Genetic Analysis of \textit{At4CL} Gene Regulation and \textit{AtMyb} subfamily 14 Functional Characterization in \textit{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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<td>ACC synthase</td>
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To all who taught me
To my family
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)

<table>
<thead>
<tr>
<th>Gene Family</th>
<th>Predicted Gene Number</th>
<th><em>Arabidopsis</em></th>
<th><em>Drosophila</em></th>
<th><em>C. elegans</em></th>
<th><em>S. cerevisiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Myb</td>
<td>~130</td>
<td>35</td>
<td>16</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>AP2/EREBP</td>
<td>150</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NAC</td>
<td>105</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>bHLH/MYC</td>
<td>100</td>
<td>61</td>
<td>38</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>bZIP</td>
<td>100</td>
<td>24</td>
<td>18</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>HD</td>
<td>90</td>
<td>113</td>
<td>88</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Z-C2H2</td>
<td>85</td>
<td>352</td>
<td>138</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>MADS</td>
<td>80</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>WRKY</td>
<td>75</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ARF</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dof</td>
<td>41</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

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 downregulate 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 hypermethylated. 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 (Pf'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 sill. 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 sill 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 sill-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 AtPAL gene family and AtPAL1, 2 and 4 have demonstrated de-amination activity (Cochrane et al., 2004). Only one AtC4H 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 *At4CL3* is not affected but the other *At4CL* 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 al., 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
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 kinasel (WPKI) 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, 4CL, C4H, 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* subfamily #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 days at 4°C on germination medium. Then, seeds were germinated at 20 °C either on soil (SunsHine 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 the plates 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 rifampicillin (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, rifampicillin, 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® 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 32P-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 phosho-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.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1kb4CL1-Forward</td>
<td>5' - GGTCTCCAAAGTTGAATTAATGGTTGTAG</td>
</tr>
<tr>
<td>GUS-Reverse</td>
<td>5' - TCGCGATCCAGACTGAATGCCCAC</td>
</tr>
<tr>
<td>4CL1/ORF-Forward</td>
<td>5' - CTCTTGTTAAAACACAACCTGGTTTCGA</td>
</tr>
<tr>
<td>4CL1/ORF-Reverse</td>
<td>5' - GTTTCCTAACGCAAAGCTGGTCAGGGGC</td>
</tr>
<tr>
<td>-2504CL2-Forward</td>
<td>5' - ATGTCGACAGCATGGATAATGATAGTAGAGTAGGC</td>
</tr>
<tr>
<td>84Eco-F</td>
<td>5' - AAGAATTCGGATCCAAGATCGAAGATCAAGAAGCTGG</td>
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<tr>
<td>84 Rev primers</td>
<td>5' - ATGGTACCAATCGATTTGAATCAGAATACAGAGAGGC</td>
</tr>
<tr>
<td>LBbl</td>
<td>5' - GCGTGGACCGCTTGCTGCAACT</td>
</tr>
<tr>
<td>4CL1/RT-F</td>
<td>5' - CTAATGCGCAAACGCGTTGTCAGGGGATAC</td>
</tr>
</tbody>
</table>

