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Lignification and cinnamyl alcohol dehydrogenase activity in developing stems of tomato and poplar: a spatial and kinetic study through tissue printing

Ronelle Roth[1], Alain M. Boudet and Rafael Pont-Lezica[2]

UMR 5546 Conseil National de la Recherche Scientifique, Université Paul Sabatier, 118, route de Narbonne, F-31062 Toulouse Cédex, France

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Abstract

Cinnamyl alcohol dehydrogenase (CAD) is an enzyme involved in the synthesis of lignin monomers. Tissue prints were used to localize CAD activity in tomato and poplar tissues. In parallel, lignin deposition was estimated by classical histochemical tests. Image analysis was also used to quantify the amount of CAD activity and the extent of lignification throughout the development of stems. Tomato stems showed a limited lignification. CAD activity, restricted to the xylem, reached a maximum during the early stages of development and showed a patched pattern on the xylem ring. In contrast, CAD activity was much stronger in poplar stems and closely associated with all lignified areas: starting with localized areas within the xylem and finally completely covering the xylem ring. The enzyme showed a significant activity in primary and secondary xylem, and both lignin deposition and CAD activity increased as the plant matured. These results show that CAD activity, which is important for lignification, can easily be detected in plant tissues by tissue prints. They also reveal that tomato (herbaceous stems) and poplar (woody stems) dramatically differ in the developmental pattern of CAD within their lignified tissues.

Key words: Tomato, poplar, lignification, CAD activity, developing stems.

Introduction

Cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) is an NADP(H) specific oxidoreductase catalysing the reversible conversion of cinnamyl aldehydes to the corresponding alcohols or monolignols (Mansel *et al.*, 1974; Wyrambik and Griesebach, 1975; Boudet *et al.*, 1995). Polymerization of the three monolignols: *p*-coumaryl, sinapyl and coniferyl alcohols lead to lignin. The enzyme is closely related to lignification, and chemical inhibition of its activity reduces the synthesis of lignins (Moesbacher *et al.*, 1990; Mauch-Mani and Slusarenko, 1996). This enzyme has been purified and characterized from several herbaceous species, such as soybean, wheat and tobacco, or woody species like *Forsythia*, *Acer*, *Quercus*, *Picea*, *Pinus*, *Populus*, and *Eucalyptus* (Boudet *et al.*, 1995). Several studies have shown that CAD may be polymorphic with isoforms that differ not only on substrate affinity, but on molecular mass (Mansell *et al.*, 1974, 1976; Luderitz and Griesebach, 1981; Sarni *et al.*, 1984; Pillonel *et al.*, 1992; Halpin *et al.*, 1992; Goffner *et al.*, 1992; Boudet *et al.*, 1995). Some of these isoforms seem to be associated with the synthesis of defence lignins (Mitchell *et al.*, 1994).

In previous studies the presence of enzymes involved in lignification have not only been shown in tissues undergoing active lignin synthesis (Baudracco *et al.*, 1993; Boudet *et al.*, 1995), but have also been associated with non-lignified tissues (O'Malley *et al.*, 1992) or poorly lignified systems such as cell suspension cultures (Wyrambik and Griesebach, 1975; Galliano *et al.*, 1993), suggesting that CAD may be associated to other secondary metabolites in addition to lignin. The localization of this enzyme at tissue level has only recently been demonstrated through standard histochemical and microscopic methods in herbaceous stems and roots (Baudracco *et al.*, 1993). In addition to the well-known lignified areas, developing

[1] Present address: Department of Plant Sciences, University of Oxford, Oxford OX1 3WB, UK.
[2] To whom correspondence should be addressed. Fax: +33 5 61 55 62 10. E-mail: lezica@cict.fr

xylem and phloem fibres, CAD activity was also found in the epidermal and subepidermal cells of root and shoot in *Phaseolus* as shown by Baudracco *et al.* (1993). However, the localization of CAD activity has not been undertaken in woody species, where lignification is an important regulated process. On the other hand, the expression of one gene encoding *Eucalyptus* CAD was studied in transgenic poplar, by the transfer of a chimeric gene fusion containing the CAD promoter and the β-glucuronidase (GUS) reporter gene. The expression of such construction was studied through GUS activity detected in parenchyma cells differentiating between the xylem-conducting elements and the xylem ray cells (Feuillet *et al.*, 1995).

In order to provide new information on the tissue location of CAD and to evaluate the degree of correlation of CAD activity and lignification, the fast and sensitive method of tissue printing was selected (Cassab and Varner, 1987; Reid *et al.*, 1992) to compare CAD activity during the developmental process of lignification in an herbaceous (tomato) and a woody (poplar) plant.

Materials and methods

Plant material

Stems from tomato (*Lycopersicon esculentum* L.) and poplar (*Populus* × *euramericana* clone I 214) were used to localize CAD activity. Tomato seeds were germinated in vermiculite under 14 h photoperiod (14 h light, 25 °C, 70% relative humidity, and 10 h darkness, 18 °C, 80% relative humidity). Stem and root sections for tissue printing, were made from 4–5-week-old plants. Actively growing poplar branches were taken from 1-year-old plants, collected from the field in July and August.

