STRUCTURE AND EXPRESSION OF PHENYLALANINE AMMONIA-LYASE-ENCODING GENES IN HYBRID POPLAR

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We accept this thesis as conforming to the required standard

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Date Dec. 21, 1995
Abstract

Genomic PAL sequences were isolated from a hybrid poplar genomic library. Three PAL genes were identified, two similar to each other (PAL1 and PAL2) and one diverged (PAL3). PAL1 and PAL2 were placed onto a poplar linkage map. The 3' and 5' regions of these two genes were sequenced, compared, and examined for functional motifs. An interesting motif was a promoter sequence conserved between PAL1 and PAL2, which is similar to AC boxes found in the promoters of other phenylpropanoid genes. The single intron was identified in both PAL1 and PAL2 and surrounding sequences compared for the two genes. The intron's position is conserved when PAL1 and PAL2 are compared to each other and to other plant PAL genes. However, PAL1 and PAL2 possess introns of the same size, which has not been observed for any other plant PAL gene family.

Expression of these genes was also examined. The promoters of PAL1 and PAL2 were fused to the reporter gene GUS and transformed into tobacco, where they directed essentially identical patterns of expression in vascular tissues and in ovules. No evidence of differential expression of PAL1, PAL2, or PAL3 was found in these transgenic tobacco or in comparative Northern blot analyses of RNA extracted from various poplar tissues. Seasonal variation in levels of PAL protein and PAL mRNA in bud/leaf and secondary xylem of poplar was investigated using Western and Northern blot analysis. PAL mRNA levels were highest in leaves in March, and PAL protein levels were highest in leaves in April. In secondary xylem, PAL protein levels were highest in May.
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1. General introduction

1.1 Phenylpropanoid metabolism

In plants, the phenylpropanoid pathway is responsible for the formation of several classes of chemical compounds including coumarins, flavonoids, lignin, stilbenes, suberin and simpler phenolic molecules. These chemicals are active biologically as plant pigments, as defensive molecules, in inter-organismal signalling, and as structural materials within the plant body.

Phenylpropanoid metabolism consists of a central pathway (Figure 1) in which three enzymes convert phenylalanine to an activated substrate (the CoA esters of cinnamic acid and its derivatives). This activated substrate is then used in a number of different possible branch pathway reactions leading to the biosynthesis of the various classes of phenylpropanoid compounds.

![Figure 1. The central pathway of phenylpropanoid metabolism.](image)

The first enzyme in the central pathway is phenylalanine ammonia-lyase (PAL) which diverts fixed carbon in the form of phenylalanine from primary metabolism into phenylpropanoid metabolism, forming cinnamic acid by a deamination of phenylalanine. The next enzyme in the pathway, cinnamate-4-hydroxylase (C4H), hydroxylates the 6-membered aromatic ring of cinnamic acid at the para-position, making 4-coumarate. This compound is the substrate for 4-coumarate-Coenzyme A ligase (4CL). The activity of this enzyme catalyzes the addition of Coenzyme A to form a thio-ester derivative.
Since PAL acts as the gateway from primary to phenylpropanoid secondary metabolism, the regulation of its activity at both the level of gene expression and at the level of enzyme activity is of great interest. The structure and expression of PAL genes have been extensively studied in herbaceous angiosperms, but relatively little was known about such genes in woody plants when this thesis project was initiated. This thesis describes the structure and expression of two PAL genes isolated from poplar, a model organism for the molecular biology and genetics of woody plants.

### 1.2 Products of phenylpropanoid metabolism

Phenylpropanoid metabolism gives rise to several important classes of chemical compounds including the flavonoids, lignin and other cell-wall associated phenolics, coumarins, and stilbenes. These molecules serve a wide variety of functions as phytoalexins, UV-protectants, nod-gene inducers, anthocyanin pigments, and structural components. This section will review two classes of phenylpropanoid compounds, lignin and the flavonoids, which are of particular significance in poplar. Lignin is a major structural component of all woody perennials including poplar, and flavonoid compounds have been implicated as possible defense-related molecules for poplars; evidence for this latter point is presented in section 1.4. Coumarins, lignans, and tannins will not be discussed.

#### 1.2.1 Lignin

Lignin is a complex polymer found in the secondary cell walls of higher plants as well as ferns and club mosses (Higuchi, 1985). Lignin serves two general purposes: one is structural and the other is water-proofing. Lignin is also produced around sites of wounding or pathogen attack as a healing or blocking agent (Smith et al., 1994).

The three monomers of the lignin polymer are derived from para-coumaric acid by a series of successive hydroxylations and methylations at the 3, 4, and 5 positions on the phenolic ring by hydroxylases and O-methyltransferases. Activated thioester derivatives are formed by 4CL,
and then the thioesters are reduced to aldehyde groups by the action of a reductase. Finally, the aldehydes are converted to the alcohols by cinnamyl alcohol dehydogenase (CAD). Thus, para-coumarate gives rise to para-coumaryl alcohol, ferulate to coniferyl alcohol, and sinapate to sinapyl alcohol (Lewis and Yamamoto, 1990). (Figure 2)

These monolignols are probably produced in association with the endomembrane system. They may be stored as glycosides in the central vacuole and then exported to the site of polymerization at the secondary cell wall through the activity of endomembranous vesicles. At the secondary wall, they undergo an apparently randomized polymerization after being subjected to the activity of peroxidase or laccase, which causes them to become reactive free radicals (Higuchi, 1985). Recent analyses indicate that dehydration of the cell wall as it matures may confer some topological organization to the phenolic rings of the lignol moieties in the wall (Terashima et al., 1993).

Different species form different types of lignin; that is, lignin which contains a preponderance of one or several of the three possible classes of monolignols. The taxonomic distribution of lignin types has been reviewed in detail (Lewis and Yamamoto, 1990). In brief, ferns, gymnosperms, monocots, and dicots contain lignin, as do the club mosses. Gymnosperms contain largely guaiacyl lignins which consist mainly of the monolignol coniferyl alcohol. However, this is a generalization and there are exceptions. Angiosperms contain lignins derived from both coniferyl and sinapyl alcohols; again, this is a generalization. Graminaceous monocots contain lignins made up of all three monolignols. That is, monocots incorporate a substantial proportion of
para-coumaryl lignols into their lignin. The cell walls of grasses also contain esterified hydroxycinnamic acids.

Lignin composition also differs between the different cell types within a single organism. For example, compression wood in gymnosperms seems to contain more para-coumaryl moieties than does normal wood (Higuchi, 1985). Separate studies on different species of poplar (Terashima et al., 1986; Wolter et al., 1974) have demonstrated that the early vessel walls of developing aspen wood contain guaiacyl moieties, whereas fibres developing later contain more syringyl moieties.

Differences in the pattern of incorporation of the different monolignols are found even at the subcellular level. In both angiosperms and certain conifer species, guaicyl-rich lignin tends to be deposited early, in the primary wall, in the middle lamella, and in the cell corners. Secondary walls tend to contain mostly syringyl alcohol derivatives. The exception to this pattern are the tracheids of conifers whose secondary walls contain only guaicyl lignin (Lewis and Yamamoto, 1990; Wolter et al., 1974).

1.2.2 Flavonoids

Flavonoids and anthocyanins are also derivatives of the phenylpropanoid pathway. Their structure may be described as two aromatic rings joined via a 3-carbon bridge. They are formed by the condensation of the phenylpropanoid derivative para-coumaryl CoA with three malonyl-CoA units, catalyzed by the enzyme chalcone synthase (Figure 3).

![Figure 3. Action of the enzyme chalcone synthase.](image-url)
Flavonoids are often found in plants as glycosides. They are often coloured and/or light absorbant, thus functioning as plant pigments (Given et al., 1988; Taylor and Briggs, 1990). The anthocyanin class of pigments has been characterized in many plant species, for example, maize, carrot, Arabidopsis seedlings, strawberry fruit, Petunia, and Antirrhinum, and in many different biological situations. The genetic and molecular bases of anthocyanin biosynthesis have been well characterized in some systems, especially maize and Antirrhinum (Dooner and Robbins, 1991).

Flavonoids absorb UV light and this property allows them to function as UV protectants. In parsley, UV-protective flavonoids accumulate in the epidermis of the leaf; about 20 different flavonoid derivatives have been observed (Lois et al., 1989; Hahlbrock and Scheel, 1989).

Flavonoids are also known to function as inter-organismal signal molecules, particularly in the context of legumes and their Rhizobium symbionts (Maxwell and Phillips, 1990; Recourt et al., 1992). In addition, flavonoids extracted from several plant species have been demonstrated to function as phytoalexins (chemicals synthesized by plants possessing antibiotic properties). Of particular note are isoflavonoid derivatives glyceollin, phaseollin, and pisatin from Glycine max, Phaseolus vulgaris, and Pisum sativum respectively (Preisig et al., 1991).

1.3 Functions of phenylpropanoid products

1.3.1 Developmental functions

During the growth of higher plants, phenylpropanoid derivatives are required to fulfill a variety of functions. As mentioned above, lignin accumulates in the cell walls of certain plant tissues as they differentiate, particularly in the cell walls of vessel elements and tracheids of angiosperm and gymnosperm water conduction systems, and in the walls of schlerenchymal derivatives, for example the fibre bundles of phloem. Lignin serves to waterproof the water conductive vasculature, strengthens the protective fibre bundles, and contributes to the overall structural rigidity of the plant body, enabling land plants to attain the upright growth habit necessary for effective collection of light energy for photosynthesis (Raven, 1986). Smaller
phenolic molecules are also found esterified to the cell walls of many plant cell types (Davin and Lewis, 1992).

Suberin is another phenylpropanoid-derived polymer with a structural role. Its most notable occurrence is in the Casparian strips around each of the endodermis cells in the roots of most vascular plants. This waxy substance blocks passage of water and minerals through extracellular spaces and forces all materials to be taken up into the stele through the cytoplasm of the endodermis cells, thus ensuring specificity of uptake (Raven, 1986). The protective, waterproof, outer phellem layers of bark are also heavily suberized (Esau, 1978), as is the abscission layer which seals off the plant stem from the external environment upon leaf-drop (Raven, 1986).

The flavonoid pigments described in section 1.2.2 may function in reproductive processes to attract pollinators to flowers, or herbivores to fruits to assist in dissemination of plant seeds (Raven, 1986). In addition, these compounds may serve to absorb light energy and warm the pigmented tissues, thus allowing physiological function and/or survival at lower temperatures (Lovelock, 1991).

Constitutively present, visibly colored pigments may also absorb stressful UV radiation in the same way as induced flavonoids (Lois et al., 1989), preventing evolution of harmful radicals within the cytoplasm of the exposed cells.

Some phenolic derivatives may also function as signalling molecules. For example, dehydro-diconiferyl alcohol glycoside derived from Vinca tissue cultures is able to mimic cytokinin activity, suggesting a signalling role in growth regulation (Lynn et al., 1987). Flavonol 3-O-glycosides confer the ability to germinate upon pollen of conditionally-male petunia plants (Vogt and Taylor, 1995). Flavonoid-derived molecules produced by soybean and alfalfa function in signalling interactions with symbiotic bacteria (Recourt et al., 1992; Maxwell and Phillips, 1990).
1.3.2 Functions associated with environmental stress

As sessile organisms, plants are unable to flee environmental stresses. Thus, they germinate only in environments that will be hospitable to them or they attempt to adapt biochemically and developmentally to their situation. The activity of the phenylpropanoid pathway is implicated in the response of plants to stresses such as attack by pathogenic viruses, bacteria, and fungi, ultraviolet light stress, wounding, and grazing by insects and other animal herbivores. The induction of phenylpropanoid metabolism in general, and specifically of PAL enzymatic activity and transcription, in response to various environmental stresses has been observed in many systems.

Resistance to pathogen attack

Historically, there are two terms applied to types of resistance to pathogens observed in plants. Vertical or qualitative resistance is an all-or-none response which is usually conditioned by a single genetic locus. The possession of a single dominant allele at this locus generally confers complete and effective resistance of that plant to that race of the pathogen. On the other hand, horizontal (or quantitative) resistance confers a general resistance to a pathogenic species. The physiological and biochemical bases of these two types of resistance are, of course, of great interest. The activity of the phenylpropanoid pathway is involved in both types of resistance.

With the above definitions in mind, there are four possible plant responses to potential pathogens (Staskawicz et al., 1995). One is the interaction of the non-host plant with a pathogen which is not in fact a pathogen of that plant (for example, a potato plant being exposed to a tomato pathogen). The potato plant in this case is completely resistant to the pathogen. Thus, either its constitutive components of resistance are sufficient to render that pathogen non-effective, or a rapidly induced resistance response (for example, a hypersensitive response, a localized and rapidly developing necrotic spot which contains the pathogen's advance through the plant) efficiently combats pathogen ingress.
The second type of interaction involves a host plant, for example a tomato plant, challenged by a tomato pathogen. When this tomato is susceptible to that particular race of pathogen, its horizontal or constitutive components of resistance are insufficient to overcome that pathogen's capacity to infect, and disease results. This is a compatible reaction.

In the third sort of interaction, involving vertical or qualitative resistance, a host plant challenged by a pathogen race recognizes the pathogen race and responds with a resistance reaction often characterized by a hypersensitive response. This type of interaction, termed an incompatible interaction, requires the presence of a specific allele at a plant resistance locus and a corresponding avirulence-conferring allele in the pathogen race attempting infection.

Another sort of response may be observed in concert with certain of the above interactions; this response is called systemic acquired resistance (SAR). When a part of a plant is challenged by a pathogen, the rest of the plant may experience a switching-on of various components of biochemical defense, resulting in a generalized heightened resistance to many pathogens throughout the plant body (Staskawicz et al., 1995).

The defensive biochemistry evoked during these interactions include phytoalexin production, formation of physical barriers, and the production of proteins such as chitinases, beta-1,3-glucanases, and protease inhibitors which may have an actively deleterious effect on the invasive organism. Phytoalexins (compounds with anti-fungal or anti-bacterial affects) include molecules of furanocoumarin origin in parsley, isoflavonoids in legumes, the diterpenoid casbene in castor bean and sesquiterpenoids in the Solanaceae (Collinge and Slusarenko, 1987; Preisig, 1991). Physical blocking of pathogen ingress, as mentioned above, may also play a part in the resistance response. The localized synthesis of lignin, callose and suberin has been observed as a part of this physical blocking process in some plants (Kombrink et al., 1986; Nicholson and Hammerschmidt, 1992).

The activity of phenylpropanoid metabolism is an important component of certain aspects of plant defense. Many of these defense responses involving production of phenylpropanoid derivatives are preceded by activation of the genes encoding various enzymes of the pathway. For example, in response to elicitor treatment or fungal infections, an increase in PAL enzyme activity and transcriptional activation have been observed in bean, alfalfa, pea, Arabidopsis,
soybean, potato, parsley, tomato, tobacco, and in the suspension cell cultures of pine and poplar (Bell et al., 1986; Dalkin et al., 1990; Yamada et al., 1992; Huang et al., 1994; Ward et al., 1989; Fritzemeier et al., 1987; Lois et al., 1989; Lee et al., 1992; Pellegrini et al., 1994; Campbell and Ellis, 1992; Moniz de Sa et al., 1992).

Phenylpropanoid products are involved in the hypersensitive response (HR) typical of incompatible reactions. In the initial, extremely fast component of the response (in less than three minutes), the necrotic spot of the hypersensitive response is initiated (Nicholson and Hammerschmidt, 1992). Pre-formed phenolics may take part in this reaction, converting from non-toxic to toxic phenolic moieties at the site of the HR, or participating in cross-linking reactions with the cell wall. Ferulic acid is one example of a phenolic compound taking part in these sorts of reactions (Nicholson and Hammerschmidt, 1992). *De novo* synthetic activity on the part of the phenylpropanoid pathway is not implicated in this first, fast sub-class of the hypersensitive response.

However, within three hours of the initiation of an incompatible interaction, activity of the phenylpropanoid pathway is often observed. Enzyme activities are induced and in fact, the genes encoding those enzymes are often also transcriptionally activated, resulting in the formation of more enzyme and thus of newly synthesized phenolic compounds to inhibit the spread of the pathogen. Deposition of lignin (particularly in Graminaceous species) and suberin and synthesis of phytoalexins are observed in the region surrounding the necrotic region. Typically, this class of reaction takes place within the time frame of about 6 to 24 hours; transcription is usually reduced after that point. However, there are a number of studies demonstrating activation of genes within three minutes of inoculation with pathogen or elicitor (Lawton and Lamb, 1987).

Salicylic acid is also suspected to be synthesized *de novo* by the phenylpropanoid pathway in response to pathogen attack. Salicylic acid is postulated to be a signal to the rest of the plant that this type of attack is taking place; constitutive resistance elements are induced, resulting in systemic acquired resistance (SAR) effective for that time against a wide range of pathogens (Nicholson and Hammerschmidt, 1992; Staskawicz et al., 1995). However, a recent study may indicate that salicylic acid is not the communicating compound itself, but rather that the amount of salicylic acid is key in establishing SAR (Lamb, personal communication).
Protection against light stress

When plants are exposed to potentially harmful fluxes of UV light, they may respond by manufacturing UV-absorbing molecules to augment the protection afforded by the constitutively present compounds. The highly conjugated rings of phenolic derivatives make them ideal UV absorbants; the accumulation of UV protectant phenolic compounds is best documented in parsley systems. Flavonoid glycosides are synthesized in the epidermal cells of intact parsley leaves when the plant is irradiated with UV light. The wavelength 350 nanometers has been found to be maximally effective in inducing this response (Hahlbrock and Scheel, 1989). When potato tuber discs are illuminated, they accumulate chlorogenic acid but the function of this compound in this context is unknown (Lamb and Rubery, 1976). In addition, dark-grown spruce suspensions experience a 30-fold increase in extractable PAL activity in response to UV irradiation, and this increase in activity is probably involved in the synthesis of UV-protectant compounds (Messner et al., 1991).

Response to wounding

Phenolic compounds are also involved in plants' responses to wounding, either grazing by herbivores or other mechanical wounding. There are two functional components to the responses to the wounding; one is to seal up or seal off the wound site, and the other is to combat other organisms, either the grazing herbivore or potential pathogens which may attempt to invade the plant through the convenient opening. Suberin accumulates around the wound site, as does lignin, to prevent entry of potential pathogens into otherwise healthy plant tissue (Raven, 1986; Cottle and Kolattukudy, 1982). Other phenolic compounds also accumulate in the immediate area of the wound and systemically throughout the plant, particularly in response to insect grazing (Harley and Fim, 1989). When potato tubers are wounded, they accumulate chlorogenic acid. As in the response to light, the function of this compound in this context is unknown (Hahlbrock and Schell, 1989). The bitterness of many phenolic derivatives may serve as an
antifeedant, especially for mammalian herbivores. The accumulated phenolics may also be phytoalexins synthesized as part of the disease-resistance-like compendium of responses serving to protect the plant from pathogens entering through the wounded site. Other components of this response include the production of pathogenesis-related (PR) proteins, hydroxyproline-rich proteins, chitinases, glucanases, and proteinase inhibitors (Parsons et al., 1989).

1.4 Poplar as a model woody plant

Commonly known as poplar, aspen, or cottonwood, the genus *Populus* is found throughout North America. These small hardwoods grow quickly and many species hybridize readily in nature (Heilman and Stettler, 1985). As a genus, *Populus* is gaining importance both as a pulp source for paper and as a useful laboratory organism for the study of physiology, genetics, and molecular biology of a woody perennial.

Although lignin extraction procedures may give varying results, hardwoods, on the average, seem to contain five to ten percent less lignin by dry weight than do softwoods and thus can provide a fibre stock which is easier and less expensive for the pulp and paper industry to process. Figures from several studies are presented in Table 2. In addition, those processed papers in which hardwood pulps are the major component are whiter and smoother, although of a lower strength than those containing a larger proportion of softwood pulps, since the hardwood fibres are shorter (Personal communication, P. McAuliffe, Scott Paper). Poplars are increasingly being used as preferred pulp source in Canada, since they grow quickly on marginal land unuseable for other agricultural purposes and are an easily renewable resource. Poplars are amenable to culture in short rotation intensive cultivation (SRIC plantations) particularly in the Pacific Northwest and the biomass produced is used as feedstock for pulp and paper manufactures. In response to this economic demand, several productive hybrids have been developed by the breeding programs of Stettler et al. in Washington state during the last three decades (Heilman and Stettler, 1985; Stettler et al., 1988). These F1 hybrids resulted from
crosses between various female western black cottonwoods (*Populus trichocarpa*) and male eastern cottonwoods (*Populus deltoides*). Of particular interest are F1 individuals designated

<table>
<thead>
<tr>
<th>Species</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Jack pine</td>
<td>29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>White spruce</td>
<td>29</td>
<td>29.4</td>
<td>-</td>
</tr>
<tr>
<td>Red maple</td>
<td>24</td>
<td>22.4 to 23.3</td>
<td>-</td>
</tr>
<tr>
<td>Red alder</td>
<td>-</td>
<td>23.9</td>
<td>-</td>
</tr>
<tr>
<td>White birch</td>
<td>-</td>
<td>18.2</td>
<td>-</td>
</tr>
<tr>
<td>Trembling aspen</td>
<td>16</td>
<td>-</td>
<td>17.1 to 19.9</td>
</tr>
<tr>
<td>Black cottonwood</td>
<td>-</td>
<td>21.4</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 1.** Lignin content of selected softwood and hardwood species, reported as percentage of dry weight. (References: 1. MacDonald, 1969; 2. Isenberg, 1980; 3. Einspahr, 1967)

53-242 (male) and 53-246 (female). These superior clones in the F1 family 53 exhibit many desirable growth qualities. The genetic bases of these growth characteristics are under study using quantitative locus mapping, placing them into an increasingly refined poplar linkage map (Bradshaw *et al.*, 1994). Genetically defined material of this three-generation pedigree, illustrated in Figure 4, (parental clones 93-968 and ILL-129, the described F1, and F2 and B1 generations) are under culture here in the Northwest, and are available for genetic and molecular studies.

The superior performance of 53-242 and related hybrids make them good candidates for SRIC in British Columbia and for further genetic improvement through biotechnological methods. Characters which might usefully be improved by these means include reduced or altered lignin as well as greater or altered resistance to rust infections such as those caused by *Melampsora* species (Hsiang and van der Kamp, 1985).

Like all trees, the secondary xylem (wood) of poplar contains relatively large amounts of lignin. The control of the process of wood formation, which utilizes a large proportion of the carbon fixed annually, is a vitally important process. As more is understood about PAL’s role in lignogenesis, perhaps the process can be controlled to suit the desires of humans, for example by altering the quantity or type of lignin deposited in the secondary walls of wood tracheary elements.
Relatively little is known about poplar defense reactions, particularly those which could affect susceptibility to rust. However, some aspects of poplar defense against pathogens may involve phenylpropanoid derivatives. An unidentified phenolic glycoside is produced by aspen in response to exposure to pathogenic fungus (Flores and Hubbes, 1980). Pinocembrin, a flavonoid secreted by the leaves of eastern cottonwood, is a major constituent of the leaf resin of young leaves of that species, and has been demonstrated to have antibiotic properties (Shain and Miller, 1982). When poplar suspension cultures are treated with elicitors, phenylpropanoid biosynthesis increases rapidly, and soluble and cell-wall bound phenolic substances, which may have defensive roles, accumulate (Moniz de Sa et al., 1992). One soluble glycoside was characterized by spectroscopic methods and was identified as a glucose ester of caffeic acid, the aglycone of which has strong anti-fungal properties in vitro (Douglas et al., 1992). An understanding of this involvement of phenylpropanoid metabolism in defense and possible biotechnological manipulation of defense responses leading to increased rust resistance would be useful since plantations often involve large numbers of identical, high-yielding clones growing in close proximity. Such an arrangement is susceptible to extensive damage and economic loss if virulent rust infects the plantation.

Poplar is a useful organism in which to investigate the structure and regulation of plant genes in a woody perennial species. It is an economically important species and breeding
programs have made multi-generation pedigrees available. In addition, poplar is transformable and regenerable (Leple et al., 1992; Kajita et al., 1994), lends itself well to tissue culturing techniques, and has a conveniently small genome size (at least in terms of trees), approximately $2C = 1.2$ picograms of DNA, or about $3.6 \times 10^5$ kilobases (Bradshaw et al., 1994).

1.5 Phenylalanine ammonia-lyase (PAL) - the enzyme

Phenylalanine ammonia-lyase (PAL) activity (EC 4.3.1.5) was first demonstrated in barley tissues (Koukol and Conn, 1961). This enzyme is found in plants and in fungi, but not in animals, and it is one of the most intensively studied enzymes of plant secondary metabolism. The enzyme deaminates the aromatic amino acid phenylalanine to form cinnamic acid, thus diverting carbon from primary metabolism into secondary metabolic pathways in plants. As implied, secondary metabolism in plants is an anabolic pathway; in fungi, PAL may serve a catabolic rather than an anabolic function (Marusich et al., 1981).

1.5.1 PAL activity detected or purified

This enzyme activity has been described in many plants, including monocotyledonous and dicotyledonous species, gymnosperms, and a fern (for example, Camm and Towers, 1973; Jangaard, 1974; Jones, 1984; Campbell and Ellis, 1992c; Whetton and Sederoff, 1992).

The enzyme has been purified from a number of plants to the point that antibodies could be raised against the protein; these species include bean (Bolwell et al., 1986), pine (Whetton and Sederoff, 1992), and sweet potato (Tanaka et al., 1977).
1.5.2 Characteristics of the enzyme

Structural characteristics

Information regarding structure of the PAL enzyme is available from a wide variety of sources. In most of the plant species studied, the PAL monomer has a molecular weight of 77 to 83 kD. For example, isoforms of bean PAL with MW of 77 and 83 kD have been reported (Bolwell and Rodgers, 1991). Amino acid sequences deduced from nucleic acid sequences support these observations, typically predicting PAL open reading frames of about 700 amino acids. PAL from different species forms enzymatically active tetramers ranging in size from 300 to 330 kD (Camm and Towers, 1974; Jones, 1984). Iso-electric points for PAL are generally found to be in the acidic range; for example, two different forms of bean PAL are reported to possess pI's of 4.8 and 5.4 (Bolwell and Rodgers, 1991) and in alfalfa, pI's for several isoforms are reported as falling between 5.1 and 6.1 (Jorrin and Dixon, 1990). Two poplar PAL isoforms with different pI's are demonstrated to be expressed in a baculovirus-expression system from a single cDNA (McKegney et al., in press). PAL from alfalfa is reported to be highly hydrophobic (Jorrin and Dixon, 1990). The PAL enzyme has been observed to be associated with carbohydrate by several authors (for example, Havir 1979; Bolwell and Rodgers, 1991). One study involving wounded potato tubers indicates that the PAL enzyme may have to be glycosylated in order to be active; treatment with tunicamycin inhibited glycosylation of PAL subunits and reduced levels of extractable PAL activity with no effect on rates of enzyme synthesis or degradation (Shaw et al., 1990). There is also some evidence for other post-translational modification, specifically an N-terminal blockage of attempted N-terminal sequencing, although these results may be artifactual. This type of blockage has been observed both in PAL produced by a eukaryotic expression system (McKegney et al., in press) and in extractions of PAL protein from bean cell cultures (Bolwell and Rodgers, 1991).
Functional characteristics

The PAL enzyme's functional characteristics have also been extensively studied. Typical $K_m$ values reported in the older literature range from 30 micromolar to 15 millimolar (Camm and Towers, 1974). Examples from more recent articles include 40, 70 and 110 micromolar for the three studied isoforms of alfalfa PAL (Jorrin and Dixon, 1990), and 27 micromolar for the PAL isolated from Pinus taeda (Whetton and Sederoff, 1992); these results are in keeping with earlier observations. Optimal pH for PAL activity has been observed to be moderately alkaline, between pH 8.5 and pH 9.5 (Camm and Towers, 1974). Negative co-operativity has sometimes been observed (Jones, 1984) but is now mostly discounted as a phenomenon derived from the co-purification of multiple isoforms (which may or may not participate in heterotetramer formation) with different affinities for phenylalanine (Bolwell et al., 1985; Jorrin and Dixon, 1990; Whetton and Sederoff, 1992). Often the partially purified or purified enzyme demonstrates activity against tyrosine as well as phenylalanine, particularly those preparations derived from Graminaceous species (for example, Reid et al., 1972; Jangaard, 1974). This activity is usually observed to be more labile than the PAL activity (Camm and Towers, 1973).

