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Immunohistochemical localization of enzymes related to lignin biosynthesis in the primary xylem of hybrid aspen

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Abstract We have investigated the spatial regulation of the accumulation of enzymes involved in the biosynthesis of shikimate and lignin during differentiation of primary xylem from the apical meristem via procambium in hybrid aspen (*Populus sieboldii* × *Populus grandidentata*). Immunohistochemical staining revealed that, in the top part of shoots, lignification began in a single or just a few adjacent vessel elements and subsequently spread to neighboring cells. The spatial localization of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), which is one of the key enzymes in the shikimate pathway, was tightly correlated with the cell-specific deposition of lignin in the primary xylem. We also found that the spatial localization of enzymes in the general phenylpropanoid pathway and in the lignin-specific pathway was closely associated with the cell-specific deposition of lignin and the accumulation of DAHPS. Our data suggest that enzymes that act in the shikimate, general phenylpropanoid, and lignin-specific pathways are initially produced and function coordinately in a single or a few adjacent elements at the start of primary xylem development.

Key words Immunohistochemical localization · Lignin deposition · Phenylpropanoid pathway · Primary xylem · Shikimate pathway

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Introduction

As a plant grows, the differentiation and maturation of various tissues and cells with specific functions are important for the viability of the plant itself. For example, differentiation of xylem tissue is essential to the viability of vascular plants, a process that is one of the most extensively studied aspects of plant development. The morphological features of this process and a variety of biochemical and molecular features have been explored as tools for analyzing the differentiation of xylem.[1,2] Morphogenetic studies have been concerned primarily with differentiating the vessel elements in xylem tissues. These vessel elements are characterized by the formation of a secondary cell wall with annual, spiral, or reticulate thickening.[3] Upon completion of differentiation, the vessel elements are able to provide a pathway for the transport of water and nutrients that support plant growth.

To clarify the physiological roles of xylem tissue and the details of the mechanism of wood formation, it is important to establish the details of the control of the formation of the secondary wall of xylem cells during their differentiation. The molecular aspects of lignification during xylem differentiation are also of interest, as modifications of the lignin biosynthetic pathway should contribute to improving the availability of biomaterials derived from woody plants. The culture of cells isolated from *Zinnia*, during which tracheary elements can be induced to differentiate from mesophyll cells in vitro, has proved to be a powerful tool for analyzing the differentiation of xylem cells.[4] Studies of this model system have revealed a number of cytological, biochemical, and molecular aspects of the sequence of events during differentiation of tracheary elements.[5–7] This system has also provided useful information about lignin biosynthesis and the thickening of secondary walls during cell differentiation.[8,9]

Despite elegant studies of the *Zinnia* system, questions remain about the spatial and temporal regulation of the various enzymes involved in lignin biosynthesis during the differentiation of intact xylem cells. Thus, in the present

study we examined the spatial patterns of induction of six enzymes involved in differentiation of primary xylem cells of trees: 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), which acts in the shikimate pathway;[10] phenylalanine amonia-lyase (PAL), caffeate *O*-methyltransferase (COMT), and 4-coumarate:CoA ligase (4CL), which act in the general phenylpropanoid pathway; and cinnamyl alcohol dehydrogenase (CAD) and peroxidase (POX), which act in the lignin biosynthetic pathway.[11] We performed a detailed immunohistochemical analysis using antisera raised against recombinant partial peptides of the enzymes. In this report, we describe evidence for the coordinate regulation of the shikimate pathway and the deposition of lignin during development of the xylem. We also discuss a possible regulatory mechanism that might underlie the tightly coordinated activation of a long sequence of reactions during lignin biosynthesis at the start of the development of the xylem in trees.

Materials and methods

Plant material

Tissue samples (shoots) were harvested from hybrid aspen (*Populus sieboldii* × *Populus grandidentata*) growing under field conditions in Fuchu, Japan. The samples were obtained in June and divided into various tissues. Samples of shoot apices were fixed prior to histochemical and immunohistochemical analyses. Crude preparations of enzymes for Western blotting analysis were prepared from shoot internodes.

Histochemical staining by the Wiesner reaction

To detect lignin in primary xylem tissue, thin sections (10–20 μm) from the highest internode were allowed to react with the Wiesner reagent.[12] Sections were fixed in 4% glutaraldehyde for 60 min, rinsed with water, and then incubated in a mixture of phloroglucinol (5% in ethanol) and 12 M HCl (1:1, v/v) for 1 min. All procedures were performed at room temperature (RT). Photographs of samples were taken with a brightfield microscope (Nikon, Tokyo, Japan).

Ultraviolet microscopy

For ultraviolet (UV) microscopic observations, sections from the highest internodes with primary xylem tissue were fixed in a freshly prepared solution of 0.1% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at RT for 2 days. After washing and dehydration through a graded alcohol series, tissues were embedded in epoxy resin. Ultrathin sections (1 μm) of tissue were cut with a glass knife on an ultramicrotome. Sections were mounted on quartz slides and sealed under coverslips with glycerin. UV microphotographs were obtained at 280 nm under an ultraviolet microspectrometer (Carl Zeiss UMSP80).[13]

