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Short Communication

Immunological detection and cellular localization of the phenylalanine ammonia-lyase of a hybrid aspen

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Abstract The cellular-localization pattern of promoter activities of two phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) genes, *palg1* and *palg2b*, of a hybrid aspen, *Populus kitakamiensis*, was determined using the GUS reporter system. The strong activities of *palg2b* promoter were detected in lignified tissues such as xylem and phloem fiber cells of the aspen stem, suggesting the specific function of the PALg2b in lignin biosynthesis. Immunoelectron microscopy and sub-cellular fractionation of xylem cells showed that the PAL activity was detected in both the plastid and cytosol of the xylem ray-parenchyma cells and the cytosol in the developing xylem cells of the aspen mature stem. Our results of the biochemical characterization of xylem PAL protein suggested that the PAL localization was varied during cell differentiation of the aspen xylem to function in lignin biosynthesis.

Key words: Hybrid aspen, phenylalanine ammonia-lyase.

Woody plants are valuable resources, making it important to identify their physiological functions and metabolism. A large part of assimilated carbon sources during photosynthesis is accumulated and stored as phenylpropanoid metabolites, especially as lignin in woody plants (Boudet et al. 2003). Phenylpropanoid metabolism, which includes lignin biosynthesis, has been extensively studied due to its essential and valuable functions, such as mechanical supports, protection against biotic and abiotic stresses, pigments, and signalling molecules in plants (Boerjan et al. 2003; Dixon and Paiva 1995; Holton and Cornish 1995; Weisshaar and Jenkins 1998; Whetten and Sederoff 1995). Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is a key enzyme, which catalyses the deamination of L-phenylalanine to give cinnamate and mediates carbon flux into the phenylpropanoid biosynthetic pathway in plants (Hahlbrock and Scheel 1989). Many studies showed the regulatory mechanisms for various PAL isoforms in response to different plant conditions. PAL transcription is controlled by environmental and developmental signals in plant cells (Liang et al. 1989; Shufflebottom et al. 1993; Kumar and Ellis 2001) and also PAL protein is posttranslationally modified (Allwood et al. 1999; Bolwell 1992). Poplar PAL protein is subject to phosphorylation by the *Arabidopsis* calcium dependent protein kinase in maize protoplasts (Allwood et al. 1999; Cheng et al. 2001). Moreover, PAL subunits showed variable subcellular localization pattern. Tobacco PAL1 and bean PAL were localized in both soluble and microsomal fractions detected by the protein gel blot analysis (Rasmussen and Dixon 1999). Recently, co-localizaition of tobacco PAL1 and cinnnamate 4-hydroxylase (C4H) to endoplasmic reticulum (ER) has been shown in transgenic plants using confocal microscopy, and the metabolic channelling of PAL and C4H complexes may allow for the efficient reaction (Achnine et al. 2004). Furthermore, the immunocytochemical study of *Populus* PAL and C4H using electronmicroscopy showed that these enzymes localized in the rough-endoplasmic reticulum (r-ER) and the Golgi apparatus and PAL also localized in cytosol (Takabe et al. 2001; Sato et al. 2004). These studies indicate that the characterization of PAL subunits is modified by different environmental and developmental signals, which may affect the protein organization and

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localization in plant cells and provide increased efficiency and advantages to the reaction.

We have analyzed the expression pattern of three *pal* genes of a hybrid aspen in previous study (Osakabe et al. 1995a, b). We showed that *palg1* is expressed in young stem tissues, whereas *palg2a* and *palg2b* genes are expressed mainly in older stems, suggesting that *palg2a* and *palg2b* play an important role in the maturation of hybrid aspen stem (Osakabe et al. 1995b). We have also determined the immunocytochemical localization of PAL proteins at the cellular level during the development of hybrid aspen (Osakabe et al. 1996). Metabolomics and proteomics analysis of woody plants would give valuable resources for molecular breeding and application. To gain a better understanding of enzyme function, it is important to identify reaction loci for each isoenzyme in plant tissues and cells. For this purpose, we chose a key enzyme in phenylpropanoid biosynthesis, PAL. In this study, we used the promoter regions of *pal* genes to detect the localization pattern of gene expression and identified that the tissue-specific localization pattern of the gene family. We also analyzed the PAL subunits by isoelectric focusing and localization of PAL with both of immunoelectron microscopy and sub-cellular fractionation using an antiserum made against the PAL peptide.

