The 4-Coumarate:Coenzyme A Ligases from *Nicotiana tabacum* and *Arabidopsis thaliana*: Characterization of cDNA Clones, Gene Families, Recombinant Proteins, and Antisense Transgenic-Plants

by

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Abstract

The cDNAs encoding 4-coumarate:coenzyme A ligase (4CL), an enzyme in the general phenylpropanoid pathway, were cloned from *Nicotiana tabacum* and *Arabidopsis thaliana*. In tobacco, 4CL was encoded by a gene family and northern blot analysis demonstrated that the steady-state RNA levels were highest in stems, ovaries, and non-pigmented portions of the corolla. Two *4CL* cDNAs, which were 80% identical to each other at the nucleotide level, were expressed in *E. coli*. The relative abilities of the recombinant-4CL proteins to utilize 4-coumarate, ferulate, and caffeate as substrates were comparable to that of the 4CL activity found in tobacco-stem extracts. Both recombinant-4CL proteins utilized cinnamate as a substrate, an activity not observed in tobacco extracts. This activity towards cinnamate was inhibited by a modifying-component found in tobacco extracts and the evidence suggests that the substrate specificity of 4CL is, in part, determined by post-translational modification such as phosphorylation.

In *Arabidopsis*, 4CL was shown to be encoded by a single gene. Northern blot analysis indicated that, like tobacco, *4CL* steady-state RNA levels were highest in the bolting stem. The *Arabidopsis-4CL* cDNA was inserted in antisense orientation behind the CaMV 35S or parsley *4CL1* promoters and introduced into *Arabidopsis*. Transgenic plants were analyzed by western blot analysis and plants with severely suppressed-4CL protein levels were further analyzed. One transgenic-*Arabidopsis* line had greater than 90% decrease in 4CL enzyme activity and accumulated significantly less (50%) lignin in the bolting stem as compared to wild-type untransformed plants. Despite the decrease in *4CL* mRNA, 4CL protein, and 4CL enzyme activity, anthocyanin accumulation was unaffected in the antisense-*4CL* *Arabidopsis*-lines. Mature, fully-expanded *Arabidopsis*-leaves were wounded and this resulted in a coordinated increase in RNA transcripts from genes encoding enzymes in the oxidative pentose phosphate pathway, the shikimic acid pathway, and the general

phenylpropanoid pathway. The coordinated activation of gene expression was observed in the wild-type and antisense-*4CL* transgenic lines suggesting that wound-induced gene expression is not dependent on the carbon flow through the 4CL-catalyzed step.

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Abbreviations

35S	Cauliflower mosaic virus 35S promoter
4CL	4-Coumarate:coenzyme A ligase
6PGDH	6-phosphogluconate dehydrogenase
At4CL	cDNA encoding 4-Coumarate:coenzyme A ligase from *Arabidopsis thaliana*
BSA	Bovine serum albumin
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CAF	Caffeic acid
CCR	Cinnamoyl-CoA reductase
CHS	Chalcone synthase
CIN	Cinnamic acid
CIP	Calf intestinal alkaline phosphatase
COL	Columbia ecotype of *Arabidopsis*
COU	4-Coumaric acid
DAHPS	3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase
DFR	Dihydroflavonol 4-reductase
EPSPS	5-*Enol*pyruvylshikimate-3-phosphate synthase
F3H	Flavanone 3-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
FER	Ferulic acid
G6PDH	Glucose-6-phosphate dehydrogenase
IPTG	Isopropyl-ß-D-thiogalactopyranoside
kb	Kilobase pair
kDa	Kilodalton
K_m	Michealis-Menten constant
MJ	Methyl jasmonate
MS	Murashige and Skoog
Nt4CL	cDNA encoding 4-Coumarate:coenzyme A ligase from *Nicotiana tabacum*
PAL	Phenylalanine ammonia-lyase
Pc4CL-P	Parsley *4CL1* promoter
pQE-1	QIAexpressionist plasmid containing the Nt4CL-1
pQE-19	QIAexpressionist plasmid containing the Nt4CL-19
RLD	RLD ecotype of *Arabidopsis*
SDS	Sodium dodecyl sulphate
V_{max}	Maximum velocity

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Chapter 1

Introduction

1.1 Phenylpropanoid Metabolism

1.1.1 Introduction

The flow of carbon from primary metabolism into the biosynthesis of an array of phenylpropanoid secondary products involves a minimum of three enzymatic steps, catalyzed by the actions of phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL) which collectively form the general phenylpropanoid pathway (Figure 1.1). The phenylpropanoid products formed by the action of specific downstream branch pathways include coumarins, flavonoids, lignin, suberin, tannins, and other phenolic compounds which serve diverse functions as phytoalexins, UV-protectants, floral and fruit pigments, structural components of cell walls, and signaling molecules (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). The biosynthetic pathways leading to the formation of flavonoids and lignin have been relatively well characterized and they involve key

enzymes like chalcone synthase (CHS) and chalcone isomerase (CHI) for flavonoid biosynthesis; and cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) for lignin biosynthesis.

Phenylpropanoid metabolic pathways are coordinately regulated. In bean hypocotyls, mechanical wounding and infection with pathogenic fungi causes an increase in *PAL*, *CHS*, and *CHI* mRNA transcripts (Lawton and Lamb, 1987). Similarly, treatment of bean cell cultures with elicitor causes a coordinate activation of *PAL*, *CHS*, and *CHI* expression (Cramer *et al.*, 1985a,b) and in some studies, gene activation was initiated as rapidly as 5 minutes post-elicitation (Lawton and Lamb, 1987). The increase in phenylpropanoid-specific enzyme activity is correlated with the increase in isoflavonoid phytoalexins (Robbins *et al.*, 1985). In parsley cell cultures (Chappell and Hahlbrock, 1984) and parsley protoplasts (Dangl *et al.*, 1987), treatment with UV-light resulted in an increase in *PAL*, *4CL* and *CHS* RNA levels followed by an increase in flavonoids. Treatment with elicitor caused an increase in *PAL*, *4CL* and *BMT* (bergaptol *O*-methyltransferase, an enzyme involved in furanocoumarin biosynthesis) RNA levels concomitant with increases in coumarins (Chappell and Hahlbrock, 1984; Dangl *et al.*, 1987). *In situ* hybridization demonstrated the presence of *PAL*, *4CL*, and *CHS* RNAs in the epidermal layer of young parsley-leaves where flavonoids accumulate. In the epithelial cells of oil-ducts where furanocoumarins accumulate, parsley *PAL*, *4CL*, and *BMT* RNA transcripts were detected (Wu and Hahlbrock, 1992). These results demonstrate that phenylpropanoid gene expression is coordinately regulated and is temporally- and spatially- specific to the accumulated end-products. Coordinate up-regulation of phenylpropanoid genes or enzymes has also been demonstrated in other plant systems (Hahlbrock and Scheel, 1989) such as soybean (Börner and Grisebach, 1982), potato (Fritzemeier *et al.*, 1987), alfalfa (Dalkin *et al.*, 1990), *Arabidopsis* (Kubasek *et al.*, 1992), poplar (Moniz de Sá, 1992), cactus (Paré *et al.*, 1992), and tobacco (Ellard-Ivey and Douglas, 1996). The coordinate up-regulation of many of the

genes in the general phenylpropanoid and specific branch pathways may be an efficient way of controlling carbon flow through these pathways into product formation.

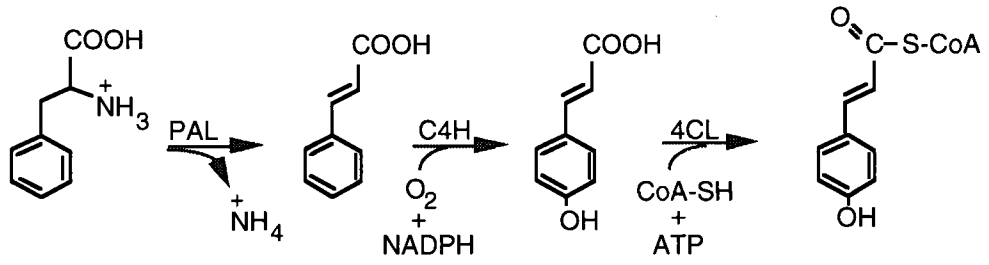
Phenylpropanoid metabolism is developmentally regulated. Genes encoding phenylpropanoid enzymes have been shown to be activated in a cell-specific manner in cells of the developing xylem, epithelial cells of oil-ducts, and the epidermal cells of flower petals (Schmelzer *et al.*, 1989; Wu and Hahlbrock, 1992; Drews *et al.*, 1992; Reinold *et al.*, 1993). The activation of phenylpropanoid genes in these cell-types and tissues may be important for the biosynthesis of lignin, furanocoumarins, and anthocyanins respectively. Promoter-*GUS* fusion analysis in transgenic tobacco and transgenic *Arabidopsis* showed that the promoters of *PAL* (Bevan *et al.*, 1989; Liang *et al.*, 1989; Ohl *et al.*, 1990), *4CL* (Hauffe *et al.*, 1991; Lee *et al.*, 1995a), and *CHS* (Schmid *et al.*, 1990; Drews *et al.*, 1992) direct complex patterns of GUS staining in the flower, the leaf vein, and the root vasculature. These results show that phenylpropanoid expression occurs in a tissue-specific and temporally-regulated manner that correlates with the time and place where phenylpropanoid compounds accumulate.

Wounding and pathogen attack activates phenylpropanoid metabolism. In bean hypocotyls, infection with an incompatible fungus causes an increase in *PAL* and *CHS* RNA (Cramer et *al.*, 1985a,b; Lawton and Lamb, 1987) and enzyme activity (Robbins *et al.*, 1985). In potato leaves, infection with a fungal pathogen results in the accumulation of *PAL* and *4CL* transcripts (Fritzemeier *et al.*, 1987). In parsley, infection with a non-pathogenic fungus and wounding both cause the accumulation of phenylpropanoid gene transcripts at the infection/wound site (Schmelzer *et al.*, 1989). Similarly, *Arabidopsis PAL* (Ohl *et al.*, 1990; Wanner *et al.*, 1995) and *4CL* (Lee *et al.*, 1995a) RNA levels increase during wounding and during infection by *Pseudomonas syringae*. The induction of phenylpropanoid gene expression at the wound or infection site is believed to be for the biosynthesis of phytoalexins, phenolic compounds, and lignin-like subunits which are deposited at wound/infection sites (reviewed in Nicholson and Hammerschmidt, 1992). These phenylpropanoid products

prevent pathogen invasion by inhibiting pathogen growth (phytoalexins) or by inhibiting pathogen spread (deposition of cell wall components). It should be noted that pathogen attack also activates a systemic response which includes the expression of the *PR* (Pathogenesis-Related) genes. Some of the PR proteins are of unknown function while others encode chitinases, hydroxyproline-rich proteins, and protease inhibitors. These enzymes/proteins increase the plants systemic resistance against pathogens (SAR; systemic acquired resistance). The systemic response may be mediated in part by salicylic acid, a compound derived from the product of the PAL-catalyzed reaction. Thus, in addition to the activation of phenylpropanoid gene expression, pathogen attack may also activate another class of genes, the *PR* genes, via salicylic acid which is derived from phenylalanine.

Phenylpropanoid metabolism has also been shown to be induced by other conditions such as ozone (Sharma and Davis, 1994; Eckey-Kaltenbach *et al.*, 1994), cytokinins (Deikman and Hammer, 1995), nutrient starvation (Dixon and Paiva, 1995), and cold treatment. (Leyva *et al.*, 1995). The roles of phenylpropanoid compounds under these stresses have not been clearly defined (reviewed in Dixon and Paiva, 1995).

In vivo footprinting and deletion analysis have defined putative *cis*-elements that are conserved in the promoters of phenylpropanoid genes. In particular, conserved sequences like the P-, A-, and L- boxes (Lois *et al*, 1989; Logemann *et al.*, 1995), and putative Myb-binding sequences (Sablowski *et al.*, 1994; Douglas, 1996) have been detected in the promoters of *PAL, 4CL, CHS*, and *CAD*. BPF-1, a DNA-binding protein from parsley (da Costa e Silva *et al.*, 1993), and PBP, a flower-specific Myb protein from tobacco (Sablowski *et al.*, 1994) have been shown bind to the P-box. Gel-retardation assays have identified nuclear proteins from parsley and tobacco which bind to the parsley *4CL1* promoter (Hauffe *et al.*, 1993). Thus, the presence of these conserved *cis*-elements and the activities of the *trans*-acting factors provide a mechanism in which the phenylpropanoid genes may be coordinately regulated.



Phenylalanine | Cinnamic acid | 4-Coumaric acid | 4-Coumaroyl:CoA

Figure 1.1: The general phenylpropanoid pathway. The three core reactions in the general phenylpropanoid pathway are catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:coenzyme A ligase (4CL).

1.1.2 General Phenylpropanoid Metabolism

The three enzymes PAL, C4H and 4CL comprise the general phenylpropanoid pathway (Figure 1.1). Products derived from the general phenylpropanoid pathway enter branch pathways which synthesize specific end-products such as furanocoumarins, flavonoids and lignin. Thus, PAL, C4H and 4CL are important in that they are the core enzymes from which all other phenylpropanoid compounds are made. These enzymes and the genes encoding them are described below.

PAL is the first enzyme in the general phenylpropanoid pathway and it is one of the most well characterized enzymes in phenylpropanoid metabolism (Jones, 1984). PAL catalyzes the deamination of phenylalanine to form cinnamic acid (Figure 1.1). Cinnamic acid can be used for the biosynthesis of other phenolic acids (coumaric acid, caffeic acid), phenolic esters, benzoic acid derivatives (salicylic acid, gallic acid), coumarins, B-ring-deoxy flavonoids and other cinnamyl derivatives (Lewis, 1993; Lee *et al.*, 1995b; Liu *et al.*, 1995). As the first enzyme in general phenylpropanoid metabolism, PAL has been suggested to catalyze the rate-limiting step which controls

the flux of carbon compounds into phenylpropanoid biosynthesis. Chemical analogs of phenylalanine like α-aminooxy-β-phenylpropionic acid (AOPP) and 1-amino-2-(phenylethyl)phosphonic acid (APEP) inhibit PAL activity. In buckwheat seedlings, red-cabbage seedlings, morning-glory flowers, and other tissues, the use of AOPP causes a decrease in anthocyanin accumulation (Amrhein and Hollander, 1979). Mungbean seedlings treated with AOPP have collapsed xylem vessels and lack lignin-like components as demonstrated by phloroglucinol/HCL staining and nitrobenzene oxidation (Amrhein *et al.*, 1983; Smart and Amrhein, 1985). In tobacco leaves, sense suppression of *PAL* causes a decrease in chlorogenic acid and rutin accumulation (Bates *et al.*, 1994). In contrast, lignin content in tobacco stems decreases only when PAL activity was suppressed by 75% to 80% compared to wild-type levels (Bates *et al.*, 1994). These results confirm that the biosynthesis of phenylpropanoid compounds require PAL activity and that it is the major control point in the biosynthesis of chlorogenic acid and rutin. However, with respect to lignin biosynthesis, the endogenous levels of PAL in tobacco stems are in excess relative to the amount of lignin made in normal development.

The genes and cDNAs encoding *PAL* have been isolated from over 25 plants (Genbank) including bean (Cramer *et al.*, 1989), parsley (Lois *et al.*, 1989), poplar (Subramaniam *et al.*, 1993), tobacco (Pellegrini *et al.*, 1994) and *Arabidopsis* (Wanner *et al.*, 1995). In most plants, PAL is encoded by a small gene family of 3-5 genes; however, notable exceptions include the PAL from loblolly pine which may be encoded by a single gene (Whetton and Sederoff, 1992) and the PAL from potato which is represented by a family of 40-50 genes (Joos and Hahlbrock, 1992). Members of the *PAL* gene family are differentially expressed. In parsley, *PAL2* is expressed constitutively in the roots while *PAL3* is expressed in response to wounding (Lois and Hahlbrock, 1992). In potato (Joos and Hahlbrock, 1992), *PAL1* is expressed more rapidly than *PAL2* in response to infection by a virulent fungus (compatible interaction). The bean *PAL2* gene is expressed after elicitor treatment whereas the

PAL3 gene is not (Cramer *et al.*, 1989). In agreement, transgenic studies with the bean *PAL* promoters fused to *GUS* demonstrate that the bean *PAL* promoters direct different patterns of tissue-specific GUS staining and direct differential GUS-staining in response to wounding and elicitor treatment (Shufflebottom *et al.*, 1993).

Like other phenylpropanoid genes, *PAL* expression is developmentally regulated, stress inducible and coordinately regulated. In parsley (Wu and Hahlbrock, 1992), *in situ* localization shows that *PAL* RNA levels accumulate in a tissue-specific fashion that is coordinately regulated with the presence of RNA from *4CL*, *CHS* (in the epidermis) or *BMT* (in the oil ducts). In parsley cell cultures, treatment with UV-light or elicitor cause a coordinate increase in *PAL* and *4CL* RNA levels (Ragg *et al.*, 1981; Chappell and Hahlbrock, 1984; Schmelzer *et al.*, 1985). Inoculation of parsley leaves with a non-pathogenic fungus causes a localized increase in *PAL* RNA at the site of attempted infection and this expression is coordinated with the expression of *4CL* and other pathogen-related (*PR*) and elicitor-related (*ELI*) genes (Schmelzer *et al.*, 1989). Developmental and stress-regulated *PAL* expression also occurs in bean cell cultures (Edwards *et al.*, 1985), potato (Fritzemeier *et al.*, 1987), *Arabidopsis* (Kubasek *et al.*, 1992) and poplar (Subramaniam *et al.*, 1993).

The second enzyme in the general phenylpropanoid pathway is C4H. C4H is a cytochrome P-450 monoxygenase and it catalyzes the hydroxylation of cinnamate at the *para*-position to produce 4-coumarate using O_2 and NADPH. Because of its instability and its microsomal localization, purification and characterization of C4H has been limited to a few plants. The C4H enzymes from potato (Tanaka *et al.*, 1974), wheat (Maule and Ride, 1983), Jerusalem artichoke (Gabriac *et al.*, 1991), and soybean (Kochs *et al.*, 1992) have been examined. In Jerusalem artichoke tubers, C4H enzyme activity increases after wounding (Benveniste *et al.*, 1977) and pea seedlings which have been adapted to darkness exhibit increases in C4H activity when exposed to light (Benveniste *et al.*, 1978). In sweet-potato roots, wound-induced C4H activity is paralleled by the increase in PAL activity (Tanaka *et al.*, 1974) and in

wounded wheat-leaves, the increase in C4H activity is coordinated with the increase in 4CL activity (Maule and Ride, 1983).

Full-length cDNAs encoding C4H have been cloned from mung bean, Jerusalem artichoke, alfalfa, and *Catharanthus roseus* (Mizutani *et al.*, 1993; Teutsch *et al.*, 1993; Farhendorf and Dixon, 1993; Hotze *et al.*, 1995) and the isolation of partial-length *C4H* clones from other plants has been reported (Genbank). The expression pattern of *C4H* has not been well characterized but in the few plants in which it has been examined, *C4H* expression appears to be inducible and occurs in a manner analogous to that of *PAL* and *4CL*. In parsley cell cultures, parsley leaves, and parsley roots, UV-light treatment, elicitor treatment, and wounding cause an increase in *C4H* RNA levels with very similar kinetics as the induction of parsley *PAL1, PAL2, PAL3*, *4CL1* and *4CL2* (Logemann *et al.*, 1995). In *Catharanthus roseus* cell cultures, *C4H* RNA levels increase after elicitation (Hotze *et al.*, 1995) and in alfalfa cell cultures, elicitation causes an increase in *C4H* RNA that is coordinated with the increase in *CHS* transcripts (Fahrendorf and Dixon, 1993). Although limited, these results suggest that *C4H* is induced by the same treatments as those which activate *PAL* and other phenylpropanoid genes.

4CL is the last enzyme in the general phenylpropanoid pathway and it has been detected in many higher plants including monocotyledons, dicotyledons and gymnosperms (Gross *et al.*, 1975; Wallis and Rhodes, 1977; Kutsuki *et al.*, 1982b). Using ATP and coenzyme A (CoA), 4CL converts 4-coumaric acid and other hydroxy- or methoxy- derivatives of cinnamic acid, such as caffeic acid, ferulic acid, and sinapic acid, to form the corresponding CoA esters (Figure 1.2). These esters serve as substrates for entry into various branch-pathways (Hahlbrock and Scheel, 1989). Because of its terminal position in the general phenylpropanoid pathway, and its ability to utilize a number of differently substituted hydroxy-cinnamic acids, it has been hypothesized that 4CL could control the partitioning of carbon into different branch-pathways through the activity of distinct 4CL-isoforms (Knobloch and Hahlbrock, 1975;

Grand *et al.*, 1983). For example, 4CL may direct 4-coumaroyl:CoA into flavonoid biosynthesis via CHS and CHI. 4CL may regulate the amounts of 4-coumaroyl, feruloyl and sinapoyl:CoA esters that enter lignin biosynthesis via the CCR and CAD reactions (Figure 1.2). In support of this, physically-distinct 4CL isoforms have been reported from soybean, petunia, poplar, maize, and parsley (Knobloch and Hahlbrock, 1975; Ranjeva *et al.*, 1976; Grand *et al.*, 1983; Vincent and Nicholson, 1987; Lozoya *et al.*, 1988). Furthermore, in soybean, petunia, and poplar, partially-purified 4CL isoforms exhibit different substrate specificities towards substituted cinnamic acids. The soybean 4CL1 isoenzyme is able to use sinapate as a substrate whereas 4CL2 can not (Knobloch and Hahlbrock, 1975). Based on their substrate specificity, the three petunia 4CL-isoforms are named caffeate:CoA ligase, sinapate:CoA ligase, and ferulate:CoA ligase for their high activities, respectively, towards caffeate, sinapate and ferulate as substrates (Ranjeva *et al.*, 1976). In poplar, 4CL1 is able to react with 4-coumarate, ferulate, and sinapate as substrates; 4CL2 can use 4-coumarate and ferulate as substrates; and 4CL3 can use 4-coumarate and caffeate as substrates (Grand *et al.*, 1983). The distribution of the poplar 4CL-isoenzymes correlates with the types of phenylpropanoid products formed, suggesting that the activities of the 4CL isoenzymes control the types of products made (Grand *et al.*, 1983). In contrast, a single 4CL form was purified from loblolly pine (Voo *et al.*, 1995), and two slightly different 4CL isoforms with similar substrate preferences are encoded by two parsley *4CL* genes (Lozoya *et al.*, 1988), suggesting that 4CL does not participate in the selective distribution of metabolites in these species.

The genes and cDNAs encoding 4CL have been cloned from 9 plants (Douglas *et al.*, 1987; Lozoya *et al.*, 1988; Zhao *et al.*, 1990; Becker-André *et al.*, 1991; Uhlmann and Ebel, 1993; Voo *et al.*, 1995; Yazaki *et al.*, 1995; Allina and Douglas, 1994; Lee *et al.*, 1995a; Lee and Douglas, 1996b). 4CL is encoded by multiple, divergent genes in some plants like rice, soybean, poplar, *Lithospermum erythrorhizon*, and tobacco (Zhao *et al.*, 1990; Uhlmann and Ebel, 1993; Allina and Douglas, 1994; Yazaki *et al.*,

1995; Lee and Douglas, 1996b); by very similar duplicated genes as in the case of parsley and potato (Lozoya *et al.*, 1988; Becker-André *et al.*, 1991); and by apparently single genes as is the case in *Arabidopsis* and pine (Lee *et al.*, 1995; Voo *et al.*, 1995). In conjunction with earlier biochemical studies, it is possible that the divergent *4CL* genes in plants such as soybean and poplar may encode 4CL enzymes with distinct enzymatic properties. This hypothesis has not yet been tested. Other than the study of two nearly-identical parsley-*4CL* genes and their encoded proteins (Lozoya *et al.*, 1988), there has been no published information on the properties of recombinant-4CL proteins from divergent members of *4CL* gene families.

4CL gene expression, like that of many of the phenylpropanoid genes, is regulated developmentally and is activated by external stimuli such as pathogen infection, elicitor treatment, wounding, and UV-light irradiation (Douglas *et al.*, 1987; Schmelzer *et al.*, 1989; Wu and Hahlbrock, 1992). In tobacco flowers, *in situ* hybridization shows that endogenous tobacco *4CL* transcripts and those of an introduced parsley *4CL1* gene accumulate in a cell-type specific manner, and that the patterns of accumulation are generally consistent with the sites of phenylpropanoid natural-product accumulation (Reinold *et al.*, 1993). As well, *4CL* expression in tobacco is activated by wounding, light, and methyl jasmonate treatment (Douglas *et al.*, 1991; Ellard-Ivey and Douglas, 1996). The *4CL* genes from parsley and soybean are differentially regulated. *4CL-2* from parsley is preferentially expressed in the flowering stem and is light inducible, whereas *4CL-1* is wound inducible in roots (Lois and Hahlbrock, 1992). In soybean, *4CL-16* is inducible by fungal infection whereas *4CL-14* is not (Uhlmann and Ebel, 1993).

The 4CL reaction converts the carboxylic acid group into a thioester group, thereby "activating" the molecule for further biochemical reactions. In the biosynthesis of lignin, the CoA esters are reduced to form alcohols whereas in flavonoid biosynthesis, the CoA esters are condensed with malonyl CoA. Some of the products made from hydroxy- and methoxy- cinnamoyl:CoA esters are described below.

1.2 Biosynthesis of Phenylpropanoid Products

1.2.1 Lignin

Lignin is a three-dimensional polymer composed of cinnamyl alcohol derivatives linked in covalent bonds. Lignin is deposited in the walls of specialized cells of the xylem and phloem including the tracheids, vessel members, sclereids, and fibers. Lignin provides rigidity and hydrophobicity to the cell walls, properties which are particularly important for cells which conduct water. Thus, the evolution of lignin has been considered a major step for the emergence of terrestrial vascular plants (Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995). The analysis of lignin is relatively difficult because the polymer is large and resists extraction. The methods used to extract lignin usually degrade the polymer such that analysis of the polymer in its native form is near impossible. Many methods of lignin detection and extraction are available and each have advantages and disadvantages (reviewed in Lewis and Yamamoto, 1990).

The deposition of lignin is developmentally regulated. Lignin is deposited in the developing vasculature of leaf veins and roots. In perennials, lignin deposition in secondary growth is activated by favorable environmental condition (Raven *et al.*, 1987). Lignin deposition is also inducible. During infection by pathogens, plants may deposit lignin and lignin-like polymers at the infection site. It is believed that lignin may serve as a physical barrier to attenuate pathogen invasion. The deposition of lignin and lignin-like components during wounding and pathogen attack is relatively rapid and is likely derived from pre-existing phenolic compounds (Nicholson and Hammerschmidt, 1992). *Zinnia elegans* (Fukuda and Komamine, 1982) and pine cell cultures (Campbell and Ellis, 1992) have been useful in studying the biochemistry of lignin formation since these cultures can be induced to undergo lignification.

Deposition of lignin is a multi-component procedure consisting of: 1) biosynthesis of lignin monomers, 2) storage or transport of lignin monomers, and 3) polymerization of monomers. Sequential hydroxylation and methylation of the

aromatic ring in the C3 and C5 positions of 4-coumaric acid produces caffeic acid (3, 4-dihydroxycinnamic acid), ferulic acid (3-methoxy 4-hydroxycinnamic acid), 5-hydroxyferulic acid (3-methoxy 4, 5-dihydroxycinnamic acid), and sinapic acid (3,5-dimethoxy 4-hydroxycinnamic acid). These ring modifications are catalyzed by coumarate 3-hydroxylase (C3H), bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT), and ferulate 5-hydroxylase (F5H). 4CL converts some, or all of these modified cinnamic acids to form their corresponding CoA esters (Figure 1.2). An alternative pathway in which the hydroxylation and methylation steps occur after CoA activation has been suggested (Figure 1.2). In support of this, an enzyme activity (CCo3H) from parsley cell cultures which hydroxylates 4-coumaroyl:CoA to form caffeoyl:CoA has been described (Kneusel *et al.*, 1989). In carrot cell cultures, caffeoyl:CoA 3-*O*-methyltransferase (CCoAOMT) which methylates caffeoyl:CoA to feruloyl:CoA has been described (Kühnl *et al.*, 1989) and this enzyme activity has been purified and characterized from parsley cell cultures (Pakusch *et al.*, 1989). The cDNA encoding CCoAOMT has been cloned from parsley and *Zinnia* (Schmitt *et al.*, 1991; Ye *et al.*, 1994). In parsley, *CCoAOMT* RNA levels increase in a manner analogous to the increase in *PAL* RNA levels during elicitation. Crude extracts from *Zinnia* have been shown to methylate caffeoyl:CoA and 5-hydroxyferuloyl:CoA to form feruloyl:CoA and sinapoyl:CoA respectively (Ye *et al.*, 1994). The temporal and spatial expression of *CCoAOMT* from *Zinnia* coincides with tissues undergoing lignification (Ye *et al.*, 1994). These results suggests that cinnamoyl:CoA derivatives may be generated by at least 2 pathways and in *Zinnia*, both the COMT- and CCoAOMT-mediated pathways appear to be functional (Ye and Varner, 1995).

4-coumaroyl:CoA, feruloyl:CoA, and sinapoyl:CoA are then reduced to their corresponding alcohols via the actions of CCR and CAD. CCR and CAD use NADPH as an electron donor to reduce the thioesters to form alcohols. The ability of CCR and CAD to reduce 5-hydroxyferuloyl:CoA to its corresponding alcohol *in vivo* has been implied by the presence of 5-hydroxyferulyl lignin subunits in transgenic plants

(Antanassova *et al.*, 1995) and the CCR from soybean uses 5-hydroxylferuloyl:CoA as a substrate *in vitro* (Wengenmayer *et al.*, 1976). In most plants, lignin monomers are of the *trans* conformation; however, lignin from the bark of the American beech tree is composed of the *cis*-isomers (Lewis *et al.*, 1988). Radiolabelled feeding experiments show that *trans/cis* isomerization occurs at the level of the alcohol (Lewis *et al.*, 1987). Although UV-light induced isomerization occurs *in vitro*, *trans/cis* isomerization of monolignols probably involves an isomerase *in vivo* (Lewis *et al.*, 1987). Once made, the monolignols are glycosylated at the 4-hydroxy position and then transported to the site of lignin deposition or storaged as monolignol glucosides. Monolignols are glycosylated by UDP-glucose:monolignol glucosyltransferases and de-glycosylated by ß-glucosidases. The mechanism of monolignol transport is not well understood; however, it can be partially explained with the endomembrane theory. The theory suggests that the lignin precursors are mobilized from the cytoplasm in phospholipid vesicles that are derived from the Golgi body or the endoplasmic reticulum and then deposited outside the cell membrane in a manner similar to exocytosis (Pickett-Heaps, 1968). In conifers, it has been hypothesized that monolignol glucosides are stored in the vacuole, transported out of the cytoplasm, and then de-glycosylated in the cell walls (Whetten and Sederoff, 1995). In pine, a coniferin ß-glucosidase has been localized to the differentiating xylem where peroxidase activity is also found (Dharmawardhana *et al.*, 1995). Peroxidases and/or laccases convert the monolignols into free radicals and the free radicals undergo a free-radical polymerization reaction. Evidence suggests that free-radical polymerization may be ordered and controlled since polymerization appears to begin at the middle lamella and progress inwards towards the lumen. Polymerization also appears to start at the "corners" of cell walls first and then progress away from the corners, suggesting that a mechanism exists to direct the polymerization event (reviewed in Takabe *et al.*, 1989).

There is a general trend in the types of lignin found in plants. Lignin from gymnosperms contains predominantly guaiacyl subunits (from feruloyl:CoA). In

angiosperms, the lignin is composed of guaiacyl and syringyl (from sinapoyl:CoA) subunits and lignin from grasses contains syringyl, guaiacyl and 4-hydroxyphenyl subunits. How lignin composition is regulated is unclear; however, it is likely controlled by the enzymes involved in the biosynthesis or transport of monolignols. The substrate specificity of lignin biosynthetic enzymes may control the types of monolignols formed. For example, COMT enzymes from gymnosperms are more active towards caffeic acid whereas COMT enzymes from angiosperms are more active towards 5-hydroxyferulic acid (reviewed in Whetten and Sederoff, 1995). In soybean (Knobloch and Hahlbrock, 1975), petunia (Ranjeva *et al.*, 1976), and poplar (Grand *et al.*, 1983), 4CL isoforms with different substrate specificities have been identified and the activities of these isoforms may define the kinds of monolignols formed (see section 1.1.2). In a similar manner, the preferential reduction of different cinnamoyl:CoA derivatives by CCR and CAD may control which kinds of monolignols are made for polymerization. CCR can reduce sinapoyl:CoA, feruloyl:CoA, and 4-coumaroyl:CoA (Sarni *et al.*, 1984; Goffner *et al.*, 1994); however, CCR has a preference for feruloyl:CoA as a substrate (Wengenmayer *et al.*, 1976; Lüderitz and Grisebach, 1981). Lüderitz and Grisebach (1981) demonstrated that CCR from spruce is predominanty active towards feruloyl:CoA whereas CCR from soybean uses both feruloyl:CoA and sinapoyl:CoA, suggesting that the activities of CCR may control the kinds of lignin found in gymnosperms and angiosperms. CAD activities with different substrate specificities have been found in wheat (Pillonel *et al.*, 1992), and *Eucalyptus gunnii* (Goffner *et al.*, 1992). Kutsuki *et al.* (1982a) examined CAD activity from a number of gymnosperms and angiosperms and showed that CAD enzymes from gymnosperms prefer the ferulyl substrate whereas CAD enzymes from angiosperms use both ferulyl and sinapyl substrates. These results suggest that the types of lignin found in plants may be regulated by the substrate specificities of COMT, 4CL, CCR, and CAD; however, more targeted studies are required before the role of each of these enzymes in regulating lignin quality can be assigned. The transport of monolignol

glucosides either for storage or for polymerization is not well characterized. However, conceptually, it is possible that lignin composition may be regulated by the selective activity of UDP-glucose:monolignol glucosyltransferases and ß-glucosidases. The purified ß-glucosidase from lodgepole pine uses both coniferin and syringin *in vitro*, suggesting that the activity of this particular enzyme does not control lignin quality in pine (Dharmawardhana *et al.*, 1995).

The isolation of mutants and reverse genetics using cloned genes has demonstrated the importance of certain steps in the lignin biosynthetic pathway. Sense suppression of *PAL* in tobacco results in lower levels of lignin, demonstrating that the amount of lignin made can be controlled in the early steps of phenylpropanoid metabolism (Elkind *et al.*, 1990). However, quantitative differences in lignin accumulation are observed only when PAL activities were suppressed by >75%, suggesting that under normal conditions, PAL activity is not a limiting factor (Bates *et al.*, 1994). Antisense-RNA technology has been used to down-regulate COMT activity in tobacco (Dwivedi *et al.*, 1994; Atanassova *et al.*, 1995), and poplar (Van Doorsselaere *et al.*, 1995) resulting in lignin with higher guaiacyl or 5-hydroxyferulyl subunits relative to the wild-type counterparts, suggesting that lignin composition is regulated, in part by, *COMT* expression. It is interesting to note that the syringyl-subunit levels were lowered when COMT activity was suppressed, suggesting that carbon flow into sinapate synthesis is preferentially blocked. Significant differences in the S:G (syringyl to guaiacyl) ratio were observed when COMT activity was suppressed by >80% (Atanassova *et al.*, 1995; Van Doorsselaere *et al.*, 1995). The *bm3* mutants of maize are disrupted at the *COMT* gene and possess altered lignin quality (Vignols *et al*, 1995) and quantity (Grand *et al.*, 1985). These results suggest that COMT activity has a role in controlling lignin quality, and perhaps a role in controlling lignin quantity as well. In poplar, F5H activity was preferentially localized in sclerenchyma, a tissue which also has high levels of syringyl lignin (Grand, 1984). The *fah1* (*sin1*) mutant of *Arabidopsis*, which lacks F5H activity, has been identified and

these plants did not accumulate sinapate derivatives including syringyl lignin (Chapple *et al.*, 1992). Thus in *Arabidopsis*, the deposition of syringyl lignin requires F5H function and it has been suggested that the lack of F5H activity in gymnosperms may be the reason for the high guaiacyl and low syringyl lignin in these plants (reviewed in Campbell and Sederoff, 1996; Grand, 1984). The reduction of CAD activity by antisense RNA resulted in tobacco plants with higher ratio of aldehyde/alcohol lignin monomers as compared to control plants (Halpin *et al*, 1994). The increase in aldehyde/alcohol monomers was evident when CAD activity was decreased by >84%. The increase in sinapyl aldehydes was more pronounced than the increase in coniferyl aldehydes. The *bmr*6 mutant of *Sorghum* has lower levels of CAD activity and also deposits the aldehyde forms of the lignin subunits. However, the phenotype of the *bmr6* mutant should not be compared directly to the antisense-*CAD* plants since *bmr6* mutant also has decreased COMT activity. In the *bmr6* mutant, there was a pronounced increase in the coniferyl (and not sinapyl) aldehyde monomers (Pillonel *et al.*, 1991). Overproduction of a lignin-specific anionic peroxidase resulted in an increase in lignin accumulation in transgenic tobacco (Lagrimini, 1991) and transgenic tomato (Lagrimini *et al.*, 1993). The authors suggest that in pith tissues, the low levels of lignification is due to the limiting levels of endogenous anionic peroxidase (Lagrimini, 1991).

