* promoter fragments tested, and that the *At4CL2* transcribed region might contain such an element (LWRE) directing the late wound response. While the *At4CL2*-cDNA::*GUS* line did not show any wound responsiveness (Figures 4-5, b) GUS expression in lines harboring the full-length construct or the *At4CL2*-intron # 2&3::*GUS* construct showed clear wound-induced GUS expression by 72 h or earlier (Figures 4-5, a & c). This late response was weaker in *At4CL2*-intron # 2&3::*GUS* lines (Figures 4-5, c) than in the full-length lines (Figures 4-5, a). Thus, this suggests that a LWRE resides in intron # 2 and/or # 3 of the *At4CL2* gene (Figure 4-9) and that there may be another LWRE in intron 1, directing a stronger late wound response as strong as seen in full-length lines (Figures 4-5, a). Interestingly, there is an AG-motif at position + 61 of intron 3, which was found in the promoter of the *NtMyb2* gene, a wounding and elicitor stress induced regulator of tobacco *PAL* gene expression (Sugimoto *et al.*, 2003).

4.3.5 Conclusion

The wound-induced accumulation of *At4CL2* mRNA is in agreement with work of others, showing an early and transient wounding response of this gene (Ehlting *et al.*, 1999), and a second, later wound response characteristic of other phenylpropanoid genes (Logemann *et al.*, 1995; Batard *et al.*, 2000). As well, *At4CL1* and *At4CL2* promoter-GUS fusions directed developmental expression to predicted sites in the vascular system, where the 4CL enzymes play roles in the biosynthesis of lignin precursors. A simple model would predict the localization of positive regulatory elements that direct developmental and stress-induced expression of these genes in the respective upstream promoter regions. Instead, our dissection of the regulatory elements associated with the *At4CL2* gene suggests a highly complex regulatory structure, with positively and negatively acting elements scattered at different locations both upstream of and within the transcribed region (Figure 4-10). This unexpected complexity may reflect the interplay of multiple signals that direct both developmental expression to specific cell types and a complex pattern of stress-induced expression of this gene. Further work will be required

to fully characterize the specific *cis*-regulatory elements that direct these complex expression patterns.

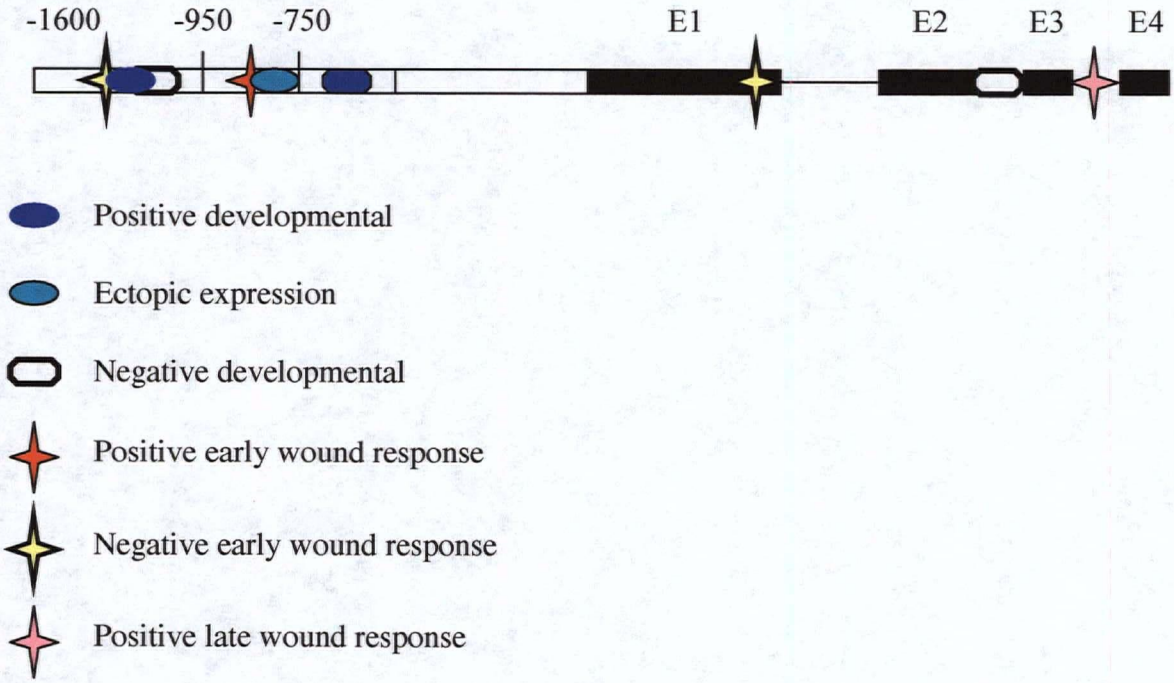


Figure 4-10 Schematic presentation of the locations of putative regulatory elements in the *At4CL2* gene

CHAPTER 5

REVERSE GENETIC ANALYSIS OF AtMYB SUBFAMILY 14

5.1 Introduction

MYB transcription factors belong to some of the largest plant transcription factor families (Romero *et al.*, 1998; Riechmann *et al.*, 2000). The MYB gene family is comprised of more than 125 members divided in 24 subfamilies in *Arabidopsis* (Stracke *et al.*, 2001). There is evidence that MYB transcription factors play important roles in the regulation of phenylpropanoid metabolism in *Arabidopsis* and other plants (Sablowsky *et al.*, 1994; Borevitz *et al.*, 2000, Jin *et al.*, 2000; Preston *et al.*, 2004), as well as regulating other biochemical and developmental pathways. Some plant MYB transcription factors recognize a core-binding site (Grotewold *et al.*, 1994) that is found in the promoters of *PAL*, *4CL* and other genes encoding enzymes in phenylpropanoid metabolism (Douglas, 1996) leading to lignin biosynthesis. Dr. Wang in our lab, studying *AtMYB68*, showed that this gene has a strong root-specific expression pattern. Fusion of the *AtMyb68* promoter to GUS revealed the predominant expression of this gene in the xylem pole pericycle cells of the root in the transgenic *Arabidopsis* seedlings. She also identified and characterized an *AtMyb68* T-DNA knock out line. However, a phenotype has not been detected so far in this line (Wang, 2003). Functional redundancy with a related *AtMyb* gene is a possible reason for the lack of an obvious phenotype. From a phylogenetic tree the AtMyb transcription factors, it is known that *AtMyb36*, *AtMyb68*, *AtMyb84* and *AtMyb87* are closely related in subfamily #14 (Figure 5-1) (Stracke *et al.*, 2001). In this chapter, I focused on *AtMyb84*, since it was found to be the closest subfamily member to *AtMyb68*. I generated *AtMyb84* RNAi knock down and T-DNA knockout lines to look for potential *AtMyb84*-related phenotypes. In addition, I developed a method for creating double RNAi knock down lines, and created double *AtMyb68/AtMyb84* knock out / knock down lines to study their potential functional redundancy.

5.2 RESULTS

5.2.1 *In-silico* analysis of *AtMyb* subfamily #14

According to the alignments shown in Figure 5-1, and phylogenetic analysis done by Stracke et al. (2001) for all the *Arabidopsis* Myb genes, *AtMyb68* and *AtMyb84* are the most closely related *AtMyb* genes in the subfamily #14 and their amino acid sequences show more than 82% identity. Using the *Arabidopsis* genome sequence, I also identified the genes surrounding *Myb* sub family # 14 genes on their linkage groups. I found that the genomic regions around *AtMYB68* and *AtMyb84* show the highest levels of apparent synteny. The gene immediately upstream of *AtMyb68* encodes branched-chain amino acid transaminase 5 (BCAT5) while immediately downstream in reverse orientation is a putative aminocyclopropane-1-carboxylate synthase (ACC synthase) encoding gene. The gene upstream of *AtMyb84* also encodes a branched-chain amino acid transaminase (BCAT3) and, likewise in reverse direction downstream of *AtMyb84*, there is again a putative ACC synthase gene. Such a strong synteny was not observed between these two *AtMyb* genes and the others in the subfamily.