According to studies on the related enzyme histidase (Taylor et al., 1991), the active sites of histidase and of PAL apparently contain a dehydroalanine residue formed from a highly conserved, post-translationally modified serine. This electrophilic dehydroalanine may be conjugated with a Schiff's base or other double bonds within the enzyme structure so as to activate the phenylalanine amino group to form a better leaving-group. These studies used NaB$_3$H$_4$ or $^{14}$CN$^-$ to label the active site; when the protein is then hydrolyzed, either tritiated alanine or $[^{14}$C] aspartic acid are the released radioactively labelled amino acids. Several models have been proposed to explain the role of this dehydroalanine in the deamination of phenylalanine by PAL. The accepted model (Hanson and Havir, 1970) postulates a nucleophilic attack by the amino group of the substrate upon the dehydroalanine as the initial step in the deamination. A recent paper presents an alternative model (Schuster and Retey, in press) in which the dehydroalanine is attacked by electrons from the ortho position in the phenyl ring to initiate the process. Both models revolve around the generation of an intermediate from which the
$\textit{Si}$ hydrogen can be removed easily, and the effective stabilization of the carbanionic intermediate generated by the abstraction of the $\textit{Si}$ hydrogen from the beta carbon.

1.5.3 Recombinant PAL

The availability of cDNA clones encoding PAL from various organisms has made possible the expression of large quantities of easily purified enzyme in several heterologous systems, including \textit{E. coli} and baculovirus. This large-scale synthesis of PAL protein facilitates some types of studies and makes others possible. For example, since the recombinant PAL preparations generally contain a single PAL isoform, the characteristics of that particular form can be determined without encountering the problems associated with purifying the isoform away from other isozymes.

Recombinant expression has been used by some researchers to confirm identity of cDNA clones from an initial screen of cDNA libraries. cDNA clones isolated from a sweet potato library were confirmed by expression in \textit{E. coli} followed by examination of the molecular weight of the major product (77 kD, which is appropriate for sweet potato PAL) and PAL enzyme assay using labelled phenylalanine as substrate (Tanaka et al., 1989). The same sort of confirmation was applied to putative PAL cDNA clones from loblolly pine except that crude extracts were subjected to western analysis (Whetton and Sederoff, 1992).

Other studies have been carried out to test various hypotheses regarding aspects of PAL enzyme activity. A single PAL cDNA from parsley was expressed in \textit{E. coli} and was capable of converting phenylalanine to cinnamic acid, demonstrating that the functional catalytic center can be formed in a bacterial system. This means that either the formation of the center is auto-catalytic or that the exogenous factors required for its formation are available both in plants and in bacteria (Schulz et al., 1989).

The cDNA clones encoding all four PAL genes from parsley were expressed by producing and cleaving glutathione S-transferase fusions (Appert et al., 1994). An apparent turnover rate for active sites was determined to be $1/22$ seconds, and other enzymic characteristics for single pure parsley PAL isoforms such as $K_m$'s, pH and temperature optima, a low but detectable activity
towards tyrosine, and inhibition $K_i$ values were also measured. No negative co-operativity was observed in this system, supporting the idea that this phenomenon may be caused by the co-purification from plant tissues of multiple isoforms with different affinities for the substrate. The authors conclude that the specific expression patterns of parsley $PAL$ genes observed in planta may be significant not so much for the expression of isoforms (derived from different loci) possessing different kinetic properties, but for expression of uniquely post-translationally modified isoforms possessing different kinetic properties, or of isoforms participating in formation of heterotetramers with unique functions as suggested by Bolwell et al. in 1985, or of isoforms participating in multienzyme complexes with unique functions (Appert et al., 1994).

Poplar $PAL$ was expressed in a eukaryotic system using baculovirus (McKegney et al., in press). The construct directed expression of a protein exhibiting $PAL$ activity, and site-directed mutagenesis changing the proposed active-site serine$^{202}$ to an alanine resulted in loss of this activity. This latter result supports previous work indicating serine$^{202}$ as a key component of the active site (Schulz et al., 1989). Observations regarding $K_m$, inhibition by cinnamic acid, and activity towards tyrosine were all in accord with previous reports of the characteristics of $PAL$ enzymes in other plant species.

1.6 Regulation of $PAL$ activity

Since the various classes of compounds produced by the activity of the phenylpropanoid pathway have significance in many different biological situations, the regulation of phenylpropanoid metabolism at all levels must be sensitive and specific. The regulation of carbon flux through the gateway activity of $PAL$ is particularly interesting and can be regulated on many levels.

Changes in extractable $PAL$ activity have been observed in response to plant hormones, photoperiod, light of various wavelengths, elicitor and pathogenic attack and wounding, endogenous levels of various phenolic products, and tissue-specific developmental signals.
1.6.1 Developmental and hormonal regulation of PAL activity

PAL enzyme activity is regulated during plant development so that the accumulation of phenolic compounds necessary for the normal elaboration of the plant's structure and its functions are synthesized in appropriate cells at appropriate times. For example, PAL activity increases from undetectable levels to a peak soon after germination of seeds of many species examined, and then falls to lower levels (Camm and Towers, 1973).

This enzyme activity has also been shown to have highly specific patterns of regulation when different plant tissues are compared. In 1968, Rubery and Northcote reported that extractable PAL activity was observed only in tissues which were actively lignifying, in a variety of tissues sampled from maple, pea, bean, and parsley (Rubery and Northcote, 1968). PAL activity is also observed in tissues accumulating other phenylpropanoid derivatives as part of a developmental process. For example, the appearance of the anthocyanin compounds which pigment mustard and tomato seedlings during development is preceded by an increase in PAL activity (Beggs et al., 1987; Goud et al., 1991).

This enzyme activity has been demonstrated to be responsive to both endogenous and exogenously supplied plant hormones. The level of PAL activity associated with lignification and xylogenesis can be manipulated with combinations of auxins and cytokinins, for example in differentiating lettuce pith explants (Miller et al., 1985) and in differentiating pine tissue culture systems (Ramsden and Northcote, 1987). Gibberellins have been shown to increase PAL activity, both in maize seedlings (Reid et al., 1972) and in the development of pigmentation in Petunia corollas (Weiss and Halevy, 1989). This latter study identified the stamens as the in planta source of the gibberellic acid.

Evidence for the involvement of plant hormones as signalling molecules in plant responses to pathogen attack is accumulating as well. Endogenously produced ethylene participates in the induction of PAL activity in parsley suspension cells treated with fungal elicitor (Chappell et al., 1984), and seems to play a similar role in the defense responses mounted by rice plants challenged by blast fungus (Haga et al., 1988). Both auxin and ethylene augment PAL activity when bean suspensions are elicited (Hughes and Dickerson, 1990), and intact soybean
seedlings of a resistant cultivar become susceptible to fungal pathogen when treated with abscisic acid. This susceptibility is accompanied by a loss of the normally observed increase in PAL activity (Ward et al., 1989). Thus, abscisic acid inhibits PAL induction in this system.

In bean suspension cultures treated with fungal elicitor, the addition of cinnamic acid and derivatives thereof has been shown to have an inhibitory effect on levels of extractable PAL activity, on amounts of newly synthesized PAL protein, and on transcription rates of PAL mRNA (Bolwell et al., 1986; Bolwell et al., 1988). Similar results have been reported for alfalfa and sunflower hypocotyl systems (Orr et al., 1993; Jorrin et al., 1990). The suggestion has been made that the reduction in PAL activity when cinnamic acid concentration is high is caused by the activation of a PAL degradation system (Bolwell et al., 1986). However, a transgenic tobacco system up-regulated with respect to C4H expression (in theory thus reducing the effective cinnamate pool) does not exhibit the predicted increased PAL expression (Dixon, personal communication). Further experimentation is required to reconcile these results.

1.6.2 Environmental regulation of PAL activity

External stimuli such as challenge by a pathogen, wounding, and light are all environmental factors known to effect PAL enzyme activity. In bean, parsley, pine, and poplar suspension cultures, treatment with an appropriately derived fungal elicitor results in an increase in extractable PAL activity (Cramer et al., 1985; Davis and Hahlbrock, 1987; Campbell and Ellis, 1992; Moniz de Sa et al., 1991). In several cases, increases in phenolic compounds implicated in defense processes, such as lignin and coumarin or isoflavonoid phytoalexins, have been observed concomitant with the increase in PAL activity (Campbell and Ellis, 1992; Davis and Hahlbrock, 1987; Cramer et al., 1985).

When a suspension culture of a resistant tomato cultivar was co-cultivated with avirulent strain of Verticillium albo atrum, increased PAL activity was accompanied by inhibition of fungal growth, while growth was not inhibited when a near-isogenic susceptible tomato line was used (Bernards and Ellis, 1989). Related whole-plant studies show a suppression of extractable PAL activity (as compared to the resistant line) upon infection of the susceptible tomato line by the
virulent fungal pathogen. As well, less suberin coating accumulates in the conductive tissues of
the susceptible line (Lee et al., 1992b). When parsley seedlings undergo a non-host defensive
response to a soybean pathogen, PAL protein accumulates immediately around the small
hypersensitive lesions (Jahnen and Hahlbrock, 1988).

Increases in extractable PAL activity in response to wounding of plant tissues are well-
documented. For example, increases in PAL protein and in extractable PAL activity are observed
in wounded potato tubers (Ishizuka et al., 1991; Shaw et al., 1990). Immunolocalization of PAL in
wounded bean hypocotyls revealed that PAL protein accumulates largely in the epidermal cell
layer (Smith et al., 1993). In another system, whole birch plants subjected to caterpillar grazing
and simulated grazing showed increases in PAL activity locally and systemically (Hartley and
Firn, 1989).

Light also plays a major role in regulating PAL enzyme levels. Regulation of PAL activity
by light may be in response to either developmentally required light cues or stressful levels of
light irradiation. PAL activity has also been demonstrated to be responsive to light as part of plant
circadian and diurnal rhythms. UV light-treated parsley suspension cultures were one of the first
systems in which up-regulation of PAL activity by light was demonstrated (Hahlbrock and
Schroder, 1975). Tissue cultures of carrot (Takeda, 1990) and Gomphrena globosa (Casteneda
and Quintero, 1991) respond to irradiation by various wavelengths of light with increased
extractable PAL activity, and accumulation of anthocyanins or of betalains. These responses
probably mimic normal developmental processes.

Whole plant systems can also be used to demonstrate responses of PAL to light. In pea,
mustard, and tomato seedlings, PAL activity is up-regulated in response to illumination, and is
associated with accumulation of lignin or pigments during normal development (Wilkinson and
Butt, 1992; Beggs et al., 1987; Lercari et al., 1986). In the tomato system, red/far-red illumination
studies indicate that phytochrome mediates the accumulation of PAL activity (Lercari et al., 1986;
Goud et al., 1991).

Studies on several Lemnaceae taxa show that PAL activity levels are regulated in an
innate circadian rhythm under continuous illumination of the intact plant. In addition, under normal
day/night illumination, the plants demonstrate a different, diurnal rhythmicity of PAL activity levels,
with a single peak each day (Gordon and Koukkari, 1978; Knypl et al., 1986). Other plants, including amaranth and beet, also show circadian patterns of PAL activity levels under continuous light (Bopp and Meier, 1973).

1.6.3 Mechanisms regulating levels of PAL enzyme activity

As discussed above, PAL levels are influenced by a myriad of stimuli. Increases in effective PAL activity might result from increased de novo synthesis of PAL, from increased availability of substrate, from activation of inactive forms of the enzyme, by stimulation of higher rates of enzymatic activity, or from decreased rates of degradation of active enzyme. Down-regulation may be achieved through the inactivation and/or degradation of PAL enzyme. In addition, reduction in de novo transcription and translation may contribute to reducing PAL activity over time. Finally, a decrease in available substrate may reduce effective activity in planta.

De novo synthesis of PAL transcripts has been demonstrated by run-on experiments (Cramer et al., 1985; Lawton and Lamb, 1987; Lois et al., 1989) to precede increases in PAL activity in suspension cell cultures. Inhibitor studies using actinomycin D indicate that transcription is necessary for an observed increase in PAL activity in response to various stimuli (for example, Hahlbrock and Ragg, 1975; Goud et al., 1991). However, the higher levels of PAL activity observed in resistant lines of tomato as compared to those of near-isogenic susceptible lines was shown to be due not to increased PAL mRNA synthesis in the resistant line, but rather to suppressed transcription in the susceptible line (Lee et al., 1992). The transcriptional regulation of PAL gene regulation is discussed in further detail in section 1.9.

In concert with transcriptional regulation of PAL gene expression, de novo protein synthesis has been shown to be necessary for observed increases in PAL activity by 35S-labelling of newly synthesized PAL or by the use of cycloheximide to inhibit protein synthesis (Lawton et al., 1983; Hahlbrock and Schroder, 1975; Hahlbrock and Ragg, 1975; Goud et al., 1991). However, in at least one instance, an effective increase in PAL activity upon stimulation was shown to be due not to an increase in rate of synthesis, but rather to a decrease in the rate of degradation of PAL enzyme (Zucker, 1971). As well, a single publication exists reporting that
activation of newly synthesized but inactive PAL protein occurs during the hypersensitive response of bean cells to fungal infection (Da Cunha, 1987).

Although a large body of evidence indicates that de novo synthesis of PAL mRNA and protein is the major mechanism whereby the cell increases PAL activity in response to various stimuli, substrate supply and feed-forward activation may also play some part in the regulation of PAL activity. This hypothesis and supporting arguments and evidence were reviewed by Margna (1977). The most compelling components of the argument are as follows: (1) the ability of PAL to deaminate phenylalanine is very high when compared to typically observed rates of formation of phenylpropanoid derivatives, (2) an exogenously supplied excess of phenylalanine often results in an increased rate of synthesis of phenylpropanoid derivatives (accompanied by a decrease in PAL activity), (3) free phenylalanine levels are extremely low in plant cells, and (4) the processes of protein synthesis and phenylpropanoid synthesis are frequently observed to be exclusive of each other and thus perhaps competitive for this low supply of free phenylalanine.

Down-regulation of PAL activity by the cell has also been addressed in the literature. Several studies show that accumulation of cinnamic acid can reduce PAL levels and activity in elicited bean suspensions through the activation of a degradation system (Bolwell et al., 1986; Bolwell et al., 1988). One study has indicated that cAMP-dependent phosphorylation may be a significant factor in down-regulating PAL activity by tagging the enzyme for degradation. Phosphorylated PAL subunits are of smaller size, suggesting degradation (Bolwell, 1992). Finally, a proteinaceous factor released from sunflower leaf plastids has been demonstrated to inhibit activity of a fungal PAL enzyme (Gupta and Creasy, 1991). This factor behaves as an enzyme and irreversibly destroys the ability of PAL to catalyze the de-amination of phenylalanine, but not its capacity to bind the substrate; the factor appears to cleave away part of the PAL enzyme but does not disrupt the active site itself. Other plant-derived inhibitors of PAL activity have been described in the literature. A sweet potato inhibitor (Tanaka et al., 1978) behaves similarly to that of the sunflower, but others, such as that from barley, differ in that their action is reversible (Podstolski, 1983).
1.6.4 Interactions with other enzymes of the phenylpropanoid pathway

Evidence for the existence of multi-enzyme complexes

There is some evidence that PAL, in a multi-enzyme complex with other phenylpropanoid enzymes, is associated with the membrane of the endoplasmic reticulum (Hrazdina and Wagner, 1985; Northcote, 1985; Deshpande et al., 1993). This conclusion is based on detection of high MW forms of the various enzymes (corresponding to units integrated into the complex) as well as kinetic studies, substrate channelling, and trypsin susceptibility.

Co-ordinate regulation of PAL and other phenylpropanoid activities

PAL activity is regulated in response to various stimuli in co-ordination with other phenylpropanoid enzymes. The three central-pathway activities (PAL, C4H, and 4CL) were originally observed to be co-ordinately regulated in parsley suspension cell cultures subjected to transfer stress (Hahlbrock and Wellman, 1973). These same enzymes are co-ordinately regulated in a developmental context during light-stimulated lignogenesis in etiolated pea seedlings (Wilkinson and Butt, 1992). PAL and enzymes participating in the flavonoid-specific branch pathway have also been observed to be co-ordinately regulated. In response to elicitor treatment, bean PAL, chalcone synthase, and chalcone isomerase levels increase co-ordinately (Cramer et al., 1985), and in illuminated mustard seedlings accumulating anthocyanin pigments, PAL and chalcone isomerase activities are co-ordinately regulated (Beggs et al., 1987). Co-ordinate regulation of PAL with other phenylpropanoid enzymes is observed at the level of transcription as well; these experiments are discussed in section 1.9.

Some experiments indicate that different transduction routes activate different components of the central phenylpropanoid pathway and related branch pathways. For example, studies comparing inducibility of PAL activity and of anthocyanin accumulation in various photomorphogenetic mutants of tomato show that PAL activity is induced by a different pool of phytochrome than is the accumulation of anthocyanin (Goud et al., 1991). During lignification of
lettuce pith explants, the addition of an ethylene antagonist induced PAL activity but inhibited both cell-wall-bound peroxidase activity and lignification. Ethylene is therefore proposed to be involved in induction of the peroxidase but not of PAL (Miller et al., 1985). Both of these examples illustrate that different pathways branching out from the central pathway of phenylpropanoid metabolism may be activated by different signals, even if the co-regulated enzymes respond co-ordinately at the level of gene expression or enzyme activity.

1.7 PAL-encoding genes in plants

PAL genes have been isolated from many, mostly cultivated, species of plants, as well as two trees and a weed. Taxonomically, a coniferous gymnosperm, a grass, several members each from the legume, aster, and nightshade families, and lone representatives of the cucurbit and morning glory families are those plant species for which PAL genes have been described (Table 1). In approximate chronological order, the species in which these genes have been characterized are French bean, parsley, rice, sweet potato, alfalfa, soybean, potato, pine, tomato, poplar, subterranean clover, melon, tobacco, and Arabidopsis.

PAL genes described in the literature have been isolated in a variety of ways. The first technique involves the use of anti-serum raised against PAL protein which is used to screen an expression library of cDNAs raised from mRNA of the species and tissue of choice. The second method involves the use of heterologous cDNAs previously isolated from the cDNA libraries of other species. These cDNAs are used as probes to isolate cDNAs from libraries of the organism of interest. The third method involves the PCR amplification of conserved homologous sequences which may then be used as probes. The latter two methods in particular are highly dependent on sequence homology between the species to be screened and the species used as source of probe. Only those PAL genes which are quite similar in sequence will be detected and isolated from the screened species; highly divergent (and potentially interesting) genes will not be detected.

PAL is encoded in most plants by a small gene family. There are two exceptions to this: one is the large gene family in potato (Solanum tuberosum) which consists of 40 to 50 genes, at least ten of which are transcriptionally active (Joos and Hahlbrock, 1992). The other exception is
<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
<th>cDNA</th>
<th># of Genes</th>
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<td>Lois <em>et al.</em>., 1989</td>
<td>+</td>
<td>4</td>
<td>3 - 7</td>
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<td>77.7 kD</td>
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<td>Cramer <em>et al.</em>., 1989</td>
<td>+</td>
<td>3</td>
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<td>-</td>
<td>4 - 8</td>
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<tr>
<td><em>Pisum sativum</em></td>
<td>Yamada <em>et al.</em>., 1992</td>
<td>+</td>
<td>4</td>
<td>N/A</td>
<td>724/725 a.a.</td>
</tr>
<tr>
<td><em>Populus hybrids</em></td>
<td>Subramaniam <em>et al.</em>., 1993</td>
<td>+</td>
<td>2</td>
<td>3-8 (***)</td>
<td>2.4 kb</td>
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<tr>
<td></td>
<td>Osakabe <em>et al.</em>., 1995</td>
<td>+</td>
<td>3</td>
<td>3-6</td>
<td>715 a.a.</td>
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<td>714 and</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>682 a.a. (**)</td>
</tr>
<tr>
<td><em>Cucumis melo</em> (L) var. reticulatus</td>
<td>Dallinas &amp; Kanellis, 1994</td>
<td>+</td>
<td>&quot;small - gene family&quot;</td>
<td>2.4 kb RNA detected.</td>
<td></td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em> (L)</td>
<td>Pellegrini <em>et al.</em>., 1994</td>
<td>+</td>
<td>2-4</td>
<td>4-6</td>
<td>2.14 kb</td>
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<td>712 a.a.</td>
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<td>76.5 kD</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>694 a.a. (**)</td>
</tr>
</tbody>
</table>
**PAL** sequences have been also been submitted to genebanks for -
1) *Persea americana*, 2.00 kb, D. Prusky.
2) *Stylosanthes humilis*, 2.40 kb, J.M. Manners.

*) *Medicago sativa* is reported to be an autotetraploid.

**) Faint bands in these genomic Southernblots may be *Pinus taeda* or are *Populus hybrids* representative of diverged PAL genes.

# *Trifolium subterraneum* possesses PAL genes as a clustered family.

@ Shortest and highly divergent PAL genes.

Table 2. A listing of **PAL** genes isolated from plants. Species and references are indicated. The (+) signs indicate that **PAL** cDNA's have been obtained for this species. Estimated number of genes in the gene family is reported, as well as the number of bands on a genomic Southern blot from which the number of genes was estimated. Sizes of genes have been reported by researchers as open reading frames (ORF's) in kilobases, deduced amino acid (a.a.) sequences, or molecular weights of proteins (kD).
the single PAL gene in the only conifer species (Pinus taeda) characterized thus far (Whetton and Sederoff, 1992). However, lighter bands in the genomic Southern blots may correspond to highly diverged PAL genes. The suggestion that highly diverged and possibly differentially regulated PAL genes exist has been made for several species including the conifer just mentioned, as well as poplar (Osakabe et al., 1995) and Arabidopsis (Wanner et al., 1995).

The sizes of these genes are presented in various ways. In general, they are between 2100 and 2400 base pairs long, or around 700 to 720 amino acids in length in terms of the polypeptide encoded. There are a few reports of shorter genes; two examples are the divergent PAL genes in poplar (682 a.a. in length, Osakabe et al., 1995) and in Arabidopsis (694 a.a. in length, Wanner et al., 1995). In poplar, this shortening of the protein is due to a 5' truncation of the encoding gene.

PAL-encoding genes in plants possess only one intron in the 5' end of the gene which when spliced out generates a conserved arginine codon; the single documented exception to this rule is an Arabidopsis gene called PAL3 which possesses a second intron, 103 basepairs in length, further downstream in the gene (Wanner et al., 1995).

1.8 The structure of PAL promoters

The structure of PAL promoters has been studied in several species, and in certain cases functions have been assigned to the identified sequence motifs. In vivo footprinting of the portion of the parsley PAL promoter between -300 and the TATA box was carried out with DMS treatment and DNA methylation (Lois et al., 1989). This experiment determined the location of footprints in that region responsive to elicitor and UV light treatment of parsley cell cultures. Three footprints were identified, centered at -110, -163, and -181 upstream from the transcription start site. The footprint centered at -163 seems to be specific to elicitor responsiveness and the other two respond both to UV light and to elicitor treatments.

A variety of nomenclatures has been applied to these boxes, including the specific reference systems "Box 1" etc., (Ohl et al., 1990) and "P/A/L Boxes" (Da Costa e Silva et al.,
1993), and most recently the more general reference system "AC boxes" (Hatton et al., 1995). This latter nomenclature seems most convenient since it refers to the AC richness of the two stress-responsive motifs and this richness makes the two boxes interconvertible given only a few bases' degeneracy. These motifs are found in the promoters of all phenylpropanoid enzyme-encoding genes which have been found to be responsive to stress, including PAL, 4CL, and CHS from various species (e.g. Lois et al., 1989). When data bases of plant genes were scanned for these motifs, they were found only in three other plant promoters, so the AC/L box seems to be quite specific to the responsiveness of phenylpropanoid-associated genes to stress.

These AC-rich motifs have been demonstrated to interact with a petal-derived Myb protein-like putative transcription factor (Sablowski et al., 1994), and in separate experiments with a pathogen-induced DNA-binding protein in parsley (da Costa e Silva et al., 1993). In addition to the in vivo footprinting work mentioned above, AC boxes have been demonstrated to play a role in conferring complex, tissue-specific patterns of gene regulation in transgenic tobacco containing constructs derived from a bean PAL promoter (Hatton et al., 1995).

Several other conserved sequences are found in the promoters of PAL and other phenylpropanoid genes. These include the "H" box, an AC-like element seen in the bean CHS15 promoter (Loake et al., 1992) and the "G" box. This latter element was first identified in the promoter for the small subunit of ribulose bisphosphate carboxylase (Giuliano et al., 1988) and confers responsiveness to light. Finally, a 7 bp putative xylem-specific motif has been identified in the promoters of other plant genes such as the gene encoding a vascular-specific glycine-rich protein (Keller et al., 1991). This element has also been noted in the promoters of two loblolly pine genes which are preferentially expressed in the differentiating xylem of that tree (Loopstra and Sederoff, 1995).
1.9 Regulation of plant PAL gene expression

1.9.1 Developmental and environmental regulation of plant PAL gene expression

PAL activity can be regulated by many stimuli at the levels of both the enzyme and the gene. Developmental and tissue-specific signals, plant hormones, external stimuli such as interactions with other organisms (pathogenic or symbiotic), light, and wounding all affect PAL activity, mRNA abundance, and transcription (Hahlbrock and Scheel, 1989).

Specific methods for demonstrating PAL gene regulation include the comparison of mRNA populations extracted from various tissues or treatment regimes by northern blot analysis, the amount of reporter gene expression from PAL promoter: GUS reporter fusions in transgenic plants, *in situ* hybridization, run-on transcription, and indirect methods such as inhibition of RNA synthesis by actinomycin D or cordycepin treatments.

Tissue-specific and developmentally regulated expression of PAL genes has been studied in a variety of systems. For example, the PAL genes of rice were observed to be responsive to light during the greening of seedlings (Minami et al., 1989), and PAL gene expression is up-regulated during development of Arabidopsis seedlings, specifically in association with the synthesis of anthocyanin pigments (Kubasek et al., 1992). In Cucumis, PAL gene expression increases during fruit ripening (Diallinas and Kanellis, 1994). Various complex patterns of tissue-specific PAL gene expression have been documented in species such as Arabidopsis (Wanner et al., 1995), Medicago sativa (Gowri et al., 1991), Petroselinum crispum (Lois and Hahlbrock, 1992; Wu and Hahlbrock, 1992), Phaseolus vulgaris (Bevan et al., 1989, Leyva et al., 1992, Liang et al., 1989), Pisum sativum (Yamada et al., 1992), and Populus hybrids (Subramaniam et al., 1993; Osakabe et al., 1995). Although a comprehensive summary of the collective results will not be given, many of these systems showed increased PAL transcript abundance in association with the development of vascular tissue. The specific histology of this response and of responses of other associated phenylpropanoid genes will be discussed in section 1.9.3. In addition, increased PAL gene expression has been noted during the differentiation of tracheary elements in cultured Zinnia mesophyll cells (Lin and Northcote, 1990).
The activation of PAL gene expression is an integral part of plant defense against pathogen attack, and the possible involvement of PAL and of phenylpropanoid metabolism in the hypersensitive response (HR) of resistant host plants to pathogens is particularly interesting. *In situ* hybridization of tobacco leaves infected by TMV or injected with elicitor indicate that PAL transcripts accumulate in the zone immediately adjacent to the the necrotic lesion of the HR (Pellegrini *et al.*, 1994). Similar results were reported in parsley exhibiting nonhost resistance (Schmelzer *et al.*, 1989) and earlier, by northern blot analysis of micro-dissected tissues of resistant bean seedlings inoculated with fungal pathogen (Bell *et al.*, 1986). The time course of the latter accumulation suggested that PAL mRNA began to increase in the inoculated region before the necrotic lesion became visible. The speed of this response is further illustrated by experiments with bean suspension cultures which begin to accumulate detectable quantities of newly synthesized PAL mRNA five minutes after the initiation of elicitor treatment (Lawton and Lamb, 1987). Also, northern blot analysis and *in vitro* translation of polysomes from elicited alfalfa suspensions show that levels of translatable PAL mRNA increase substantially less than an hour after treatment (Dalkin *et al.*, 1990). As well, PAL gene expression is rapidly induced by elicitor treatment in parsley (Dangl *et al.*, 1987, Lois *et al.*, 1989), in bean (Cramer *et al.*, 1985; Edwards *et al.*, 1985; Shufflebottom *et al.*, 1993), and in poplar cell suspension systems (Moniz de Sa *et al.*, 1992).