Preparation of antisera

We used pQE expression vectors (Qiagen; Chatsworth, CA, USA) to produce partial peptides from fragments of cDNA for DAHPS, 4CL, and CAD. A fragment of cDNA for DAHPS of hybrid aspen, encoding a peptide of 167 amino acids,[14] was inserted into pQE30. The resultant plasmid was transferred to *Escherichia coli* JM109 for synthesis of the partial peptide. A fragment of cDNA encoding part of the 4CL of hybrid aspen (i.e., a carboxy-terminal peptide of 260 amino acids)[15] was inserted into pQE30. The peptide was produced in *E. coli* SG13009. A cDNA fragment encoding part of the CAD of hybrid aspen (303 amino acids)[16] was inserted into the expression vector pQE32, and the peptide was produced in *E. coli* SG13009. Each partial peptide was purified according to the instructions from Qiagen and was then used as an antigen to raise an antiserum in rabbits as described previously.[17] Rabbit antisera raised against recombinant partial peptides derived from PAL, COMT, and POX were described previously.[17–20] Serum from nonimmunized rabbits was used as the control antiserum. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blotting analysis were performed as described previously.[17]

Immunohistochemical analysis

Sections of shoot apices and of internodes with primary xylem tissues were fixed and embedded in LR White hydrophilic resin (London Resin, Surrey, UK) as described previously.[17] Ultrathin sections (1 μm) of tissue were cut with a glass knife on an ultramicrotome. For immunostaining prior for light microscopy, ultrathin sections were blocked with 0.8% bovine serum albumin (BSA), 1% normal goat serum, and 0.1% IGSS-quality gelatin in phosphate buffer (pH 7.4) that contained 0.15 M NaCl. They were then incubated with rabbit antiserum raised against purified recombinant peptides derived from DAHPS, PAL, COMT, 4CL, CAD, or POX by previously described methods.[17] Sections that had been incubated with specific antisera or the control antiserum were immersed, and then silver enhancement was performed for 10 min with IntenSEM (Amersham, Buckinghamshire, UK) in accordance with the manufacturer's instructions.

Results

Western blotting analysis with specific antisera

We tested the various antisera by Western blot analysis to confirm the antigenic specificity of each antiserum. As expected, each recombinant peptide produced in *E. coli* was recognized specifically by the corresponding antiserum (data not shown). We next tested the antisera by Western blotting analysis with crude extracts of proteins prepared from young shoots of a hybrid aspen tree. The results indicated that each antiserum, with the exception of that against

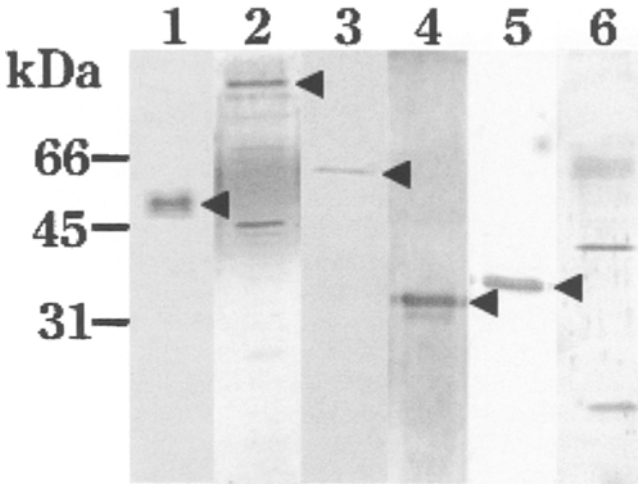

Fig. 1. Western blotting analysis with peptide-specific antisera. *Arrowheads* indicate the immunological detection of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS, lane *1*), phenylalanine ammonia lyase (PAL, lane *2*), 4-coumarate: coenzyme A ligase (4CL, lane *3*), caffeate-*O*-methyltransferase (COMT, lane *4*), cinnamyl alchol dehydrogenase (CAD, lane *5*), and peroxidase (POX, lane *6*), respectively. See text for details

PAL and POX antisera, recognized the corresponding polypeptide(s); and the molecular mass of the reactive polypeptide(s) were consistent with predicted values (Fig. 1). The anti-DAHPS, anti-4CL, and anti-CAD sera reacted specifically with a single protein, and anti-OMT serum recognized two isoforms of the enzyme, as reported previously.[19]

The antiserum recognized a protein of PAL isoform whose molecular mass is approximate 76.5 kDa (Fig. 1, lane 2), as reported in our previous work.[17] In addition, a few other minor signals, derived from peptides of lower molecular weight, were also detected with this antiserum. These other signals might have been mainly due to degradation of the mature PAL protein, as the PAL peptide seems to be inherently unstable in vitro, as reported by Bolwell et al.[21,22] When we used antiserum raised against POX, several clear, broad bands were observed after immunostaining (Fig. 1, lane 6), suggesting that this antiserum recognized distinct POX peptides. There are, in fact, several distinct forms of POX with different molecular masses in the xylem tissue of *Populus* trees,[23,24] so this result can be explained satisfactorily. It is also possible that some of the signals observed by Western blotting with the anti-PAL and anti-POX sera were derived from nonspecific interactions between these antisera and distinct proteins. However, these antisera could strongly inhibit PAL[17] and POX (data not shown) activities, respectively. Thus, it appears that the PAL- and POX-specific antisera include polyclonal antibodies that can react with each aspen protein(s). Based on the results described above regarding the anti-PAL and anti-POX sera, we concluded that it was appropriate to use both antisera for immunolocalization of each protein during further analyses, even though these antisera might have had broad specificity.

