Phenylalanine Ammonia-Lyase in Woody Plants:
A Key Switch of Carbon Accumulation in Biomass

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ABSTRACT

Phenylalanine ammonia-lyase (PAL) is a key enzyme of phenylpropanoid metabolism and catalyses the first step from primary metabolism to secondary metabolism. The transcriptional activation of *PAL* genes is controlled during plant growth and development and by abiotic and biotic stresses. Recent studies of biochemical characterization of PAL isoforms suggest that PAL protein is posttranslationally modified and this regulation might module the PAL metabolic networks.

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INTRODUCTION

Plant species are able to convert massive quantities of carbon dioxide into biomass by the process of photosynthesis in secondary tissues. Dicotyledonous plants have secondary growth originating from the vascular cambium forms between the xylem and phloem in the vascular bundles. The vascular cambium cells divide to produce secondary xylem, 'wood', and secondary phloem. During the development of secondary xylem and phloem, the cells first undergo differentiation processes, which involve cell division, expansion, maturation, lignification, secondary cell wall thickening, and programmed cell death. The cell differentiation and growth respond to environmental and seasonal changes presumably through cell-to-cell communication. It is important to identify the mechanisms that regulate the developmental processes of secondary cell walls, which are major compartments of wood.

In woody plants, a majority of assimilated carbon sources is accumulated and stored in the secondary cell wall, especially as a phenylpropanoid compound, lignin. Phenylpropanoid metabolism is derived from the shikimic acid pathway, which includes lignin, flavonoid, coumarin, and stilbene biosyntheses. It has been extensively studied and shown to play important roles such as mechanical support (Chabannes *et al*. 2001; Jones *et al*. 2001), protection against biotic and abiotic stresses (Dixon and Paiva 1995), the source of pigments (Holton and Cornish 1995) and signalling molecules in plants [e.g. flavonoids as nodulation factors (Xie *et al*. 1995)]. A number of studies suggest that the biosynthetic enzymes of various phenylpropanoid isoforms are regulated in response to different plant conditions (Boerjan *et al*. 2003; Boudet *et al*. 2003; Raes *et al*. 2003). Genomic studies in both poplar and *Arabidopsis* have identified a number of genes involved in the phenylpropanoid biosynthesis, especially some transcription factors regulating this biochemical process by controlling gene expression.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyses the first metabolic step from primary metabolism into secondary phenylpropanoid metabolism, deamination of L-phenylalanine to produce cinnnamate (Hahlbrock and Scheel 1989). Cinnamic acid is further converted into *p*-coumaric acid by hydroxylase. *p*-Coumaric acid is modified by additional hydroxylation and methylation, and the CoA esters of the derivative compounds are then precursors in lignin biosynthesis (Boudet *et al*. 2003). In this review, we highlight recent findings in the functional analysis of PAL to understand the mechanisms that regulate lignin biosynthesis in secondary vascular tissues. Applications of the study of the genes coding for phenylpropanoid biosynthesis enzymes will provide insight into the molecular biology of woody species.

EXPRESSION PATTERN AND PHYSIOLOGICAL ROLES OF PAL IN PLANT

The expression of *PAL* genes is controlled by environmental and developmental signals in plant cells (Liang *et al*. 1989; Shufflebottom *et al*. 1993; Kumar and Ellis 2001). Typically,

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PAL genes are encoded by a small gene family and multiple PAL isoforms have been detected in *Phaseolus vulgaris* (Bolwell *et al*. 1985), *Solanum tuberosum* (Joos and Hahlbrock 1992), *Arabidopsis* (Cochrane *et al*. 2004), and *Populus kitakamiensis* (Osakabe *et al*. 2006). In the *Arabidopsis* genome, PAL is encoded by four genes, *AtPAL1*, *AtPAL2*, *AtPAL3*, and *AtPAL4* (Raes *et al*. 2004; Rohde *et al*. 2004). The transcription of *AtPAL1* and *AtPAL2* was detected in an organ-specific manner; these genes were expressed mainly in roots and stem, and the expression of these genes was increased during the later stages of inflorescence stem development. The activity of the *AtPAL1* promoter-GUS was detected in the vascular tissues (Ohl *et al*. 1990; Leyva *et al*. 1995). Rohde *et al*. (2004) analyzed the single mutant of *AtPAL1* or *AtPAL2*, and the *AtPAL1/AtPAL2* double mutant. Each single mutant did not show clear phenotypic alterations, whereas the *AtPAL1/AtPAL2* double mutant showed decreased PAL activity in the inflorescence stems, and the lignin content of the double mutant was significantly reduced and the syringyl/guaiacyl ratio of lignin monomers was increased. These results suggested that AtPAL1 and AtPAL2 contribute to the production of lignin precursors. Furthermore, L-Phe was overaccumulated and this caused a severe imbalance in the levels of many other amino acids. Interestingly, the disruption of PAL protein resulted in the adaptation of the transcriptome in not only phenylpropanoid biosynthesis (e.g. 4-coumarate:CoA ligase and carboxymethylenebutenolidase genes), but also carbohydrate metabolism (e.g. invertase and β-1,3-glucanase genes) and amino acid metabolism (e.g. chorismate mutase, glycine dehydrogenase and tryptophan synthase β chain genes), suggesting a complex interaction of these pathways.