To identify the cellular-localization of PAL genes in aspen, we constructed the transgenic aspen plants expressing the *palg1* and *palg2b* promoter fragments fused with the GUS reporter gene. The 1.56 kb fragment (between the *Bgl* II site and −1 nt from the ATG) of *palg1* and the 1.89 kb fragment (between the *Hind* III site and −1 nt from the ATG) of *palg2b* were amplified by PCR and separately inserted into the pBI101 binary vector to construct the *pal* fusions, These promoter-GUS cassettes were introduced into the hybrid aspen (*Populus sieboldii*×*Populus grandidentata*) grown as described previously (Osakabe et al. 1995a, b) via *Agrobacterium*. Fifteen independent transgenic aspens for each construct were obtained by the antibiotic selection. The GUS activities of these transgenic aspen plants were detected using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) as the substrate (Jefferson 1989). The GUS activities of all lines of each construct were detected in a similar manner. Figure 1 shows the expression pattern of the promoter-GUS in the young shoot of the hybrid aspen. *palg1*-GUS showed higher expression in the whole young leaf, the epidermis tissues of shoot bud and stem, but no expression was found in the vascular tissues (Figure 1 A, B). The GUS activity of the *palg2b* promoter was detected strongly in the xylem and phloem fiber cells in the older stem of the transgenic aspen (Figure 1C, D). These expression patterns showed that PALg2b catalyze phenylalanine to cinnamic acid in the conducting tissues, suggesting that the activity of

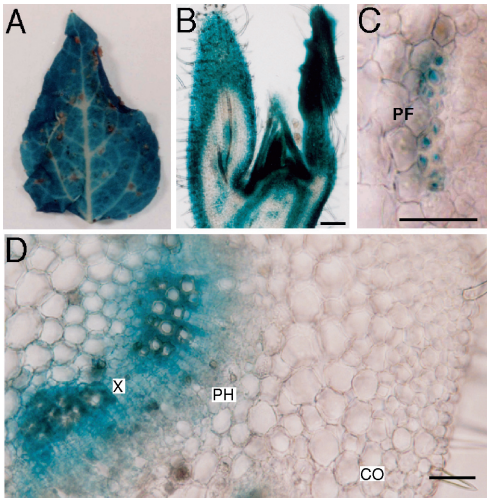

Figure 1. Histochemical localization of *palg1* (A, B) and *palg2b* (C, D) promoter-GUS activities. (A) young leaf of the transgenic aspen of the *palg1* promoter-GUS expression vector. (B) longitudinal section of a young stem of the transgenic aspen of the *palg1* promoter-GUS vector. (C) The GUS activity was detected in phloem fiber cells of the cross section of a young stem of the transgenic aspen of the *palg2b* promoter-GUS expression vector. (D) the cross section of a young stem of the transgenic aspen of the *palg2b* promoter-GUS vector. The phloem fiber cells were not developed at this stage of the section. CO: cortex, PH: phloem, PF: phloem fiber X: xylem. Magnification: ×100. Bar=100 μm.

PALg2b is important to catalyze this compound into the lignin biosynthesis pathway, since conducting cells were known as the most lignified tissues. On the contrary, the localization of PALg1 was associated with mainly in the surface of the aspen young tissues, indicating that the function of PALg1 relates to biosynthesis of the other compounds, such as flavonoids or lignans, which may protect aspen tissues from environmental stimuli. These results of promoter-GUS analysis also correlated closely with the results of Northern blot and S1 nuclease assays, which showed the *palg1* expression to be higher in young stems, while transcripts of *palg2*-class genes were accumulated mainly in the mature stems (Osakabe et al. 1995a, b). Taken together, the expression and localization of PAL are tightly associated with their function in aspen tissues and controlled by the *cis*-regulatory elements in the *PAL* gene promoters during aspen developmental stages.