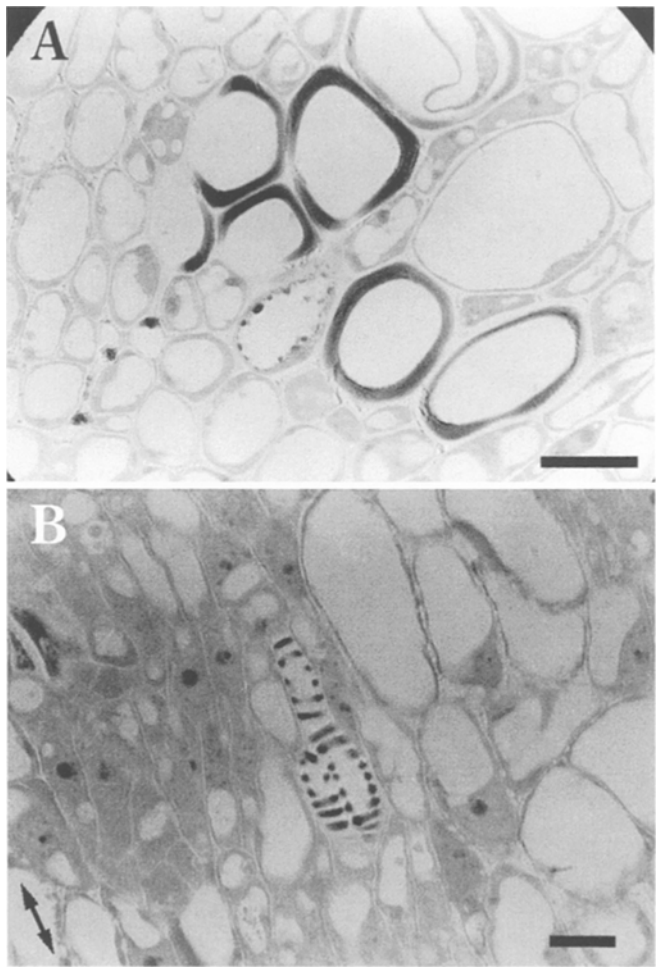

Fig. 2A,B. Photomicrographs of differentiating primary xylem tissues, seen under ultraviolet (UV) light (280 nm). **A** Cross section of tissue. **B** Longitudinal section of tissue. *Arrow* corresponds to the longitudinal axis of the tree. Bars 10 μm (**A**), 20 μm (**B**)

Vessel-specific deposition of lignin at the thickened spiral secondary wall of primary xylem tissues differentiated from apical meristem

Figure 2 shows UV microphotographs of tissues close to a young shoot tip of a hybrid aspen. In cross sections of tissue from under the apical meristem (about 3 mm below the top of the shoot apex), UV absorption was observed at single and adjacent vessel elements with a thickened secondary wall, indicating that lignification was in progress in these cell walls. In addition, staining with Wiesner's reagent revealed that deposition of lignin in the thickened secondary wall was in almost the same area as that associated with significant absorption of UV light (Fig. 3A). In longitudinal sections UV absorption and signals due to chemical staining were observed only in the thickened spiral secondary walls of vessel elements, as was the case in the cross sections (Figs. 2B, 3B,C).