In the *Populus* genome, PAL genes also belong to a small gene family. From a hybrid poplar (*Populus trichocarpa* x *Populus deltoides*), the *PtdPAL1/2* promoter-GUS activity (Gray-Mitsumune *et al*. 1999) was detected in young stems and leaves. Two types of PAL cDNA, *PtPAL1* and *PtPAL1*, were isolated from another *Populus* species, quaking aspen (*Populus tremuloides* Michx.). The expression of *PtdPAL1/2* were regulated in a tissue-specific manner, thus *PtPAL1* was expressed in young tissues and *PtPAL2* was expressed in heavily lignified structural cells of shoots (Kao *et al*. 2002). It has been shown that *PtPAL1* and *PtPAL2* showed sequence homology with *PkPALg1* and *PkPALg2b* from hybrid aspen (*Populus kitakamiensis*) (Osakabe *et al*. 1995a, 1995b), respectively. The expression patterns of *PkPALg1* and *PkPALg2b* showed similar as those of *PtPAL1* and *PtPAL2*, thus *PkPALg1* was expressed in epidermis of young tissues and *PkPALg2b* was expressed in lignified tissues (Osakabe *et al*. 1995b, 1996, 2006). These data indicate that the *PALg1*-class genes function mostly in young stem tissues and leaves, suggesting that the physiological roles of *Populus* PALs were controlled at their transcriptional levels.

Phenylpropanoid compounds, such as flavonoids, stilbens, monolignols, and lignans are used in antimicrobial defence responses in plants (Dixon 2001). Lignin is also synthesized during plant defence responses and used as a physical barrier against pathogen invasion. Increased production of these compounds enhances resistance in various plant species (Felton *et al*. 1999; He and Dixon 2000). In the signalling mechanism of defence responses in plants, MAPK cascade is an important upstream component for the induction of plant defence genes (Pedley and Martin 2005). In rice, the suppression of *OsMAPK6* by an RNAi strategy, showed a significantly reduced induction of PAL upon the inoculation with the sphingolipid elicitor from *Magnaporthe grisea*, suggesting that *OsMAPK6* is required for pathogen-induced *PAL* gene expression (Lieberherr *et al*. 2005). Furthermore, the OsMAPK6 protein level and the activated kinase activity of OsMAPK6 by sphingolipid elicitors were decreased in rice cells, in which expression of a small GTPase gene, *OsRac1*, was silenced (Lieberherr *et al*. 2005). These results suggest that a signalling cascade from heterotrimeric G protein to OsRAC1, then to OsMAPK6, and presumable a MAPK cascade, throughout which OsMAPK6 activates the transcription of *PAL* gene regulates the defence response in rice cells. In transgenic tobacco (*Nicotiana tabacum* cv. 'Xanthi'), the suppression of *PAL* gene expression resulted in an increased susceptibility to the fungal pathogen *Cercospora nicotianae*, and it was associated with a strong decrease in phenylpropanoid pools (Maher *et al*. 1994). Phytohormone salicylic acid (SA) plays an important role in plant defence. SA is capable of increasing pathogenesis related (PR) gene expression and inducing systemic acquired resistance (SAR) in plants to different pathogens. In tobacco, SA biosynthesis is mainly derived from the phenylpropanoid pathway. It is also known that the silencing of *PAL* in tobacco resulted in the inhibition of the induced production of SA and did not establish SAR against tobacco mosaic virus (Pallas *et al*. 1996).

