Immunocytochemical Localization of Phenylalanine Ammonia-Lyase and Cinnamyl Alcohol Dehydrogenase in Differentiating Tracheary Elements Derived from Zinnia Mesophyll Cells

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Secondary wall thickening is the most characteristic morphological feature of the differentiation of tracheary elements. Isolated mesophyll cells of Zinnia elegans L. cv. Canary Bird in differentiation medium are converted to tracheary elements, which develop lignified secondary wall thickenings. Using this system, we investigated the distribution of two enzymes, phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) and cinnamyl alcohol dehydrogenase (CAD) (EC 1.1.1.195), by both biochemical and immunological methods. Both PAL and CAD appear to be key enzymes in the biosynthesis of lignin precursors, and they have been shown to be associated with the differentiation of tracheary elements. Cultured cells were collected after various times in culture. The culture medium was separated from cells by centrifugation and designated fraction (1), the extracellular fraction. The collected cells were homogenized and separated into four fractions: (2) cytosol; (3) microsomes; (4) cell walls (loosely bound material); and (5) cell walls (tightly bound material). PAL activity was detected in each fraction. The extracellular fraction consistently had the greatest PAL activity. Moreover, PAL activity in the cytosolic fraction increased rapidly prior to lignification, as it did in both the microsomal and the cell wall (tightly bound) fractions during lignification. Antiserum against PAL and against CAD detected the proteins with molecular masses that corresponded to those of PAL and CAD in Zinnia. Immuno-electron microscopy revealed that, in differentiating tracheary elements, PAL was dispersed in the cytoplasmic matrix and was located on Golgi-derived vesicles and on the secondary wall thickenings. "Cell-free" immuno-light microscopy supported the putative distribution of PAL on lignifying secondary walls. The pattern of distribution of CAD was similar to that of PAL. Thus, both PAL and CAD seemed to be localized in secondary wall thickenings. From the results of both biochemical assays and immunocytochemical staining, it appeared that at least two types of PAL and CAD are present in differentiating cells. One type of each enzyme is distributed in the cytosol, while the other is secreted from the Golgi apparatus and transported by Golgi-derived vesicles to the secondary wall thickenings.

Key words: Cinnamyl alcohol dehydrogenase (CAD) (EC 1.1.1.195) — Immuno-electron microscopy — Lignification — Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) — Tracheary element differentiation — Zinnia elegans.

The accumulation of lignin is a conspicuous feature of the formation of secondary walls in vascular plants. The result of this process is the production of tracheary elements, which both transport water efficiently and provide structural support and protection to the plant from microbial attack, such as infection by fungi. Many enzymes are involved in the biosynthesis of monolignols. Phenylalanine ammonia-lyase (PAL) and cinnamyl alcohol dehydrogenase (CAD) are among the most important of these enzymes. The biosynthetic pathways from carbohydrate to monolignols are well understood (Higuchi 1985). However, information about the enzymes involved in the biosynthesis of monolignols at the sub-cellular level remains limited. Thus, it is necessary to investigate both the pathways for the synthesis of monolignols and the localization of the enzymes that are involved in lignification at the sub-cellular level.

Rapid-freezing and substitution techniques have recently been introduced that allow investigations of the dynamic state of plant cells (Nishizawa and Mori 1989, Inomata et al. 1992). Compared to conventional preparative procedures, these techniques have greatly improved the preservation of ultrastructural features, both in terms of physical immobilization and in terms of the preservation of the antigenic reactivity of specific cellular components. Thus, even minor chemical components can be visualized with a high degree of resolution and specificity by immuno-electron microscopy (Nicolas and Bassot 1993).

Fukuda and Komamine (1980) established an experimental system in which Zinnia mesophyll cells differentiate directly into tracheary elements at high frequency and with a high degree of synchrony. This experimental system has proved very useful for physiological and biochemical studies of mechanisms of formation of tracheary elements, as well as of other questions related to cytodifferentiation in higher plants.

Using this system, we were able to localize two important enzymes, PAL and CAD, by immuno-electron micro-
copy in the present study. PAL is thought to catalyze the initial conversion of phenylalanine to cinnamic acid (Rubery and Northcote 1968, Rubery and Fosket 1969, Fukuda and Komamine 1982), while CAD is involved in the last step in the formation of monolignols, by catalyzing the reduction of cinnamyl aldehydes to their alcohols (4-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) in the presence of NADPH. Thus, it appears that the phenolic compounds generated by CAD are destined to serve as monolignols, and CAD is considered to be an important enzyme in lignification (Mansell et al. 1974).