It has been shown that *Populus* PAL genes were classified into two groups; *palg1*-class and *palg2a*/*b*-class genes. The *PtdPAL1/2* from a hybrid poplar (*Populus trichocarpa*×*Populus deltoids*) and *PtPAL1* from *Populus tremuloides* (Kao et al. 2002) showed higher similarity with *palg1* (*palg1*-class genes). The *PtdPAL1/2* promoter-GUS activity (Gray-Mitsumune et al. 1999) and the in situ hybridization of *PtPAL1* (Kao et al. 2002) analyses showed that these genes are expressed in young tissues. These data consistent with our results and suggest that the *palg1*-class genes

function mostly in young stem tissues and leaves. On the contrary, *PtPAL2* (*palg2a*/*b*-class genes) from *Populus tremuloides* was expressed in lignified tissues (Kao et al. 2002), suggesting that a *palg2a*/*b*-class gene has an important role in the lignin biosynthesis of the mature stem. The expression pattern of two 4-coumarate: CoA ligase (4CL) genes, *Pt4CL1* and *Pt4CL2* has been shown using a promoter-GUS assay in tobacco: *Pt4CL1* was expressed in the xylem tissue, whereas *Pt4CL2* was expressed in epidermal cells (Hu et al. 1998). The similarity of expression pattern of *PAL* and *4CL* gene family suggests that the transcription of *Populus* phenylpropanoid biosynthetic genes was controlled under the similar mechanisms, which determine the physiological role in phenylpropanoid metabolism. In *Populus* species, which maintain highly differentiated cells throughout their long lifetime, functional chemical compounds may be catalyzed strictly in the cells by the specific gene regulatory mechanisms.

To characterize PAL protein, which function in lignification of the aspen xylem cells, we then partially purified the extracts from aspen mature stem. The aspen stem tissues were grind with mortar and pestle in liquid nitrogen and suspended in KP buffer [100 mM potassium phosphate, pH 7.7, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Soluble fraction of the stem extracts were then fractionated with ammonium sulfate, dialyzed, resuspended in KP buffer. The crude extracts were then purified by ion exchange chromatography on DEAE-Toyopearl (Amersham Pharmacia, Piscataway, NJ, USA) in KCl gradient. Ion exchange chromatography of the crude extracts showed the presence of one major peak of PAL activity eluted at 0.2 M KCl (Figure 2A). We carried out western blot of the partially purified PAL fractions using an anti-PAL antiserum and one strong band for 76.5 kDa PAL protein was detected in the fractions with high PAL activity (Figure 2B, lane 4,5). The smaller band was also strongly detected in the eluted fractions and it should be a degraded PAL protein accumulated during the purification, because it was detected only in the PAL fractions and disappeared when the stem crude protein was used in western blot immediately after the extraction (Figure 2B, lane 8). The PAL fraction was then analyzed by isoelectric focusing using PhastGel IEF 4-6.5 (Amersham Pharmacia). The results of the western blot showed that the PAL fraction from ion exchange chromatography was separated three further fractions whose pI values were 5.0, 5.1, and 5.2, respectively (Figure 2C). The result suggests that various PAL isoforms existed in aspen stem tissues and may have important roles on lignification of the aspen xylem cells. The possibility of degraded PAL proteins might be separated into the different pI fractions was not excluded in this experiment and further experiments were needed to resolve this phenomenon.