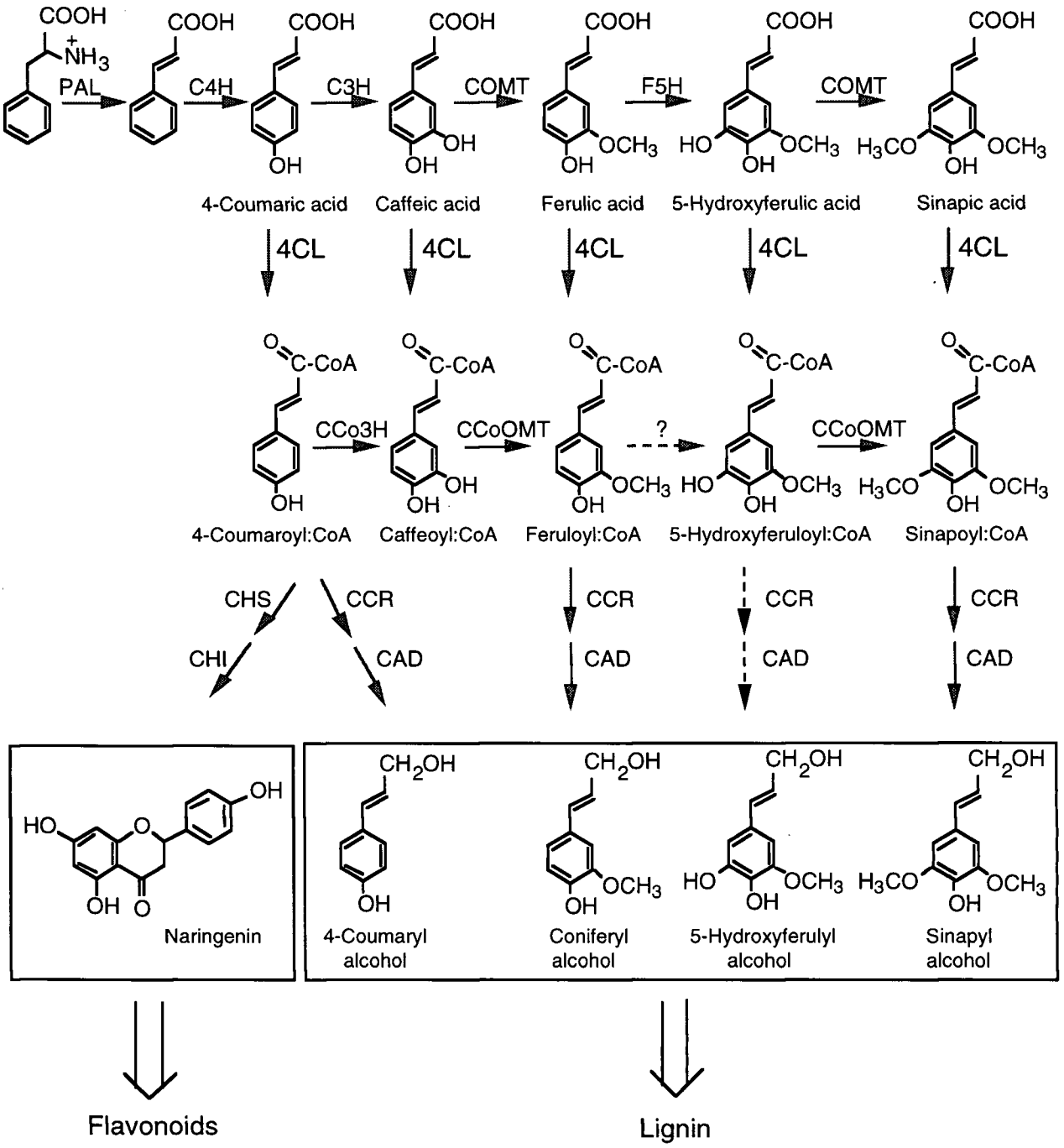


Figure 1.2: Schematic diagram of the biosynthesis of flavonoid- and lignin- precursors. The identification of novel enzymes suggests that the biosynthesis of CoA esters from 4-coumarate may proceed through a number of enzymatic steps potentially resulting in a metabolic grid rather than a linear pathway. Genetic engineering and the isolation of mutants demonstrate the flow of phenolic compounds through the metabolic grid (see section 1.2.1). Enzymes are: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase, C3H, 4-coumarate 3-hydroxylase, COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; 4CL, 4-coumarate:CoA ligase; CCo3H, 4-coumaroyl:CoA 3-hydroxylase; CCoOMT, caffeoyl:CoA O-methyltransferase; ?, uncharacterized metabolic step; CHS, chalcone synthase; CHI, chalcone isomerase; CCR, cinnamoyl-CoA reductase; and CAD, cinnamyl alcohol dehydrogenase. Dashed arrows represent enzymatic steps which have not been clearly demonstrated.

1.3.2 Flavonoids

Flavonoids are compounds structurally-based on the flavanone-skeleton (Figure 1.3A). One benzene ring (A ring) is derived from the condensation of three malonyl:CoA units and the second benzene ring (B ring) is derived from 4-coumaroyl:CoA. The oxidation state of the central heterocyclic ring (C ring) is used to group the flavonoids into classes (Figure 1.3A). Within each class of flavonoids, members differ by the hydroxylation or methoxylation on the A- and B- rings (Figure 1.3B). For example, flavanones and flavones are two classes of flavonoids which differ by the absence and presence, respectively, of a double bond between carbons 2 and 3 of the C-ring (Figure 1.3A). Pelargonidin, cyanidin and delphinidin are all members of the anthocyanidins and they differ by the hydroxylation on the 3', 4' and 5' carbons of the B-ring (Figure 1.3B). Naturally-occurring flavonoids are usually glycosylated or modified at one or more of the hydroxyl groups. Sugars can be hexoses or pentoses and can be monosaccharides, disaccharides or trisaccharides in α or ß linkages (Swain, 1965). The unglycosylated flavonoids are referred to as aglycones. In particular, anthocyanins are the glycosylated forms whereas the aglycones are called anthocyanidins. The term "leucoanthocyanidins" is sometimes used to refer to flavan-3,4-diols since they are the colorless precursors to anthocyanidins (Haslam, 1982). Condensed proanthocyanidins (also called condensed tannins) are composed of oligomeric polymers of flavan-3-ols and these are referred to as "proanthocyanidins" because treatment of the polymer with acids results in the releases of anthocyanidins (Haslam, 1982). In addition to the flavonoids listed in Figure 1.3, other minor flavonoids such as neoflavonoids, biflavonoids and triflavonoids exist (Geiger and Quinn, 1982) and will not be discussed here.

Flavonoids absorb light, the quality of light absorbed depending on the number and location of double bonds, hydroxyl groups, and ketone groups. Flavonoids with few double bonds and few hydroxyl groups (Figure 1.3A, flavan-3-ols) absorb high energy UV-light and are colorless, but they may confer a cream or ivory hue.

Flavonoids with many double bonds and hydroxyl groups (Figure 1.3, anthocyanidins and aurones) absorb UV-light and visible light and can be red, blue or violet in color. The color that flavonoids impart largely depends on the presence of other co-pigments, the pH of the environment, their compartmentalization, and the presence of chelating metals (Swain, 1965).

Flavonoids are usually found in the epidermal or subepidermal layers and accumulate in the vacuole (Salisbury and Ross, 1978). Flavonoids are most obvious and predominanty found in the flowers and fruits of plants where they attract pollinators and creatures which aid in seed dispersal. Flavonoids are also present in leaves, roots and tubers; however, the high levels of chlorophyll in the leaves tend to mask the flavonoids. The colors contributed by flavonoids are sometimes evident in very young leaves and senescing leaves where chlorophyll content is lower than that found in mature leaves (Swain, 1965). The flavonoids in leaves likely have a role in absorbing UV-light thereby protecting proteins and nucleic acids from UV-induced damage (Caldwell *et al.*, 1983; Kootstra, 1994; Stapleton and Walbot, 1994). Some flavonoids such as the condensed proanthocyanidins (condensed tannins) may also serve as feeding deterrents since they have an astringent taste and have the ability to precipitate proteins and polysaccharides (Haslam, 1982). Flavonoids have been implicated as signal molecules between plants and symbiotic microorganisms (Long, 1989). For example, flavonoids released from alfalfa have the ability to induce the expression of nodulation genes (Peters *et al.*, 1986) and enhance the growth of *Rhizobium meliloti* (Hartwig *et al.*, 1991). Some flavonoids, especially the isoflavonoids, have antimicrobrial activities and may serve as phytoalexins (Adesanya and Roberts, 1995). Recently, it has been determined that flavonols are required for proper pollen and pollen tube development in maize, petunia, and tobacco (Mo *et al.*, 1992; Ylstra *et al.*, 1992; Ylstra *et al.*, 1994). However, this physiological role is not ubiquitous to all plants since mutants of *Arabidopsis* which lack flavonoids are self fertile (Burbulis *et al.*, 1996). The first enzyme in the flavonoid biosynthetic pathway,

chalcone synthase (CHS) will be discussed in detail and subsequent enzymes will be mentioned briefly.

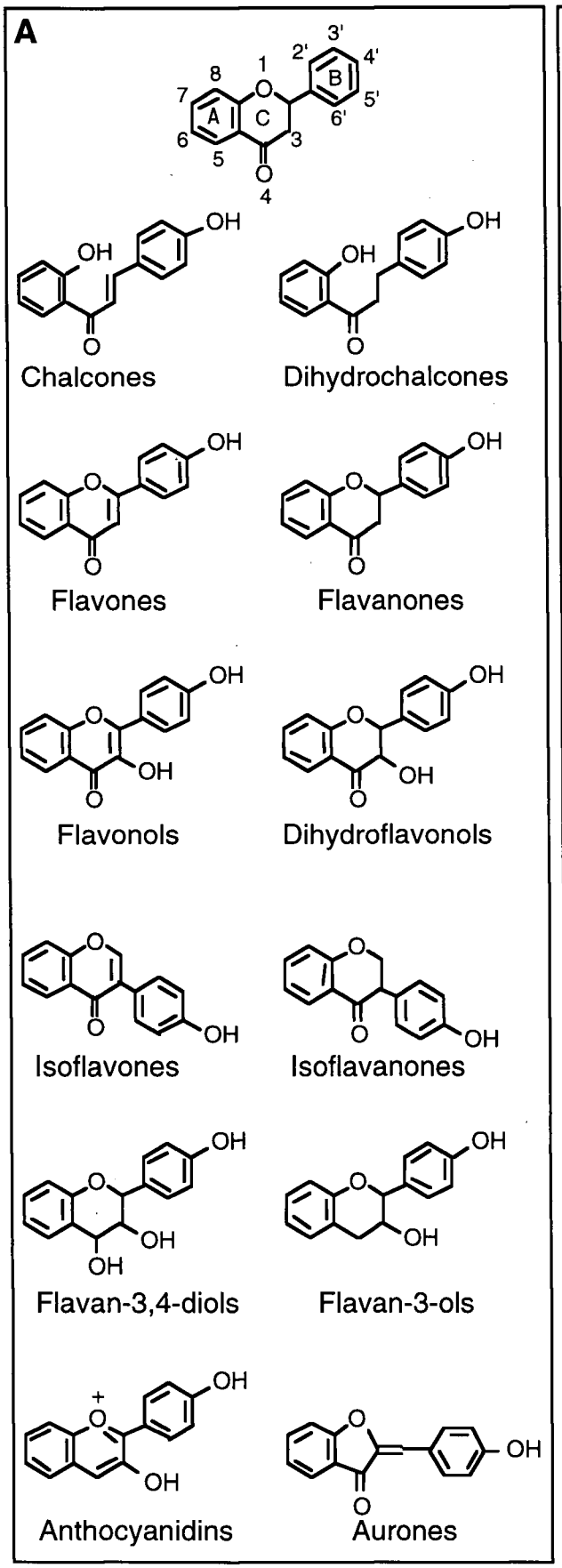


Figure 1.3: Flavonoids. A. Classes of flavonoids are listed with a representative member of each class. Note that the oxidation state of the C-ring defines the flavonoid class. Modifications on the A- and B- rings are not shown. B. Anthocyanidins. Note that members are differentiated by the hydroxylation and methoxylation on the B-ring.

CHS catalyzes the first committed step in flavonoid biosynthesis and it condenses 4-coumaroyl:CoA sequentially with three acetate units derived from malonyl:CoA to produce a chalcone (Figure 1.4). The enzyme has been purified and characterized from a few sources. In parsley cell cultures, CHS has been reported to only use 4-coumaroyl:CoA as a substrate (Hrazdina *et al.*, 1976); however, in soybean, CHS uses 4-coumaroyl:CoA and caffeoyl:CoA as substrates (Welle and Grisebach, 1987) and the CHS from petunia anthers uses 4-coumaroyl:CoA, caffeoyl:CoA, and feruloyl:CoA as substrates (Sütfeld *et al.*, 1978). Characterization of CHS from *Haplopappus gracilis* and parsley cell cultures at different pHs demonstrated that both 4-coumaroyl:CoA and caffeoyl:CoA can be used as substrates when the reaction was performed at the appropriate pH-optima (Saleh *et al.*, 1978). More recently, CHS from *Cephalocereus senilis* (old-man-cactus; Paré *et al.*, 1992) and *Pinus sylvestris* (Scots pine; Sandermann Jr. *et al.*, 1989; Fliegmann *et al.*, 1992) have been shown to use cinnamoyl:CoA as a substrate. Expression of Scots pine and *Sinapis alba CHS* in *E. coli* resulted in CHS proteins which use both 4-coumaroyl:CoA and cinnamoyl:CoA as substrates (Fliegmann *et al.*, 1992). The use of cinnamoyl:CoA by CHS is likely for the biosynthesis of B-ring-deoxy flavonoids (Paré *et al.*, 1992; Liu *et al.*, 1995) such as pinocembrin (a flavanone), cephalocerone (an aurone), and baicalein (a flavone). Because of the presence of B-ring specific hydroxylases and *O*-methyltransferases, it has been suggested that CHS probably only uses 4-coumaroyl:CoA as a substrate *in vivo* (Ebel and Hahlbrock, 1982); however, there is increasing evidence suggesting that CHS can metabolize other cinnamoyl:CoA derivatives in addition to 4-coumaroyl:CoA (Welle and Grisebach, 1987; Sütfeld *et al.*, 1978; Sandermann Jr. *et al.*, 1989; Paré *et al.*, 1992; Fliegmann *et al.*, 1992).

Genes and cDNAs encoding CHS have been cloned from more than 30 plants (GenBank). CHS appears to be encoded by a single gene in *Antirrhinum majus*, parsley, and *Arabidopsis thaliana* (Sommer and Saedler, 1986; Herrmann *et al.*, 1988; Feinbaum and Ausubel, 1988), by a small gene family of 2 - 3 gene members in maize

and tomato (Franken *et al.*, 1991; O'Neill *et al.*, 1990), and by a large gene family of 5 - 10 members in bean, petunia, soybean, pea, and clover (Ryder *et al.*, 1987; Koes *et al.*, 1989; Akada *et al.*, 1990; An *et al.*, 1993; Howles *et al.*, 1995). In petunia, members of the *CHS* gene family are developmentally regulated. *CHS A* and *CHS J* expression are observed in the corollas, the tubes, and the anthers but not in the leaves, the roots and the pistils (Koes *et al.*, 1989). Petunia *CHS* genes are differentially expressed such that only *CHS A* and *J* are expressed in the flowers whereas *CHS A, J, G* and *B* are inducible by UV-light (Koes *et al.*, 1989). The *CHS* members from bean are also differentially expressed during light treatment, elicitor treatment, wounding and infection with compatible and incompatible fungi (Ryder *et al.*, 1987).

The developmental and cell-specific expression of *CHS* has been demonstrated by *in situ* hybridization and promoter-*GUS* fusion analysis. *In situ* hybridization showed that *CHS* transcripts are localized in the epidermal layer of parsley leaves where flavonoid end-products also accumulate (Schmelzer *et al.*, 1988; Wu and Hahlbrock, 1992). In tobacco, *CHS* is highly expressed throughout the anthers, and *CHS* transcripts, localized to the epidermal cells, were 10 times higher in the limb of tobacco flowers (where anthocyanins accumulate) as compared to the flower tube (Drews *et al*, 1992). In transgenic tobacco, the bean *CHS8* promoter directed high *GUS* expression in the inner-epidermal layer of pigmented petal regions and in the apical tip of roots (Schmid *et al.*, 1990). The *CHS* promoter from *Antirrhinum* directed highest *GUS* expression in the developing seeds (3-6 weeks post-fertilization) and in callus from transgenic tobacco. GUS activity was also high in the pigmented portion of flower petals and in 4-week-old roots; however, comparative studies in tobacco and *Antirrhinum* showed no endogenous *CHS* transcripts associated with roots (Fritze *et al.*, 1991). The activity of the petunia *CHS- A, J, B*, and *G* promoters was examined by promoter-*GUS* fusions in transgenic petunia (Koes *et al.*, 1990). *CHS-A* and *CHS-J* directed *GUS* expression in corollas, flower tubes,

flower stems, ovaries, and seedpods. Only *CHS-A* directed *GUS* expression in anthers. *CHS-B* directed low expression in the same floral tissues whereas no GUS staining was observed in *CHS-G::GUS* transgenics. GUS staining was observed at the inner epidermis as directed by *CHS-A* and *J* promoters and was correlated with the location of anthocyanin accumulation. GUS staining was also observed in cell types that did not accumulate anthocyanins and it was suggested that in these tissues CHS functions to synthesize colorless flavonols such as kaemferol and quercetin (Koes *et al.*, 1990).

Like the genes of general phenylpropanoid metabolism, expression of *CHS* is developmentally regulated and inducible. Treatment of parsley cell cultures with UV-light causes a temporal increase in *CHS* RNA levels that was coordinate with the expression of *PAL* (Kreuzaler *et al.*, 1983; Chappell and Hahlbrock, 1984). In *Arabidopsis*, irradiation with blue light, white light, or UV-light induces endogenous *CHS* expression and *GUS* expression in *CHS* promoter-*GUS* transgenic plants (Feinbaum and Ausubel, 1988; Feinbaum *et al.*, 1991). *CHS* expression is induced in bean (Ryder *et al.*, 1984; Cramer *et al.*, 1985ab) and alfalfa (Dalkin *et al.*, 1990) cell cultures after elicitation and in bean hypocotyls after wounding and infection with fungi (Lawton and Lamb, 1987). In these leguminous species, CHS activity is probably used for the biosynthesis of isoflavonoids which have antimicrobial activities (Robbins *et al.*, 1985, Dalkin *et al.*, 1990).

Many of the *CHS* promoters from clover have the putative UV-inducible and elicitor-inducible *cis*-elements (P-box; Howles *et al.*, 1995) which have been described in other phenylpropanoid genes (Lois *et al.*, 1989). P- and L- boxes have also been detected in the *CHS* promoters from bean, *Antirrhinum*, maize, *Arabidopsis* and parsley (Lois *et al.*, 1989). The maize *CHS* promoters also contain putative Myb- and Myc- binding *cis*-elements as well as two UV-boxes which may confer UV-light responsiveness (Fraken *et al.*, 1991). Some of the genes which regulate maize *CHS* expression have been cloned (reviewed in Holton and Cornish, 1995) and they

include the genes at the *C1* (Cone *et al.*, 1986) and *R* (Ludwig *et al.*, 1989) loci. The product of the *C1* locus has homology to Myb transcription factors (Paz-Ares *et al.*, 1987) while products of *R* gene members have homology to Myc transcription factors (Ludwig *et al.*, 1989; Consonni *et al.*, 1993). Members of the Myc and Myb gene families are both required for anthocyanin biosynthesis and evidence suggests that these regulatory gene products interact with one another (Goff *et al.*, 1992).

Suppression of *CHS* by antisense RNA (van der Krol *et al.*, 1988) and sense suppression (Napoli *et al.*, 1990; van der Krol *et al.*, 1990) resulted in transgenic plants with lower pigmentation in the flower petals. Some flowers completely lacked pigments while other flowers acquired a pattern of alternating colored and uncolored sectors (van der Krol *et al.*, 1988; Napoli *et al.*, 1990; van der Krol *et al.*, 1990). White flower sectors regained pigmentation when naringenin-chalcone was supplied to the tissue suggesting that the suppression was specific to the CHS step of anthocyanin biosynthesis (van der Krol *et al.*, 1988). The *c2* and *whp* mutants of maize (reviewed in Holton and Cornish, 1995), the *nivea* mutant of *Antirrhinum* (Spribille and Forkmann, 1982), and the *tt4* mutant of *Arabidopsis* (Shirley *et al.*, 1995) all have defective *CHS* genes.

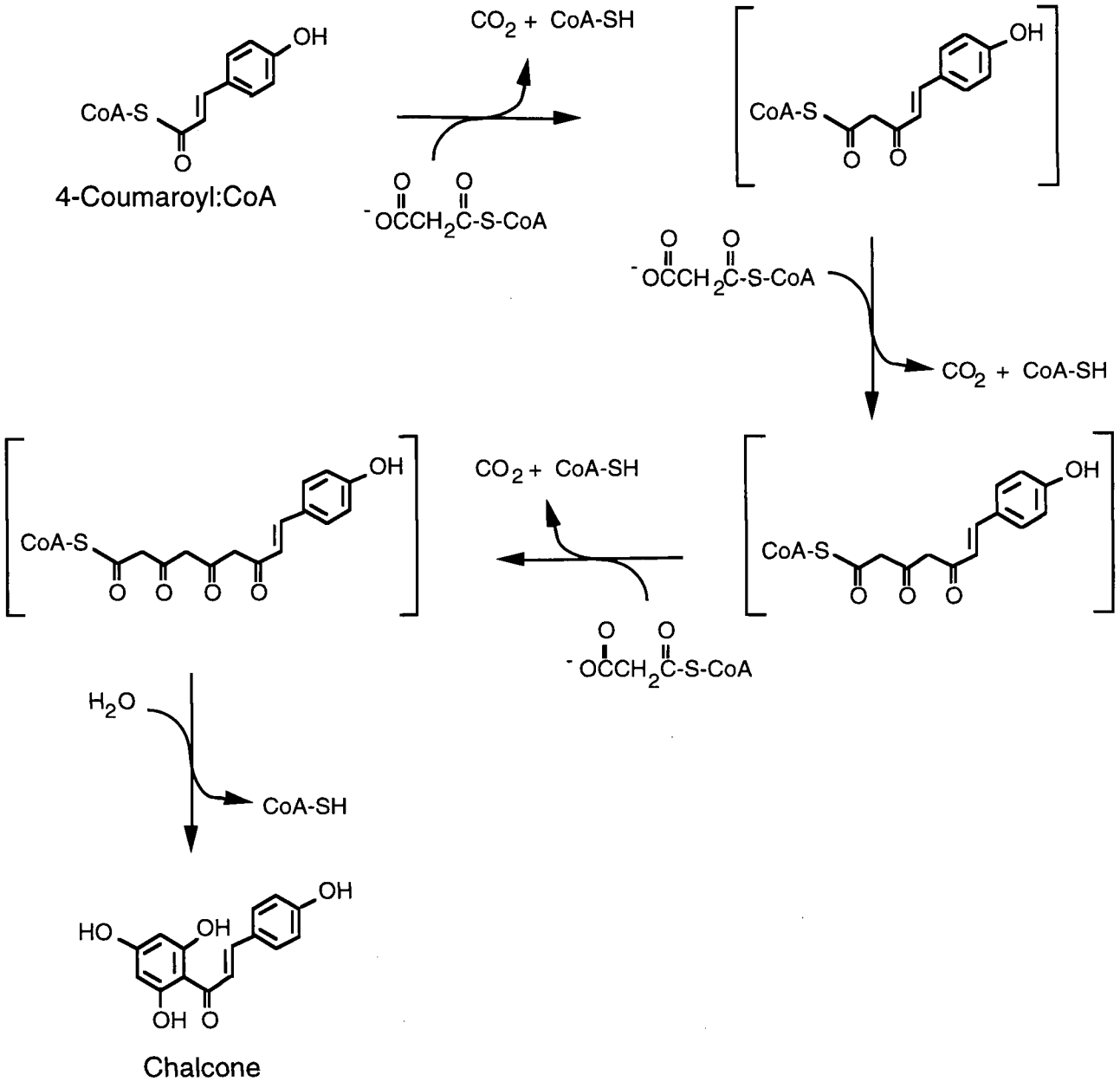


Figure 1.4: Reaction catalyzed by chalcone synthase. Structures in square brackets represent reaction intermediates.

The product of the CHS reaction is a chalcone. Once made, the chalcone is modified at the A-, B-, and C-rings to produce a variety of different flavonoids. The biochemical relationship between the different classes of flavonoids is shown in Figure 1.5; however, in some cases, the enzyme catalyzing the given reactions has not been well characterized. Chalcones can be converted into flavanones by the action of

chalcone isomerase (CHI; reviewed in Holton and Cornish, 1995). Flavanones can be modified to form three different classes of flavonoids. Oxidation of flavonones by a proposed flavonoid oxidase (Ebel and Hahlbrock, 1982) produces flavones. Mobilization of the aryl group, a reaction catalyzed by isoflavone synthase (IFS; reviewed in Barz and Welle, 1992), produces isoflavones. Isoflavones in turn can be reduced by isoflavone reductase (IFR; reviewed in Barz and Welle, 1992) to produce isoflavanones. Hydroxylation of flavanones at the number 3 position by flavanone 3-hydroxylase (F3H; reviewed in Holton and Cornish, 1995) produces dihydroflavonols. The action of flavonol synthase (FLS; reviewed in Holton and Cornish, 1995) produces a double bond between carbons 2 and 3 of dihydroflavonols to give flavonols. Dihydroflavonols can also be reduced by dihydroflavonol-4-reductase (DFR; reviewed in Holton and Cornish, 1995) to produce flavan-3,4-diols. Flavan-3,4-diols can then oxidized and dehydrated by the actions of anthocyanidin synthase (ANS; reviewed in Holton and Cornish, 1995) to produce anthocyanidins. Members of each class of flavonoids can also be hydroxylated, methoxylated and glycosylated to produce a large number of compounds. For example, the actions of flavonoid 3'-hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) results in hydroxylation of the B-ring of flavonoids. By far the most well characterized class of flavonoids is the anthocyanin class, probably because they are colored and therefore the most obvious. The genetics and biochemistry of anthocyanin biosynthesis have recently been reviewed by Holton and Cornish (1995) and will not be discussed here.

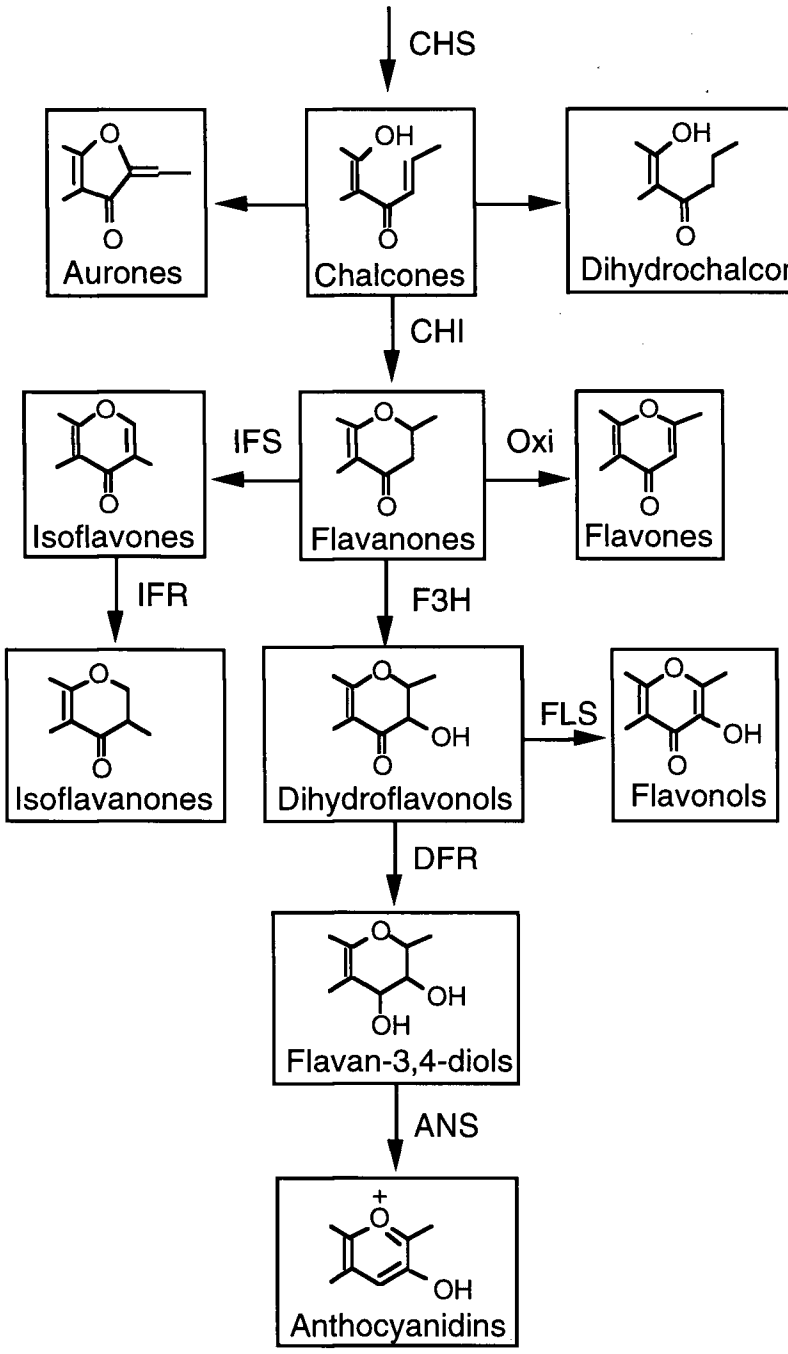


Figure 1.5: Biochemical relationship between classes of flavonoids. Only the C-ring is shown. Enzymes are: CHS, chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; IFR, isoflavone reductase; Oxi, flavonoid oxidase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; and ANS, anthocyanidin synthase.

1.2.3 Other Phenylpropanoid Molecules

In addition to lignin and flavonoids, many other phenylpropanoid compounds are found in plants. These will be described briefly (Figure 1.6). Lignans are "dimeric" compounds composed of two monolignol subunits linked in a stereospecific manner (Figure 1.6). Lignans have antimicrobial, antifungal, and antifeedant properties and

may have defense related roles *in planta* (Davin and Lewis, 1992). The biosynthetic pathway leading to lignan synthesis has not been well characterized; however, since lignans are optical active, it is likely that the mechanism of synthesis is different from that of lignin biosynthesis which is non-stereospecific (Davin and Lewis, 1992).

Suberin is a waxy, protective layer composed of phenolic compounds linked in ester bonds to long-chain fatty acids (Salisbury and Ross, 1985). The phenolic component of suberin can be phenolic acids (like ferulic acid) or phenolic alcohols (like 4-coumaryl alcohol) and the lipid-like component can be 16 to 28 carbons long (Davin and Lewis, 1992). Suberin is deposited at wound-sites, in the Casparian strips of roots, in the walls of bundle-sheath cells of grasses, and other cell types (Salisbury and Ross, 1985).

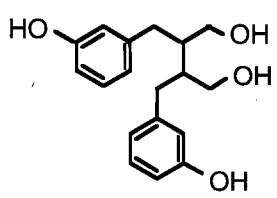
Stilbenes are similar to flavonoids in that they are synthesized from 4-coumaroyl:CoA and three molecules of malonyl:CoA; however, the cyclization mechanism is different resulting in resveratrol instead of naringenin (Figure 1.6). Stibene synthase and chalcone synthase have relatively high sequence homology and can be differentiated by the products of the catalyzed reaction (Fliegmann *et al.*, 1992). It has been proposed that the activities of stilbene synthase and chalcone synthase depend on a conserved cysteine residue. Site-directed mutagenesis of amino acids prior to the conserved cysteine did not convert chalcone synthases into stilbene synthases or vice versa suggesting that the inherent enzymatic activities are defined by more than the few amino acids examined (Schröder and Schröder, 1992). Stilbenes have antifungal properties and are capable of preventing fungal spore germination and mycelial growth (Dercks *et al.*, 1994).

Coumarins are volatile lactones derived from hydroxylated phenylpropanic acids (Figure 1.6). The early steps in coumarin biosynthesis have not been well characterized, but involve hydroxylation at the C2- (*ortho*) position of the phenolic ring, *trans/cis* isomerization of the propane side-chain, followed by spontaneous cyclization (Lewis, 1993). Coumarins can be further modified by hydroxylation, methylation, and

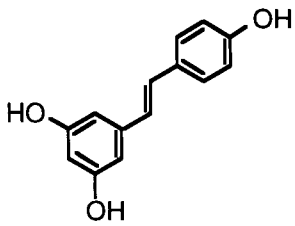
glycosylation. The glycosylated forms are nonvolatile (Salisbury and Ross, 1985). Furanocoumarins are of particular interest since they have been shown to have antifungal properties (Beier and Oertli, 1983). Furanocoumarins can be linear or angular (Figure 1.6) and, in both cases, the furan ring is derived from dimethylallyl pyrophosphate. Furanocoumarins are also modified by hydroxylation and methylation and in parsley, the activity of bergaptol *O*-methyltransferase (BMT) has been used as a marker for furanocoumarin biosynthesis (Wu and Hahlbrock, 1992). In parsley cell cultures, treatment with fungal elicitors resulted in an accumulation of furanocoumarins (Tietjen *et al.*, 1983) and in parsley leaves, infection with fungi caused an accumulation of furanocoumarins at the site of infection (Jahnen and Hahlbrock, 1988). These results suggest that, in parsley, furanocoumarins may have a role in pathogen defense. In uninfected parsley plants, furanocoumarins accumulate in the lumen of oil ducts and these compounds may represent storage forms of the defense molecules (reviewed in Hahlbrock and Scheel, 1989).

Benzoates (C_6-C_1) are not strictly phenylpropanoids since they lack the three-carbon propane side-chain (Figure 1.6); however, they may be derived from phenylpropanoids and are therefore sometimes considered products of phenylpropanoid metabolism. The biosynthesis of benzoates is still under debate; however, three mechanism have been proposed. Benzoates may be converted directly from dehydroshikimate (an intermediate of the shikimic acid pathway) or benzoates may be converted from phenylpropane units. The latter mechanism may be comparable to "ß-oxidation", using CoA esters of hydroxycinnamate derivatives, or "non-oxidative", using the hydroxycinnamic acids themselves (reviewed in Lewis, 1993). Radiolabelled feeding-experiments with ^{14}C-phenylalanine (Yazaki *et al.*, 1991) and ^{14}C-cinnamate (Yalpani *et al.*, 1993) suggest that benzoates can be derived from phenylpropanoids. Furthermore, benzoates can be synthesized in the absence of ATP and coenzyme A, suggesting that the non-oxidative mechanism is occurring and that CoA-activation of the cinnamic acid derivatives is not required

(French *et al.*, 1976; Yazaki *et al.*, 1991; Schnitzler *et al.*, 1992). More recently however, using an improved assay system, Löscher and Heide (1994) provide evidence that the biosynthesis of 4-hydroxybenzoate occurs via 4-coumaroyl:CoA. Hydroxylated benzoates like 4-hydroxybenzoate, salicylic acid (2-hydroxybenzoate), and gallic acid (3,4,5-trihydroxybenzoate) are of particular interest. 4-hydroxybenzoates are deposited in plant cell walls and are biosynthetic intermediates (Schnitzler *et al.*, 1992). Salicylic acid has been implicated as the intercellular signal in systemic acquired resistance (Malamy *et al.*, 1990; Métraux *et al.*, 1990). Gallic acids are often conjugated to sugars or other phenolic molecules and are components of "hydrolysable tannins" (Lewis, 1993).

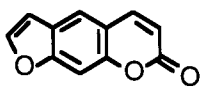


Enterodiol
(Lignan)

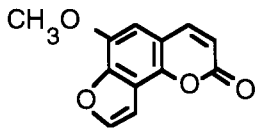


Resveratrol
(Stilbene)

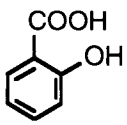
Figure 1.6: Phenylpropanoid Compounds.



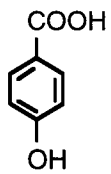
Psoralen
(Linear Furanocoumarin)



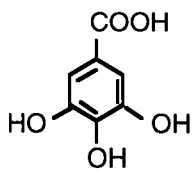
Angelicin
(Angular Furanocoumarin)



2-Hydroxybenzoate
(Salicylic Acid)



4-Hydroxybenzoate



3,4,5-Trihydroxybenzoate
(Gallic Acid)

1.3 Carbon Flow into Phenylpropanoid Metabolism

Phenylalanine, the primary metabolite from which all phenylpropanoid compounds are derived, is made via the shikimic acid pathway (Ireland, 1990). The first enzyme in the shikimic acid pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHP synthase), condenses phospho*enol*pyruvate (from glycolysis) with erythrose 4-phosphate (from the oxidative pentose phosphate pathway) to produce DAHP (Figure 1.7). DAHP is then converted to chorismate by the actions of 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, shikimate kinase, 5-*enol*pyruvylshikimate 3-phosphate synthase (EPSP synthase) and chorismate synthase (Herrmann, 1995). Chorismate can then enter two branch-pathways for the biosynthesis of tryptophan (via anthranilate synthase) or tyrosine and phenylalanine (via chorismate mutase). A number of studies have shown that the actions of DAHP synthase and chorismate mutase increase after elicitor treatment and wounding (McCue *et al.*, 1989; Muday and Herrmann, 1992; Kuroki and Conn, 1988); however, it is only recently that the genes encoding DAHP synthase, EPSP synthase, and chorismate synthase have been shown to be transcriptionally activated during similar stress conditions including light-treatment and pathogen attack (Dyer *et al.*, 1989; Keith *et al.*, 1991; Henstrand *et al.*, 1992; Görlach *et al.*, 1995). These results suggest that environmental stresses which induce the production of phenylpropanoid compounds also cause the up-regulation (gene expression and extractable enzyme activity) of components of the shikimic acid pathway.

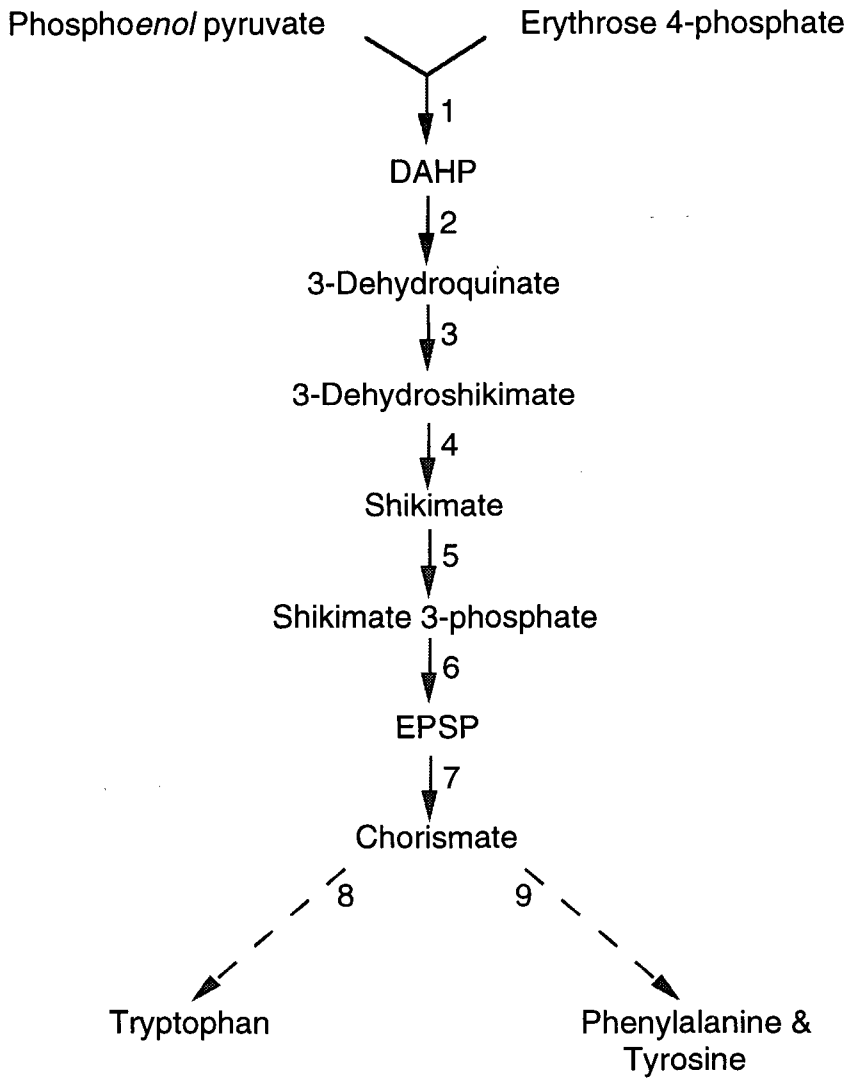


Figure 1.7: Schematic diagram of the shikimic acid pathway. The aromatic amino acids are synthesized from the shikimic acid pathway. Abbreviations and enzymes are: DAHP, 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate; EPSP, 5-*enolpyruvyl shikimate* 3-phosphate; 1, DAHP synthase; 2, 3-dehydroquinate synthase; 3, 3-dehydroquinate dehydratase; 4, shikimate dehydrogenase; 5, shikimate kinase; 6, EPSP synthase; 7, chorismate synthase; 8, anthranilate synthase; and 9, chorismate mutase. Dashed arrows represent multiple enzyme steps. Adapted from Herrmann (1995).