PAL gene expression has also been shown to be activated in response to pathogen attack upon intact plants in *Arabidopsis* (Huang *et al.*, 1994), soybean (Estabrook and Sengupta-Gopalan, 1991), and potato (Fritzmeier *et al.*, 1987). The two former experiments are particularly interesting since they address the relative activity of PAL genes in compatible versus incompatible interactions. PAL transcripts are preferentially induced in *Arabidopsis* by treatment with an incompatible strain of bacterial pathogen, and in soybean different PAL genes are activated by the initiation of symbiotic versus pathogenic interactions. In susceptible and resistant lines of tomato interacting with a wilt fungus, there is an increase in the PAL enzyme activity accompanied by an observed increase in deposited suberin as a barrier to further fungal invasion (Lee *et al.*, 1992b). *De novo* synthesis of PAL mRNA is suppressed in the susceptible line and there is less suberin deposition observed in those lines.
When a bean *PAL* gene was inserted into the genome of tobacco, co-suppression of the expression of the native tobacco *PAL* genes was observed, resulting in a decrease in PAL activity and of constitutive levels of chlorogenic acid (Bate *et al.*, 1994). Chlorogenic acid is thought to be a relatively non-toxic precursor molecule which is altered upon infection to form a highly toxic compound in those cells which are infected or are immediately adjacent to the infection site. In the transgenic tobacco plants with decreased PAL activity and chlorogenic acid, a more rapid spread of disease upon exposure to a pathogen was observed and larger disease lesions were formed in response to that infection (Maher *et al.*, 1994). These results indicate that phenylpropanoid metabolism synthesizes precursor molecules necessary for the effective containment of pathogens, and that any interference with *PAL* transcription, such as co-suppression, may also adversely affect the plant's defensive capacities.

*PAL* gene expression is both up- and down-regulated by plant hormones. During the interaction of soybean with fungal pathogen, exogenous ABA represses the accumulation of *PAL* mRNA and of the putative phytoalexin glyceollin, as well as the phenotype of resistance (Ward *et al.*, 1989). Both auxin and ethylene treatments augment *PAL* mRNA levels accumulated in response to elicitor by bean suspension cultures (Hughes *et al.*, 1989, Hughes *et al.*, 1990). Carrot suspension cultures are induced to accumulate *PAL* mRNA by ethylene treatment (Ecker *et al.*, 1987), but in other experiments, a slow, anthocyanin-associated increase in *PAL* mRNA was partially repressed by treatment of carrot suspensions with exogenous 2, 4-D (Ozeki *et al.*, 1990, Takeda *et al.*, 1993).

The light responsiveness of *PAL* genes has been examined in several plants. In *Arabidopsis* seedlings, accumulation of both *PAL* mRNA and anthocyanin pigment was observed to be responsive to illumination by either UVB or blue light (Kubasek *et al.*, 1992) and earlier experiments indicated that the *Arabidopsis PAL1* promoter drives GUS expression in response to light treatment of transgenic plants (Ohl *et al.*, 1990). Up-regulation of parsley *PAL* gene expression in response to illumination has been observed in parsley cell suspensions and whole plants. Parsley protoplasts respond to UV irradiation by accumulating *PAL*, *4CL*, and *CHS* mRNA's, the corresponding enzyme activities, and UV-protectant flavonoids (Dangl *et al.*, 1987). *In situ* hybridizations of parsley seedlings grown in the dark and in the light show that *PAL* mRNA
accumulates particularly in tissues exposed to light and specifically in the leaf epidermis of illuminated seedlings (Wu and Hahlbrock, 1992). A bean PAL2 promoter fused to the GUS reporter gene and transferred into tobacco responded to white light and near-UV by specifying GUS expression in the epidermis and subepidermis of etiolated plant material (Liang et al., 1989). As well, there was a lesser response in the vascular-associated tissues. Finally, in both dark-grown callus and mature green leaves of hybrid poplar, PAL mRNA was observed to accumulate after UV irradiation (Osakabe et al., 1995).

Plant PAL genes are responsive to wounding stimuli as well. This response has been observed in many plants including melon (Diallinas and Kanellis, 1994), sweet potato (Tanaka et al., 1989), Arabidopsis (Ohl et al., 1990), and parsley (Lois and Hahlbrock, 1992). In addition, the tuber tissues of potato accumulate PAL mRNA in response to cut-wounding followed by aerobic incubation, (Ishizuka et al., 1991; Rumeau et al., 1990) and in response to impact-wounding (Rickey and Belknap, 1991). This latter response was demonstrated to be transcriptionally regulated through the use of nuclear-runoff experiments.

Exposure to heavy metals also activates PAL gene expression. For example, Arabidopsis PAL genes respond to treatment by HgCl2 (Ohl et al., 1990), and CuCl2 exposure causes the accumulation of PAL mRNA in pea (Preisig et al., 1991).

Since the transcriptional activity of plant PAL genes has previously been demonstrated to be regulated by various developmental and environmental signals, during the course of this work poplar PAL promoters were transformed into tobacco to characterize development- and wounding-specific patterns of reporter-gene expression driven by those promoters.

1.9.2 Co-ordinate regulation of PAL and other phenylpropanoid genes

The expression of PAL genes has been observed to be co-ordinately regulated with that of other genes, both those encoding other phenylpropanoid-associated enzymes and those encoding stress-related proteins. Co-ordinate regulation of PAL and other phenylpropanoid gene transcription has been best documented in parsley. The kinetics of PAL and 4CL mRNA accumulation in parsley suspension cultures responding to environmental stresses are very
similar (Ragg et al., 1981). That is, the patterns of accumulation of the two mRNA species were identical in the context of each stress considered. However, the accumulation of both mRNA's was slower and smaller under UV light stress as compared to the accumulation patterns in response to elicitor treatment (Lois et al., 1989). Other studies show that under UV treatment, the parsley CHS gene is co-ordinately regulated with 4CL and thus by inference with PAL (Dangl et al., 1987). In situ localization of PAL, 4CL, and bergapto-O-methyl transferase (BMT) mRNA's in parsley leaves infected with a non-host pathogen also show that PAL and 4CL transcripts appear simultaneously, while the BMT mRNA appears somewhat later (Schmelzer et al., 1989).

Similar co-ordinate regulation of PAL and 4CL mRNA's in response to elicitor has been reported for poplar, (Moniz de Sa et al., 1992), potato (Fritzemeier et al., 1987), and alfalfa (Dalkin et al., 1990). In the latter case, CHS is also regulated in co-ordination with the two central-pathway enzymes. In untreated carrot suspension cultures, expression of PAL and CHS mRNA's is co-ordinately induced five days after transfer to fresh medium when anthocyanin pigments synthesis begins in the culture. This accumulation is inhibited by the addition of 2, 4-D to the culture, indicating that the mRNA's are co-ordinately repressed by the hormone in this system (Ozeki et al., 1990).

1.9.3 Tissue-specific localization of PAL and other phenylpropanoid enzymes associated with lignogenesis

During seasonally regulated cycles of activity, the cambium of woody dicotyledons is periodically stimulated to become active and to generate xylem elements and tracheids, fibres, and ray parenchymal tissues centripetal to the cambial zone. After division is complete, those fusiform initials destined to become xylem elements and tracheids undergo a differentiation process known as xylogenesis. This involves the development of polyploidy and elaboration of endomembranes and microtubules within the cell, vacuolization and enucleation concomitant with the laying down of primary and secondary cell walls in various patterns, and finally death of the cell, leaving the conductive wall components in place (Esau, 1977; Raven et al., 1986). Lignin
deposition is an integral part of the formation of the secondary wall, and the activity of the phenylpropanoid pathway and thus of PAL is indispensable to this process.

The exact histological location and timing of the appearance of PAL and other phenylpropanoid-associated enzyme activities within these cambial derivatives can pinpoint exactly when and where lignification takes place, and which cells participate in the process. Enzymes of interest include those of the central pathway (PAL, C4H, and 4CL) as well as other enzymes participating in the synthesis of the lignol moieties (for example, cinnamyl alcohol dehydrogenase (CAD) and the O-methyl-transferases (OMT)), or in their polymerization. These activities can be localized rather roughly by micro-dissection of the various tissues, extraction of the tissues, and examination of the RNA or protein populations by northern blot analysis, by enzyme assay, or by western blot. In situ hybridization or immunolocalization may also be used to visualize mRNA's or proteins within the tissues of interest. Finally, the promoters of genes encoding various enzymes may be fused to a reporter gene such as GUS and the developmentally regulated expression driven by these promoters in transgenic tissues assayed. Thus, the location and relative timing of activity of each of the studied enzymes participating in the synthesis and polymerization of lignin precursors may be determined within the larger process of xylem differentiation.

A large body of information on the location of PAL protein is available. PAL activity was first localized to the xylem tissue of several species (maple, pea, and celery) by microdissection and enzyme assay; this tissue contained both differentiating and differentiated xylem (Rubery and Northcote, 1968). The same technique was used to localize PAL activity in different tissues of poplar (Grand and Ranjeva, 1979). PAL was found to be highest in xylem tissues (again composed of a mixture of differentiated and differentiating material) and was also found to be reduced in lower portions of the stem in which lignification was already well advanced. In situ localization of PAL mRNA in developing parsley seedlings reveals PAL mRNA accumulation is limited to developing xylem cells (Wu and Hahlbrock, 1992). Three different studies using the promoters of bean PAL genes fused to the reporter gene GUS in transgenic tobacco show that PAL promoters specify expression in differentiated and lignified xylem elements (Shufflebottom et al., 1993), in prexylem non-lignified cells (Liang et al., 1989), or in ray parenchyma cells adjacent
to differentiated xylem (Bevan et al., 1989). This latter result was confirmed by immunolocalization of PAL protein in differentiating vascular tissues of bean hypocotyl. PAL was found in parenchyma cells adjacent to differentiating and differentiated tracheary elements, as well as in the cytoplasm of committed prexylem cells (Smith et al., 1994).

The second enzyme in the central pathway has also been immunolocalized in developing bean hypocotyl; cinnamate-4-hydroxylase was also found in xylem parenchyma adjacent to differentiating xylem with secondary thickenings visible (Smith et al., 1994).

Accumulation of 4CL protein has been studied by microdissection and enzyme assays of several tissues dissected out from differently aged regions of a young poplar stem (Grand and Ranjeva, 1979). Activity was only detectable in sections of the stem which were undergoing or had undergone substantial differentiation of xylem, and was highest specifically in that dissected tissue. When in situ hybridization and immunolocalization were carried out to locate 4CL mRNA and protein in young parsley seedlings, both techniques indicated gradual decreases when young developing vascular tissue was compared to older mature vasculature (Wu and Hahlbrock, 1992). Tobacco transgenic for a parsley 4CL promoter fused to a GUS reporter gene exhibited GUS activity in the files of xylem ray parenchyma (Hauffe et al., 1991).

Caffeic acid O-methyl transferase (OMT) activity, required for the formation of lignols, was also studied by microdissection and enzyme assay in aspen. OMT activity was highest in that section of the stem which was undergoing active xylem differentiation, and was also highest in dissected xylem (Grand and Ranjeva, 1979). Tissue-printing and immunolocalization of an OMT specific to both caffeic acid and 5-hydroxyferulic acid reveal that this enzyme is localized to developing xylem in very young aspen stem. When tissues were microdissected from a 3 to 4 year-old aspen, OMT gene expression was limited to and was very high in stem scrapings consisting of a mixture of differentiated and differentiating xylem in 3 to 4 year-old aspens (Bugos et al., 1991).

Cinnamyl alcohol dehydrogenase (CAD) is required for the synthesis of cinnamyl alcohols (lignols). A CAD activity specific for the formation of coniferyl alcohol was found to be highest in basipetal-most sections of poplar stems which contain mostly substantially differentiated wood tissues, and was extremely high in the xylem tissue (Grand and Ranjeva, 1979). Poplars
transgenic for a construct consisting of a eucalyptus CAD gene promoter fused to the GUS reporter gene showed strong activity in the parenchymal cells both in differentiating and differentiated xylem tissues (Feuillet et al., 1995).

A beta-glucosidase activity specific to the deglycosylation of coniferin in lodgepole pine has been localized to that region of the xylem which is rapidly differentiating and undergoing substantial lignification using an in situ enzyme assay (Dharmawardhana et al., 1995). In addition, the same study shows peroxidase activity restricted to this zone (Dharmawardhana et al., 1995). When a cationic cell wall-associated peroxidase was immunolocalized in differentiating hypocotyl of bean, it was associated with differentiating xylem and specifically with the secondary thickenings and the middle lamella of those differentiating cells (Wu and Hahlbrock, 1992).

In summary, CAD activity appears to be highest in tissues that are older and undergoing the final stages of differentiation, while PAL activities are highest in younger tissues still undergoing elongation. Since PAL and related activities early in the phenylpropanoid pathway create a pool of substrate for the later activity of CAD to act upon, these observations make intuitive sense. As well, PAL mRNA and protein levels measured by in situ localization are observed in differentiating xylem and in ray parenchymal cells adjacent to differentiating xylem. Plants transgenic for PAL promoter : reporter gene fusions express GUS in ray parenchymal cells and in both differentiated and differentiating xylem elements. Studies with CAD parallel these findings.

Results regarding the localization of other enzymes involved in synthesizing and polymerizing lignols during xylogenesis also concur with intuitive models of lignification. OMT can be localized to the layer of differentiating xylem (containing both differentiated and differentiated cells) and the latterly-acting enzymes beta-glucosidase and peroxidase are associated with tissues which are lignifying. The peroxidase is immunolocalized to secondary wall thickenings and middle lamella of lignifying xylem elements.

From these results, PAL (and by implication, the other enzymes in the general phenylpropanoid pathway) seems to be highly expressed in differentiating xylem elements and in neighboring ray parenchymal cells. Since the activity of CAD has also been co-localized to
those cells, all intervening activities are probably also present. Finally, the peroxidase is localized in the immediate vicinity of the final polymerization of the lignin subunits, as would be predicted from the consensus model of lignin biosynthesis.

There have been several contradictory models proposed for the site of synthesis of lignin precursors. Freudenberg (1965) suggested that the cambium was the site for this synthesis and the precursors were then passed centripetally to the lignifying xylem elements. This seems to be disproven since little phenylpropanoid-associated enzyme activity of any sort is localized to the cambium. Rubery and Northcote (1968) modified Freudenberg’s idea and suggested that lignols are synthesized in the more recently derived initials within a file and then passed centripetally to older and more differentiated elements in which lignification was taking place. On the other hand, Wardrop and Bland (1959) believed that precursors were synthesized by and deposited into the cell wall of each differentiating cell individually. None of the present evidence is incompatible with either of these hypotheses.

However, the repeated finding that PAL, 4CL, and CAD mRNA’s and proteins are localized to the ray parenchyma in differentiating xylem tissue, implies that these cells are also involved in producing and passing precursors to the lignifying cells. This suggests that the model could be extended to include ray parenchymal cells as lignol sources. Like Wardrop and Bland, Terashima et al. (1993) state that individual, isolated cells are competent to produce their own precursors and undergo lignification, based on observations of lignification of isolated *Zinnia* cells (Fukuda and Komamine, 1982) and conclude that synthesis of lignin precursors in one cell followed by transport of lignols to another, actively lignifying cell is unlikely (Terashima et al., 1993). However, the experimental finding simply demonstrates that isolated cells in vitro are able to lignify appropriately, and does not at all address the possibility of intercell exchanges of precursor in planta. No experiment has demonstrated passage of lignols from younger initial or parenchymal cell to older lignifying cell, but the localization of high levels of synthetic enzymes to those young tissues raises the possibility of such exchange.

Since poplar is a woody perennial, it is a convenient organism in which to examine phenylpropanoid gene activation and enzyme activity with respect to lignification. In this thesis, reporter gene activity driven by the two poplar PAL promoters in the secondary stem tissues of
tobacco was examined for localization of \( PAL \) expression. In addition, the relative roles of the three poplar \( PAL \) genes in lignification of secondary xylem was investigated through the use of differential probes.

### 1.9.4 Differential regulation of \( PAL \) gene family members

Differential regulation of individual genes within \( PAL \) gene families has been documented in several plants. Differences in expression with respect to both developmentally regulated tissue-specificity and various stress treatments have been observed. In *Arabidopsis*, \( PAL3 \) is shown to have lower expression levels than the other \( PAL \) genes in all tissues examined (Wanner *et al.*, 1995). In parsley, \( PAL2 \) expression alone is responsible for the high \( PAL \) expression in roots, while \( PAL3 \) is most responsive to wounding (Lois and Hahlbrock, 1992). The promoters of bean \( PAL2 \) and \( PAL3 \) direct different, complex patterns of tissue-specific expression in transgenic tobacco (Shufflebottom *et al.*, 1993). The transgenic tobacco examined for differential expression of bean \( PAL2 \) and \( PAL3 \) promoters in a tissue-specific context also exhibit differential expression driven by those promoters in response to wounding and elicitor treatment (Shufflebottom *et al.*, 1993). In addition, the kinetics of accumulation of transcripts from different bean \( PAL \) genes differ in response to elicitor treatment, in degree of inhibition by cinnamic acid treatment in the context of elicitor, and in speed of recovery from cinnamic acid treatment (Mavandad *et al.*, 1990). In poplar one \( PAL \) gene is reported to be preferentially expressed in young stem as compared to young leaf; a second gene is largely responsible for expression in young leaf (Osakabe *et al.*, 1995). Carrot suspension cultures exhibit a slow transcriptional activation of one class of \( PAL \) genes associated with the biosynthesis of anthocyanins and this response is uniquely repressible by 2, 4-D treatment (Ozeki *et al.*, 1990). In contrast, the rapid accumulation of a second class of \( PAL \) transcripts is associated with transfer stress (Ozeki *et al.*, 1990). Different members of the soybean \( PAL \) gene family respond to symbiotic versus pathogenic interactions (Estabrook and Sengupta-Gopalan, 1991). Finally, in compatible interactions of potato with a pathogen, the kinetics of mRNA accumulation differ for \( PAL1 \) and \( PAL2 \) (Joos and Hahlbrock, 1992).
Poplar PAL genes exist as a small gene family, and some evidence exists for differential regulation of those genes. Therefore, in this work differential probes were used to attempt to confirm those results.

1.9.5 Expression of poplar PAL genes - previous work

Sequence of PAL-encoding genes from poplar was reported in 1993 (Subramaniam et al., 1993) and some work on tissue-specific expression of PAL was done; when the cDNA PAL 7 was used as a probe on northern blots, expression was observed in buds and young leaves and in the young stem. Low levels of expression were also observed in the stem-scrape of mature stem and in the bark of the stem, but essentially no expression was seen in mature leaf. In situ hybridization of cDNA PAL 7 antisense RNA to sections of very young leaves showed that expression in this organ is localized to the developing vascular tissue and to the layer of subepidermal cells which later differentiates into the palisade or spongy parenchymas.

In a recent publication (Osakabe et al., 1995), a diverged poplar PAL gene referred to as pal g2a is shown to be preferentially expressed (as compared to levels of its expression in young leaf) in the young green stems of poplar, approximately internodes 3 through 7, with some expression in the region including internodes 8 through 10. (These regions correspond quite closely to zones I and II defined in Populus x euramericana (Dode) c.v. 1214; PAL activity in these zones was reported to be about double that in the next lower zone (Grand and Ranjeva, 1979).) Another PAL clone, referred to as pal g1, is expressed strongly in young leaves and other young tissues and is expressed more weakly in the lower stems. This latter isolate may correspond to the clones studied by Subramaniam et al.. Both pal g1 and pal g2a appear to be stimulated at the transcriptional level by exposure to U.V. radiation, particularly in dark grown callus. The response is much less noticeable in mature leaves (Osakabe et al., 1995).
Environmental and hormonal cues contribute to seasonal variations in perennial phenology and physiology

As large woody perennials, trees in temperate zones may undergo many repeated annual cycles of alternating growth and dormancy triggered by environmental signals such as changes in daylength, light intensity, and temperature (Raven, 1986). The buds laid down in the autumn break in the early spring, the leaves flush out, and the cambium becomes active as the growing season progresses, detectable phenologically as the phenomenon of bark slippage. In the late summer the type of xylem laid down becomes different in character in many species (late-versus earlywood) and buds for the following year begin to develop. Bark slippage decreases. By the late fall or early winter the tree is once more dormant with little physiological activity taking place (Coleman et al., 1992). After suitable chilling, the organism enters a quiescent state from which it may be activated by exposure to suitably warm temperatures (Hanninen, 1990). These yearly phenological patterns are echoed at cellular and molecular levels by similarly rhythmic annual changes, such as those observed for carbohydrate content in *Nothofagus* leaves (Alberdi et al., 1989), for willow bark storage-protein vacuoles (Greenwood et al., 1989), and for RNA levels of O-methyltransferase in the secondary xylem of poplar (Chiang et al., 1991).

Breaking dormancy is a complex and not-completely understood process varying from species to species. The most important environmental signal in enabling budbreak is an exposure to cold temperatures followed by warmer temperatures (Hanninen, 1990). On the other hand, phenological events in the autumn such as growth cessation, cold hardiness, and dormancy, seem to be linked more to length of night and photoperiod (Coleman et al., 1991). Plants' perceptions of the environment are communicated to relevant responding tissues through the transport and action of plant hormones such as abscisic acid, auxins, cytokinins, gibberellic acid, and ethylene (Raven, 1986).

In trees, the control of the seasonal activity of the vascular cambium (and thus of the processes of xylogenesis, lignification, and related phenylpropanoid metabolic activities) by light and by hormones is of great interest. In some systems, particularly conifers, auxin and abscisic acid have been demonstrated to play a role in regulating cambial activity, especially in the
transition from early- to late-wood (Savidge 1983, Sundberg et al., 1987). In young spruce, transfer into a photoperiodic regime with a night-length of 7 1/2 to 8 hours is sufficient to signal a cessation of cambial activity within about four weeks (Bjornseth, 1981). This response is independent of the cessation of terminal growth, which occurs in response to a night-length of 4 to 5 hours at that latitude; in this species at least, the cambial response is not keyed by hormonal signals such as auxins sent from the dormant apex, but rather by perceived photoperiod. Several studies on woody perennial angiosperms correlate various aspects of auxin physiology or phenology with cambial activity. In beech, the speed of the descending wave of auxin is shown to be comparable to the speed of the wave of cambial activation under field conditions, a point which was formerly under dispute (Lachaud and Bonnemain, 1982). A study on young Populus deltoides indicates that the highest levels of endogenous auxin are found in that internode which is undergoing a transition from the production of primary to the production of secondary xylem (DeGroote and Larson, 1984). Short-day treatments were found to decrease the levels of endogenous auxin, and this transition zone was shifted into younger internodes closer to the source of auxin. Quiescent plants placed under long-day conditions experienced a marked increase in endogenous auxin at 9 and 14 days after introduction into long-days and showed the same correlation between highest auxin levels and transition zone. Twelve-year-old ash trees were studied to compare phenological stages of the plant with cambial activities (Atkinson and Denne, 1988). Expanding vessels in stem and branches were first observed 3 weeks before budbreak, and the first mature vessels were seen in the upper stem just before leaves emerged from the buds.

Tissue-culture and other simple systems yield information regarding the relative roles of plant hormones and light in the process of stimulating differentiation of vascular tissues and more specifically in activating various components of phenylpropanoid metabolism. Much of this literature has been reviewed in previous sections addressing light and hormone regulation of PAL enzyme activity (section 1.6) and PAL gene expression (in section 1.9). However, only a few experiments have addressed the exact nature of the interaction of light and hormones with enzymes and genes controlling lignification. For example, when etiolated pea seedlings are illuminated, they show a 5-fold increase in PAL enzyme activity followed by a 10-fold increase in
lignin content within twenty-four hours (Wilkinson and Butt, 1992). In xylogenic cultures of pine, specific combinations of sugar, auxin, and kinetin were observed to induce PAL activity in conjunction with the appearance of tracheids with mature secondary walls containing substantial quantities of lignin (Ramsden and Northcote, 1987). Similar observations have been made with several angiosperm systems, for example, *Zinnia* (Lin and Northcote, 1990). Ethylene has been found to be involved in the latter stages of lignification in experiments with lignifying pith explants from lettuce, possibly through activation of cell-wall associated peroxidases. However, PAL activity was actually increased by the addition of silver, which acts as an ethylene antagonist (Miller *et al*., 1985). This indicates that ethylene may down-regulate PAL activity.

Some work has been done characterizing aspects of seasonally-regulated phenylpropanoid metabolism. An aspen bi-specific O-methyltransferase (OMT) cDNA encoding an enzyme active in the latter part of the metabolic pathway producing lignols for incorporation into the cell wall, has been cloned and characterized (Bugos *et al*., 1991). Tissue printing and northern blot analysis showed that this gene is expressed specifically in developing secondary xylem. Seasonal patterns of OMT RNA accumulation show two peaks, one in mid-June and the other highest peak in late July; the RNA is undetectable before May 19 and after September 8. This bi-phasic pattern was observed repeatedly over three separate growing seasons. OMT activity and CAD activity were monitored and were also found to peak in July. The biphasic nature of OMT gene expression and the high peak of both enzyme activities may be related to their involvement in the formation of early- and late-woods (these are not highly differentiated from each other in *Populus*, except that late-wood is richer in highly lignified fibers). A single study regarding seasonality of PAL enzyme activity in trees has been reported. In *Robinia pseudoacacia* L., quarterly samples taken in Germany showed that the highest PAL activity was in the highly pigmented heartwood of that species in November, in addition to being detectable in association with the formation of lignin at the outermost growth ring at other times of the year. High CHS activity was also observed in the former tissue (Magel *et al*., 1991).

These data indicate that the activity of poplar PAL genes may be subject to regulation by seasonal changes in light, temperature, and/or transmitted plant hormones. In the portion of this
work dealing with seasonality of PAL, the baseline annual variations in PAL protein and RNA amount are described and associated with phenological and environmental changes.

1.11 Proposal of thesis

When this work was initiated, little was known about the structure and expression of *PAL* genes in woody plants. The seasonal nature of gene activation in poplar is also interesting, since, like other perennials, this organism undergoes annual cycles of growth and dormancy. In addition, the characterization of poplar's responses to the repeated pathogenic challenges which a perennial organism must endure would be valuable. Finally, the study of phenylpropanoid-involved genes in poplar may lead to the development of strategies useful in economic improvement of poplars or of other woody species through the transfer of altered genes to those species.