TRANSCRIPTIONAL REGULATION OF PAL GENE

Mechanisms underpinning the spatial and temporal control of phenylpropanoid biosynthesis have been revealed by the investigation of the transcriptional regulation of many of genes (Demura and Fukuda 2007). Promoter analysis showed that the AC elements of phenylpropanoid pathways genes such as *PAL*, *4CL* (4-coumarate:CoA ligase), *CCR* (cinnamoyl-CoA reductase) and *CAD* (cinnamyl alcohol dehydrogenase) genes are important for the transcription of these genes in xylem lignification. Members of the R2R3-MYB family transcription factors may regulate lignification through their interaction with AC elements. In woody xylem tissue, several R2R3-MYB family genes have been isolated in several tree species (**Table 1**).

Nine poplar MYB transcription factors involved in secondary vascular tissue formation were screened and isolated from the poplar EST collection (Karpinska *et al*. 2004). One of the poplar MYB genes, *pttMYB21a* was restrictedly expressed in the secondary wall formation zone of xylem and in the phloem fiber. *pttMYB21a* belongs to Arabidopsis MYB R2R3 subgroup 21 and shows homology to *AtMYB52*.

Table 1 Transcription factors that control phenylpropanoid biosynthesis in woody plants.

Transcription factor	Species	Location of gene expression	Regulated down stream genes	Function	Reference
PtMYB4 (R2R3-MYB type; homologous to AtMYB83, 46)	*Pinus taeda*	Stem epidermal layer, Early differentiating xylem, Phloem fiber	\<upregulated\> C3H, CCoAOMT, COMT, CCR \<downregulated\> PAL	Ring modification of monolignol	Patzlaff *et al*. 2003
EgMYB2 (R2R3-MYB type; homologous to AtMYB83, 46)	*Eucalyptus grandis*	Stem differentiating secondary xylem	\<upregulated\> HCT, CCR, CAD, C3H, F5H, CCoAOMT, COMT	Side-chain and ring modification of monolignol	Goicoechea *et al*. 2005
PtMYB21a (R2R3-MYB type; homologous to AtMYB52)	*Populus* sp.	Root differentiating xylem, central veins	\<downregulated\> CCoAOMT	Ring modification?	Karpinska *et al*. 2004
NtMYBJS1 (R2R3-MYB type; homologous to AtMYB13, 14, 15)	*Nicotiana tabacum*	Vascular tissues	\<upregulated\> PAL, C4H, C3H, 4CL	General phenylpropanoid biosynthesis	Galis *et al*. 2006