CAD activity on tissue prints

Free-hand cross-sections were made with a clean, sharp razor blade, from the first six internodes, under the meristem of tomato and poplar shoots. Four tissue prints were made of each internode on pieces of nitrocellulose (0.45 μm pore size, Schleicher and Schull). The fresh tissue prints were washed in cold 20 mM TRIS–HCl (pH 8.8) for 15 min to eliminate all small hydrophilic metabolites that do not bind the membrane, and then maintained on ice to preserve the stability of the enzyme.

CAD activity was detected on prints as described by Baudracco *et al.* (1993) for histochemical studies, with some modifications. Activity was developed by incubating the printed membranes (1 h at 37 °C) with the following reaction medium (complete medium): 0.35 mg ml^{-1} nitro blue tetrazolium (NBT), 1 mg ml^{-1} $NADP^+$, 0.4 mg ml^{-1} phenazine methosulphate (PMS), 3.6 mg ml^{-1} coniferyl alcohol in 20 mM TRIS–HCl (pH 8.8). The reaction was stopped by washing the membranes in H_2O for 10 min. The histochemical assays on nitrocellulose prints started within 1 h of printing, due to a progressive decrease of CAD activity on the prints. In control prints, coniferyl alcohol, NADP or both were omitted. In some assays *N*(*O*-hydroxyphenyl) sulphinamoyl-tertiobutylacetate (OH-PAS, 3.3 mM), a specific inhibitor of CAD (Grand *et al.*, 1985), was added to the medium. Once the prints were completely air-dried, the activity of CAD could be localized by observing the formation of the blue, insoluble, formazan product.

Lignin staining

Lignins were detected in stem sections by the Weisner (phloroglucinol-HCl) reaction (Monties, 1984). Phloroglucinol reacts with all aromatic aldehydes, in addition to those included in lignin. However, in the conditions used here the reaction was located in the xylem area and tightly linked to the occurrence of lignins (Monties, 1989). Free-hand cross-sections (± 100 μm) were made from the same internodes as for the tissue prints. The sections were incubated for 3 min in phloroglucinol and mounted in a drop of HCl on clean microscope slides. The red lignin stain was observed with a binocular microscope. Photographic records of tissue prints and lignin-stained sections were made with an Olympus binocular microscope equipped with an Olympus camera.

Image analysis of CAD activity and lignification

To correlate CAD activity to lignification on different stem internodes, image analysis was performed. The photographs obtained from the tissue prints and from the sections stained for lignin were transformed in a video image using a CCD camera (Panasonic WV-CD15/B) and stored as a 512 × 512 pixel image into a Synapse digital framestore (Synoptics Ltd). Computer analysis was performed with a Dell System 433/ME fitted with a high resolution graphic board, an 80287 math co-processor, and Semper6Plus software for image analysis. The areas of CAD activity and lignification were measured in pixels by cutting out the stained areas with a mouse. The number of pixels for each section was added in order to obtain the total number of pixels for the section. This provides an estimation of the CAD activity and lignification areas as a function of age. CAD activity in tomato prints showed similar intensities for different ages, only the area showing activity changed. By contrast, poplar prints showed quite different intensities in CAD activity with age. An integrated value was used involving the surface and the intensity of the stain (Image-Pro Plus software, Media Cybernetics).

Results

Specificity of the CAD reaction on tissue prints

Cinnamyl alcohol dehydrogenase activity on tissue prints was tested by coupling the reverse reaction of CAD (*in vivo* CAD reduces the coniferyl aldehyde to alcohol) to the reduction of NBT into formazan:

$$\text{coniferyl alcohol} + NADP^+ \Leftrightarrow \text{coniferyl aldehyde} + NADPH + H^+$$

$$NADPH + H^+ + NBT \Rightarrow \text{formazan} + NADP^+$$

In the complete medium a blue-violet stain corresponding to the xylem area was observed in poplar and tomato stems (Plate 1A, D). Controls were done to confirm that the staining observed was due to CAD activity. Without substrate (coniferyl alcohol, or coniferyl alcohol and $NADP^+$), no staining was observed (Plate 1B, E). The colour reaction was strongly reduced when a specific inhibitor of CAD (OH-PAS), was added to the complete medium (Plate 1C, F). The same results were obtained with tomato root (data not shown). This assay will not discriminate between CAD isoforms if the substrate utilized can be used by the different isoenzymes.