Therefore, poplar *PAL* genes were isolated and their structure characterized so that they could be compared to each other and to the *PAL* genes of other, herbaceous and coniferous plant species. With the isolation of these genes, the functioning of their coding and promoter regions could also be studied by various researchers and compared to that of other *PAL* genes.

In this thesis, several experiments were performed to characterize the function of the poplar *PAL* promoters. First, the developmentally specified and wounding-responsive patterns of poplar *PAL* gene expression were analyzed. Potential differential expression of the members of the poplar *PAL* gene family was investigated. Seasonal changes in levels of PAL protein and *PAL* mRNA in various poplar tissues were characterized. Finally, individual results from several experiments were examined to elucidate the possible role(s) of the several poplar *PAL* gene family members in lignogenesis associated with the biosynthesis of wood.
2. Materials and Methods

2.1 Maintenance and manipulation of bacterial and bacteriophage strains

The *Escherichia coli* strain DH5-alpha was used to harbor all plasmid vectors used. The *Agrobacterium tumefaciens* strain LBA4404 was used as a host for binary vector constructions to transform SR1 tobacco, and the *A. tumefaciens* strain A281 was transformed with binary vector constructions to generate strains for the transformation of 93-968 poplar. Original genomic sequences were cloned in Lambda GEM12 bacteriophage, and these bacteriophage were grown on the *E. coli* host strain KW251.

All bacteria were grown on LB media (made by adding 25 grams BDH commercial LB mixture per litre of distilled water and autoclaving; solid media were made by adding 7 grams agar/litre) supplemented with appropriate antibiotics (100 mg/L ampicillin or 50 mg/L kanamycin for *E. coli* strains, and 100 mg/L kanamycin for *A. tumefaciens* strains) unless otherwise noted.

*A. tumefaciens* strains were sometimes selected on AB minimal medium which was made as follows: melted sterile AB agar at 60 degrees Celsius (7.5 grams agar was made up to 400 ml with distilled water and autoclaved) was mixed with 100 ml sterile Minimal A salts (2.25 grams KH2PO4, 5.26 grams K2HPO4, 0.5 grams (NH4)2SO4, and 0.25 grams sodium citrate were mixed with distilled water to make 100 ml and autoclaved) 1 ml sterile1 M MgSO4, and 5 ml 20% w/v filter-sterilized glucose. This AB medium was supplemented with antibiotic when appropriate.

*E. coli* strains were grown at 37 degrees Celsius, and *A. tumefaciens* strains at 28 degrees Celsius.

Introduction of DNA into bacterial strains was accomplished by heat shock, triparental mating, or electroporation. Heat shock transformation of *E. coli* was achieved by thawing a 100 microlitre aliquot of heat shock-competent bacteria on ice and then adding DNA (most often a ligation reaction) in 40 microlitres or less. The combination was incubated on ice for 30 minutes to an hour and then was shocked for 1.5 to 2 minutes at 42 degrees Celsius. The mixture was then promptly chilled in ice water. The mixture of bacteria and DNA was transferred into a sterile culture tube containing 1 or 2 ml of cold LB. This culture mix was shaken gently at 37 degrees Celsius for
one hour; then 200 microlitres were removed and spread on an LB plate with appropriate selective antibiotics and 25 microlitres of 40 mg/ml X-gal (5-bromo-4-chloro-3-indoyl-B-galactoside) and allowed to grow overnight at 37 degrees Celsius. Large regular colonies were picked individually for minipreparation and confirmation of transformation.

Triparental mating was used to introduce plasmids from *E. coli* into *A. tumefaciens* (Ditta *et al.*, 1980). Using sterile toothpicks, DH5-alpha harboring the plasmid to be transferred, the recipient strain *A. tumefaciens* LBA4404, and the *E. coli* helper strain pRK2073 were mixed in a small section of a new LB plate and allowed to grow together for several days at 28 degrees Celsius. When the bacterial mass was well established, streaks were transferred onto an AB plate with appropriate antibiotic selection and grown at 28 degrees Celsius for several days. A negative control consisting of transformed *E. coli* was inoculated onto a similar plate at the same time so that transformed LBA4404 could be identified. Large regular colonies were picked individually for confirmation of transformation.

Electroporation was used to transform both *E. coli* and *A. tumefaciens* strains according to Bio-Rad instructions. A 40 microlitre aliquot of electroporation-competent bacteria (suspended in a non-ionic buffer such as 10% glycerol in distilled water) was thawed on ice. The Bio-Rad Gene Pulser was adjusted to 2.5 kV, 25 micro-farads, and 200-400 ohms, and the slide tray was placed in the freezer to pre-chill for half an hour. Plasmid DNA in sterile distilled water was introduced into the bacterial aliquot and the mixture gently swirled. The 42 microlitre mixture was then placed into the electroporation cell by pasteur pipet, tapped to settle the bacteria, wiped of condensation, and placed into the slide tray. After administration of the shock, the mixture was immediately transferred into a sterile culture tube containing 1 ml SOC medium and allowed to recover with shaking at either 37 degrees or 28 degrees Celsius for one hour. SOC medium was made by combining 20 g bacto-tryptone, 5 g bacto-yeast extract, and 0.5 g NaCl in 950 ml of distilled water. Then 10 ml of a 250 mM KCl solution was added (1.86 g KCL was dissolved into a final volume of 100 ml with distilled water), pH adjusted to 7 with NaOH, volume made up to one litre with distilled water, and the medium autoclaved. After cooling, 5 ml sterile 2 M MgCl and 20 ml of sterile 1 M glucose were added (Sambrook *et al.*, 1989). For *E. coli*, 200 microlitres were then removed and spread on an LB plate with appropriate selective antibiotics and 25 microlitres of 40
mg/ml X-gal and allowed to grow overnight at 37 degrees Celsius. Large regular colonies were picked individually for minipreparation and confirmation of transformation. For *A. tumefaciens*, 200 microlitres of culture were spread onto an AB plate with appropriate antibiotic selection and grown at 28 degrees Celsius for several days. Large regular colonies were picked individually for confirmation of transformation.

Electroporation-competent cells of both species were prepared according to Bio-Rad instructions by inoculating a sterile 250 ml aliquot of LB in a shaker flask with the appropriate strain and shaking overnight at the appropriate temperature. The culture was then chilled for half an hour in ice water and centrifuged in sterile bottles for fifteen minutes at 4000g at 4 degrees Celsius. The bacterial pellet was rinsed free of contaminating culture components and ions by two successive resuspensions into cold sterile distilled water; the first wash volume was 250 ml, and the second wash volume was 125 ml. Each wash was followed by a centrifugation as above. After the second wash and spin cycle, the pellet was resuspended in 5 ml cold 10% v/v sterile glycerol in distilled water and centrifuged once more. Finally, the pellet was resuspended in 750 microlitres of cold 10% v/v sterile glycerol in distilled water, and aliquoted in individual Eppendorfs in volumes of 40 microlitres and immediately frozen in liquid nitrogen and stored at -90 degrees Celsius.

Medium-term storage of transformed bacterial strains was effected by storage of sealed plates at 4 degrees Celsius for up to two months. Longterm storage was carried out by making glycerol stocks (*Maniatis et al.*, 1982) by growing a 5 ml culture in LB and appropriate antibiotics overnight at appropriate temperatures, and mixing 0.85 ml culture with 0.15 ml sterile glycerol in a sterile vial by vortexing. The vial was then frozen in liquid nitrogen and placed in a -90 degree Celsius freezer.
2.2 Molecular methods

2.2.1 Isolation of DNA and RNA

Poplar leaf RNA and genomic DNA

Young leaf RNA and genomic DNA were isolated from young poplar leaves which were 0.5 to 3 centimeters in length, bright green, shiny, and malleable. This method was also used to extract RNA from mature leaves throughout the growing season (Parsons et al., 1989). Leaf tissue frozen in liquid nitrogen was ground with sand to a fine powder in a mortar and pestle. Approximately 3 to 4 minutes of grinding were required depending on the size of the sample. For each gram of sample, 1 ml of extraction buffer, 100 mM Tris, 20 mM EDTA, 0.5 M NaCl, 0.5% SDS, 0.5% 2-mercapto-ethanol, pH 8 (prewarmed to 60 degrees Celsius) was aliquoted into an appropriately sized sterile plasticware tube along with 1/5 volume of buffer-equilibrated phenol. The extraction buffer was made by mixing 10 ml 1M Tris pH 8, 4 ml 0.5 M EDTA, 2.9 g NaCl, 2.5 ml 20% SDS, and making up to 100 ml with distilled water, and autoclaving, after which 0.5 ml beta-mercapto-ethanol was added. For RNA preparations, 100 microlitres of DEPC (di-ethyl pyrocarbonate) was mixed in thoroughly and the solution was allowed to rest overnight before autoclaving. The ground sample was added to the buffer and phenol and the mixture was vortexed thoroughly. Then one volume of 24 : 1 chloroform : isoamyl alcohol (v/v) was added and the tube centrifuged at 3400g for 10 minutes at room temperature. The aqueous phase was transferred, extracted with one volume of 25 : 24 : 1 phenol : chloroform : isoamyl alcohol (v/v/v), and then transferred and re-extracted with one volume of 24 : 1 chloroform : isoamyl alcohol. One fifth volume of 10 M LiCl2 was added to the aqueous phase in baked glass centrifuge tubes and incubated on ice for 10 minutes to overnight to precipitate RNA. The RNA was centrifuged down at 10,000g for 10 minutes at room temperature. DNA was precipitated from the supernatant by the addition of one volume of isopropanol. The RNA pellet was dissolved in DEPC-treated water and re-precipitated by the addition of 2/3 volume of 5M ammonium acetate and two volumes of ethanol.
Tobacco genomic DNA

One gram of young leaf tissue (leaves 4 centimeters or less in length) frozen in liquid nitrogen was ground with sand to a fine powder in a mortar and pestle; approximately 3 to 4 minutes of grinding were required depending on the size of the sample. After grinding, the tissue was mixed with a 7.5 ml aliquot of CTAB isolation buffer, 2% w/v CTAB, 1.4 M NaCl, 0.2% v/v 2-mercapto-ethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8 preheated to 60 degrees Celsius (Doyle et al., 1990). One hundred ml CTAB isolation buffer was made by combining 2 grams CTAB (hexadecyltrimethylammonium bromide), 8.2 grams NaCl, 4 ml 0.5 M EDTA stock, and 10 ml 1 M Tris stock, making up the volume to 100 ml with distilled water and autoclaving. After autoclaving 200 microlitres of beta-mercapto-ethanol were added. The mixture was incubated at 60 degrees Celsius for thirty minutes and then extracted with an equal volume of 24 : 1 chloroform : isoamyl alcohol (v/v). The phases were separated by centrifuging at 1600g for 15 minutes at room temperature. The aqueous supernatant was removed to a fresh tube and mixed with a two thirds volume of cold isopropanol and centrifuged at 500g for 15 minutes. The nucleic acid pellet was drained and rinsed with a wash buffer, 76% v/v ethanol, 10 mM ammonium acetate, for twenty minutes. An NH4OAc stock was made by dissolving 0.077 grams of the compound into a final volume of 100 ml with distilled water; 24 ml of this stock was combined with 76 ml of 95% ethanol to make the wash buffer. Then the pellet was centrifuged again at 1600g for 10 minutes, drained and air-dried briefly. It was resuspended in 1 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), in a 1.5 ml microcentrifuge tube and RNAase A was added to a concentration of 10 micrograms/ml and incubated for thirty minutes at 37 degrees Celsius. Then the mixture was transferred to a 15 ml sterile tube and 1 ml TE was added along with 0.75 ml of 7.5 M NH4OAc and 5 ml of chilled 95% ethanol. The DNA was centrifuged at 10, 000g for 10 minutes at 4 degrees Celsius, rinsed with 70 % ethanol, dried, and resuspended in TE.
Lambda bacteriophage DNA

DNA from the poplar genomic clones in the LambdaGEM-12 vector was prepared using a combination of methods from Sambrook et al., 1989 and the Promega LambdaGEM-12 Technical Bulletin as follows: 500,000 plaque-forming units in a volume of 100 microlitres of SM buffer without gelatin (0.58g NaCl, 0.2 g MgSO$_4$$\cdot$H$_2$O, 5 ml 1M Tris pH 8, were mixed and made up to a volume of 100 ml and autoclaved for 15 minutes) were mixed with 500 microlitres of an overnight culture of KW251 grown at 37 degrees Celsius in 5 ml of LB enriched with 50 microlitres each of 1M MgSO$_4$ and 20% sterile maltose. This mixture was incubated for twenty minutes at 37 degrees Celsius and then aseptically introduced into a pre-warmed 50 ml aliquot of LB enriched with 500 microlitres of 1M MgSO$_4$ in a 250 ml flask. The flask was then shaken hard for 7 hours at 37 degrees Celsius. One half ml of chloroform was added to the flask to lyse the remaining bacteria and the mixture shaken for an additional 15 minutes. The lysate was then poured into a 50 ml Falcon tube and centrifuged at 8000g for 10 minutes. The supernatant was decanted into a sterile 50 ml Falcon tube and stored overnight at 4 degrees Celsius. It was then returned to room temperature and pancreatic DNase I and RNAse were added to digest bacterial nucleic acids, to a final concentration of 1 microgram/ml, from stocks of 1000 micrograms/ml, and the lysate incubated at 37 degrees Celsius for 30 minutes. Solid NaCl was added to a final concentration of 1M (that is, 2.9 grams of NaCl were added to the 50 ml lysate). The NaCl was allowed to dissolve and the mixture was incubated for one hour on ice. It was then centrifuged for 10 minutes at 11,000g at 4 degrees Celsius. The supernatant was transferred into another 50 ml Falcon tube and solid PEG 8000 was added at a ratio of 1 gram per 10 ml and dissolved. This mixture was cooled in ice water and incubated for one hour on ice. It was then centrifuged for 10 minutes at 11,000g at 4 degrees Celsius in sterile glass tubes; then the supernatant was gently drained away and the faint opaque deposit of intact bacteriophage was resuspended into 500 microlitres of SM without gelatin by gentle vortexing and the debris centrifuged down at 8000g for 2 minutes at 4 degrees Celsius. Immediately the SM and bacteriophage were pulled away from the pellet of debris with a sterile 1 ml pipet and transferred into 1.5 ml microcentrifuge tubes. The bacteriophage were extracted twice with 1 volume of 25:24:1 phenol:chloroform:isoamyl alcohol (v/v/v) pH 8 by
vortexing vigorously for one minute followed each time by a 10 minute centrifugation in a microfuge at top speed. This was followed by a single extraction with 24:1 chloroform:isoamyl alcohol (v/v) and a 5 minute centrifugation in a microfuge at full speed. The supernatant was transferred and an equal volume of isopropanol was added and incubated for 20 minutes at -20 degrees Celsius to precipitate DNA. The DNA was centrifuged down in a microfuge for 10 minutes at 4 degrees Celsius at full speed. The supernatant was discarded and the pellet rinsed with 70% ethanol, air dried for 30 minutes, and resuspended in 250 microlitres of TE. The DNA was allowed to resuspend overnight at 4 degrees Celsius, and the absorbance at 260 nanometers was taken on a Beckman DU-64 spectrophotometer to determine concentration.

**Bacterial DNA minipreparation**

A 1.5 ml aliquot of a 2 ml overnight bacterial culture grown in LB with appropriate antibiotic selection was poured into a 1.5 ml microcentrifuge tube and centrifuged for 15 to 20 seconds. Supernatant was aspirated away and bacterial pellet resuspended in 100 microlitres TE. Two hundred microlitres of fresh lysis buffer, 0.2 N NaOH, 1% SDS, was added to each microcentrifuge tube, mixed, and incubated on ice for five minutes. Then 150 microlitres 3 M NaOAc, pH 4.8 was added and mixed gently by inversion. This mixture was incubated 45 minutes on ice and centrifuged full speed in an Eppendorf centrifuge for 10 minutes. The supernatant was transferred to a new microcentrifuge tube containing 1 ml 95% ethanol, mixed, and centrifuged full speed in an Eppendorf centrifuge for 10 minutes. The supernatant was removed and the nucleic acid pellet resuspended in 200 microlitres of 0.1 M NaOAc, pH 4.8 and 500 microlitres of 95% ethanol was added, mixed, and the mixture centrifuged full speed in an Eppendorf centrifuge for 10 minutes. The pellet was rinsed with 70% ethanol, air dried, and resuspended in 20 to 50 microlitres of TE.
Fast bacterial DNA minipreparation

A five ml LB aliquot with suitable antibiotic selection was inoculated with a bacterial strain and shaken overnight at 37 degrees Celsius. An aliquot of this culture was transferred to a microcentrifuge tube and centrifuged for 20 seconds. The supernatant was discarded and the bacterial pellet resuspended in 100 microlitres of freshly prepared STET buffer, 8% w/v sucrose, 5% w/v Triton X-100, 50 mM EDTA, 50 mM Tris HCl, pH 8. The resuspended pellet was boiled for two minutes and then centrifuged in a microfuge at 4 degrees Celsius at full speed for 15 minutes. The supernatant was transferred to another microcentrifuge tube and 100 microlitres of isopropanol added to precipitate DNA. Then the tube was centrifuged in a microfuge at 4 degrees Celsius at full speed for 15 minutes. The supernatant was discarded and the pellet rinsed with 70% ethanol, and air-dried on the bench or in a speed vacuum centrifuge. Finally, the pellet was resuspended in 40 microlitres of sterile distilled water or of TE, and stored at 4 degrees Celsius (Gatermann et al., 1988).

Preparation of total *A. tumefaciens* DNA

An aliquot of a well-grown overnight 2 ml culture of *A. tumefaciens* grown in LB with appropriate antibiotic selection at 28 degrees Celsius was centrifuged for 20 seconds in a 1.5 ml microcentrifuge tube. The supernatant was aspirated away and the bacterial pellet resuspended in 400 microlitres TE by vortexing. Twenty microlitres 20% w/v sodium dodecyl sulfate (SDS) and 50 microlitres 20 mg/ml Proteinase K were added and the mixture incubated at 65 degrees Celsius for 20 minutes. Then 57 microlitres 5 M NaCl were added and vortexed into the viscous solution thoroughly to mix, and incubated at 65 degrees Celsius for 20 minutes. Five hundred microlitres phenol were added, mixed thoroughly, and centrifuged full speed in a microfuge for 5 minutes. The upper aqueous phase was removed into a new microcentrifuge tube, extracted with 500 microlitres chloroform and centrifuged full speed in a microfuge for 5 minutes. The upper phase was transferred again to another microcentrifuge tube containing 1 ml cold 95% ethanol and centrifuged full speed in a microfuge for 5 minutes. The supernatant was removed, and the pellet...
air dried and resuspended in 200 microlitres TE. Twenty microlitres 3 M NaOAc, pH 4.8 and 500 microlitres 95% ethanol were added to precipitate the nucleic acids. The tube was centrifuged full speed in a microfuge for 5 minutes, the supernatant was removed, and the pellet was rinsed with 70% ethanol and air dried. The pellet was then resuspended in 50 microlitres TE.

**Bacterial DNA maxipreparation**

A 500 ml aliquot of LB (containing appropriate selective antibiotics) was inoculated with a 5 ml overnight culture of desired bacterial strain and shaken at 37 degrees Celsius overnight. The bacteria were then centrifuged down at 4000g for 10 minutes at 4 degrees Celsius in sterile bottles and the bacterial pellets resuspended in approximately twice their volume of suspension buffer, 50 mM glucose, 25 mM Tris HCl, 10 mM EDTA, pH 8. The suspension buffer was made by combining 0.9 grams glucose, 0.37 grams EDTA, and 5 ml 1 M Tris pH 8 stock. The volume made up to 100 ml and autoclaved. Lysozyme was added to a final concentration of 5 mg/ml and mixed vigorously; the mixture was then incubated at room temperature for five minutes. A 2X volume of cold, newly made lysis buffer, 0.2 N NaOH, 1% SDS, was added and the solution was mixed by gentle inversion. Then the tube was incubated for 10 minutes on ice. 3 ml cold 3 M / 5 M potassium acetate (for 60 ml acetate mixture 28.5 ml distilled water and 29.4 grams KOAc were combined. 11.5 ml glacial acetic acid were then added and the mixture autoclaved) was added. The solution was gently inverted to mix and incubated 10 minutes on ice. Then it was centrifuged 30 minutes at 20,000g at 4 degrees Celsius, and the supernatant transferred to sterile 15 ml Corex tubes. Iso-propanol, 0.6 volumes at room temperature, was added, mixed, and incubated at room temperature for 15 minutes, followed by centrifugation for 30 minutes at 17,000g at room temperature. The supernatant was discarded, and the DNA pellet was rinsed with 70% ethanol. If necessary, the tube was centrifuged briefly to settle the pellet to facilitate removal of 70% ethanol. The pellet was air dried and dissolved in 5 ml TE.

A combination of 1 gram anhydrous CsCl and 80 microlitres 10 mg/ml EtBr (ethidium bromide) stock for each ml of the DNA and TE solution was added to the DNA solution (Maniatis *et al.*, 1982), and the resulting solution (1.55 g/ml CsCl) loaded into heat-sealed tubes and
centrifuged at 40,000 g overnight at room temperature. The plasmid band was removed using a sterile 18 gauge needle and sterile 3 cc syringe. EtBr was removed by successive extractions with water-saturated butanol in a sterile 15 ml Sarstedt tube and then the salts were dialyzed away in two successive baths of 600 ml each of TE, pH 8, gently stirred. For two litres, 20 ml 1 M Tris pH 8 stock and 4 ml 0.5 M EDTA pH 8 stock were made up to 2 L with distilled water, followed by an overnight dialysis bath with gentle stirring in 800 ml of TE at 4 degrees Celsius. The dialysed DNA solution was removed into sterile 1.5 ml microcentrifuge tubes.

2.2.2 Construction and screening of a poplar genomic library

A genomic library was constructed from genomic DNA extracted from the hybrid poplar 53-242 (a gift from Brian Watson and Dr. H. Bradshaw). Partial MboI digests using serial enzyme dilutions were run (according to Maniatis et al., 1982) in 50 microlitre final volumes; the three best digests (giving an apparent mean molecular weight of 15 to 20 kb) were individually extracted once with one volume of 25:24:1 phenol:chloroform:isoamyl alcohol and once with one volume of 24:1 chloroform:isoamyl alcohol, then ethanol-precipitated by the addition of 1/10 volume 3M NaOAc pH 4.8 and 2 volumes 95% ethanol. Each sample was incubated 10 minutes on ice, centrifuged, rinsed once with 70% ethanol, and air dried for one hour on the bench. The pellets were resuspended in 20 microlitres TE at 4 degrees Celsius overnight. Recovery was tested by visual examination of 1 microlitre of the 20 microlitre resuspension diluted into 10 microlitres TE and 1 microlitre of loading buffer and run on a 0.7% agarose gel.

The MboI sites were then partially filled in with "A" and "G" nucleotides (Pharmacia) at a final concentration of 1 mM using 5 units of Klenow from DuPont and 3 microlitres BRL-React 1 buffer, all added to the 20 microlitre resuspensions to a final volume of 30 microlitres. This reaction ran for 30 minutes at 37 degrees Celsius. The reaction volume was extracted with one volume of 25:24:1 phenol:chloroform:isoamyl alcohol (v/v/v) and once with one volume of 24:1 chloroform:isoamyl alcohol (v/v), then ethanol-precipitated by the addition of one tenth volume 3M NaOAc pH 4.8 and 2 volumes 95% ethanol. Each sample was incubated for 10 minutes on ice, centrifuged, rinsed once with 70% ethanol, and air dried for one hour on the bench. The pellets
were resuspended in 20 microlitres TE. Recovery was tested by visual examination of 1 microlitre of the 20 microlitre resuspension diluted into 10 microlitres TE and 1 microlitre of loading buffer and run on a 0.7% agarose gel.

Three quarters of a microlitre from each of the three parallel treatments were pooled for a total of approximately 1.5 micrograms of cut and filled-in genomic DNA. This was combined with 2.5 micrograms (5 microlitres) of XhoI Half Site Arms from the Promega Lambda GEM12* XhoI Half-Site Arms Cloning Kit for a molar ratio of 1 : 2.5 insert : arms. T4 ligase, 0.5 microlitres, 0.8 microlitres ligation buffer, and 0.25 microlitres distilled water were added for a final ligation volume of 8 microlitres. This reaction ran for 45 minutes at 25 degrees Celsius. Successful ligation was tested by visual examination of 1 microlitre of the 8 microlitre ligation mixture diluted into 10 microlitres TE and 1 microlitre of loading buffer and run on a 0.7% agarose gel.

The ligation was then packaged using the Gigapack II Gold packaging protocol from Stratagene, using half of the ligation mixture (approximately 3.5 microlitres) giving 1.5 micrograms participating in the packaging. Five hundred microlitres SM (0.58g NaCl, 0.2 g MgSO4, 5 ml 1M Tris pH 8), were mixed and made up to a volume of 100 ml and autoclaved for 15 minutes) were added, followed by 20 microlitres of chloroform, and the mixture was centrifuged briefly and put at 4 degrees Celsius for storage.

Bacterial strains for titration and screening of the genomic library were prepared as follows: strain KW251 from the Promega Lambda GEM12 XhoI Half-Site Arms Cloning Kit was streaked and allowed to grow overnight at 37 degrees Celsius on an NZCYM plate. This medium consisted of 10 grams NZamine, 5 grams NaCl, 5 grams bacto-yeast extract, 1 gram casamino acids, 2 grams MgSO4.H2O dissolved in 950 ml of deionized water, adjusted to pH 7.0 with 5 M NaOH, adjusted to 1 litre and made into plate agar by the addition of 15 grams of agar, and autoclaved. Single colonies were picked into prewarmed sterile aliquots of 50 ml LB in 250 ml shaker flasks supplemented with 0.5 ml each of 20% w/v filter-sterilized (0.22 micron syringe filter) maltose and sterile 1M MgSO4. The flasks were then shaken for 6 hours at 37 degrees Celsius; the cultures were then poured gently into new sterile 50 ml Falcon tubes, centrifuged at 1000g for 10 minutes at 25 degrees Celsius, and gently resuspended in 25 ml of sterile 10 mM MgSO4. The prepared bacteria were stored at 4 degrees Celsius and used within 48 hours, particularly for initial platings.
This library was screened twice for genomic clones of poplar PAL using the PAL 7 cDNA as a probe (this in turn had been isolated from a poplar cDNA library using the 3' 1.5 kb end of a potato PAL cDNA (Subramaniam et al., 1993); 14 positive isolates were obtained.

2.2.3 Restriction analysis, fragment isolation, and ligation

The bacteriophage DNA for most of these clones was prepared as in section 2.2.1 and the genomic inserts mapped by restriction digest and hybridization to 5' and 3' probes derived from cDNA PAL 7. Restriction digests were carried out according to manufacturers' instructions using commercially supplied enzymes and buffers.

Fragments to be studied further were isolated from agarose gels by running the fragment onto DE81 paper (Whatman DEAE Ion Exchange Chromatography Paper) prepared by soaking for five minutes in 2M NaCl made up with sterile TE pH 8, rinsing twice with TE pH 8, and storing in sterile TE pH 8. The portion of the DE81 with DNA adhering was cut away and placed in a 0.5 ml sterile microcentrifuge tube with a hole punched in the bottom, centrifuged so that all liquid was removed into the larger Eppendorf tube (2 minutes at full speed in an Eppendorf centrifuge, with the small microcentrifuge tube suspended in a 1.5 ml microcentrifuge tube), and then incubated for 30 minutes under 30 microlitres of 1M NaCl in TE pH 8 saturated with urea. The DNA was released into the solution, which was then centrifuged off as above into a new sterile 1.5 ml microcentrifuge tube. The solution was cleared of urea by processing through a G-50 spin column. This method was also used to isolate fragments to be used as templates for labelled probes.