The first goal of this study was to assay PAL activity in order to clarify the chemical characteristics of the enzyme. The second goal was to visualize PAL and CAD at the sub-cellular level. Using rapid-freezing and substitution techniques instead of chemical fixation prior to immunocytochemical staining, we were able to achieve enhanced preservation of ultrastructural features, as well as the immobilization of cellular components at their original sites.

Materials and Methods

Preparation and culture of single cells—Mesophyll cells of the first true leaves of 14-day-old seedlings of *Zinnia elegans* L. cv. Canary Bird (Takii Shubyo Co., Kyoto, Japan) were isolated and cultured as described previously (Fukuda and Komamine 1980). The culture medium contained 0.1 mg liter⁻¹ naphthaleneacetic acid and 1.0 mg liter⁻¹ benzyladenine as growth regulators. The culture medium contained 0.1 mg liter⁻¹ naphthaleneacetic acid and 1.0 mg liter⁻¹ benzyladenine as growth regulators. The culture medium contained 0.1 mg liter⁻¹ naphthaleneacetic acid and 1.0 mg liter⁻¹ benzyladenine as growth regulators.

Preparation of single cells—Mesophyll cells were collected at various times during their culture (at 24, 48, 60, 72 and 96 h). The culture medium was filtered through a Whatman GF/C filter, and the filtrate was designated the extracellular fraction. The cells were homogenized at 0°C with a Branson Sonifier (Branson Sonic Power Co., Danbury, Connecticut, U.S.A.) in a solution of 50 mM Tris-HCl buffer (pH 7.5) plus 1 mM dithiothreitol (DTT) (four times the fresh weight of the cells) to which polyvinyl polypyrrolidone (Nacalai Tesque, Inc., Kyoto, Japan), and Amberlite XAD-2 (Organo Co. Ltd., Tokyo, Japan) had been added (0.5 g each per g of cells). After 10 min, the mixture was centrifuged at 500 x g for 5 min each. The supernatant was diluted with the buffer and centrifuged at 105,000 x g for 60 min. The supernatant was collected and designated the cytosolic fraction. The resuspended pellet was designated the microsomal fraction. The pellet after centrifugation of 500 x g was washed six times with the above-mentioned buffer that contained 1% Triton X-100. Then, it was extracted with 1-2 ml of buffer that contained 3 M NaCl for 12 h at 4°C. The resultant supernatant after centrifugation twice at 500 x g for 5 min each was dialyzed against buffer for 24 h, and it was designated the cell wall (loosely bound) fraction. PAL was assayed by the method of Fukuda and Komamine (1982), and PAL activity was monitored at 290 nm. A calibration curve was constructed and PAL activity was expressed in terms of nanomoles of cinnamic acid produced under the conditions of the reaction. All experiments were repeated five times to confirm the reliability and reproducibility of the results.

Antisera—Rabbit antisera against tobacco PAL was provided courtesy of Prof. M. Okazaki (Faculty of Textile Science and Technology, Shinsyu University, Ueda, Japan). Rabbit antisera against CAD from *Aralia cordata* was provided by courtesy of Dr. T. Hihino (Mitsui Plant Biotechnology Research Institute, Tsukuba, Japan).

Western blotting analysis—The cross-reactivity of the antibodies, which had been raised against PAL from tobacco and CAD from *Aralia cordata* respectively, with PAL and CAD from cultured *Zinnia* cells was examined by immunoblotting of proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell extracts. Memophyll cells were collected at various times during culture (at 24, 48, 60, 72 and 96 h), washed with fresh hormone-free medium and homogenized by the same preparative procedure as described above. Each homogenate was rotated at 30 rpm for 10 min at 0°C and filtered through Whatman No. 1 paper. The filtrate was centrifuged at 500 x g for 10 min. The supernatant was stored at −20°C and used for Western blotting analysis. Electrophoresis was performed on a 10% polyacrylamide gel (12 x 10 cm). Molecular masses were estimated by comparing electrophoretic mobilities to most of standard proteins (94, 67, 43, 30 and 20 kDa (Electrophoresis Calibration kit; Protein Standards, Uppsala, Sweden). Proteins were transferred to a nitrocellulose filter (ClearBlot Membrane-P; Atto Corp., Tokyo, Japan) in a horizontal blot apparatus according to the protocol from the manufacturer (AE-6675; Atto Corp.). After incubation with antibody against PAL or against CAD, filters were incubated with 1-nm gold-labeled goat antibodies against rabbit IgG (H+L) (Auro Probe™ One GAR; Amersham International plc, Amersham Place, U.K.) and then silver enhancement (Intense™ SL silver enhancement kit; Amersham International plc) was performed according to the protocol from Amersham. Treatment against antibody, filters were incubated with 1-nm gold-labeled goat antibodies against rabbit IgG (H+L) (Auro Probe™ One GAR; Amersham International plc, Amersham Place, U.K.) and then silver enhancement (Intense™ SL silver enhancement kit; Amersham International plc) was performed according to the protocol from Amersham.