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/25 μl 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 Alphalmager 1220.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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<tr>
<td>4CL1/RT-F</td>
<td>5'-CTAATGCCAACACTCGGTAGGAGATAC</td>
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<tr>
<td>4CL1/RT-R</td>
<td>5'-CTCTTGTAACACGACACCTGTTTGAC</td>
</tr>
<tr>
<td>4CL2/RT-F</td>
<td>5'-CCCAACGCAAAGCTTGGTCAGGGCTATGGG</td>
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<td>4CL2/RT-R</td>
<td>5'-CACCAAGCATCTTTCTTCATGGCGACG</td>
</tr>
<tr>
<td>4CL3/RT-F</td>
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<td>4CL3/RT-R</td>
<td>5'-CTGGATTTACATCTTTTCATGTAGT</td>
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<td>4CL4/RT-F</td>
<td>5'-CGAGGTATCAGTACTCCTTTGAT</td>
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<td>4CL4/RT-R</td>
<td>5'-GGTTTGATAGTTGGATCGAGCGACTG</td>
</tr>
<tr>
<td>Actin-F</td>
<td>5'-GCGCAAATGGAATTCGGAAT</td>
</tr>
<tr>
<td>Actin-R</td>
<td>5'-GGTAGCATGTGGAGTTGCATACC</td>
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</tbody>
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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 37°C 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.
<table>
<thead>
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<th>Primer name</th>
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<td>- 110 F</td>
<td>5'-ATGTCGACATATGGAAACACTGATCATCATGC</td>
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<td>- 250 F</td>
<td>5'-ATGTCGACAGCATTGGATATGATAGTAGAGTAGC</td>
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<td>- 420 F</td>
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<td>- 750 F</td>
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<td>-1.6kF</td>
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<td>2Cod-R</td>
<td>5'-ATCCCATGGGGGTTCATTAATCCTTCTTCTTCTTAG</td>
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<td>2ORF-F</td>
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</tr>
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</tr>
<tr>
<td>+ 1000 cod-F</td>
<td>5'-TACCATGGTTAAGTCTGAGCGAGCTCCTC</td>
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<tr>
<td>+ 1500 cod-F</td>
<td>5'-TGACCATGGACTGTATTTTAACCTTTTAGAGGAGGTCCAC</td>
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<tr>
<td>+ 2000 cod-F</td>
<td>5'-GGGCCCATGGGAATGACAGAGCGAGGTCCCGG</td>
</tr>
<tr>
<td>-1kb4CL1-For</td>
<td>5'-GGTCTCCAAAGTGAATTAAATGTTGTA</td>
</tr>
<tr>
<td>GUS-Reverse</td>
<td>5'-TCGCGATCCAGACTGAATGCCCAC</td>
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<tr>
<td>4CL1/ORF-Re</td>
<td>5'-GTTTCCTAAGCAGCCAGTCTGAGTCAAGGG</td>
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<td>pHannibal-F</td>
<td>5'-CCCACATCTCTCGCAAGACCC</td>
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<td>5'-CAACGTGCAACACGAGAATTGAAAGG</td>
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<td>Hanni-intron</td>
<td>5'-CATACTAATTAACATCACCCTAAAC</td>
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<tr>
<td>reverse</td>
<td></td>
</tr>
<tr>
<td>Hanni-intron</td>
<td>5'-CATGTCATTGTGTTATCATTGT</td>
</tr>
<tr>
<td>forward</td>
<td></td>
</tr>
</tbody>
</table>

**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 \textit{At4CL::GUS} plasmid constructs

Various \textit{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 \textit{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 \textit{XbaI} to eliminate 600 bp of the upstream promoter sequences to prepare the 950bp construct.

![Diagram](image.png)

Figure 2-1 Relative position of the primers used to amplify \textit{At4CL2} promoter fragments. \textit{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 \textit{At4CL2} gene from \textit{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 \textit{XbaI} and \textit{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 \textit{4CL2::GUS} construct (Figure 2.2-A & -B). Using \textit{XbaI} and \textit{SacI} restriction enzymes, the full-length \textit{4CL2::GUS} construct was transferred to the pBAR binary vector.
Figure 2-2 Schematic representation of \textit{At4CL2} promoter and transcribed regions fused to GUS. A) Locations of primers used to amplify \textit{At4CL2} promoter plus transcribed regions. B) Structure of \textit{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 \textit{At4CL2} promoter::\textit{At4CL2}-cDNA::GUS construct, the \textit{At4CL2}-cDNA was amplified using 2ORF-F and 2Cod-R primers (Figure 2.2-A), both containing Ncol sites (Table 2.3). Using Ncol and SacII 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 SacI and XbalI restriction enzymes, the 950 bp promoter::4CL2-cDNA::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 Nhel. 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 NcoI-XbaI restriction fragment containing this construct was transferred to the pCambia1300 binary vector. To prepare the intron 1 construct, a 2950-bp AvrII-PstI 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 BamH1 and KpnI 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 Clal, and cloned into pHannibal which already had the 400-bp sense arm (Figure 2.3-B).
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M68-EcXbH-F</td>
<td>5'-TTGAATTCTAGAAGCTTCAGCACAACACTCACCATTACCAAAC</td>
</tr>
<tr>
<td>M68-Bam-F</td>
<td>5'-AAAGGATCCCCTTGTATCATGAGCAGCAGC</td>
</tr>
<tr>
<td>M68-Kp-CL R</td>
<td>5'-ATGGTACCACATCGATTGCGCATTGAAAGTAATCTTGC</td>
</tr>
<tr>
<td>M68-Ec-Ba-F</td>
<td>5'-AAGAATTCTGGATCCAAGATCGAGATCAAAGAAGCTGG</td>
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<tr>
<td>M68-Xba-F</td>
<td>5'-GATCTAGAACTGGGAGAAAAACACCTCAC</td>
</tr>
<tr>
<td>M68-Kp-Cl-R</td>
<td>5'-ATGGTACCAATCGATTGAAATCGAATAAACAGAGAGC</td>
</tr>
<tr>
<td>Gus-Xb-Ec-F</td>
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<td>Gus-Xho-F</td>
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<tr>
<td>Gus-Kp-Cl-R</td>
<td>5'-TCGGTACCTATCGATTGACCCACACTTTGCGG</td>
</tr>
<tr>
<td>M84-R-Kpn</td>
<td>5'-TACGCTACCTACAAATTATAATAATGATGTC</td>
</tr>
</tbody>
</table>