I found signatures of *AtMYB84* gene expression in germinating seedlings and also in the inflorescence as well as root and leaves using the MPSS (http://mpss.udel.edu/at/GeneQuery.php) database. Signatures of *AtMYB68* gene expression were found in the root and inflorescence. I also searched *Arabidopsis* Functional Genomics Tools (http://bbc.botany.utoronto.ca) web site at the University of Toronto for expression data related to *Myb* subfamily # 14. The results showed that if the expression level of *AtMyb* genes in the rosette is considered zero, the expression levels of *AtMyb36, AtMyb68* and *AtMyb84* are first down regulated (-4 to -6) in the germinating seedlings and then highly up regulated (+3 to +8) in the seedling root. Based on these data *AtMyb68* and *AtMyb84* are also up regulated (+2 to +4) in the hypocotyl and up regulated (+2) in the flowers. Meanwhile both are down regulated in the leaf (- 0.75). Exceptionally and in several array reports, *AtMyb84* is up regulated (+1) in the shoot apex. Overall, compared with *AtMyb36* and *AtMyb87*, data collected from more than 80 microarray experiments show that expression of *AtMyb68* and *AtMyb84* genes is more coordinately regulated than that of other *AtMyb* subfamily #14 genes, supporting the possibility that they have similar functions.

```
AtMyb36   1   MGRAPCCDKA NVKKGPWSPE EDVKLKDYID KYGTGGNWIA LPQKIGLKRC
AtMyb68   1   MGRAPCCDKA NVKKGPWSPE EDAKLKDYIE NSGTGGNWIA LPQKIGLRRC
AtMyb84   1   MGRAPCCDKA NVKKGPWSPE EDAKLKSYIE NSGTGGNWIA LPQKIGLKRC
AtMyb87   1   ma--------- -VKKGPWSTE EDAVLKSYIE KHGTGNNWIS LPQRIGIKRC

AtMyb36  51   GKSCRLRWLN YLRPNIKHGG FSEEEDRIIL SLYISIGSRW SIIAAQLPGR
AtMyb68  51   GKSCRLRWLN YLRPNIKHGG FSEEEDNIIC NLYVTIGSRW SIIAAQLPGR
AtMyb84  51   GKSCRLRWLN YLRPNIKHGG FSEEEENIIC SLYLTIGSRW SIIAAQLPGR
AtMyb87  42   GKSCRLRWLN YLRPNLKHGG FTDEEDYIIC SLYITIGSRW SIIASQLPGR

AtMyb36 101   TDNDIKNYWN TKLKKKLLGR Qkqmnrqdsi tdstennlsn nnnnkspqnl
AtMyb68 101   TDNDIKNYWN TRLKKKLLNK QRKEFQEARM KQE--MVMMK RQ--------
AtMyb84 101   TDNDIKNYWN TRLKKKLINK QRKELQEACM EQQemMVMMK RQ--------
AtMyb87  92   TDNDIKNYWN TRLKKKLLSK QGKAF----- ---------- ----------

AtMyb36 151   snsalerlql HMQLQNLQSP FSSFYNNPIL -----WPKL- -----HPLLQ
AtMyb68 141   ---------- QQGQgqgqsn gstdlylnnm fgsspWPLlp qlpppHHQIP
AtMyb84 143   ---------- HQQQQIQTSF MMRQDQTMFT -----WPLH- -----HHNVQ
AtMyb87 117   ---------- HQQLn----- ---------- ---------- ----------

AtMyb36 190   STTTNQNpkl asqesfhplg v--------- ---------- -NVDHQHnnt
AtMyb68 181   LGMMEPTSCN YYqttpsCNL EQ[redacted]TLKN MVKIEE[redacted]R [redacted]DHHH---
AtMyb84 172   VPALFMNQTN SF-----CDQ EDV[redacted]KN MVKIED[redacted]LR K[redacted]HHH---
AtMyb87 122   ---------- ---------- ---------- ---------- ----------

AtMyb36 270   vskelfqvgn efeltngssw wseeVELERK TTSSSSwgsa svldqttegm
AtMyb68 228   ---------- ---------- ---------- ---------- ----------
AtMyb84 214   ---------- ---------- ---------- ---------- ----------
AtMyb87 122   ---------- ---------- ----VKFERG TTSSSSsqnq iqifhdentk

AtMyb36 320   vmlq------ ---------- ---------- ---------- -------DYA
AtMyb68 228   ---------- ---------- -----QDSVT NPFDF-SFSQ LLLDPNY---
AtMyb84 214   ---------- ---------- -----QDSMT NAFDhlSFSQ LLLDPNH---
AtMyb87 148   snqtlynqvv dpsmrafame eqsmiKNQIL EPFSW-EPNK VLFDVDYDAA

AtMyb36 327   QMSYHsv--- ---------- ---------- ---------- ----------
AtMyb68 249   ----Y[redacted]GG GGEGDFAIMS SSTNSP---L PNTSSDQHPS QQQEILQWFG
AtMyb84 236   ----N[redacted]E EGFSMNSILS ANTNSP---L LNTSNDn--- ------QWFG
AtMyb87 197   ASSYHHHASP SLNSMSSTSS IGTNNsslqM SHYTVNHNDH DQPDMFFMDG

AtMyb36 334   ---------- ---------- ---------- ---------- ----------
AtMyb68 292   SSNFQTEAIN DMFINNNnni vnletientk vygdasvaga avraalgGGT
AtMyb84 270   --NFQAETVN lfs------- ---------- ---------- -------GAS
AtMyb87 247   FENFQAELFD EIANNNTven gfdgteilin nnyldh---- ----------

AtMyb36 334   ---------- ---------- ---
AtMyb68 342   TSTSADQSTI SWEDITSLVN SEDASYFnap nhv
AtMyb84 284   TSTSADQSTI SWEDISSLVY SDSKQFF--- ---
AtMyb87 283   ---------- ---DISSFID yplydne--- ---
```

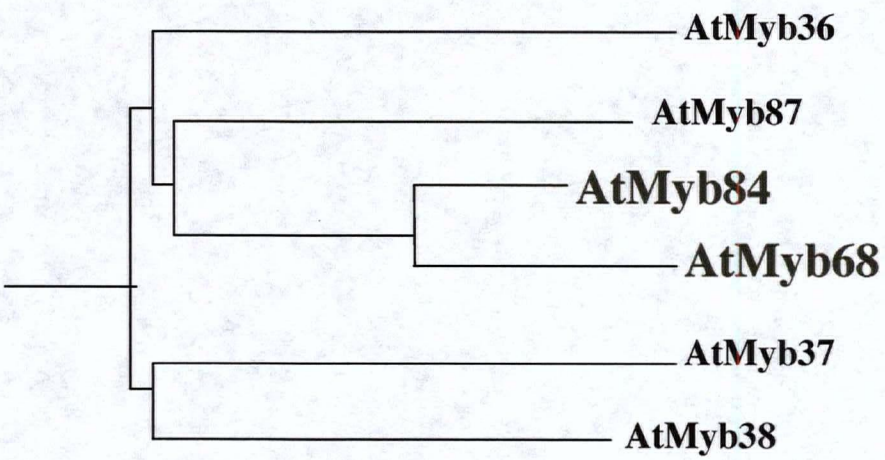


Figure 5-1 Alignment of AtMyb subfamily #14 members and related phylogenetic tree, taken from Stracke et al. (2001)

5.2.2 The *AtMyb84* knock down line preparation

Based on a revised phylogenetic tree (Figure 5-1) (Stracke *et al.*, 2001), and as discussed above, *AtMyb84* was considered the best candidate for a gene with hypothesized functional redundancy to *AtMyb68*. Since a T-DNA insertion line was not available at the time, I generated an *AtMyb84*-specific RNAi construct (Figure 5-2) to repress the expression of this gene (gene knock down).