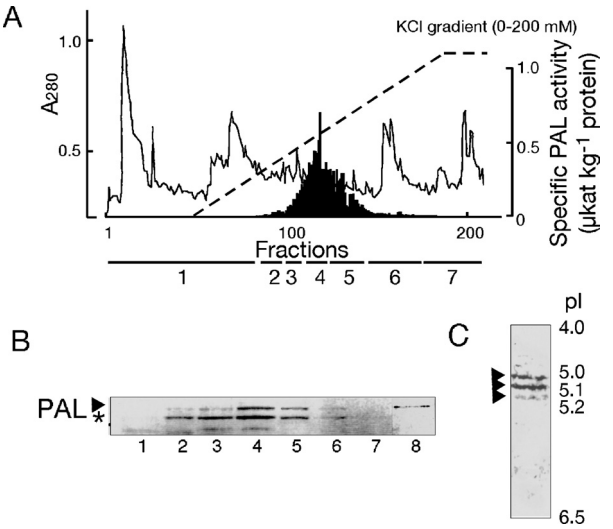

Figure 2. Identification of PAL subunits from the aspen stem. (A) PAL elution pattern of aspen stem extracts from DEAE-Toyopearl column (2.5×20 cm). Approximately 200 g of aspen stem tissues were ground with liquid nitrogen and suspended in 100 ml of KP buffer. The filtrate through one layer of Miracloth (Calbiochem, LaJolla, CA) was centrifuged at 20,000 g for 30 min and the supernatant were then fractionated by ammonium sulfate. The fraction precipitated in 40–70% saturation was then used for further purification by ion exchange chromatography on DEAE-Toyopearl in 300 ml of KCl gradient. The measurement of PAL activity was carried out as described previously (Osakabe et al. 1996). (B) Western blot analysis to detect the PAL protein from eluted fractions (lane 1–7; same fractions in A). The 76.5 kDa PAL protein (arrow) and the degraded protein (asterisk) were detected in the fractions, which have the PAL activity. The degraded protein was not detected when the crude protein was used immediately after the extraction (lane 7). (C) Western blot of the PAL subunits of the eluted fractions separated by isoelectric focusing using PhastGel IEF 4-6.5 (Amersham Pharmacia).

The identification of subcellular localization gives significant information to understand the enzyme functions. We then carried out immunocytochemical detection of PAL protein using PAL polyclonal antibody to identify the localization of PAL proteins in the cell component. We used the differentiating xylem tissues to detect the subcellular localization of PAL protein. Fixation and embedding with LR White (London Resin Co., UK) of the mature stem section from three aspen plants were carried out as previously described (Osakabe et al. 1996). Ultra-thin sections of the stem tissues mounted on a nickel grid were labelled with the PAL antibody followed by 10 nm colloidal gold secondary antibody. Counter-staining was carried out with uranyl acetate and lead citrate before observation by electron microscopy. In Figure 3A–C, the immunological detection of PAL protein with gold particles showed that abundant labeling of PAL proteins are localized mainly in the plastids of the ray parenchyma cells of xylem. The PAL proteins were also observed in the cytosol in the mature xylem cells (Figure 3D).

We then isolated the plastid fraction from the stem

tissues of the hybrid aspen and measured the PAL activity. The plastid fraction was isolated from the stem tissues of the hybrid aspen using a Percoll gradient including 1 mM PMSF and 10 mM iodoacetic acid. The chlorophyll content was determined according to the method of Lamppa (Lamppa 1995). The marker enzyme activities were determined according to the methods of Hageman and Hucklesby (1971) and Storrie and Madden (1990). Table 1 showed that the activities of PAL and marker enzymes, such as catalase for peroxisome, NADH-nitrate reductase for cytosol, and cytochorme *c* oxidase for mitochondria, and the chlorophyll contents of the plastid fraction and the supernatant from the Percoll gradient of the stem extract. The marker enzyme activities showed that the plastid fraction was isolated in pure form. PAL activity was found in both the supernatant and plastid fractions. Western blot analysis also showed that PAL protein was detected in both the supernatant and plastid fractions (Figure 4A). If protease inhibitors were not included in the isolation buffers, PAL protein band was not detected in the plastid fraction (Figure 4B). These assays including the isolation of plastid fraction, the enzyme assays, and the detection of PAL protein with anti-PAL antiserum, were repeated two times and the similar results were detected. These results indicate that the plastidic PAL appears to be digested by the proteases at higher efficiency than the cytosolic PAL, suggesting that the characterization of each PAL subunits might be different. PAL activity in the chloroplast fraction from spinach was reported in 1979 (Nishizawa et al. 1979). In this study, we have identified the localization of PAL in the plastid of woody plants both of immunocytochemical and biochemical analysis. Phenylalanine is synthesized in the shikimic pathway, and this pathway is localized in both plastid and cytosol (Herrmann and Weaver, 1999). The PAL localization in the plastid of aspen xylem might be a strategy to make the reaction more efficient and suggests another possibility, in which the removed ammonium ion from phenylalanine could be promptly catalyzed by glutamate synthase, which is also localized in the plastid (Tobin and Yamaya 2001).