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-EcXba-H-F) or (M68-Bam-F) and (M68-Kp-CLR) primers and cloned in pHannibal using *HindIII* and *Kpnl* restriction sites for cloning sense arm and *BamHI* and *ClaI* sites for cloning the antisense arm (Figure 2-4, A). I chose ~350 bp of the downstream 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 *XhoI* and *Kpnl* restriction enzymes and cloned into pHannibal as the sense arm. The 312-bp antisense arm was also amplified using (Gus-Xbal-Eco-F) and (Gus-Kp-Cl-R) primer. PCR products were cut by *XbaI* and *ClaI* 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 XhoI and EcoRI 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 EcoRI and BamHI 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 NotI 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 dhlA 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 (dhlA) gene (Janssen et al., 1989) works as a negative selection marker in Arabidopsis (Naested et al., 1999) when dhlA-expressing plants are treated with a haloalkane pro-toxin substrate, such as 1,2-dichloroethane (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 \textit{At4CL} EXPRESSION AND EPIGENETIC SILENCING OF 4\textit{CL} 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).

\textit{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 \textit{et al}, 2003). \textit{At4CL1}, \textit{At4CL2}, \textit{At4CL3} (Lee \textit{et al}, 1995; Ehlting \textit{et al}, 1999 and \textit{At4CL4} (Hamberger \textit{et al}, 2004) encode the complete 4\textit{CL} gene family in \textit{Arabidopsis}. \textit{At4CL1} and \textit{At4CL2} share 83\% amino acid sequence identity and 74\% cDNA identity in cDNA coding regions (Ehlting \textit{et al}, 1999; Hamberger \textit{et al}, 2004).

\textit{At4CL1} and \textit{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 \textit{et al}, 1995, Mizutani \textit{et al}, 1997, Ehlting \textit{et al}, 1999), similar to the parsley (\textit{Petroselinum crispum}) 4\textit{CL1} gene, the first 4\textit{CL} gene cloned (Hauffe \textit{et al}, 1991). From the analysis of organ-specific expression of \textit{At4CL1} and \textit{At4CL2} genes in \textit{Arabidopsis} it is known that both \textit{At4CL1} and \textit{At4CL2} have high expression levels in seedling roots. In adult plants, \textit{At4CL1} is the only gene family member strongly expressed in bolting stems (Ehlting \textit{et al}, 1999).

\textit{At4CL1} and \textit{At4CL2} promoters contain multiple common \textit{cis} regulatory elements that are also found in other genes encoding enzymes in the general phenylpropanoid and lignin-specific biosynthetic pathways (Ehlting \textit{et al}, 1999; Hamberger \textit{et al}, 2004). Considering the high identity of \textit{At4CL1} and \textit{At4CL2} plus common \textit{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; Martienssen, 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 \textit{At4CL::GUS} transgenic lines to isolate multiple mutant lines with reduced \textit{GUS} expression. All mutant lines showed non-Mendelian inheritance of the \textit{GUS} expression phenotypes and the majority were sensitive to treatment by the demethylating agent, 5-aza that restored the original \textit{At4CL::GUS} transgene expression in the roots, implicating alterations in DNA methylation in the loss of \textit{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 \textit{At4CL} epimutants had been isolated. \textit{At4CL1::GUS} epimutants differed from \textit{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 \textit{AT4CL} SIGNALING PATHWAY MUTANTS