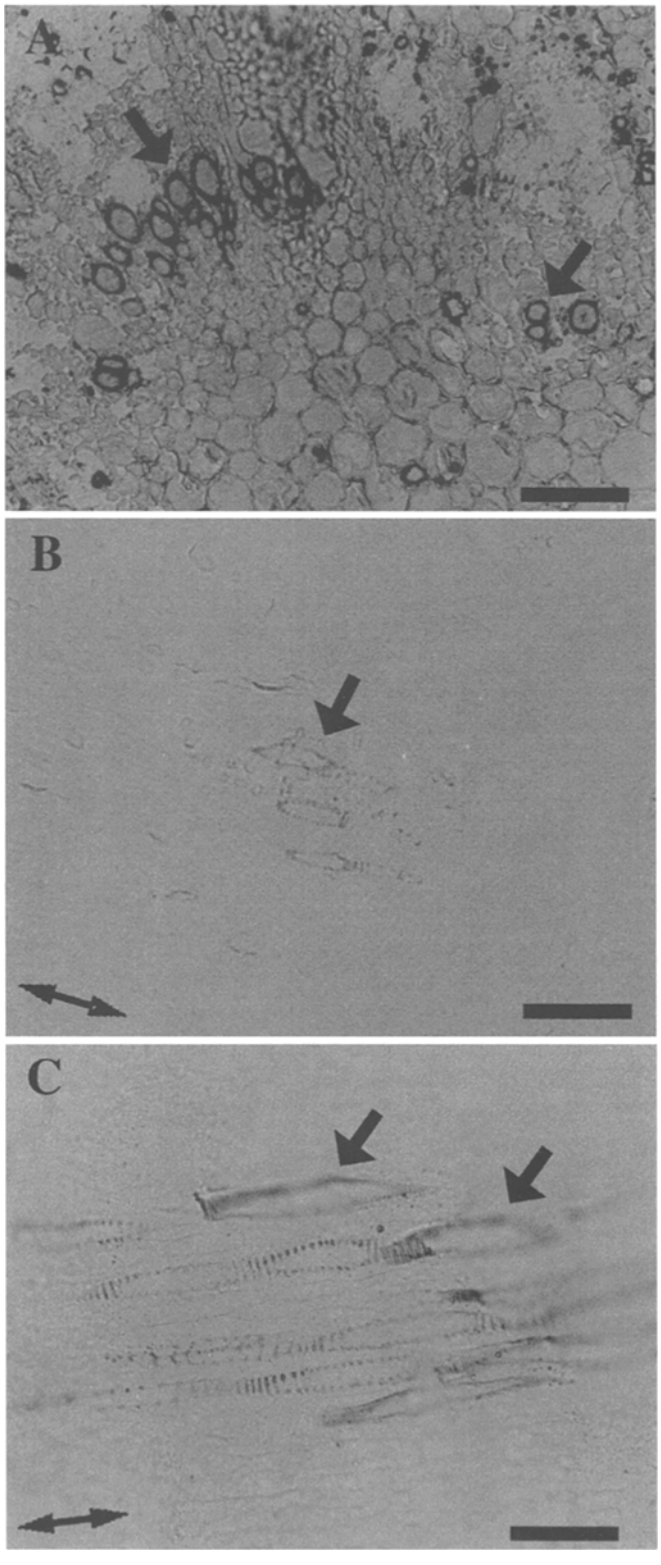

Fig. 3A–C. Histochemical staining (Wiesner's reagent) of lignin in primary xylem tissues. **A** Cross section of primary xylem tissue. **B** Longitudinal section of tissue just under the apical meristem. **C** Longitudinal section of tissue in which xylogenesis is proceeding. *Arrows* in **A** and **B** indicate stained vessel elements. *Double-headed arrows* correspond to the longitudinal axis of the tree. Bars 150 μm

Immunohistochemical localization of DAHPS in primary xylem tissues

Using tissue from near the shoot tip, we examined the immunohistochemical localization of DAHPS, which catalyzes the first step in the shikimate pathway using antiserum raised against a partial peptide of DAHPS. In cross sections, signals were detected mainly on single and adjacent vessel elements with thickened secondary walls (Fig. 4A, arrows). In longitudinal sections of the same region, DAHPS was also detected in small, round vessel elements (Fig. 4C). In tissues in which xylogenesis had proceeded further, DAHPS was also localized mainly in vessel elements that were arranged linearly (Fig. 4E). Signals due to DAHPS were hardly detectable in fiber and parenchymal cells that surrounded vessel elements in the primary xylem. These results suggested that production of DAHPS, an enzyme that represents an entry point for the flow of carbon into aromatic compounds, was closely associated with the cell-specific biosynthesis of lignin that accompanies vessel development from apical meristem via procambium.

Immunohistochemical localization of enzymes for general phenylpropanoid and lignin-specific pathways in primary xylem tissues differentiated from apical meristem

We next examined the localization of enzymes that act in the general phenylpropanoid pathway (i.e., PAL, COMT, 4CL). In cross sections that included primary xylem tissue, differentiating vessel elements were immunolabeled with the antiserum against PAL (Fig. 5A), as noted in a previous study.[17] In addition, signals due to COMT and 4CL were also observed mainly in vessel elements at the same stage of differentiation in which lignification was underway in the secondary walls (Fig. 5B,C). Furthermore, we found that enzymes that act in the lignin-specific pathway (CAD and POX) also accumulate in the differentiating vessel elements (Fig. 5D,E). The data suggest that these enzymes are produced coordinately for the biosynthesis of lignin at specific sites during vessel differentiation.

In longitudinal sections, signals obtained with antisera against PAL, COMT, 4CL, CAD, and POX were also observed in vessel elements of primary xylem (Figs. 6, 7). In younger parts of shoots, signals were concentrated in round, immature vessel elements in which lignification had begun, as in the case of the DAHPS-specific signals shown in Fig. 4C (Figs. 6A–C, 7A,B). In addition, intense signals were visible in the vessel elements of primary xylem that were elongating dramatically with extension of spiral secondary wall thickening and that were connected to one another to form water conductive tubing (Figs. 6D–F, 7C,D). These results indicate that the localization of enzymes in the general phenylpropanoid pathway (PAL, COMT, 4CL) and the lignin-specific pathway (CAD, POX) in primary xylem tissues was similar to the localization of DAHPS.