In conclusion, we identified that PALg2b has an

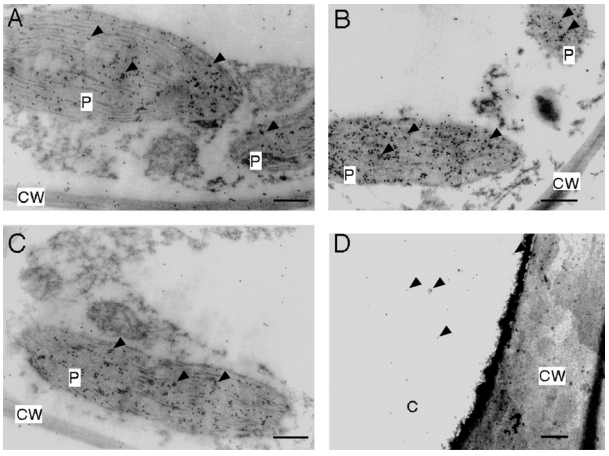

Figure 3. Immunocytochemical localization of PAL proteins in the differentiating secondary xylem observed by electron microscopy. (A–C) PAL signals were identified mostly in plastid of ray parenchyma cells in xylem. (D) PAL signals were identified in cytosol of mature xylem cells. P: plastid, CW: cell wall. Magnification: ×12000. Bar= 1 μm.

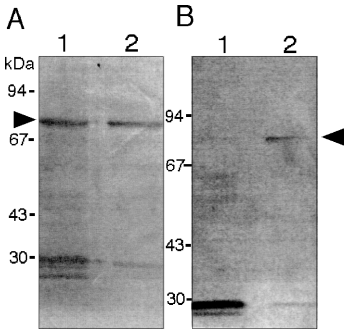

Figure 4. Western blot analysis of PAL from (1) the plastid fraction and (2) the cytosol. Proteins were extracted from stem tissues of the hybrid aspen (A) with protease inhibitors or (B) without protease inhibitors. Protein extractions were separated in SDS-PAGE gel and transferred onto nitrocellulose membranes. Arrows indicate PAL. PAL signals were detected in the plastid fraction when protease inhibitors were included in all the buffers.

Table 1. The relative activities of enzymes and chlorophyll contents of the supernatant and the intact plastid fraction separated by Percoll gradient centrifugation from aspen stem extraction. Thirty g of stem tissues were homogenized in 100 ml of 350 mM sorbitol, 25 mM Hepes-NaOH, pH 7.6, 2 mM EDTA, 2 mM sodium erythorbate (isolation buffer). The homogenate was filtered through Miracloth and centrifuged for 5 min at 1,500×*g*. The supernatant was centrifuged for 10 min at 5,000×*g*. The pellets were resuspended in isolation buffer and transferred onto Percoll gradients [50 ml of 80–10% (vol/vol) Percoll with 3% (w/v) PEG6000, 1% Ficoll, and 1% BSA in isolation buffer]. The gradients were centrifuged for 30 min at 10,000×*g* After centrifugation two organellar bands were detected. The lower band including intact plastids was collected and washed in isolation buffer and used for enzyme assays.