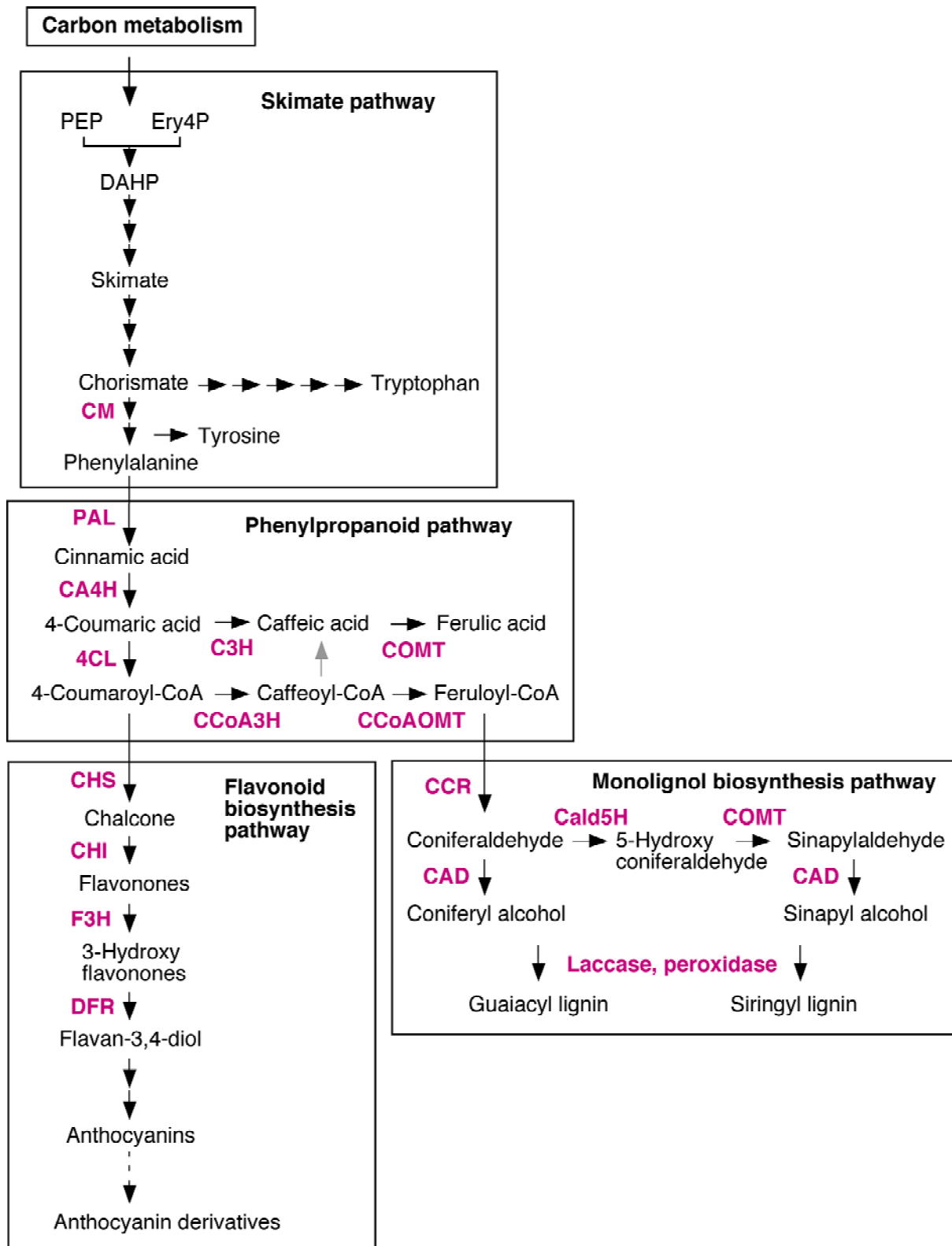
Fig. 1 Schematic diagram of skimate, phenylpropanoid, flavonoid, and monolignol biosynthesis patways. PEP: phosphoenol pyruvate, Ery4P: erythrose 4-phosphate, DAHP: 3-deoxy-D-arabinoheptulosonic acid-7-phosphate, CM: chorismate mutase, PAL: phenylalanine ammonia-lyase , CA4H: cinnamate 4-hydroxylase, 4CL: 4-coumarate:CoA ligase, C3H: 4-coumarate 3-hydroxylase, CCoA3H: 4-coumaroyl-CoA 3-hydroxylase, COMT: caffeate *O*-methyltransferase, CCoAOMT: caffeoyl coenzyme A *O*-methyltransferase, CCR: cinnamoyl-CoA reductase, Cald5H: coniferaldehyde 5-hydroxylase, CAD: cinnamyl alcohol dehydrogenase, CHS: chalcone synthase, CHI: chalcone isomerase, F3H: flavanone 3-hydroxylase, DFR: dihydroflavonol 4-reductase.

The antisense transgenic poplar of *pttMYB21a* showed higher transcript level of the *CCoAOMT* (Caffeoyl coenzyme A *O*-methyltransferase) gene in the phloem region. Therefore, Karpinska *et al.* suggested that *pttMYB21a* might work as a repressor for *CCoAOMT*. However, *pttMYB21a* did not affect the transcript level of other phenylpropanoid pathway genes such as *PAL*, *4CL*, *CAD*, *OMT* and *C4H* (cinnamate 4-hydroxylase gene).

In *Pinus taeda*, a R2R3-MYB family gene *PtMYB4* was isolated and showed to be expressed in all lignified cells such as early differentiating xylem, phloem fiber and the epidermal layer of stem (Patzlaff *et al.* 2003). The predicted

*Pt*MYB4 protein is closely related to Arabidopsis R2R3-MYB protein, *At*MYB46 and *At*MYB83. Recombinant *Pt*MYB4 protein could bind with three different AC elements, AC-I, AC-II and AC-III, which are important *cis*-sequences in the transcriptional regulation of the lignin biosynthetic pathway. Overexpression of *PtMYB4* in transgenic tobacco upregulated the expression of *C3H*, *CCoAOMT*, *COMT* (caffeate *O*-methyltransferase), *CCR* and *CAD*, which are believed to be involved in the 'monolignol-specific' portion of the lignin biosynthetic pathways (**Fig. 1**). However, the general phenylpropanoid pathway's gene expression was not up-regulated by *PtMYB4*, and in fact *PAL* transcription was down-regulated.