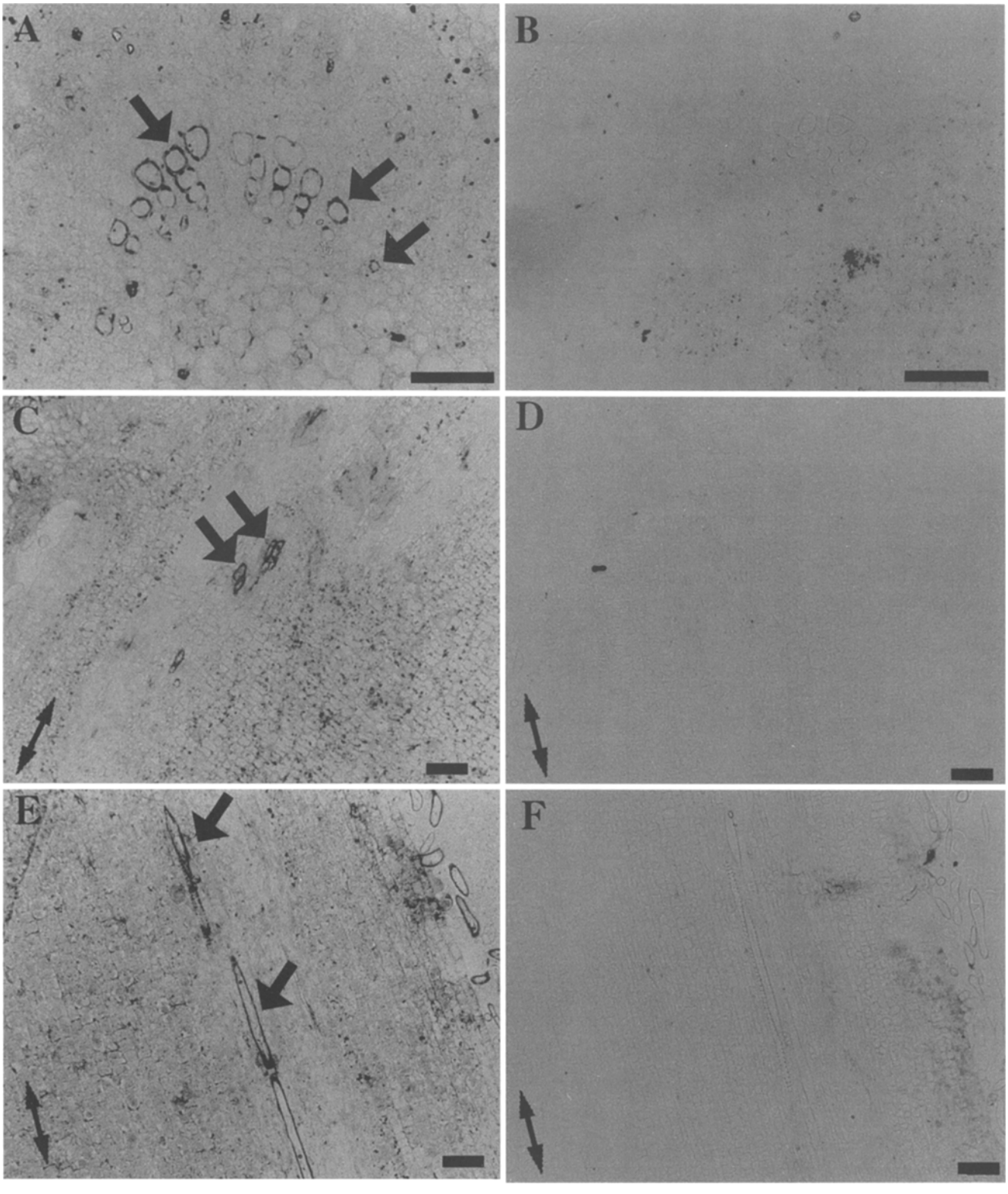

Fig. 4A–F. Immunolocalization of DAHPS in primary xylem tissue. **A** Localization of DAHPS in a cross section of primary xylem tissue. **B** Control immunostaining (using serum from a non-immunized rabbit) of a cross section of the same region as in **A**. **C** Localization of DAHPS in a longitudinal section of tissue near the apical meristem. **D** Control immunostaining as in **B** of a longitudinal section of tissue in the same region as in **C**. **E** Localization of DAHPS in a longitudinal section of tissue in which xylogenesis is proceeding. **F** Control immunostaining as in **B** of tissues in the same region as in **E**. *Arrows* indicate xylem cells that reacted with the specific antiserum. *Double-headed arrows* correspond to the longitudinal axis of the tree. Bars 150 μm