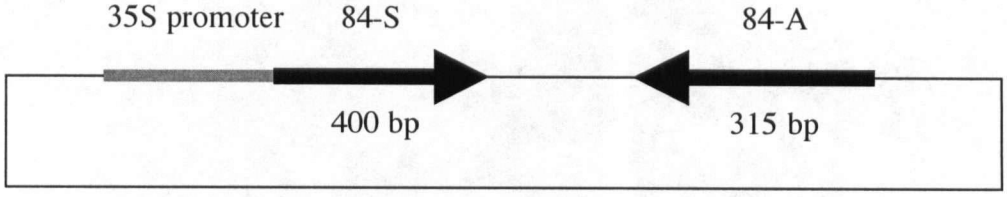


Figure 5-2 RNAi construct for *AtMyb84*

More than 10 independent homozygous lines were generated and semi-quantitative RT-PCR was performed on 10 days old seedlings of these lines to find those with down regulated *AtMyb84* gene expression (Figure. 5-3).

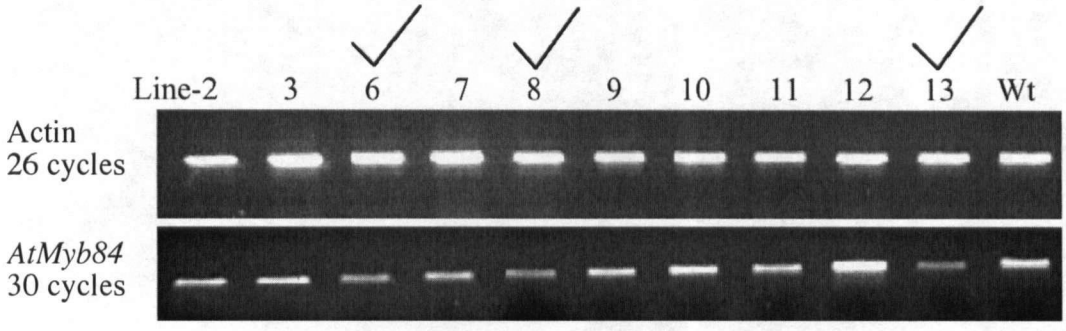


Figure 5-3 RT-PCR on *AtMyb84* RNAi knock down lines. Cycle numbers for the target gene and actin control genes that generated products in non-saturated levels are given. Multiple independent lines were tested and those with reduced *AtMyb84* mRNA levels relative to the actin control are indicated by checks.

Three of these lines (lines # 6, #8 and #13) reproducibly showed lower gene expression in several repetitions of the experiment. Seedlings of lines 3, 6, 12, 13 and wild type controls were grown vertically in order to identify possible phenotypes at the seedling level. At this stage (Figure 5-4) and later developmental stages, no morphological difference between the lines with lower *AtMyb84* mRNA levels (#6 and #13) and wild type plants or RNAi lines with normal *AtMyb84* expression levels (#3 and #12) were detected.

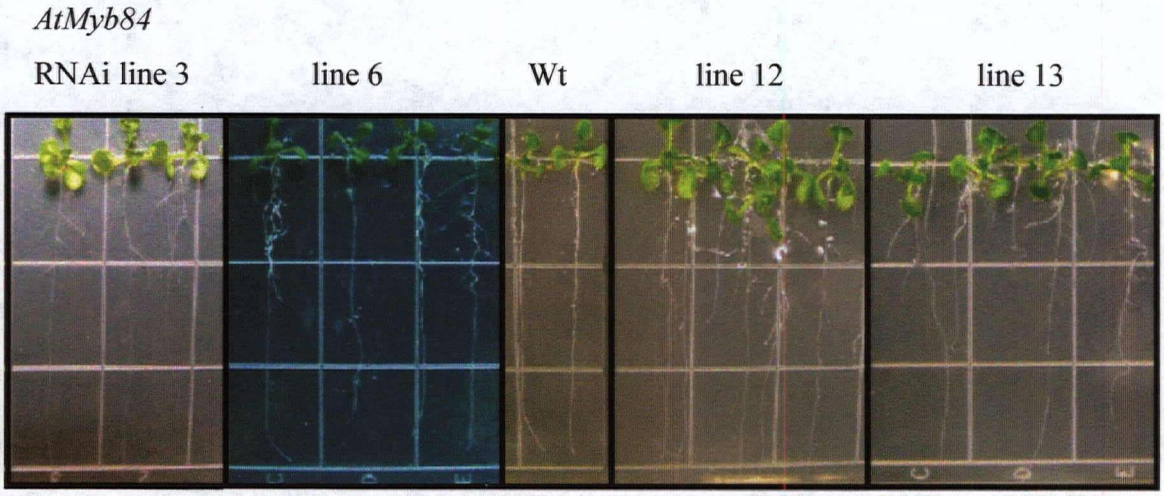


Figure 5-4 Seedling growth and morphology of *AtMyb84*-RNAi knock down lines.

5.2.3 *AtMyb84* knock down line preparation using a multiple arm-RNAi construct

I prepared dual RNAi constructs containing two arms specific to separate genes in order to simultaneously down regulate both genes. Constructs are shown in Figure 2-4. To test the functionality of this strategy, test dual constructs were generated containing RNAi specific to *GUS* and to *AtMyb84* (Figure 5-5).

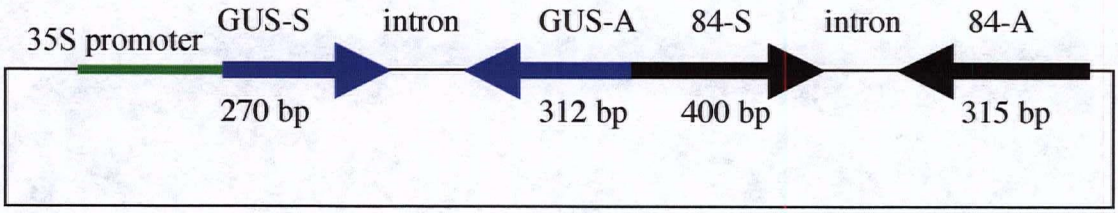


Figure 5-5 Dual RNAi construct specific for *GUS* and *AtMyb84* genes

A transgenic 35S::GUS line was transformed with this dual RNAi construct. More than 20 independent T1 lines were tested for GUS expression using the histochemical assay and many of them showed apparent GUS down regulation (Figure 5-6).