The oxidative pentose phosphate pathway produces two intermediates which are needed in phenylpropanoid metabolism: erythrose 4-phosphate, a substrate for DAHP synthase, and NADPH, reducing equivalents which are used in a number of reactions. NADPH is a key player in phenylpropanoid metabolism and NADPH is associated with enzymes in the general phenylpropanoid pathway (C4H), enzymes in the flavonoid biosynthetic pathway (IFS, IFR, F3'H, F3'5'H, DFR), and enzymes in the lignin biosynthetic pathway (CCR, CAD). The majority of the NADPH reducing-power

used in lignin biosynthesis has been shown to come from the oxidative pentose phosphate pathway (Pryke and Rees, 1977). NADPH is produced in the first-two steps of the oxidative pentose phosphate pathway: the steps catalyzed by glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). Studies have shown that G6PDH enzyme activity increases during pathogen attack (Börner and Grisebach, 1982) and elicitation (Robbins *et al.*, 1985; Daniel *et al.*, 1990). Very recently, the genes encoding G6PDH and 6PGDH have been shown to be transcriptionally induced during elicitation of alfalfa cell cultures (Fahrendorf *et al.*, 1995).

Wounding and parasitic infection causes an increase in respiration that is referred to as "wound respiration" and "infection-induced respiration" (Uritani and Asahi, 1980). Increase in oxygen consumption and the breakdown of carbohydrates is probably to supply ATP and precursors for secondary metabolism (Uritani and Asahi, 1980). There is evidence that phosphofructo kinase and other enzymes from glycolysis are enzymatically induced during wounding (Kahl, 1974; Uritani and Asahi, 1980); however, the wound-induced expression of genes encoding glycolytic enzymes has not been well characterized.

Taken together, these results suggest that genes encoding key enzymes in the oxidative pentose phosphate pathway, the shikimic acid pathway, the general phenylpropanoid pathway, and the branch pathways leading to specific secondary-product formation may all be coordinately regulated (Figure 1.8). The flow of carbon through these metabolic pathways may depend on 1) the activities of the enzymes leading to product formation and 2) the activities of branch-point enzymes which divert carbon away from product formation (Stephanopoulos and Vallino, 1991). Stephanopoulos and Vallino (1991) proposed the concept of "network rigidity" and suggested that successful redistribution of carbon via genetic manipulation ("metabolic engineering") depends on understanding the regulation of carbon flow, particularly at biochemical branch-points ("nodes"). Kholodenko and Westerhoff (1995) suggested

that regulation of cellular metabolism cannot be easily predicted since subcellular ("microworld") interactions render the systems more complex ("non-ideal") than that proposed by traditional biochemical studies. Two examples of molecular interactions which affect flux through a pathway, but are not apparent in purified enzyme ("ideal") systems, are 1) metabolic channelling between enzymes, and 2) compartmentalization of enzymes or metabolites (Kholodenko and Westerhoff, 1995). The metabolic control theory (Reder, 1988; Kell and Westerhoff, 1986) has been used to describe the role of PAL in carbon flux through phenylpropanoid metabolism (Bate *et al.*, 1994). There is a direct relationship between PAL activity and chlorogenic acid accumulation in sense-suppressed tobacco plants, pointing to PAL as a major control point in regulating carbon flux into this compound. However, the accumulation of rutin (a flavonoid) and lignin is relatively unaffected by the same perturbations in PAL activity in these plants. Other control points downstream of PAL (e.g. CHS, or the release of glycosylated lignin monolignols from vacuolar pools) are likely important for the regulation of carbon flux into these products.

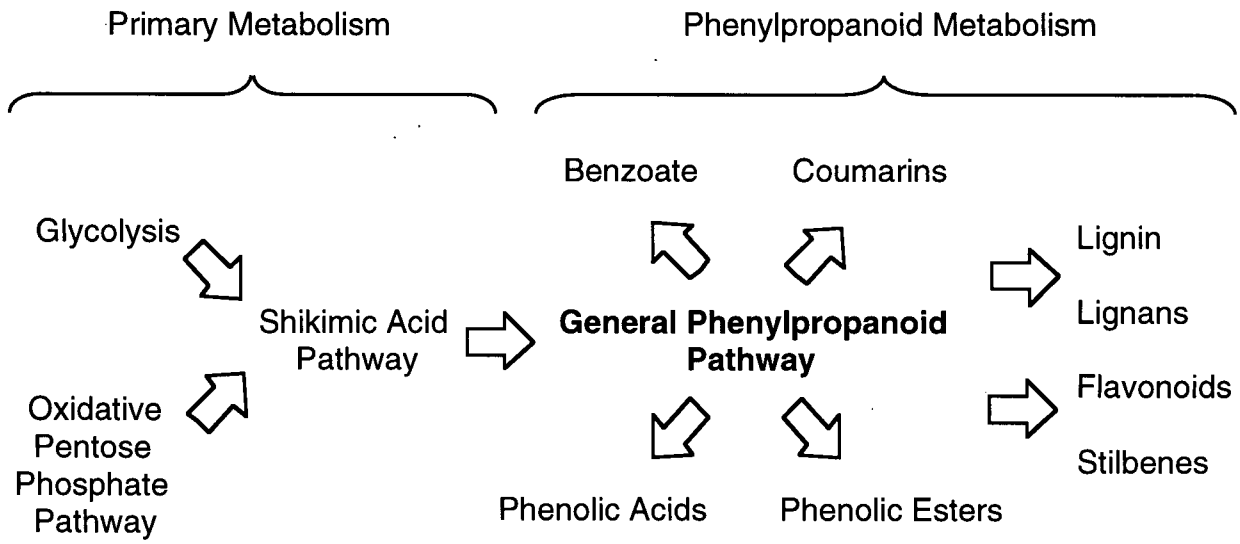


Figure 1.8: Carbon flow through the general phenylpropanoid pathway. Phospho*enol*pyruvate, from glycolysis, and erythrose 4-phosphate, from the oxidative pentose phosphate pathway, enter the shikimic acid pathway which, through a serious of metabolic steps is converted to phenylalanine. Phenylalanine enters the general phenylpropanoid pathway via the action of PAL and is converted into diverse phenylpropanoid products. Adapted from Dixon and Paiva (1995).

1.4 Research Objectives and Approaches

4CL may have a pivotal in controlling the flow of carbon from primary metabolism into secondary metabolism. I will examine 1) the nature of the *4CL* cDNAs and gene-families in tobacco and *Arabidopsis*, 2) the potential role of 4CL in regulating carbon-flow into phenylpropanoid branch-pathways, and 3) the consequences of blocking 4CL function. Chapter 3 describes the cloning and characterization of cDNAs encoding 4CL from tobacco and *Arabidopsis*. Using the cDNAs as hybridization probes, the size of the *4CL* gene-families and the expression patterns of *4CL* are characterized. To determine if divergent *4CL* cDNAs from tobacco encoded proteins with distinct enzyme activities, the cDNAs were expressed in *E. coli*. Chapter 4 describes the enzymatic characterization of the endogenous and recombinant tobacco 4CL proteins. In chapter 5, antisense-RNA technology was used to suppress *4CL*-expression in *Arabidopsis*. The morphological, biochemical, and molecular consequences of suppressing *4CL* in *Arabidopsis* is described.

Antisense RNA technology can be used to down-regulated the expression of specific genes in transgenic organisms. The mechanism by which antisense-RNA suppression occurs is still not clearly understood. Accumulated evidence suggests that, in plants, the mode of action is post-transcriptional and may involve the formation of RNA:RNA duplexes (Simons, 1988; Bourque, 1995). In the nucleus, the RNA duplex may be inefficiently processed, it may be unstable and rapidly degraded, or it may be poorly transported into the cytoplasm for translation. In the cytoplasm, the RNA duplex may again be rapidly degraded, or it may not be efficiently translated due to interference with ribosome binding. In animals, there is evidence that antisense RNA may affect transcription by forming localized RNA:DNA triplexes (Hélène and Toulmé, 1990; Bourque, 1995). In general, inhibition of gene expression by antisense RNA does not appear to target one specific step in the flow of information from DNA to RNA to protein, but rather to affect any one of a number of steps necessary for gene expression.

Tobacco and *Arabidopsis* are chosen as the experimental organisms for this work. Transgenic studies with the parsley *4CL-1* promoter fused to *GUS* (Hauffe *et al.*, 1991; 1993) and *in situ* hybridization (Reinold *et al.*, 1993) have demonstrated the expression-pattern of *4CL* in tobacco. However, the nature of the tobacco *4CL* gene-family has not been analyzed and the work described here contributes to the existing knowledge of tobacco *4CL* genes and the tobacco-4CL proteins. Tobacco is also chosen as the experimental organism because it is easy to transform and regenerate. *Arabidopsis* is a powerful model-system for genetic and molecular analysis because it has a small genome, it has relatively little repetitive DNA, it is self fertile (each plant producing thousands of seeds), it is has a short generation-time, and it is also relatively easy to transform and regenerate (Estelle and Somerville, 1986; Meyerowitz, 1987). An additional advantage with using *Arabidopsis* is that there is a large scientific community working on this crucifer and resources (electronic newsletter, mutant lines, seed stocks, linkage maps, expressed sequence tags) are readily available through the *Arabidopsis thaliana* Database (AtDB) in the internet (http://genome-www.stanford.edu/arabidopsis/). Virtually nothing is known about the *Arabidopsis 4CL* and the work described here represents the first detailed analysis of 4CL in *Arabidopsis*.

Chapter 2

Materials and Methods

2.1 Plant Growth-Conditions, Tissues, and Experimental Treatments

Nicotiana and *Arabidopsis* plants were grown in soil in growth chambers at 23°C with ~120 $\mu E\ s^{-1}m^{-1}$ of light, and an 8 h dark /16 h light regime. Under tissue-culture conditions, seeds were germinated and grown at 23°C under ~150 $\mu E\ s^{-1}m^{-1}$ of constant light. Light source was from General Electric catalogue number F20T12/CW.

Tobacco (*Nicotiana tabacum* L. cv. Xanthi SR1) seeds were from Dr. C. Douglas whereas *Nicotiana sylvestris* and *Nicotiana tomentosiformis* seeds were kindly donated by S.E.I.T.A. (Institut Expérimental du Tabac, Bergerac, France). Protein extracts for enzyme assays were prepared from tobacco-stem sections approximately

1 cm in diameter, between the third- and sixth- node above the base of mature tobacco plants. Protein extracts used for western blot screening of transgenic tobacco were prepared from 17-day-old seedlings that had been grown in MS plates supplemented with 100 mg/L kanamycin. Tobacco RNA was extracted from tissues described below. Tobacco young shoot tips were between 4 cm and 8 cm in length. Young stems, approximately 2 cm in length, were harvested just below the apical meristems. Old stems, greater than 1 cm in diameter and approximately 6 cm in length, were harvested from the base of mature plants. Floral tissues were harvested from developmental-stages 1 to 6 as described by Reinold *et al.* (1993). The floral tissues were divided into sectors as described by Drews *et al.* (1992): the limb (corresponding to the pigmented part of the corolla), the tube (corresponding to the white part of the corolla), the base (corresponding to the corolla that is surrounded by the sepals) and the sepal.

Seeds of the RLD ecotype of *Arabidopsis* were kindly donated by Dr. L. Kunst (Department of Botany, University of British Columbia, British Columbia, Canada) whereas the Columbia ecotype was supplied by Dr. J. Dangl (Max-Delbrück Labor, Max-Planck-Insititut, Köln, Germany). Protein extracts (for western blot analysis and 4CL enzyme assays) and lignin were extracted from *Arabidopsis* stems that were 15 cm in height. *Arabidopsis* RNA was isolated from tissues described below. *Arabidopsis* seedlings, Columbia ecotype, were grown on MS plates and harvested after 2, 3, 4, 5, 7, and 10 days post-germination. Seedlings were harvested either as whole-seedlings, or dissected into shoot- and root- sectors before freezing in liquid nitrogen. *Arabidopsis* leaves were mature and fully expanded, and bolting-stems were 3 cm, 6 cm, 12 cm, and 20 cm in height. *Arabidopsis* flowers, prior to anthesis, were harvested as a cluster containing one open flower with the remaining flower buds still enclosed by the sepals.

Wound treatments were performed as follows. Mature, fully-expanded tobacco or *Arabidopsis* leaves were wounded by slicing the leaves into 1-2 mm-wide strips and

placing them on a piece of Whatman filter paper moistened with MS liquid media. Tobacco leaves were wounded for 24 h before they were used for RNA isolation, whereas *Arabidopsis* leaves were wounded for 0.5 h, 1 h, 2 h, 4 h and 6 h before they were used for RNA isolation. For 0 h of wounding, leaves were detached from the plants and immediately frozen in liquid nitrogen.

Methyl jasmonate treatments were carried out by spraying tobacco plants with 1 mM methyl jasmonate (in 1% Triton-X-100) until the solution ran off the leaves. The treated plants were covered with a bell-jar and placed under constant light for 24 h. Mature, fully-expanded leaves were then excised and used for RNA isolation. For untreated RNA samples, mature, fully-expanded tobacco leaves were detached from the plants and immediately frozen in liquid nitrogen for control, untreated RNA samples.

High-intensity light treatments were performed by exposing 25-day old *Arabidopsis* plants to high-intensity white-light (~900 $\mu E\ s^{-1}m^{-1}$) for 24 h. The leaves were harvested and used for anthocyanin and RNA isolation.

2.2 Hybridization Probes

Hybridization probes used in Southern and northern blot analysis were generated from cDNAs, genes, or expressed sequence tags (ESTs, Newman *et al.*, 1994) as described in Table 2.1 and 2.2. The ESTs were selected from the *Arabidopsis* EST database (dbEST) based on the sequence homology between the EST and previously cloned genes. The 5' and 3' portions of the ESTs were sequenced to confirm their identity. ^{32}P-radiolabeled probes were generated using the Random Primers DNA Labeling System (Gibco BRL, Gaithersburg, MD) according to the manufacturer's specifications.

Table 2.1: cDNA and gene fragments used for generating hybridization probes.

Probe	Enzyme for Fragment Excision	Fragment Size (kb)	Reference
St4CL	*Eco*RI	2	Becker-André *et al.*, 1991
StPAL	*Eco*RI of pCP61.13	0.6	Joos and Hahlbrock, 1992
	*Eco*RI of pCP63.15	0.9	
AtDSH1	*Eco*RI of pBK 5.70-R	1.8	Keith *et al.*, 1991
AtPAL	*Hind*III of *PAL10-3*	0.5	Wanner *et al.*, 1995
AtC4H	*Sal*I, *Not*I	2	gift from C. Chapple
rRNA	*Hind*III of pHA2	8.7	Jorgensen *et al.*, 1982

Table 2.2: ESTs used for generating hybridization probes.

Probe	Putative Encoded Enzyme	Homology (%, organism)	Size (kb)	EST Accession Number
AtHEXO	Hexokinase	98%, *A. thaliana*	1.3	84G1T7
AtPFK	Phosphofructo kinase	77%, *B. vulgaris*	1.3	146I12T7
AtG6PDH	Glucose 6-phosphate dehydrogenase	82%, *S. tuberosum*	1.3	35D3T7
At6PGDH	6-Phosphogluconate dehydrogenase	79%, *M. sativa*	2	11B4T7P
AtEPSPS	5-*Enol*pyruvyl-shikimate 3-phosphate synthase	98%, *A. thaliana*	1.3	131D24T7
AtCAD	Cinnamyl alcohol dehydrogenase	95%, *A. thaliana*	1.3	90C21T7
AtCHS	Chalcone synthase	99%, *A. thaliana*	1.2	YAP097T3

2.3 Cloning of *4CL* cDNAs

A tobacco cDNA library was constructed in λZAPII (Stratagene, La Jolla, CA) using poly(A)+ RNA isolated from tobacco young shoot-tips (S. Lee and C. Douglas, unpublished). The primary library, consisting of approximately 10^7 independent recombinants, was amplified and approximately 10^5 plaque forming units (p.f.u.) were screened using XL1-Blue cells. Putative *4CL* cDNA clones were generated as pBluescript I SK plasmids via *in vivo* excision using the helper bacteriophage R408 as specified by the manufacturer.

The *Arabidopsis* λYES cDNA library, kindly donated by Dr. R. W. Davis (Stanford University, Stanford, California), was made with poly(A)+ RNA from aerial portions of plants ranging in size from those which had just opened their primary leaves to plants which had bolted and were flowering. The primary library, containing

10^7 independent recombinants was amplified and approximately 2 x 10^5 plaque forming units (p.f.u.) were screened using LE392 pMC9 cells. Putative clones were generated as plasmids via *in vivo* excision using BNN132 cells and the *Arabidopsis 4CL* cDNA was then subcloned into the *Xho*I site of pBluescript I KS.

Tobacco- and *Arabidopsis*- cDNA libraries were screened using a 2-kb potato *4CL* cDNA (Becker-André *et al.*, 1991) as a hybridization probe. The hybridization filters were washed at low stringency (2 x SSC, 0.1% SDS, 65°C) as described by Sambrook *et al.* (1989).

2.4 Restriction Map-Analysis, Subcloning, Sequencing and Sequence Analysis of Tobacco and *Arabidopsis* cDNA Clones

All putative tobacco-*4CL* cDNA clones were partially sequenced at the 5' and 3' ends using the T7 Sequencing Kit™ (Pharmacia, Piscataway, NJ). Clones with high sequence-homology to the parsley and potato *4CL* cDNAs (Lozoya *et al.*, 1988, Becker-André *et al.*, 1991) were classified into groups according to their restriction maps. Overlapping subclones of the two longest cDNA clones, Nt4CL-1 and Nt4CL-19, were constructed in pBluescript II KS for sequencing. A restriction map was determined for the putative *Arabidopsis 4CL* cDNA, At4CL, and overlapping subclones were constructed in pBluescript I KS.

Nt4CL-1 and Nt4CL-19 subclones were sequenced by the University of British Columbia Nucleic Acid-Protein Service Unit using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Foster City, CA). At4CL subclones were sequenced manually using the T7 Sequencing Kit™. Overlapping nucleotide sequences were aligned and contiguous cDNA-sequences were reconstructed. Accuracy of the sequencing results was confirmed by the complementarity between the top- and bottom- strand DNA-sequences and by translation of the sequences into amino acid sequences comparable to those from previously cloned *4CL*s.

DNA and predicted amino acid sequences were analyzed using BESTFIT and PILEUP computer programs from the University of Wisconsin Genetics Computer Group software. Predicted phosphorylation- and glycosylation- sites, isoelectric points, and molecular weights were determined using computer programs PRO-SITE and pI/MW from the University of Geneva (Geneva, Switzerland).

2.5 Southern Blot and Northern Blot Analysis

Genomic DNA was isolated using CTAB (hexadecyltrimethylammonium bromide) as described by Doyle and Doyle (1990). Ten μg of genomic DNA were digested with restriction enzymes, electrophoresed in a 0.8% agarose gel, and blotted onto Hybond™ nylon-membranes (Amersham, Oakville, Ont, Canada). Hybond™ nylon-blots were hybridized in 6 x SSC, 0.5% SDS, 5 x Denhardts, and 0.1 mg/mL denatured salmon-sperm DNA (Sambrook *et al.*, 1989). Southern blots were washed at high (0.2 x SSC, 0.1% SDS, 65°C) or low (2 x SSC, 0.1% SDS, 65°C) stringency.

RNA was extracted using guanidinium hydrochloride as described by Logemann *et al.* (1987). Ten μg of RNA were electrophoresed in 1.2% agarose gels containing 2.2 M formaldehyde, the gel was rinsed in water for one hour, and then blotted onto Zeta-Probe® GT Genomic Tested Blotting Membranes (Bio-Rad Laboratories, Hercules, CA) or Hybond™ nylon-membranes. Wound-induced and high-intensity white-light induced RNA samples were blotted onto Zeta-Probe® membranes. All other RNA samples were blotted onto Hybond™ membranes. Zeta-Probe blots were hybridized in 0.5 M Na_2HPO_4 pH 7.2, and 7% SDS as specified by the manufacturer. Hybond nylon-blots were hybridized as described above. Northern blots hybridized to the tobacco *4CL* probes were washed at moderate stringency (0.5 x SSC, 0.1% SDS, 65°C). Northern blots hybridized to the potato *PAL* probe were washed at low stringency (2 x SSC, 0.1% SDS, 65°C). All *Arabidopsis* northern blots were washed at high stringency (0.2 x SSC, 0.1% SDS, 65°C). Blots were stripped by

pouring boiling 0.1% SDS onto the blots and allowing the solution to cool to room temperature. This process was done twice.

2.6 Cloning of Tobacco-*4CL* cDNAs into *E. coli* Expression-Vectors

PCR was used to engineer *Sph*I restriction-sites into the 5' ends of the Nt4CL-1 and Nt4CL-19 cDNAs using a T7 primer and cDNA-specific oligonucleotides (5'-GGGGCATGCCAATGGAGACTACTAC-3' for Nt4CL-1 and 5'-GGGGCATGCATGG AGAAAGATACAAAACAG-3' for Nt4CL-19). Nt4CL-1 and Nt4CL-19 PCR products were digested with *Sph*I and *Xho*I and cloned into the *Sph*I and *Sal*I restriction-sites of the expression-plasmids pQE-32 and pQE-30 respectively (QIAexpressionist™ Kit, QiaGen Inc., Chatsworth, CA). The 5' and 3' portions of the DNA constructs were sequenced to ensured fidelity of the PCR and to confirm that the cDNAs were in frame with the ATG from the pQE expression-vectors. The center portion of each PCR product was excised and replaced with the corresponding portion from the original cDNA (*Sac*I/*Kpn*I fragment of Nt4CL-1 and the *Bst*BI fragment of Nt4CL-19) to eliminate the possibility of single base-pair changes from the PCR. All DNA manipulations were performed in XL1-Blue cells. Expression of the cDNAs were performed in *Escherichia coli* strain M15 as recommended by the manufacture. The expression-plasmid (pQE-30), without a cDNA insert, was generated in M15 as a negative control.

2.7 Protein Extraction

Recombinant-4CL proteins were generated by inducing 10 mL of a bacterial culture ($OD_{600} \approx 0.7$) containing the expression-plasmids with 2 mM IPTG for 4 h. After centrifugation the bacterial-pellet was resuspended in 2 mL of 200 mM Tris, pH 7.8 and the cells were disrupted in a French Press. Cellular debris was removed by centrifugation and then glycerol was added to the supernatant to a final concentration

of 30%. Samples were frozen in liquid nitrogen and stored at -80°C until further analysis.

Plant proteins, for western blot analysis, were extracted by first grinding the tissue in liquid nitrogen and then resuspending the powder in 50 mM Tris, pH 8, and 5 mM $MgCl_2$. The homogenate was centrifuged twice at 4°C to remove cellular debris and the final supernatant was used for western blot analysis.

For enzyme assays, plant extracts were prepared as described by Knobloch and Hahlbrock (1977). Briefly, stem- and leaf- tissues were ground into a fine powder in liquid nitrogen; the powder was resuspended in 200 mM Tris, pH 7.8 and 15 mM ß-mercaptoethanol; the suspension was rotated, in the presence of 10% (w/w) Dowex, at 4°C for 15 minutes; the mixture was centrifuged to remove debris; the supernatant was concentrated using Microsep™ 10K cut-off micro-concentrators (Filtron, Northborough, MA); the protein extract was made to 30% glycerol, frozen in liquid nitrogen, and then stored at -80°C until further analysis.

Protein content was quantified by the Bradford (1976) method using the Bio-Rad Protein Assay Kit™ (Bio-Rad, Hercules, CA) with BSA as a standard.

2.8 Western Blot Analysis

Western blot analysis was performed as described by Sambrook *et al.* (1989). Plant (25 µg) or bacterial (5 µg) proteins were electrophoresed in 10% SDS-polyacrylamide separating gels (Laemmli, 1970) and then blotted onto Hybond™ nylon-membranes at 4°C for 3 h using 25 mM Tris and 250 mM glycine. The blots were blocked with 5% (w/v) non-fat powdered milk, reacted with a 1:5000 dilution of a rabbit antiserum raised against the parsley 4CL (Ragg *et al.*, 1981), reacted with a 1:2500 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase, (Gibco BRL, Gaithersburg, MD), and then visualized using 4.5 mM Fast-Red R and 2.5 mM Naphthol AS-MX phosphate dissolved in 50 mM Tris, pH 8 as substrates (Sigma, St. Louis, MO).

2.9 4CL Enzyme Assay

4CL enzyme activity was measured spectrophotometrically at room temperature as described by Knobloch and Hahlbrock (1977). The 4CL reaction mixtures contained protein extracts, 5 mM ATP, 5 mM $MgCl_2$, 0.33 mM coenzyme A, and 0.2 mM cinnamic acid derivatives. The blank (reference) mixtures contained the same components but without the coenzyme A. The change in OD of the reaction mixtures relative to the blank mixtures was monitored at wavelengths of 311 nm, 333 nm, 346 nm, 345 nm, and 352 nm, which are the absorption maxima for cinnamoyl:CoA, 4-coumaroyl:CoA, caffeoyl:CoA, feruloyl:CoA, and sinapoyl:CoA, respectively (Stöckigt and Zenk, 1975). Calculations were performed using 22000 L $mol^{-1}cm^{-1}$, 21000 L $mol^{-1}cm^{-1}$, 18000 L $mol^{-1}cm^{-1}$, 19000 L $mol^{-1}cm^{-1}$, and 20 000 L $mol^{-1}cm^{-1}$ as extinction coefficients for cinnamoyl:CoA, 4-coumaroyl:CoA, caffeoyl:CoA, feruloyl:CoA, and sinapoyl:CoA, respectively (Stöckigt and Zenk, 1975).

2.10 Generation of Constructions to Antisense and Sense *4CL*

All plasmids were propagated in *E. coli* strain DH5α, and manipulations were performed by conventional molecular biology methods. The plasmids used to generate the *4CL* sense and antisense constructs are as follows. The 2-kb potato *4CL* cDNA used in some constructions was propagated in pBluescribe, whereas At4CL was cloned and propagated in pBluescript I KS. pRT101 is a plasmid containing the cauliflower mosaic virus 35S promoter, a convenient multicloning-site followed by the 35S polyadenylation/termination sequences (Töpfer *et al.*, 1987). Plasmid 35-31 contains the 1.5 kb parsley *4CL1* promoter in pBluescribe (Hauffe *et al.*, 1991) and pBin19 (Bevan, 1984) is a binary vector containing the right- and left- borders used in *Agrobacterium*-mediated plant-transformations. Relevant portions of selected plasmids are shown in Figure 2.1.

pRT101-Pc4CL–P, in which the 35S promoter of pRT101 was replaced with the parsley-*4CL1* promoter, was constructed in three steps. First, plasmid 35-31 was

digested with *Pst*I and *Eco*RI to release the 1.5-kb parsley-*4CL1* promoter-fragment. Second, the 0.4 kb 35S promoter was excised from pRT101 by complete *Eco*RI and partial *Pst*I digestion (Töpfer *et al.*, 1987). Lastly, the parsley-*4CL1* promoter-fragment was ligated into the modified pRT101 to produce a DNA construct consisting of the parsley-*4CL1* promoter, a convenient multicloning-site followed by the 35S polyadenylation/termination sequences. Both pRT101 and pRT101-Pc4CL-P were used to generate antisense- and sense- *4CL* DNA constructs.

The full-length potato *4CL* cDNA (St4CL) was inserted in sense- and antisense-orientations behind the 35S promoter by ligating the 2-kb *Eco*RI cDNA-fragment into the *Eco*RI site of pRT101. The two orientations were distinguished by digestion with *Bam*HI. A partial-length potato-*4CL* cDNA, the *Eco*RI/*Kpn*I fragment, was cloned in sense orientation into the *Eco*RI/*Kpn*I sites of pRT101. For a partial-length potato-*4CL* cDNA in antisense orientation, St4CL was completely digested with *Xba*I and partially digested with *Kpn*I and then the released 1.6-kb fragment was ligated into the *Xba*I/*Kpn*I sites of pRT101. The partial-length St4CL constructs lacked the polyA tail and the equivalent of 23 amino acids at the carboxy-terminal of the predicted potato 4CL polypeptide.

The *Arabidopsis 4CL* cDNA was inserted in sense- and antisense- orientation behind the 35S or the parsley-*4CL* promoters in plasmids pRT101 and pRT101-Pc4CP-P, respectively. Sense orientation constructs were made by digesting At4CL with *Xho*I/*Bgl*II, or *Kpn*I/*Bgl*II, and ligating the fragments into the *Xho*I/*Bam*HI, or *Kpn*I/*Bam*HI sites, of pRT101 and pRT101-Pc4CL-P respectively. Antisense constructs were made by first generating an At4CL cDNA clone in the opposite orientation as the one diagrammed in Figure 2.1 with respect to the polylinker. This construct, At4CL*, had the *Xba*I restriction-site on the 5' end of the cDNA. At4CL* was digested with *Xba*I/*Bgl*II and subcloned into the *Xba*I/*Bgl*II sites of pRT101 and pRT101-Pc4CL-P. The antisense At4CL DNA constructs were digested with *Xba*I and *Cla*I, and then Klenow was used to blunt-end the termini. Re-ligation of the plasmid produced a

construct which had the At4CL cDNA in antisense orientation and this construct lacked the multicloning site derived from Bluescript I KS. Digestion of the *Arabidopsis 4CL* cDNA with *Bgl*II removes the polyA tail and the equivalent of 10 amino acids at the carboxy-terminal of the predicted *Arabidopsis* 4CL polypeptide.

The above DNA constructs were subcloned as a cassette into the *Hind*III site of pBin19. The potato-*4CL* constructs were partially digested with *Hind*III since there are internal *Hind*III sites within the cDNA. All DNA constructs were verified by diagnostic restriction-digest analysis after each subcloning step to confirm the orientation of the cDNA and to insure that all portions of the construct were not lost or rearranged.

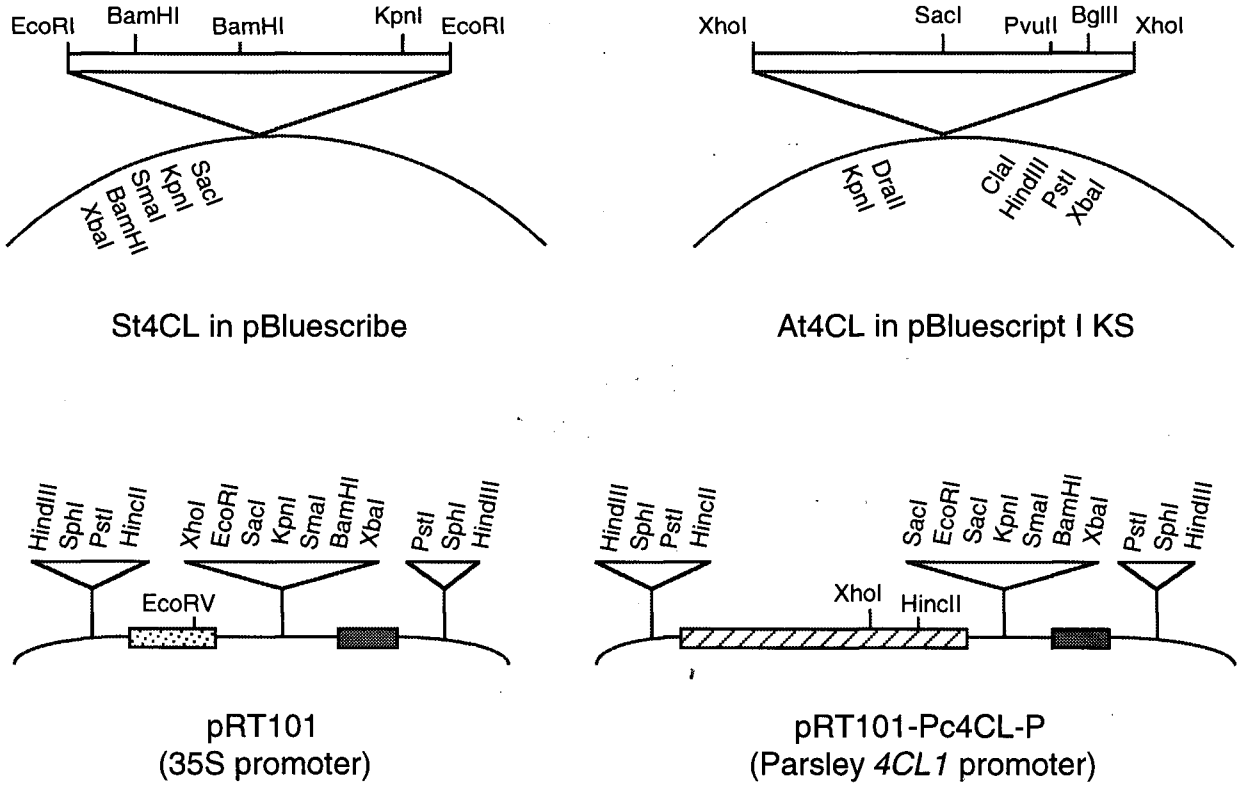


Figure 2.1: Plasmids used in generating *4CL* sense- and *4CL* antisense- DNA constructions. Abbreviations are: St4CL, potato *4CL* cDNA; At4CL, *Arabidopsis 4CL* cDNA; and 35S promoter, cauliflower mosaic virus 35S promoter. Open bars represents cDNAs, stippled bar represents the 35S promoter, slashed bar represents the parsley *4CL1* promoter, and gray bars represents the 35S polyadenylation/termination sequences.

2.11 Plant Transformation

Agrobacterium-mediated transformations of tobacco and *Arabidopsis* were done as described by Lee and Douglas (1996a). The pBin19 constructs were mobilized from *E. coli* into *Agrobacterium tumefaciens* strain LB4404 by tri-parental mating using the helper-plasmid pRK2013. For transformation into tobacco, overnight *Agrobacterium* cultures were diluted 1:10 with Murashige and Skoog (MS) liquid media while, for *Arabidopsis* transformations, overnight *Agrobacterium* cultures were diluted 1:20 with callus induction media (CIM). Details of media components are listed in Figure 2.2.

Nicotiana tabacum SR1 seeds were surface-sterilized and grown under axenic-conditions in Magenta GA7 boxes containing MS media until the tobacco leaves were 4-5 cm in length. Tobacco leaves were cut into pieces, approximately 1 cm x 1 cm in size, incubated in the diluted *Agrobacterium* cultures for 1-5 minutes, dabbed dry on a piece of sterilized Whatman filter paper, and then placed upside-down on MS media supplemented with 1 mg/L 6-benzylaminopurine and 0.2 mg/L α-napthaleneacetic acid. After 2 - 3 days, the leaf-pieces were transferred onto MS media supplemented with 1 mg/L 6-benzylaminopurine, 0.2 mg/L α-napthaleneacetic acid, 100 mg/L kanamycin, and 300 mg/L carbenicillin. Approximately 2 - 3 weeks later, shoots protruding from the calli were excised and transferred into Magenta GA7 boxes containing MS media supplemented with 100 mg/L kanamycin and 300 mg/L carbenicillin. When roots were visible, the plants were transferred into soil and were maintained until seed set.

Arabidopsis RLD and Columbia seeds were surface-sterilized and grown under axenic-conditions in a 250 mL flask containing 50 mL liquid MS media. The flasks, containing 5 - 10 seeds, were shaken at 22°C, on a rotary shaker at 50 rpm under constant-light for 3 - 4 weeks until a mass of roots accumulated. The roots were excised from the shoots, spread onto CIM plates and pre-incubated for 3-days after which they were incubated in the diluted *Agrobacterium* culture for approximately 5

minutes. The roots were then dabbed dry on a piece of sterilized Whatman filter paper and placed on CIM plates. After 2 days, the bacteria were removed by washing the roots in liquid CIM supplemented with 500 mg/L carbenicillin. The roots were then dabbed dry, cut into 0.5 cm pieces, spread onto shoot induction media (SIM), and maintained by transferring them onto fresh SIM plates weekly. After 3 - 4 weeks, green calli were visible and, after a number of weeks, shoots emerged from the calli. These shoots were excised from the calli, placed in a sterile, 15 cm, glass test-tube containing 2 mL root induction media (RIM). The transformants were allowed to mature and set seed in the test-tubes.

1000 x MS Vitamins

10 g myo-inositol
0.1 g nicotinic acid
0.1 g pyridoxine HCl
1.0 g thiamine HCl
adjust to 100 mL
filter sterilize

MS Media

1 MS Salt Mixture
1L package
Gibco, Cat.# 11117
30 g sucrose
1 mL 1000 x MS vitamins
KOH to pH 5.7
adjust to 1L
8 g phytoagar
autoclave

B5 Media

1 Gamborg's B-5 Medium
1L package
Gibco, Cat. # 21153
0.5 g MES
KOH to pH 5.7
adjust to 1L
8 g phytoagar
autoclave

CIM

B5 media
0.5 mg/L 2,4-D
0.05 mg/L kinetin

SIM

B5 media
5.0 mg/L 2ip
0.15 mg/L IAA
50 mg/L kanamycin
500 mg/L carbenicillin

RIM

MS media
1 mg/L NAA
50 mg/L kanamycin

Figure 2.2: Media used in plant transformation and regeneration. Liquid media were made by omitting the phytoagar. Phytohormones are: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2ip, N^6-(2-isopentenyl)adenine; IAA, indole-3-acetic acid; and NAA, α-napthaleneacetic acid. Abbreviations are: MS, Murashige and Skoog; CIM, callus induction media; SIM, shoot induction media; and RIM, root induction media.

2.12 Lignin and Anthocyanin Extraction

Lignin was extracted as described by Bruce and West (1989). Approximately 50 mg of tissue was accurately weighed and then homogenized in methanol. The homogenate was filtered through GF/A glass-microfiber discs (Whatman International Ltd., Maidstone, England) and allowed to air-dry overnight. The dried alcohol-insoluble residue (AIR, approximately 10 mg) was weighed and then transferred to a 13 mL Sarstedt tube (Newton, NC). The sample was incubated in 1 mL 2N HCl and 0.5 mL thioglycolic acid (Fisher Scientific, Fair Lawn, NJ), with intermittant shaking, at 100°C for 4 h. The mixture was centrifuged at maximum speed; the pellet was rinsed with 1 mL water and then transferred to a 2-mL screw-cap eppendorf tube; and then the solid material was extracted by shaking in 1 mL 0.5 M NaOH for 18 h at room temperature. The mixture was centrifuged and the thioglycolic acid-derived lignin was precipitated from the supernatant at 4°C for 4 h with 0.2 mL concentrated HCl. The sample was centrifuged 10 minutes at maximum speed and then the pellet was dissolved in 1 mL 0.5 M NaOH and quantified spectrophotometrically at 280 nm. Absorbance readings were calculated per mg AIR or per mg fresh weight starting material.