Rapid freezing and substitution—Mesophyll cells (at 24, 48, 72 and 96 h) were carefully placed on a small copper loop (2-3 mm in diameter) that had been coated with formvar and poly-L-lysine (0.1%). After the medium had been soaked up with filter paper, each specimen was plunged into liquid propane that had been cooled with liquid nitrogen and then stored in liquid nitrogen. For substitution, samples were incubated for 2 days in a mixture of 0.5% tannic acid and 0.5% glutaraldehyde dissolved in acetone at −80°C. After washing and dehydration, they were embedded in LR White resin (London Resin Co. Ltd., Basingstoke, U.K.).

Visualization by immuno-electron microscopy of PAL and CAD—Ultra-thin sections mounted on nickel grids were used for immuno-electron microscopy. All preparations were subjected to the following treatments: (1) incubation for 30 min in 2% glycine in PBS (pH 7.4); (2) incubation for 1 h at 37°C in blocking buffer, which contained 0.8% bovine serum albumin (BSA), 0.1% high-quality gelatin (Amersham International plc), 0.5% normal goat serum, and 2 mM NaN₃ in PBS; (3) three washes, for 5 min each, in washing buffer that contained the same components as blocking buffer with the exception of goat serum; (4) overnight incubation at 4°C with rabbit antisera against tobacco PAL (diluted 1:200 in blocking buffer), or rabbit antisera against CAD from *Aralia cordata* (diluted 1:500); (5) three washes, for 10 min each, in washing buffer; (6) incubation for 3 h with 20-nm colloidal gold-conjugated goat antibodies against rabbit IgG (Auro Probe EM; GAR20; Amersham International plc; diluted 1:25 in blocking buffer); (7) three washes, for 15 min each, in washing buffer and then three washes, for 5 min each, in PBS; (8) postfixation for 10 min in 2% glutaraldehyde; and (9) three washes, for 5 min each, in PBS and then three washes, for 5 min each, in excess water. All treatments were performed in drops of liquid on Parafilm™ (American National Can, Greenwich, Ohio).
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U.S.A.). Control sections were treated in the same way with the exception that rabbit pre-immune serum or incubation buffer alone was used instead of rabbit antiserum against tobacco PAL or against CAD from *Aralia cordata*. All sections were stained with 2% uranyl acetate for 30 min and then with Reynolds' lead citrate for 1 min, and finally they were examined under a transmission electron microscope (JEM-100C; JEOL, Tokyo, Japan) operated at 100 kV.

To eliminate possible errors in the identification of immunogold particles, we developed a filtering method. First, tracing paper (very thin paper) was laid on an immuno-electron photomicrograph and then the immunogold particles in the frame were copied manually onto the paper. In this way, the immunogold particles were completely separated from other features of cells, allowing us to assess their distribution accurately.