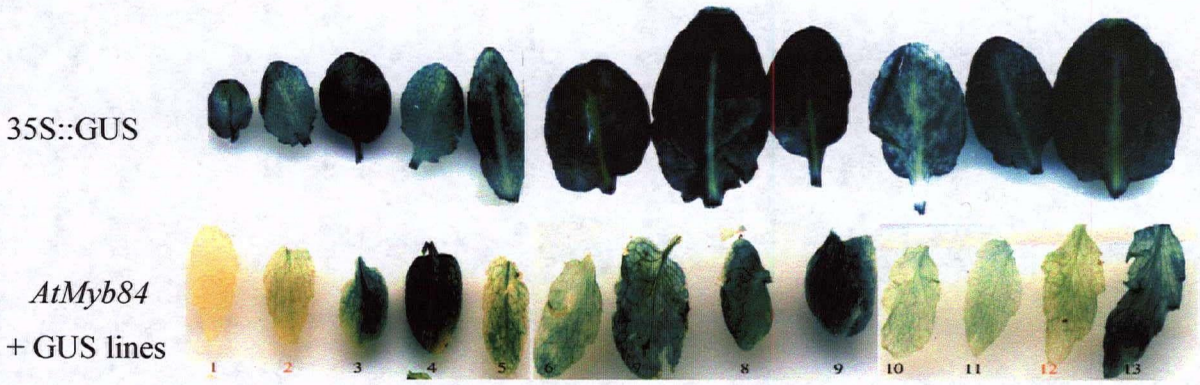


Figure 5-6 GUS expression in transgenic plants generated by transformation of a *35S::GUS* line with a *Myb84+GUS* double RNAi construct. Top, histochemical assay of GUS expression in leaves of a control *35S::GUS* line. Bottom leaves of similar age from 13 independent lines transgenic for the *Myb84+GUS* double RNAi construct in the *35S::GUS* background.

I performed RT-PCR on these lines to estimate *AtMyb84* expression levels and found both *GUS* and *AtMyb84* down regulation in some of the lines (Figure 5-7) with lines #2 and #10 showing strong down regulation of the *AtMyb84* gene along with strong reduction in GUS activity. No *AtMyb84*-associated phenotype has been detected in these knock down lines so far.

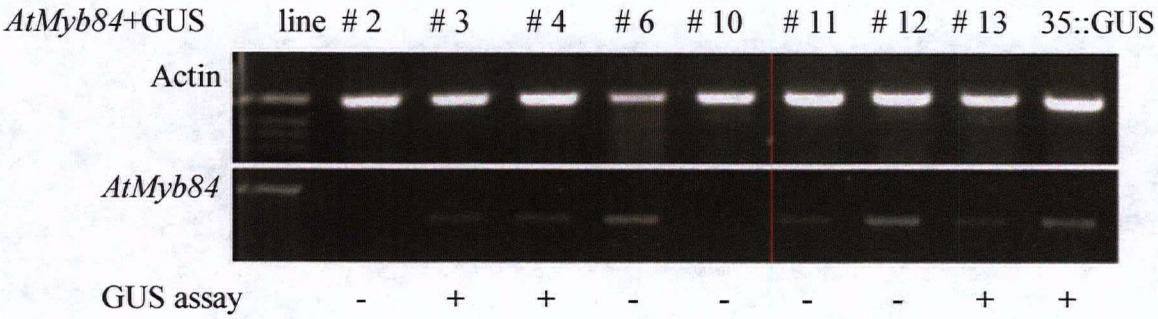


Figure 5-7 RT-PCR analysis of *AtMYB84* expression in *Myb84*+GUS double RNAi lines. Results of histochemical GUS assays performed on 21 days old plants are shown below; "-" indicates substantially reduced GUS expression. PCR reactions were performed for 28 cycles, within the linear range of *AtMyb84* amplification.

Ten transgenic lines were prepared containing the dual *AtMyb68* and *AtMyb84* RNAi construct shown in Figure 2-4. No morphological phenotype was detected in the vertically grown seedlings of these lines (Figure 5-8) or later stages of plant development (data not shown).

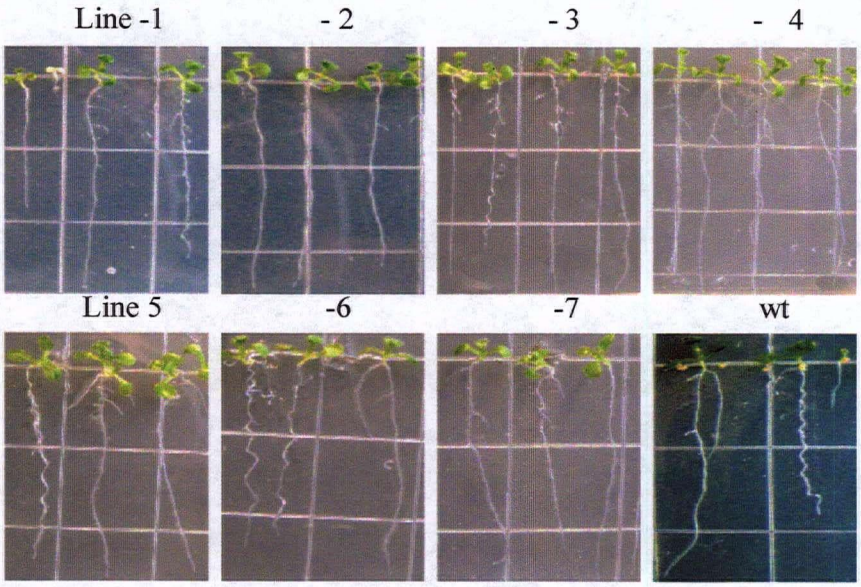


Figure 5-8 Seedling growth and morphology of transgenic lines containing double RNAi *AtMyb68* +*AtMyb84* constructs. Plants were 10 days old.

5.2.4 Transformation of *AtMyb68* knock out line using the *AtMyb84* RNAi construct

The *AtMyb68* knock out line previously characterized by Wang (2003) was transformed with the *AtMyb84* specific RNAi construct. Vertical growth of homozygous transformants in parallel to other controls did not reveal any morphological differences at the seedling stage (Figure 5-9).

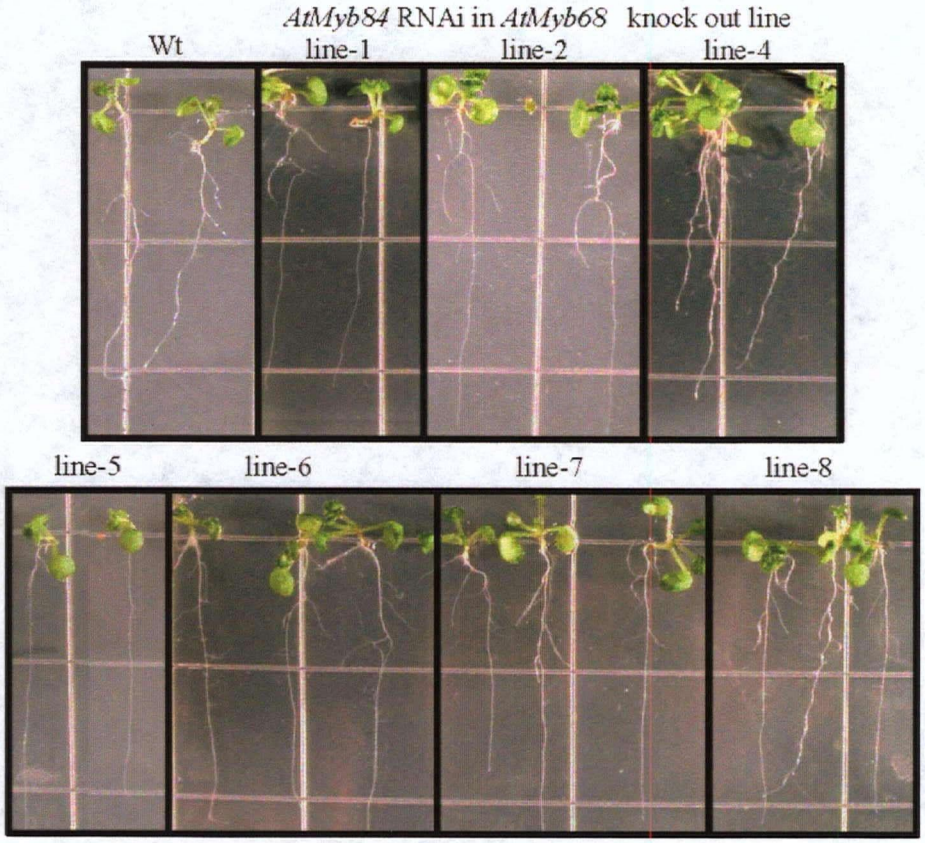


Figure 5-9 Seedling growth and morphology of *AtMyb84* RNAi knock down lines in the *AtMyb68* knock out background. Seven representative RNAi lines are shown.