3.2.1.1 Generation of transgenic \textit{Arabidopsis} lines containing 4CL::Reporter transgenes

I, intended to screen for mutants in genes required for activation of developmentally regulated \textit{At4CL} expression in mutagenized transgenic lines containing \textit{At4CL::GUS} or \textit{At4CL::dhlA} transgenes. Transgenic \textit{Arabidopsis thaliana} (Ler) lines containing 1.0-kb \textit{At4CL1}, 1.6-kb \textit{At4CL2} and 0.6-kb \textit{At4CL3} promoter fused to \textit{GUS} transgenes, were generated by Dr. J. Ehlting. The \textit{At4CL1::GUS} and \textit{At4CL2::GUS} transgenic lines showed GUS expression throughout the plant vasculature while \textit{At4CL3::GUS} line showed the expression of GUS in areas other than the vasculature (Figure 3-1). Both representative \textit{At4CL1::GUS} and \textit{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 \textit{At4CL1::GUS} and \textit{At4CL2::GUS} transgenic lines were used in
two parallel genetic screens aimed at identifying mutants affected in developmental regulation of \textit{At}4\textit{CL}1 and \textit{At}4\textit{CL}2, using \textit{GUS} expression as a visual phenotype.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3-1.png}
\caption{Developmentally regulated GUS expression in \textit{At}4\textit{CL}1::\textit{GUS}, \textit{At}4\textit{CL}2::\textit{GUS} and \textit{At}4\textit{CL}3::\textit{GUS} lines (data from Dr. J. Ehlting, unpublished).}
\end{figure}

The dehalogenase-A (\textit{dhlA}) gene, when driven by the CaMV35S promoter, has been shown to be an effective negative selectable marker in \textit{Arabidopsis} plants fed with the pro-toxin substrate 1,2-dichloroethane (DCE) (Janssen et al., 1994; Naested et al., 1999). In collaboration with Dr. Ehlting, I explored the use of \textit{dhlA} to select for mutants affected in developmental regulation of \textit{At}4\textit{CL} promoter activity. To establish a negative selection system for \textit{At}4\textit{CL} promoter activity, Dr. Ehlting fused the \textit{dhlA} gene to \textit{At}4\textit{CL}1, \textit{At}4\textit{CL}2 and \textit{At}4\textit{CL}3 promoters, and introduced the \textit{At}4\textit{CL}1::\textit{dhlA}, \textit{At}4\textit{CL}2::\textit{dhlA} and \textit{At}4\textit{CL}3::\textit{dhlA} fusions into \textit{At}4\textit{CL}1::\textit{GUS}, \textit{At}4\textit{CL}2::\textit{GUS} and \textit{At}4\textit{CL}3::\textit{GUS} transgenic plants, respectively, to generate lines harboring both \textit{At}4\textit{CL}::\textit{GUS} and \textit{At}4\textit{CL}::\textit{dhlA} transgenes. This would allow dual screening for mutants impaired in \textit{At}4\textit{CL} 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 \textit{At}4\textit{CL}1::\textit{dhlA}, \textit{At}4\textit{CL}2::\textit{dhlA}, and \textit{At}4\textit{CL}3::\textit{dhlA} transgenic lines. The
majority of these lines showed monohybrid ratios for the \textit{At4CL::dhlA} transgenes inheritance. Using \textit{At4CL} promoter specific and \textit{dhlA/ORF} specific reverse primers, the presence of \textit{At4CL::dhlA} transgenes in the plants was confirmed.

\subsection*{3.2.1.2 Negative selection system optimizing for \textit{At4CL::dhlA} lines}

I first optimized the system to establish the highest amount of DCE that was not toxic for wild type \textit{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 \textit{At4CL::dhlA} lines in the appropriate \textit{At4CL::GUS} background were treated with DCE under conditions optimized for negative selection of a control \textit{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 \textit{35S::dhlA} seeds or seedlings. Germination and growth of \textit{At4CL::dhlA} lines in the \textit{At4CL::GUS} background, were also delayed but DCE treatment was not lethal as their growth resumed after treatment. In all of the \textit{At4CL::dhlA} lines with \textit{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).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3-2.png}
\caption{Treatment of \textit{At4CL::dhlA} lines with DCE and comparison of their sensitivity to the \textit{35S::dhlA} line.}
\end{figure}
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](image)

**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::dhIA* lines

I examined *At4CL::GUS* transgene expression in the *At4CL::dhIA/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::dhIA/At4CL::GUS* transgenic plants. A) *At4CL1::dhIA* lines in the *At4CL1::GUS* background. B) *At4CL3::dhIA* 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.](attachment:image.png)
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.