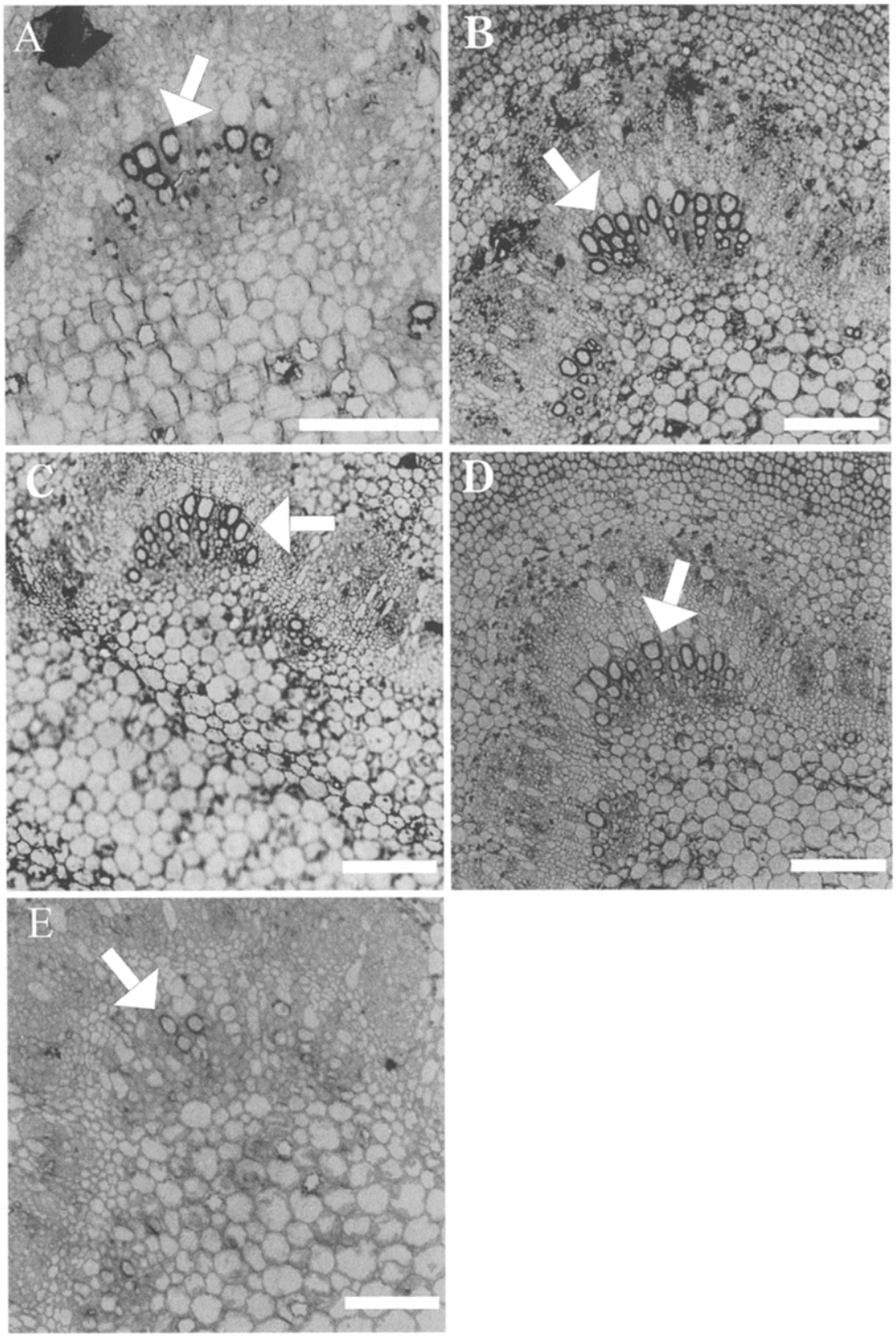

Fig. 5A–E. Immunolocalization of enzymes in the general phenylpropanoid pathway (PAL, COMT, 4CL) and the lignin-specific pathway (CAD, POX) in cross sections of primary xylem tissues. **A** PAL. **B** COMT. **C** 4CL. **D** CAD. **E** POX. *Arrows* indicate xylem cells that reacted with each specific antiserum. Bars 150 μm

Discussion

We immunohistochemically identified the spatial localization of enzymes that act in the shikimate, general phenylpropanoid, and lignin biosynthetic pathways at an early stage of development of the primary xylem. We were able to visualize lignification in the cell wall during the differentiation of primary xylem cells from procambium, by UV microscopy and chemical staining. At an extremely early stage, deposition of lignin was observed specifically in individual isolated and adjacent immature vessel elements with thickened spiral secondary walls (Figs. 2, 3). Our observations suggest that lignification of the cell walls of vessel elements in primary xylem might begin with lignification of a single cell and then proceed gradually to surrounding