However, rosettes of some of these lines such as line #1 and #3 were smaller than others in the first round of phenotypic analysis and lines 1, 2, and 3 were delayed in development of the primary inflorescence shoot (Figure 5-10). In this figure, while line 4 has developed a bolting stem and even siliques, line 2 just started to develop and lines 1 and 3 are severely delayed in bolting.

Figure 5-10 Rosette stage growth and development of four *Myb84* RNAi lines in the *Myb68* knock out background. Plants were homozygous for both transgenes and of the same age but their rosettes showed different sizes and stages of development.

Preliminary analysis of more individuals of these transgenic lines after another week of growth suggested that in lines #1, #3 and #7 development of the primary inflorescence shoot was delayed relative to control plants and other RNAi lines (Figure 5-11). While lines 4, 5 and 8 had developed primary inflorescence shoot with developing siliques, lines 1 and 3 were still at the rosette stage and line 7 had just started to develop an inflorescence. Mature plants of these lines did not show any phenotypic difference, compared to wild type plants (Figure 5-12). Overall, a consistent pattern of delay in emerging inflorescence was observed in this phenotypic analysis.

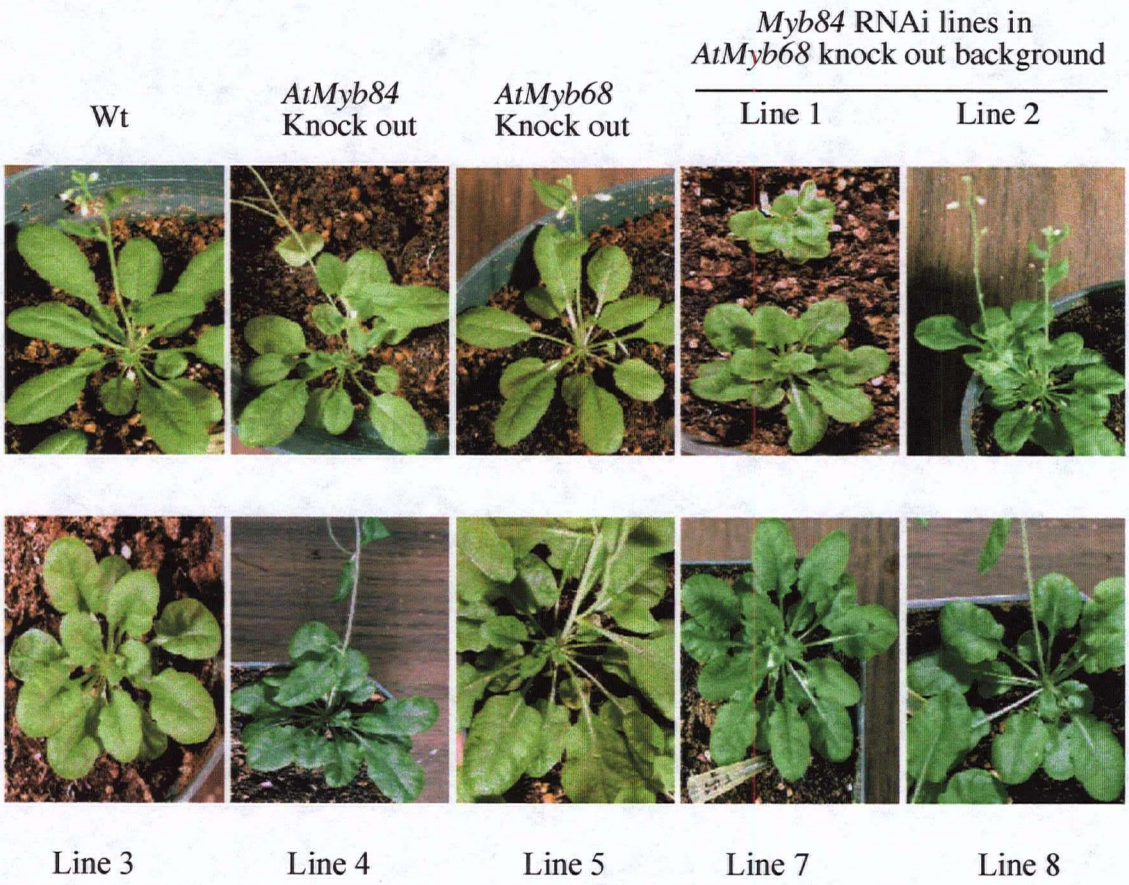


Figure 5-11 Rosettes with emerging primary shoot in seven *Myb84* RNAi lines in the *Myb68* knock out background. These lines are compared to Wt, single *Myb68* knock out, and single *Myb84* knockout control lines one week younger in age.