Anthocyanins were extracted as described by Feinbaum and Ausubel (1988). Briefly, 0.5 mg of frozen leaf-tissue was homogenized in 2 mL 1% HCl (w/v in methanol) and shaken overnight at room temperature. After the addition of 1 mL of water, the mixture was extracted with chloroform and the pink, aqueous-phase was quantified spectrophotometrically at 530 nm and 657 nm. Anthocyanin content was calculated by subtracting the absorbance due to chlorophyll using the equation $OD_{530} - OD_{657}$.

Chapter 3

Cloning and Characterization of the *4CL* cDNAs from *Nicotiana tabacum* and *Arabidopsis thaliana*

3.1 Cloning and Characterization of the *Arabidopsis-4CL* cDNA

cDNAs encoding *4CL* were cloned from *Arabidopsis* by screening an *Arabidopsis thaliana* cDNA library using the potato *4CL* cDNA (Becker-André *et al.*, 1991) as a hybridization probe. Five putative *Arabidopsis 4CL* clones were isolated but plasmid Southern blot analysis of the cDNA inserts showed that only one insert strongly and reproducibly hybridized to the potato *4CL* probe. This clone, At4CL, contained an insert approximately 2-kb in size and sequencing of the cDNA demonstrated that it had high (63% to 71%) homology to other known *4CL* sequences (Table 3.1). A putative initiation ATG-codon from the At4CL insert was located only five nucleotides from the 5' end of the cDNA insert. It is possibile that additional in-frame methionine codons are located further upstream; however, because the putative initiation ATG-codon is in a favorable sequence-context for the initiation of translation (Joshi, 1987; Kozak,1986) and because amino acid homology to other 4CL proteins begins shortly after this methionine (Figure 3.1), we consider it likely that this is the

initiator codon and that the cDNA is missing most of the 5' untranslated portion of the *Arabidopsis 4CL* gene.

The predicted amino acid sequence from At4CL was between ~60% to ~70% identical to the predicted amino acid sequences from tobacco, potato, *Lithospermum erythrorhizon*, parsley, soybean, pine and rice *4CL* cDNAs. At4CL shared highest amino acid identity (78%) with the *4CL14* clone from soybean. The At4CL cDNA sequence had an open reading-frame predicted to encode a protein of 562 amino acids with a molecular mass of 61.0 kDa and an isoelectric point of 5.25 (not shown). The predicted polypeptide (Figure 3.1, *Arab*-1) contained the conserved "GEICIRG" amino acid motif believed essential for 4CL activity (Becker-André *et al.* 1991) and an AMP-binding signature ([LIVMFY]-X_2-[STG]-G_2-[ST]-[STE]-[SG]-X_2-[PALIVM]-K; Schröder, 1989). The predicted *Arabidopsis* 4CL peptide also contained four of the five cysteine-residues reported in other cloned *4CL*s (Figure 3.1, Uhlmann and Ebel, 1993). The high sequence homology, the conserved amino acid motifs, and the good sequence-aligment suggest that the cDNA cloned, At4CL, encodes 4CL from *Arabidopsis*.

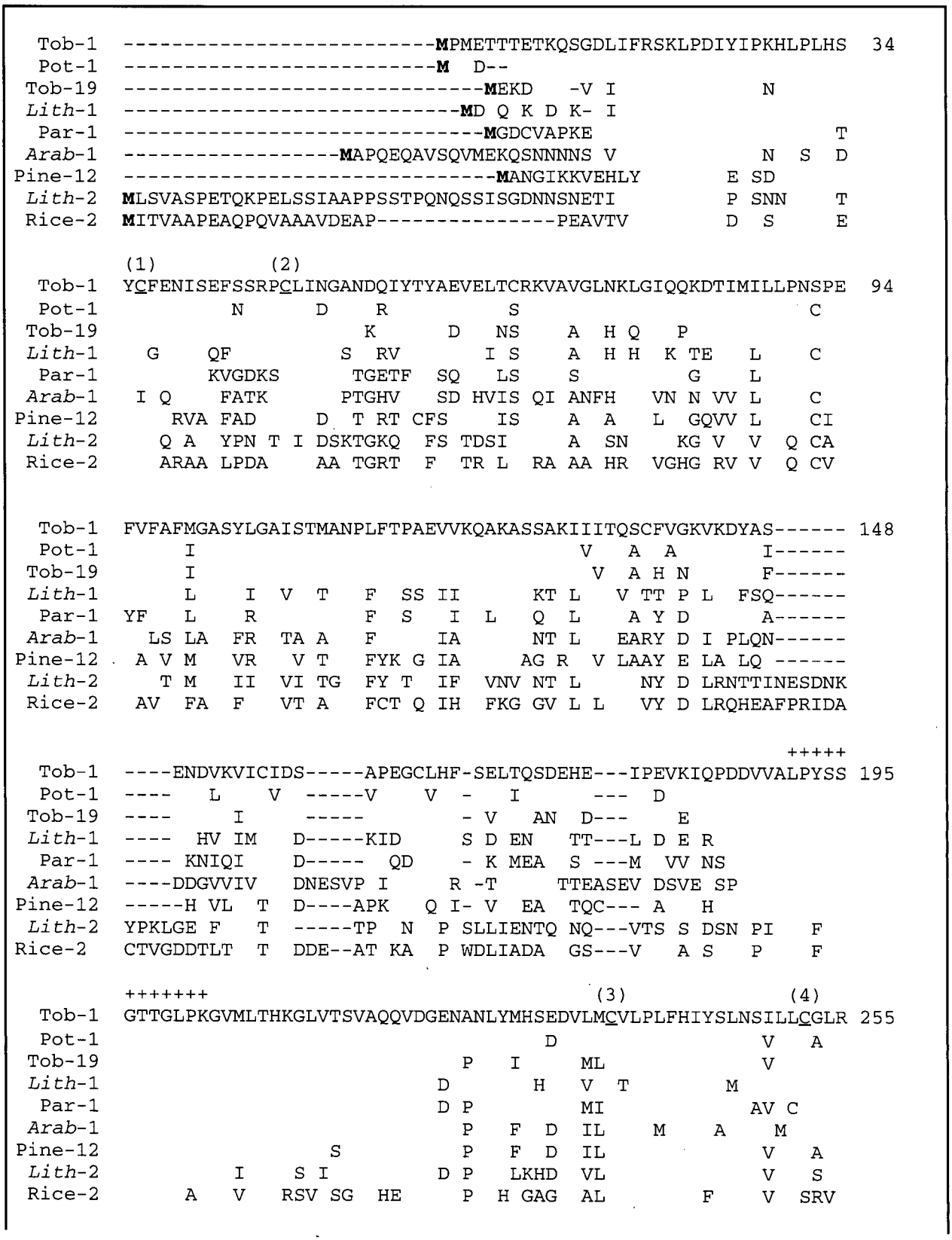
In order to estimate the number of *4CL* genes in the *Arabidopsis* genome, genomic Southern blots were hybridized to the *Arabidopsis 4CL* cDNA. A simple hybridization pattern was observed when *Arabidopsis* genomic DNA was digested with several different enzymes (Figure 3.2) which, with the exception of *Eco*RI, did not cut within the cDNA. In each case, a single hybridizing genomic restriction-fragment was observed when the blot was washed under stringent conditions. The cDNA contains two *Eco*RI sites which are approximately 150 bp and 300 bp from the 5' end of the cDNA clone. Based on the location of conserved exon-intron boundaries in the parsley (Lozoya *et al.*, 1988), potato (Becker-André *et al.*, 1991), and rice (Zhao *et al.*, 1990) *4CL* genes, these *Eco*RI sites would be within the first exon. It is likely that a genomic fragment containing the flanking DNA 5' to the first *Eco*RI site was not detected on the blot due to a weak signal from this small portion of the probe, and that

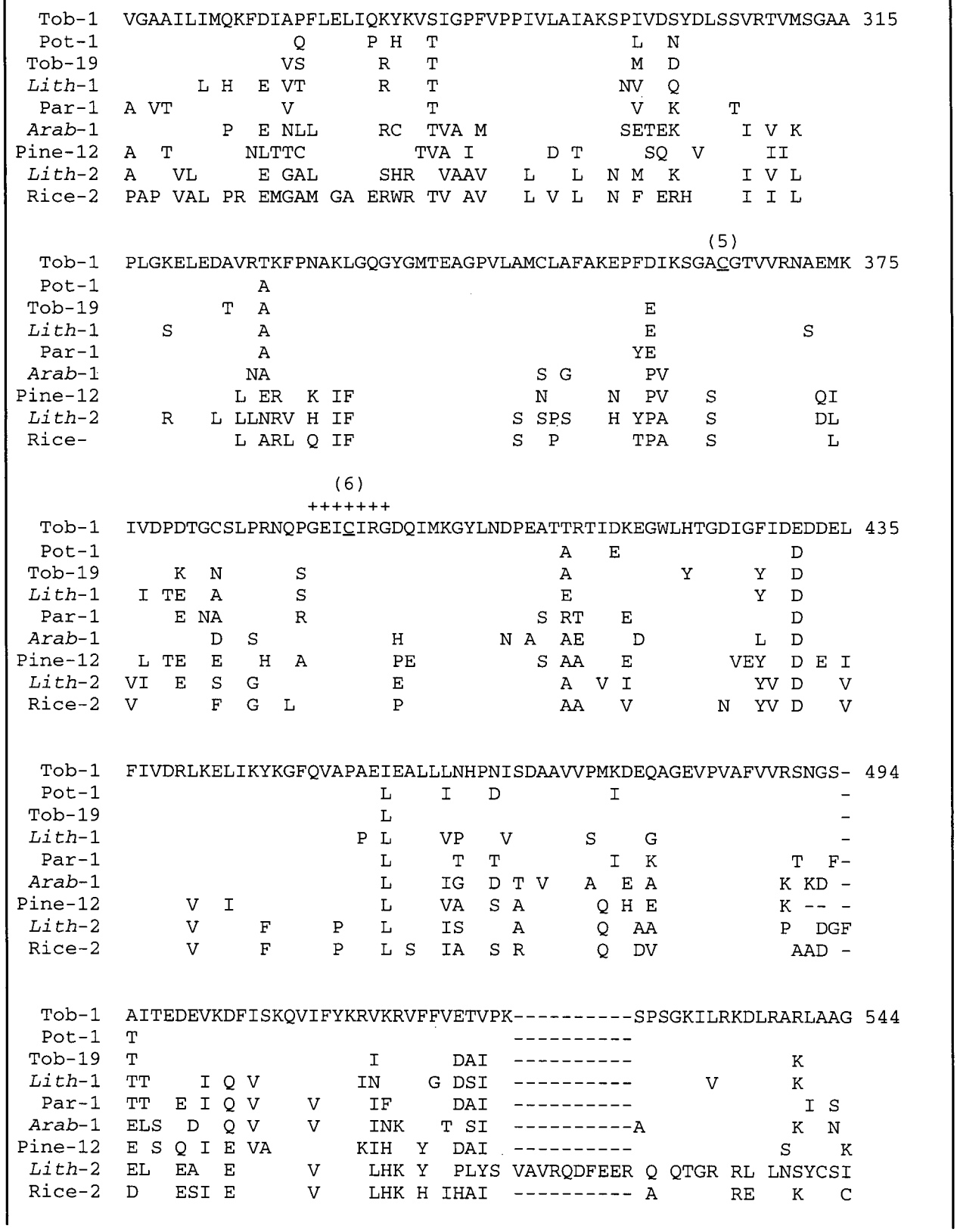
the predicted 150-bp *Eco*RI fragment was too small to be detected on the blot. Identical patterns of hybridization were observed when blots were hybridized under non-stringent conditions (not shown). Thus, consistent with previously reported results (Trezzini *et al.*, 1993), 4CL appears to be encoded by a single gene in *Arabidopsis.*

Table 3.1: Comparison of At4CL, Nt4CL-1, and Nt4CL-19 nucleotide (coding region) and predicted amino acid sequences to each other and to other *4CL* sequences.

	At4CL			Nt4CL-1			Nt4CL-19		
	% DNA Iden.	% Amino Acid Iden.	% Amino Acid Sim.	% DNA Iden.	% Amino Acid Iden.	% Amino Acid Sim.	% DNA Iden.	% Amino Acid Iden.	% Amino Acid Sim.
Tobacco-1	67	66	80	-	-	-	82	81	89
Potato-1	67	71	85	88	86	92	80	80	88
Tobacco-19	70	69	82	82	81	89	-	-	-
Lithospermum-1	67	70	82	75	80	89	75	81	90
Parsley-1	69	69	82	75	73	83	76	72	81
Soybean-14	71*	78*	87*	73*	73*	83*	73*	73*	83*
Arabidopsis	-	-	-	67	66	80	70	69	82
Soybean-16	70*	70*	84*	66*	62*	80*	69*	65*	79*
Pine-12	64	65	81	65	63	79	66	65	80
Lithospermum-2	66	61	77	64	62	80	64	62	79
Rice-2	63	60	78	63	60	80	59	61	80

* Comparisons based on partial sequences.


Tob-1 ----------MPMETTTETKQSGDLIFRSKLPDIYIPKHLPLHS 34
Pot-1 ----------M D--
Tob-19 ----------MEKD -V I N
Lith-1 ----------MD Q K D K- I
Par-1 ----------MGDCVAPKE T
Arab-1 ----------MAPQEQAVSQVMEKQSNNNNS V N S D
Pine-12 ----------MANGIKKVEHLY E SD
Lith-2 MLSVASPETQKPELSSIAAPPSSTPQNQSSISGDNNSNETI P SNN T
Rice-2 MITVAAPEAQPQVAAAVDEAP----------PEAVTV D S E
(1) (2)
Tob-1 YCFENISEFSSRPCLINGANDQIYTYAEVELTCRKVAVGLNKLGIQQKDTIMILLPNSPE 94
Pot-1 N D R S C
Tob-19 K D NS A H Q P
Lith-1 G QF S RV I S A H H K TE L C
Par-1 KVGDKS TGETF SQ LS S G L
Arab-1 I Q FATK PTGHV SD HVIS QI ANFH VN N VV L C
Pine-12 RVA FAD D T RT CFS IS A A L GQVV L CI
Lith-2 Q A YPN T I DSKTGKQ FS TDSI A SN KG V V Q CA
Rice-2 ARAA LPDA AA TGRT F TR L RA AA HR VGHG RV V Q CV
Tob-1 FVFAFMGASYLGAISTMANPLFTPAEVVKQAKASSAKIIITQSCFVGKVKDYAS------ 148
Pot-1 I V A A I------
Tob-19 I V A H N F------
Lith-1 L I V T F SS II KT L V TT P L FSQ------
Par-1 YF L R F S I L Q L A Y D A------
Arab-1 LS LA FR TA A F IA NT L EARY D I PLQN------
Pine-12 A V M VR V T FYK G IA AG R V LAAY E LA LQ ------
Lith-2 T M II VI TG FY T IF VNV NT L NY D LRNTTINESDNK
Rice-2 AV FA F VT A FCT Q IH FKG GV L L VY D LRQHEAFPRIDA
+++++
Tob-1 ----ENDVKVICIDS-----APEGCLHF-SELTQSDEHE---IPEVKIQPDDVVALPYSS 195
Pot-1 ---- L V -----V V - I --- D
Tob-19 ---- I ----- - V AN D--- E
Lith-1 ---- HV IM D-----KID S D EN TT---L D E R
Par-1 ---- KNIQI D----- QD - K MEA S ---M VV NS
Arab-1 ----DDGVVIV DNESVP I R -T TTEASEV DSVE SP
Pine-12 -----H VL T D----APK Q I- V EA TQC--- A H
Lith-2 YPKLGE F T -----TP N P SLLIENTQ NQ---VTS S DSN PI F
Rice-2 CTVGDDTLT T DDE--AT KA P WDLIADA GS---V A S P F
+++++++ (3) (4)
Tob-1 GTTGLPKGVMLTHKGLVTSVAQQVDGENANLYMHSEDVLMCVLPLFHIYSLNSILLCGLR 255
Pot-1 D V A
Tob-19 P I ML V
Lith-1 D H V T M
Par-1 D P MI AV C
Arab-1 P F D IL M A M
Pine-12 S P F D IL V A
Lith-2 I S I D P LKHD VL V S
Rice-2 A V RSV SG HE P H GAG AL F V SRV


Tob-1 VGAAILIMQKFDIAPFLELIQKYKVSIGPFVPPIVLAIAKSPIVDSYDLSSVRTVMSGAA 315
Pot-1 Q P H T L N
Tob-19 VS R T M D
Lith-1 L H E VT R T NV Q
Par-1 A VT V T V K T
Arab-1 P E NLL RC TVA M SETEK I V K
Pine-12 A T NLTTC TVA I D T SQ V II
Lith-2 A VL E GAL SHR VAAV L L N M K I V L
Rice-2 PAP VAL PR EMGAM GA ERWR TV AV L V L N F ERH I I L
(5)
Tob-1 PLGKELEDAVRTKFPNAKLGQGYGMTEAGPVLAMCLAFAKEPFDIKSGACGTVVRNAEMK 375
Pot-1 A
Tob-19 T A E
Lith-1 S A E S
Par-1 A YE
Arab-1 NA S G PV
Pine-12 L ER K IF N N PV S QI
Lith-2 R L LLNRV H IF S SPS H YPA S DL
Rice- L ARL Q IF S P TPA S L
(6)
+++++++
Tob-1 IVDPDTGCSLPRNQPGEICIRGDQIMKGYLNDPEATTRTIDKEGWLHTGDIGFIDEDDEL 435
Pot-1 A E D
Tob-19 K N S A Y Y D
Lith-1 I TE A S E Y D
Par-1 E NA R S RT E D
Arab-1 D S H N A AE D L D
Pine-12 L TE E H A PE S AA E VEY D E I
Lith-2 VI E S G E A V I YV D V
Rice-2 V F G L P AA V N YV D V
Tob-1 FIVDRLKELIKYKGFQVAPAEIEALLLNHPNISDAAVVPMKDEQAGEVPVAFVVRSNGS- 494
Pot-1 L I D I -
Tob-19 L -
Lith-1 P L VP V S G -
Par-1 L T T I K T F-
Arab-1 L IG D T V A E A K KD -
Pine-12 V I L VA S A Q H E K -- -
Lith-2 V F P L IS A Q AA P DGF
Rice-2 V F P L S IA S R Q DV AAD -
Tob-1 AITEDEVKDFISKQVIFYKRVKRVFFVETVPK----------SPSGKILRKDLRARLAAG 544
Pot-1 T ----------
Tob-19 T I DAI ---------- K
Lith-1 TT I Q V IN G DSI ---------- V K
Par-1 TT E I Q V V IF DAI ---------- I S
Arab-1 ELS D Q V V INK T SI ----------A K N
Pine-12 E S Q I E VA KIH Y DAI ---------- S K
Lith-2 EL EA E V LHK Y PLYS VAVRQDFEER Q QTGR RL LNSYCSI
Rice-2 D ESI E V LHK H IHAI ---------- A RE K C

```
  Tob-1   VPN*                                                          547
  Pot-1   IS *
 Tob-19   L  *
 Lith-1   FL GPTTNVVPNGGNVAKDNVPNGVSNGVSKANGGVAKEGVANGVPTDGD
  Par-1   DLPK*
 Arab-1   L*
Pine-12   *
 Lith-2    A*
 Rice-2   *


 Lith-A   YGVATKGVANGISNGVYKQVSNGVVSNGVANGIVSNGIANGVHN*
```

Figure 3.1: Comparison of the deduced amino acid sequences with that of 4CL from other plants. Amino acid sequences from Nt4CL-1 (Tob-1), Nt4CL-19 (Tob-19), and At4CL (*Arab*-1) are aligned to those from potato *4CL1* (Pot-1), *Lithospermum 4CL1* (*Lith*-1), parsley *4CL1* (Par-1), pine *4CL12* (Pine-12), *Lithospermum 4CL2* (*Lith*-2), and rice *4CL2* (Rice-2). Amino acids are only shown when they differ from the predicted amino acid sequence of Nt4CL-1. Dashes indicate gaps introduced to maximize alignment. Putative initiation methionines are in bold; conserved cysteine-residues in 4CL proteins are underlined and are numbered above the sequence; conserved motifs postulated to be involved in 4CL enzyme activity are marked (+++) above the sequence; translational stops are indicated with an asterisk; and numbers on the far right indicate the predicted amino acid position of Nt4CL-1.

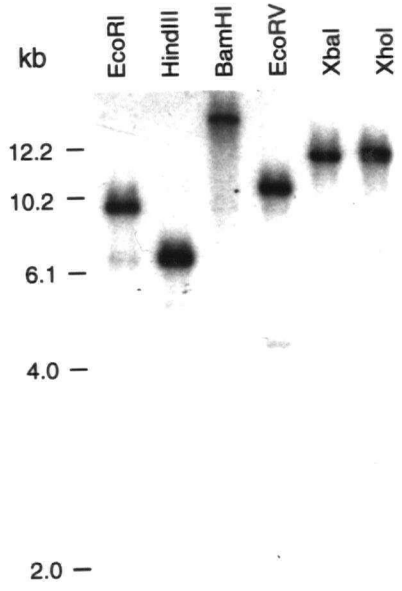


Figure 3.2: *Arabidopsis* genomic Southern blot analysis. *Arabidopsis* Columbia genomic DNA (10 µg) was digested, separated on an agarose gel, transfered to a nylon membrane, hybridized to the At4CL probe, and washed at high stringency (0.2 x SSC, 0.1% SDS, 65°C). The migration of size standards is shown to the left of the blot.

3.2 Expression of the *Arabidopsis 4CL*

The developmentally-regulated expression of the *Arabidopsis 4CL* gene was examined by northern blot analysis. *Arabidopsis* seeds were germinated and grown on MS plates under constant light at 23°C. Under these conditions, the seedlings

underwent normal growth and development and exhibited very little anthocyanin-pigmentation (indicative of light-induced stress) in the hypocotyls and cotyledons. Seedlings were harvested after 2, 3, 4, 5, 7, and 10 days of germination and, by 3-days post-germination, it was possible to separate the seedlings into root- and shoot-sectors. These tissues were used for RNA isolation. Northern blot analysis (Figure 3.3) showed that *Arabidopsis 4CL* mRNA accumulation was not detectable in 2-day old seedlings but was detectable subsequent to 3-days post-germination. Low, but detectable, levels of *4CL* mRNAs were found in the shoots whereas the roots contained higher levels of *4CL* transcripts compared to the shoots. *4CL* mRNA levels appeared to remain fairly constant in these tissues from day-3 to day-10 seedlings.

In mature *Arabidopsis* plants (Figure 3.4), northern blot analysis indicated that *4CL* was expressed at low levels in mature leaves and in flower buds (prior to anthesis). In contrast, *4CL* transcripts accumulated to high levels in bolting stems that were between 3 cm to 20 cm in height.

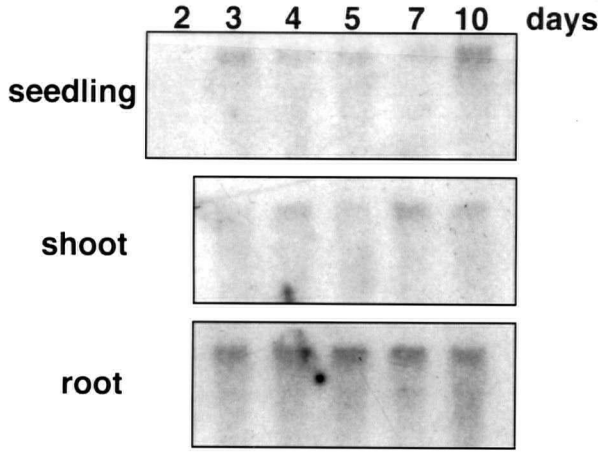


Figure 3.3: Northern blot analysis of *4CL* RNA levels in *Arabidopsis* seedlings. Seedlings were harvested 2-10 days post-germination and RNA was extracted from whole-seedlings, seedling-roots and seedling-shoots (hypocotyls and cotyledons). Total RNA (10 μg) was separated on a formaldehyde gel, transferred to a nylon membrane, and hybridized to an At4CL probe. The gel was stained with ethidium bromide before transfer to the nylon membrane to ensure equal loading of RNA, and the blot was washed at high stringency (0.2 x SSC, 0.1% SDS, 65°C).

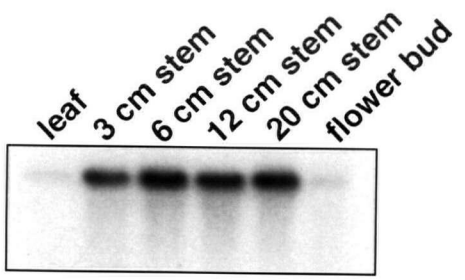


Figure 3.4: Northern blot analysis of *4CL* RNA levels in mature *Arabidopsis*. RNA was isolated from mature, fully-expanded leaves, bolting stems (3 to 20 cm in height), and flower buds. Total RNA (10 μg) was separated on a formaldehyde gel, transferred to a nylon membrane, and hybridized to the At4CL probe. The gel was stained with ethidium bromide before transfer to the nylon membrane to ensure equal loading of RNA, and the blot was washed at high stringency (0.2 x SSC, 0.1% SDS, 65°C).

3.3 Cloning and Characterization of the Tobacco-*4CL* cDNAs

Tobacco *4CL* cDNAs were cloned by screening a tobacco shoot-tip cDNA library using the potato *4CL* cDNA (Becker-André *et al.*, 1991) as a hybridization probe. Eleven clones encoding 4CL were isolated and preliminary sequence-analysis and restriction digests showed that the cDNAs could be placed into four groups, represented by Nt4CL-1, Nt4CL-5, Nt4CL-17, and Nt4CL-19 (Table 3.2). The cDNAs within each group had identical 3'-sequences suggesting that although they were of different size, they were likely products of the same gene. Detailed restriction-maps of clones 1, 5, 17, and 19 (Figure 3.5), showed that the four cDNAs fell into 2 classes. One class (Nt4CL-1 and Nt4CL-17) had distinctive *Pst*I and *Kpn*I sites while the other class (Nt4CL-19 and Nt4CL-5) had *Ava*II, *Bam*HI, and *Sac*I sites. Sequencing of the 5' region of these clones showed that restriction-site polymorphisms between Nt4CL-17 and Nt4CL-1 and between Nt4CL-19 and Nt4CL-5 were due to single base-pair changes. The 3' sequences of the four cDNAs were compared (Figure 3.6) and the results demonstrate that the four cDNAs again fell into two classes with Nt4CL-1 and Nt4CL-17 in one class (94% sequence identity) and Nt4CL-19 and Nt4CL-5 in a second class (97% sequence identity). In contrast, the 3' sequences of Nt4CL-1 and Nt4CL-19 were only 67% identical; comparison of any other pair of clones between the two classes gave similar results (<70% sequence identity, not shown). It should be noted that although Nt4CL-19 and Nt4CL-5 were 97% identical at the 3' end, there is a 36 base- pair region within Nt4CL-5 which is absent in Nt4CL-19. One clone from

each of the two *4CL*-cDNA classes was selected for further analysis. The two longest clones, Nt4CL-1 and Nt4CL-19, were subcloned and completely sequenced.

The nucleotide and predicted amino acid sequences of Nt4CL-1 and Nt4CL-19 were compared to each other and to other *4CL* sequences available in the EMBL database (Table 3.1). In general, the predicted amino acid sequences from Nt4CL-1 and Nt4CL-19 had the highest identity (≥ 80%) to the predicted 4CL amino acid sequences from potato and *Lithospermum erythrorhizon*. The tobacco 4CL amino acid sequences were also homologous, but with decreasing percent identity (≤73%), to the predicted amino acid sequences from the parsley, soybean, *Arabidopsis*, pine and rice *4CL* sequences. An exception is the *4CL-2* clone from *Lithospermum* which shared significantly lower amino acid identity (62%) to the tobacco 4CL polypeptides as compared to the *Lithospermum 4CL-1* clone (~80%). Interestingly, Nt4CL-1 shared a slightly higher amino acid homology to the potato 4CL sequence (85%) than to the prediced amino acid sequence from Nt4CL-19 (81%). A putative initiation ATG-codon was identified in the 5' end of Nt4CL-1 and Nt4CL-19. Upstream of this ATG-codon was approximately 70 nucleotides which included translational-stop codons that were in-frame with the predicted polypeptides (not shown); therefore, these cDNAs likely encode the entire tobacco-4CL proteins.

The Nt4CL-1 cDNA had an open reading frame predicted to encode a protein of 547 amino acids with a molecular mass of 59.8 kDa and an isoelectric point of 5.40. The predicted polypeptide from Nt4CL-19 was 542 amino acids in length with a molecular mass of 59.4 kDa and an isoelectric point of 5.69. Both predicted peptides (Figure 3.1) contained the conserved "GEICIRG" amino acid motif believed essential for 4CL activity (Becker-André *et al.* 1991), an AMP-binding signature ([LIVMFY]-X_2-[STG]-G_2-[ST]-[STE]-[SG]-X_2-[PALIVM]-K; Schröder, 1989), and five other characteristic cysteine-residues that have been reported in other predicted 4CL amino acid sequences (Figure 3.1, Uhlmann and Ebel, 1993).

The high sequence homology, the conserved amino acid motifs, and the good sequence-aligment suggest that Nt4CL-1 and Nt4CL-19 encode tobacco 4CL proteins. Clones Nt4CL-1 and Nt4CL-19 were both used for further analysis since they represent members of the two divergent classes of tobacco *4CL* cDNAs cloned. It is interesting to note that tobacco is an allotetraploid which arose from the hybridization of two *Nicotiana* species related to present-day *N. sylvestris* and *N. tomentosiformis*. Whether the two divergent classes of *4CL* cDNAs originated from the two progenitor-species and how the *4CL* genes are inherited is addressed in the following section.

Clone Grouping	Homologous Clones	Size (kb)
Nt4CL-1	1	2
	6	2
	11	1.7
	14	1.7
	16	0.9
Nt4CL-5	5	1.5
	13	0.7
	18	1.4
Nt4CL-17	17	1.6
	4	1.3
Nt4CL-19	19	2

Table 3.2: Summary of tobacco- *4CL* cDNA clones. The eleven isolated 4CL cDNA clones can be placed into four groups.

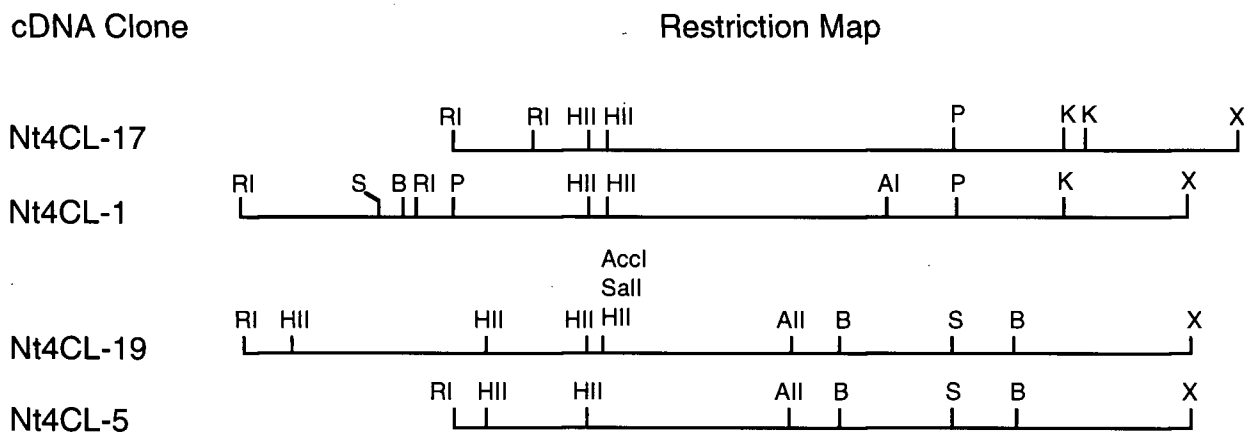


Figure 3.5: Restriction maps of representative tobacco-*4CL* cDNA clones. Results demonstrate that the 4 cDNAs fall into two classes. Restriction enzymes are: AI, *Aval*; AII, *AvaII*; B, *Bam*HI; HII, *Hinc*II; K, *Kpn*I; P, *Pst*I; RI, *Eco*RI; S, *Sac*I; and X, *Xho*I.

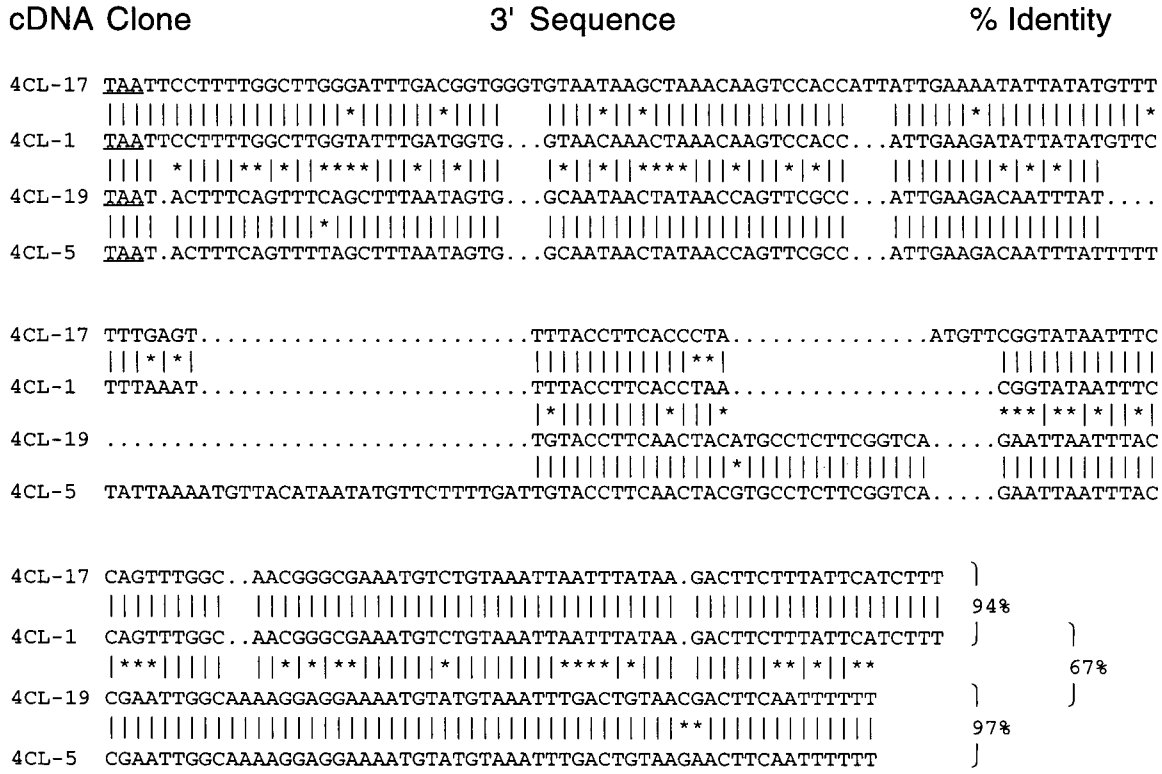


Figure 3.6: 3' sequences of representative tobacco-*4CL* cDNA clones. Translation stop codons are underlined. Vertical lines indicate identical nucleotides, asterisks indicate different nucleotides and dots indicate gaps introduced to maximize alignment. All sequences terminate with a poly(A) tail (not shown) with the exception of Nt4CL-17 which extends for an additional 136-bp and Nt4CL-19 which extends for an additional 6 nucleotides prior to polyadenylation. The percent sequence identity between pairs of clones is shown at the bottom right.

3.4 Inheritance of the Tobacco-*4CL* Genes

Southern blot analysis was used to assess the size of the *4CL* gene-family in tobacco. Cytogenetic (Smith, 1968), biochemical (Gray *et al.*, 1974; Obokata *et al.*, 1990; Takahashi *et al.*, 1991), and more recently, molecular studies (van Buuren *et al.*, 1992; Pellegrini *et al.*, 1993; Hua *et al.*, 1993; Kroneberger *et al.*, 1993) strongly suggest that *N. tabacum* (tobacco) arose from the hybridization of gametes from two species of *Nicotiana* follwed by chromosome duplication. The gametes are thought to be from ancestors of *N. sylvestris* and *N. tomentosiformis*. Thus, we also used Southern blot analysis to determine if the *4CL* genes corresponding to the cloned cDNAs, Nt4CL-1 and Nt4CL-19, were present in the progenitor species and how they

were inherited into tobacco. In a preliminary experiment, restriction-digested Nt4CL-1 and Nt4CL-19 cDNAs were blotted and hybridized separately to probes generated from the insert of each plasmid. Figure 3.7 shows that at moderate stringency (0.5 X SSC, 0.1% SDS, 65°C), little cross-hybridization was observed, demonstrating that the cDNAs could be used as gene-specific probes for distinguishing the presence of Nt4CL-1-like and Nt4CL-19-like sequences in tobacco.

Genomic Southern blots prepared with DNA from *N. sylvestris*, *N. tomentosiformis*, and *N. tabacum* were hybridized sequentially to Nt4CL-1 and Nt4CL-19 probes. Figure 3.8 shows that both probes hybridized to restriction fragments from the progenitors and from tobacco, and that each probe hybridized to a unique set of restriction fragments in the three species. This demonstrates that at least two divergent *4CL* genes, represented by the Nt4CL-1 and Nt4CL-19 cDNA clones and which we designate *4CL1* and *4CL2*, are both present in the progenitors as well as in tobacco.

The small number of fragments which hybridized to each probe in *N. sylvestris* and *N. tomentosiformis* suggests that there are 1-2 tobacco *4CL1*-like and *4CL2*-like genes in these progenitor species (Figure 3.8). The DNA hybridization-patterns in *N. tabacum* were more complex, as would be predicted from the acquistion of a set of *4CL1*-like and *4CL2*-like genes from each progenitor. Indeed, Nt4CL-1-hybridizing (*4CL1*) restriction fragments in *N. tabacum* can be directly traced from the restriction fragments of the parental species. For example, a single 9-kb Eco*RV* *4CL1* fragment was observed in the *N. sylvestris* genome, three Eco*RV* fragments (1.3 kb, 3 kb, and 4 kb in size) were observed in *N. tomentosiformis*, and all four Eco*RV* fragments were observed in the *N. tabacum* genome (Figure 3.8, probe Nt4CL-1). Nt4CL-19-hybridizing fragments (*4CL2*) in *N. tabacum* also appeared to be derived from a combination of the *N. sylvestris* and *N. tomentosiformis* *4CL2* gene-complements, but about half of the *N. tabacum* *4CL2* restriction fragments were polymorphic with respect to fragments in the progenitor species (Figure 3.8, arrows).

Restriction-fragments hybridizing to both *4CL1* and *4CL2* were detected when the genomic Southern blot was hybridized to Nt4CL-19 and washed at low stringency (Figure 3.8, Nt4CL-19 low stringency). In addition, however, additional hybridizing restriction fragments were observed (Figure 3.8, asterisks) which were not apparent after high-stringency washes with either probe. These bands may represent additional *4CL*-genes divergent from *4CL1* and *4CL2*.