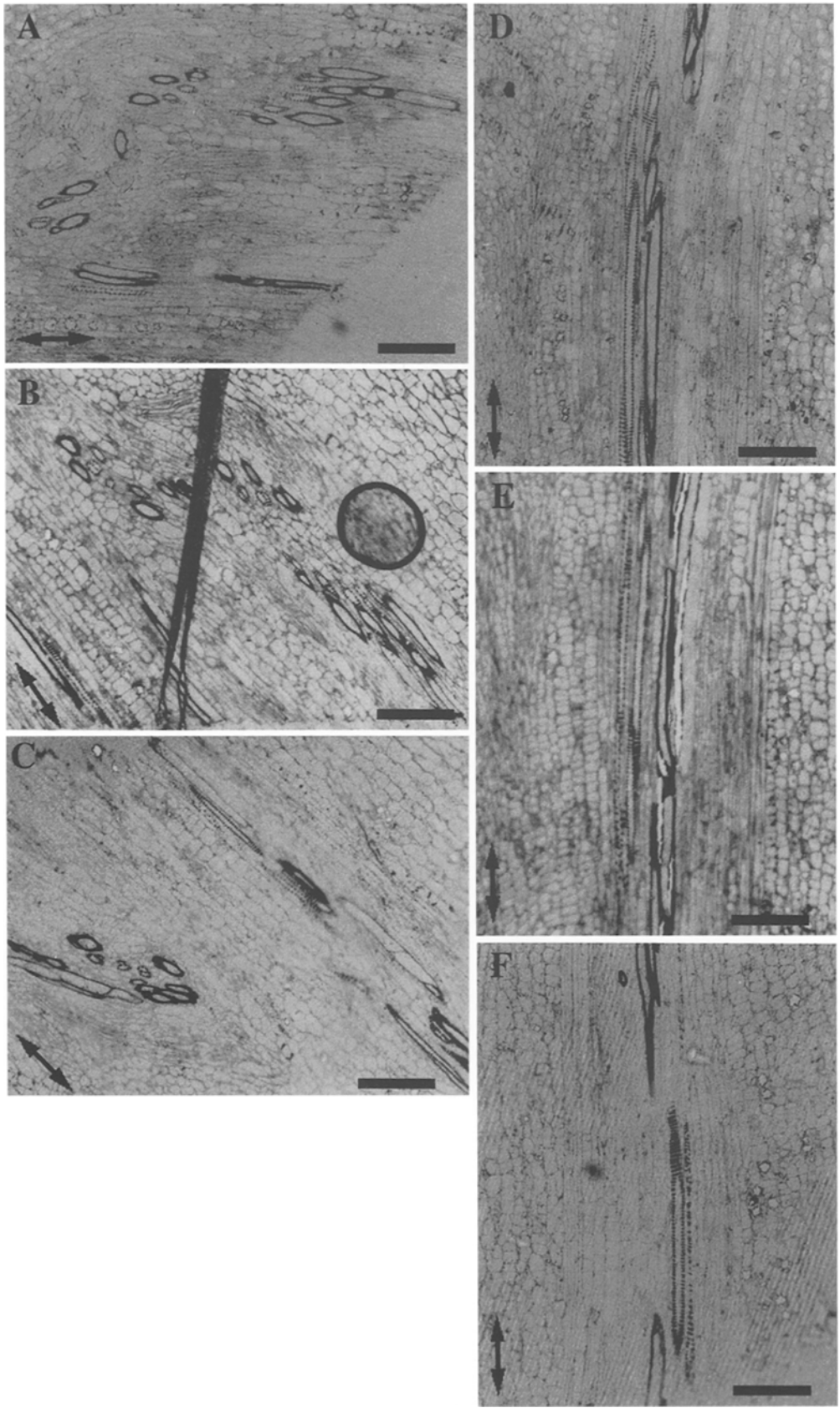

Fig. 6A–F. Immunolocalization of enzymes in the general phenylpropanoid pathway in longitudinal sections of primary xylem tissues. **A**, **B**, **C** Localization of PAL, COMT, and 4CL, respectively, in tissue near the apical meristem. **D**, **E**, **F** Localization of PAL, COMT, and 4CL, respectively, in tissue in which xylogenesis is proceeding. *Double-headed arrows* correspond to the longitudinal axis of the tree. Bars 150 μm

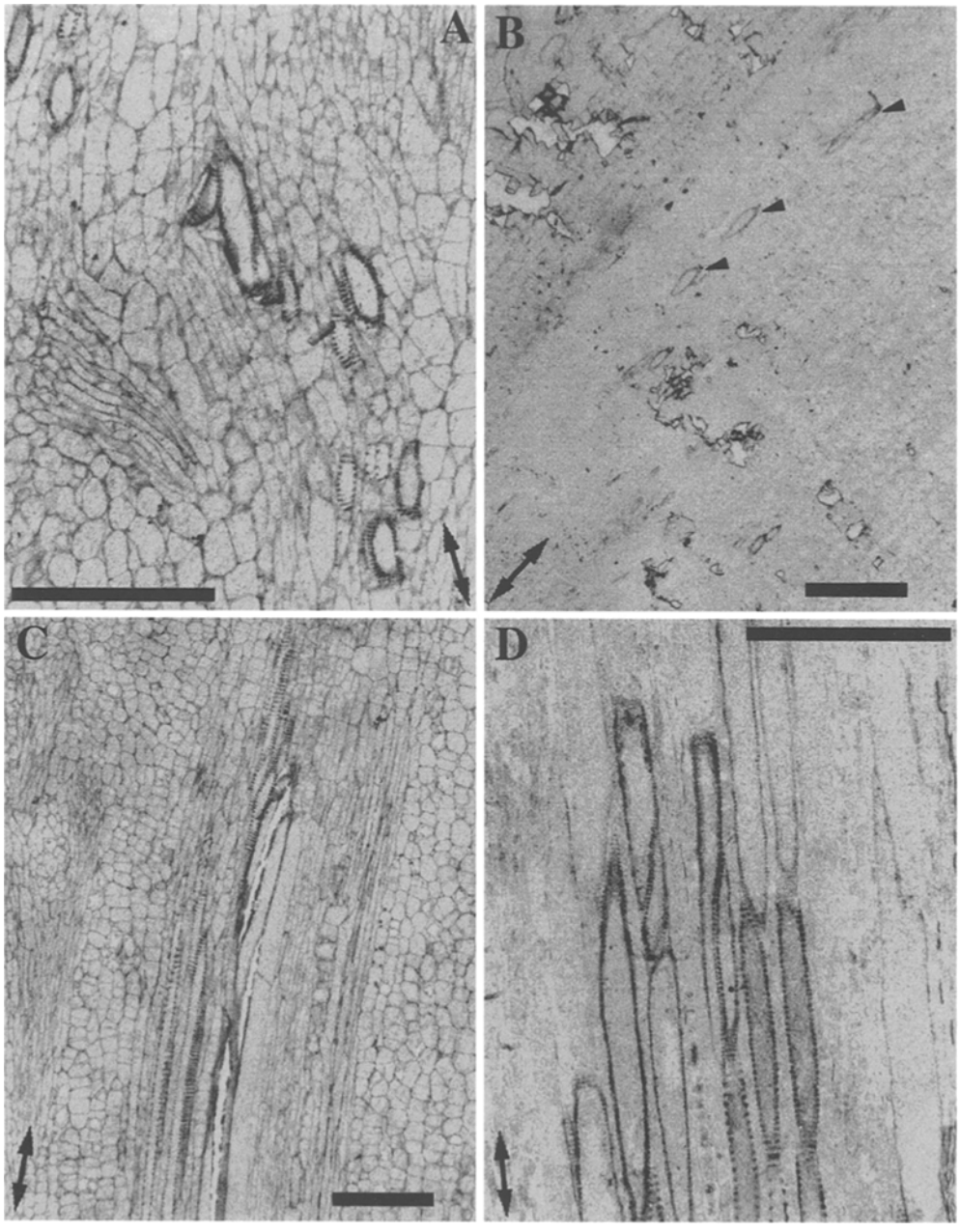

Fig. 7A–D. Immunolocalization of enzymes in the lignin-specific pathway in longitudinal sections of primary xylem tissue. **A**, **B** Localization of CAD and POX (*arrowheads*), respectively, in tissue near the apical meristem. **C**, **D** Localization of CAD and POX, respectively, in tissue in which xylogenesis is proceeding. *Double-headed arrows* correspond to the longitudinal axis of the tree. Bars 150 μm