Visualization by *cell-free* immuno-light and immuno-fluorescence microscopy of PAL—Cultured cells (at 72 h) were washed with fresh hormone-free medium and sectioned at 20-μm thickness with a sliding microtome equipped with a freezing device. Then specimens were rinsed well in water and layered on coverslips as described previously (Falconer and Seagull 1985). All preparations were subjected to the following treatments: (1) extraction for 1 h with 1% (w/v) Triton X-100 in PBS (pH 7.4); (2) three washes, for 3 min each, in PBS; (3) incubation for 1 h at 37°C in blocking buffer, which contained 0.5% (w/v) Tween 20, 5% skimmed milk, and 2 mM NaN₃ in PBS; (4) three washes, for 3 min each, in PBS; (5) incubation for 2 h in rabbit antiserum against tobacco PAL (diluted 1:500 in blocking buffer); (6) three washes, for 5 min each, in PBS; (7) incubation for 1 h in 1-nm colloidal gold-labeled goat antibodies against rabbit IgG (H+L) (Auro Probe™ One GAR; Amersham International plc; diluted 1:50 in blocking buffer) for immuno-light microscopy under a phase-contrast microscope (BHS-N; Olympus, Tokyo, Japan) or, alternatively, in fluorescein isothiocyanate-labeled goat antibodies against rabbit IgG (H+L) (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada; diluted 1:50 in PBS) for immuno-fluorescence microscopy (BH-RFL; Olympus); (8) six washes, for 3 min each, in PBS; (9) incubation for 10 min with reagents from an IntenSE™M silver enhancement kit (Amersham International plc) for immuno-light microscopy; and finally (10) three washes, for 3 min each, in distilled water. Then the specimens were mounted in a mixture of glycerol and PBS (1:1, v/v). Control sections were treated in the same way with the exception that rabbit pre-immune serum or incubation buffer alone was used instead of rabbit antiserum against tobacco PAL.

**Results**

**Time courses of the formation of tracheary elements and lignification**—In this culture system, deposition of polysaccharides in secondary wall thickenings is initially detectable under the electron microscope at 60 h and then proceeds rapidly (Nakashima et al. unpublished). Tracheary elements were formed at a very high frequency and eventually accounted for up to 40% of the cultured cells. There was a high degree of synchrony in the formation of tracheary elements after 60 h of culture. Photomicrographs of differentiating tracheary elements taken under UV light at 280 nm, revealed that the secondary wall thickenings began to absorb UV light weakly at 72 h, with a gradual increase as differentiation proceeded. The strongest UV absorption was noted at 96 h, when the differentiation of tracheary elements was complete (Nakashima et al. unpublished). The UV absorption spectra of secondary wall thickenings revealed that the absorbance at around 280

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![Fig. 1](https://academic.oup.com/pcp/article/38/2/113/1873897/pdf)  
Fig. 1 Changes in PAL activity in cultures of isolated *Zinnia* mesophyll cells in differentiation medium. White and black dots represent mean values from five replicates and vertical bars denote standard deviations.

![Fig. 2](https://academic.oup.com/pcp/article/38/2/113/1873897/pdf)  
Fig. 2 Changes in PAL activity in various fractions from isolated *Zinnia* mesophyll cells during culture in differentiation medium. The results in each column represent mean values from five replicates.
Fig. 3 Time courses of changes in levels of PAL (a) and CAD (b) in a culture of differentiating *Zinnia* cells. Proteins were extracted from cells at the times indicated and separated by electrophoresis on a 10% polyacrylamide gel. Proteins on gels were blotted onto nitrocellulose membranes. The membranes were incubated with antiserum against tobacco PAL or with against CAD from *Aralia cordata*, and proteins were visualized by silver enhancement after incubation with 1-nm gold-labeled goat antibodies against rabbit IgG. Many polypeptide bands were observed when the polyacrylamide gel was stained with Coomassie brilliant blue (CBB), as shown on the extreme left (72 h).

nm, which corresponded to the accumulation of lignin, became detectable at 72 h, with a gradual increase with differentiation until a maximum was reached at 96 h. Therefore, it seemed that lignification of secondary wall thickenings began almost 12 hours later than the deposition of polysaccharides and then proceeded continuously to completion at 96 h. Measurements of PAL activity—The changes in total PAL activity (including and excluding the activity in the extracellular fraction) during culture are shown in Figure 1. The extracellular fraction, represented by the difference between the two graphs, consistently contained the greatest amount of PAL activity, which accounted for up to 70% of the total PAL activity. The changes in PAL activity in various extracts during culture (excluding the extracellular fraction) are shown in Figure 2. There were two phases of changes after 60 h. First, the activity in the cytosolic fraction increased six-fold between 60 and 72 h, remaining stable thereafter. Second, the activity in the cell wall tightly bound fraction increased six-fold both between 60 and 72 h and between 72 and 96 h. Moreover, the latter increase coincided with that in the microsomal fraction, which increased as much as 13-fold between 72 and 96 h.