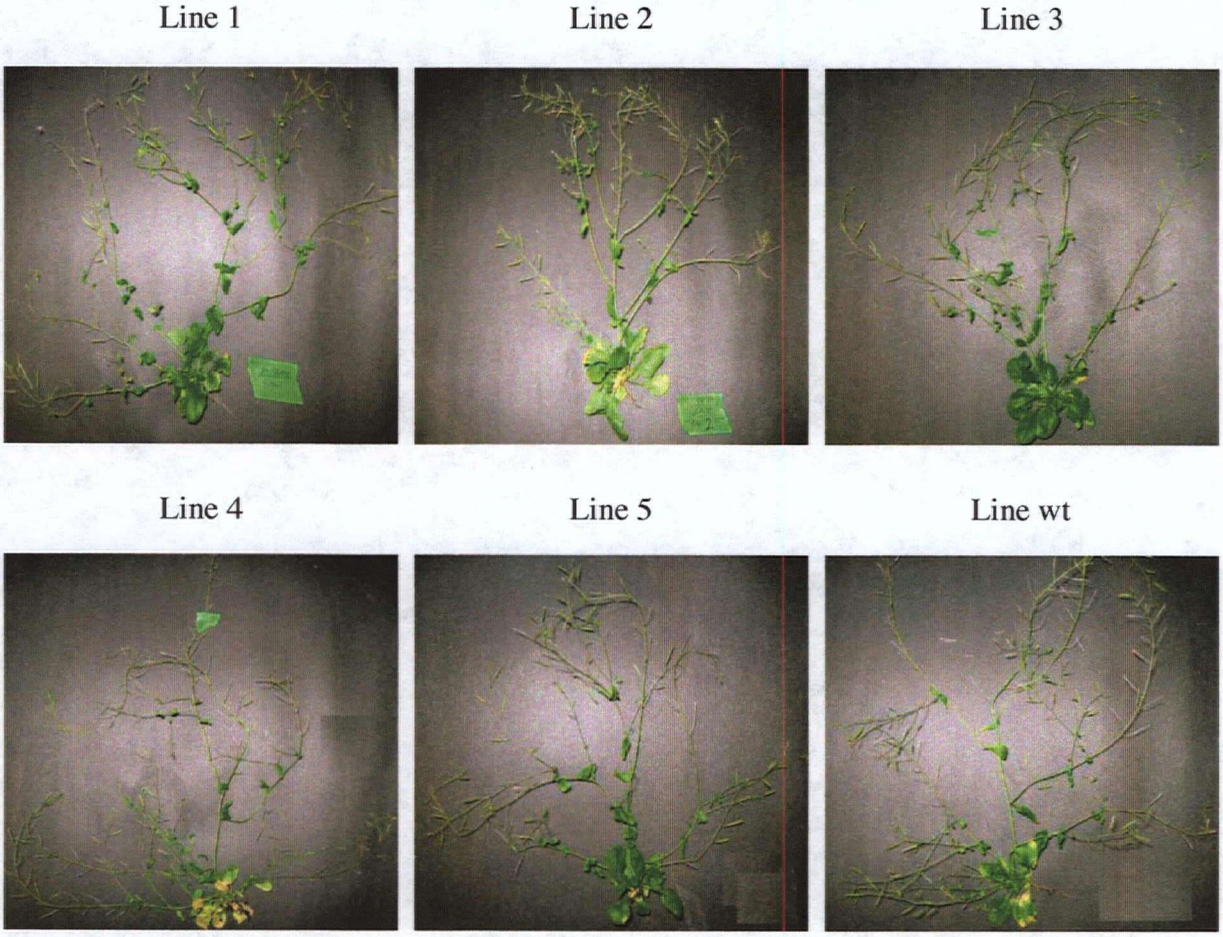


Figure 5-12 Mature plants of *AtMyb84* RNAi lines in the *AtMyb68* knock out background compared to Wt control.

5.2.5 The *AtMyb84* knock out line preparation

Subsequent to the above work, a potential *AtMyb84* T-DNA knock out line became available in the Salk collection and was obtained. This line was no longer kanamycin resistant, but the presence of a T-DNA insertion in the *AtMyb84* gene (Figure 5-13) was confirmed by PCR, using T-DNA left border specific (LBb1) and *AtMyb84* specific (84Eco-F) primers (Table 2.1).

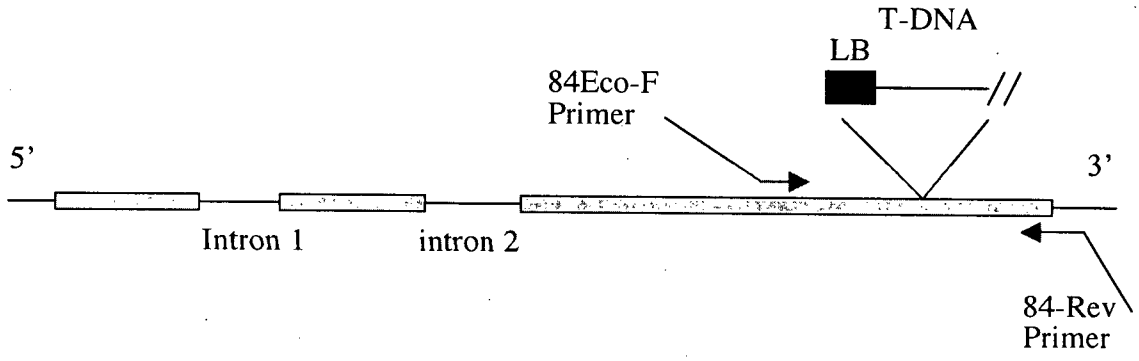


Figure 5-13 Schematic of T-DNA insertion within *AtMyb84* knock out lines from Salk collection. Blue boxes indicate exons and arrows indicate the primers used in the PCR reactions. LB indicates left border of the T-DNA.

This PCR reaction generated the expected 100-bp PCR product from the third exon of the gene into which the T-DNA had been integrated. However, using two *AtMyb84* specific primers, 84Eco-F and 84-Rev (Table 2.1), an additional 300-bp wild-type PCR product indicated that the original line was heterozygous. A homozygous *AtMyb84* knock out line was identified in the next generation and was confirmed by multiple PCR tests. Preliminary RT-PCR confirmed *AtMyb84* knock out phenotype (data not shown). Seedlings of this *AtMyb84* knock out line were vertically grown in parallel with *AtMyb68* knock out line and wild type plants but no significant morphological difference was detected at this stage (Figure 5-14, A). Also no morphological difference was detected at the rosette stage (Figure 5-14, B) and mature plants (Figure 5-14, C) of this line compared to wild type and *AtMyb68* knock out lines. The obvious next step to cross *AtMyb68* and *AtMyb84* knock out lines to generate a double knock out has been initiated, but analysis of progeny from this cross is beyond the scope of this thesis.

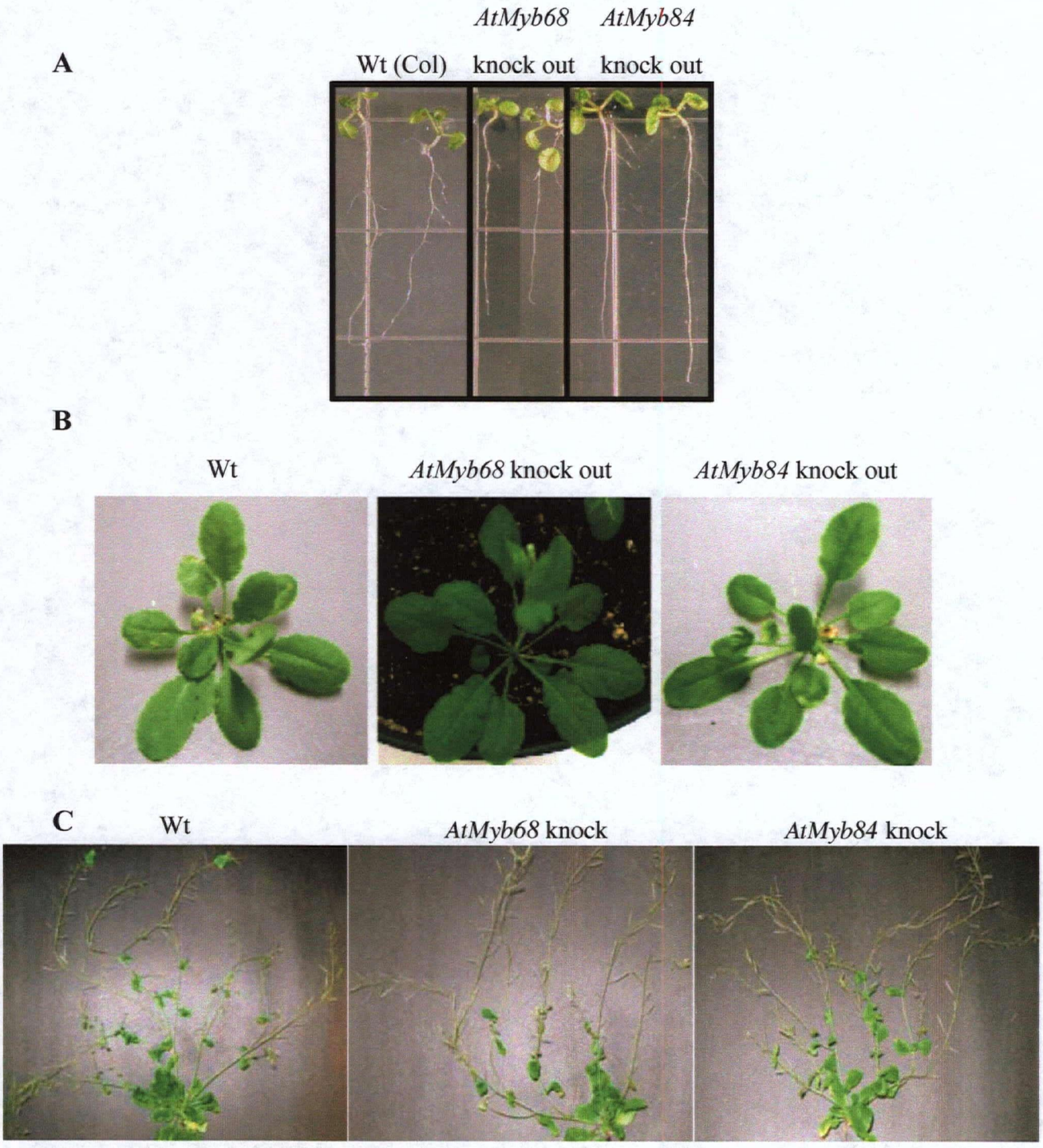


Figure 5-14 Comparison of *AtMyb68* and *AtMyb84* knock out lines and wild type plant, **A)** 10 days old seedlings, **B)** Rosettes, **C)** Mature plants