Isolated fragments were mixed in appropriate relative molar concentrations of ligatable ends and ligated using commercially available enzymes and buffers. Ligation reactions usually proceeded at 12 to 16 degrees Celsius overnight.
2.2.4 Electrophoretic separation and analysis of nucleic acids

Agarose gels were made by mixing 0.6 to 1.0 grams of agarose per 100 ml of 1X TAE buffer, microwaving to a boil and then shaking occasionally until agarose was dissolved and mixture had cooled to approximately 60 degrees Celsius. 50X TAE stock was made by combining 242 grams Tris base, 57.1 ml glacial acetic acid, and 100 ml 0.5 M EDTA pH 8 and making up to one litre with distilled water. Then one or two microlitres of 10 mg/ml EtBr stock per 100 ml TAE/agarose was added and the gel was poured. Gels were run at 30 to 100 volts in 1X TAE buffer.

Southern analysis

DNA was size-fractionated on agarose gels and denatured by one hour gentle shaking under alkaline denaturation solution, 1.5 M NaCl, 0.5 M NaOH. The gel was then neutralized in neutralization buffer, 1 M Tris HCl, pH 8, 1.5 M NaCl, for one hour and transferred onto Hybond by overnight transfer (Maniatis et al., 1982) using 10X SSC as transfer buffer. The 20X SSC stock was made by combining 175.3 grams NaCl with 88.2 grams sodium citrate, the pH adjusted to pH 7 with concentrated HCl, and the volume made up to one litre with distilled water. Alternatively, denaturation, neutralization, and transfer were effected using the Pharmacia LKB2016 Vacugene vacuum blotting system according to manufacturers' instructions. For some genomic DNAs, a pretreatment of five minutes exposure to a de-purination solution (0.25 M HCl) served to break higher molecular weight strands into smaller fragments, thus facilitating effective transfer to the filter.

Blots were prehybridized at 65 degrees Celsius for 2 hours in 10 ml of prehybridization solution, 6X SSC, 0.5% SDS, 5X Denhardt's solution, 100 micrograms / ml single-stranded DNA, for every 150 to 200 cm² of filter. Prehybridization buffer was made by mixing 3 ml 20X SSC stock, 0.25 ml 20% SDS, 100 microlitres single-stranded DNA, 1 ml 50X Denhardt's solution stock (five grams each Ficoll, polyvinylpyrrolidone, and Pentax Fraction V BSA were combined with water to make a final volume of 500 ml), and 5.5 ml distilled water to make a final volume of 10 ml.
Blots were then transferred to a hybridization solution made in the same manner with the addition of 200 microlitres 0.5 M EDTA per 10 ml hybridization solution to a final concentration of 0.01 M EDTA, and a \(^{32}\)P-labelled probe, and allowed to hybridize overnight.

Blots were then rinsed twice for five minutes each in 2X SSC at room temperature, washed for one hour at 65 degrees Celsius in a wash of 2X SSC for low stringency and 0.5X SSC for high stringency, and then promptly wrapped in Saran wrap, monitored with a handheld Geiger counter, and exposed to X-ray film at 25 degrees Celsius or at -90 degrees Celsius with amplifying screens based on the Geiger readings. X-ray films were developed in a Kodak M35A X-OMAT Processor.

**Northern analysis**

Northern analysis was performed in the same manner as Southern analysis, except that 0.1% SDS was added to the rinse buffers to reduce background.

**2.2.5 Labelling of probes**

Labelling of DNA fragments was carried out according to the BRL Random Primers DNA Labeling System instructions. Approximately 25 nanograms of template was denatured by heating to 100 degrees Celsius for six minutes and chilled in ice-water. The denatured template, in a volume of 10 microlitres or less, was combined with 2 microlitres each of appropriate cold nucleotides, 15 microlitres buffer, and a volume of distilled sterile water to bring the total volume to either 45 (if two labelled nucleotides were being used) or 47 (if only one labelled nucleotide was being used) microlitres. One microlitre of Klenow enzyme was added on ice, and then 2 microlitres of each \(^{32}\)P-labelled nucleotide to be incorporated was added behind shielding, for a final reaction volume of 50 microlitres. The reaction ran for 1 to 3 hours, and the labelled probe was separated from unincorporated label by adding 50 microlitres TE and centrifuging the mixture through a Sephadex G-50 column at 1550g for 3 minutes into a sterile 1.5 ml screw-cap microcentrifuge tube. A one microlitre aliquot was taken for scintillation counting to document incorporation, and the
remainder was heated to 100 degrees Celsius for 6 minutes to denature the strands, and chilled on ice water in preparation for use.

2.2.6 Sequencing

35S sequencing - reactions and gels

Some sequence information was obtained by in-laboratory sequencing using 35S label and the T7 Sequencing Kit from Pharmacia. Plasmid DNA was prepared using the "Fast bacterial minipreparation" method in section 2.2.1. Two micrograms of DNA in less than eight microlitres volume was combined with 2 microlitres 2 M NaOH and a volume of sterile distilled water to bring the final volume to 10 microlitres in a 1.5 ml microcentrifuge tube. Denaturation was allowed to proceed for 10 minutes at room temperature; then 3 microlitres 3M NaOAc and 7 microlitres sterile distilled water was added to each tube and mixed. Then 60 microlitres of cold 95% ethanol were added and the denatured DNA precipitated overnight at -20 degrees Celsius. It was then microfuged for 10 minutes at 4 degrees Celsius, rinsed with cold 70% ethanol, vacuum-dried, and resuspended in 10 microlitres sterile distilled water. On ice, 2 microlitres of appropriate primer were added, followed by 2 microlitres of annealing buffer. Annealing of primer took place for 20 minutes at 37 degrees Celsius. Then the tubes were cooled at room temperature for 10 minutes, and then an Enzyme Premix of 6 microlitres was added to each tube and incubated for 5 minutes at room temperature. For one reaction, the Premix was composed by combining 1 microlitre sterile distilled water, 3 microlitres Labelling Mix, 2 microlitres diluted T7 Polymerase (equivalent to 3 units for each reaction, from an 8 unit/microlitre stock made by diluting 1 microlitre T7 Polymerase stock into 5 microlitres cold Enzyme Dilution Buffer), and 2 microlitres labelled nucleotide. In a series of 4 microcentrifuge tubes for each reaction, 2.5 microlitres of the 4 nucleotide Mixes (A, G, C, T) were aliquoted and preheated to 37 degrees Celsius for at least one minute. Then 4.5 microlitres of the hot master tubes were transferred into each of the individual tubes and incubated for 5 minutes at 37 degrees Celsius. Five microlitres of Stop Buffer were added to each tube, and the samples
were denatured by heating to 80 degrees Celsius for 2 minutes, chilled on ice water, and loaded onto a gel, 2 microlitres per well.

Most sequencing gels were run as 6% polyacrylamide 33 x 40 cm gels. Both plates were washed thoroughly and rinsed with distilled water and allowed to dry. The larger plate was treated with a mixture of 20 ml 95% ethanol, 600 microlitres of 10% acetic acid, and 60 microlitres methacryloxypropyltrimethoxysilane and allowed to dry; this was the hydrophilic plate. The other, smaller plate was treated with about 30 ml of dimethyl dichlorosilane solution (supplier BDH) as the hydrophobic plate. Both plates were polished when they were dry, the large plate with 20 ml iso-propanol and the smaller plate with 10 ml distilled water. When both plates had dried from this treatment, they were taped together with 0.5 mm spacers and comb with yellow electrician tape. For a 6% gel, 31.5 grams urea was mixed with 30 ml distilled water, heated to dissolve the urea, and cooled to room temperature. Then 11.5 ml of acrylamide stock was added (38 grams of acrylamide were combined with 2 grams N,N'-methylene bisacrylamide and distilled water to a final volume of 100 ml) and this mixture was de-ionized with 0.5 gram analytical grade mixed bed resin (Bio-Rad AG 501-X8 (D) Resin, 20-50 mesh) by stirring for 5 minutes. The resin was then filtered out through a vacuum flask apparatus with a Whatman 1 filter paper disk; 7.5 ml 10X TBE buffer was also filtered through, care being taken to avoid the resin beads. 10X TBE was composed of 54 grams Tris base, 27.5 grams boric acid, and 20 ml of 0.5 M EDTA stock made up to 500 ml final volume with distilled water. If necessary, this solution was filtered through Whatman 1 filter paper, autoclaved, and stored in a bottle rinsed thoroughly with 0.5 M EDTA. This mixture was de-gassed under vacuum for five minutes, and then 500 microlitres of freshly made 10% ammonium persulfate and 16 microlitres of TEMED (N, N, N', N' tetramethyl ethylenediamine) were added to initiate polymerization. This mixture was rapidly poured between the prepared plates, the comb was inserted upside down at the top of the gel, and the plates were clamped along the sides.

The gel was allowed to polymerize for 1 to 2 hours, and was then stripped of tape and clamps, rinsed and comb inserted, and set to preheat for 45 minutes at about 95 watts on a Bio-Rad Model 3000Xi Computer Controlled Electrophoresis Power Supply, in a Bio-Rad Model S2 rig with 1X TBE buffer. Wells were rinsed and samples were loaded and run for an appropriate
time. The rig was drained and the plates were pulled apart; the large plate and the adhering gel were soaked for 20 minutes in 10% acetic acid to remove the urea; then the gel was rinsed and dried at 60 degrees Celsius in a blowing oven. The gel was cooled and loaded into a cassette with X-ray film to develop images.

**UBC Nucleic Acid and Protein Sequencing (NAPS) sequencing - DNA isolation and reactions**

Other sequencing was done by preparing plasmid DNA and sending out reactions to the NAPS laboratory for fluorescence sequencing.

DNA was prepared by a modified alkaline lysis / PEG precipitation procedure; bacteria were first grown in TB broth overnight at 37 degrees and then centrifuged down for 20 seconds in 1.5 ml microcentrifuge tubes. TB broth was made by adding 100 ml of sterile 0.17 M KH$_2$PO$_4$ and 0.72 M K$_2$HPO$_4$ to 900 ml sterile base broth composed by mixing 12 g bacto-tryptone, 24 g bacto-yeast extract, and 4 ml glycerol, making up the volume to 900 ml with distilled water and autoclaving. Three volumes of culture were centrifuged down consecutively in each microcentrifuge tube so as to triple this low-yield procedure. The pellet was resuspended in 200 microlitres of GTE buffer, 50 mM glucose, 25 mM Tris HCl, 10 mM EDTA, pH 8, (0.9 grams glucose, 2.5 ml 1M Tris HCl pH 8, 2 ml 0.5M EDTA pH 8 were combined and made up to 100 ml with distilled water, and autoclaved) Then 300 microlitres of fresh lysis buffer, 0.2 N NaOH, 1% SDS was added to each tube, mixed by inversion, and allowed to sit on ice for five minutes. Three hundred microlitres of 3M KOAc pH 4.8 were added and mixed by inversion, and incubated on ice for five minutes. The mixture was then centrifuged in a centrifuge at full speed for ten minutes at room temperature and the supernatant transferred to a new microcentrifuge tube with 14 microlitres of 1 mg/ml DNase-free RNase A stock and incubated for 20 minutes at 37 degrees Celsius. Next, 400 microlitres of chloroform were added and mixed by inversion for 30 seconds; the mixture was centrifuged for one minute and the aqueous phase was transferred and re-extracted with another 400 microlitres of chloroform. An equal volume of isopropanol was added to the transferred aqueous phase and the DNA was centrifuged down for 10 minutes at room
temperature. The pellet was rinsed with 70% ethanol and air dried on the bench for half an hour; then it was redissolved in 32 microlitres of sterile distilled water. Next, 8 microlitres of 4M NaCl were added and mixed, and 40 microlitres of 13% PEG8000 were added and mixed in. The DNA was centrifuged for 15 minutes at 4 degrees Celsius. The supernatant was carefully removed from the delicate pellet, the pellet rinsed with 0.5 ml 70% ethanol, air dried, resuspended in 20 microlitres sterile distilled water, and stored at -20 degrees Celsius.

Approximately one microgram of this DNA was mixed with 3.2 picomoles of appropriate primer, made up to 10.5 microlitres with sterile distilled water, and taken to the NAPS Unit for processing; a four-color sequencing printout produced by the Dye-Deoxy Terminator process (Perkin Elmer Applied Biosystems) was returned.

Alignment and comparison of sequences

When required, sequences were typed into the GCG Sequence Analysis Software program at cansnd.cistl.nrc.ca in Ottawa, Canada. They were aligned using the "bestfit" command with the default values of Gap Weight equal to 5.000 and Length Weight equal to 0.300. This software could also be used to search for sequence motifs of interest, using the command "motifs".

2.2.7 Polymerase chain reaction (PCR) used to amplify a diverged PAL gene

PCR was used to amplify a fragment of a diverged PAL gene from H-11 genomic DNA (Figure 5). The PCR primers were a gift to the Douglas laboratory from the laboratory of Dr. Brian Ellis, and the amplification, subcloning, and partial sequencing of the PCRPAL16 clone corresponding to the hybrid poplar pedigree's version of a diverged poplar PAL gene identified in *Populus kitakamiensis* (Osakabe *et. al.*, 1995) were carried out by Shirley Duong. She used one primer corresponding to nucleotides 1172 through 1196 (a.a. RTSPQWL) of cDNA PAL7, and a second primer corresponding to nucleotides 1550 through 1569 (a.a. HVQSAEQ) to amplify out a 400 bp fragment.
Figure 5. PCR strategy for subcloning of a fragment equivalent to a divergent $pal\ g2a$, or $PAL3$.

The primers from the Ellis laboratory were used to amplify the analogous fragment from the PAL7 cDNA using the following reaction mixture and amplification conditions on an MJ Research MiniCycler Programmable Thermal Controller, Model PTC-150: in 40 microlitres, 0.25 microlitres 10mM dNTP mix, 5 microlitres each 20 pmol/microlitre primer stocks, 1 microlitre 10 nanograms/microlitre template stock, 22 microlitres sterile distilled water, 1.2 microlitres 50 mM MgCl$_2$ stock, 4 microlitres 10X PCR buffer, and 0.4 microlitre of Taq polymerase were combined. (The latter three components were supplied in a Taq DNA Polymerase kit by Gibco BRL.) The mixture was overlain with a drop of mineral oil and allowed to amplify by a program of 5 minutes at 94 degrees Celsius, followed by 35 repetitions of the following cycle: 50 seconds at 94 degrees Celsius, 50 seconds at 55 degrees Celsius, and 70 seconds at 72 degrees Celsius. The tube was frozen and the mineral oil removed and a 25% aliquot of the reaction mixture was run on an agarose gel to verify the amplified fragments.
2.3 Plant methods

2.3.1 Poplar methods

Poplar maintenance

Poplar clones H-11, 53-242, 53-246, 93-968, ILL-129, and DT 49-177 were grown from one foot cuttings in standard potting mix in 6" or 8" pots in the greenhouse at approximately 20 degrees Celsius and seasonal light, or in a Conviron CMP 3244 growth chamber at 20 degrees Celsius and 23 hours light, or in the field in light and temperature seasonal to Vancouver, British Columbia.

Collection of field samples

DT 49-177 material was collected from the Scott Paper nursery at Agassiz, British Columbia during the growing season of 1993, from February through October, from two adjacent clones of DT 49-177. These materials consisted of bud or leaf, and scrapes of secondary xylem from a branch about two to four years old. In summary, for each month, a total of four samples (leaf and xylem from trees 1 and 2) were collected and frozen in liquid nitrogen on the site and stored at -90 degrees Celsius until extracted.

2.3.2 Tobacco methods

Tobacco maintenance

Nicotiana tabacum strain SR-1 plants were grown in sterile culture in a Conviron growth chamber with continuous light at 23 degrees Celsius. The plants grew in Magenta boxes with 40 ml solid MS medium. This medium was composed by mixing one package of Gibco BRL
Murashige and Skoog Salt Mixture (Murashige and Skoog, 1962) into 750 ml of distilled deionized water with 30 grams of sucrose, adding 1 ml of 1000X vitamins (100 ml of this stock was made by combining 10 grams inositol, 1 gram thiamine HCl, and 0.1 gram each of nicotinic acid and pyridoxine HCl and making up to 100 ml with distilled deionized water) and adjusting the pH to 5.7 with dilute KOH. The volume was made up to one litre with distilled deionized water and the solution was autoclaved. Solid medium was made by the addition of 7.5 g agar before autoclaving.

**Tobacco transformation**

Tobacco plants were transformed (Horsch et al., 1985) by infection with an *A. tumefaciens* strain called LBA4404 containing appropriate BIN19-based constructs by cutting one cm² sections from between the veins of leaves about 4 to 5 centimeters long from axenically maintained SR1 plants. The infection was carried out as follows. A 2 ml culture of the appropriate LBA4404 strain was grown overnight at 25 degrees Celsius in LB-kanamycin. This culture was diluted under sterile conditions into 20 ml of sterile liquid MS in a sterile 100 x 15 mm disposable petri plate. The sections of tobacco leaf were immersed in the diluted culture for about 3 minutes and then placed upside-down on solid TC- plates. TC medium consisted of MS liquid medium with 4 grams of agar/500 ml and added phytohormones 5 ml/500 ml of 100 mg/L BAP stock (benzylamino-purine, Sigma) and 1 ml/500 ml of 100mg/L NAA stock (naphthalene-acetic acid, Sigma). The bacteria and plant sections were co-cultivated for 2 to 3 days in a Conviron chamber at 23 degrees Celsius under continuous light until a film of bacteria was seen starting to grow out from the explants. The explants were then transferred to TC+ plates. TC+ plates are TC-medium with the addition of 500 microlitres/500 ml of 100mg/ml kanamycin stock and 500 microlitres/500 ml of 300mg/ml carbenicillin stock to select transformed shoots and to select against bacteria.

Explants/calli were transferred to new TC+ plates every week and were examined regularly for the development of leafy shoots. These shoots were excised to Magenta boxes containing 40 ml of solid MS-kanamycin/carbenicillin (concentrations of antibiotics as in TC+.
plates) to encourage the formation of roots. When a healthy root system had developed the plantlets were removed from sterile culture and placed into individual 6 inch pots with potting medium and allowed to grow either in growth chamber or in greenhouse conditions.

**Collection of transformed seed and manipulation of T1 seedlings**

When seed pods and their stems had turned brown, transgenic T1 seeds were collected into 1.5 ml microcentrifuge tubes by pinching off the tip of the pod and shaking the contents into the tube. These tubes were stored in the dark in a dry cool place.

Sterile seeds were generated by shaking in 70% ethanol in a 60 X 15 mm disposable Petri dish for one minute, and then in 3 or 4 successive washes of distilled sterile water. The seeds were allowed to dry in the sterile hood and were stored in the sealed petri dishes.

Sterile seeds were germinated on MS-kan plates (concentration of kanamycin as in TC+ plates above) and ratios of kanamycin-resistant to kanamycin-susceptible T1 for each transformant were determined after about two weeks of growth in the Conviron chamber as above; susceptible seedlings only germinate, do not develop true leaves, and generally have a yellowed appearance in comparison to their kanamycin resistant siblings. A plate of negative control, non-transformed SR1 seeds were germinated at the same time to demonstrate the susceptible phenotype. Estimated number of insertional loci were then calculated from the ratios of resistant to susceptible seedlings for each transformant. In order to determine if concatenated inserts were present, Southern analysis of DNA of transformed tobaccos was performed to determine the number of borders between plant and inserted DNA.
2.4 Histochemical methods

2.4.1 Toluidine blue staining of poplar stem sections

To visualize stem tissues subjected to analysis of seasonal variation of PAL levels, poplar branches harvested in May from the Agassiz plantation were wrapped in moistened towelling and brought back to the laboratory in Vancouver where hand sections were taken using a Wilkinson Sword Classic Sword-Edge razor blade; these sections encompassed the bark, cambium, and underlying differentiating and differentiated wood tissue. The sections were immersed in 0.05% w/v toluidine blue dissolved in sterile distilled water for ten minutes and then rinsed in sterile distilled water and mounted for microscopic examination and photography, using Tungsten Ektachrome film in a Zeiss MC 100 camera on a Zeiss Axioskop microscope.

2.4.2 Assay of GUS expression associated with development

Transformed tobacco plants were assayed using the histochemical assay for GUS activity driven by the poplar promoters by hand-sectioning tissues (leaves, stems, and flowers of various ages) with a Wilkinson Sword Classic Sword-Edge razor blade and immediately placing the sections into 1.5 ml microcentrifuge tubes containing a solution of 0.5 mg/ml 5-bromo-4-chloro-3-indol-beta-D-glucuronic acid (X-Gluc.) in 100 mM NaPO₄ buffer, pH 7 and incubating with shaking or periodic inversion at 37 degrees Celsius for 1 to 18 hours (Jefferson, 1987). The buffer was made by mixing 30.5 ml of 0.2 M Na₂HPO₄ with 19.5 ml of 0.2 M NaH₂PO₄ and making up the volume to 100 ml with distilled water.

The X-Gluc-Phosphate buffer was pipetted off and replaced with 95% ethanol for 2 to 24 hours at 25 degrees Celsius to clear the tissues. The ethanol was then replaced stepwise with 1:1 glycerol:distilled water as a medium for mounting and microscopic evaluation. Representative sections were photographed as above.

In order to examine GUS expression in T1 seedlings, sterile seeds were germinated for several of the strongest-expressing transformants for both vectors, and several seedlings were
removed from the plate for each timepoint and immersed in the above staining solution overnight at 37 degrees Celsius. They were then cleared, mounted, and photographed as described above.

2.4.3 Assay of GUS expression associated with wounding

For the wounding experiments, 6 circular sections were cut out of 10 to 15 cm leaves of GUS-responsive T1 individuals for each of the transformed constructs (representing the promoters of PAL1 and PAL2). The sections were incubated for 24 hours in a Conviron chamber at 23 degrees Celsius under continuous light in petri plates on sterile Whatman paper moistened with MS- medium. This constituted the wounding treatment. The circular edges constituted the wounded site. Then the sections were cut in half to create two half circles and were incubated for 24 hours at 37 degrees Celsius in X-Gluc/ NaPO4 buffer as above to stain tissues in which GUS was active. The straight edges constituted a control to determine if staining was due solely or partially to the capacity of the medium to penetrate the sections via a cut edge. The sections were then cleared, mounted and examined microscopically as described above.

2.5 Protein methods

2.5.1 Extraction of total protein from poplar tissues

For each extraction, 5 to 6 cm² of leaf, (about 0.5 grams) or 3 to 4 buds, or about 0.5 grams of xylem or bark tissue were ground in a mortar and pestle using sand and liquid nitrogen for 2 to 3 minutes. The ground extracts were stored under liquid nitrogen until 4 extracts had been processed. Then each extract was tapped into a 1.5 ml microcentrifuge tube containing chilled 1 ml aliquots of combined PVPP and extraction buffer 50mM Tris, 5 mM MgCl₂ Extraction buffer consisted of 2.5 ml of 1M Tris pH 8 mixed with 10 ml of 25 mM magnesium chloride and made up to 50 ml with distilled water. About 0.1 g PVPP was added to each microcentrifuge tube. The tubes were shaken thoroughly and then immediately centrifuged for 10 minutes at 4 degrees
Celsius at maximum speed in an Eppendorf microfuge. The supernatant was transferred onto a
G-50 column as described for the purification of restriction fragments, except that the G-50 was
suspended in extraction buffer. These columns were centrifuged for 3 minutes and the spin-
through was collected and subjected to Bradford analyses.

After Bradford quantification, appropriate volumes (containing 20 micrograms of total
protein) were aliquoted and dried down in a speed vacuum apparatus and stored at -20 degrees
Celsius.

2.5.2 Bradford assay

Bradford protein assay was performed by first making a stock solution of Bovine Serum
Albumin (Fraction V, Sigma) at a concentration of 1000 micrograms/ml in distilled sterile water; this
stock was stored at -20 degrees Celsius. It was used to make a suitable dilution series, usually
ranging from 0.0 to 20.0 microlitres of stock diluted into a total of 800 microlitres with sterile distilled
water. Two hundred microlitres of Bradford reagent (Bio-Rad Protein Assay Dye Reagent
Concentrate) were added to each tube and allowed to rest at 25 degrees Celsius for
approximately 20 minutes. Suitable volume aliquots from protein samples were diluted and
treated in the same manner. Then all samples were read for absorbance at 595 nanometers and
protein concentrations were determined using the Protein Assay Soft-Pac Module (program 6) in
the Beckman DU Series 60 Spectrophotometer (Bradford, 1976).

2.5.3 Electrophoretic separation of proteins

Stacking gels were prepared (according to Sambrook et al., 1989), using the Bio-Rad
Model 360 Mini Vertical Slab Gel apparatus with 1.5 mm spacers. A single pouring rig was
cleaned and assembled in the flow hood with yellow tape around the bottom three sides and red
clamps tightly fastened. A 15 ml aliquot of resolving gel was assembled in a 15 ml sterile Sarstedt
tube by combining 5.9 ml distilled water, 5.0 ml 30% w/w acrylamide mix (29 grams acrylamide
and 1 gram bis-acrylamide (N, N'-methylene bis-acrylamide) were combined with 50 ml warm
distilled water to dissolve, made up to 100 ml with distilled water, filtered through Whatman 1 filter paper, and stored in a dark glass bottle at 4 degrees Celsius), 3.8 ml 1.5 M Tris base pH 8.8 mix (18.16 grams Tris base mixed with 75 ml distilled water, adjusted to pH 8.8 with HCl, made up to 100 ml and autoclaved), and 70 microlitres of 20% w/w sodium dodecyl sulfate in distilled water (autoclaved), with the final addition of 300 microlitres of freshly made 10% w/v ammonium persulfate in sterile distilled water and 6 microlitres TEMED (N, N, N', N'-tetramethylethylene diamine). The assembled mix was quickly loaded into the gel apparatus using a 9 inch Pasteur pipet to a mark 0.75 cm below the final position of the teeth of the loading comb. The gel was overlain with water-saturated butanol to prevent oxidation and allowed to polymerize for 30 minutes at 25 degrees Celsius. Then the butanol was rinsed away with distilled water, and the internal surfaces of the rig were carefully dried with strips of Kimwipe. A 4 ml aliquot of stacking gel mix was assembled in a sterile 15 ml Sarstedt tube as follows: 2.7 ml distilled water, 0.67 ml 30% w/w acrylamide mix, 0.5 ml 1.0 M Tris base pH 6.8 mix (12.11 grams Tris base were mixed with 75 ml distilled water, adjusted to pH 6.8 with HCl, made up to 100 ml and autoclaved), and 40 microlitres of sterile 20% w/w sodium dodecyl sulfate in distilled water were combined, and 100 microlitres of freshly made 10% w/v ammonium persulfate in sterile distilled water and 4 microlitres TEMED were added. The assembled mix was very quickly loaded into the gel apparatus using a 9 inch Pasteur pipet and the comb was inserted carefully so as to minimize bubbles. The gel was allowed to polymerize for 30 minutes at 25 degrees Celsius.

Samples were prepared for loading by resuspending the pre-quantified aliquots of 20 micrograms total protein (prepared as in section 2.5.1) in 16 microlitres of distilled sterile water and adding 8 microlitres of "sample combination" made as follows: for 10 samples, 27 microlitres of 1M dithiothreitol were mixed with 88 microlitres of 3X SDS loading buffer, 150 mM Tris-HCl, pH 6.8, 300mM dithiothreitol, 6% SDS, 0.3% bromphenol blue, 30 % glycerol. For 500 microlitres of 3X loading buffer, 75 microlitres 1M Tris base pH 6.8, 150 microlitres 20% SDS, and 150 microlitres glycerol were mixed with 125 microlitres distilled water, and 0.3% w/v bromphenol blue was added. The samples were then denatured by heating to 90 degrees Celsius for five minutes and cooling rapidly on ice; suitably aliquoted molecular weight markers such as BRL Prestained Protein Molecular Weight Standards were processed and loaded in the same way. All samples
were then loaded into the rinsed wells and the rig was run with 1X running buffer, 25 mM Tris base, 250 mM electrophoresis grade glycine pH 8.3, 0.1% SDS for about 60 minutes at 170 to 180 volts, until the blue leader dye had just run out of the bottom of the gel. 5X running buffer was made by combining 15.1 g Tris base, with 94 g glycine pH 8.3 in 900 ml distilled water, 50 ml of 10% SDS is added and the final volume of one litre was made up with distilled water (Sambrook et al., 1989).