**Western blotting analysis of PAL**—Many polypeptide bands were observed when polyacrylamide gels were stained with Coomassie brilliant blue (CBB) after proteins in extracts had been separated by electrophoresis. The cross-reactivity of the antiserum against PAL with proteins in each extract (at 24, 48, 60, 72 and 96 h) was investigated by Western blotting. The antiserum immunoreacted with a single polypeptide with a relative molecular mass \((M_r)\) of 79,000 at 60 h, 72 h (strong reaction), and 96 h (Fig. 3a). No polypeptide bands were detected when filters for Western blotting were incubated with rabbit pre-immune serum or with buffer alone.

**Visualization of PAL by immuno-electron microscopy**—Figure 4a shows an immuno-electron micrograph of PAL in a differentiating tracheary element after cells had been cultured for 72 h. The immunocytochemical labeling of PAL in such cells indicated that the distribution of PAL was not uniform in each tracheary element. Immuno-gold particles seemed to be localized on the cytoplasm and on the secondary wall thickenings. Part of the same cell as shown in Figure 4a is shown at higher magnification in Figure 4b. To generate Figure 4c, we used tracing paper to filter out the immuno-gold particles from other features of cells, so that we could focus exclusively on the distribution of the particles. The immuno-gold particles, which are indicated by the small dots in Figure 4c, are distributed throughout the panel. In this and similar micrographs, the particles were located on the cytoplasm and on the secondary wall thickenings. The particles were more densely distributed on Golgi-derived vesicles and on secondary wall thickenings than on the cytoplasmic matrix (arrows in Fig. 4b, c).

Quantitative analysis of the distribution of the immu-
Table 1  Distribution of gold particles after immuno-gold labeling of PAL in differentiating tracheary elements of Zinnia at 72 h

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Distribution (%)</th>
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<tbody>
<tr>
<td>Cytoplasmic matrix</td>
<td>22.9</td>
</tr>
<tr>
<td>On membranes total</td>
<td>33.0</td>
</tr>
<tr>
<td>On Golgi-derived vesicles</td>
<td>23.9</td>
</tr>
<tr>
<td>On plasma membranes</td>
<td>9.1</td>
</tr>
<tr>
<td>On cell walls total</td>
<td>44.1</td>
</tr>
<tr>
<td>Primary walls</td>
<td>1.8</td>
</tr>
<tr>
<td>Secondary walls</td>
<td>42.3</td>
</tr>
</tbody>
</table>

* The values represent results for 6,954 gold particles on 20 electron micrographs.

Localization of PAL and CAD in Zinnia

Fig. 6 Localization of PAL in differentiating tracheary elements after culture for 72 h. These micrographs were taken under a light (left) and a phase-contrast (right) microscope. Micrograph a shows a fragmented tracheary element incubated with antiserum against PAL. Micrograph b shows a tracheary element that escaped fragmentation incubated with antiserum against PAL. Micrograph c shows a fragmented tracheary element incubated with rabbit pre-immune serum, as a control.

sectioning with the microtome, some of the tracheary elements were cracked or fragmented. The cytoplasm of these elements had disappeared during the washing procedure. Fragmented cell walls were easily recognized from the pattern of secondary wall thickenings. When these tracheary elements were incubated with antiserum against PAL, they were intensively immuno-labeled. Immunolight microscopy revealed such labeling as silver particles
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Fig. 7 Immuno-fluorescence micrographs of PAL in differentiating tracheary elements after culture for 72 h. Micrograph a shows a cracked tracheary element, and micrographs b and c show fragmented tracheary elements incubated with antiserum against PAL. An arrow indicates the cracked portion of the tracheary element in micrograph a. Micrograph d shows tracheary elements that escaped fragmentation incubated with antiserum against PAL. Micrograph e shows a fragmented tracheary element incubated with buffer alone as a control. Bar = 20 μm.

(Fig. 6a), whereas immuno-fluorescence microscopy revealed labeled protein as a fluorescent green color (Fig. 7a, b, c). In the same preparation, those tracheary elements escaped fragmentation showed no labeling (Fig. 6b, 7d). The auto-fluorescence of the secondary wall thickenings, shown in Figure 7d, was strong but the color of the autofluorescence differed from that due to specific immuno-labeling of PAL. No immuno-labeling was observed on control samples, even when the tracheary elements were fragmented, as seen in the case of samples treated with rabbit pre-immune serum (Fig. 6c) or with buffer alone (Fig. 7e).