![Wild-type At4CL1::GUS, Weak GUS Activity, Patchy GUS Activity, No GUS Activity](image)

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 \((\text{At}\text{4CL1}::\text{GUS})\) 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 \(4\text{CL1}::\text{GUS}\) mutant lines were crossed to the non-mutant \(4\text{CL1}::\text{GUS}\) 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

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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 \textit{At4CL1::GUS} line (T14) crossed to wild type showed root specific lack of \textit{GUS} expression. These results suggest that the putative mutants affected in \textit{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 \textit{At4CL::GUS} transgene DNA methylation in the mutant lines, I grew three mutant lines in the \textit{At4CL1::GUS} background (1-A, 1-B, 1-C) and one mutant in the \textit{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 \textit{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 MspI/HpaII 
and MboI/Sau3A1 to restrict the genomic DNA from mutants 1-A and 2-8 respectively. 
MspI is only inhibited when the outermost cytosine in its recognition site (CCGG) is 
methylated, allowing detection of methylation in a non-CG context, and is otherwise is
able to cut at the recognition site. However, the *HpaII* 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 *MspI/HpaII* (TCCGGT) restriction site in the 1 kb *At4CL1::GUS* promoter, whose cleavage in combination with cleavage in the T-DNA and *GUS MspI/HpaII* 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 *MspI* or *HpaII* 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 *HpaII* and *MspI* digestion, showing that the *MspI/HpaII* 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 *HpaII* restricted DNA of line 1-A is clearly thicker and more intense than the band of similar size of the same DNA restricted by *MspI*, 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 *HpaII* and *MspI* 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 *HpaII* restricted DNA and also a band bigger than 1200 bp that was unique to the *HpaII* 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 *HpaII* to cut its recognition site within the *At4CL1::GUS* and/or endogenous *At4CL1* promoters. Methylation of other
Mspl sites upstream of the At4CL1::GUS transgene in the T-DNA may create 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 MspI and HpaII 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/Sau3AI isoschizomer pair, with Sau3A 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/Sau3AI recognition site in the At4CL2 promoter. This analysis showed that digestion of mutant 2-8 genomic DNA with Sau3A generated an At4CL2 promoter fragment unique to the mutant. The size of this fragment (1165bp, Figure 3-14) is consistent with failure of Sau3A to cut at the MboI/Sau3AI recognition site in At4CL2 promoter, resulting of the fusion of 979 bp and 186 bp fragments to yield a 1165 bp Sau3A fragment in the mutant. This fragment was not observed in Sau3A 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 At4CL2 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/Sau3AI 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 $\sim$ 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.

![Northern blot analysis of *At4CL1* gene expression in *At4CL1::GUS* lines.](image)

**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 sill-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-specific probe. Again, the results were consistent among multiple experiments, but in contrast to the At4CL1 lines, showed that the endogenous At4CL2 gene was significantly downregulated 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 sill 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-homocysteine 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 sill 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 sill-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 \textit{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 \textit{et al.}, 1999; Chandler, 2000). The down regulation of endogenous \textit{At4CL2} gene expression in the mutant 2-8 was specific to \textit{At4CL2} and \textit{At4CL1} was not affected (Figure 3-16 line 7). Conversely, \textit{At4CL2} gene expression was not affected in the mutant 1-A, which affected \textit{At4CL1::GUS} expression (Figure 3-17 line 5). To confirm the specificity of \textit{At4CL::GUS} silencing, I crossed mutant 1-A to the \textit{At4CL2::GUS} line and mutant 2-8 to the \textit{At4CL1::GUS} line to see if silencing of the \textit{At4CL1::GUS} transgene could trigger silencing of \textit{At4CL2::GUS} and vice versa. F1 seedlings showed GUS expression in both crosses suggesting that silencing of \textit{At4CL1::GUS} may not affect the expression of the \textit{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.