	Chlorophyll (g g^{-1} protein)	Catalase[a]	NADH-nitrate reductase[b]	Cytochrome *c* oxidase[c]	PAL (μk kg^{-1} protein)
		(units min^{-1} mg^{-1} protein)			
Supernatant	0.2	72	50	5.82	7.36
Plastid	2.4	0.01	0.02	0.001	2.03

[a] Catalase activity was measured as a marker for the peroxisome fraction.
[b] NADH-nitrate reductase activity was measured as a marker for the cytosol fraction.
[c] Cytochrome c oxidase activity was measured as a marker for the mitochondria fraction.

important role for lignification of xylem tissues and the cellular localization of PAL proteins by immunoelectron microscopy and sub-cellular fractionation in xylem; PAL localization and activity were found in both the plastid and the cytosol during lignification of the xylem tissues of the hybrid aspen. The association of PAL with membrane fraction has been previously reported (Wagner and Hrazdina 1984; Hrazdina and Wagner 1985; Rasmussen and Dixon 1999; Achnine et al. 2004; Sato et al. 2004). Tobacco PAL1 was found in both soluble and microsomal fractions, whereas tobacco PAL2 is not detected in the microsome (Rasmussen and Dixon 1999). The colocalizaition of tobacco PAL1 and cinnnamate 4-hydroxylase (C4H) to endoplasmic reticulum (ER) indicates the metabolic channelling of PAL and C4H complexes (Achnine et al. 2004). *Populus* PAL and C4H colocalized in the r-ER and the Golgi apparatus and PAL also localized in cytosol (Sato et al. 2004). Furthermore, Cheng et al. (2001) suggested that phosphorylation might target PAL protein to membranes. These findings suggested that each member of the PAL gene family was localized to a specific cell compartment depending on its structure and function as a result of environmental stimuli and/or plant developmental signals. Our results also suggested the possibility, in which the differences in the PAL structure and post-translational modification may contribute to the efficiency of digestion by proteases and in turn affect the transport of PAL into the plastid. Although the N-terminal extended sequences of PALg2b appeared to be specific amino acid sequences, this region is not characteristic of a typical transit peptide. This suggested that there are several possibilities in the targeting mechanisms of the proteins, which do not have a transit peptide, such as alternative splicing, post-transcriptional and -translational regulation (Soll 2002; Silva-Filho 2003; Jarvis and Robinson 2004). Further experiments focused on these possibilities will elucidate the regulation of aspen PAL function in lignin biosynthesis.

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References

Achnine L, Blancaflor E, Rasmussen S, Dixon RA (2004). Co-localization of L-phenylalanine ammonia-lyase and cinnamate 4-hydroxylase for metabolic channeling in phenylpropanoid biosynthesis. *Plant Cell* 16: 3098–3109

Allwood EG, Davies DR, Gerrish C, Ellis BE, Bolwell GP (1999) Phosphorylation of phenylalanine ammonia-lyase: evidence for a novel protein kinase and identification of the phosphorylated residue. *FEBS Lett* 457: 47–52

Allwood EG, Davies DR, Gerrish C, Bolwell GP 2002 Regulation of CDPKs, including identification of PAL kinase, in biotically stressed cells of French bean. *Plant Mol Biol* 49: 533–544

Boerjan W, Ralph J, Baucher M (2003) Lignin Biosynthesis. *Annu Rev Plant Biol* 54: 519–546

Bolwell GP (1992) A role for phosphorylation in the down-regulation of phenylalanine ammonia lyase in suspension-culture cells of French bean. *Phytochemistory* 31: 4081–4086

Boudet AM, Kajita S, Grima-Pettenati J, Goffner D (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. *Trends Plant Sci* 8: 576–581

Cheng SH, Sheen J, Gerrish C, Bolwell GP (2001) Molecular identification of phenylalanine ammonia-lyaseasa substrate of a specific constitutively active Arabidopsis CDPK expressed in maize protoplasts. *FEBS Lett* 503: 185–188

Gray-Mitsumune M, Molitor EK, Cukovic D, Carlson JE, Douglas CJ (1999) Developmentally regulated patterns of expression directed by poplar *PAL* promoters in transgenic tobacco and poplar. *Plant Mol Biol* 39: 657–669

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* 7: 1085–1097

Hageman RH, Hucklesby DP (1971) *Methods in Enzymology* 23A: 491

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 40: 347–369

Herrmann KM, Weaver LM (1999) The shikimate pathway. *Annu Rev Plant Physiol Plant Mol Biol* 50: 473–503

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7: 1071–1083

Hrazdina G, Wagner GJ (1985) Metabolic pathways as enzyme complexes: Evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes. *Arch Biochem Biophys* 237: 88–100