2.5.4 Coomassie staining for total protein

Duplicate protein gels were stained with Coomassie to ensure that protein was loaded evenly. The gel was placed in a staining solution on a slowly rotating rotary shaker at room temperature and allowed to stain overnight. To make the staining solution, 0.25 grams Coomassie Brilliant Blue R250 were dissolved in a mixture of 45 ml distilled water, 45 ml methanol, and 10 ml glacial acetic acid, filtered through a Whatman 1 filter, and stored in a dark bottle. The gel was then destained in four to six successive half hour washes of the solute mixture above (4.5 distilled water : 4.5 methanol : 1 glacial acetic acid, v/v/v) with no stain added to it, until the gel was clear and the protein bands were visible with good contrast (Sambrook et al., 1989).

2.5.6 Western detection of poplar PAL subunits

When the evenness of loading had been confirmed by Coomassie staining, a duplicate gel was run using duplicate aliquots of appropriate samples and transferred to a membrane for western blot analysis.

The gel was rinsed of SDS for five minutes in 50 ml 1X transfer buffer, 25 mM Tris base, 250 mM electrophoresis grade glycine. The 5X transfer buffer stock was made by combining 15.1 grams Tris base with 94 grams glycine, making up the volume to one litre with distilled water and autoclaving. The left corner was clipped for convenient reference, and the proteins were transferred to Hybond N using an in-house electroblotting system and the blotting setup described by Sambrook et al., 1989; in order from the negative electrode, the transfer setup
consisted of two precut Whatman 3 mm Chr sheets, the gel, the precut Hybond N, and two more sheets of precut Whatman 3 mm Chr paper, all prewetted in 1X transfer buffer. Transfer was carried out at 195 milliAmperes for 3.5 to 4 hours and was confirmed by inspection of the Hybond N for transfer of the prestained molecular weight markers. The filter was allowed to air dry and was stored in a Whatman 3 mm Chr folder until use in western analysis.

The filter was then rewetted by floating and immersion in distilled water. It was blocked for one hour at room temperature in 5% IGA skim milk powder (in distilled water), 25 ml for a 6 x 8 cm filter, shaking at one rotation per second on a rotary shaker.

The block was drained away and the filter was shaken in 25 ml of 5% skim milk containing 1:1000 dilution (25 microlitres) of primary antibody (polyclonal anti-poplar PAL, a gift from Grant McKegey and Brian Ellis) for one hour as above. It was then rinsed three times in 25 ml aliquots of 1X PBS (phosphate-buffered saline) with 25 microlitres of Tween 20 added, followed by a final rinse in 1X PBS alone. The 10X PBS stock was made by dissolving 8 grams of NaCl, 0.2 grams of KCl, 1.44 grams of Na2HPO4, and 0.24 grams of KH2PO4 in distilled water, adjusting to pH 7.4 with HCl, and making up the volume to one litre with distilled water. The solution was then autoclaved.

The filter was incubated for one hour as above with secondary antibody (Gibco BRL Goat Anti-Rabbit IgG (H+I)-Alkaline Phosphatase Conjugate (Human Adsorbed)) at a 1:300 dilution, about 10 microlitres in 25 ml of 5% skim milk powder. It was then rinsed as above with 3 rinses of PBS-Tween and one rinse of PBS alone. The filter was equilibrated in 50 ml of 50 mM Tris HCl pH 8 for five minutes on the rotary shaker and then the equilibration volume was drained away. A reaction solution was applied, having been mixed as follows IMMEDIATELY before application: 0.1 gram Fast Red TR (Sigma) and 0.05 grams Naphthol AS-MX Phosphate (sodium salt) (Sigma) were dissolved into 50 ml of 50 mM Tris HCl pH 8. With shaking, signal appeared within 3 to 25 minutes. The reaction was then stopped by shaking for ten minutes in distilled water. The filter was allowed to air dry and was stored in a Whatman 3 mm Chr folder.
3. Structure of poplar PAL-encoding genes

3.1 Introduction

In order to investigate the number, structure, and expression of PAL genes in a woody angiosperm, genomic poplar PAL sequences (including promoter, coding, intron, and 3' regulatory sequences) had to be obtained. Genes encoding PAL have been cloned from a number of plant species. The first PAL cDNA was isolated from a cDNA library derived from elicited bean suspension cultures in 1985 (Edwards et al., 1985). PAL is generally encoded by a small gene family in the species examined. The structure of these gene families, and of the PAL genes themselves, were discussed in detail in section 1.7, and a listing of plant PAL genes described in the literature was presented in Table 2. When this work was begun, there were no reports of PAL genes isolated from woody perennials.

A cDNA encoding a full-length poplar PAL gene, 2.4 kb. in length, was isolated from a young-leaf cDNA library constructed using RNA from H-11, a hybrid related to 53-242 and 53-246 (Subramaniam, et al., 1993). The library was screened using a 3'-specific 1.5 kb. potato PAL fragment as a probe. Several other cDNA clones were also obtained (from this library and from a library derived from 53-246 young leaf RNA), restriction mapped, and sequenced at the 3' ends. The restriction maps and 3' sequences were compared and two classes of clones identified, one class possessing a pair of HindIII sites generating a 1800 bp. HindIII fragment, and the other missing these sites (Subramaniam, et al., 1993). The existence of two classes of cDNAs suggested that PAL might be encoded by a gene family in poplar. The full-length poplar cDNA, named cDNA PAL 7, possessed the pair of HindIII sites and was used to screen a genomic library in order to obtain genomic poplar PAL sequences. This library was constructed from DNA isolated from an F1 generation hybrid poplar (53-242) from Stettler's pedigree described in Section 1.4.
3.2 Results

Since this thesis work was carried out over several years, portions of the research have been published (Subramaniam et al., 1993). In addition, some results were obtained in collaboration with other researchers, and a full understanding of other results can only be reached when other researchers’ results are considered. Therefore, an explicit acknowledgment of each contribution is necessary to clarify the relationships of the results presented in this chapter and in the Subramaniam et al. publication.

The poplar genomic library was made by this candidate with the guidance of Dr. Harvy Bradshaw and Dr. John Davis, and was screened for genomic PAL sequences by this candidate. Those genomic PAL sequences were analyzed by this candidate.

cDNA libraries were screened in collaboration with Gopal Subramaniam. Southern analysis of inheritance of PAL genes in hybrid poplar was carried out in collaboration with Dr. Carl Douglas, Brian Watson, and Dr. Harvey Bradshaw.

Placement of the two PAL loci on the poplar linkage map was carried out by Dr. Carl Douglas, Brian Watson, and Dr. Harvey Bradshaw. Northern analysis of poplar tissues was performed by Gopal Subramaniam, and in situ hybridizations of PAL to poplar tissues were made by Susanne Reinold.

3.2.1 Isolation and characterization of genomic clones

Fourteen genomic clones were isolated from a Lambda GEM12 library of hybrid 53-242 genomic DNA, identified by hybridization to cDNA PAL 7. The clones were restriction-mapped and the orientation and location of PAL genes within each clone were determined by Southern hybridization to 5' and 3' probes derived from cDNA PAL 7. The clones fell into two classes based on their maps. The most important difference was that the regions containing the PAL genes either contained internal HindIII sites releasing a 2400 bp. HindIII fragment, or else they lacked these sites, and this fragment was therefore not released. In addition, there were differences in the relative position of the 5'-noncoding BamH1 and 3'-noncoding EcoRV and XbaI
sites, as well as substantial differences in restriction sites in the 3 kb genomic region 3’ to the genes. Representative clones possessing the longest 5’ noncoding regions (promoters) are illustrated in Figure 6.

![Restriction maps of two poplar genomic clones containing PAL genes.](image)

**Figure 6.** Restriction maps of two poplar genomic clones containing PAL genes. (B, BamHI; RI, EcoRI; RV, EcoRV; H, HindIII; S, SacI X, XbaI.)

The largest representatives (clone #18, derived from locus PAL2; and clone #23, derived from locus PAL1) from each of the two classes of Lambda GEM12 phage clones were selected for further analysis; the sizes of their respective 5’ regions were the main factors in their selection since one of the main foci of this research was the analysis of 5’-controlled promoter activity.

The two PAL genes were subcloned into pBluescript II KS- as illustrated in Figure 7 and transformed into the host strain DH5-alpha so that large quantities of DNA could be isolated, and so that some of the extraneous genomic sequence could be excluded from further manipulations. Both genes were subcloned using a 5’ *SalI* site in the phage polylinker and an *XbaI* site in nearly identical locations downstream of the coding regions of the two genes, giving 6.5 kilobase subclones in each case. The *PAL1* 5’ upstream region is approximately 1.1 kilobases in length, and the equivalent region in *PAL2* is about 1.7 kilobases long.

The restriction patterns of the PAL genes within each of the genomic clones were also compared to those of the cDNA PAL 7 to determine similarity to the cDNA. The sites were mostly colinear with two exceptions. First, as mentioned, one class of genomic clones (represented in Figure 6 by genomic isolate #23) lacked *HindIII* sites and thus fell into the other class of cDNAs.
Secondly, fragments including the region which in PAL genes from other plants contains the intron (typically splitting a conserved arginine-encoding codon) differed in length by about 600 bp when equivalent genomic and cDNA fragments were compared. For example, the cDNA PAL 7 releases an internal HindIII fragment about 1800 bp long, but the equivalent HindIII fragment from the genomic clone #18 is about 2400 bp in length.

3.2.2 Structure of the poplar PAL gene family

Inheritance of PAL1 and PAL2

The restriction maps of the genomic clones were compared to Southern blots of DNA extracted from individuals in the poplar T x D pedigree described in section 1.4 and probed with cDNA PAL7. The inheritance of PAL-associated RFLPs through that pedigree was examined, and the non-HindIII-containing group of isolates was identified as the locus PAL1, and the HindIII-containing group as the locus PAL2 (Subramaniam, et al., 1993).
To summarize those findings, there is a *HindIII* fragment (about 23 kb) present in the maternal (93-968) *HindIII* digest in Figure 8A which hybridizes to both 3' and 5' *PAL* probes (Subramaniam *et al.*, 1993) and thus contains a complete *PAL* gene. Likewise, in the corresponding lane of paternal DNA (ILL 129) there is a *HindIII* doublet around 20 and 19 kb, each fragment containing a complete *PAL* gene. These three fragments are inherited as three alleles at a single locus in the following F1, F2, and B1 generations (Subramaniam *et al.*, 1993). Since the *PAL* gene at this locus contains no internal *HindIII* sites, the class of genomic isolates (and of cDNAs) possessing no internal *HindIII* sites are presumed to be derived from this locus, named *PAL1*.

In the maternal (93-968) *HindIII* digest in Figure 8A there is a pair of *HindIII* fragments (about 7.5 and 6.5 kb respectively), which hybridized only to a 3' probe and thus contained only the 3' portion of a *PAL* gene. In the paternal (ILL 129) *HindIII* digest there is a single fragment at about 4.5 kb, which again hybridized only to a 3' probe. These three fragments are inherited as three alleles at a second *PAL* locus (Subramaniam *et al.*, 1993). Finally, the 2.5 kb *HindIII* fragment found in all *HindIII* digests in the pedigree should be noted. This common fragment hybridizes to a 5' probe and contains most of the 5' region of those *PAL* alleles segregating at the second locus, since they are co-inherited without exception. Genomic and cDNA clones containing internal *HindIII* sites are derived from this locus, called *PAL2*.

The two genomic clones studied in detail, #23 containing a single *PAL* allele at locus *PAL1* and #18 a single *PAL* allele at locus *PAL2*, were isolated from F1 hybrid poplar 53-242. The parental origin of these two isolated alleles was determined by comparison of RFLPs. The key fragment demonstrating allelic derivation of #23 (*PAL1*) was the *BamHI* fragment 6.5 kb in length containing the whole *PAL* gene from clone #23 (Figure 6). This *BamHI* fragment hybridizes to cDNA PAL 7 and is present only in the Southern blot of the paternal (ILL 129) DNA and not in the maternal DNA (Figure 8A). The parental origin of the second genomic clone, #18 (corresponding to an allele at locus *PAL2*) was determined by comparing *HindIII* polymorphisms specific to the 3' ends of *PAL2*. Digestion of genomic isolate #18 by *HindIII* releases a 4.5 kb fragment which is only observed in the paternal *HindIII* genomic digest (Figure 8A). Thus, both *PAL* alleles isolated from 53-242, one from each locus, are inherited from the male parent, ILL 129.
Evidence for a third \textit{PAL} gene

When genomic Southern blots hybridized to cDNA PAL 7 were over-exposed, faint bands were noticeable. (Figure 8B) The possibility that those faint bands might represent a third, divergent poplar PAL gene was investigated using a PCR approach. A pair of degenerate primers specific to the second exon of \textit{PAL2} was used to amplify \textit{PAL} sequences from H-11 genomic DNA, and PCR products were cloned and sequenced (S. Duong, S. Allina, and C. Douglas, unpublished data). Partial sequencing of one clone called PCRPAL16 (193 basepairs at the 3' end) showed that this fragment was 73\% identical with cDNA PAL 7, suggesting it was highly divergent from \textit{PAL1} and \textit{PAL2}. However, the sequence showed 90\% nucleotide identity with \textit{pal g2a}, a diverged \textit{PAL} gene identified in \textit{Populus kitakamiensis} (Osakabe \textit{et al.}, 1995). The clone PCRPAL16 also contains an open reading frame with substantial similarity to that of cDNA PAL 7, with 88.7\% identity and 95.2\% similarity at the amino acid level. These results suggest that this is a fragment of an authentic \textit{PAL} gene. No complete cDNA or genomic clones representing this third poplar \textit{PAL} gene have yet been characterized from 53-242 or relatives.

When PCRPAL 16 was hybridized to the stripped genomic Southern blot shown in Figure 8A, it hybridized exclusively to a subset of the faint bands observed previously (Figure 8C), suggesting that these bands represent a third type of divergent poplar \textit{PAL} gene.
Figure 8. Genomic Southern blots of parental and hybrid poplars. (A) 2 day exposure (-90 degrees Celsius) of a genomic Southern blot of DNA from the parents 93-968 and ILL-129, probed with cDNA PAL 7, 2X SSC. (B) 5 day over-exposure (-90 degrees Celsius) of the same blot probed with 5' portion of cDNA PAL 7. (C) 7 day exposure (-90 degrees Celsius) of the same blot, stripped and probed with PCRPAL16, 0.5X SSC. B, BamH1; E, EcoRV; H, HindIII; X, Xbal. Fragments discussed in review of inheritance of PAL1 and PAL2 are indicated by arrows in panel A. Representative "faint bands" in panel (B) hybridizing to PAL3 probe in panel (C) are denoted by asterisks. Fragment sizes are reported in kb.
3.2.3 Analysis of the promoter regions of PAL1 and PAL2.

The presence of multiple PAL genes in poplar suggested that they could be differentially regulated. Since the genomic sequences for PAL1 and PAL2 were available, this pair of genes was investigated. If the two poplar PAL genes are differentially regulated, their promoters may have different structures and sequence motifs reflecting their differing functions. Therefore the structure and sequences of the 5' regions of these two genes were compared.

The entire available 5' upstream region of the PAL2 gene present in clone #18 was subcloned and positions of restriction sites determined. A 1.7 kb 5' fragment from PAL2 was subcloned with relative ease, using the same 5' Sall site used to transfer the PAL2 gene from Lambda Gem12 into pBluescript II KS - (as in Figure 9) and a convenient HindIII site located just upstream of the ATG translation start site; this fragment was inserted into the 5' Sall and 3' HindIII sites of the vector pBluescript II KS- for sequencing and further manipulation.

![Figure 9. Restriction strategy for subcloning PAL2 promoter into pBluescript II KS-](image)

The PAL1 promoter region was restriction-mapped in less detail. The subcloning of a 1.1 kb PAL1 5' region was more difficult since there were no useful restriction sites in the required locations. Therefore, the region was amplified by PCR using the 6.5 kb genomic subclone as a template. The commercial T3 primer was used as the 5' PCR primer and a PAL1-specific primer, derived from PAL1 sequence, as the 3' primer. The PAL1-specific primer was designed to amplify a fragment starting at the same relative position as does the promoter subclone for PAL2. This PCR fragment has the Sall site contained in its 5' end and an Xbal site designed into the 3' end.
The amplified product was cut with these enzymes, cloned into pBluescript II KS-, and its authenticity confirmed by restriction digest and by comparison of sequence of the two ends to original genomic sequence. Then the large, central (unsequenced) portion of this amplified region was removed using Ncol and Xhol sites within the confirmed ends, and the equivalent fragment from the genomic clone was inserted in its place. This precludes any PCR-associated sequence errors in the final promoter fragment. The PCR amplification step extended the confirmed promoter sequence inwards from both ends of the fragment of interest to encompass convenient but otherwise unusable cloning sites (Ncol and Xhol sites) in that promoter fragment.

Figure 10. PCR and restriction strategy for subcloning of PAL1 promoter. Dark region is original genomic sequence subcloned into light PCR-derived region.

The restriction maps of the two promoter regions are presented for comparison in Figure 11, aligned with respect to the putative ATG start of translation. At this level the two promoters appear to possess no similarity. Portions of the cloned promoters were sequenced as indicated in Figure 12.

About 350 base pairs of the two promoter regions of the PAL1 and PAL2 genes were sequenced. These sequences are which is shown in Figure 12. When a total of 365 base pairs are compared, there is 81.6% identity (Table 3), considering only mismatches. When one takes into account the gaps forced by the program to attain maximal alignment, that identity is reduced to 64.7%. Percent identity at the nucleotide level of this and other non-coding regions are presented
in Table 3. Examination of the two promoter sequences presented in Figure 12 suggests the location of a putative TATA box. In addition, a sequence motif is found in both promoters which is nearly identical to the "P" and "L" boxes identified by Lois et al. in the promoters of parsley PAL and other phenylpropanoid genes (Lois et al., 1989).

Figure 11. Restriction maps for the promoter regions of PAL1 and PAL2. Sequenced regions are indicated by underlining.

Table 3. Percentage nucleotide identity for non-coding regions of PAL1 and PAL2. Thin bars represent non-coding regions, and thick bars represent exons. Shaded areas have been sequenced and compared for the two genes.
Figure 12. A comparative alignment of 350 basepairs of sequence for the promoters of \textit{PAL1} and \textit{PAL2} upstream of the putative ATG codon. Putative TATA boxes, "AC/L" boxes, and ATG start sites of translation are indicated by horizontal brackets. Alignment carried out by GCG Sequence Analysis Software.
These motifs are also referred to as "AC" boxes, a general category of promoter motif conserved in phenylpropanoid genes (Hatton et al., 1995). The putative TATA boxes of the two genes are centered 172 base pairs upstream of the translational start site of \textit{PAL1} and 143 base pairs upstream of the ATG translational start site of \textit{PAL2}. The AC box is centered around positions -240 and -195 for \textit{PAL1} and \textit{PAL2} respectively. The putative TATA and the AC boxes are labelled in Figure 12.

The promoter sequences were searched for CAAT boxes, for additional motifs conserved among other phenylpropanoid promoters, such as "G" boxes (Giuliano et al., 1988) and the putative xylem specific element identified in loblolly pine promoters (Loopstra and Sederoff, 1995). Neither of these motifs was found.

3.2.4 Restriction and sequence information from 3' ends of \textit{PAL1} and \textit{PAL2}

Convenient and (at the restriction site level) identical 0.56 kb \textit{EcoRI} 3' fragments from the two genes were subcloned into pBluescript II KS- and sequenced completely so as to compare 3' translated and untranslated sequences and to identify functional motifs in those regions.

![Figure 13. Restriction strategy for subcloning of 3' regions from \textit{PAL1} and \textit{PAL2}.](image)

The 3' non-coding regions of genes within a gene family tend to be more different than the coding regions of those genes since there is less selective pressure to conserve the sequence of
the non-coding regions. Comparison of those regions can therefore give an idea of how divergent two members of a gene family are from each other.

When the 3' non-coding regions of the multiple cDNAs isolated from young leaf cDNA libraries are compared to each other (Subramaniam et al., 1993), they fall into two classes corresponding to PAL1 and PAL2 as defined by inheritance of HindIII RFLPs in the T X D poplar pedigree. Examples of 3' ends of cDNAs are presented as a modified figure from Subramaniam et al. in Figure 14.

When comparing the sequences of cDNAs allelic at PAL1 to cDNAs allelic at PAL2, there is a 77% identity at the nucleotide level for the 130 base pairs of 3' non-coding sequence available to be compared. However, when one compares within an allelic class (that is, cDNAs corresponding to one locus such as H11-7 and H11-2), the 3' regions have an identity of 93.5% (Figure 14). The appropriate 3' regions from the two genomic clones were also compared to each other (Figure 15) and to the two cDNA classes represented in Figure 14 and displayed the same nucleotide identities described above, indicating that they too are alleles at poplar PAL loci 1 and 2. In the cDNA and genomic alleles examined so far, the two classes use different stop codons; the codon TGA is used by those alleles derived from PAL1 and the codon TAA is used by alleles derived from PAL2. The stop codons are marked in the sequences presented in Figure 14 for the 3' ends of the cDNAs and in Figure 15 for the 3' ends of the two genomic clones.
Figure 14. 3' non-coding sequences of several *PAL* cDNAs isolated from young leaf-derived cDNA libraries from individuals H-11 and 53-246. The sequences are aligned on the terminal stop codon. Putative alleles at *PAL2* comprise the upper bracketed set of sequences and putative alleles at *PAL1* the lower bracketed set of sequences. Asterisks indicate mismatches when a given sequence is compared to that of clone H11-7 (cDNA PAL7).
Figure 15. Alignment of sequences for the 3' 0.56 kb EcoRI fragments subcloned from *PAL1* and *PAL2*. Stop codons are indicated by a horizontal bracket, and a broad arrow points to the polyadenylation site for *PAL2*. Putative poly-adenylation-associated motifs (variations on "AAUAAA") are also labelled with the abbreviation "PPAA".
In considering the homology of the 3' non-coding regions, two 560 bp EcoRI fragments common to PAL1 and PAL2 were sequenced completely. These sequences contain stop codons, 3' untranslated regions, and putative polyadenylation-associated motifs (Figure 15). Alignment of these sequences showed a 72.8% identity at the nucleotide level for the 3' non-coding regions, about 360 bp (Table 3).

3.2.5 Sequence information from intron-containing subclones

In order to compare the sequences of the intron-containing regions of the two genes, PCR primers were used to amplify appropriate portions of the genomic subclones and of the cDNA PAL 7. These amplifications were carried out by Stefanie Butland in the laboratory of B. E. Ellis, and the resulting PCR products were a gift from her. The amplified fragments were digested using engineered EcoRI and Xbal sites at the 5' and 3' ends respectively, and cloned into pBluescript II KS- (Figure 16).

Several independently-obtained amplified fragments were sequenced and a consensus sequence determined for each gene to remove PCR-related artifactual errors. The two sequences
were then compared. As seen in Table 3, there was 34.2% nucleotide identity for the 441 (about half of the intron) basepairs compared. When the 50 bp regions closest to the ends of the introns are compared between the two genes, the 5' end shows 62% identity, and the 3' end shows 52% identity. These regions are usually somewhat conserved presumably because of function during splicing. For example, although the intron sequence of two potato PAL genes is described as "very dissimilar" to each other, the 43 basepairs 3'-most in the intron are 85% identical (Joos and Hahlbrock, 1992).

The sequences immediately surrounding the splice junctions are presented for comparison in Figure 17; since the PAL7 cDNA is derived from an allele at locus PAL2, the fact that its coding sequence is identical to that of the PAL2 gene is not surprising. An arrowhead indicates the point in the PAL7 cDNA sequence at which the intron is excised from between the second and third nucleotides of an arginine codon. The PAL1 coding sequence differs slightly from PAL2 and cDNA PAL 7 sequences; differing nucleotides are starred. As mentioned above, the sequences of the introns themselves differ substantially more than do the coding sequences. The dicotyledonous intron splice junction consensus (White et al., 1992) of an intron-associated 5' "GT" and 3' "AG" is present and indicated by underlining.

**PAL 7 cDNA**

(nucleotide #515)

...GAAGGAACTTATTAGATTCTTGAATGCTGG...

**PAL II**

...GAAGGAACTTATTAG_ATTCTTGAATGCTGG...

GTGAGCAA...TCTTTCCCCCTTTTCAG

**PAL I**

...*AAGGGAACTCATTAG_ATTCTTGAATGCTGG...

GTGAGTTA...CTCCCCCTTTTCAG

Figure 17. Sequences surrounding intron splice sites for cDNA PAL 7, PAL1, and PAL2. The intron splice site is indicated by an arrowhead in the cDNA PAL 7 sequence, conserved splice sites are underlined, and coding nucleotides differing between PAL1 and PAL2 are starred.
3.2.6 Comparison of coding regions of PAL1 and PAL2

To continue the structural comparison of the two isolated poplar PAL genes, portions of coding regions of both genomic clones sequenced during this work were compared (Table 4). Comparisons between the two loci can be made at the nucleotide and the amino acid levels. These comparisons are summarized in Table 4.

Exon I of poplar PAL is 510 bp long and exon II is 1910 bp in length (Subramaniam et al., 1993). Regions indicated in Table 4 were sequenced in both genes and compared. About two thirds of the sequence of exon I were obtained and compared for PAL1 and PAL2. The compared regions were determined to be 88.9% identical at the nucleotide level, 88.5% identical at the amino acid level, and 90.5% similar at the amino acid level. About one fifth of exon II was sequenced and compared for the two genes. For these regions, identities of 91.1% and 93.8% at nucleotide and amino-acid levels respectively were observed, as well as an amino acid similarity of 97.6%.

Thus, although PAL1 and PAL2 were not sequenced in their entirety, a total of about 700 basepairs may be compared between the two genes' coding regions, about one third of the coding sequence. Of those nucleotides, 90.1% are identical, and at the amino acid level, an identity of 91.4% and a similarity of 94.4% are observed. All coding sequence information is presented in Table 4.

<table>
<thead>
<tr>
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<th>Exon I</th>
<th>Exon II</th>
<th>Total</th>
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<tr>
<td>Nucleotide identity</td>
<td>88.9 %</td>
<td>91.1 %</td>
<td>90.1 %</td>
</tr>
<tr>
<td>Amino acid identity</td>
<td>88.5 %</td>
<td>93.8 %</td>
<td>91.4 %</td>
</tr>
<tr>
<td>Amino acid similarity</td>
<td>90.5 %</td>
<td>97.6 %</td>
<td>94.4 %</td>
</tr>
</tbody>
</table>

Table 4. Similarity and identity at nucleotide and amino acid levels for coding regions of PAL1 and PAL2. Dark regions were sequenced.
3.3 Discussion

In this work, poplar PAL genes were cloned and studied to determine the structure both of the gene family and of the genes themselves. Based on comparison of 3' sequences of cDNAs (Figure 14) and of genomic clones (Figure 18) there are two classes of PAL sequence easily isolated from poplar using the potato PAL sequence provided. These results indicate that PAL is encoded in poplar, as in most other plants examined (Table 1), as a small gene family with at least two relatively similar members. The comparative structure of this pair of genes (PAL1 and PAL2) was studied in detail, and their mode of inheritance determined. In addition, their loci were mapped onto a poplar linkage map (Subramaniam et al., 1993), and they were shown to be unlinked. However, since the HindIII restriction fragment upon which the locus PAL1 is carried (Figure 8) is so large, above 20 kb for all observed alleles, the possibility that there are in fact two closely linked and possibly very recently diverged PAL genes carried in tandem on that fragment has not been disproven.