Similarly, transgenic tobacco overexpressing *EgMYB2* (*Eucalyptus grandis*) showed increased expression of *C3H* (*p-coumarate 3-hydroxylase*), *F5H* (*ferulate 5-hydroxylase*), *CCoAOMT* and *COMT* (Goicoechea *et al.* 2005). The predicted *Eg*MYB2 protein shares the highest homology with Poptr1:49071 (a *Populus trichocarpa* MYB protein), *Pt*MYB4 (*Pinus taeda*) and *At*MYB83. These MYB proteins have a conserved C-terminal motif except from *Pt*MYB4, which is from a gymnosperm. Transcription of *PAL* was not significantly affected by overexpression of *EgMYB2*. Thus, the transcriptional regulation of *PAL* genes for lignification in woody species has not yet been clarified. Perhaps, several transcription factors might be involved to regulate the synthesis of each general phenylpropanoid, side-chain modification and ring modification of monolignol biosynthesis. Gális *et al.* (2006) showed that the overexpression of *NtMYBJS1* in tobacco BY-2 cells caused induction of the transcription level of *PAL*, *C4H* and *4CL*. The R2R3 MYB *NtMYBJS1* has the conserved motifs of three corresponding Arabidopsis MYB genes (*AtMYB13*, *AtMYB14* and *AtMYB15*). This type of MYB transcription factor is clearly related with the regulation for general phenylpropanoid synthesis.

Recently, the key transcription factors for differentiation of vessel elements and for the regulation of secondary wall synthesis in fiber cells have been discovered. All of these transcription factors are the members of the NAC [NAM ('no apical meristem'), ATAF1/2, CUC2 ('cup-shaped cotyledons 2')] domain protein, which are plant-specific transcription factors characterized by a conserved NAC domain located at the protein N-terminal (Olson *et al* 2005). Kubo *et al.* (2005) found two transcription switches *VASCULAR-RELATED NAC-DOMAIN* (*VND6*) and *VND7* for protoxylem and metaxylem differentiation respectively from microarray analysis with a newly established Arabidopsis *in vitro* xylem vessel element formation system. In the microarray analysis, *AtMYB83* and *AtMYB46* showed up-regulated expression during the actively formation of xylem vessel elements.

Recently, two NAC transcription factors, *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1* (*SND1*)/ *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) and *NST3* were discovered to be regulators for fiber secondary wall formation (Zhong *et al.* 2006; Mitsuda *et al.* 2007). The overexpressor of *SND1* showed increased transcription of monolignol biosynthesis pathway genes *4CL* and *CCoAOMT* (Zhong *et al.* 2006). *NST1* overexpression led to up-regulated expression of the laccase gene involving the last step of lignification (Mitsuda *et al.* 2005). These NAC domain proteins regulate downstream transcription factor genes, such as MYB family proteins, for lignin biosynthesis of secondary wall formation.