5.3 DISCUSSION

5.3.1 Bioinformatics data indicated high homology and overlapping expression of *AtMyb68* and *AtMyb84* genes.

Based on a the Phylogenetic tree of the AtMyb sub-family # 14 shown in Figure 5-1 (Stracke *et al.*, 2001), *AtMyb84* was considered the best candidate for a gene with hypothesized functional redundancy to *AtMyb68*. Conserved synteny and also highly similar cDNA sequences and high homology of protein sequences of these two genes (Figure 5-1) indicate that the DNA blocks containing *AtMyb84* or *AtMyb68* belong to duplicated regions of the *Arabidopsis* genome and that *AtMyb68* and *AtMyb84* may be functionally redundant. We were thus expecting overlapping expression patterns for both of these genes, and the MPSS database as well as *Arabidopsis* Functional Genomics Tools on the University of Toronto BBC web site (http://bbc.botany.utoronto.ca/) indicated such an overlapping expression pattern for these two genes in several organs and developmental stages. These two genes also showed overlapping expression with other members of AtMyb subfamily #14, especially with *AtMyb36* that is co-expressed with *AtMyb68* and *AtMyb84* in the root. This suggests that *AtMyb68* and *AtMyb84* may have similar functions in the shoot. On the other hand, these two genes as well as *AtMyb36* could encode partially redundant root functions.

5.3.2 No phenotype has been observed for *AtMyb84* knock down and knock out lines.

In the *AtMyb68* knock out line no phenotype has been detected so far (Wang, 2003; Figure 5-14). From RNA blots and GUS assays performed on *AtMyb68::GUS* transgenic plants (Wang, 2003) it is known that *AtMyb68* has a root preferred expression pattern. From the microarray data it is also known that *AtMyb36, AtMyb68* and *AtMyb84* show overlapping expression in the root. Thus, we hypothesized that if only *AtMyb68* and *AtMyb84* genes are functionally redundant, we may be able to see a phenotype in the root when both are knocked down/out in the same line. If *AtMyb84* is not functionally redundant to any other *Myb* gene in the subfamily, a single gene knock down/out of *AtMyb84* should show a phenotype.

I generated more than 10 independent homozygous *AtMyb84* RNAi knock down lines and also *AtMyb84+GUS* double RNAi knock down lines, performed semi-quantitative RT-PCR, and identified lines with down regulated *AtMyb84* gene expression (Figure 5-3 and 5-7). Consistent with our hypothesis, no morphological differences between vertically grown seedlings of the candidate *AtMyb84* knock down lines (line #6 and #13) and the control plants were detected (Figure 5-4). Also, *AtMyb84 + GUS* double RNAi lines (#2 and # 10) in the Figure 5-7 were grown vertically on MS media and soil and compared with the recipient 35S::GUS line as control but no phenotypic differences between these two lines and the control were found (data not shown).

I later found a putative *AtMyb84* T-DNA knock out line in the Salk collection and a homozygous *AtMyb84* knock out line was identified in the next generation. Although we still need to confirm this knock out phenotype through analysis of *AtMyb84* RNA levels, vertically grown seedlings of this line also did not show any differences with control plants (Figure 5-14, A). I also detected no differences between the rosettes and mature plants of these knock out line and those of control plants (Figure 5-14, B and C).

The tomato *Blind* gene is an R2R3 class *MYB* gene that is closely related to members of AtMyb subfamily #14 (Schmitz *et al.,* 2002). In the *blind* mutants, the initiation of lateral meristems during the shoot and inflorescence development is blocked leading to reduction of the number of lateral axes, which is manifested in reduction of shoot and inflorescence branching. I also examined the *Arabidopsis* shoot branching in the *AtMyb84* knock down and knock out lines and did not see differences between the candidate lines and the controls (Figure 5-14, C).

5.3.3 Preliminary phenotype for double *AtMyb*68 - *AtMyb*84 knock down/out lines.

As the accumulated data indicated that *AtMyb68* and *AtMyb84* have at least partially redundant functions, I used different strategies to knock both *AtMyb68* and *AtMyb84* genes down and/or out in the same plant. First, The *AtMyb68* knock out line was transformed using the *AtMyb84* specific RNAi construct (Figure 5-2). Second, I considered that double mutants may be lethal. To avoid full knock out of both genes I made *AtMyb68* plus *AtMyb84* double gene knock down lines using a double RNAi construct. In advance, functionality of dual RNAi constructs was first examined using

dual test construct of GUS plus *AtMyb84* RNAi (Figure 5-5). In terms of the technical advance, I found that using two RNAi molecules in a tandem arrangement to down regulate two genes at the same time works at a reasonably high level, such that one RNAi arm (*AtMyb84* specific) will not prevent second RNAi arm (GUS specific) from functioning to initiate RNAi mediated RNA degradation (Figure 5-6 & 5-7 line 2 and 10). We plan to use this method to down regulate all the genes in the subfamily #14 to further investigate the redundancy relationships of its members.

Vertical growth of homozygous lines generated using the first and second strategies did not reveal any mutant phenotypes at the seedling stage (Figure 5-8 and 5-9). The lines of *AtMyb84* RNAi knock down line in the *AtMyb68* knock out background were transferred to soil in order to search for the possible phenotypes in the aerial parts of the mature plants. In some of these lines, such as lines #1 and #3, I found smaller rosettes (Figure 5-10). They also were delayed in shoot development (Figure 5-11). Comparison of these lines (Figure 5-10), showed that when lines 1 and 3 were still at an early stage of rosette development, line 2 was more advanced and line 4 had already developed siliques. All these lines were transplanted from agar plates at the same age and grown in identical conditions. Also plants from the same homozygous line grown in each pot showed the same phenotypes. Overall, the severity of the rosette-stage phenotype varies between the individual lines generated by this approach, as judged by the sizes of mutant plants shown in Figure 5-10, and timing of the development of inflorescence meristems (Figure 5-11) relative to control plants. This variability could be due to the variability in the degree of *AtMyb84* knock down between lines, which will be tested by measurement of relative RNA levels by RT-PCR.

Based on the phenotype of tomato *blind* mutants, I searched for the reduction of shoot and inflorescence stem branching in the *Arabidopsis AtMyb68*, *AtMyb84*, and *AtMyb68- AtMyb84*double knock out/down lines. I did not see a difference between the candidate lines and the controls (Figure 5-12 & 5-14). Once it is confirmed that *AtMyb84* RNA is down-regulated or absent in double knock out/down lines, this data would indicate either that *AtMyb68* and *AtMyb84* do not share analogous functions with the *BLIND* gene, despite their close phylogenetic relationships (Schmitz *et al.*, 2002), or that

additional genes within AtMyb subfamily 14 have redundant functions with *AtMyb68* and/or *AtMyb84* with respect to control of lateral bud formation.