xylem cells. In the vessel elements of primary xylem, we observed the deposition of lignin in the thickened secondary wall but not in the primary wall or the middle lamella (Fig. 2). Fukushima et al.,[25] who studied the primary xylem of *Magnolia kobus*, described similar results. Extensive studies have led to the general view that deposition of lignin in the secondary xylem starts at the corners of cells and then proceeds to the middle lamella and the secondary wall.[26–30] Thus, lignin deposition restricted to the thickened spiral secondary walls of vessel elements in primary xylem suggests differences between the mechanisms that control the sequential events of lignin deposition in primary and secondary xylem tissues.

The cell-specific lignification of vessel elements in primary xylem raised the question of how the vessel-specific initiation of lignin biosynthesis might be related to the induction and accumulation of the enzymes required for lignin biosynthesis[31–33] and the question of how the various enzymes in the general phenylpropanoid and lignin biosynthetic pathways might act in harmony with those in the shikimate pathway, which operates upstream of the former pathways.[10,34–36] Our localization of five enzymes (PAL, COMT, 4CL, CAD, POX) that act in the general phenylpropanoid or lignin biosynthetic pathways demonstrates that each enzyme in the primary xylem of young shoots is restricted almost entirely to the vessel elements (Figs. 5–7). Thus, our data suggest that lignification and accumulation of these enzymes during differentiation and maturation of the vessel elements might be tightly coordinated. The patterns of accumulation of these enzymes in the

vessel elements closely resemble those of PAL, CAD, and POX in the differentiating tracheary elements in the *Zinnia* system.[37–39] Thus, the enzymes essential for lignin biosynthesis may be produced cooperatively and may accumulate similarly in vivo and in vitro.

To our knowledge, our analysis is the first to reveal the immunohistochemical localization of DAHPS during the differentiation of vessel elements. As indicated in Fig. 4, DAHPS in young shoot tissue appeared to be restricted to just a few juvenile vessel elements, as was the case for the other enzymes analyzed in this study. Although DAHPS should be produced at a level sufficient for the synthesis of aromatic amino acids in each cell, our data indicate that the extent of DAHPS accumulation in the vessel elements in primary xylem might be greater than in other cells, and that the accumulation of DAHPS might be closely correlated with lignin deposition in the vessel elements. Jones et al.[40] reported that lignification of the stems of some transgenic potato plants that harbored a chimeric antisense gene construct for DAHPS was reduced, indicating that this enzyme plays a critical role in lignin biosynthesis as well as in the biosynthesis of aromatic amino acids. It is likely that, for vessel-specific lignification, DAHPS must be accumulated cooperatively in cells together with PAL, COMT, 4CL, CAD, and POX. Because the cooperative accumulation of these enzymes, including DAHPS, appeared to start singly, as shown in Figs. 4–7, these enzymes might be produced in each cell undergoing lignification.

In addition to immature vessel elements, immuno-gold signals are observed on the cell walls of other vessel elements that may have already been dead owing to autolysis (Figs. 4–7). This result slightly contradicts data reported by Takeuchi et al.[41] Their data indicated that COMT and POX were localized in the cytosolic fraction of the cells in secondary xylem of poplar but not in the cell wall. These findings suggest that immuno-gold signals obtained in our present study include nonspecific signals derived from interactions between the antisera and nonantigenic component(s) in the cell wall. In fact, it is well known that cell wall component(s) such as lectin can be bound to rabbit immunoglobulin G (IgG).[42] Thus, based only on our present data with the nonpurified antisera, we cannot eliminate the possibility that some of the immuno-gold signals were derived from the interaction between the nonantigenic wall component(s) and antisera. However, there is no doubt that these signals were derived, in part at least, from specific interactions between the antisera and antigenic proteins, as no apparent signals were observed on the cell wall using the control serum (Fig. 4B,D,F); and each antiserum, except for the anti-PAL and anti-POX sera, specifically recognized each protein in the Western blot analysis. Immunohistochemical and immunocytochemical analyses with antibody purified from each antiserum by affinity chromatography of each antigenic protein can contribute to obtaining further information on the localization of each protein. In future work, we will try again, with the purified IgG fraction, to analyze the immunohistochemical localization of each protein.

Conclusions

We have described a close spatial relation between lignification and the accumulation of various enzymes. For specific lignification in primary xylem tissue, our present data indicate that a tightly coordinated mechanism must exist for the accumulation of enzymes in the shikimate, general phenylpropanoid, and subsequent lignin-specific pathways during the development of primary xylem in hybrid aspen. Our data also suggest that the deposition of lignin that accompanies the differentiation and maturation of vessel elements is probably controlled in a cell-specific manner.

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