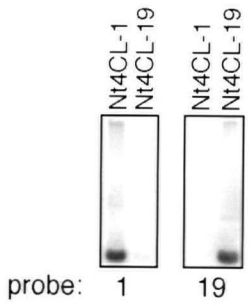


Figure 3.7: Plasmid Southern blot analysis. Southern blot analysis of tobacco- *4CL* cDNAs hybridized to either Nt4CL-1 or Nt4CL-19 probes (1 or 19). Blots were washed at moderate stringency (0.5 x SSC, 0.1% SDS, 65°C).

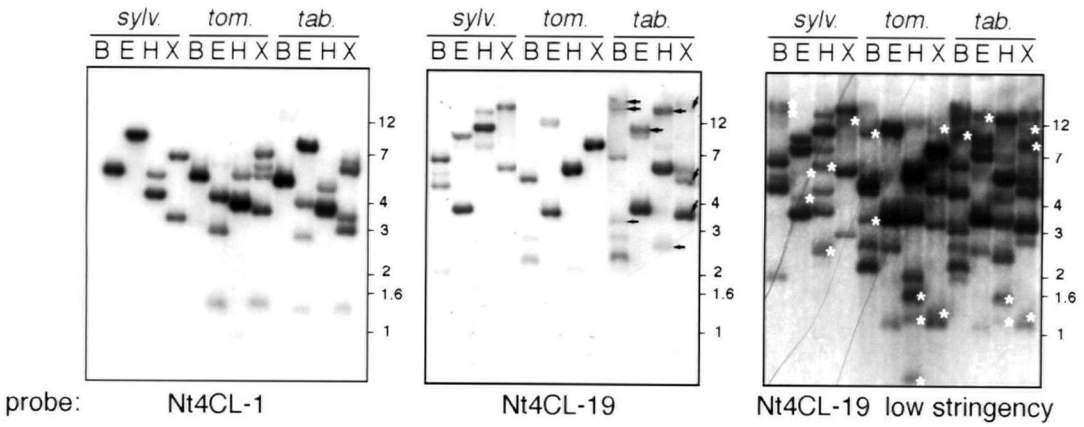


Figure 3.8: Tobacco genomic Southern blot analysis. Southern blots of genomic DNA (10 µg) from *N. sylvestris* (*sylv.*), *N. tomentosiformis* (*tom.*), and *N. tabacum* (*tab.*) digested with different restriction-enzymes. A single blot was hybridized sequentially with Nt4CL-1 and Nt4CL-19 probes and washed at high stringency (0.2 x SSC, 0.1% SDS, 65°C). Arrows indicate hybridization restriction-fragments that are unique to *N. tabacum* and not found in the progenitors. Asterisks indicate unique hybridization restriction-fragments that are observed when the blot was probed with Nt4CL-19 cDNA and washed at low stringency (2 x SSC, 0.1% SDS, 65°C) but not observed at high stringency washes. Restriction enzymes are: B, *Bgl*II; E, *Eco*RV; H, *Hind*III; and X, *Xba*I.

3.5 Expression of the Tobacco-*4CL* Genes

Characterization of the tobacco *4CL* cDNAs demonstrated that there were at least two classes of divergent *4CL* genes. To determine if these divergent forms of *4CL* were differentially expressed, northern analysis was performed using RNA isolated from wounded tobacco-leaves, methyl jasmonate treated tobacco-leaves, and various organs from tobacco plants. Blots were hybridized to Nt4CL-1 or Nt4CL-19 probes under conditions which allowed little cross-hybridization between probes (Figure 3.7). Subsequent stripping and re-hybridization of the blots to a pea rRNA gene probe confirmed equal RNA-loading between samples and between gels. Figure 3.9 demonstrates that the expression of both classes of *4CL* genes was inducible by wounding and methyl jasmonate treatment. In this and similar experiments, however, *4CL2* was less responsive to methyl jasmonate treatment than *4CL1*, but was approximately equivalent in its response to wounding. The steady-state levels of both *4CL1* and *4CL2* mRNA transcripts were highest in old stems followed by young stems and ovaries. RNA levels were lowest in shoot tips, the pigmented limb of the petals, and untreated, mature leaves (Figure 3.9). No major differences in the expression of *4CL1* and *4CL2* were observed, although *4CL2* RNA was reproducibly less abundant in young stems and ovaries relative to *4CL1*.

The low expression of *4CL* in the pigmented limb of tobacco petals was unexpected since transgenic studies using the parsley *4CL-1* promoter, bean *PAL* and *CHS* promoters, and a tobacco *CHS* promoter fused to the *GUS* reporter-gene showed high GUS-activity in the floral limbs (Bevan *et al.*, 1989; Schmid *et al.*, 1990; Hauffe *et al.*, 1991, Drews *et al.*, 1992). This led us to examine *4CL* expression in petals more closely. Tobacco flowers from developmental-stages 1 through 6 (at stage 1, the corolla is just emerging from the sepals; pigmentation of limb-tissue is

detectable at stage 4, and at stage 6 anthers are dehiscing; Reinold *et al.*, 1993) were harvested, and RNA was isolated from the sepals, and the base, tube, and limb-sectors of the corolla (see section 2.1). Figure 3.10 shows a northern blot of this RNA hybridized to an Nt4CL-19 probe. *4CL2* mRNA accumulation was highest in the unpigmented portions of the petals (tube and base), where its accumulation was temporally regulated. High transcript-levels were first evident in stage 4 flowers, and *4CL2* mRNA levels had decreased significantly in stage 6 flowers. An identical pattern of expression was observed when blots were hybridized to a Nt4CL-1 probe (results not shown). To determine whether *PAL* gene expression was coordinately regulated with that of *4CL* in petals, a northern blot was hybridized to a potato *PAL* probe (Figure 3.10, StPAL). Although the heterologous probe did not hybridize strongly, an identical pattern of *PAL* expression was observed, with high expression in tube- and base-segments of stage 4 and stage 5 flowers.

To complement the results from the northern blots, western blot analysis was performed using an antiserum raised against the parsley 4CL. Similar to the RNA levels, 4CL protein was most abundant in the tube- and base- segments of tobacco petals (Figure 3.10, anti-4CL). 4CL protein levels were highest in stage 5 and stage 6 petals, consistent with the accumulation of 4CL protein subsequent to *4CL* RNA accumulation. Western blot analysis also revealed that 4CL proteins were also present in the limbs and in the sepals, although RNA was only weakly detectable in these organs. These results show that, during flower development, phenylpropanoid gene expression and enzyme accumulation are very active in the unpigmented portions of tobacco-petals and appears to be much higher than in pigmented floral-limbs.

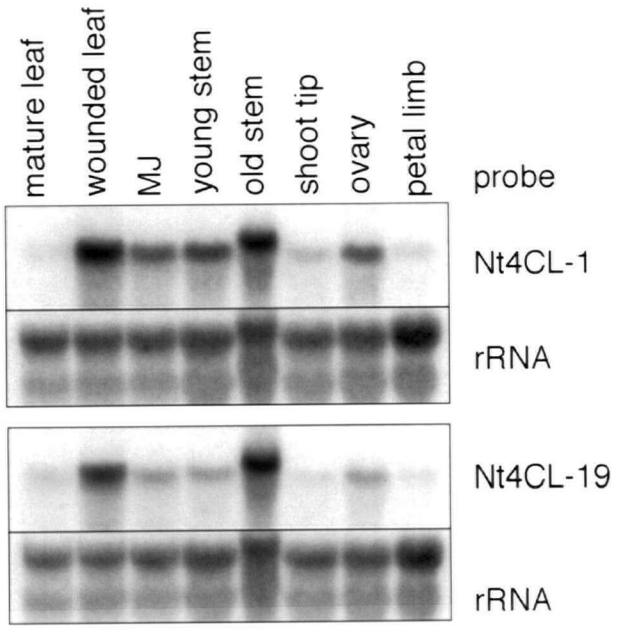


Figure 3.9: Northern blot analysis of *4CL* RNA levels in tobacco. RNA was isolated from fully-expanded leaves (mature leaves), or fully-expanded leaves which had been wounded (wounded leaf) or treated with methyl jasmonate (MJ) and other tissues as indicated. Duplicate blots of total RNA (10 µg) from various organs were separated on formaldehyde gels, blotted to nylon membranes, hybridized to Nt4CL-1 or Nt4CL-19 probes and washed at moderate stringency (0.5 x SSC, 0.1% SDS, 65°C). The blots were subsequently stripped, and hybridized to rRNA probes to demonstrate evenness of loading.

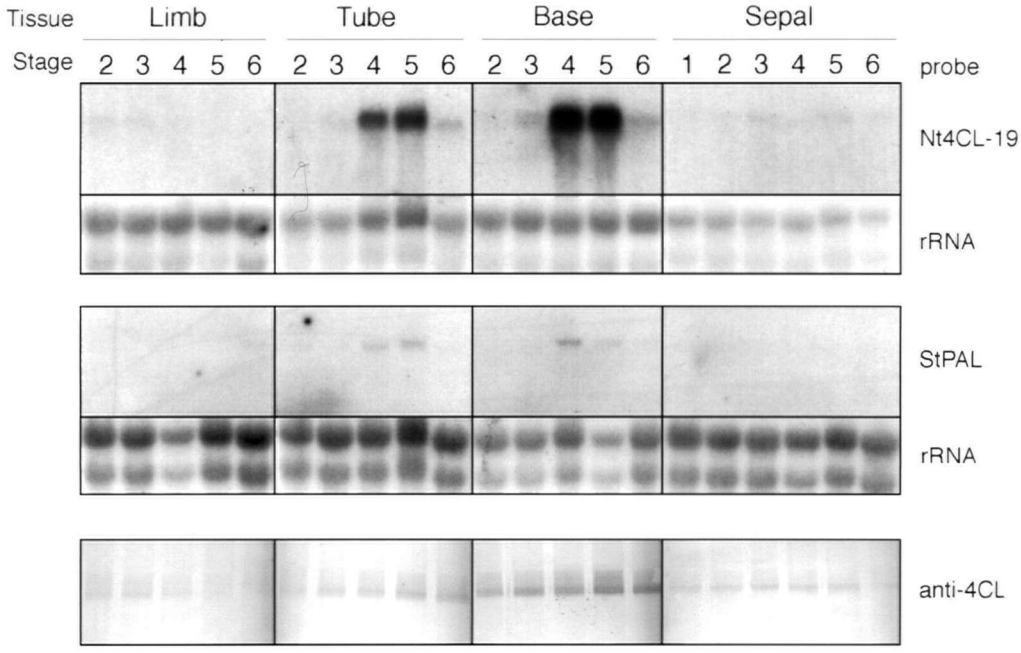


Figure 3.10: Steady-state levels of *4CL* RNA, *PAL* RNA and 4CL proteins in tissues from developing petals and sepals of tobacco. Tobacco flowers in developmental stages 1 through 6 were harvested and dissected into four sectors: the limb, the tube, the base and the sepals (see section 2.1). Duplicate northern blots prepared using 10 µg total RNA from these samples were hybridized to Nt4CL-19 and potato PAL (StPAL) probes and washed at moderate (0.5 x SSC, 0.1% SDS, 65°C) and low (2 x SSC, 0.1% SDS, 65°C) strigency respectively. The blots were subsequently stripped and re-hybridized to rRNA probes to demonstrate evenness of loading. The bottom panel shows western blot analysis of protein extracts from the petal and sepal samples described above. The blot was reacted with a polyclonal antibody raised against parsley 4CL.

3.6 Discussion

High nucleotide and predicted amino acid homology, and good alignment of the putative initiation-methionine to known 4CL sequences, suggests that At4CL, Nt4CL-1, and Nt4CL-19 are near full-length cDNAs encoding 4CL from *Arabidopsis* and tobacco. In *Arabidopsis*, 4CL appears to be encoded by a single gene, a result also suggested by Trezzini *et al.* (1993), who identified a putative, although poorly characterized, *Arabidopsis 4CL* genomic clone. 4CL is encoded by a single functional gene in pine (Voo *et al.*, 1995) and by very similar, duplicated genes in parsley (Lozoya *et al.*, 1988) and potato (Becker-André *et al.*, 1991). Since *Arabidopsis* 4CL is encoded by a single gene, it is likely that the promoter of the *4CL* gene is responsive to all developmental signals and environmental signals which dictate the need for 4CL activity. In contrast, the tobacco 4CL proteins are encoded by a divergent gene-family analogous to that found in soybean (Uhlmann and Ebel, 1993), *Lithospermum* (Yazaki *et al.*, 1995), rice (Zhao *et al.*,1990; EMBL accession number L43362), and poplar (Allina and Douglas, 1994). Nt4CL-1 and Nt4CL-19 were only ~80% identical at the amino acid level and the nucleotide level; however, despite their divergence, the corresponding genes were apparently not differentially expressed. To date, differential expression of *4CL* genes has only been demonstrated in soybean and parsley. In soybean, fungal infection and elicitation of cell cultures caused the increase in *4CL-16* while the RNA levels of *4CL-14* remained the same. The soybean *4CL* cDNAs are partial-length and the truncated polypeptides are predicted to be 65% identical to one another (Uhlmann and Ebel, 1993). In parsley, *4CL-1* is wound-inducible in the roots whereas *4CL-2* is light-inducible in cell cultures. *4CL-2* is also preferentially expressed in the flowering stem (Lois and Hahlbrock, 1992). The predicted amino acid sequences from the parsley *4CL* cDNAs are 99.5% identical (Lozoya *et al.*, 1988).

Genomic Southern blot analysis (Figure 3.8) demonstrates the presence of tobacco *4CL* gene-family members in *Nicotiana sylvestris* and *N. tomentosiformis*, the

Nicotiana species most closely related to the presumed progenitors of tobacco, strongly suggesting that *4CL* gene-duplication and gene-divergence had occurred in *Nicotiana* prior to the emergence of these species. The sequences of near full-length cDNA clones specific to two of these genes, which we call *4CL1* and *4CL2*, show that they are relatively divergent, sharing about 80% nucleotide and amino acid sequence identity (Table 3.1). Consistent with the allotetraploid nature of the *N. tabacum* genome, genomic Southern blots (Figure 3.8) show that the tobacco *4CL* gene-family contains copies of *4CL1* and *4CL2* from both parental species, for a total of at least 4 genes. This is further supported by the existence of two classes of polymorphic cDNA clones, each class containing two cDNA types (Figures 3.5 and 3.6). Formally, it is possible that Nt4CL-1 / Nt4CL-17, and Nt4CL-19 / Nt4CL-5, are allelic to one another and represent the *4CL* genes inherited from one or the other progenitor species. However, since we isolated eleven *4CL* cDNA clones and they all fell into 4 groups (Table 3.2), it is more likely that Nt4CL-1 & 17 and Nt4CL-19 & 5 are not allelic but are the *4CL1*- and *4CL2*- genes originating from the two progenitor species. In this case, the polymorphism observed between clones 1 &17, and clones 19 & 5, are due to the different orgin of these genes from the two progenitor species. Similar evolutionary schemes have been proposed for the origins of tobacco gene-families encoding nitrite reductase (Kroneberger *et al.*, 1993), psbP (Hua *et al.*, 1993) and endochitinases (van Buuren *et al.*, 1992).

When the tobacco genomic Southern blot was washed at low stringency, the Nt4CL-19 probe hybridized to numerous restriction-fragments in tobacco and its progenitors which were distinct from the hybridization fragments observed at high-stringency washes (Figure 3.8, low stringency, asterisk). These DNA sequences may represent a third class of *4CL* genes, distinct from *4CL1* and *4CL2*. Three tobacco *4CL* cDNA sequences have recently been deposited in the EMBL data bank. The sequence of one partial-length clone, TOBTCL2 (EMBL accession number D50033), is 99% identical to Nt4CL-1 at the nucleotide level. A second clone, full-length in size,

TOB4CCAL (EMBL accession number D43773), has high homology to the 5' (97% identical) and 3' (100% identical) end of Nt4CL-5 and includes the 36-bp stretch specific to Nt4CL-5 (Figure 3.6). TOBTCL2 and TOB4CCAL are likely the same clones as Nt4CL-1 and Nt4CL-5 with single base-pair differences due to sequencing descrepencies or natural polymorphism between individual plants. In contrast, a third partial-length tobacco *4CL* clone, TOBTCL6 (accession number D50034) is only 75% and 74% identical to Nt4CL-1 and Nt4CL-19 respectively. Thus, TOBTCL6 may represent a member of the third, highly-divergent class of tobacco, *4CL3*, which I had detected under low-stringency hybridization.

The tobacco *4CL* nucleotide and amino acid sequences (Table 3.1) deduced from Nt4CL-1 and Nt4CL-19 were most similar to the potato *4CL*, followed by the *4CL-1* sequence from *Lithospermum*. Tobacco (order Polemoniales), potato (order Polemoniales) and *Lithospermum* (order Lamiales) are all in the Asteridae class of plants and thus, it is not surprizing that the *4CL* sequences reflect this relatedness (Figure 3.11). In addition, tobacco and potato are in the same family of plants (Solanaceae) and, as expected, have *4CL* sequences of even greater homology to each other. The tobacco *4CL* sequences are increasingly divergent (Table 3.2) from the *4CL* sequences in plants of the orders Umbellales (parsley), Fabales (soybean), Papaverales (*Arabidopsis*), Pinales (pine), and Poales (rice). Although there are but a few *4CL* sequences available, this pattern of sequence-divergence is consistent with proposed taxonomic relationships between these groups (Heywood, 1978; Figure 3.11). An exception is the *4CL-2* clone from *Lithospermum* which does not comply to the expected taxonomic ranking (Table 3.2). Yazaki *et al.* (1995) suggests that *4CL-2* from *Lithospermum* may be a highly-divergent form of 4CL which has an organellar localization. Whether a similar situation is found with members of the putative *4CL3* gene-family from tobacco (detected by genomic Southern analysis under low-stringency washes) is unknown and further analysis is required before the evolutionary relationship between these divergent *4CL* genes is understood.

Interestingly, the Nt4CL-1 cDNA (*4CL1*) and amino acid sequences are more similar to potato *4CL* than to Nt4CL-19 (*4CL2*). This suggests that *4CL1* may be homologous to an ancestral *4CL*-gene from which all Solanaceous *4CL*-genes (including potato *4CL*) evolved, while *4CL2* may represent a *4CL* gene which had duplicated in the *Nicotiana* evolutionary line and has undergone greater divergence from the ancestral *4CL*-gene. *4CL1* restriction-fragments are conserved in *N. tabacum* with respect to its proposed progenitors *N. sylvestris* and *N. tomentosiformis* (Figure 3.8, Nt4CL-1 probe) whereas many of the *4CL2*-specific restriction-fragments are polymorphic (Figure 3.8, Nt4CL-19 probe, arrows). This is consistent with a more rapid divergence of the *4CL2*-locus than the *4CL1*-locus subsequent to the hybridization event giving rise to tobacco. The interspecies hybridization event is thought to have occurred within the last 6 million years (Okamuro and Goldberg, 1985).

Northern blot analysis was used to examine the expression pattern of *4CL* in *Arabidopsis* and tobacco. In young *Arabidopsis* seedlings, *4CL* was developmentally regulated and accumulation of the mRNA was not visible until the third day post-germination (Figure 3.3). The timing of this expression-pattern, as demonstrated by basic fuchsin staining (Lee *et al.*, 1995a) and confocal laser scanning microscopy (Dharmawardhana *et al.*, 1992), correlates well with the onset of lignin deposition in the differentiated tracheids of the cotyledons and roots. In other studies, high expression of *Arabidopsis PAL*, *CHS* and other anthocyanin biosynthetic genes was reported in 3- to 4-day-old seedlings, and was correlated with the accumulation of anthocyanin pigments (Kubasek *et al.*, 1992; Ohl *et al.*, 1990]. Here however, under the light conditions in which the seedlings were grown, no anthocyanin pigmentation was observed, suggesting that *4CL* gene expression is not associated with anthocyanin biosynthesis, rather, it is associated with the biosynthesis of lignin during vascular differentiation. The higher levels of *4CL* transcripts in seedling-roots relative to shoots may be important for the biosynthesis of additional phenylpropanoid-derived

to shoots may be important for the biosynthesis of additional phenylpropanoid-derived compounds in roots such as suberin. *4CL* and *PAL* promoter-*GUS* fusion studies also report high GUS-activity in non-vascular root tissues as compared to shoot tissue (Lee *et al.*, 1995a; Ohl *et al.*, 1990). In mature *Arabidopsis* plants, *4CL* expression was highest in bolting stems (Figure 3.4). A comprehensive characterization of lignin deposition in *Arabidopsis* demonstrates that the stem tissues are highly lignified (Dharmawardhana *et al.*, 1992). Therefore, *Arabidopsis 4CL* expression appears to be associated with lignin deposition and may be useful as a marker for xylem-differentiation during vascular system development. Analogous to *Arabidopsis*, tobacco *4CL1* and *4CL2* expression is highest in old stems where it likely functions for the biosynthesis of monolignol-precursors (Figure 3.9).

Tobacco *4CL1* and *4CL2* are both wound inducible and methyl jasmonate inducible (Figure 3.9). Methyl jasmonates have been implicated as the intracellular signal in response to wounding (Farmer and Ryan, 1992; Ellard-Ivey and Douglas, 1996) and, in agreement with the results presented here, studies utilizing heterologous probes in tobacco have shown that *4CL* genes are activated by wounding and methyl jasmonate treatment (Douglas *et al.*, 1991; Ellard-Ivey and Douglas, 1996). The high levels of induced *4CL* transcripts likely have a role in the biosynthsis of defense-related compounds such as phenylpropanoid-derived phytoalexins and cell wall components which may serve as physical barriers against pathogen invasion (reviewed in Hahlbrock and Scheel, 1989; Nicholson and Hammerschmidt, 1992). While both *4CL1* and *4CL2* appeared to be inducible, the levels of *4CL2* (Nt4CL-19) mRNA appeared to be lower than that of *4CL1* (Nt4CL-1) in methyl jasmonate treated leaves as well as in the ovaries and young stems (Figure 3.9). Although these differences were reproducible, the significance of the 2 - 3 fold

lower levels of *4CL2* transcripts is unclear. Other than these quantitative differences, *4CL1* and *4CL2* does not appear to be differentially regulated despite the high divergence between members of the *4CL1* and *4CL2* gene-family. These results suggest that *4CL1* and *4CL2* gene-families may be functionally redundant. However, since the ovary is a complex organ and *4CL* is expressed in the ovules, the carpel walls, and in the nectaries (Hauffe *et al.*, 1991; Reinold *et al.*, 1993), we cannot exclude the possibility that the two genes are differentially regulated at the tissue-level in this organ. Further experiments using gene-specific probes and *in situ* hybridization will be necessary to resolve this issue.

The high levels of *4CL1* and *4CL2* expression in the unpigmented corolla tube and base-portions of tobacco flowers, and the very weak expression in the pigmented limb (Figure 3.10) are in striking contrast to the heterologous *PAL-GUS*, *CHS-GUS*, and *4CL-GUS* fusions which direct cell-specific expression in the limb-portions of transgenic-tobacco flowers (Bevan *et al.*, 1989; Liang *et al.*, 1989; Schmid *et al.*, 1990, Hauffe *et al.*, 1991). The spacial accumulation of tobacco *4CL* transcripts in the flowers does not correlate with the accumulation of tobacco *CHS* transcripts and does not correlate with the GUS activity (directed by tobacco *CHS-GUS* fusions) found in the epidermal cells of the limb where anthocyanin pigments accumulate (Drews *et al.*, 1992). However, the temporally-regulated expression of *4CL* in the petal tube and base is consistent with previous results which showed, by *in situ* hybridization, that tobacco *4CL* mRNA accumulates to high levels in epidermal and mesophyll cell-layers of stage 4 and stage 5 tobacco flowers (Reinold *et al.*, 1993). Furthermore, the results in Figure 3.10 show that tobacco *PAL* mRNA, detected using a heterologous potato *PAL* probe, accumulates in a similar manner, and that 4CL protein accumulates to higher levels in the tube and base of the flower than in the limb of the flower. The presence of 4CL proteins in the limbs and sepals may represent the basal-level of pre-existing 4CL that is present before the developmentally-induced *4CL1* and *4CL2* transcripts observed during stage 4 and stage 5 of tobacco flower development. It is not clear whether theses pre-existing 4CL proteins are products of the *4CL1* and *4CL2* genes or products of other divergent *4CL* genes. The biosynthesis of anthocyanins in the pigmented portion of tobacco flowers may be due to these pre-existing proteins. Taken together, these results clearly indicate that phenylpropanoid metabolism is strongly activated in the unpigmented petal during flower development.

A possible function of 4CL in the unpigmented portions of tobacco petals is to synthesize colorless flavonols like kaempferol and quercetin which accumulate in tobacco-flower petals (Holton *et al.*, 1993). Recent observations indicate that colorless flavonoids are abundant in the base- and tube- regions of tobacco flowers (Reinold, 1995) and, in petunia, *CHS* expression in unpigmented petal cells may be correlated with the accumulation of uncolored flavonoids (Koes *et al.*, 1990). The white petals of *Arabidopsis* also accumulate kaempferol and quercetin (Shirley *et al.*, 1995) yet, *4CL* mRNA levels were low in the flower buds (Figure 3.4). The reason for this discrepancy may be because the flower buds used for northern analysis corresponded to an early developmental stage (see section 2.1) in which flavonoid biosynthesis may not have yet begun. In agreement with this, *PAL* promoter-*GUS* fusion studies reported GUS staining in mature *Arabidopsis* flowers but no GUS activity in early flower development (Ohl *et al.*, 1990). Another possiblity is that *4CL* transcripts may be diluted out since the *Arabidopsis* flowers used for northern analysis were still enclosed by sepals. A more detailed study of *Arabidopsis* flower development and *4CL* expression is required before this can be clarifled. Another role for PAL and 4CL in the unpigmented corolla tube of tobacco flowers may be to synthesize yet uncharacterized phenylpropanoid-products such as colorless flavonoids (see section 1.2.2). Currently, there is no evidence of this, nonetheless, it is a formal possiblity that should be considered and investigated.

In summary, the expression pattern of At4CL and Nt4CL, as demonstrated by northern analysis, is consistent with the expression of other phenylpropanoid genes and with the expression as described by promoter-*GUS* studies. A striking difference between the endogenous tobacco-*4CL* expression-pattern as compared with *GUS* reporter-gene analysis is the high levels of *4CL* transcripts in the unpigmented portion of the petal. We have shown *4CL* to be encoded by a single gene in *Arabidopsis* and by divergent gene-families in tobacco. We predict that the *Arabidopsis 4CL* gene would be responsive to all developmental and environmental signals which direct *4CL* expression. Potentially, the divergent *4CL* genes from tobacco may respond uniquely to developmental and environmental signals; however, we have not shown the tobacco *4CL* genes to be differentially regulated at the level of RNA accumulation. The divergence of the tobacco *4CL* genes may confer different enzymatic properties to the encoded 4CL-isoforms. The possiblity of divergent 4CL-isoforms regulating

carbon flow into phenylpropanoid metabolism at the level of substrate specificity is examined in the following chapter using recombinant tobacco-4CL proteins.

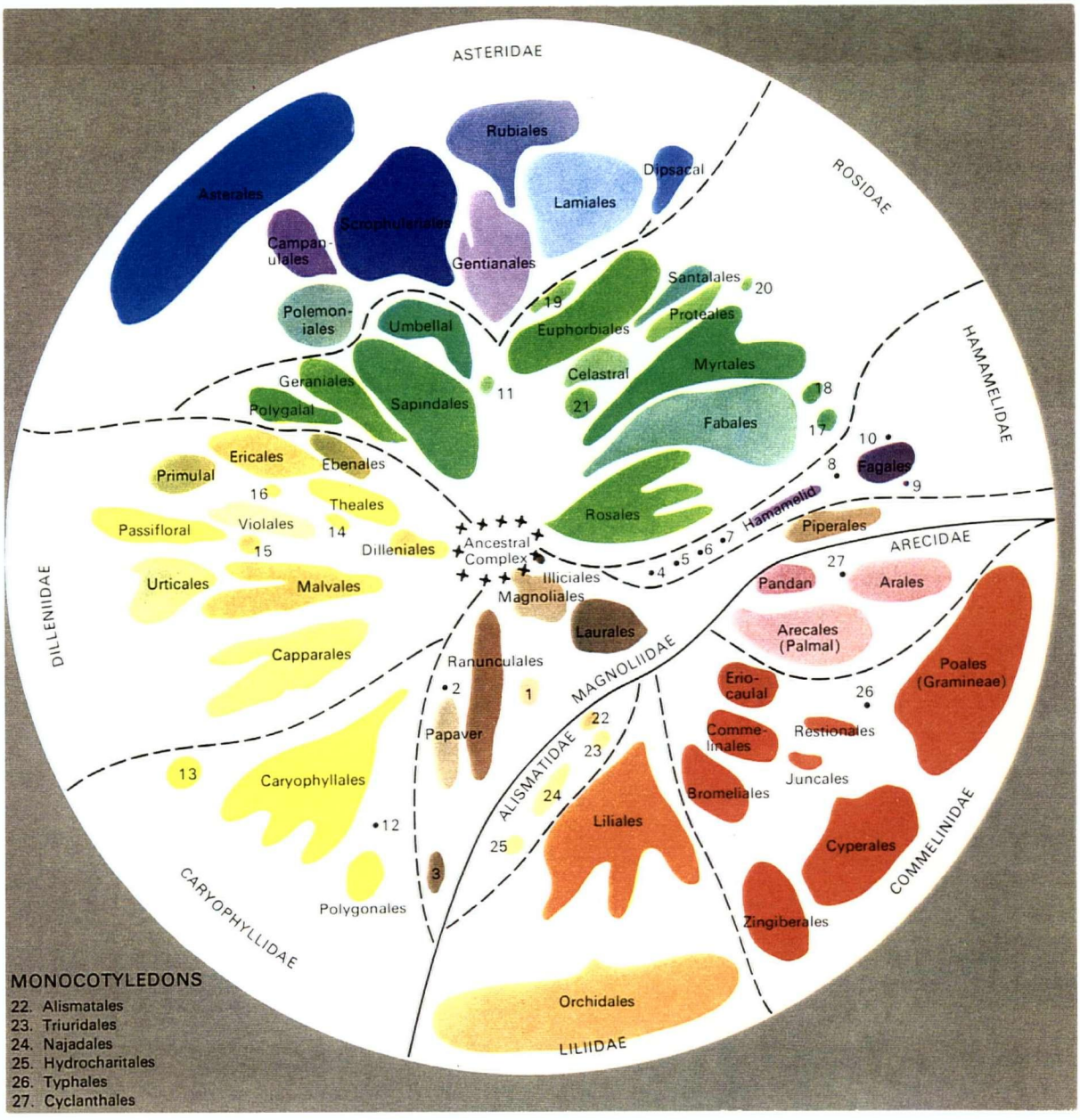


Figure 3.11: Diagram showing the relatedness of the orders in the angiosperms. Of particular interest are the orders Polemoniales (tobacco and potato), Lamiales (*Lithospermum*), Umbellales (parsley), Fabales (soybean), Papaverales (*Arabidopsis*), and Poales (rice). Taxa are: 1, Nymphaeales; 2, Sarraceniales; 3, Aristolochiales; 4, Trochodendrales; 5, Cercidiphyllales; 6, Didymeleales; 7, Eupteleales; 8, Eucommiales; 9, Casuarinales; 10, Leitneriales; 11, Juglandales; 12, Batales; 13, Plumbaginales; 14, Lecythidales; 15, Salicales; 16, Diapensiales; 17, Podostemales; 18, Haloragales; 19, Cornales; 20, Rafflesiales; and 21, Rhanmales. From Heywood, 1978.

Chapter 4

Recombinant 4CL

4.1 Expression of Tobacco-*4CL* cDNAs in *E. coli.*

Nt4CL-1 and Nt4CL-19, two cDNAs encoding tobacco-4CL proteins, were ~80% identical at the nucleotide and predicted amino acid sequence levels. To determine if the encoded enzymes had different biochemical characteristics, recombinant 4CL1 and 4CL2 were produced in *Escherichia coli.* The cDNA-inserts were cloned into pQE expression-plasmids, designated pQE-1 and pQE-19 for Nt4CL-1 and Nt4CL-19 respectively, and transformed into *E. coli.* pQE-1 and pQE-19 each contain an engineered translational start codon, a histidine-tag and a multicloning site such that the recombinant proteins have 13 (MRGSHHHHHHGIR) and 14 (MRGSHHHHHHGSAC) additional amino acids prior to the initiation methionine encoded by the cDNA-inserts. pQE-19-transformants expressed 4CL protein efficiently and a protein-band corresponding to the recombinant 4CL2 was detected by Coomassie Blue staining on a SDS-polyacrylamide gel after 2 hours of IPTG-induction. pQE-1-transformants expressed 4CL protein less efficiently and a Coomassie Blue stained protein-band corresponding to the recombinant 4CL1 was visible only after 4 hours of IPTG-induction (results not shown). In addition, the bacterial strain harboring pQE-19 was grown in LB media whereas the bacterial strain

containing pQE-1 was grown in Super Media, a more nutritious medium recommended for generating higher levels of recombinant proteins (QIAexpressionist™ Kit, QiaGen Inc., Chatsworth, CA). After 4 hours of IPTG-induction, pQE-19-generated protein (4CL2) was present in both cytosolic fractions and membrane bound fractions whereas pQE-1-generated protein (4CL1) was localized in the cytosol (results not shown). The recombinant proteins were approximately 60 kDa in size, reacted with the antibody raised against parsley-4CL, and migrated to the same location in an SDS-polyacrylamide gel as the 4CL protein(s) found in tobacco-stem extracts (Figure 4.1). No 4CL protein (Figure 4.1, lane 1) or 4CL activity (not shown) was detected in the bacterial strain containing the pQE-30 plasmid lacking a *4CL* cDNA insert.

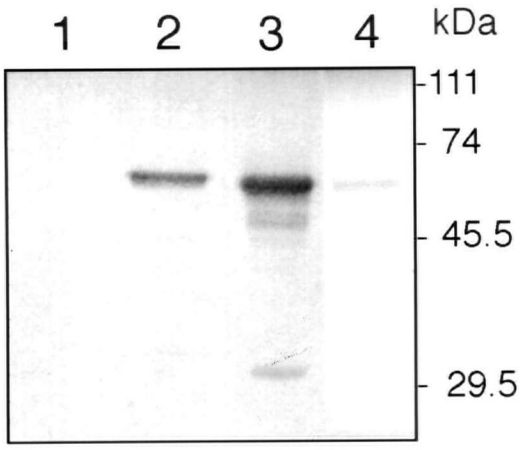


Figure 4.1: Western blot analysis of recombinant tobacco-4CL expressed in *E. coli*. Crude, bacterial protein-extracts (5 µg) were separated by SDS-PAGE, blotted onto a nylon membrane, and the blot reacted with an antibody raised against parsley-4CL. Lane 1, extract from bacteria harboring the expression-plasmid pQE-30 without an insert; lane 2, extract from bacteria harboring pQE-1, expressing Nt4CL-1-encoded protein (4CL1); lane 3 extract from bacteria harboring pQE-19, expressing Nt4CL-19-encoded protein (4CL2); lane 4, extract of total protein (25 µg) from tobacco old-stem. Molecular mass standards (in kDa) are shown on the right.

4.2 Enzymatic Characterization of 4CL

The recombinant proteins present in crude bacterial-extracts expressing pQE-1 and pQE-19 were tested for their relative abilities to utilize differently-substituted hydroxycinnamic acids as substrates. Initially, enzyme activities of recombinant 4CL1 and recombinant 4CL2 were determined using 0.025 mM to 0.5 mM cinnamic acid, 4-coumaric acid, caffeic acid, ferulic acid, and sinapic acid as substrates (Figures 4.2 and 4.3). Double-reciprocal transformation of the data (Lineweaver-Burke plot; Table 4.1) was used to estimate the Michealis-Menten constant (K_m) and the maximum

velocity of the reaction (V_{max}). The recombinant proteins did not react with sinapate and exhibited enzyme kinetics comparable to one another when cinnamate, 4-coumarate, caffeate, and ferulate were used as substrates (Figure 4.3). Both recombinant proteins experienced substrate-inhibition at concentrations above 0.2 mM of 4-coumarate, caffeate, or ferulate and these data points were not used in K_m and V_{max} determination. The K_m and V_{max} values of recombinant 4CL2 towards caffeate could not be estimated with reasonable accuracy from the data obtained. In parallel, crude extracts prepared from tobacco-stems were assayed for 4CL activity and found to have an approximate K_m and V_{max} of 0.0095 mM and 0.45 mmol s^{-1} kg^{-1} for 4-coumarate (Figure 4.4). The K_m of endogenous tobacco 4CL towards 4-coumarate is comparable to the K_m of recombinant 4CL1 (0.017 mM) and 4CL2 (0.0087 mM) towards 4-coumarate (Table 4.1). 4CL enzyme activity from crude tobacco-stem extracts was also inhibited by concentrations of 4-coumarate above 0.2 mM (Figure 4.4, arrow). K_m and V_{max} values for other substrates were not determined because the 4CL enzyme activity in crude tobacco-stem extracts was low, especially when low concentrations of substrate were used, and approached the limit of detection.

Since preliminary data (above) suggests that the enzymatic properties of the recombinant proteins appear relatively similar to one another, the substrate specificities of these enzymes were characterized more thoroughly at one concentration of substrate. A substrate concentration of 0.2 mM was chosen since at this concentration, there was little or no apparent substrate-inhibition of the recombinant 4CL proteins and of the endogenous tobacco-stem 4CL proteins (Figures 4.3 and 4.4) and, with the exception of cinnamate, this concentration represents near-saturating levels of substrate (Figure 4.3). Crude extracts prepared from tobacco stems had relative enzyme-activities of 100%, 17%, and 60% when 4-coumarate, caffeate, and ferulate (respectively) were used as substrates; no detectable activity towards cinnamate as a substrate; and a small amount of activity (4%) towards

sinapate as a substrate. Recombinant 4CL1 had relative 4CL-activities of 100%, 21%, 17%, and 73% when 4-coumarate, cinnamate, caffeate, and ferulate were used as substrates (respectively), while extracts of recombinant 4CL2 had relative activities of 100%, 29%, 25%, and 62% (Figures 4.2 and 4.5). Taken together, these results show that the two recombinant-4CL proteins have nearly identical substrate specificities, and that these are comparable to those of the 4CL from tobacco-stem extracts. However, the lack of activity when sinapate was used as a substrate and relatively high activity when cinnamate was used as a substrate (cinnamate is not generally regarded as a cellular target for the 4CL enzyme) distinguished the recombinant enzymes from the native 4CL-protein(s) in stem extracts.

Subsequent experiments focused on recombinant 4CL2 since the two recombinant proteins appeared to be relatively similar to one another and 4CL expression from pQE-19 was more efficient than expression from pQE-1 (section 4.1). The ability of chemical analogs (Figure 4.2) and down-stream phenylpropanoid-products to inhibit recombinant-4CL2 activity was analyzed. When 4-coumarate was used as a substrate, recombinant-4CL2 activity was strongly inhibited by 0.2 mM sinapic acid and 3,4-methylenedioxycinnamic acid and weakly inhibited by the same concentration of 5-hydroxyferulic acid. Coniferin (coniferyl alcohol glucoside), derived from feruloyl:CoA, did not inhibit recombinant-4CL2 activity whereas 1 mM naringenin, a flavanone derived from 4-coumaroyl:CoA, did (Figure 4.6). Similar results were observed with crude 4CL-preparations from tobacco stems (not shown).