Western blotting analysis of CAD—The cross-reactivity of the antiserum against CAD with proteins in each extract (at 24, 48, 60, 72 and 96 h) was indicated by immunostaining of a major protein with \( M_r \) of 40,000 and a minor protein with \( M_r \) of 39,000 at 60 h, 72 h (strong reaction), and 96 h (Fig. 3b). No polypeptides were visualized when the rabbit antiserum against CAD from Aralia cordata was replaced by rabbit pre-immune serum or buffer alone.

Visualization of CAD by immuno-electron microscopy—When ultra-thin sections of differentiating tracheary elements after culture for 72 h were immunostained with the antiserum against CAD, the immuno-gold particles were generally observed in patterns similar to those in specimens that had been treated with the antiserum against PAL. That is, immuno-gold particles were dispersed on the cytoplasm and the secondary wall thickenings. The particles were densely distributed on Golgi-derived vesicles (Fig. 8). In control sections that had been incubated with pre-immune rabbit serum, hardly any immuno-gold particles were observed on the tracheary elements (data not shown).

Discussion

As shown in Figure 1, the greatest amount of PAL activity was consistently found in the extracellular fraction. At the beginning of culture, the extracellular PAL activity might have been induced by injury to the cells. However, a large amount of extracellular PAL activity was detected even at 96 h, suggesting an unknown mechanism for the transport and/or secretion of PAL from cells to the liquid medium through the cell wall.

The changes in total PAL activity shown in Figure 2 support the results of Fukuda and Komamine (1982). Moreover, we obtained more detailed information about PAL activity after fractionation of the culture. The most prominent feature in Figure 2 is that the activity of the cytosolic fraction increased rapidly prior to lignification, as did the PAL activity in both the microsomal and the cell wall (tightly bound) fractions during lignification.

Western blotting analysis of PAL revealed a single
Fig. 8  Immuno-electron micrographs of CAD in a differentiating tracheary element after culture for 72 h. This ultra-thin section was incubated with rabbit antibodies against CAD from *Aralia cordata* and then with 20-nm colloidal gold-conjugated goat antibodies against rabbit IgG. Arrows indicate Golgi-derived vesicles. MT, Microtubules; PI, plastid; SW, secondary wall thickening; V, vacuole.
band of a polypeptide with $M_r$ of 79,000 at 60 h, 72 h (strong) and 96 h (Fig. 3a). PAL catalyzes the first step in the biosynthetic pathways to polyphenolic compounds, such as lignin, cinnamate esters, and flavonoids, and it is one of the key regulatory enzymes in the metabolism of all these compounds. Therefore, while we measured the PAL activity of all types in the experiments for which results are shown in Figures 1 and 2, in Figure 3a, we provide information about just one or some of the isoenzymes of PAL that cross-reacted with the antiserum against tobacco PAL. Furthermore, the latter type(s) of PAL seemed to be closely associated with differentiation. According to Lin and Northcote (1990), PAL with $M_r$ of 80,000, which might possibly be involved in lignification, is detected when cells are cultured for more than 60 h in the present system. Thus, the single polypeptide that we detected might be a subunit of PAL that plays a critical role in the lignification of tracheary elements in Zinnia.

Immuno-electron microscopy clearly revealed the localization of PAL at the sub-cellular level in the differentiating tracheary elements of Zinnia.

At 72 h, PAL activity increased rapidly (Fig. 1), and lignification of secondary wall thickenings began (Nakashima et al. unpublished). Thus, we were able to investigate the localization of PAL that is involved in lignification. Our filtering method clearly demonstrated the distribution of immuno-gold particles, and Figure 4 indicates that some of the immuno-gold particles that corresponded to PAL were dispersed on the cytoplasm. Moreover, a large number of these particles were localized on the Golgi apparatus, the Golgi-derived vesicles, and the secondary wall thickenings. These observation indicate that PAL was secreted from the Golgi apparatus and transported to the lignifying secondary wall thickenings by the Golgi-derived vesicles. Quantitative analysis of the distribution of PAL tended to support these results (Table 1). However, the distribution of immuno-gold particles indicated in Table 1 does not reflect the measurements of PAL activity in each fraction at 72 h that are shown in Figure 2. The discrepancy between the two sets of results might be due to the following factors. In Table 1, we focused only on the differentiating tracheary elements (which account for 40% of the total cells, at most). However, in Figure 2, we show the PAL activity measured using the total cells. Furthermore, in Figures 1 and 2, we show the PAL activities of all types, while in Table 1 we only show results for one or some of the isoenzymes of PAL that cross-reacted with the antiserum against tobacco PAL.