\subsection*{3.3.4 Silencing of \textit{At4CL1} and \textit{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 \textit{et al.}, 2002), specific methylation of multiple identical promoters of (trans) genes and 5-aza-restorable expression point to TGS versus PTGS of \textit{At4CL::GUS} transgenes. To evaluate the critical characteristics of the transgene loci that could have directed TGS of \textit{At4CL::GUS} and \textit{At4CL::dhIA} 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 \textit{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 \textit{At4CL2::GUS} line with the additional \textit{At4CL2::dhlA} construct, the \textit{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 \textit{At4CL2::GUS} plants until the introduction of the second transgene (\textit{At4CL2::dhlA}) with a common \textit{At4CL2} promoter and 5'-UTR sequence. We suggest that 5'-UTR common to both transcripts was detected by a \textit{At4CL2} gene-specific threshold sensing system, causing \textit{trans}-silencing of \textit{At4CL2::reporter} transgenes and the endogenous \textit{At4CL2} gene in the \textit{At4C2::dhlA} and mutant 2-8 lines (Figure 3-17). Sporadic up regulated \textit{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 \textit{At4CL2} promoter as well as the \textit{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 \textit{At4CL2} 5'-UTR sequences in \textit{At4CL2::GUS} plants under the control of a strong promoter other than \textit{At4CL2} promoter. We would predict this to result in a co-suppression like phenomenon (Napoli et al., 1990).

Contributions of aberrant promoter transcripts to \textit{trans}-TGS of un-linked transgene has also been reported (Mette \textit{et al.}, 1999, and 2000; Sijen \textit{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 \textit{At4CL1} and \textit{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 \textit{At4CL2::GUS} and \textit{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 *hogl* and *sill* 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 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-UTRs 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 \textit{At4CL3} mRNA levels are not affected (Ehlting \textit{et al.}, 1999). The response of \textit{At4CL4} to wounding is yet unknown.

A typical approach used to identify \textit{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 \textit{et al.}, 1991; Zhang \textit{et al.}, 1994; Gidekel \textit{et al.}, 1996; de Boer \textit{et al.}, 1999; Ito \textit{et al.}, 2003).

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

Here, I show that \textit{Arabidopsis thaliana} (Ler) \textit{At4CL1} and \textit{At4CL2} promoter::GUS fusions direct developmental GUS expression to the vasculature of leaves, stems, and roots, whereas the \textit{At4CL4} promoter does not direct detectable developmentally regulated GUS expression. I report the locations of \textit{cis}-regulatory elements controlling developmental and wound-induced expression of the \textit{At4CL2} gene. Using promoter deletions, I identified the locations of \textit{cis}-elements directing developmental expression in upstream promoter regions where they were expected. RNA analysis has shown rapid wound responsiveness of the \textit{At4CL2} gene (Ehlting \textit{et al.}, 1999), and data presented here show that \textit{At4CL2} expression is activated in a biphasic manner, with early and late responses. However, \textit{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 \textit{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 \textit{At4CL2} gene. We also report lack of developmental GUS expression driven by the \textit{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 \textit{At4CL} Promoter::GUS fusions

Transgenic plants containing \textit{At4CL1}, \textit{At4CL2}, \textit{At4CL3}, and \textit{At4CL4} promoter::GUS constructs (Figure 4-1, C) were generated and introduced into \textit{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 \textit{At4CL1}::GUS and \textit{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 \textit{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 \textit{et al.}, 1999). Developmental GUS expression in the mature leaves of \textit{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 \textit{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 \textit{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 \textit{At4CL3}::GUS line showed no wound response while the \textit{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.
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<th>72 h post Wounding</th>
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**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](image-url)
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-bp 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. \textit{At4CL1} and \textit{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). \textit{At4CL4} transcript levels also increased by 2.5 h post-wounding but remained elevated for at least 12 h. \textit{At4CL3} expression was rapidly down regulated in response to wounding, but recovered to basal levels by 4 h post wounding. Thus, \textit{At4CL} gene family members display a diverse set of responses to wounding.

![Diagram showing accumulation of At4CL mRNA after wounding](image)
**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 (Narvéz-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 undetected 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 \textit{At4CL2}-cDNA::\textit{GUS} and \textit{At4CL2}-intron 2\&3::\textit{GUS} lines (Figures 4-2 and 4-5).