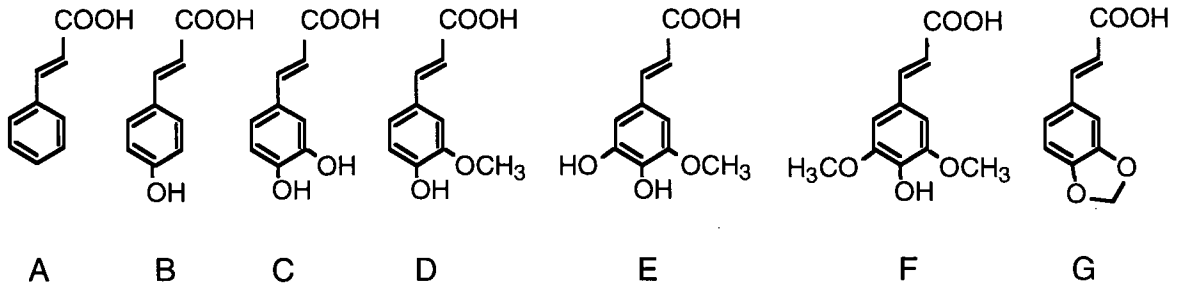


Figure 4.2: Chemical analogs used as substrates and inhibitors of 4CL activity. The chemical structures of cinnamic acid derivatives used to characterize 4CL is listed: cinnamic acid (A); 4-coumaric acid (B); caffeic acid (C); ferulic acid (D); 5-hydroxyferulic acid (E); sinapic acid (F); and 3,4-methylenedioxycinnamic acid.

Table 4.1: Estimated K_m and V_{max} values for recombinant 4CL1 and recombinant 4CL2 from crude bacterial-extracts.

	Recombinant 4CL1			Recombinant 4CL2		
Substrate	K_m (mM)	V_{max} (mmol s^{-1} kg^{-1})	V_{max}/K_m *	K_m (mM)	V_{max} (mmol s^{-1} kg^{-1})	V_{max}/K_m *
Cinnamic acid	0.70	21	30 (1)	0.27	17	63 (1)
4-Coumaric acid	0.017	25	1500 (50)	0.0087	26	3000 (48)
Caffeic acid	0.38	13	34 (1.1)	-	-	-
Ferulic acid	0.044	22	500 (17)	0.017	17	1000 (16)

* relative ratios are presented in parenthesis.

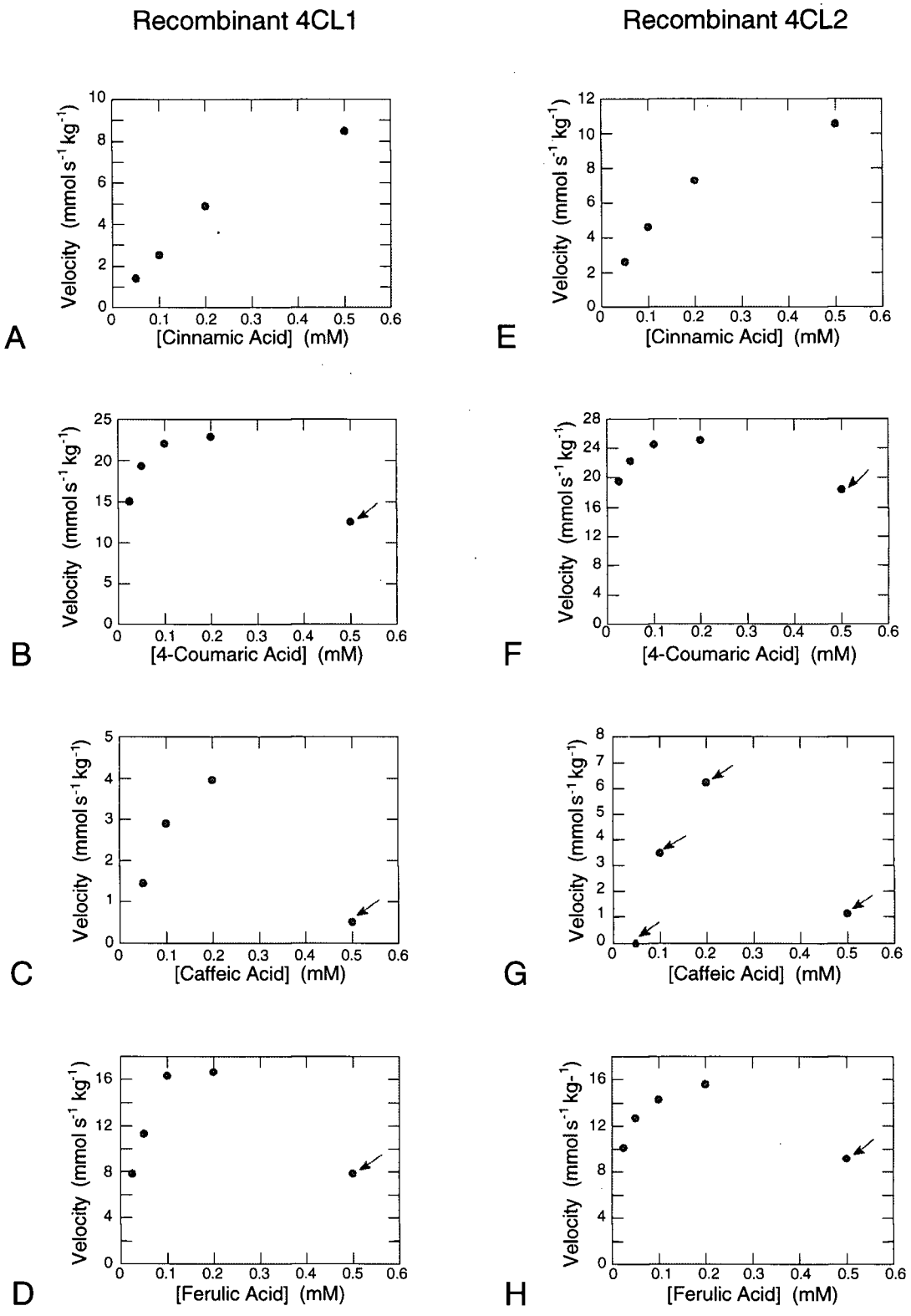


Figure 4.3: Recombinant-4CL1 and recombinant-4CL2 activities as a function of substrate concentration. Recombinant 4CL1 (A-D) and recombinant 4CL2 (E-H) were assayed for enzyme activity using varying concentrations (0.025 mM to 0.5 mM) of cinnamic acid (A, E), 4-coumaric acid (B, F), caffeic acid (C, G), and ferulic acid (D, H). Enzyme activity was measured at room temperature spectrophotometrically. Arrows indicate data points which were not used for K_m and V_{max} determination.

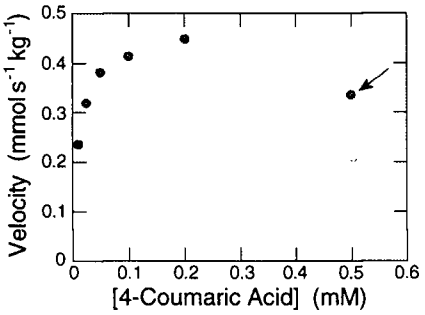


Figure 4.4: 4CL enzyme activity from crude tobacco-stem extracts plotted as a function of substrate concentration. Crude tobacco-stem extracts were assayed for enzyme activity using varying concentrations (0.025 mM to 0.5 mM) of 4-coumaric acid. Enzyme activity was measured at room temperature spectrophotometrically. Arrow indicate the data point which was not used for K_m and V_{max} determination.

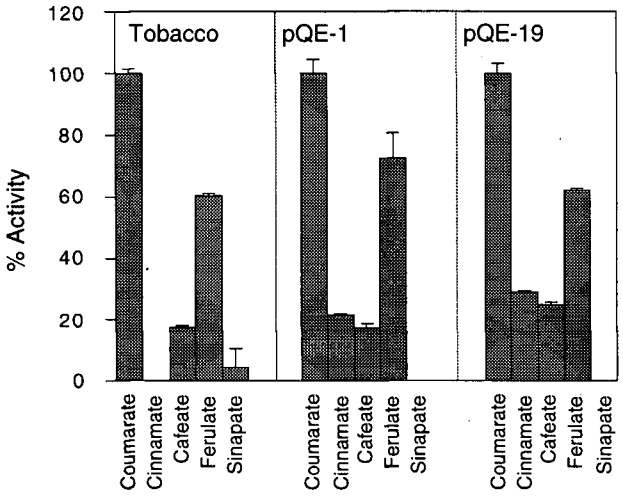


Figure 4.5: Substrate specificities of tobacco-4CL and recombinant-4CL proteins. 4CL enzyme activity was measured from crude tobacco-stem extracts (tobacco), crude bacterial-extracts expressing Nt4CL-1 (pQE-1) and crude bacterial-extracts expressing Nt4CL-19 (pQE-19). 4CL activity is expressed as a percentage of the activity of the preparation using 4-coumarate as a substrate. One hundred percent activity represents 0.33 mmol s^{-1} kg^{-1} (tobacco), 23 mmol s^{-1} kg^{-1} (pQE-1) and 25 mmol s^{-1} kg^{-1} (pQE-19). Results are averaged from three determinations using 0.2 mM of the hydroxycinnamate substrates. Error bars represent standard deviation.

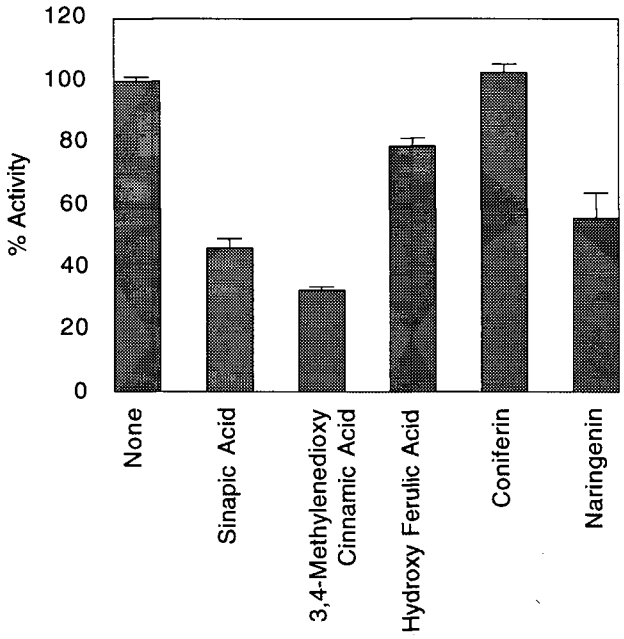


Figure 4.6: Inhibition of recombinant-4CL2 activity by phenylpropanoid metabolites. Recombinant-bacterial extracts expressing Nt4CL-19 (pQE-19) were assayed for 4CL enzyme activity using 0.2 mM 4-coumarate as substrate and the presence of 0.2 mM sinapic acid, 0.2 mM 3,4-methylenedioxycinnamic acid, 0.2 mM hydroxyferulic acid, 1 mM coniferin, or 1 mM naringenin. Activity is expressed as a percentage of the 4CL activity in the absence of added inhibitors. Results are averaged from three determinations; error bars represent standard deviations.

4.3 Modification of Recombinant-4CL Activity

Comparisons between recombinant 4CL1, recombinant 4CL2 and 4CL from crude tobacco-stem extracts suggested that the recombinant proteins have enzymatic characteristics like those of native tobacco-4CL. However, a striking difference between the recombinant-4CL proteins and endogenous tobacco-4CL is the ability of the recombinant proteins to use cinnamate as a substrate (Figure 4.5). We considered this phenomena particularly enigmatic and considered possible explanations for this discrepancy. Conceivably, the bacterial extracts which were used to assay recombinant-4CL activity could contain an activity capable of modifying cinnamate (e.g. by hydroxylation), leading to an apparent activity towards this substrate. This was tested by assaying 4CL activity, using cinnamate as a substrate, in tobacco extracts to which crude extracts of the bacterial strain harboring the empty expression-plasmid (pQE-30) had been added. No 4CL activity towards cinnamate was detectable, indicating that the bacterial extract itself was not the cause of the apparent 4CL activity towards cinnamate (results not shown).

We next considered the possibility that recombinant-4CL protein has bona fide activity toward cinnamate as a substrate, but that the native tobacco-protein is post-translationally modified, or interacts with other proteins, repressing its ability to use cinnamate as a substrate *in planta.* To test this, crude tobacco-stem extracts were incubated together with crude bacterial-extracts containing recombinant 4CL2 and the mixture assayed for activity towards cinnamate as a substrate. Figure 4.7 shows that the ability of recombinant 4CL2 to use cinnamate as a substrate decreased exponentially in the presence of increasing amounts of tobacco-stem extract, consistent with the hypothesis that such extracts indeed contain a component capable of modifying the substrate specificity of recombinant 4CL. Furthermore, this apparent modification was specific to the activity towards cinnamate as a substrate. The ability of recombinant-4CL2 to use 4-coumarate, caffeate or ferulate as substrates was unchanged (Figure 4.8). Boiled tobacco-stem extract was no longer capable of

modifying the activity of recombinant 4CL2 (Figure 4.8). In contrast, desalting the tobacco-stem extract by chromatography through a G-50 Sephadex column did not abolish its ability to modify recombinant-4CL2 activity towards cinnamate as a substrate (Figure 4.8). Neither plant-extraction buffer alone (Figure 4.8) nor BSA (not shown) decreased the ability of recombinant-4CL2 to use cinnamate as a substrate. While these experiments were performed using pQE-19 extracts containing recombinant-4CL2 protein, a similar effect of tobacco-stem extracts on recombinant 4CL1 in extracts of the pQE-1-expressing *E. coli* strain was also observed (results not shown). Taken together, these results suggest that tobacco-stem extracts contain a large heat-labile component, possibly a protein which inhibits the ability of recombinant-4CL to use cinnamate, thereby modifying its substrate specificity.

The distribution of the 4CL-modifying component was determined. Preliminary results (Figure 4.9) show that crude tobacco-extracts from old leaves, wounded leaves, methyl jasmonate treated leaves, and shoot tips were also able to decrease the ability of recombinant-4CL2 to use cinnamate as a substrate. This decrease in activity towards cinnamate was not observed when equivalent levels of BSA was added to the reaction mixture (not shown). In general, it appeared that less than 50 μg of tobacco-stem protein was required to decrease the recombinant-4CL2 activity (towards cinnamate as a substrate) by 50% whereas in excess of 200 μg of protein from the other tobacco-extracts was required for 50% inhibition (Figure 4.9). 4CL in these tobacco extracts did not use cinnamate but did use 4-coumarate as a substrate. 4CL activity was highest in tobacco stems and significantly lower in shoot tips, wounded leaves, methyl jasmonate treated leaves, and old leaves (Figure 4.10). Although these experiments were performed once and the results should be considered as preliminary data, there appears to be a good (but not absolute) correlation between the concentration of the modifying-component and the levels of endogenous tobacco-4CL activity.

The identity of the modifying-component and the modification it performs is unknown; however, since the 4CL enzyme-assay mix includes ATP, it was postulated that the modification event may involve phosphorylation. To test this, recombinant 4CL2, tobacco-stem extracts, and alkaline phosphatase were incubated together prior to assaying for 4CL activity towards cinnamate as a substrate (Figure 4.11). In the presence of alkaline phosphatase, the inhibition of recombinant-4CL2 activity towards cinnamate by tobacco-stem extract was partially alleviated (Figure 4.11, X X X X). This suggests that the modifying-component in tobacco functions through phosphorylation since the presence of a phosphatase reduces its affect. The complementary experiment using NaF, a phosphatase inhibitor, showed that the inhibition of recombinant-4CL2 activity towards cinnamate by tobacco-stem extracts was intensified with added NaF (Figure 4.11, ——). The affect of NaF was specific since an equivalent amount of NaCl did not elicit the same response (not shown) and control assays, performed in the presence of NaF and alkaline phosphatase, demonstrate that the concentration of NaF used was effective in blocking exogenously-applied alkaline phosphatase (Figure 4.11, · · · ·). Although the affect of NaF was small, it was reproducible and suggests that, under the physiological state from which the stems were harvested, endogenous tobacco-phosphatases are a minor component of the proposed 4CL phosphorylation-dephosphorylation regulatory-system. Under normal conditions, 4CL from tobacco-stem extracts do not use cinnamate as a substrate (Figures 4.5, 4.10 and 4.11); however, preliminary results show that in the presence of 20 units of alkaline phosphatase, 4CL from crude tobacco-stem preparations displayed a slight activity towards cinnamate as a substrate (not shown). These results suggests that tobacco extracts contain an activity, possibly a kinase, which regulates 4CL substrate-specificity through phosphorylation and that this regulation occurs *in vivo* with the endogenous tobacco-4CL as well as *in vitro* with the recombinant 4CL proteins.

Computer analysis of the encoded Nt4CL-1 and Nt4CL-19 peptide-sequences predicted numerous potential sites of phosphorylation ([S/T]-X-[R/K], [S/T]-X_2-[D/E], [R/K]-$X_{(2-3)}$-[D/E]-$X_{(2-3)}$-Y; Patschinsky *et al.*, 1982; Hunter, 1982; Woodgett *et al.*, 1986). Eleven putative serine-threonine phosphorylation-sites and one tyrosine kinase phosphorylation-site were found in both Nt4CL-1 and Nt4CL-19 predicted amino acid-sequences (Figure 4.12, underlined). Furthermore, two putative serine-threonine phosphorylation sites were conserved in all predicted 4CL-sequences analyzed in Table 3.1 (Figure 4.12, +++). To determine if the recombinant-4CL proteins were directly phosphorylated by a putative kinase found in tobacco extracts, radiolabelling experiments using ^{32}P γ-ATP were performed. Crude bacterial-extracts expressing pQE-19 were incubated with tobacco-stem extracts in the presence of ^{32}P γ-ATP and then chromatographed through a nickel column to enrich for the recombinant 4CL by virtue of the engineered histidine-residues at the amino terminus (see section 4.1). The eluant was electrophoresed on an SDS-polyacrylamide gel and then exposed to autoradiographic film. Results showed three radioactive signals, one of which was approximately 60 kDa in size; however, the intensity of the 60 kDa signal was conspicuously weak (results not shown) and since the nickel column did not purify the recombinant 4CL to homogeneity, we cannot yet conclude that 4CL is directly phosphorylated.

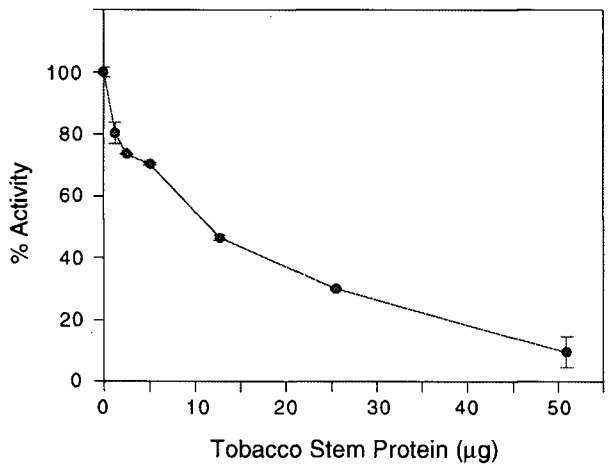


Figure 4.7: Effect of tobacco-stem extracts on recombinant-4CL2 activity towards cinnamate as a substrate. Recombinant bacterial-extracts expressing Nt4CL-19 (pQE-19) were assayed for 4CL enzyme activity using 0.2 mM cinnamate as substrate in the presence of different amounts of a tobacco-stem extract. Activity is expressed as a percentage of the 4CL activity towards cinnamate as a substrate in the absence of added tobacco extract. Results are averaged from three determinations; error bars represent standard deviations.

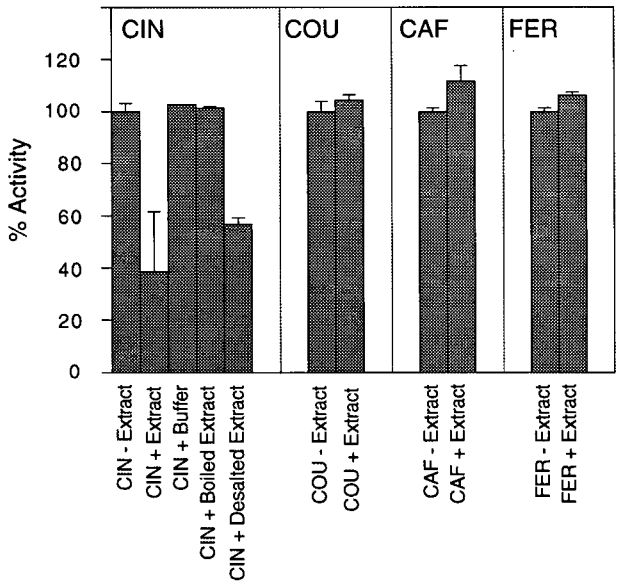


Figure 4.8: Characterization of the 4CL-modifying activity in tobacco-stem extracts. Recombinant 4CL2 (pQE-19) was assayed for 4CL activity towards cinnamate (CIN), 4-coumarate (COU), caffeate (CAF), or ferulate (FER) as substrates in the absence (- Extract) or presence (+ Extract) of 25 µg tobacco-stem extract. The tobacco-stem extracts used in the assays with cinnamate as a substrate were either untreated (CIN + Extract), heated at 100°C for 15 minutes (CIN + Boiled Extract), or passed through a Sephadex G-50 column (CIN + Desalted Extract) before being added to the enzyme assays. Activity is expressed as a percentage of the 4CL activity towards each hydroxycinnamic acid in the absence of added tobacco extract. Results are averaged from three determinations; error bars represent standard deviations.

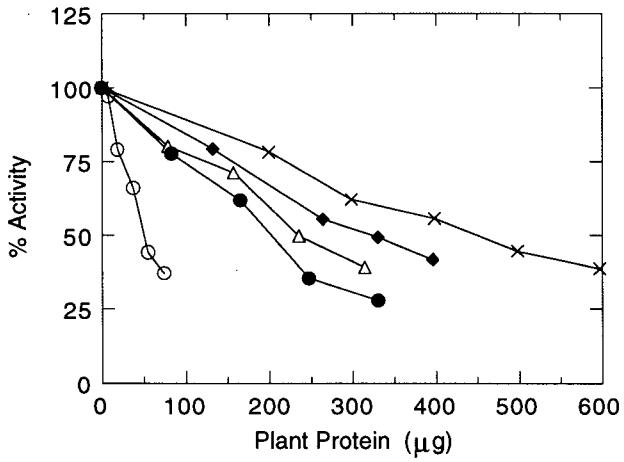


Figure 4.9: Distribution of the 4CL-modifying activity in tobacco organs. Recombinant-bacterial extracts expressing Nt4CL-19 were assayed for 4CL enzyme activity using 0.2 mM cinnamate as substrate in the presence of different amounts of tobacco-stem extract (-O-O-), tobacco shoot-tip extract (-•-•-), wounded tobacco-leaf extract (-Δ-Δ-), old tobacco-leaf extract (-◆-◆-), or methyl jasmonate treated tobacco-leaf extract (-X-X-). Activity is expressed as a percentage of the 4CL activity towards cinnamate as a substrate in the absence of added tobacco extract.

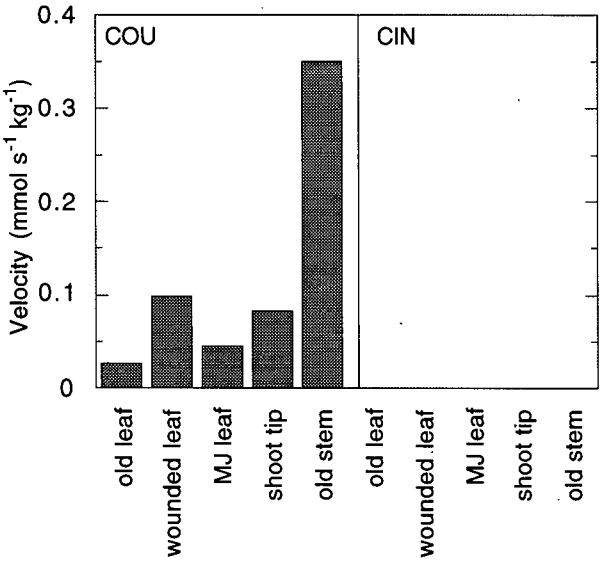


Figure 4.10: Distribution of endogenous tobacco-4CL activity. Crude tobacco-extracts from tobacco stems, tobacco shoot-tips, wounded tobacco-leaves, old tobacco-leaves, or methyl jasmonate treated tobacco-leaves (MJ) were assayed for 4CL enzyme activity using 4-coumarate (COU) or cinnamate (CIN) as substrates.

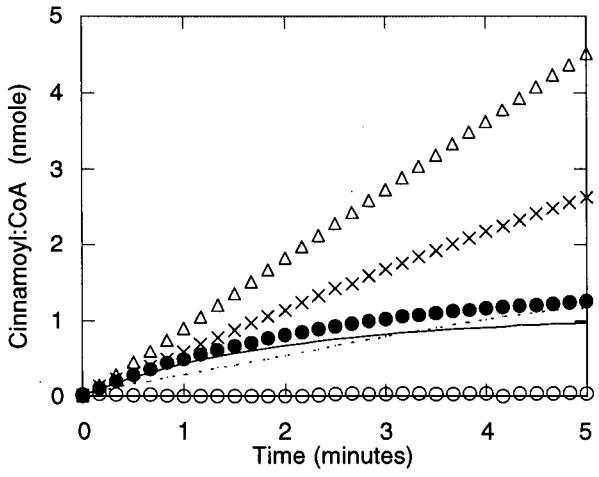


Figure 4.11: Affect of alkaline phosphatase and NaF on the 4CL-modifying activity from tobacco-stem extracts. Recombinant-bacterial extracts expressing Nt4CL-19 (pQE-19) were assayed for 4CL enzyme activity using 0.2 mM cinnamate as substrate (Δ Δ Δ). Recombinant-4CL activity was assayed in the presence of 25 µg of tobacco-stem extract (• • •); in the presence of 25 µg of tobacco-stem extract and 20 units of calf intestinal alkaline phosphatase (X X X); in the presence of 25 µg of tobacco-stem extract and 50 mM NaF (——); or in the presence of 25 µg of tobacco-stem extract, 20 units of calf intestinal alkaline phosphatase and 50 mM NaF (····). Open circles (o o o) represent 4CL enzyme activity of 25 µg tobacco-stem protein toward cinnamate as a substrate. The data represent the results of a typical experiment.

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Nt4CL-1     MPMETTTETKQSGDLIFRSKLPDIYIPKHLPLHSYCFENISEFSSRPCLI   50
Nt4CL-19        MEKDTKQ-VDIIFRSKLPDIYIPNHLPLHSYCFENISEFSSRPCLI   45

Nt4CL-1     NGANDQIYTYAEVELTCRKVAVGLNKLGIQQKDTIMILLPNSPEFVFAFM  100
Nt4CL-19    NGANKQIYTYADVELNSRKVAAGLHKQGIQPKDTIMILLPNSPEFVFAFI   95

Nt4CL-1     GASYLGAISTMANPLFTPAEVVKQAKASSAKIIITQSCFVGKVKDYASEN  150
Nt4CL-19    GASYLGAISTMANPLFTPAEVVKQAKASSAKIIVTQACHVNKVKDYAFEN  145

Nt4CL-1     DVKVICIDSAPEGCLHFSELTQSDEHEIPEVKIQPDDVVALPYSSGTTGL  200
Nt4CL-19    DVKIICIDSAPEGCLHFSVLTQANEHDIPEVEIQPDDVVALPYSSGTTGL  205
                  +++.
Nt4CL-1     PKGVMLTHKGLVTSVAQQVDGENANLYMHSEDVLMCVLPLFHIYSLNSIL  250
Nt4CL-19    PKGVMLTHKGLVTSVAQQVDGENPNLYIHSEDVMLCVLPLFHIYSLNSVL  245

Nt4CL-1     LCGLRVGAAILIMQKFDIAPFLELIQKYKVSIGPFVPPIVLAIAKSPIVD  300
Nt4CL-19    LCGLRVGAAILIMQKFDIVSFLELIQRYKVTIGPFVPPIVLAIAKSPMVD  295
                 +++ .
Nt4CL-1     SYDLSSVRTVMSGAAPLGKELEDAVRTKFPNAKLGQGYGMTEAGPVLAMC  350
Nt4CL-19    DYDLSSVRTVMSGAAPLGKELEDTVRAKFPNAKLGQGYGMTEAGPVLAMC  345

Nt4CL-1     LAFAKEPFDIKSGACGTVVRNAEMKIVDPDTGCSLPRNQPGEICIRGDQI  400
Nt4CL-19    LAFAKEPFEIKSGACGTVVRNAEMKIVDPKTGNSLPRNQSGEICIRGDQI  395

Nt4CL-1     MKGYLNDPEATTRTIDKEGWLHTGDIGFIDEDDELFIVDRLKELIKYKGF  450
Nt4CL-19    MKGYLNDPEATARTIDKEGWLYTGDIGYIDDDDELFIVDRLKELIKYKGF  445

Nt4CL-1     QVAPAEIEALLLNHPNISDAAVVPMKDEQAGEVPVAFVVRSNGSAITEDE  500
Nt4CL-19    QVAPAELEALLLNHPNISDAAVVPMKDEQAGEVPVAFVVRSNGSTITEDE  495

Nt4CL-1     VKDFISKQVIFYKRVKRVFFVETVPKSPSGKILRKDLRARLAAGVPN*    547
Nt4CL-19    VKDFISKQVIFYKRIKRVFFVDAIPKSPSGKILRKDLRAKLAAGLPN*    542
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Figure 4.12: Predicted phosphorylation-sites of polypeptides encoded by Nt4CL-1 and Nt4CL-19. Computer analysis of the predicted amino acid sequence from Nt4CL-1 and Nt4CL-19 revealed numerous putative phosphorylation-sites, some of which are found in both Nt4CL-1 and Nt4CL-19 (underlined) and two of which (+++) are found in all the 4CL sequences listed in Table 3.1. Dashes indicate gaps introduced to maximize alignment, translational stops are indicated with an asterisk, and numbers on the far right indicate the predicted amino acid position.

4.4 Discussion

The expression of two divergent tobacco-*4CL* cDNAs, Nt4CL-1 and Nt4CL-19, in *E. coli* allowed us to examine the properties of recombinant-4CL proteins. The K_m and V_{max} values of recombinant 4CL1 and recombinant 4CL2 were estimated; however, these kinetic characteristics should be considered as preliminary data since the experiments were performed only once and were performed with unpurified enzymes. Given these limitations, the K_m of recombinant-4CL activity towards 4-coumarate (~10 μM to ~20 μM) is within the range of that reported for the 4CL enzymes from *Brassica* (14 μM; Rhodes *et al.*, 1973), soybean (17 μM; Knobloch and Hahlbrock, 1975), parsley (14 μM; Knobloch and Hahlbrock, 1977), spruce (11 μM; Lüderitz *et al.*, 1982), and poplar (10 μM for 4CLII and 13 μM for 4CLIII; Grand *et al.*, 1983). However, the K_m values towards other cinnamic acid derivatives were not directly comparable between the recombinant-4CL proteins and the 4CL activities from other plants. The endogenous concentration of cinnamate in bean cell cultures is between 4 μM to 25 μM (Mavandad *et al.*, 1990) and is much lower than the estimated K_m of the recombinant 4CL proteins towards cinnamate. However, uptake experiments using ^{14}C-cinnamic acid showed that cinnamate concentrations after elicitation can be as high as 550 μM (Mavandad *et al.*, 1990; Edwards *et al.*, 1990). This suggest that although the K_m values of the recombinant 4CL towards cinnamate is high, it is not impossible that endogenous cinnamate concentratrions reach these levels. Authors also suggest that the presence of specific metabolic pools and interactions between cinnamate and proteins may cause high, localized, concentrations of cinnamate (Mavandad *et al.*, 1990).

The relative substrate-specificities as determined by the V_{max}/K_m ratios (Table 4.1, values in parenthesis) demostrate the almost identical substrate-specificities of recombinant 4CL1 and recombinant 4CL2. The relative substrate-specificity as measured by the percent enzyme activity using 0.2 mM cinnamic acid derivatives (Figure 4.5) also demonstrates the similarity in enzyme activites of 4CL1 and 4CL2. In

general, the activities of the recombinant-4CL proteins were most similar to the activities of the parsley-4CL proteins which had maximal activity towards 4-coumarate, followed by ferulate, caffeate, and cinnamate as substrates (Lozoya *et al.*, 1988).

By using a series of chemical analogs as substrates and inhibitors (Figure 4.2), the nature of the substrate binding site of recombinant 4CL2 was investigated. 4CL activity was higher towards 4-coumarate as compared to cinnamate as substrates, suggesting that the hydroxyl group on position 4 of the aromatic ring may be important for electrostatic interactions between the substrate and enzyme. In contrast, a hydroxyl group on position 3 of the aromatic ring seemed to decrease the activity of recombinant 4CL towards caffeate as a substrate; however, methylation of the 3-hydroxyl group caused ferulate to be more efficiently metabolized. This suggests that the substrate binding site of 4CL may contain a hydrophobic pocket such that non-polar groups at the number-3 position are better tolerated. Since the chemical group modified by 4CL is the carboxylic acid group on the propane chain distal to the aromatic ring, it is likely that the hydroxylation and *o*-methylation modifications at the aromatic ring affects substrate binding and the observed differences in K_m values is in support of this (Table 4.1). However, it should be noted that the carboxylic acid group is apart of the conjugated double-bond system so that its chemical reactivity may be affected by ring modifications. One might predict that sinapic acid, 3,4-methylenedioxycinnamic acid, and 5-hydroxyferulic acid may act as competitive inhibitors that bind to 4CL and prevent catalysis of 4-coumarate (Figure 4.6). However, preliminary kinetic-studies using sinapate as an inhibitor and 4-coumarate as a substrate showed that the mechanism of sinapate inhibition was quite complex. Increasing the 4-coumarate concentration did not alleviate the inhibition caused by sinapate and the K_m and V_{max} of recombinant 4CL2 towards 4-coumarate as a substrate were both altered by the presence of sinapate (results not shown).

It has been hypothesized that, in some plants, different forms of 4CL may play a role in controlling the biosynthesis of different phenylpropanoids by their substrate

specificities (Knobloch and Hahlbrock, 1975; Grand *et al.*, 1983). The results reported here suggest that the recombinant proteins do not have different enzymatic properties and, in fact have kinetic properties very similar to one another (Table 4.1, Figures 4.3 and 4.5). Thus, it is unlikely that the proteins encoded by the Nt4CL-1 and Nt4CL-19 cDNAs have a role in modulating the types of phenylpropanoid-products made in tobacco. This conclusion is consistent with the expression patterns of Nt4CL-1 and Nt4CL-19 which show no differential expression of the two genes at the level of mRNA accumulation (Figure 3.9). 4CL is encoded by a divergent gene-family in soybean (Uhlmann and Ebel, 1993) and two isoforms of 4CL with different substrate specificities have been identified in soybean (Knobolch and Hahlbrock, 1975). The soybean-*4CL* cDNAs were partial-length so that the enzymatic activity of the encoded proteins were not tested. In poplar, three 4CL-isoenzymes with distinct enzyme activities have been found and two significantly different *4CL* cDNAs have been cloned (Allina and Douglas, 1994). Preliminary evidence suggests that the poplar-*4CL* cDNAs encode enzymes with indistinguishable activities (S. Allina and C. Douglas, unpublished). Two parsley *4CL* cDNAs were expressed in *E. coli* and the recombinant proteins had enzymatic properties very similar to one another. This however, was not unexpected since the predicted amino acids sequences were 99.5% identical. Thus, despite a number of reports in which 4CL forms exhibiting distinct substrate specificities were partially purified (Grand *et al.*, 1983; Wallis and Rhodes, 1977; Ranjeva *et al.*, 1976; Knobloch and Hahlbrock, 1975), there is yet to be a documented example of recombinant-4CL isoforms having different substrate specificities.

Preliminary results show that the recombinant-4CL activities were inhibited by substrate concentrations above 0.2 mM (Figure 4.3). Substrate inhibition of 4CL by 4-coumarate was also reported in soybean and parsley cell cultures (Knobloch and Hahlbrock, 1975; 1977) whereas the 4CL purified from loblolly pine xylem was not inhibited by cinnamate, coumarate, ferulate, caffeate or sinapate (Voo *et al.*, 1995).

Inhibition of 4CL by naringenin has been reported here with recombinant 4CL2 (Figure 4.6) as well as in the 4CL proteins from loblolly pine and petunia (Voo *et al.*, 1995; Ranjeva *et al.*, 1976). Naringenin, made from 4-coumaroyl:CoA, is one of the first compounds produced in the flavonoid biosynthetic pathway and, as suggested by Voo *et al.* (1995) and Ranjeva *et al.* (1976), its ability to inhibit 4CL activity towards 4-coumarate as a substrate may represent regulation by feedback-inhibition. Similiar to the 4CL in *Vanilla planifolia* (Funk and Brodeolius, 1990), recombinant 4CL2 and endogenous tobacco-stem 4CL were inhibited by 3,4-methylenedioxycinnamic acid. Recombinant-4CL activity towards 4-coumarate as a substrate was also inhibited by sinapate. Thus not only does recombinant 4CL2 not use sinapate as a substrate, sinapate also serves as an inhibitor. Recently, it has been shown that tobacco stems contain syringyl lignin (Halpin *et al.*, 1994). How is this type of lignin made when the two recombinant-4CL proteins examined here do not use sinapate as a substrate? One possibility is that a third form of 4CL exists in tobacco and this form is capable of converting sinapate into sinapoyl:CoA for syringyl lignin biosynthesis. If this is true, then the ability of sinapate to inhibit recombinant 4CL2 suggests that sinapate may be a key metabolite in the fine-tuning of 4CL activity in tobacco. For example, during developmental stages where sinapyl alcohol is in high demand, the actions of hydroxylases and *o*-methyltransferases may generate relatively high levels of sinapate which in turn down-regulate the 4CL isoforms which do not use sinapate as a substrate. As the sinapyl alcohol demand is supplied, the metabolic-pool of sinapate would decrease and susceptible forms of 4CL would be released from sinapate inhibition. Currently there is no evidence to support this hypothesis; however, in addition to the *4CL*-cDNAs cloned here, there appears to be another class of *4CL* genes in tobacco (Figure 3.8 and Genebank sequences) and crude tobacco-stem extracts do exhibit low 4CL activity towards sinapate. This data suggest that alternate forms of *4CL* genes and 4CL activities exist in tobacco but whether these forms are the ones which metabolize sinapate will be the topic of future research.