Hu W-J, Kawaoka A, Tsai C-J, Lung J, Osakabe K, Ebinuma H, Chiang VL (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate:CoA ligase genes in aspen (*Populus tremuloides*). *Proc Natl Acad Sci USA* 95: 5407–5412

Jarvis P, Robinson C (2004) Mechanisms of protein import and routing in chloroplasts. *Curr Biol* 14: 1064–1077

Jefferson RA (1989) The GUS Reporter Gene System. *Nature* 342: 837–838

Kao YY, Harding SA, Tsai CJ (2002) Differential expression of two distinct phenylalanine ammonia-lyase genes in condensed tannin-accumulating and lignifying cells of quaking aspen. *Plant Physiol* 130: 796–807

Kumar A, Ellis BE (2001) The phenylalanine ammonia-lyase gene family in *Rubus idaeus*: Structure, expression and evolution. *Plant Physiology* 127: 230–239

Lamppa GK (1995) *Methods in Plant Molecular Biology*, 153–156. Cold Spring Harbor Laboratory Press, USA

Liang X, Dron M, Cramer CL, Dixon RA, Lamb CJ (1989) Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. *J Biol Chem* 264: 14486–14492

Nishizawa AN, Wolosiuk RA, Buchanan BB (1979) Chloroplast phenylalanine anamonia lyase from spinach leaves: Evidence for light mediated regulation via the ferredoxin/thioredoxin system. *Planta* 145: 7–12

Osakabe Y, Ohtsubo Y, Kawai S, Katayama Y, Morohoshi N (1995a) Structures and tissue-specific expression of genes for

phenylalanine ammonia-lyase from a hybrid aspen, *Populus kitakamiensis*. *Plant Sci* 105: 217–226

Osakabe Y, Osakabe K, Kawai S, Katayama Y, Morohoshi N (1995b) Characterization of the structure and determination of mRNA levels of the phenylalanine ammonia-lyase gene family from *Populus kitakamiensis*. *Plant Mol Biol* 28: 1133–1141

Osakabe Y, Nanto K, Kitamura H, Kawai S, Kondo Y, Fujii T, Takabe K, Katayama Y, Morohoshi N (1996) Immunocytochemical localization of phenylalanine ammonia-lyase in tissues of *Populus kitakamiensis*. *Planta* 200: 13–19

Rasmussen S, Dixon RA (1999) Transgene-mediated and elicitor-induced perturbation of metabolic channeling at the entry point into the phenylpropanoid pathway. *Plant Cell* 11: 1537–1552

Shufflebottom D, Edwards K, Schuch W, Bevan M (1993) Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction patterns. *Plant J* 3: 835–845

Sato T, Takabe K, Fujita M (2004) Immunolocalization of phenylalanine ammonia-lyase and cinnamate-4-hydroxylase in differentiating xylem of poplar. *Comptes Rendus Biologies* 327: 827–836

Silva-Filho MC (2003) One ticket for multiple destinations: dual targeting of proteins to distinct subcellular locations. *Curr Opin Plant Biol* 6: 589–95

Soll J (2002) Protein import into chloroplasts. *Curr Opin Plant Biol* 5: 529–535

Storrie B, Madden EA (1990) *Methods in Enzymology* 182: 203–225

Takabe K, Takeuchi M, Sato T, Ito M, Fujita M (2001) lmmunocytochemical localization of enzymes involved in lignification of the cell wall. *J Plant Res* 114: 509–515

Tobin AK, Yamaya T (2001) Cellular compartmentation of ammonium assimilation in rice and barley. *J Exp Bot* 52: 52591–52604

Wagner GJ, Hrazdina G (1984) Endoplasmic reticulum as a site of phenylpropanoid and flavonoid metabolism in *Hippeastrum*. *Plant Physiol* 74: 901–906

Weisshaar B, Jenkins GI (1998) Phenylpropanoid biosynthesis and its regulation. *Curr. Opin. Plant Biol* 1: 251–257

Whetten R, Sederoff R (1995) Lignin biosynthesis. *Plant Cell* 7: 1001–1013