Figure 4-9 Summary of deduced positions of positive and negative regulatory elements in the \textit{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, \textit{At4CL4}::\textit{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 \textit{At4CL4} mRNA accumulation in wounded leaves (Figure 4-7). In both experiments levels of wounding induced \textit{At4CL4} mRNA accumulation and \textit{At4CL4}-driven GUS expression were distinguishable from the control even after 10 min post wounding (data not shown). This characteristic distinguishes \textit{At4CL4} from the other \textit{At4CL} genes, and suggests the presence of strong wound responsive \textit{cis}-regulatory elements in the 1-kb \textit{AtCL4} promoter used in these experiments.

4.3.4 \textit{Intronic cis}-regulatory elements are involved in the late wounding response of \textit{At4CL2} gene expression.

The line expressing the 1-kb promoter \textit{At4CL1}::\textit{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.
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).

![RNAi construct for *AtMyb84*](image)

**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).

![RT-PCR on *AtMyb84* RNAi knock down lines](image)

**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.

*AtMyb84*

| RNAi line 3 | line 6 | Wt | line 12 | line 13 |

![Figure 5-4 Seedling growth and morphology of AtMyb84-RNAi knock down lines.](image)

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).
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).

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](image)

**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).
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 \textit{AtMyb68} and \textit{AtMyb84} genes.

Based on a the Phylogenetic tree of the AtMyb sub-family # 14 shown in Figure 5-1 (Stracke \textit{et al.}, 2001), \textit{AtMyb84} was considered the best candidate for a gene with hypothesized functional redundancy to \textit{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 \textit{AtMyb84} or \textit{AtMyb68} belong to duplicated regions of the \textit{Arabidopsis} genome and that \textit{AtMyb68} and \textit{AtMyb84} may be functionally redundant. We were thus expecting overlapping expression patterns for both of these genes, and the MPSS database as well as \textit{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 \textit{AtMyb36} that is co-expressed with \textit{AtMyb68} and \textit{AtMyb84} in the root. This suggests that \textit{AtMyb68} and \textit{AtMyb84} may have similar functions in the shoot. On the other hand, these two genes as well as \textit{AtMyb36} could encode partially redundant root functions.

5.3.2 No phenotype has been observed for \textit{AtMyb84} knock down and knock out lines.

In the \textit{AtMyb68} knock out line no phenotype has been detected so far (Wang, 2003; Figure 5-14). From RNA blots and GUS assays performed on \textit{AtMyb68::GUS} transgenic plants (Wang, 2003) it is known that \textit{AtMyb68} has a root preferred expression pattern: From the microarray data it is also known that \textit{AtMyb36}, \textit{AtMyb68} and \textit{AtMyb84} show overlapping expression in the root. Thus, we hypothesized that if only \textit{AtMyb68} and \textit{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 \textit{AtMyb84} is not functionally redundant to any other \textit{Myb} gene in the subfamily, a single gene knock down/out of \textit{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 AtMyb68 - AtMyb84 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 $\textit{At4CL}$ genes.

### 6.2 FUNCTIONS OF $\textit{AtMYB}$ SUBFAMILY 14 MEMBERS

No phenotype related to $\textit{AtMyb68}$ 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. $\textit{AtMyb84}$ was found to be the closest family member to the $\textit{AtMyb68}$. No phenotype was detected in $\textit{AtMyb84}$ knock out line, supporting the functional redundancy hypothesis. Double $\textit{AtMyb68}$ knock out / $\textit{AtMyb84}$ knock down lines were generated using an $\textit{AtMyb84}$ 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 $\textit{AtMyb68}$ and $\textit{AtMyb84}$ 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 $\textit{AtMyb84}$ expression in these lines ($\textit{AtMyb68}$ 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 $\textit{AtMyb36}$ and $\textit{AtMyb87}$ genes in the subfamily 14, could also be functionally redundant with $\textit{AtMyb68}$ and $\textit{AtMyb84}$ genes, it may be necessary to create triple knockout lines in order to observe strong phenotypes. $\textit{AtMyb68}$ expression is primarily associated with roots and $\textit{AtMyb36}$ also shows root abundant expression (Wang, 2003), but these genes are also related to the tomato $\textit{Blind}$ 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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