The inability of crude tobacco-stem extracts to utilize cinnamate was unexpected since recombinant-4CL proteins used cinnamate as a substrate (Figure 4.5) and northern analysis demonstrated high levels of Nt4CL-1 and Nt4CL-19 transcripts in this organ (Figure 3.9). One formal possibility is that this unique property of the recombinant proteins is due to the presence of the additional amino acids at the amino terminus of the recombinant proteins, a consequence of the bacterial system we used to express Nt4CL-1 and Nt4CL-19 cDNAs (see section 4.1). However, we considered this possibilty unlikely since several kinetic properties of the recombinant proteins were similar to those of endogenous 4CL from tobacco-stem extracts (see section 4.2). Also, unmodified recombinant poplar-4CL protein produced in an eukaryotic (baculovirus) expression-system also used cinnamate as a substrate, an activity not found in poplar extracts (A. Pri-Hadash, S. Allina, B. Ellis, and C. Douglas, unpublished). Results shown in Figures 4.7, 4.8 and 4.9 suggest that the discrepancy between the recombinant-4CL and endogenous-4CL in their abilites to use cinnamate as a substrate may be attributed to the presence of a 4CL-modifying factor(s) present in tobacco extracts. The factor(s) in tobacco-stem extracts specifically affects the ability of recombinant-4CL to use cinnamate as a substrate, reducing it by five to ten-fold (Figure 4.7), but has no effect on activity towards other substrates (Figure 4.8). Thus, we hypothesize that the *in vivo* enzymatic properties of 4CL1 and 4CL2 in tobacco are partially determined post-translationally by interaction with factor(s), absent in the *E. coli* host used to produce the recombinant proteins.

What is the nature of the 4CL-modifying activity in tobacco stems? We have accumulated evidence that the 4CL-modifying activity is present in high quantities in tissues which have high 4CL activity (Figure 4.9 and 4.10). Thus the presence of the modifier correlates well with the predicted location where its function would be most required. Studies with alkaline phosphatase and NaF, a phosphatase inhibitor, suggest that the 4CL-modifying activity may be a protein kinase (Figure 4.11). At least three mechanisms can be postulated by which 4CL activity could be post-

translationally modified by a putative kinase. The most simple explanation, is that 4CL is covalently altered by phosphorylation and that this renders the enzyme inactive towards cinnamate as a substrate. This model (Figure 4.13, A) is supported by the fact that there are putative phosphorylation-sites in the predicted 4CL1 and 4CL2 amino acid sequences (Figure 4.12); however, using ^{32}P-γ–ATP, we have not been able to clearly demonstrate phosphorylation of the recombinant-4CL proteins (not shown). Reversible phosphorylation-dephosphorylation as method of regulating enzyme activity has been extensively characterized in animal systems (Cohen, 1980) and has been demonstrated in plants (Ranjeva and Boudet, 1987). In particular, phosphorylation-dephosphorylation of nitrate reductase (Kaiser and Huber, 1994) and phospho*enol*pyruvate carboxlase (Jiao and Chollet, 1991) has been well characterized and putative kinases which mediate this modification have been identified and partially purified (Bachmann *et al.*, 1995; Wang and Chollet, 1993b; Bakrim *et al.*, 1992). Light-mediated phosphorylation of spinach nitrate reductase at serine-543 (Bachmann *et al.*, 1996) leads to inactivation of the enzyme and thereby serves as an "on/off" switch. In contrast, phosphorylation of a serine residue (Bakrim *et al.*, 1992) at the far N-terminus of the phospho*enol*pyruvate carboxlase leads to changes in enzyme kinetics. The phosphorylated form of phospho*enol*pyruvate carboxlase is less sensitive to feedback-inhibition by L-malate and exhibits higher enzyme activities at a suboptimal pH range (Nimmo *et al.*, 1987; Bakrim *et al.*, 1992). PAL has been shown to be phosphorylated but this was associated with enzyme degradation and not changes in enzyme activity (Bolwell, 1992).

A second method in which 4CL may be regulated by a kinase is by the interaction of 4CL with another protein which is phosphorylated (Figure 4.13, B). In such a model, when crude tobacco-extracts are added to recombinant 4CL, the interacting-protein (phosphorylated) interacts with the recombinant 4CL and alters its abiity to use cinnamate as a substrate. In the presence of alkaline phosphatase, the phosphate group is removed from the 4CL-interactor resulting in complex dissociating

and the re-acquisition of recombinant-4CL activity towards cinnamate as a substrate. This model is supported by the fact that immunoprecipitation experiments suggest that enzymes of phenylpropanoid biosynthesis pathways may form multienzyme complexes *in vivo* (Hradzina and Wagner, 1985; Deshpande *et al.*, 1993). However, if this model is true, the putative interactors must be present in the tobacco-stem extracts in great excess. This is because in the co-incubation experiments described above, the concentration of recombinant 4CL is approximately 50-75 times greater than that of endogenous tobacco-4CL protein (based on enzyme activity). Although possible, it is unlikely that tobacco-stem extracts would contain 4CL-interactors that are 50-75 fold in excess compared to the levels of endogenous tobacco-4CL.

A third possibility is that 4CL is regulated by a cascade of events which includes a phosphorylation step (Figure 4.13, C). It is unlikely that this model is accurate since the results presented in this section suggest that recombinant-4CL activity is modified by reversible phosphorylation-dephosphorylation. The reversibility of a signal cascade, as presented in Figure 4.13, C is difficult to envision.

The potential regulation of 4CL substrate specificity by phosphorylation would be more meaningful if the occurance and the biological significance of 4CL activity towards cinnamate as a substrate was understood. We have not identified a developmental stage, a physiological state, or an experimental treatment where endogenous tobacco 4CL uses cinnamate as a substrate. In the presence of gibberellic acid, the 4CL activity found in carrot cell cultures does not use cinnamate as a substrate. However, carrot cell cultures grown in medium lacking gibberellic acid accumulate large amounts of anthocyanins and contain a 4CL activity which uses cinnamate as a substrate (Heinzmann *et al.*, 1977). It is not clear whether this difference in substrate specificity is due to post-translational modification, as might be the case of the tobacco 4CL described here, or due to the presence of divergent *4CL* gene-products. Nevertheless, these results suggest that, in carrot cell cultures, 4CL activity toward cinnamate as a substrate is induceable and may be important in

phenylpropanoid metabolism. If tobacco 4CL uses cinnamate as a substrate *in planta*, what kinds of products can be made from cinnamoyl:CoA? The 4CL from *Cephalocereus senilis* (old-man-cactus; Liu *et al.*, 1993) uses cinnamate as a substrate probably for the formation of B-ring deoxy flavonoids such as pinocembrin (a flavanone), cephalocerone (an aurone), and baicalein (a flavone). Pinocembrin (Shain and Miller, 1982) and cephalocerone (Pare *et al.*, 1991) have antimicrobial activities. Although similar compounds have not been identified in tobacco, pinocembrin has been found in *Nierembergia hippomanica*, a member of the Solanaceae family (Pomilio and Gros, 1979), suggesting that such B-ring deoxy flavonoids may exist in tobacco. Salicylic acid is made from cinnamate (Yalpani *et al.*, 1993) and it has been hypothesized that the conversion of C_6-C_3 to C_6-C_1 may involve a mechanism analogous to "ß oxidation of fatty acids" (Lewis, 1993; Löscher and Heide, 1994). If this suggestion is true, then cinnamoyl:CoA would be a prime candidate as the precursor to salicylic acid. The biosynthesis of salicylic acid is still under debate but results suggest that salicylic acid biosynthesis occurs through a "non oxidative" route thereby by-passing the 4CL-catalyzed step (French *et al.*, 1976; Yazaki *et al.*, 1991; Schnitzler *et al.*, 1992).

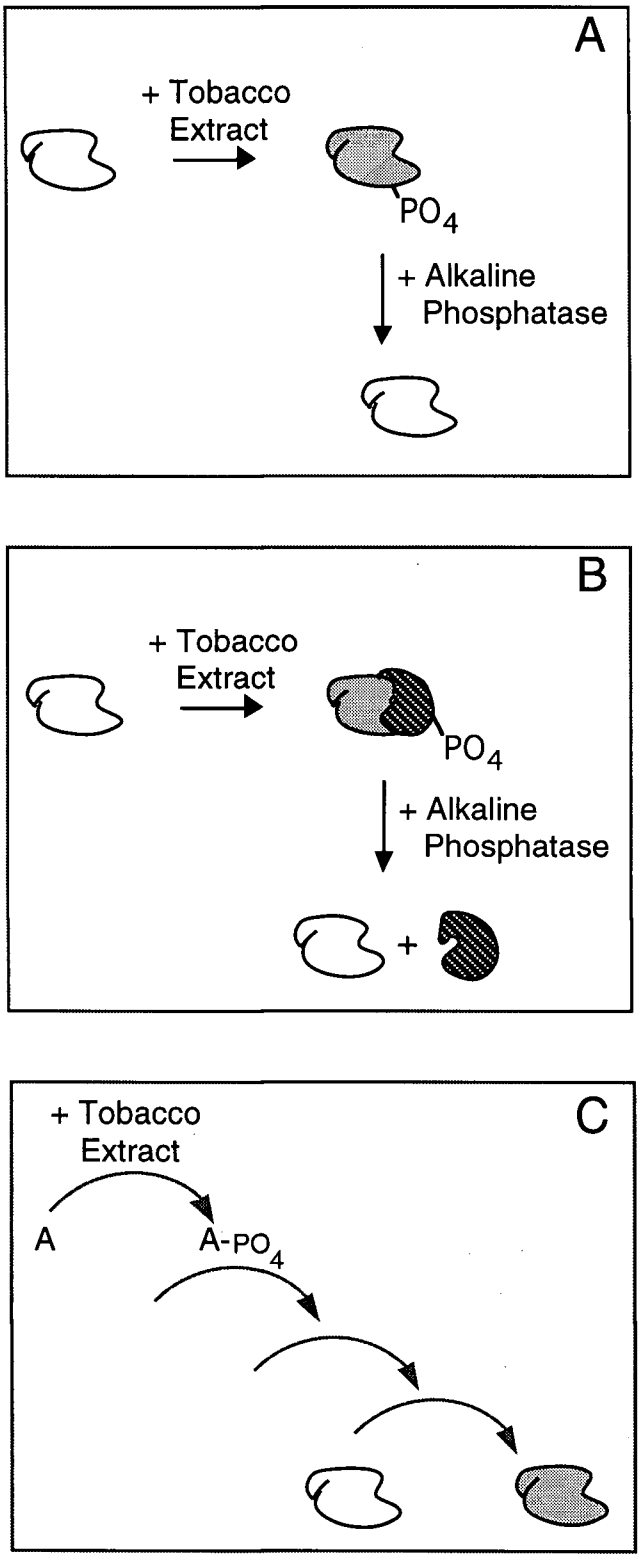

Figure 4.13: Three models describing the involvement of phosphorylation-dephosphorylation in the regulation of 4CL. A: 4CL is modified by phosphorylation. B: 4CL interacts with another protein which is phosphorylated. C: 4CL substrate specificity is regulated by a signal cascade which includes a phosphorylation step. White globular structures represent recombinant-4CL proteins which use cinnamate as a substrate. Grey structures represent modified recombinant-4CL proteins which do not use cinnamate as a substrate. Black structures represent protein(s) which interact with 4CL. PO_4 represents phosphate group; its location on the proteins is schematic. A and A-PO_4 represent components of a signal cascade.

Chapter 5

4CL-Suppressed Transgenic Plants

5.1 Introduction

One of the ways in establishing the developmental significance or physiological role of a given gene is to examine the consequences of its absence. Since mutants lacking 4CL activity have not been identified, we used sense suppression and antisense RNA to down regulate *4CL* expression. The mechanism by which sense suppression functions is still unclear (Jorgensen, 1995); however, it has been successfully used to down-regulate, most notably, the expression of *CHS* in petunia (Napoli *et al.*, 1990; van der Krol *et al.*, 1990) and *PAL* in tobacco (Elkind *et al.*, 1990). A major disadvantage of sense suppression is that it can be genetically-unstable giving "revertants" in subsequent generations and, sometimes, within sectors of the primary transformant (Jorgensen, 1995). Antisense RNA has been widely used to down-regulate genes in primary and secondary metabolism. For example, genes encoding ACC-oxidase (ethylene-forming enzyme; Hamilton *et al.*, 1990), DAHP

(Jones *et al.*, 1995), COMT (Ni *et al.*, 1994; Dwivedi *et al.*, 1994; Atanassova *et al.*, 1995; Van Doorsselaere *et al.*, 1995), and CAD (Halpin *et al.*, 1994) have been successfully down-regulated by antisense RNA. However, antisense RNA also has limitations (reviewed in Lee and Douglas, 1996a), one of them being that it rarely produces transgenics completely suppressed at the target-gene.

The physiological role of 4CL in tobacco and *Arabidopsis* plants was examined by generating transgenic plants suppressed in *4CL* expression. In 1992, when this work was initiated, the *4CL* cDNA clones from tobacco and *Arabidopsis* had not been isolated; thus antisense RNA and sense suppression were attempted in tobacco using the cDNA encoding 4CL from potato (Becker-André *et al.*, 1991). By 1993, the *Arabidopsis 4CL* cDNA had been cloned and well characterized (Lee *et al.*, 1995a) and the endogenous *4CL*-gene family in tobacco was better understood (Lee and Douglas, 1996b). Since 4CL appeared to be encoded by a single gene in *Arabidopsis*, as opposed to tobacco where 4CL is encoded by a diverse gene-family of 4 or more members, and because *Arabidopsis* is a good model-system for molecular and genetic analysis (Estelle and Somerville, 1986; Meyerowitz, 1987), we chose to concentrate our studies on the *Arabidopsis* system. Furthermore, since sense suppression has been shown to be unstable (Bates *et al.*, 1994; Jorgensen, 1995), we limited our analysis to the antisense-suppressed plants. This decision was appropriate especially since *Arabidopsis* has a short generation-time and it is inevitable that our research would span over a few *Arabidopsis* generations.

This chapter is a summary of the work done in tobacco and *Arabidopsis* using antisense-RNA suppression and sense suppression. Some sections (tobacco and sense-suppressed transgenic lines) contain results laying the groundwork which will serve as a foundation for further analysis by other researchers. The antisense *4CL* *Arabidopsis* transgenic-plants are analyzed in detail and are the main topic of discussion in this chapter. The questions we would like to address using the antisense *4CL* plants are as follows. First, what are the phenotypic consequences of

blocking *4CL* expression? Second, how does blocking *4CL* expression affect the biosynthesis of phenylpropanoid-products? And lastly, if we are successful in blocking carbon flow into phenylpropanoid biosynthesis, what is the affect of blocking *4CL* expression on stress-activated gene expression?

5.2 *4CL*-Suppressed Tobacco Plants

5.2.1 Generation of *4CL*-Suppressed Tobacco Plants

The potato-*4CL* cDNA was subcloned behind the 35S promoter in sense or antisense orientation (Figure 5.1) and transformed into tobacco using *Agrobacterium*-mediated transformation. The regenerated plants (T0-generation) were allowed to set seed (T1-generation); however, approximately 9% (8 out of 93) of the primary transformants were sterile so that propagation of these transgenic lines and analysis of the progeny was not possible (Table 5.1). Half of these sterile transgenic-tobacco plants (4 out of 8) appeared to have filaments that projected far beyond (exserted) the stigma such that the pollen-bearing anthers were removed from the stigma. Attempts to pollinate the stigma by manually placing pollen from the same plant onto the stigma were not successful.

To ensure that the T1-generations contained the transgene and to assess the copy-number of the transgenes in each plant, the seeds from the T1-plants were screened for the presence of the *nptII* selectable-marker; namely, the seeds were surface-sterilized, plated on MS plates supplemented with kanamycin and scored for their ability to grow in the presence of kanamycin (Table 5.2). Results demonstrate that most of the transgenic tobaccos contained one locus of the transgene (KanR/KanS ratio of ~3:1). Some transgenic lines contained 2 (KanR/KanS ratio of ~15:1) or more transgene loci. Approximately 8% (7 out of 85) of the seedlots were nonviable, and of the seeds that were viable, approximately 15% (12 of 78) had particularly low germination frequencies or produced seedlings with unusual characteristics such as short roots or chlorotic, yellow leaves (Table 5.1 and 5.2).

These seedlings, however, were not correlated with low levels of 4CL proteins as determined by western blot analysis (not shown).

Using the antibody raised against the parsley 4CL to detect tobacco 4CL, T1 transgenic-tobacco seedlings were screened by western blot analysis to determine which transgenic lines had decreased levels of 4CL protein. Seedlots were surface-sterilized, germinated on MS plates supplemented with kanamycin and 17-day old kanamycin-resistant seedlings were pooled for protein extraction. A representative western blot is shown in Figure 5.2. Many of the transgenic tobacco lines had lower levels of 4CL protein as compared to the wild-type plants. To ensure that these results were accurate, screening by western blot analysis was performed twice on all ~80 transgenic lines and duplicate gels were electrophoresed and stained with Coomassie Blue to ensure eveness of protein-loading (not shown). The protein extracts used in the western blot screening represent a pool from seedlings of homozygous and heterozygous genotype. Individuals homozygous for the transgene were identified by examining the T2-generation of selected lines. Transgenic lines which consistently showed lower levels of 4CL compared to that in wild-type seedlings were allowed to self and set seed (T2-generation). The T2-seeds were plated on MS media supplemented with kanamycin and seedlots which had 100% kanamycin-resistant seedlings, and were thus likely homozygous for the antisense transgene, were stored for future analysis (Table 5.3). The 4CL-protein levels and 4CL enzyme activities of these putative homozygous lines have not been determined due to time constraints.

Transgenic tobacco plants with lower 4CL-protein levels, as demonstrated by western blot analysis, appeared relatively normal in size and growth habits compared to that of wild-type SR1 tobacco plants. The number of flowers and the color of the petals in the transgenic plants were similar to that of the wild-type tobacco plants. One exception is that transgenic-tobacco plants with lower 4CL-protein levels appeared to have curved leaf-margins (repand) whereas wild-type tobacco leaf-blades tend to be planar. One transgenic line, containing 1.6-kb of the potato *4CL* cDNA in sense

orientation (Eco-Kpn Sense, transgenic line #9.1), had severely curved leaf-margins and the intervening tissue between the secondary veins were buckled (Figure 5.3).

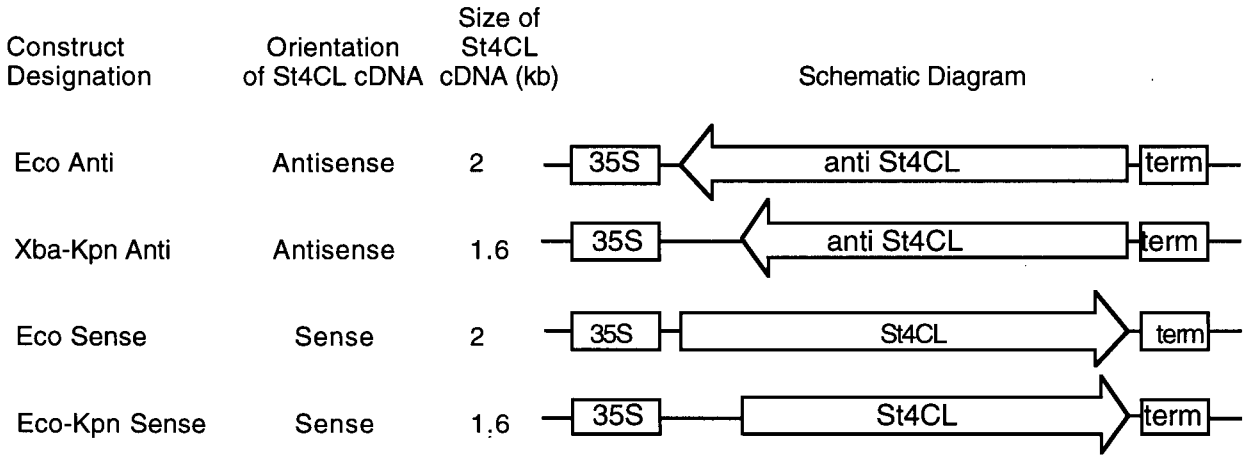

Figure 5.1: Summary of potato-*4CL* cDNA constructs. The potato *4CL* cDNA (St4CL) was subcloned in sense and antisense orientation behind the CaMV 35S promoter (35S). The DNA constructs included the 35S polyadenylation/termination sequences (term). Designation of the DNA-constructs stem from the restriction enzymes used during the subcloning process (see section 2.10 for details).

Table 5.1: Characteristics of primary transgenic-tobacco plants and their seeds.

Construct Designation	# Primary Transformants	# Fertile Transformants	# Transformants with Non-viable Seeds	# Unusual Seedlots*
Eco Anti	17	15	1	2
Xba-Kpn Anti	29	27	3	5
Eco Sense	19	18	0	2
Eco-Kpn Sense	28	25	3	3
TOTAL	93	85	7	12

* Unusual seedlots are ones which had low germination frequencies or produced seedlings with short roots or chlorotic leaves.

Table 5.2: The proportion of kanamycin-resistant (KanR) to kanamycin-sensitive (KanS) T1 tobacco-seedlings.

Eco Anti				Eco Sense			
Transgenic Line	# KanR plants	# KanS plants	KanR:KanS ratio	Transgenic Line	# KanR plants	# KanS plants	KanR:KanS ratio
1	26	5	5.2	1	33	10	3.3
2	34	7	4.8	2	38	18	2.1
3	35	2	17.5	3	47	0	∞*
4	21	11	1.9	4	31	14	2.2
6	41	16	2.5°	5	0	40	0
8	17	12	1.4	6	41	21	1.9
9	28	10	2.8	7	47	1	47
10	24	6	4	9	40	8	5
11	28	13	2.1	10	37	20	1.9
12	22	7	3.1	11	42	15	2.8
14	28	0	∞°	12	53	0	∞
15	18	10	1.8	13	28	16	1.8
16	50	0	∞	14	38	14	2.7
17	37	10	3.7	15	60	4	15
				16	39	14	2.8†
				17	53	0	∞
				18	24	18	1.3
				19	24	5	4.8

Xba-Kpn Anti				Eco-Kpn Sense			
Transgenic Line	# KanR plants	# KanS plants	KanR:KanS ratio	Transgenic Line	# KanR plants	# KanS plants	KanR:KanS ratio
1	53	9	5.8	1	64	0	∞
2	16	13	1.2*	2	61	20	3°
3	39	18	2.2	3	21	21	1*
4	56	4	14	4	55	14	4
5	14	6	2.3*	5	42	17	2.5
6	54	0	∞	6	57	6	9.5
8	27	15	1.8	7	49	17	2.9
9	28	3	9.3	8	58	21	2.8
10	48	5	9.6	9	58	7	8.3
11	54	7	7.7	10	26	14	1.9*
13	49	5	9.8	11	23	7	3.3
14	0	62	0	13	37	18	2
15	38	12	3.2	14	45	14	32
16	22	18	1.2	18	39	11	3.5
17	45	4	11.3	20	55	4	13.8
18	41	9	4.6†	21	48	20	2.4
20	19	17	11	22	24	16	1.5
21	32	2	16*	23	39	25	1.6
22	41	3	14	24	31	7	4.4
24	29	12	2.4	26	39	19	2
26	0	45	0	27	59	15	4
27	30	13	2.3	28	38	0	∞
28	38	9	4.2*				
29	44	6	7.3				

†Seedlings with short roots.
° Seedlings with chlorotic, yellow leaves.
* Seedlots with low germination frequency.

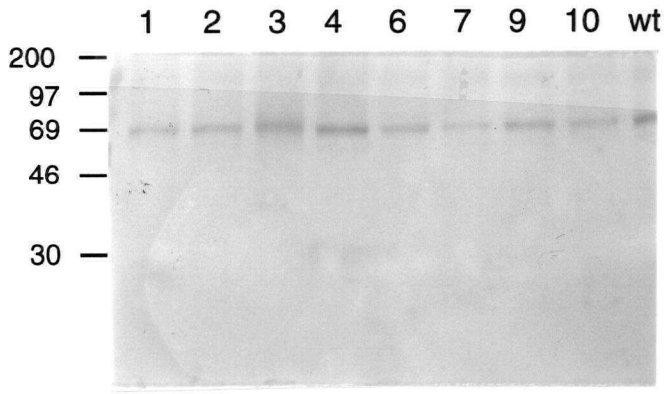

Figure 5.2: An example of a western blot used to screen transgenic tobacco plants. Protein extracts are from pools of T1-tobacco plants transgenic for the 2-kb potato *4CL* cDNA subcloned in sense orientation behind the CaMV 35S promoter (Eco Sense). Numbers above the figure refer to the transgenic line whereas wt refers to proteins from wild-type SR1 seedling. Molecular mass standards (in kDa) are on the left.

Construct Designation	Transgenic Lines with Lower 4CL Protein	Putative Homozygous Individuals (100% KanR T2 Seedlings)				
Eco Anti	9	9.2	9.3	9.6	9.7	9.8
	10	10.4	10.5	10.7	10.8	
	11	11.1	11.2	11.7	11.8	
	16	16.8	16.9			
Xba-Kpn Anti	1	1.3	1.6	1.8		
	15	15.2	15.6	15.8		
	17	17.2	17.3	17.4	17.5	17.7
	29	-				
Eco Sense	1	1.1	1.2			
	7	7.2				
	10	10.7	10.8			
	14	14.4	14.6	14.7		
	15	15.2	15.8			
Eco-Kpn Sense	2	2.2	2.3	2.4	2.5	2.8
	9	-				
	10	10.4	10.5	10.7		
	18	18.6	18.7	18.8		

Table 5.3: Summary of *4CL*-suppressed transgenic-tobacco lines. Transgenic lines with lower 4CL-protein levels as demonstrated by western blot analysis are listed together with putative homozygous individuals within each transgenic line.

Figure 5.3: An extreme case of altered leaf morphology in *4CL*-suppressed transgenic tobacco. Transgenic tobacco "Eco-Kpn Sense 9.1" is heterozygous for the 1.6-kb potato-*4CL* cDNA, subcloned behind the CaMV 35S promoter, transgene. Note the curving of the leaf-margin and the buckling of the tissue between the secondary veins.

5.2.2 Discussion

Approximately 9% of the primary transformants were sterile and have since died, making further examination of them impossible. Whether the sterility was caused by low 4CL activity is unknown; however, it is conceivable that suppression of *4CL* may cause sterility since flavonols, downstream-products of the 4CL-catalyzed reaction, have been shown to be important in some plants for pollen development and pollen-tube growth (Yistra *et al.*, 1992; 1994). Sense-suppressed *PAL* tobacco-plants had abnormal flowers containing stamens with short filaments as compared to those of wild-type tobacco plants (Elkind *et al.*, 1990). This is in contrast to the *4CL*-suppressed tobacco plants reported here which have unusually long filaments. How the down-regulation of *4CL* (or *PAL*) leads to structural differences in the stamen is not immediately obvious and remains to be resolved in the future. Sense-suppressed *PAL* plants had white or faded-pink flowers, an observation not paralleled here in the *4CL*-supressed tobacco plants. This latter result was unexpected since anthocyanins are downstream-products of the 4CL-catalyzed reaction so that one would predict that a block in 4CL should cause a block in anthocyanin accumulation. Without direct measurement of the residual 4CL enzyme activity remaining in the transgenic

tobaccos, it is difficult to speculate on the mechanism in which anthocyanins are made in these plants. It should be noted that antisense-*4CL Arabidopsis* plants with ~8% residual 4CL activity accumulated anthocyanins in a manner analogous to wild-type *Arabidopsis* plants (see section 5.3.2).

The cause of the unusual leaf morphology in antisense-*4CL* tobacco plants is unknown. However, one possibility is that the lower levels of 4CL activity may result in lower levels of accumulated lignin and that this may cause an abnormal rate of cell expansion between the highly lignified trachery elements in the vasculature as compared to the parenchyma-cells in the intervening tissue. Sense suppression of *PAL* in tobacco (Elkind *et al.*, 1990) also produced transgenic plants with altered leaf morphology; however, the leaves were described as "spoon-like" and "epinastic" in nature. Sense-suppressed *PAL* plants accumulated lower levels of lignin compared to wild-type plants (Elkind *et al.*, 1990; Bate *et al.*, 1994) thereby supplying a possible link between the biochemical lesions with the observed morphology. The antisense-*DAHP* potato plants were reported to have structural differences in stem-diameter, stem-length, and growth habits but these results are puzzling since the levels of residual DAHP synthase activity did not correlate with the levels of lignin (Jones *et al.*, 1995). However, in other studies, transgenic tobacco (Ni *et al.*, 1994), transgenic potato (Jones *et al.*, 1995; Yao *et al.*, 1995), and transgenic *Arabidopsis* (see section 5.3.2) plants had lower levels of lignin but did not exhibit differences in leaf- or stem-morphology.

The effect of antisense *4CL* on transgenic tobacco needs to be investigated further; however, I have chosen to concentrate on the *Arabidopsis* system and the results of these studies are described below.

5.3 *4CL*-Suppressed *Arabidopsis* Plants

5.3.1 Generation of *4CL*-Suppressed *Arabidopsis* Plants

In order to generate *Arabidopsis* plants with suppressed *4CL* levels, approximately 1.6-kb of the *Arabidopsis 4CL* cDNA (Lee *et al.*, 1995a) was subcloned behind the parsley-*4CL1* promoter or the 35S promoter in sense or antisense orientation (Figure 5.4) and transformed into *Arabidopsis* using *Agrobacterium*-mediated root-transformation. Two *Arabidopsis* accessions were used; the RLD ecotype, which has been reported to be easier to transform and regenerate (Dr. L. Kunst, personal communication), and the Columbia ecotype, which is one of the more commonly studied varieties of *Arabidopsis*. As predicted, the RLD variety of *Arabidopsis* was more efficiently transformed and regenerated such that 27 antisense *4CL* transgenic RLD-lines were obtained whereas only 8 antisense-*4CL* transgenic Columbia-lines were obtained (Table 5.4). The primary transformants (T0) were allowed to self and set seed while in tissue culture (see section 2.11). The T1-seeds were selected for the presence of the *nptII* transgene and then allowed to self and set seed. The T2-generation was screened to isolate seedlots with 100% kanamycin-resistance seedlings and these putative homozygous lines were used for western blot analysis. Protein extracts from wild-type and antisense-*4CL* transgenic *Arabidopsis* stems were used to screen for plants with decreased levels of 4CL protein, using an antibody raised against the parsley 4CL. Screening by western blot analysis was performed twice to ensure their accuracy and duplicate gels were electrophoresed and stained with Coomassie Blue to ensure eveness of protein-loading (not shown). More than 50% of the transgenic RLD-lines had lowered 4CL-protein levels and the efficacy of the parsley *4CL1* promoter (8 suppressed lines out of 12) was comparable to that of the 35S promoter (9 suppressed lines out of 15) given the small sample-size. Although only a few transgenic Columbia-lines were obtained, many of them had lower levels of 4CL protein and both the parsley-*4CL1* promoter (4 suppressed lines out of 6) and the 35S promoter (2 suppressed lines out of 2) were effective in causing

antisense RNA suppression. An example of a western blot is shown in Figure 5.5. *Arabidopsis* lines transformed with the sense constructs were not examined but are stored as T2-seeds.

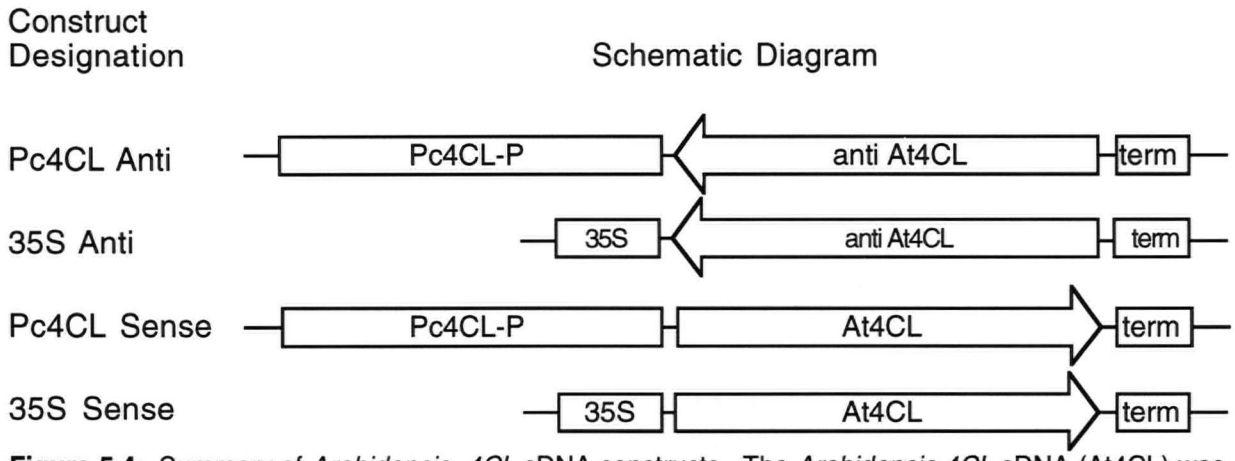

Figure 5.4: Summary of *Arabidopsis -4CL* cDNA constructs. The *Arabidopsis 4CL* cDNA (At4CL) was subcloned in sense and antisense orientation behind the CaMV 35S promoter (35S) or the parsley-*4CL1* promoter (Pc4CL-P). The DNA constructs included the 35S polyadenylation/termination sequences (term).

Construct Designation	Ecotype	# Primary Transformants
Pc4CL Anti	RLD	12
35S Anti	RLD	15
Pc4CL Sense	RLD	22
35S Sense	RLD	21
Pc4CL Anti	Columbia	6
35S Anti	Columbia	2

Table 5.4: Summary of transgenic *Arabidopsis* plants generated.

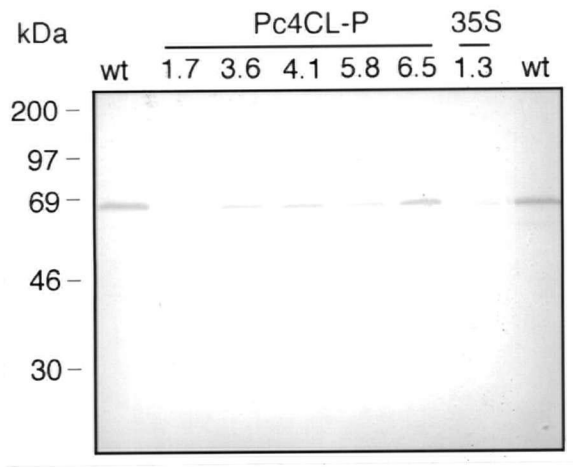

Figure 5.5: An example of a western blot used to screen transgenic *Arabidopsis* plants. Protein extracts are from stems of *Arabidopsis* plants (Columbia ecotype) transgenic for the *Arabidopsis 4CL* cDNA subcloned in the antisense orientation behind the parsley-*4CL1* promoter (Pc4CL-P) or the CaMV 35S promoter (35S). Numbers above the figure refer to the transgenic line whereas wt refers to proteins isolated from the stems of wild-type plants. Molecular mass standards (in kDa) are on the left.

5.3.2 Characteristics of *4CL*-Suppressed Lines

Based on western blot analysis, four antisense-*4CL Arabidopsis* lines with lowest levels of 4CL protein were selected for detailed analysis. The four transgenic *Arabidopsis*-lines appeared normal with similar size and growth habits as that of wild-type plants of the same variety (Figure 5.6) and genomic Southern blot analysis indicated that the transgene was stably integrated in these *Arabidopsis* lines (Figure 5.7). Despite their normal appearance, these plants exhibited significant differences at the biochemical level. 4CL enzyme assays using *Arabidopsis*-stem extracts showed that the transgenic lines had 15% (Pc4CL Anti, RLD ecotype, homozygous line 11.3), 50% (35S Anti, RLD ecotype, homozygous line 11.1), 20% (Pc4CL Anti, Columbia ecotype, homozygous line 1.7) and 8% (35S Anti, Columbia ecotype, homozygous line 1.3) residual 4CL-enzyme activity compared to that of wild-type plants of the same variety (Figure 5.8). For simplicity, these transgenic lines were designated RLD:Pc4CL, RLD:35S, COL:Pc4CL, and COL:35S respectively.

Lignin is one of the major downstream-products of the 4CL-catalyzed reaction. To examine the affect on lignin accumulation in the *4CL*-suppressed *Arabidopsis* lines, lignin was extracted from the bolting stems using the thioglycolic acid method. Figure 5.9 show that the lignin levels, as expressed per mg of fresh weight, were reduced in the antisense lines. Similar results were obtained when the lignin content was expressed per mg dry (alcohol-insoluble residue) weight (not shown). The degree of lignin reduction corresponded well with the levels of residual 4CL-enzyme activity found in the transgenic plants (compare Figures 5.8 and 5.9). Although the lignin content followed the same pattern as the levels of residual 4CL-enzyme activity, only the plants with less than 20% residual 4CL-activity had lignin levels that were significantly less than that found in wild-type plants. When the lignin content is plotted as a function of 4CL enzyme activity (Figure 5.10), it becomes clear that lignin levels begin to drop precipitously when 4CL activity is below 20% of that found in wild-type plants. Extrapolation of the curve in Figure 5.10 could provide a method for predicting

the levels of lignin that will be accumulated in plants with a given level of 4CL activity. For example, plants with 5% residual 4CL enzyme activity would be predicted to have ~75% decrease in lignin content.

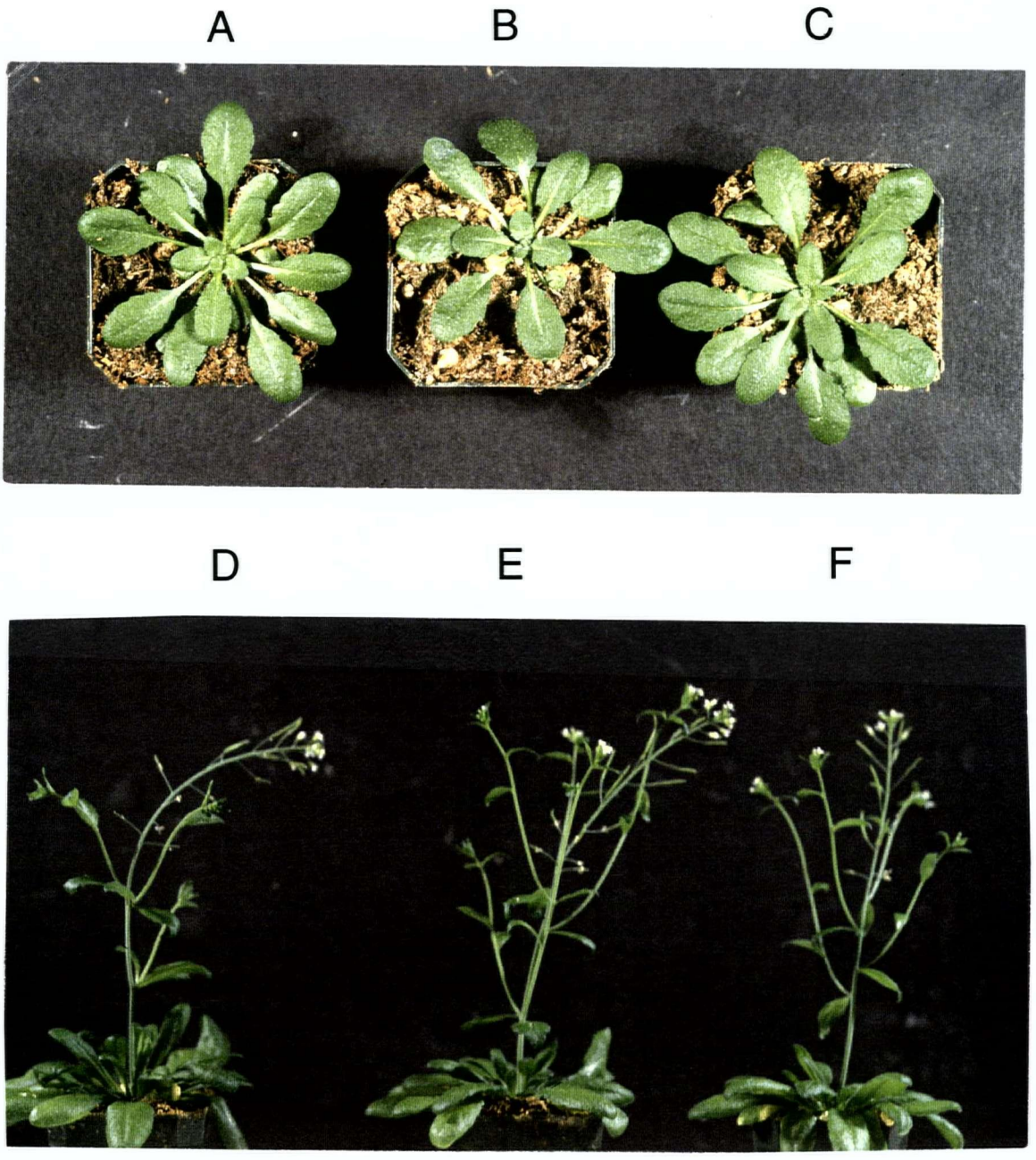

Figure 5.6: Phenotype of antisense-*4CL* suppressed transgenic *Arabidopsis*. *Arabidopsis* plants, Columbia ecotype, were transformed with the *Arabidopsis 4CL* cDNA subcloned in antisense orientation behind the parsley-*4CL1* promoter or the CaMV 35S promoter. Plants are: A and D, COL:Pc4CL; B and E, COL:35S; and C and F, wild-type.