A portion of a third and relatively divergent PAL gene was obtained by PCR, and named PCRPAL16. The patterns of hybridization observed (Figure 8) when this fragment is used to probe genomic DNA digests lends strength to the suggestion (Osakabe et al., 1995) that this DNA is part of a relatively diverged PAL gene that would hybridize only faintly to probes derived from PAL1 and PAL2. The other subset of faint bands present in Figure 8B are likely other restriction fragments of the diverged gene not detected by the PCRPAL16 probe, since it represents only a fraction of the gene. Thus, a third PAL gene, designated PAL3, appears to be present in P. trichocarpa, P. deltoides, and their hybrid progeny.

The fact that PAL3 had not been previously cloned by repeated screening with probes related to PAL1 and PAL2 is a reminder of the potential dangers of using restriction fragment-based probes to screen for new sequences. Targets are limited to sequences which are similar to that of the probe. Perhaps an additional method such as a PCR-based approach using primers based on highly conserved regions of the genes (conferring conserved functions on the encoded proteins), or a polyclonal antibody to screen an expression library could be used as a secondary method to isolate diverged gene family members. There are now several precedents for PAL gene
families with divergent genes, including Arabidopsis (Wanner et al., 1995) and poplar (Subramaniam et al., 1993; Osakabe et al., 1995). The "lighter bands" in genomic Southerns of loblolly pine may well also correspond to diverged PAL genes in that species, in which only a single PAL gene was detected (Whetton and Sederoff, 1992).

As mentioned above, the structure of the pair of relatively similar PAL genes, PAL1 and PAL2, was compared in detail, including structure of promoters and 3' sequences, the location and size of the intron, and coding region similarity. Structural components typical of eukaryotic genes as well as a 5' motif specific to phenylpropanoid promoters were identified.

The 5' non-coding regions of PAL1 and PAL2 were examined and the putative TATA box, ATG start of translation, and a conserved phenylpropanoid-specific AC box were identified in both promoters (Figure 12). However, these motifs are still only putative regulatory sequences since the transcription start site has not yet been identified for these two genes. The presence of the AC box in both promoters is particularly intriguing since this cis-element is conserved in PAL and related phenylpropanoid genes from a number of plants (Lois et al., 1989; Ohl et al., 1990; Leyva et al., 1992; Yamada et al., 1994; Logemann et al., 1995). A potential role for these elements in light and elicitor-responsiveness in parsley suspension cultures is suggested by in vivo footprinting (Lois et al., 1989). These motifs have been demonstrated to interact both with a petal-derived Myb protein-like putative transcription factor (Sablowski et al., 1994) and a pathogen-induced DNA-binding protein in parsley (da Costa e Silva et al., 1993). In addition, AC boxes play a role in conferring complex, tissue-specific patterns of gene regulation in transgenic tobacco containing constructs derived from a bean PAL promoter (Hatton et al., 1995).

Table 5 presents the consensus sequence (with asterisks representing positions allowed to vary) which was used to search the poplar PAL promoters and which identified the AC boxes present in poplar PAL1 and PAL2. These motifs are compared to selected AC boxes previously identified in other plant PAL promoters.

The 3' coding and non-coding regions of PAL1 and PAL2 were sequenced and compared to identify poly-adenylation-associated motifs in the 3' noncoding regions and to determine sequence similarity. Several putative poly-adenylation sites were identified (Figure 15) downstream of the stop codons.
Examination of relative sizes of cDNA- and genomic-derived restriction fragments reveals only one region which is larger in the genomic clones, indicating the presence of an intron. In plant PAL genes surveyed so far, this intron is conserved in position, typically splitting an arginine codon in the 5'-most half of the gene (Subramaniam et al., 1993), but the size of the intron is not conserved even among gene family members of a single species (S. Butland, personal communication). The introns of the two isolated poplar PAL genes are located in the same position as those of other PAL genes. However, the size of their introns is the same, the first time that this situation has been observed for PAL introns. Perhaps this indicates that PAL1 and PAL2 are recently diverged. In this case, the observed intron sequence identity of only 34% may indicate how rapidly DNA sequences can change if not subjected to selective pressure. The sequences of the intron-containing regions for the two genes contain conserved sequences typical of eukaryotic splice junctions (Figure 17).

As summarized in Table 4, sequenced coding regions compared between PAL1 and PAL2 possess identities at the nucleotide and amino acid levels of 90.1% and 91.4% respectively. This indicates a level of similarity between the two genes which is comparable to that observed for other sets of similar PAL gene family members in other species (for example, Cramer et al., 1989; Wanner et al., 1995; and Yamada et al., 1992). However, poplar PAL1 and PAL2 seem slightly

|        | poplar PAL1 | -248 | gGTtACCTACC- |
|        | poplar PAL1 | -205 | gGTtACCTACC- |
|        | parsley PAL1 | -119 | -TTCACCTACC- |
|        | bean PAL2   | -161 | -ACCCACCTACC- |
|        | Arabidopsis PAL1 | -61 | gGTtACCTACC- |
| CONSENSUS |         |  | -*C*CAAC*ACC- |

Table 5. Comparison of AC boxes from selected promoters. Asterisks in the CONSENSUS sequence used to search the poplar promoters indicate positions where multiple nucleotides may be substituted. Small type nucleotides in the promoter sequences indicate nucleotides which differ from the consensus (Parsley sequence, Lois et al., 1989; bean sequence, Cramer et al., 1989; Arabidopsis sequence, Ohl et al., 1990.).
more similar than are most PAL gene family members which are differentially regulated (reviewed in section 1.9.4), as listed in Table 6 and reviewed in Section 1.9.4.

However, differential regulation of gene family members would likely be conferred by diverged promoter regions. The promoters of PAL1 and PAL2 have an identity of 81.6% (when gaps are discounted), and 64.7% (when gaps are considered) for the region compared (Table 3) which may be sufficient to direct differential regulation of PAL1 and PAL2. The latter figure of 64.% approaches the percent identity calculated for the promoters of potato PAL1 and PAL2, which are differentially regulated in response to compatible interactions with pathogens (Joos and Hahlbrock, 1992). Thus, there seems to be sufficient precedent for differential regulation of PAL genes which differ structurally as described, to justify an inquiry into the possible differential regulation of poplar PAL1 and PAL2.

<table>
<thead>
<tr>
<th></th>
<th>Nucleotide % Identity</th>
<th>Amino acid % Similarity</th>
<th>Amino acid % Identity</th>
<th>Promoter % Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis PAL3 vs. PAL/12</td>
<td>92 to 95%</td>
<td>84%</td>
<td>73%</td>
<td>not alignable</td>
</tr>
<tr>
<td>Potato PAL1 vs PAL2</td>
<td>92%</td>
<td>-</td>
<td>-</td>
<td>64%</td>
</tr>
<tr>
<td>Carrot</td>
<td>82%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bean PAL2 t vs. PAL3</td>
<td>59 to 74%</td>
<td>72%</td>
<td>-</td>
<td>33%</td>
</tr>
<tr>
<td>Parsley PAL3 vs. Pal 1/2</td>
<td>91%</td>
<td>98%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Poplar PAL1 vs. PAL2</td>
<td>90.1%</td>
<td>94.4%</td>
<td>91.4%</td>
<td>64% / 81%</td>
</tr>
</tbody>
</table>

Table 6. Percentage similarities observed when comparing PAL gene family members which are differentially regulated. (Arabidopsis, Wanner et al., 1995; potato, Joos and Hahlbrock, 1992; carrot, Ozeki et al., 1990; bean, Cramer et al., 1989; parsley, Lois and Hahlbrock, 1992, Lois et al., 1989)
4. Expression of poplar PAL genes

4.1 Introduction

In the previous section, the structure and inheritance of poplar PAL genes were described. Since three poplar PAL genes had been identified, there was the possibility that the genes were differentially regulated. In addition, a unique opportunity to study the function of PAL genes of a tree was now presented. Characterization of developmentally regulated PAL gene expression in a woody perennial would be interesting for several reasons. First, the formation of lignin-rich wood would seem to require a substantial PAL activity to provide sufficient phenolic substrate for the lignin formed each year, and probably this activity is regulated to some degree at a transcriptional level. Second, PAL activity and PAL transcription must be regulated in a seasonal manner, the levels of protein and transcription varying from month to month throughout the growing season. The nature of this variation could be investigated. Several approaches were used to address these interesting issues. The first involved histochemical examination of tobacco transgenic for poplar PAL promoter : GUS reporter gene fusions; although several attempts to generate transformed poplar were made, these attempts were unsuccessful and therefore the tobacco transformants were used for analysis of tissue-specific and wounding-responsive expression patterns driven by the poplar promoters. In addition, northern blot analyses of PAL RNA extracted from various poplar tissues and western analyses of PAL protein from poplar tissues were used.
4.2 Results

4.2.1 Expression of PAL promoter : GUS constructs

**Generation of PAL promoter : GUS constructs**

In order to study the *in planta* function of the *PAL1* and *PAL2* promoters, these promoters were fused to the *GUS* reporter gene and cloned into suitable vectors for transformation into tobacco. All available 5' sequences were cloned for each promoter, 1.1 kb for *PAL1*, and 1.7 kb for *PAL2*; these fragments probably contain most or all relevant control elements.

For the promoter of *PAL2*, a convenient *XbaI* site just distal to the *HindIII* site was used in conjunction with the 5' *SalI* site to transfer the promoter region (Figure 18) from pBluescript II KS-into pBG, a vector created by subcloning the *XbaI* and *EcoRI* fragment of JDD, a reporter construct used in transient assays (Schulze-Lefert *et al.*, 1989) into the same sites in the vector BIN19 (Figure 19). The final vector used to transform tobacco was therefore a 1.7 kb fragment of the *PAL2* promoter region fused to the *GUS* gene, all contained within the BIN 19 vector. The pBG1.7 (named for its length in kb) construct, containing the promoter of *PAL2*, was transformed into *E. coli*, confirmed by restriction digest, transferred to *A. tumefaciens* strain LBA4404 by triparental mating, confirmed by restriction digest, and transformed into tobacco strain SR1 by leaf disc infection.

![Figure 18. Strategy for construction of pBG1.7.](image-url)
The promoter of $PAL_1$ was also subcloned into the pBG vector and transformed into SR1 tobacco. The 1.1 kb promoter fragment was cut out from pBluescript II KS- with $SalI$ and $XbaI$ and was inserted into the requisite sites in pBG (Figure 20). This construct was named pBG1.1, and was manipulated in the same way as pBG1.7 except that the final construct was electroporated into LBA4404 and confirmed by extraction of total DNA and Southern blot analysis using $GUS$ as a probe (results not shown).
Generation of transformed tobacco

When the pBG1.7 construct was used to transform tobacco leaf discs, eight independent transformants were recovered and examined histochemically for tissue-specific and developmentally regulated patterns of GUS expression driven by the poplar PAL2 promoter. When the pBG1.1 construct was used to transform tobacco, ten independent transformants were recovered and characterized. As a negative control, the vector pBG with no promoter insert was also transformed into tobacco using the techniques described for the PAL1 promoter. Eight independent transformants were recovered and characterized.

Analysis of T1 kanamycin resistant / susceptible ratios

For each type of tobacco transformant (pBG, pBG1.1, and pBG1.7), seeds were collected from each primary transformant and plated on kanamycin-containing selective media to confirm the presence of transgenes and to determine number of transgene loci by segregation of kanamycin resistance. A single insertion may consist of concatamerized constructs, but each insertional event will behave as a single dominant allele at a new locus created by the insertion. Thus, the initial transformant T0 can be regarded as heterozygous for each insertion, and when the T0 plant undergoes self-fertilization, the T1 progeny will inherit the dominant insertion(s) as in the second generation of a mon-, di-, or tri-hybrid cross, depending on the number of insertional loci involved. If only one insertion has occurred in the genome of a T0 plant, the expected phenotypic ratio in the T1 would be one kanamycin-sensitive to three kanamycin-resistant; with two insertions, one sensitive to fifteen resistant; and so on.

Resulting T1 segregation ratios are shown in Table 7. For each set of transformants, those apparently harboring single insertions (3:1 ratios) are listed first, followed by those apparently harboring two insertions (15:1 ratios), and so on. For those plants transformed with pBG constructs, half of the transformants were determined to contain only one transgenic locus, and half contained two (or more) loci. For the pBG1.1 transformants, three individuals appeared to contain one insertion, two contained either one or two inserts, two contained two inserts, and two individuals appeared to harbor two, three, or more transgene loci. Finally, those plants
transformed with pBG1.7 were determined to contain either one insert (three plants), two inserts (one plant), two or more inserts (one plant), three inserts (one plant), or three or more inserts (one plant).

To determine if the numbers of inserts as indicated by T1 segregation ratios were accurate, genomic DNA was extracted from each of the pBG1.7 T0 transformants, cut with XbaI to liberate the 5' end of GUS from within the inserted construct (the other end of each independently inserted construct being determined by a different XbaI site donated by the tobacco genome and thus giving rise to polymorphic XbaI fragments for the various insertional events), and Southern blots hybridized to a GUS probe. The numbers of hybridizing bands in the genomic Southern digest for each individual were consistent with the phenotypic ratios for the pBG1.7 transformants (results not shown). That is, when segregation ratios predicted that a single insertion was present in a certain individual, a single hybridizing band was visible on the Southern of that individual. Interestingly, none of the pBG1.7 transformants showed bands at 3.7 kb (the predicted size of a promoter : GUS fragment liberated from a concatamerized insertion by cutting at successive GUS-derived XbaI sites), indicating that none of the insertional loci consisted of concatamerized insertions.
Table 7. Numbers of kanamycin-sensitive T1 progeny out of total numbers screened, calculated phenotypic ratios, and estimated numbers of inserts for tobacco plants transgenic for control vector (pBG), *PAL1* promoter construct (pBG1.1), and *PAL2* promoter construct (pBG1.7).
<table>
<thead>
<tr>
<th>Transformant</th>
<th>Kan Sensitive/Total</th>
<th>Kan$^S$: Kan$^R$</th>
<th>#Inserts</th>
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<tr>
<td><strong>pBG Transformants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#viii</td>
<td>30 / 68</td>
<td>1 : 2.27</td>
<td>1</td>
</tr>
<tr>
<td>#vi</td>
<td>20 / 75</td>
<td>1 : 3.75</td>
<td>1</td>
</tr>
<tr>
<td>#iii</td>
<td>23 / 81</td>
<td>1 : 3.5</td>
<td>1</td>
</tr>
<tr>
<td>#ii</td>
<td>15 / 70</td>
<td>1 : 4.6</td>
<td>1 or 2</td>
</tr>
<tr>
<td>#iv</td>
<td>4 / 76</td>
<td>1 : 19</td>
<td>2</td>
</tr>
<tr>
<td>#v</td>
<td>6 / 83</td>
<td>1 : 13.8</td>
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<td>#1</td>
<td>1 / 50</td>
<td>1 : 50</td>
<td>2 or more</td>
</tr>
<tr>
<td><strong>pBG1.1 Transformants</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>#1</td>
<td>17 / 69</td>
<td>1 : 3</td>
<td>1</td>
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<tr>
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</tr>
<tr>
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<td>34 / 144</td>
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<td>1 or 2</td>
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<td>18 / 125</td>
<td>1 : 6.9</td>
<td>1 or 2</td>
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<td>15 / 129</td>
<td>1 : 8.6</td>
<td>2</td>
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<td>1 : 17</td>
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<td>#6</td>
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<td>1 : 81</td>
<td>3 or more</td>
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<td><strong>pBG1.7 Transformants</strong></td>
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<td></td>
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<td>3</td>
</tr>
<tr>
<td>#3</td>
<td>1 / 138</td>
<td>1 : 138</td>
<td>3 or more</td>
</tr>
</tbody>
</table>
Developmental expression of PAL1 and PAL2 promoters in transgenic tobacco

The individual tobacco transformants generated by Agrobacterium-mediated transformation with the pBG1.1 (PAL1 promoter), pBG1.7 (PAL2 promoter), and negative control (pBG) constructs were examined for expression of GUS driven by the inserted promoter fragments.

Tissues examined were stems, leaves, and flowers of various ages. Expression was largely confined to vascular tissue. This type of expression was observed in young stem sections (2-4 mm in diameter) as well as older stem sections (8-9 mm in diameter with secondary xylem), in sections of young leaf (2-5 cm long), in fully expanded leaf (20 cm long) and petioles, and in the bases and the petal vasculature of flowers of various ages. Typical results are shown in Figures 21 and 22. In Figure 21, comparable sections from tobacco transformed with pBG1.1 (PAL1 promoter) and pBG1.7 (PAL2 promoter) are presented. In the first pair of panels, young leaf in cross-section from a pBG1.1 transformant (panel A) and from a pBG1.7 transformant (panel B) are compared. GUS expression is specific to the young, differentiating xylem tissues.

In the second pair of panels, fully expanded leaf in cross-section from a pBG1.1 transformant (panel C) and from a pBG1.7 transformant (panel D) are presented. In this organ, GUS expression was still observed in the developing xylem tissue driven by both promoters, but two different expression patterns are observed which may be caused by slight age differences in the observed tissues. Panel C (pBG1.1 transformant) illustrates the first expression pattern in which GUS was apparently expressed in the ray parenchyma between the xylem element files. In panel D (pBG1.7 transformant), GUS expression was limited to the younger xylem elements within each file. In the third pair of panels, young stems in cross-section from a pBG1.1 transformant (panel E) and from a pBG1.7 transformant (panel F) are compared. GUS expression for both sets of transformants was highly localized to the immediate surroundings of clustered xylem elements. In Figure 22, additional results from microscopic observation of GUS expression patterns driven by the pBG1.7 construct are shown. These tissue-specific patterns are also seen in pBG1.1 transformants (data not shown). In panel A, a longitudinal section through the left
upper region of a floral receptacle shows *GUS* expression associated with xylem tissues in the petal and the ovary wall which both extend towards the upper left.

Figure 21. Comparison of expression of *GUS* driven by pBG1.1 and pBG1.7. (A) and (B) expression in developing vasculature of cross section of young leaf for pBG1.1 and pBG1.7 respectively. (C) and (D) expression in developing vasculature of cross section of fully expanded leaf for pBG1.1 and pBG1.7 respectively. (E) and (F) expression in developing vasculature of cross section of young stem for pBG1.1 and pBG1.7 respectively. (About 20X)
Figure 22. Typical results from microscopic observations of GUS expression driven by poplar PAL promoters in various tissues of transgenic tobacco. All results in this figure are from transformants containing pBG1.7 inserts, but are representative of results from pBG1.1 transformants as well. (A) longitudinal section of base of flower; expression in vasculature of sepal, petal, and receptacle. (B) cross-section of petal; expression in vasculature. (C) longitudinal section of ovary with many attached ovules exhibiting GUS expression on surface. (Printed here at about 20X) (D) longitudinal section of young stem; expression associate with developing vasculature. Secondary thickenings are visible in stained regions. (Printed here at about 40X.)
There was also expression within the receptacle itself, again associated with convoluted xylem tissues and possibly with the nectary. Panel B illustrates GUS expression associated with the xylem tissue in a cross-section of mature pink petal. There was no expression in the epidermal layer. The final floral tissue showing GUS expression driven by both poplar PAL promoters was the ovules. A longitudinal section of the placenta to which the ovules are attached was presented in panel C. Expression of GUS was observed on the surfaces of the ovules. Panel D shows the right-hand side of a longitudinal section of a young stem with expression associated with xylem tissue. Secondary thickenings can be observed in tracheary elements.

Figure 23. A cross-section of older stem of tobacco plant transgenic for pBG1.1 (PAl1 promoter) construct. Expression was associated with ray parenchyma. (About 20X).

In stem tissues with secondary xylem, expression driven by both promoters seemed to be localized to the ray parenchymal tissues between the files of differentiated xylem elements, as illustrated in Figure 23 which shows a cross-section of an older stem section sectioned from a tobacco plant transformed with pBG1.1. Equivalent results were observed for pBG1.7 transformants (data not shown). To summarize, when patterns of GUS expression driven by these two promoters were compared, they were essentially identical, and reproducibly observed expression was limited to the xylem and ovules.
While examining each of the organs and tissues from the transformants listed at the beginning of this section, other tissues occasionally showed GUS expression (for example, the stigma surface and pollen grains) but not in any consistent manner. Epidermal tissues of the petal and leaf also demonstrated occasional and inconsistent GUS staining. Since all sections from each organ of each transformant were incubated together in the GUS substrate, these reactions may simply have been GUS product leached from a truly active tissue into an adjacent inactive tissue.

Other developmentally regulated expression patterns were also investigated. Axenic T1 seeds from individual transformants for each line of transformants previously characterized as high expressers were germinated on MS plates supplemented with kanamycin. A number of seedlings were selected for each of several timepoints after imbibition and were subjected to staining, clearing, and microscopic examination. Expression in these seedlings was observed in association with developing vascular tissues in cotyledons, young leaves, hypocotyls, and with the stele in young radicles. Typical results for both pBG1.1- and pBG1.7-transformed T1 seedlings are illustrated in Figure 24 using pBG1.7 seedlings as examples. Panel A shows a whole mount of a seedling three days post-imbibition, which had not yet completely emerged from the seed-coat capsule. Expression was observed in vasculature of hypocotyl and radicle, as well as in the base of the cotyledons. In panel B, a seedling five days post-imbibition had strong GUS expression in the hypocotyl as well as expression associated with developing vasculature in the expanding cotyledons. The first true leaves are visible as small dense bodies at the top of the hypocotyl. In the next panel (C) these leaves are now expanding and GUS expression was associated with their developing vascular tissues by nine days post-imbibition. Panel D illustrates the continued expression of GUS in the stele of the developing root at nine days post-imbibition, and the reproducible lack of expression in the root-tip. Both promoters specified patterns of expression indistinguishable from each other.
Figure 24. Expression in whole-mounted T1 tobacco seedlings transgenic for PAL2 promoter construct. (A) seedling 3 days post-imbibition; expression in developing vasculature of stem. (B) seedling 5 days post-imbibition; expression in developing vasculature of stem and expanding cotyledons. (C) seedling 9 days post-imbibition; expression in developing vasculature of stem, expanding cotyledons and expanding first leaf. (D) root of 9 day seedling; expression in developing vasculature. (About 20X)
No expression was detected in any of the tissues of any of the individual transformants receiving the promoterless negative control construct pBG even when staining was carried out for 24 hours which was sufficient time to stain responsive tissues even in the most weakly expressing pBG1.1 and pBG1.7 transformants (results not shown).

**Wounding-associated expression of \textit{PAL1} and \textit{PAL2} promoters in transgenic tobacco**

To obtain T1 plants transgenic for the two poplar promoter constructs, sterilized T1 seeds from transformants previously characterized as high expressers were plated on MS media supplemented with kanamycin. Kanamycin-resistant seedlings were allowed to grow for several weeks and transferred to soil. Untransformed SR1 plants were also germinated on soil. When leaves were several centimeters long, one centimeter discs were cut from minimally vascularized areas of the leaf, incubated for a 24 "wounding" period, and then cut in half and incubated in X-gluc. staining buffer for several hours. They were then cleared and examined microscopically. The curved edges subjected to 24 hours incubation after wounding followed by several hours of staining were the experimental areas, and the straight edges subjected only to staining were controls for nonspecific staining of edges. Wounding treatments of 6 and 12 hours gave no detectable wounding-specific \textit{GUS} response (data not shown).

In Figure 25, panels A and B show the negative control response of untransformed tobacco plants when subjected to the experimental procedure described above. No \textit{GUS} expression was observed in any tissue of the negative controls. The second pair of panels illustrate \textit{GUS} staining before wounding (panel C) and after 24 hours wounding (panel D) in tobacco leaf discs transgenic for \textit{PAL1} promoter construct (pBG1.1). Likewise, panels E and F show \textit{GUS} responses in leaf discs transgenic for \textit{PAL2} promoter construct (pBG1.7), panel E before wounding and panel F after the wounding treatment. Before wounding, the only \textit{GUS} expression visible is associated with vascular tissues, but after 24 hours wounding, there is a strong specific response driven by both promoters along the wounded edge. Both promoters seem to respond identically. Variation in intensity of wound response was disc- and transformant-specific, not construct-specific.
Figure 25. GUS expression driven by pBG1.1 and pBG1.7 (PAL1 and PAL2 promoters) constructs in response to wounding. Both promoters drive equivalent responses specific to 24 hour wounding. Constitutive vasculature-associated expression was visible. (A) SR1 tobacco nontransformed negative control leaf disc without wounding. (B) SR1 tobacco untransformed negative control leaf disc after 24 hour wounding. (C) tobacco transformed with pBG1.1 leaf disc without wounding. (D) tobacco transformed with pBG1.1 leaf disc after 24 hour wounding. (E) tobacco transformed with pBG1.7 leaf disc without wounding. (F) tobacco transformed with pBG1.7 leaf disc after 24 hour wounding. (About 20 X)
Seasonal variation in PAL mRNA populations and PAL protein in leaf and differentiating xylem of 2 to 4 year-old poplar branch

Since poplar is a woody perennial, the seasonal up-regulation and down-regulation of gene expression may be observed in this plant. This work attempts to establish baselines of PAL amount and PAL gene expression in relation to phenological and environmental events. For experiments characterizing seasonal variation of poplar PAL protein and RNA levels, tissue samples were collected from a P. deltoides x P. trichocarpa hybrid called DT 49-177 throughout the growing season of 1993. This hybrid was produced by a cross similar to that which gave rise to the hybrid 53-242 which is the source for the PAL genes studied in this work, and therefore may be expected to possess PAL genes and alleles drawn from the same pool. Samples of bark, stem scraping (containing differentiating xylem during some months), and buds or leaves were collected each month from 2 to 4 year old branches harvested from two adjacent clones of DT 49-177. These clones were planted in an espacement trial at the Scott Paper Nursery in Agassiz, British Columbia. Phenological changes were photographically documented each month, and environmental data collected by Environment Canada at Agassiz were requisitioned.

Phenological observations

Perennial plants undergo regular seasonal cycles of activity and inactivity which are accompanied by predictable alterations in physiology and appearance. The progression of morphological changes associated with changing seasons is called phenology. The poplar hybrid 49-177 from which plant material was collected was observed to undergo the following phenological changes during the growing season of 1993 (Figure 26). In February (panel A), the buds seemed inactive. A month later in March (panel B), the buds had broken, and by April (panel C) the leaves had emerged and were about 5 centimeters in length, pale green with some reddish pigmentation visible, sticky, and highly scented. By June (panel D) the leaves were fully
expanded, dark green, had lost their sticky scented coating, and appeared fully functional as photosynthetic organs. In July (panel E) an extensive foliar infection became visible as blackened lesions surrounded by yellowed zones. In addition, large sections of leaf were eaten, probably by insect grazers. These infections and grazed sites became more numerous throughout August (panel F), September, and October, when large numbers of still-green leaves were abscising freely.