METABOLIC NETWORK AND REGULATION OF PAL PROTEIN ACTIVITY

Biochemical characterization of various PAL isoforms provides essential information in the model of PAL metabolic networks and functional regulation during plant growth and development and defense responses. Among four PAL isoforms of *Arabidopsis*, AtPAL3 has a very low activity and a lower temperature optimum compared with the other three isoforms, AtPAL1, AtPAL2 and AtPAL4 (Cochrane *et al.* 2004). These three isoforms have similar *Km* values to L-Phe, and AtPAL2 is more slightly catalytically efficacious than the others. *PAL* genes are encoded by two subfamilies in tobacco (*Nicotiana tabacum*). PAL1 and PAL2, share a 82% amino acid identity (Nagai *et al.* 1994; Pellegrini *et al.* 1994). Biochemical fractionation studies have suggested that tobacco PAL1 is localized in the ER membrane, whereas PAL2 is fractionated in the soluble fraction (Rasmussen and Dixon 1999). In transgenic tobacco, PAL1 and PAL2 were localized in a similar pattern by cell fractionation studies, while protein gel blot analysis indicated PAL1 to be localized in cytosolic and microsomal fractions and PAL2 only in the cytosolic fraction (Achnine *et al.* 2004). However, both PAL isoforms were localized in the microsomal fraction in plants overexpressing C4H. FRET (Fluorescence resonance energy transfer) analysis of PAL-GFP confirmed co-localization of C4H and PAL in tobacco ER. These results suggested that C4H organized the complex for membrane association of PAL (Achnine *et al.* 2004). Spatial organization of cooperating enzymes is a distinguished property and the enzyme complex organization gives an advantage of transfering the intermediates of biosynthesis between catalytic sites of enzymes in the complex without diffusion. This phenomenon is called "metabolic channeling" and the co-localization of C4H and PAL in tobacco ER suggested that the PAL and C4H complexes may allow an efficient reaction at the entry point into phenylpropanoid biosynthesis (Winkel 2004).

An immunocytochemical study of *Populus* PAL and C4H using electron microscopy has also shown that these enzymes are anchored in the ER and the Golgi apparatus (Takabe *et al.* 2001; Sato *et al.* 2004). Recently, biochemical fractionation and immunocytochemical studies using electronmicroscopy of PAL isoforms from a hybrid aspen have suggested that PAL isoforms are localized in both plastid and soluble fractions of xylem tissues (Osakabe *et al.* 2006). Due to the activation of *PkPALg2b* transcription in lignified tissues (Osakabe *et al.* 1995b, 1996, 2006), the PAL isoforms purified from xylem tissues contained *Pk*PALg2b.

The biochemical characterization of xylem PAL protein suggested that PAL localization varied during cell differentiation of aspen xylem. It has been shown that PAL protein is posttranslationally modified (Bolwell 1992; Allwood *et al.* 1999). In maize protoplasts, poplar PAL protein was modified by protein phosphorylation by the *Arabidopsis* calcium-dependent protein kinase (Allwood *et al.* 1999; Cheng *et al.* 2001; Allwood *et al.* 2002). These studies suggest that the characteristics of PAL subunits are modified by different environmental and developmental signals, which may affect the protein organization and localization in plant cells and provide increased efficiency and advantages to the biochemical reaction.

FUTURE PROSPECTS TOWARD CELL WALL ENGINEERING

In the post-genomic era, more and more transgenic plants with altered cell wall contents and properties will likely be produced. Recently, the entire genomic sequencing of *Populus trichocarpa* has been finished (Tsukan *et al.* 2006) and an extensive application of genomic technologies is now being applied to *Populus*. This research will certainly give valuable information to understand the detailed function of genes involved in secondary cell wall biosynthesis and help to gain knowledge of the complex developmental process of secondary cell walls as well as secondary growth increasing biomass. Furthermore, metabolome analysis of woody plants would provide profound resources for molecular breeding and application. A lower lignin content in trees could offer significant environmental benefit by reducing the amount of chemicals and energy used for wood pulping, and also provide improved lignocellulosics for production of biofuel. In the past decade, a wide variety of

transgenic plants with altered expression of gene involving lignification were produced to modify lignin quantitatively and qualitatively (Rogers and Campbell 2004). This knowledge provides a blueprint for well-designed cell walls as feedstocks.

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JAPANESE ABSTRACT

フェニルアラニンアンモニアリアーゼ（PAL）は一次代謝から二次代謝への経路の分岐の反応を触媒する重要な酵素である。PAL 遺伝子は植物の分化および発達段階、生物的および非生物的ストレスにより、転写レベルで様々に制御されることが近年の研究で明らかにされてきている。また、PAL 遺伝子の転写活性化に関与する転写因子は、PAL のみならず二次代謝生合成経路に関わる酵素遺伝子を様々に制御することが明らかにされている。最近、PAL タンパク質の細胞内局在性は、小胞体やプラスチド等多様性を示すことが明らかにされ、PAL タンパク質の翻訳後調節によって酵素活性が制御されることも示唆されている。この総説では PAL 遺伝子および酵素の制御について最近の知見を紹介するとともに、バイオマス生産の効率的な人為的制御を目的とした二次代謝生合成系酵素遺伝子群の発現調節の改変に関する最近の研究について紹介する。