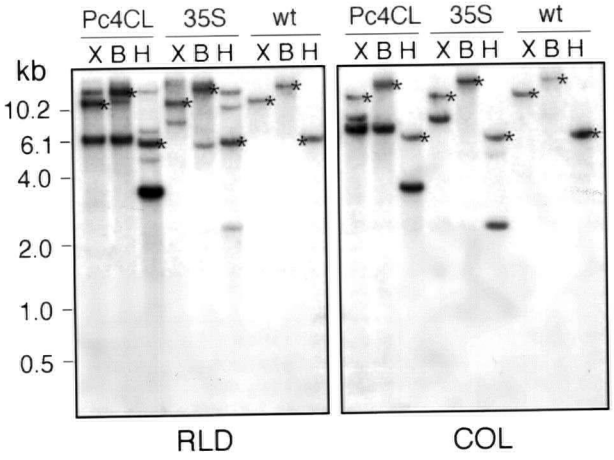

Figure 5.7: Genomic Southern blot analysis of transgenic *Arabidopsis*. Genomic DNA (10 µg) from RLD (RLD) and Columbia (COL) *Arabidopsis* plants were digested with different restriction enzymes, transferred onto nylon membranes and used in Southern blot analysis. Plants were either untransformed (wt) or transformed with the *Arabidopsis 4CL* cDNA subcloned in antisense orientation behind the parsley-*4CL1* promoter (Pc4CL), or behind the CaMV 35S promoter (35S). The blot was probed with the 2-kb *Arabidopsis 4CL* cDNA and asterisks indicate hybridization-signals arising from the endogenous *Arabidopsis 4CL* gene. *Xba*I (X) cuts the transgene to release a fragment containing the promoter and the antisense *4CL* cDNA. *Bam*HI (B) cuts the transgene once at a location 5' to the promoter. *Hind*III (H) cuts the DNA construct to release the entire transgene containing the promoter, the antisense *4CL* and the termination sequence. These restriction enzymes do not cut within the *Arabidopsis 4CL* cDNA.

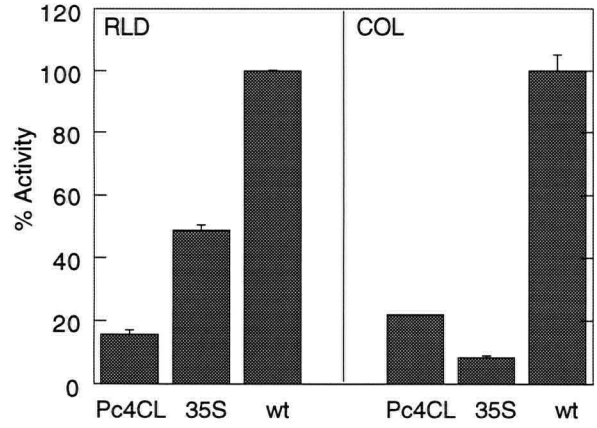

Figure 5.8: 4CL enzyme activity in transgenic and wild-type *Arabidopsis*. Proteins from the bolting stem of *Arabidopsis* plants were used for 4CL enzyme assays. Activity is expressed as a percentage of the activity in untransformed (wt) RLD or Columbia plants. Results are averaged from three determinations and error bars represent standard deviations.

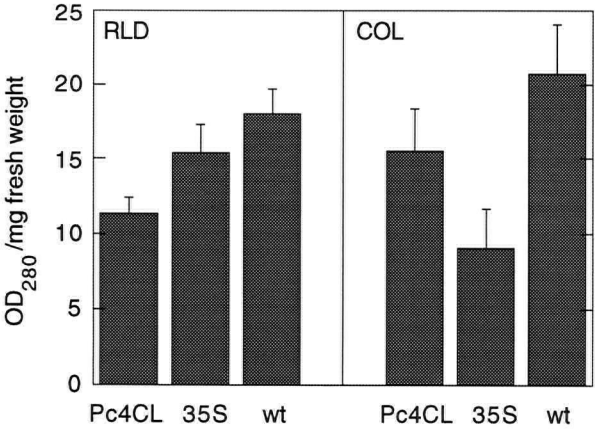

Figure 5.9: Lignin content in transgenic and wild-type *Arabidopsis*. Lignin was extracted from the bolting stem of *Arabidopsis* plants using the thioglycolic acid method and lignin amount is expressed as the optical density at 280 nm. Plants were either wild-type (wt) or transformed with the antisense *4CL* construct as indicated. Results are averaged from three determinations and error bars represent standard deviations.

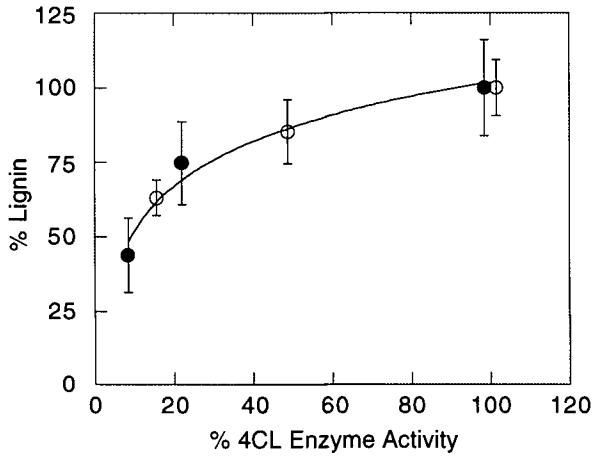

Figure 5.10: Lignin content plotted as a function of 4CL enzyme activity. The results from Figures 5.8 and 5.9 are summarized in a single plot showing the relationship between lignin accumulation and 4CL enzyme activity. Results are expressed as a percentage of the lignin content and the 4CL enzyme activity in wild-type plants. Open circles represent data from the RLD ecotype of *Arabidopsis* and closed circles represent data from the Columbia ecotype of *Arabidopsis*. Data points represent the average of three determination and the error bars represent standard deviation in lignin determination.

Another major phenylpropanoid-product thought to be derived from cinnamoyl:CoA derivatives are the anthocyanins. To examine if a block in 4CL would affect endogenous anthocyanin levels, wild-type and antisense-*4CL Arabidopsis* seedlings were visually scored to characterize pigment accumulation. Anthocyanins have been shown to accumulate in the hypocotyls and the rim of cotyledons during early development of *Arabidopsis* seedlings (Kubasek *et al.*, 1992). Antisense-*4CL Arabidopsis* seedlings appeared to accumulate purple pigments in the same manner as wild-type seedlings (not shown). There was no observable difference in pigmentation intensity nor timing or location of pigment accumulation. This result was unexpected since anthocyanins are downstream-products of the 4CL-catalyzed reaction and one would predict that a decrease in 4CL function might lead to a decrease in end-products. Flavonoids have been shown to accumulate in the petals and the seed coat of *Arabidopsis* (Shirley *et al.*, 1995) and one way to examine flavonoid content is by fluorescence microscopy. Flavonoids absorb UV-light so that tissues which contain flavonoids do not fluoresce under UV-excitation. In contrast, tissue which lack flavonoids fluoresce blue under UV-light (Shirley *et al.*, 1995). When examined under fluorescent microscopy, there were no observable differences in the petals and seeds of wild-type and transgenic *Arabidopsis* plants (not shown).

We further characterized the antisense plants by testing for differences in the induced accumulation of anthocyanin. Plants were placed under high-intensity white-light to elicit environmentally-induced anthocyanin production. After 24h of high-intensity white-light treatment, anthocyanins were extracted from the leaves of wild-type and antisense-*4CL* *Arabidopsis* plants. As previously reported (Feinbaum and Ausubel, 1988), 24 h of high-intensity white-light treatment caused an accumulation of purple pigments in the vegetative tissues (not shown). However, extraction and quantitation of anthocyanins showed that there was no significant difference in anthocyanin content between the light-treated *4CL*-suppressed plants and the light-treated wild-type plants (Figure 5.11). These results indicated that anthocyanins and flavonoids accumulate in antisense *4CL* plants to levels analogous to the levels found in wild-type *Arabidopsis* plants.

To examine the expression of *4CL* and *CHS* in antisense-*4CL* suppressed lines subsequent to high-intensity white-light treatment, northern blot analysis was performed. Total RNA was isolated from leaves of antisense-*4CL* suppressed and wild-type plants before and after high-intensity white-light treatment. Hybridization of the northern blot to a *CHS* probe showed that *CHS* steady-state RNA levels increased dramatically after light-treatment in both the antisense and wild-type plants (Figure 5.12A, CHS). Re-hybridization of the same blot to a single-stranded *4CL* riboprobe showed that the steady-state levels of *4CL* transcripts were relatively abundant in the RLD:35S transgenic and in wild-type plants. The *4CL* mRNA levels were less abundant in the RLD:Pc4CL and COL:Pc4CL transgenic lines and was almost undetectable in the COL:35S transgenic line (Figure 5.12A, 4CL). Furthermore, *4CL* transcript levels in the wild-type and the transgenic lines did not increase after high-intensity white-light treatment (Figure 5.12A, 4CL). In agreement, 4CL protein levels

as demonstrated by western analysis was highest in wild-type and RLD:35S plants; lower in RLD:Pc4CL and COL:Pc4CL plants; and very low in the COL:35S plants (Figure 5.12B). The levels of 4CL protein did not increase significantly after 24 hours of high-intensity white-light stress (Figure 5.12B). These results show that: 1) high-intensity white-light caused an increase in *CHS* mRNA levels but no apparent changes in the levels of *4CL* mRNA or 4CL protein; and 2) the levels of *4CL* mRNA and 4CL protein in the antisense-suppressed leaves parallel the levels of residual 4CL enzyme activity (Figure 5.8) and 4CL protein found in the stems.

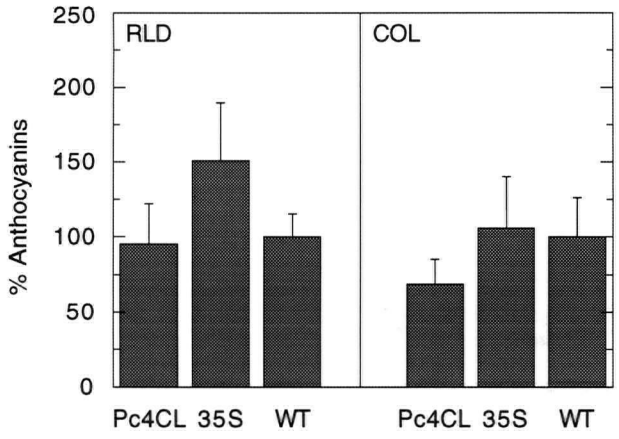

Figure 5.11: Anthocyanin levels in transgenic and wild-type *Arabidopsis* after 24 hour high-intensity white-light treatment. Anthocyanin levels are expressed as a percentage of the levels in wild-type plants (WT). Transgenic lines are as indicated. Results are averaged from three determinations and error bars represent standard deviations.

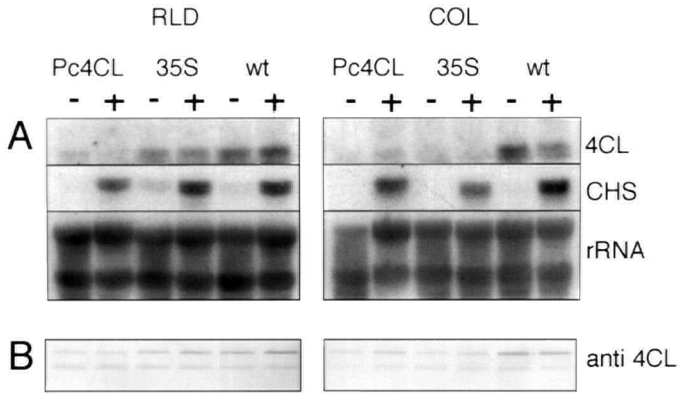

Figure 5.12: Steady-state levels of *CHS* RNA, *4CL* RNA, and 4CL protein in high-intensity white-light treated transgenic and wild-type *Arabidopsis* leaves. (A) Total RNA was isolated from leaves which were untreated (-) or had been exposed to high-intensity white-light for 24 hours (+). RNA (10 μg) was separated on formaldehyde gels, transferred onto nylon membranes and hybridized sequentially to the *Arabidopsis 4CL* (single-stranded probe) and *CHS* probes. Hybridization to a rRNA probe demonstrated evenness of loading. Blots were washed at high stringency. (B) Western blot analysis was performed using 25 μg of protein from the tissues described above. The blot was reacted with a polyclonal antibody raised against the parsley 4CL (anti 4CL).

5.3.3 Wound-Activated Gene Expression in *4CL*-Suppressed Lines

Many of the phenylpropanoid genes are coordinately regulated so that during pathogen attack and environmental stress, the genes encoding enzymes in the phenylpropanoid-biosynthetic pathways are sequentially or simultaneously induced (Ragg *et al.*, 1981; Fritzemeier *et al.*, 1987; Lois *et al.*, 1989). More recently, genes encoding enzymes in the shikimic acid pathway (Henstrand *et al.*, 1992; Görlach *et al.*, 1995) and the oxidative pentose phosphate pathway (Fahrendorf *et al.*, 1995) have also been shown to be activated in cell cultures by elicitor treatment and light treatment. The transcriptional activation of genes in these pathways likely have a role in the biosynthesis of phenylalanine or the conversion of this amino acid into a variety of phenylpropanoid-products. We used wounding as a representative stress-stimulus to determine if genes involved in primary and secondary metabolism are coordinately regulated in *Arabidopsis*. An extensive study of this nature has not been reported before in *Arabidopsis*.

Mature, fully-expanded leaves were wounded and, after 0, 0.5, 1, 2, 4, and 6 h, were harvested for RNA extraction. Total RNA was isolated for northern blot analysis (Figure 5.13). The RNA samples were electrophoresed in triplicate blots and probed with genes encoding enzymes in glycolysis (hexokinase, *HEXO*; phosphofructo kinase, *PFK*), the oxidative pentose phosphate pathway (glucose 6-phosphate dehydrogenase, *G6PDH*; 6-phosphogluconate dehydrogenase, *6PGDH*), the shikimic acid pathway (3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase, *DHS1*; 5-*Enol*pyruvylshikimate-3-phosphate synthase, *EPSPS*), the general phenylpropanoid pathway (phenylalanine ammonia-lyase, *PAL*; cinnamate 4-hydroxylase, *C4H*; 4-coumarate:CoA ligase, *4CL*), and the lignin-biosynthetic pathway (cinnamyl alcohol dehydrogenase, *CAD*). In wild-type Columbia *Arabidopsis* plants, steady-state mRNA levels for *6PGDH, DHS1*, *EPSPS*, *PAL*, *C4H* and *4CL* increased subsequent to wounding and reached maximal levels 1 to 2 hours after wounding (Figure 5.13). Wound-induced accumulation of *6PGDH*, *DHS1*, *EPSPS*, *PAL*, and *C4H* RNA was

also observed in the *4CL*-suppressed Columbia lines, COL:Pc4CL and COL:35S, despite the significantly lower levels of *4CL* transcripts, 4CL protein and 4CL enzyme activity in these lines (Figures 5.13, 5.12, and 5.8). Taking into account minor variations in RNA-loading in each lane, as determined by hybridization to a rRNA probe, the wound-induced activation of all of these genes (with the exception of *4CL* itself) occurred with the same timing and intensity in leaves from the antisense-*4CL* suppressed plants as compared to those from wild-type plants. Identical results were obtained with the wounded leaves of wild-type and antisense-*4CL* suppressed RLD-lines of *Arabidopsis* (not shown). Hybridization signals were extremely weak, or undetectable when *HEXO*, *PFK*, *G6PDH*, and *CAD* probes were used (not shown).

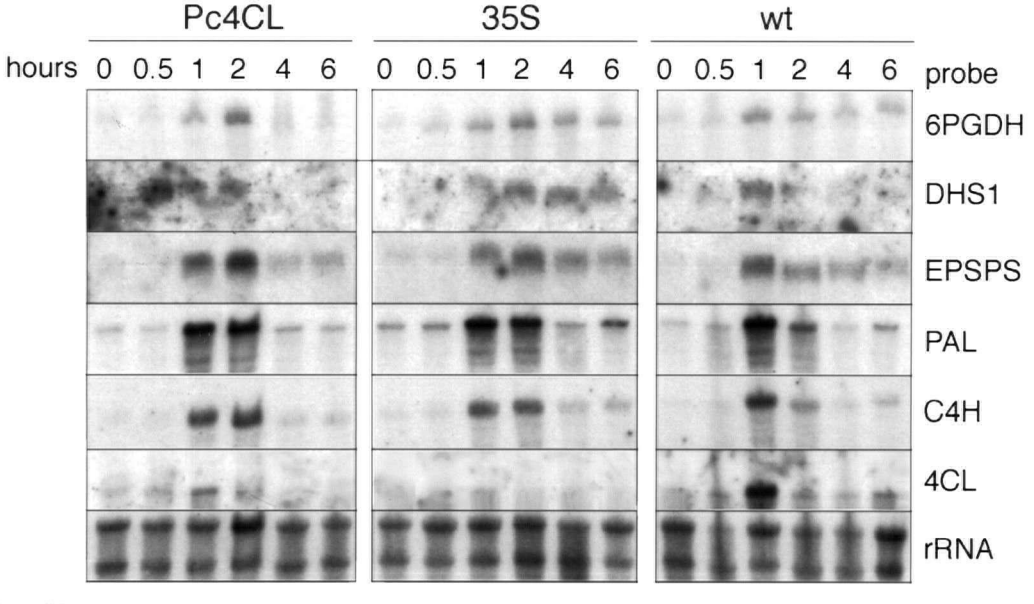

Figure 5.13: Wound-induced RNA accumulation in wild-type and antisense-*4CL* suppressed *Arabidopsis* leaves. Columbia *Arabidopsis* plants were wild-type or were transformed with the antisense *4CL* construct as indicated. Total RNA was isolated from mature, fully-expaned leaves which were wounded for 0, 0.5, 1, 2, 4 and 6 h. RNA samples (10 μg) were electrophoreses in triplicate, in formaldehyde gels, transferred to nylon membranes and hybridized to the *Arabidopsis*-genes encoding 6-phosphogluconate dehydrogenase (6PGDH), 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DHS1), 5-*enol*pyruvylshikimate-3-phosphate synthase (EPSPS), phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL, single-stranded probe). Hybridization of the blots to a rRNA probe demonstrated evenness of RNA-loading; the blot used to probe *PAL*, *C4H* and *4CL* transcripts is shown as an example (rRNA). Blots were washed at high stringency.

5.3.4 Discussion

Using antisense-RNA technology, we have generated *Arabidopsis* plants with suppressed 4CL activity such that one transgenic line, COL:35S, had 4CL enzyme activity as low as 8% compared to that of wild-type plants. The parsley-4CL antibody recognized the *Arabidopsis* 4CL and screening of the transgenic lines by western analysis was an effective method in detecting 4CL suppressed lines. The lowered levels of 4CL enzyme activity in the transgenic lines were paralleled by the lower levels of 4CL protein, as demonstrated by western blot analysis, and the lower levels of steady-state *4CL* RNA levels. Both the 35S promoter and the parsley-*4CL1* promoter were capable of suppressing *4CL*. This was not unexpected since promoter-*GUS* fusion analysis has shown the parsley-*4CL1* promoter to direct proper developmental- and wound- induced *GUS* expression in *Arabidopsis* (Lee *et al.*, 1995a). The level of *4CL* suppression observed here is comparable to the level of suppression reported in the *PAL* sense-suppressed tobacco lines which showed 90% (Maher *et al.*, 1994) to 95% (Elkind *et al.*, 1990) lower levels of PAL activity. Similarly, antisense-*CAD* tobacco plants had a 93% decrease in CAD activity (Halpin *et al.*, 1994). Some antisense *COMT* (caffeic acid 3-*O*-methyltransferase) tobacco plants had activities as low as 2% of that found in the wild-type counterparts (Atanassova *et al.*, 1995) whereas in transgenic poplar, residual COMT activity was ~5% compared to wild-type plants (Van Doorsselaere *et al.*, 1995). A transgenic line with no 4CL activity was not found and it is not clear whether this was because antisense RNA rarely causes 100% suppression, or whether a plant completely lacking 4CL activity is nonviable.

The COL:35S transgenic-line had approximately 50% lower levels of lignin but did not show any differences in morphology or growth habit. Lignin levels have also been decreased in transgenic potato plants over-expressing the tryptophan decarboxylase gene (Yao *et al.*, 1995) and transgenic tobacco plants suppressed in COMT activity (Ni *et al.*, 1994) with no reported differences in plant morphology. In

contrast, a subset of the *PAL*-suppressed tobacco plants (Elkind *et al.*, 1990) and the antisense-*DAHP* potato plants (Jones *et al.*, 1995) exhibited differences in the growth of the leaves and stems, respectively. Whether decreasing lignin levels results in abnormal plant anatomy probably depends on the percentage of lignin that has been reduced. Results reported here and in the literature (Ni *et al.*, 1994; Yao *et al.*, 1995) suggests that a decrease in lignin content of 50% or less allows the plants to maintain their structural integrity and wild-type appearance. Abnormal leaves and stunted growth of *PAL*-suppressed tobacco plants were reported to have as little as 10% lignin compared to wild-type tobacco-stems (Bates *et al.*, 1994) suggesting that highly reduced lignin levels result in irregular phenotypes. Jones *et al.* (1995) reported anomalous stem-growth in potato plants which had 35% to 75% of the lignin levels found in untransformed plants; however, the lignin content in these plants did not correlate with the levels of DAHP synthase activity, making it difficult to understand the relationship between the residual enzyme activity, the lignin content, and the resulting phenotype.

Lignin content was only significantly different from that of wild-type plants when the 4CL enzyme activity was lowered >80%. This suggests that *in vivo* levels of 4CL activity are in excess and only when the 4CL activity has been suppressed by >80% does the enzymatic reaction restrict carbon flow into lignin formation. Similar results were reported with PAL where the enzyme only becomes rate-limiting in lignin biosynthesis when PAL activity is decreased by 75%-80% compared to wild-type levels (Bates *et al.*, 1994). In light of these experiments it is likely that, under normal laboratory conditions (uninduced and non-stressed), the general phenylpropanoid enzymes are in excess compared to that that is required for lignin biosynthesis.

There have been a number of biotechnological attempts to decrease the quantity or quality of lignin for the purpose of increasing the cattle digestibility of forage crops, or to decrease the cost and environmental impact in the pulp and paper industry (Halpin *et al.*, 1994; Ni *et al.*, 1994; Dwivedi *et al.*, 1994; Atanassova *et al.*, 1995; Van

Doorsselaere *et al.*, 1995). Transgenic plants containing antisense genes encoding COMT (Dwivedi *et al.*, 1994; Atanassova *et al.*, 1995, Van Doorsselaere *et al.*, 1995) and CAD (Halpin *et al.*, 1994) did not exhibit quantitative differences in lignin accumulation; however, the monomeric composition was altered. Tobacco plants with low levels of COMT had a lower ratio of syringyl/guiacyl (S:G) subunits and this qualitative change was observed only when COMT activity was decreased by >80% compared to wild-type levels (Atanassova *et al.*, 1995; Van Doorsselaere *et al.*, 1995). *CAD*-suppressed tobacco plants accumulated hydroxy- and methoxy-cinnamylaldehyde monomers which caused the xylem to appear red-brown in color and this phenotype was observed when the CAD activity was decreased by >84% compared to that of wild-type plants (Halpin *et al.*, 1994). Genetic mutants with defects in the corresponding genes show comparable phenotypes. The *brown midrib* (*bm3*) mutant of maize has a defective *COMT* gene and the lignin in these mutants also has an altered S:G ratio (Vignols *et al*, 1995). The *bmr*6 mutant of sorghum has a lower level of CAD activity, it deposits aldehyde lignin monomers, and it has a red-brown color (Pillonel *et al.*, 1991). The *fah1* (*sin1*) mutant of *Arabidopsis* has a defective ferulate 5-hydroxylase (*F5H*) gene and it lacked sinapate-derivatives, including syringyl lignin (Chapple *et al.*, 1992). It is interesting to note that low levels of DAHP, PAL and 4CL caused a decrease in lignin quantity whereas low levels of F5H, COMT, and CAD resulted in changes in lignin quality but no differences in lignin quantity. Taken together, it is tempting to suggest that manipulation of genes early in the phenylpropanoid pathway may regulate carbon flux into lignin biosynthesis whereas manipulation of genes downsteam in the lignin-biosynthesis pathway modulate the kinds of lignin-subunits formed. This observation should be considered with caution since in one report, antisense *COMT* plants had lower levels of lignin (Ni *et al.*, 1994) and the *bm3* mutant of maize, which has a defective *COMT* gene also has lower levels of lignin (Grand *et al.*, 1985). The *bm6* mutant of *Sorghum* (Pillonel *et al.*, 1991) has lower COMT and CAD activities and it has a changed lignin composition as well as a

15%-25% decrease in lignin content. Transgenic tobacco plants over-expressing tryptophan decarboxylase had a preferential decrease in syringyl subunits as compared to the guaiacyl subunits in addition to an overall lower lignin content (Yao *et al.*, 1995). In this manuscript, suppression of *4CL* has caused a decrease in lignin content; however an unexpected affect on lignin quality may also have occurred. Studies to determine whether the *4CL* suppressed *Arabidopsis* plants have altered lignin composition in addition to lower lignin quantity are in progress. Preliminary results using Maüle staining suggest that the levels of syringyl lignin is unchanged in antisense *4CL* and wild-type *Arabidopsis* plants (C.J. Douglas, unpublished).

Under the experimental conditions described here, a significant block in 4CL function does not block anthocyanin accumulation during high-intensity white-light stress or during early *Arabidopsis* development. These results are in contrast with *PAL*-suppressed tobacco plants which have lower levels of pigments in the flowers and in the leaves (Elkind *et al.*, 1990; Bates *et al.*, 1994). Similarly, chemical inhibitors of PAL cause lower levels of anthocyanin accumulation in the flowers, seedling, and hypocotyls of a number of plants (Amrhein and Hollander, 1979; Laber *et al.*, 1986). High-intensity white-light treatment caused an increase in steady-state levels of *CHS* mRNA, but no significant increase in *4CL* transcripts or 4CL proteins (Figure 5.12). This latter result is surprizing since *PAL* and *4CL* have been reported to be light-responsive. However, careful examination of the literature reveals that, where induction has been demonstrated, *PAL* and *4CL* transcripts are induced by white-light if the plants were dark-adapted prior to light-treatment (Liang *et al.*, 1989; Ohl *et al.*, 1990; Kubasek *et al.*, 1992; Wu and Hahlbrock, 1992) or if the white-light was supplemented with UV-light (Lois *et al.*, 1989; Wu and Hahlbrock, 1992). The results presented here suggests that steady-state *4CL* RNA levels do not increase under the high-intensity white-light treatment given (~900 $\mu E\ s^{-1}m^{-2}$) when the plants were grown and maintained under normal levels of continuous light (~120 $\mu E\ s^{-1}m^{-2}$) prior to treatment. The possible induction of *4CL* RNA accumulation in dark-adapted or UV-

light treated *Arabidopsis* plants was not investigated. Thus, while we cannot yet conclude that *4CL* is not light-activated under some conditions, it is not high-intensity white light-induced in the way that *CHS* is (Figure 5.12; Feinbaum and Ausubel, 1988). In this respect, we have uncovered a differential response between *4CL* and *CHS* towards high-intensity white-light treatment and this suggests that *CHS* and *4CL* are regulated by different mechanisms.

The fact that high-intensity white-light resulted in: 1) no increase in *4CL* transcripts, 2) no increase in 4CL protein, 3) dramatic increase in *CHS* transcripts, and 4) anthocyanin accumulation, suggests that the residual 4CL enzyme activity (≥8%) in the antisense plants is sufficient to supply 4-coumaroyl:CoA esters for anthocyanin biosynthesis. Another possibility is that anthocyanins that are observed subsequent to high-intensity white-light stress are not synthesized *de novo*, but are converted from pre-existing precursors. In such a situation, the low levels of 4CL in the antisense plants may not be an impediment as long as the plants have had sufficient time to accumulate a sizable metabolic-pool. This metabolic pool may serve as a buffer such that changes in 4CL activity do not greatly affect anthocyanin production. The dramatic increase in steady-state levels of *CHS* suggest that the activity of CHS is probably involved in the *de novo* biosynthesis of at least a portion of the observed anthocyanins. It is possible that CHS is a very efficient enzyme such that the 4-coumaroyl:CoA esters made are immediately drawn into the biosynthesis of flavonoids.

During wounding, steady-state mRNA levels of *6PGDH, DHS1*, *EPSPS*, *PAL*, *C4H* and *4CL* increased to maximal levels 1 to 2 hours post-wounding. Although there has been some evidence that the genes encoding these enzymes are up-regulated coordinately, these reports are derived from different plants under different stimuli (Chappell and Hahlbrock, 1984; Davis and Ausubel, 1989; Fahrendorf *et al.*, 1995; Görlach *et al.*, 1995). In *Arabidopsis*, this is the first report demonstrating the coordinate regulation of genes encoding enzymes in the oxidative pentose phosphate

pathway, the shikimic acid pathway and the general phenylpropanoid pathway. In the *4CL* suppressed plants, the genes examined here were also coordinately regulated and responded to wounding irrespective of the significantly lower levels (≥8%) of endogenous 4CL activity. This suggests that gene activation is due to early events in the wound-signal and not a consequence of metabolic-flux through 4CL. A similar conclusion was reported with elicitor-induced gene expression in the presence of chemical inhibitors of PAL (Görlach *et al.*, 1995; Fahrendorf *et al.*, 1995). Conserved *cis*-elements like the P-, A-, and L- boxes and putative Myb-binding sequences have been identified in the promoters of genes involved in phenylpropanoid metabolism thereby potentially providing a mechanism for the coordinate, yet independent, up-regulation of gene expression (Lois *et al.*, 1989; Grotewald *et al.*, 1994; Sablowski *et al.*, 1994; Logemann *et al.*, 1995). Whether these *cis*-elements are found in the promoters of all the wound-induced genes described here is a question yet to be answered.

Using antisense-RNA technology, transgenic *Arabidopsis* lines with suppressed 4CL activity have been generated. The regulation of phenylpropanoid metabolism and the role of 4CL in carbon flow into phenylpropanoid-product formation can be characterized using these *4CL*-suppressed lines. Further and more detailed analysis of the biochemical- and stress-related phenotypes of these lines is beyond the scope of this thesis. Collaborative efforts with other laboratories have been initiated to examine the lignin, phenolic ester, and flavonoid composition of the wild-type and transgenic *Arabidopsis* lines. As well, the response to pathogen attack in the wild-type and transgenic *Arabidopsis* lines is under investigation.

Chapter 6

Conclusions and Future Directions

The work presented here represents a broad examination of 4CL from two model plants. At the level of the DNA, it was shown that 4CL was encoded by a gene-family in tobacco and by a single gene in *Arabidopsis* (chapter 3). This difference in genome-organization is interesting in that it shows that one copy of *4CL* per haploid genome is sufficient; however in plants like tobacco, multiple copies of *4CL* exist. Because 4CL is encoded by a single gene in *Arabidopsis*, it may be possible to identify genetic mutants of *Arabidopsis* that are defective at the *4CL* locus. The *Arabidopsis* research community has made available T-DNA tagged lines which may aid in the identification of *4CL* null-mutants (McKinney *et al.*, 1995), a phenotype that was not achieved in the antisense-RNA suppressed lines reported here. Sequencing of the entire *Arabidopsis* genome and anonymous cDNA clones (expressed sequence tags, ESTs; Newman *et al.*, 1994) will eventually result in the theoretical availability of all *Arabidopsis* genes and cDNAs.

Using northern blot analysis, *4CL* expression was examined at the level of the RNA (chapter 3). In tobacco, 4CL is encoded by at least 2 classes of genes; however, members of the classes did not appear to be differentially expressed. The two divergent *4CL* genes were both expressed in tobacco and were both detected in the progenitor species suggesting that multiple-copies of *4CL* exist and that it may even confer an evolutionary advantage. The expression pattern of *4CL* in tobacco and *Arabidopsis* was comparable to the expression of other phenylpropanoid genes and for the most part, can be correlated with the location where phenylpropanoid-products would be expected to accumulate. Of particular interest is the high levels of *4CL* RNA transcripts in the unpigmented portion of the petals of tobacco flowers. This result is in

contrast to the many transgenic studies where phenylpropanoid promoters directed *GUS* expression in the pigmented portion of the petals and no GUS staining in the white part of tobacco petals (Bevan *et al.*, 1989; Liang *et al.*, 1989; Schmid *et al.*, 1990, Hauffe *et al.*, 1991). However, in agreement with the results found here, *in situ* hybridization (Reinold *et al.*, 1993) clearly show accumulation of *4CL* transcripts in the white part of the flower. One area of further investigation would be to elucidate the function of 4CL and PAL in the white portion of tobacco petals. Recently, flavonoid-specific staining has shown high levels of flavonoids in the white part of tobacco petals (Reinold, 1995) suggesting that *4CL* and *PAL* may be expressed in this tissue for flavonoid biosynthesis.

The characteristics of the 4CL proteins were examined by expressing the two divergent tobacco *4CL* cDNAs in *E. coli* (chapter 4). The two recombinant-4CL proteins had almost identical enzyme activities and did not use sinapate as a substrate. This latter result was particularly enigmatic since crude tobacco-stem extracts contained 4CL activities which use sinapate as a substrate and results in the literature demonstrated high levels of syringyl lignin in tobacco stems (Halpin *et al.*, 1994). We found evidence of other divergent *4CL*-genes (genomic Southern blot analysis and Genbank sequences) and this represents an area of further investigation. Cloning and characterization of these highly divergent tobacco-*4CL* genes (*4CL3* class genes) may provide evidence of differential expression of the tobacco *4CL* genes and more importantly, expression of class 3 *4CL* cDNAs may generate 4CL isoenzymes which use sinapate as a substrate. If a sinapate-specific 4CL isoenzyme was identified, the gene(s) encoding for the isoenzyme may be used to engineer plants with higher levels of syringyl lignin, a biotechnologically desirable trait.

Expression of the *4CL* cDNAs in *E. coli* allowed us to identify a novel activity against the 4CL which may involve the post-translational modification of 4CL. The putative post-translational modification has been characterized sufficiently to suggest

that a kinase is involved in determining the substrate specificity of tobacco 4CL. Further studies might include:

1. Determine if 4CL itself is phosphorylated or if another protein is phosphorylated resulting in altered 4CL substrate-utilization.
2. Purify the putative kinase and clone the corresponding gene using generated antibodies or synthesized oligonucleotides derived from protein micro-sequencing data.
3. Determine when and where endogenous tobacco 4CL utilizes cinnamate as a sustrate and link the biochemistry with the physiology. Namely, what products are made from cinnamoyl:CoA esters and how do these downstream products relate to the biology of the plant?

Using antisense RNA, we have generated, for the first time ever, transgenic *Arabidopsis* lines with severely suppressed 4CL levels and we have begun characterizing these transgenic lines. A particularly unique result is the apparently normal accumulation of anthocyanins in antisense-*4CL* suppressed plants. Down-regulation of *PAL* by sense suppression in tobacco (Elkind *et al.*, 1990) and chemical inhibitors of PAL activity (Amrhein and Hollander, 1979) resulted in a decrease in anthocyanin accumulation. *Arabidopsis* mutants with defective *CHS* (*tt4*), *CHI* (*tt5*), or *DFR* (*tt3, ttg)* fail to accumulate anthocyanidins in the seeds, the seedlings and the leaves (Shirley *et al.*, 1995). The results presented here, show for the first time that a decrease in 4CL enzyme activity by >90% did not decrease anthocyanin accumulation. Further studies are required before we can understand the basis of this unexpected result. It is possible that alternative pathways exist for the biosynthesis of anthocyanins that by-pass the 4CL-catalyzed reaction. Antisense suppression of *COMT* did not decrease lignin levels and this provided support that an alternative pathway, involving CCoAOMT, in monolignol biosynthesis existed (Atanassova *et al.*, 1995; Van Doorsselaere *et al.*, 1995). Another possibility is that the 8% residual 4CL enzyme activity in the antisense *4CL* suppressed lines may be sufficient to allow for

wild-type levels of anthocyanins to be made. One way to resolve this would be to identify genetic mutants of *4CL* which completely lack 4CL activity. Since 4CL is encoded by a single gene in *Arabidopsis*, such a mutant can, in theory, be isolated. However, since 4CL is important in lignin biosynthesis (Figure 5.10), it is conceivable that such a mutation may be lethal.

Another area which deserves analysis is plant-pathogen interactions. *PAL*-suppressed tobacco plants have been shown to be more susceptible to pathogen invasion possibly because of diminished levels of pre-existing chlorogenic acid (Maher *et al.*, 1994). Is this phenomenon paralleled in the antisense-*4CL Arabidopsis* plants? The antisense-*4CL* plants described here are distinct from the *PAL*-suppressed plants since furanocoumarins, phenolic acids, and other phenylpropanoid derivatives are/may be made after the PAL-catalyzed step but before the 4CL-catalyzed step (see section 1.3.3) and the response of antisense-*4CL* plants to pathogens may be different from the response in *PAL*-suppressed plants. It should be noted that the pathways leading to the biosynthesis of some phenylpropanoid products (e.g. hydroxybenzoates, salicylic acid) have not yet been clearly established (French *et al.*, 1976; Yazaki *et al.*, 1991; Schnitzler *et al.*, 1992; Löscher and Heide, 1994).

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