In order to verify the localization of PAL in the secondary wall thickenings (Fig. 2, 4), we examined fragmented cells or "cell-free" specimens. Such an examination restricted the analysis to antibody-antigen reactivity within cell walls. Cells that have been cut by a microtome are cracked or fragmented and lose their cytoplasm, with resultant preparation of isolated fragments of cell walls. This phenomenon allows the direct reaction of antibodies with the secondary wall thickenings. The cracked or fragmented tracheary elements had been incubated with antiserum against PAL were strongly immunostained (Fig. 6, 7), and these results confirmed that PAL was localized in lignifying secondary wall thickenings.

The results of our biochemical assays and immuno-cytochemical staining revealed at least two types of PAL. One type is distributed in the cytoplasmic matrix and the other is secreted from the Golgi apparatus and then transported by Golgi-derived vesicles to the secondary wall thickenings. These results suggest that the conversion of phenylalanine to cinnamic acid takes place in, on, or near the Golgi-derived vesicles, as well as in the cytoplasmic matrix.

Takabe et al. (1985) reported that administration of tritiated phenylalanine to differentiating xylem cells resulted in detectable radioactivity on the Golgi apparatus and on Golgi-derived vesicles after autoradiography and transmission electron microscopy. This observation suggests that the administered phenylalanine was incorporated directly into the Golgi apparatus and was then converted to a monolignol via Golgi-derived vesicles, and it is consistent with our demonstration of PAL in Golgi-derived vesicles in differentiating tracheary elements.

Western blotting analysis of CAD revealed two polypeptides with $M_r$ of 40,000 and 39,000, respectively, at 60 h, 72 h (strong) and 96 h (Fig. 3b). According to Hibino et al. (1993), the relative masses of subunits of CAD in Aralia cordata are approximately 39,500 and 40,000. Thus, the two polypeptides detected in this study seem likely to be the subunits of CAD in Zinnia.

The N-terminal amino acid sequences of PAL that have been determined to date reveal no features characteristic of a signal peptide (Whetten and Sederoff 1992), nor does the N-terminal amino acid sequence of CAD from Aralia cordata (Hibino et al. 1993). A protein that is synthesized without a signal peptide, such as PAL and CAD, is likely to be released into the cytosol and to remain there. Even though this hypothesis might explain the consistent immuno-gold labeling of PAL and CAD in the cytosol, it cannot explain the immuno-gold particles on Golgi-derived vesicles and secondary wall thickenings. Thus, either a novel isoenzyme of PAL with a signal peptide or a new type of signal peptide related to secondary wall thickening might exist. The amino acid sequence of this isoenzyme should, thus, be fully investigated.

Immuno-electron microscopy revealed that there were at least two types of both PAL and CAD. One type of each enzyme is distributed in cytosol, and the other is secreted from the Golgi apparatus and transported by Golgi-derived vesicles to the secondary wall thickenings. The localizations of PAL and CAD were similar, and the enzymes in-
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...olved in lignification might be expected to have similar characteristics. Moreover, the toxicity of the lignin precursors should necessitate their sequestration by a protective membrane, for example, within a Golgi-derived vesicle. In such a case, details of the biosynthesis of monolignols can be proposed as follows. The biosynthesis of monolignols in Golgi-derived vesicles, on such vesicles, or near such vesicles indicates that lignin precursors are always situated in, on, or near Golgi-derived vesicles. The safe transportation of the monolignols to the lignifying secondary wall thickenings is thus ensured.

Finally, the advantages of our method for immunoelectron microscopy were demonstrated by the successful localization of PAL and CAD at the sub-cellular level for the first time in differentiating tracheary elements of Zinnia. The results of the sub-cellular visualization of PAL and CAD indicate that a more thorough and substantive explanation of the lignification of secondary wall thickenings is now necessary. A study of transport mechanisms, in particular, seems to be essential. Moreover, further investigations must be performed with monoclonal antibodies specific for PAL and CAD from Zinnia.

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References


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