In February, the cambium of the sampled branches appeared to still be inactive and there was no easy separation of the bark from the wood. To sample xylem, tissue was shaved off using razor blades to cut down to obviously differentiated wood. In following months, the cambium was active and thus gave rise to easily split-away bark (a phenomenon known phenologically as bark slippage). Upon staining and microscopic examination, the bark material included the suberized phelloderm, phellogen, and associated photosynthetic layer (not shown), a cortex containing numerous bundles of schlerenchymal fibres (stained blue in Figure 27), the phloem tissues (stained purple), and the cambial initials themselves, visible as small rectangular cells stained pale purple. Scrapings from the inner surface of this organ were collected.
Figure 26. Leaf tissues collected at Agassiz, BC through the growing season of 1993. 
(A) February - quiescent buds. (B) March - buds were breaking. (C) April - leaves were rapidly expanding, shiny, sticky, highly scented, and pigmented. (D) June - leaves were fully expanded and dark green. (E) July - leaves were now under attack by insect grazers and fungal pathogens. (F) August - insect grazing and disease lesions remain visible through to the end of the growing season.
The living material remaining on the surface of the wood of the branch consisted of differentiating tracheary elements and xylem ray parenchyma of newly formed secondary xylem. This tissue was collected by scraping the woody surface of the peeled branch. In October, bark slippage was no longer evident (probably since the cambium was inactive) and shaving was used, as in February, to collect material.

Figure 27. Cross sections of 3 to 4 year-old poplar branches, harvested in May, showing tissues collected at Agassiz, BC, stained with toludine blue. (A) Lignified tissues such as xylem- and phloem-associated schlerenchymal bundles are bright blue; phloem and the small brick-shaped cells of the cambium stain purple. (B) Cambial layers at higher magnification.

Environmental data for the growing season of 1993 at Agassiz, British Columbia

In order to determine which aspects of the environment might have an effect on the sampled poplar trees and on the expression of their PAL genes, various environmental data were collected or calculated for the site from which the poplar tissue samples were taken. Daily minima
and maxima of temperature, daily precipitation, and daily hours of sun were collected at the Agassiz, BC station of Environment Canada (Environment Canada Atmospheric Service, Agassiz CDA Observations, 1993). Average daily temperature, growing degree days, and freezing degree days were calculated for each month. Average monthly daylength was calculated for each month (List, 1966). Growing degree days, freezing degree days and average monthly daylength are presented in Figure 28.

![Figure 28](image)

**Figure 28.** Selected environmental data for Agassiz, BC. (A) Average interpolated daylength (••••) for each month for latitude 49 degrees North. (B) Calculated growing degree days (---), freezing degree days (----) for 1993 at Agassiz, BC.

**Accumulation of PAL protein in poplar tissues throughout the growing season**

Extracts of total protein were prepared from the buds/leaves and from stem scraping (primarily the differentiating secondary xylem) of two- to four-year old branches of two individuals of clone DT 49-177 for each month throughout the growing season of 1993, and PAL protein levels assessed by western blot using a polyclonal anti-poplar PAL antiserum. Loading of equal amounts of protein in all lanes was ensured by the loading of duplicate aliquots of each sample onto a second gel which was then stained by Coomassie. The aliquots were determined by Bradford assay to contact equal amounts of protein. Results shown in Figures 29 and 30 were observed for both trees sampled, although results are shown for only one tree.
PAL protein was detected in leaves in March, and by April large quantities of PAL had accumulated in the rapidly expanding leaves (Figure 29, panel A). In subsequent months, when leaves had matured, PAL protein levels were undetectable in leaf protein extracts. (Apparent low molecular weight smears in several of the lanes are artifacts derived from black and yellow oxidized materials in the samples.) Panel B in Figure 29 shows that the lanes contained approximately equal amounts of Coomassie-stained protein as previously quantified by Bradford assays of the protein extracts.

In extracts of secondary xylem, large amounts of PAL protein were detected in May, followed by reduced amounts in June and July (Figure 30, panel A). In all other months, PAL was undetectable in stem scraping extracts. Panel B in Figure 30 shows that the May lane, giving the strongest western signal, is in fact underloaded with respect to the other months, so the detected amount of PAL protein in the May sample is probably an underestimate. The molecular weights for both bud/leaf and xylem proteins appear to be somewhat less than the value which would be predicted from the size of the open reading frame (714 amino acids giving a molecular weight of about 78 kD) of cDNA PAL 7, and is less than the molecular weight observed for the protein expressed from that cDNA (McKegney et al., in press). However, the low resolution of the gel and the standards used in these experiments is such that these apparent molecular weights are not completely inconsistent with the predicted molecular weight.
Figure 29. Analysis of proteins in poplar bud/leaf throughout the growing season of 1993. (A) western blot of bud/leaf tissue. The blot was probed with an anti-poplar PAL antibody. (B) A duplicate PAGE gel showing Coomassie-stained proteins from the same extracts. 20 micrograms of protein as determined by Bradford assay were loaded in each lane. M, March; A, April; M, May; J, June; J, July; A, August; S, September; O, October. MW markers are in kD.
Figure 30. Analysis of proteins in poplar secondary xylem throughout the growing season of 1993. (A) Western blot of differentiating secondary xylem. The blot was probed with an anti-poplar PAL antibody. (B) A duplicate PAGE gel showing Coomassie-stained proteins from the same extracts. 20 micrograms of protein as determined by Bradford assay were loaded in each lane. F, February; M, March; A, April; M, May; J, June; J, July; A, August. MW markers are in kD.
Northern blot detection of *PAL* RNA accumulation in leaf

The PAL 7 cDNA was used to probe northern blots of RNA extracted from bud/leaf samples used for western analysis above for the months of February, March, and April to confirm that the presence of immuno-detectable PAL protein in those tissues (Figure 29) was accompanied (or preceded) by high steady-state levels of *PAL* mRNA. As shown in panel A of Figure 31, the probe hybridized to a single band in each lane at high stringency, demonstrating the presence of *PAL* mRNA in those tissues. Very faint signals were detected in the months of February and April, with a much higher quantity of *PAL* mRNA detectable in March. Relative loading of the lanes was assessed by stripping and re-probing the blot with an rRNA probe derived from pea, as shown in panel B.

![Figure 31. Northern blot analysis of bud/leaf tissues from February (F), March (M), and April (A) of 1993. (A) 15 micrograms of total RNA from buds/leaves from each of the three months were transferred, hybridized to cDNA PAL 7, and washed in 0.5X SSC. Exposure 14 days, -90 degrees Celsius. (B) The same blot was stripped and hybridized to a pea rRNA probe to assess relative loading (Stringency 0.5X SSC, exposure one hour room temperature). Estimated size of the hybridizing bands is given in kb.](image)

This experiment was also carried out using RNA extracted from the monthly secondary xylem samples, but no hybridization was detectable in any month (results not shown), perhaps because of the relatively low levels of *PAL* mRNA in that tissue. When the cDNA PAL 7 was used as a probe to compare *PAL* expression in secondary xylem to that in young leaf, detectable levels of *PAL* mRNA were much lower in xylem than in young leaf (Subramaniam et al., 1993).
Comparable differential probes were created for PAL3 and PAL1/2 by amplifying identical regions of PAL3 and PAL2 as described in section 2.2.7 and testing them against DNA targets. PAL1 and PAL2 were indistinguishable in expression patterns specified by their promoters in transgenic tobacco and were therefore considered as a single entity in the following northern blot analyses. When blots were washed at 0.5X SSC (moderately high stringency), the two probes effectively differentiated between PAL3, PAL2, and the pBluescript vector, 2.9 kb in length, as seen in Figure 32. In panel A, the PAL1/2 probe hybridizes to the complete 2.4 kb sequence of the PAL 7 cDNA (lane 1) and to itself (lane 2), but not to PCRPAL16, corresponding to PAL3, (lane 3) or to the pBluescript II KS- vector at approximately 3 kb in lanes 1 and 3. In panel B, the complementary experiment using PCRPAL16 as the probe demonstrates the complementary specificity.

The pair of gene-specific probes tested above and the full-length cDNA PAL 7 were used to probe triplicate filters loaded with total RNA from young green stem, May xylem scraping, May bark scraping, and young leaf tissues from poplar individual H-11, provided by Nancy Mah. Results shown were observed in two separate experiments. In panel C, the probe used was the 400 bp PAL1/2-specific probe amplified from cDNA PAL7; in panel D, the full-length cDNA PAL 7; in panel E, the 400 bp PAL3-specific probe amplified from genomic DNA. With all three probes, strong hybridization to young stem and to young leaf RNA was observed, with lesser hybridizations to differentiating xylem and to bark. Doublet bands observed in panels C and E may be caused by detection of a specific lower molecular weight degradation product by the 400 bp probes. Evenness of loading was ensured by inspection of ethidium bromide-stained gels as shown in the upper row of photographs in panels C, D, and E.
Figure 32. Evaluation of expression of PAL1/2 and PAL3.

(A) Testing of PAL1/PAL2 specific probe.
Lane 1, Plasmid digest of cDNA PAL7, liberating 2.9 kb pBluescript II KS- vector (negative control) and 2.4 kb cDNA PAL 7 (positive control).
Lane 2, 400 bp PCR fragment amplified from cDNA PAL 7.
Lane 3, plasmid digest of PCR PAL16, liberating 2.9 kb pBluescript II KS- vector and 400 bp PCR PAL16 fragment. (Wash was at 0.5 X SSC, exposure 6 hours 24 degrees Celsius.)

(B) Testing of PAL3 specific probe.
Lanes as in panel A. (Wash was at 0.5 X SSC, exposure 6 hours 24 degrees Celsius.)

(C, D, E) Triplicate RNA gels; 15 micrograms of total RNA from green stem (gs), xylem (x), bark (b), and young leaf (yl).
Top panels - ethidium-bromide-stained gels before transfer.
Lower panels -
(C) Hybridization to PAL1/PAL2 specific probe. (Wash was at 0.5x SSC, exposure 1 day -90 degrees Celsius.)
(D) Hybridization to full-length cDNA PAL 7. (Wash was at 0.5x SSC, exposure 1 day -90 degrees Celsius.)
(E) Hybridization to PAL3-derived gene-specific probe. (Wash was at 0.5x SSC, exposure 5 days -90 degrees Celsius.)
4.3 Discussion

In this section, several aspects of poplar PAL gene expression were investigated, including developmentally regulated expression, expression induced by wounding, involvement of PAL genes in lignification of secondary xylem, seasonality of PAL gene expression, and possible differential regulation of gene family members.

To examine developmentally regulated patterns of gene expression dictated by the PAL1 and PAL2 promoters, the promoters of these two genes were fused to the reporter gene GUS, transformed into tobacco, and the transgenic tobacco examined for GUS expression. Similar experiments have been carried out using the promoters of PAL genes from Arabidopsis and bean. The Arabidopsis PAL1 promoter fused to GUS was transformed back into Arabidopsis, and was observed to direct GUS expression in vascular tissues of adult plants, and in most tissues of newly germinated seedlings, excepting the root-tip (Ohl et al., 1990). The promoters of bean PAL2 and PAL3 were also fused to GUS and transformed into tobacco (Bevan et al., 1989; Liang et al., 1989), as well as into potato and Arabidopsis (Shufflebottom et al., 1993). The bean PAL2 promoter directed expression in the apical meristem and in the differentiating xylem in transgenic tobacco (Liang et al., 1989). When the expression patterns of bean PAL2 and PAL3 were compared in tobacco, potato and Arabidopsis (Shufflebottom et al., 1993), PAL2 was specific to xylem, and PAL3 to pith and endodermis of roots. Both bean promoters directed expression in root-tips, pigmented petals, and epidermal cells. However, there were a few species-specific differences in expression pattern. For example, in Arabidopsis, no expression was observed in association with the root-tip. The authors attributed these differences to species-specific variations in demand for phenylpropanoid derivatives or in available trans-acting factors (Shufflebottom et al., 1993).

When the developmentally regulated expression patterns directed by poplar PAL promoters are compared to the above systems, they seem relatively simple. Expression driven by the two poplar PAL promoters was almost entirely limited to vascular tissues in leaf, stem, and flower of adult plants and cotyledon, leaf, hypocotyl, and radicle of germinating seedlings. There were three exceptions. First, a possible nectary-associated expression was observed in
transgenic tobacco flower, which has also been observed in tobacco transgenic for parsley \textit{4CL} promoter: \textit{GUS} fusions (Hauffe \textit{et al.}, 1991). Second, a stele-associated expression observed in elongating radicles was not closely examined and may have been localized either to vascular tissues or to developing endodermis. The latter expression pattern, presumably associated with suberization of the Casparian strip, has been observed for the bean \textit{PAL3} promoter transformed into tobacco (Shufflebottom \textit{et al.}, 1993). Finally, the ovules in tobacco flowers transgenic for both \textit{PAL1} and \textit{PAL2} exhibited \textit{GUS} expression on their surfaces. This expression has been observed for parsley \textit{4CL} promoter: \textit{GUS} fusions in transgenic tobacco (Hauffe \textit{et al.}, 1991).

The external layers of the ovule develop into the seed coat of the mature seed which is pigmented in many species (Esau, 1977). Thus, this expression may be associated with the deposition of phenylpropanoid pigments. In addition, the seed coat in many species possesses a layer of highly lignified sclereids for protection against crushing (Esau, 1977) and may also be impregnated with other phenolic derivatives which serve to waterproof the seed (Marbach and Mayer, 1974).

\textit{GUS} expression patterns driven by poplar promoters in tobacco must be interpreted with caution, since poplar and tobacco have very different life histories and thus might be expected to possess different gene expression patterns. The relatively limited expression of poplar \textit{PAL1/2} promoter: \textit{GUS} fusions in the tobacco system suggests that these promoters lack the \textit{cis}-elements necessary to respond to certain tobacco-specific developmental signals. For example, poplar flowers do not possess petals and have no need for colorful, insect-attracting pigments in those tissues. The transgenic tobacco petal epidermis showed no poplar \textit{PAL} promoter-driven \textit{GUS} expression (Figure 22B), although the promoters of other species’ \textit{PAL} and other phenylpropanoid-related genes do drive \textit{GUS} expression in these typically pigmented tissues (for example, the promoters of bean \textit{PAL2} and \textit{PAL3} [Shufflebottom \textit{et al.}, 1993], and the parsley \textit{4CL} promoter [Hauffe \textit{et al.}, 1991]). In addition, the anthers of poplar are heavily pigmented, presumably with anthocyanin pigments, but no reliable detectable expression was found in transgenic tobacco anthers. Perhaps the third poplar \textit{PAL} gene is responsible for this pigmentation, or perhaps the pigment was not a phenolic derivative. Finally, \textit{in situ} hybridization with poplar \textit{PAL} anti-sense RNA detected high levels of \textit{PAL} expression in the subepidermal
layers of young poplar leaves. The large amounts of phenolic exudate synthesized by young poplar leaves probably require a correspondingly large amount of PAL activity in the subepidermal layer of the young leaf (Subramaniam et al., 1993). Since tobacco does not produce this phenolic exudate, its leaf-specific environment probably lacks the signals required to activate the poplar PAL genes in that tissue. There was no sign of this expression in the tobacco transgenic for PAL1/2 promoter: GUS fusions, although many young tobacco leaves of several developmental stages were closely scrutinized.

The response driven by the two poplar PAL promoters in response to wounding was also examined in transgenic tobacco. Both promoters responded strongly to this environmental signal in keeping with the observed regulation patterns of PAL genes in other species. PAL genes in Arabidopsis (Ohl et al., 1990), melon (Diallinas and Kanellis, 1994), sweet potato (Tanaka et al., 1989), parsley (Lois and Hahlbrock, 1992), bean (Bevan et al., 1989, Liang et al., 1989, etc.), and potato (Ishizuka et al., 1991) have all been documented as responsive to wounding.

These transgenic tobacco plants can provide information regarding the involvement of poplar PAL genes in the lignification of secondary xylem. In the photographs of PAL1 and PAL2 promoter-driven expression in older leaves (Figure 21, panels C and D) and of PAL1 promoter-driven expression in older stem (Figure 23), two types of vascular-associated expression patterns were observed. The first was associated with ray parenchymal files and the other was associated with the youngest and presumably still-lignifying elements in a file of xylem elements. Similar patterns of expression of PAL, 4CL, and CAD genes and proteins have been demonstrated in other systems (reviewed in section 1). The evidence from the literature combined with this new information localizing expression of PAL genes from a woody plant to those two cell types suggests a fusion and extension of Northcote and Rubery's, and Wardrop and Bland's hypotheses regarding precursor sources during lignification of secondary xylem. Specifically, both xylem ray parenchyma and young, differentiating xylem elements may be involved in the synthesis of lignin precursors deposited in the walls of differentiating secondary xylem elements and tracheids. The poplar PAL promoter results provide further evidence to support this extended hypothesis, but they do not address the issue of transport of lignols from one cell to another.
Northern blot analyses of tissue-specific expression of PAL1/2 and PAL3 also contain information about the role of poplar PAL genes in the formation of secondary xylem. A PAL activity specific to lignification of secondary xylem in a woody angiosperm has not been demonstrated to date. The P. kitakamiensis pal g2a gene (apparently analogous to PAL3 as discussed in section 3) was highly expressed in young green stem as compared to young leaf of P. kitakamiensis (Osakabe et al., 1995). Although young stem is composed of several tissues, this expression might be high because of expression associated with lignification of the developing secondary xylem. Intuitively, PAL activity should be high in a tissue with such an obvious demand for phenylpropanoid derivatives; most often, such a high activity would be reflected by high levels of both PAL protein and mRNA. As demonstrated previously in the literature, probes derived from both pal g2a (or PAL3) and PAL 1/2 detect expression in young leaves and in young stem (Osakabe et al., 1995; Subramaniam et al., 1993). However, only faint bands can be seen in the xylem and bark RNA's when probed with either probe at high stringency (Figure 32). Therefore, the origin of the high levels of detectable PAL protein (Figure 30) and high PAL enzyme activity extracted from differentiating secondary xylem (Subramaniam et al., 1993), remains unclear. The observed hybridizations may be specific and therefore indicate that all loci contribute some mRNA to the pool to be translated into detectable PAL protein and extractable PAL activity. Alternatively, the hybridization may be due to a very weak and non-specific detection of the expression of yet another uncharacterized PAL gene. There is also a possibility that the PAL mRNA is transcribed and translated at very high rates over a very brief time frame (hours or days) which was undetected by the once-monthly sampling points. Finally, perhaps the mRNA and/or protein is particularly stable in developing xylem and thus high levels of steady-state PAL mRNA are unnecessary.

Seasonal patterns in PAL gene expression were investigated by western blot analysis of protein levels and by northern blot analysis of RNA levels in buds/leaves and in secondary xylem collected throughout the growing season of 1993. PAL mRNA was detectable in large quantity in breaking buds in March. This was followed by an increase in PAL protein in April, when the leaves had emerged from the terminal buds and were rapidly expanding. As leaves expand, they require PAL activity to supply lignol precursors for their developing conductive
xylem. In addition, the sticky, highly scented poplar leaf exudates are complex mixtures of phenolic compounds (Shain and Miller, 1982). The transient reddish pigmentation observed in young leaves in April may also be produced by phenylpropanoid metabolism.

As the leaves mature and become significant sources of photosynthate, the cambium becomes active, initiates division, and certain cambial derivatives begin to undergo differentiation to form secondary xylem. Concomitant with the differentiation of tracheary elements in secondary xylem is the synthesis and deposition of lignin. Thus, an increase in detectable PAL protein in secondary xylem would be expected later in the growing season when the leaves can supply photosynthate to the cambium to support its activity. This is a likely explanation for the increase in levels of PAL protein in the secondary xylem observed in May (Figure 30).

Seasonal environmental phenomena may be correlated with the phenological changes and variations in PAL protein and PAL RNA reported above. The increasing temperatures in February and March (Figure 28) enabled the quiescent buds to break (Coleman et al., 1993), and by April, daily temperatures were sufficiently high that growing conditions were suitable for temperate plants, as reflected by cumulative growing degree days. The increasing daylength (Figure 28) throughout those months may also have had a role in signalling the buds to begin active growth.

There are several precedents in the literature for differential regulation of plant PAL genes, including a report that poplar PAL genes in P. kitakamiensis are differentially regulated in young poplar tissues (Osakabe et al., 1995) which indicated that the pal g2a gene (equivalent to PAL3) is more strongly expressed in young green stem than in young leaf. This thesis attempted to replicate the report documenting differential expression of P. kitakamiensis PAL genes and also compared the expression patterns driven by the promoters of poplar PAL1 and PAL2 in transgenic tobacco.

As noted above, the poplar PAL1 and PAL2 promoters directed patterns of expression which were indistinguishable from each other, both in developmental contexts and in response to wounding. Thus, the transgenic tobacco provided no evidence of differential regulation of these two poplar PAL genes. However, examination of the expression patterns driven by these
promoters when transformed back into a homologous system poplar may reveal refined patterns of regulation, including differential regulation of the two promoters.

Although the two genes studied in detail seemed not to be differentially regulated, a third poplar PAL gene (PAL3) is relatively diverged from PAL1 and PAL2. This gene would therefore seem a potential candidate for differential regulation in poplar, since divergence in the promoter may result in differential expression of that gene. However, as seen in Figure 32, differential probes derived from PAL1/2 and PAL3 detected no large differences in expression in the four tissues examined. All probes hybridized strongly to RNA from young leaf and green stem, and hybridized only weakly to RNA extracted from differentiating secondary xylem and from bark. There was no indication that either PAL1/2 or PAL3 were regulated differentially in young poplar tissues. The results presented here contradict the study by Osakabe et al. indicating that the putative P. kitakamiensis equivalent to PAL3 is more highly expressed in young stem than in young leaf.
5. Conclusions and future directions

5.1 Conclusions

This thesis demonstrated several findings regarding relatedness of PAL genes in the poplar PAL gene family. PAL in poplar is encoded by a family of at least three genes, two closely related (PAL1 and PAL2) and one diverged from the other two (PAL3). This is similar to PAL gene families found in other plants. The two related genes are unlinked. PAL1 and PAL2 are similar in coding regions sequenced so far at 90.1% nucleotide identity, 91.4% amino acid identity, and 94.4% amino acid similarity. Their promoters are 81.6% identical and 3' non-coding regions are 72.8% identical. The introns of the two genes are located in the same place as other plants' PAL genes, but the two poplar PAL introns are the same size. This has not been observed in other plant PAL gene families. Taken together, these similarities indicate that PAL1 and PAL2 may have only recently diverged from each other.

In addition, this work examined the expression of poplar PAL genes. In all ages and organs of transgenic tobacco, the promoters of both PAL1 and PAL2 directed simple and identical patterns of expression in vascular tissues, in ovules, and in response to wounding. This expression pattern is simpler than those dictated by bean (Shufflebottom et al., 1993) and Arabidopsis promoters (Ohl et al., 1990) in transgenic plants. In addition, not all predicted types of PAL expression were observed, particularly the subepidermal expression detected by anti-sense RNA in situ hybridization in young poplar leaf (Subramaniam et al., 1993). These facts suggest either that the tobacco system does not allow all potential PAL1 and PAL2 expression to be visualized, or that poplar PAL genes in addition to PAL1 and PAL2 are involved in specifying the complete pattern of PAL expression in planta.

Although there is evidence in the literature for differential expression of PAL3 (Osakabe et al., 1995), this work could detect no differential expression of the three poplar PAL genes in green stem, young leaf, secondary xylem, or bark.

Finally, this thesis presents documentation of seasonal regulation of PAL, a gene involved in lignification of a woody perennial, at levels of both protein and RNA. In buds and leaves,
poplar *PAL* genes may be up-regulated in March, resulting in a detectable increase in *PAL* RNA in that month and an increase in extractable *PAL* protein in the next month, April. This up-regulation of *PAL* is presumably necessitated by the increased demand for lignin precursors by the developing vascular tissues in the expanding leaf, and by the synthesis of the many phenolic derivatives present in and upon the young leaf. In secondary xylem, maximum levels of extractable *PAL* protein are detected in the following month, May, perhaps in association with increased demand for lignin precursors by the developing secondary xylem. This is the first instance in which variations in levels of *PAL* protein have been described on a monthly basis in several tissues of a woody perennial.

5.2 Future directions

Initially only two *PAL*-encoding genes were cloned from this pedigree but subsequently a third gene has been identified. The inheritance of this gene should be characterized by a more careful examination of the RFLPs in the hybrid pedigree and the gene should be placed onto the poplar linkage map. This gene should also be cloned in its entirety and its structure analysed by restriction-mapping and sequencing so that it can be compared in more detail to *PAL1* and *PAL2*. A detailed comparison of its structure to other diverged *PAL* genes, such as the *Arabidopsis PAL3* gene possessing a second intron (Wanner *et al.*, 1995), should also prove interesting. There may be other poplar *PAL* genes as well. A series of *PAL*-specific PCR primers is available in the laboratory of Brian Ellis. Perhaps these could be used to amplify genomic fragments from the poplar genome as an alternative method of searching for other divergent *PAL* genes. A primer based on the sequence of the highly conserved active site might be particularly effective in amplifying otherwise divergent genes. The fragments would be sequenced and compared to known *PAL* sequences, and could then be used as probes to identify cDNAs or complete genomic clones.

The promoter region of *PAL3* should be obtained by cloning of a genomic sequence so that expression patterns may be analyzed. Since expression of *PAL1* and *PAL2* promoters have already been described in tobacco, the *PAL3* promoter could be transformed into that system.
However, ideally, reporter constructs containing all three promoters would be transformed into poplar since the homologous system might report gene-specific involvement in previously described processes (such as the subepidermal $PAL$ RNA expression described by Subramaniam et al., 1993) more accurately than did the tobacco system. Because transformation and regeneration procedures have lately been developed for poplar in collaborating research groups, the constructs can now easily be tested in poplar. The possibility of differential expression of this third gene should also be pursued.

Another intriguing aspect of the $PAL1$ and $PAL2$ promoters is the presence of the conserved AC/L box found in the promoters of many phenylpropanoid genes. By altering the box (site-directed mutagenesis) or by deleting it (construction of a series of deletion mutants) and examining expression patterns directed by the altered promoters, the function of the box and of other as-yet unidentified promoter elements could be determined. If any of these poplar $PAL$ promoter elements confer xylem-specific expression, these sequences could be used to perform a Southwestern screen of a xylem expression library to identify xylem-specific transcription factors.

RNAase protection or primer extension should be carried out to determine the transcription start sites for $PAL1$ and $PAL2$.

The nature and the regulation of the seasonal variation in levels of PAL protein and $PAL$ RNA should certainly be studied in more detail. By taking leaf and stem samples more frequently, the correlation between environmental changes and variations in PAL protein and $PAL$ RNA could be refined. In addition, other phenylpropanoid genes may be regulated co-ordinately with $PAL$. $4CL$ and $CHS$ antibodies are available and could be used to characterize variation in levels of those proteins in these same tissues, particularly in the buds and leaves. These results would support the idea that high levels of PAL and other phenylpropanoid-associated enzyme activities may be required by these tissues to synthesize the flavonoid derivatives abundant on a seasonal basis in (and on) poplar leaves. Next, replicate dormant poplar plants could be treated with suitable combinations of continued cool regime (equivalent to the cooling degree days for January 1993), warming regimes equivalent to the growth degree day observations for February and 10.5 hour daylength (average daylength for February) to determine which of these
environmental changes cause the observed changes in PAL levels. Finally, the communication of perceived environmental change is controlled by the overwintered bud in some systems (Coleman et al., 1993), so removal of buds might inhibit the activation of PAL genes in cambium. Although the logistics of replicated experimentation on such large organisms is daunting, demonstration of causal links between annual environmental changes and the activation of such important genes would be rewarding.

Finally, the apparently low levels of PAL mRNA in the secondary xylem of poplar should be investigated. Perhaps the mRNA levels are sufficient to produce the observed high levels of PAL protein provided that PAL is not being degraded in that tissue. PAL degradation has been described in the literature as being associated with phosphorylation (Bolwell, 1992) and with the activity of an enzyme-like factor extracted from leaf tissue (Gupta and Creasy, 1991). Experiments could be designed to test for either of these possibilities in regulating the amount of PAL protein in poplar secondary xylem.
Bibliography