No phenotype was detected in the roots of double knock down/out lines. Assuming that *AtMyb84* expression knock down can be confirmed, we may consider *AtMyb36* as a third gene that shares redundant functions with *AtMyb68* and *AtMyb84* in the root, where all three genes show high expression. If so, triple knock outs of *AtMyb36*, *AtMyb68*, and *AtMyb84* will be required to test this hypothesis. As mentioned above, in order to reach final conclusions it is necessary that *AtMyb68* and *AtMyb84* expression in the knock out/down lines to be confirmed through RNA analysis. A lack of root and lateral branch phenotypes in the *AtMyb68* knockout/and *AtMyb84* RNAi line could result from insufficient repression of *AtMyb84* expression to generate root or lateral bud mutant phenotypes, and knock outs of both genes may be required to reveal clear phenotypes. On the other hand, in many cases and also in the case of *AtMyb68* and/or *AtMyb84*, mutations may cause no obvious morphological defect under normal growth conditions nonetheless confer phenotypes that can be detected with biochemical/molecular analysis, or by testing the mutants under a battery of stress conditions. In that sense, further genetic and phenotypic analysis of *AtMyb68* and/or *AtMyb84* mutant plants may be required to test this hypothesis.

In summary, as predicted by close phylogenetic relationships and partially overlapping expression patterns, and supported by the lack of obvious phenotypes associated with single *AtMyb68* and *AtMyb84* knockout/down lines, *AtMyb68*, *AtMyb84* *AtMyb36* may play similar and partially redundant functions in plant development. Initial information from double *AtMyb68* knockout/*AtMyb84* knockdown lines suggests redundant roles of these two genes in rosette development and timing of inflorescence stem development. Further genetic, molecular, and phenotypic analyses of double, and possibly triple mutants will be required to draw definitive conclusions regarding such functions. Also it is necessary to back cross the mutant lines to the wild type plant and try to segregate away potential unlinked mutations.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 At4CL::GUS TRANSGENE SILENCING

At4CL1::GUS and *At4CL2::GUS* transgenic plants showed predictable, overlapping patterns of xylem abundant GUS expression similar to the pattern described in transgenic *Arabidopsis* plants containing the parsley *4CL1* promoter fused to *GUS* (Lee *et al.*, 1995). Considering the presence of multiple common *cis* regulatory elements in their promoters, high identity of their cDNA sequences and overlapping expression patterns seen in northern analysis (Ehlting *et al.*, 1999), *At4CL1* and *At4CL2* genes are likely to show at least partial functional redundancy. Our finding that epimutant 2-8, in which the endogenous *At4CL2* gene was silenced, showed no phenotypic differences to non-mutant plants supports this conclusion, and this was recently confirmed by generation and analysis of a *At4CL1* and *At4CL2* double knock out (Hamberger personal communication). Our results show specific and different silencing modes for *At4CL1::GUS* and *At4CL2::GUS* transgene silencing. This silencing phenomenon in the *At4CL::dhlA* lines made our negative selection system unsuitable for mutant selection, and silencing of *At4CL::GUS* transgenes also impaired the ability to identify Mendelian mutants in components required for *At4CL* activation. To overcome these problems we suggest preparation of *At4CL::GUS* or *At4CL::dhlA* transgenic plants containing a single transgene in each line confirmed by Southern analysis, and exclusion of 5'-UTR sequences.

In terms of the modes of silencing, *At4CL1::GUS* transgene silencing was *cis* and root specific in seedlings, while *At4CL2::GUS* transgene silencing was *trans* and global. In keeping with a widely accepted model of double stranded RNA mediated transgene silencing (Bender 2004), overabundant messages containing *At4CL* 5'-UTRs was likely the trigger for silencing in both *At4CL:;GUS* and *4tCL::dhlA* lines. This could be tested by cloning of *At4CL* 5'-UTRs down stream of the 35S promoter to investigate the effect of this overabundance on transgene silencing. Investigating the presence of siRNA in the

silenced lines also may confirm the involvement of RNAi machinery in the silencing of *At4CL1::GUS* or *At4CL2::GUS* or both. To avoid transgene silencing, lines with single copy of *At4CL::GUS* transgene is more suitable for the mutant screen. Also screens for mutants with increased or ectopic transgene expression may avoid silencing artifacts. Alternatively, different regions of the endogenous *At4CL* promoter could be specifically targeted for promoter methylation with transcribed inverted repeat transgenes to map the regions necessary for expression. Regardless of the mechanism of the observed silencing, the root specific silencing of the *At4CL1::GUS* transgene is difficult to explain, using published silencing models.

6.2 *At4CL2* WOUND RESPONSE ELEMENTS

Based on northern analysis, rapid but transient accumulation of *At4CL2* mRNA is induced in response to wounding (Ehlting *et al.*, 1999). For other phenylpropanoid genes a second, later wound response has also been reported (Logemann *et al.*, 1995; Batard *et al.*, 2000). Our expression results showed early and late phases of both *At4CL1* and *At4CL2* wound responses while an immediate but longer lasting *At4CL4* response was observed. *At4CL3* expression was rapidly down regulated in response to wounding at the time that *At4CL1* and *At4CL2* were up regulated. We positioned the wound response regulatory elements associated with the *At4CL2* gene in both promoter, and in the transcribed regions. Our results suggest a highly complex regulatory structure, with positively and negatively acting elements scattered at different locations both upstream and within the transcribed region. To fully localize and characterize the specific *cis*-regulatory elements that direct these complex expression patterns, it will be necessary to create serial deletion constructs, or to mutagenize candidate *cis*-elements, and determine the effects of such specific changes, alone or in combination, on wound inducible expression.

We used *At4CL1::GUS* and *At4CL4::GUS* transgenic plants containing only 1 kb of these *At4CL* genes promoter sequences. While *At4CL1::GUS* lines only showed a late wound response, *At4CL4::GUS* lines showed a more immediate wound response. Shuffling of the *At4CL1 or At4CL2* promoter fragments with *At4CL4* promoter sequences may help to localize the *cis* elements that are responsible for the biphasic or immediate

responses of *At4CL* genes.

6.2 FUNCTIONS OF AtMYB SUBFAMILY 14 MEMBERS

No phenotype related to *AtMyb68* knock out has been found so far. Functional redundancy of this gene with the closest family member in the subfamily is a possible reason for the lack of detectable phenotype. *AtMyb84* was found to be the closest family member to the *AtMyb68*. No phenotype was detected in *AtMyb84* knock out line, supporting the functional redundancy hypothesis. Double *AtMyb68* knock out / *AtMyb84* knock down lines were generated using an *AtMyb84* RNAi construct. Preliminary results showed that rosette and primary shoot development was delayed in these knock down / out lines. Considering the lack of detectable phenotype in *AtMyb68* and *AtMyb84* single knock out lines, these putative phenotypes, not seen in either single mutant, are most likely related to the functional redundancy of these two genes. In the future, it will be essential to analyze *AtMyb84* expression in these lines (*AtMyb68* expression is already known to be almost abolished in the knock out line). It will also be necessary to document the morphological phenotypes in subsequent generations.

Since *AtMyb36* and *AtMyb87* genes in the subfamily 14, could also be functionally redundant with *AtMyb68 and AtMyb84* genes, it may be necessary to create triple knockout lines in order to observe strong phenotypes. *AtMyb68* expression is primarily associated with roots and *AtMyb36* also shows root abundant expression (Wang, 2003), but these genes are also related to the tomato *Blind* gene, which encodes a MYB transcription factor that plays a role in lateral shoot formation (Schmitz *et al.*, 2002). Thus, the specific functions of these transcription factors could be related to both shoot and root development.

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