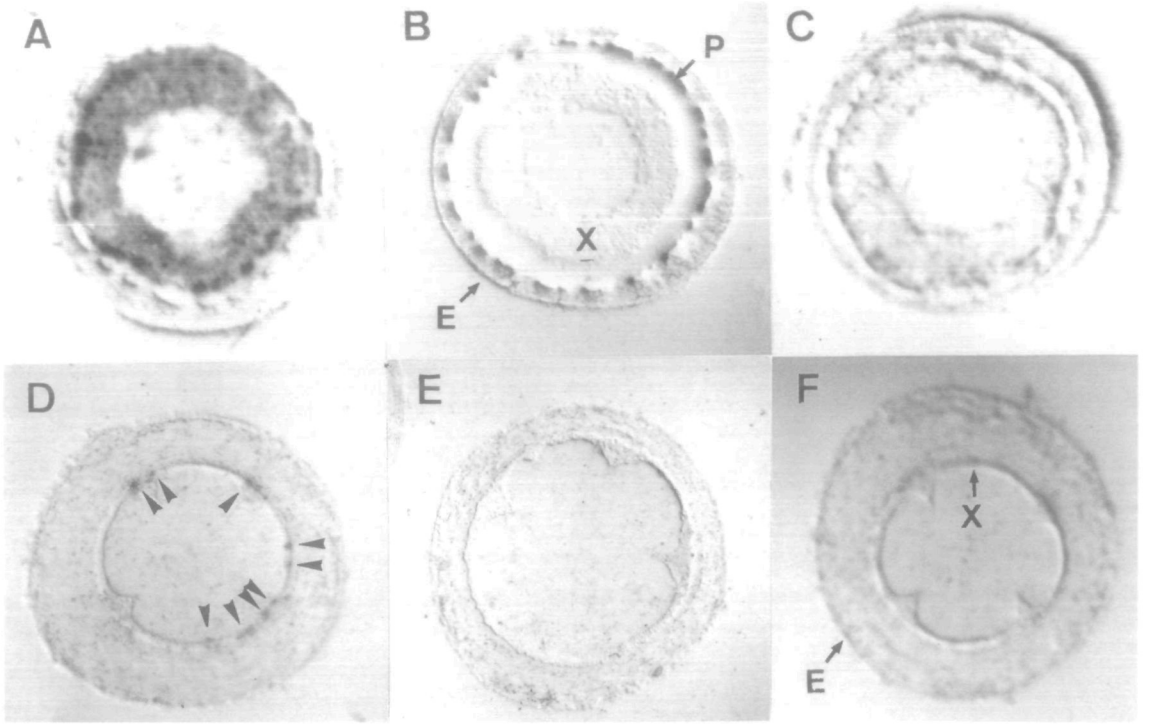

Plate 1. CAD activity on tissue prints of poplar and tomato. Tissue prints of poplar (A–C) and tomato stem (D–F) were incubated to detect CAD activity with the complete incubation medium (A, D); medium without coniferyl alcohol (B, E); and complete medium plus OH-PAS inhibitor (C, F). In tomato stem (D) the CAD activity is indicated by arrowheads Note that the dark areas around the phloem fibres in the control print of poplar (B) do not represent CAD activity, but the shadow produced by the lateral illumination of the print. P: phloem fibres; X: xylem; E: epidermis. Magnification: (A–C) ×50; (D–F) ×30.

Tissue specificity of CAD activity

When CAD activity was visualized on prints of tomato shoot or root it was confined to isolated areas on the xylem ring (Plate 2A, B). No activity was found on mature xylem vessels and fibres. A characteristic of tomato root is the presence of collateral vascular bundles. CAD activity was observed on the periphery of the vascular tissue, that is, where new phloem and xylem differentiate. No activity was detected on mature xylem vessels that fill the centre of the root (Plate 2C, D). In the stem of poplar, CAD activity was localized on developing primary and secondary xylem. This activity increased as the formation of secondary xylem increased (Plate 2E, F). Comparison of the lignified sections stained with phloroglucinol shows that CAD activity was found on all the lignified areas from poplar (Plate 2E, F), but only in particular areas of the tomato lignified regions (Plate 2A, B, C, D).

CAD activity in developing tomato and poplar stems

The development of stem tissues can be studied on a single branch by analysing the anatomy of each internode below the apical meristem. Transverse sections and tissue prints were made from different internodes of poplar and tomato stems. The first internode was the younger one located immediately below the stem apical bud, and internodes were numbered sequentially down to the older parts of the stem.

In tomato stems (herbaceous), CAD activity was not present in the first internode, where very few xylem cells had been formed. CAD activity was found on most of the developing xylem vessels from the tomato stem at the second internode below the apical meristem (Plate 3A, D). As xylogenesis increased in the older stem the activity of CAD increased (Plate 3B, E). In this tissue the activity is shown in patches, however, the stain is found through the breadth of the xylem and not only on the periphery as in the root (Plate 2C). In the more mature stem, the xylem developed to form a continuous ring. CAD activity decreased and it was restricted to isolated areas of the xylem (Plate 3C, F).

As in tomato stem, no CAD activity was found on the first two internodes from poplar. Low CAD activity was first detected in young internodes on the developing xylem (Plate 3G, J). Secondary xylem formation was initiated early in the developing stem and as more cells were laid down, CAD activity increased in intensity. This resulted in a ring of activity on the xylem of intermediate internodes (Plate 3H, K). In mature stem, CAD activity was

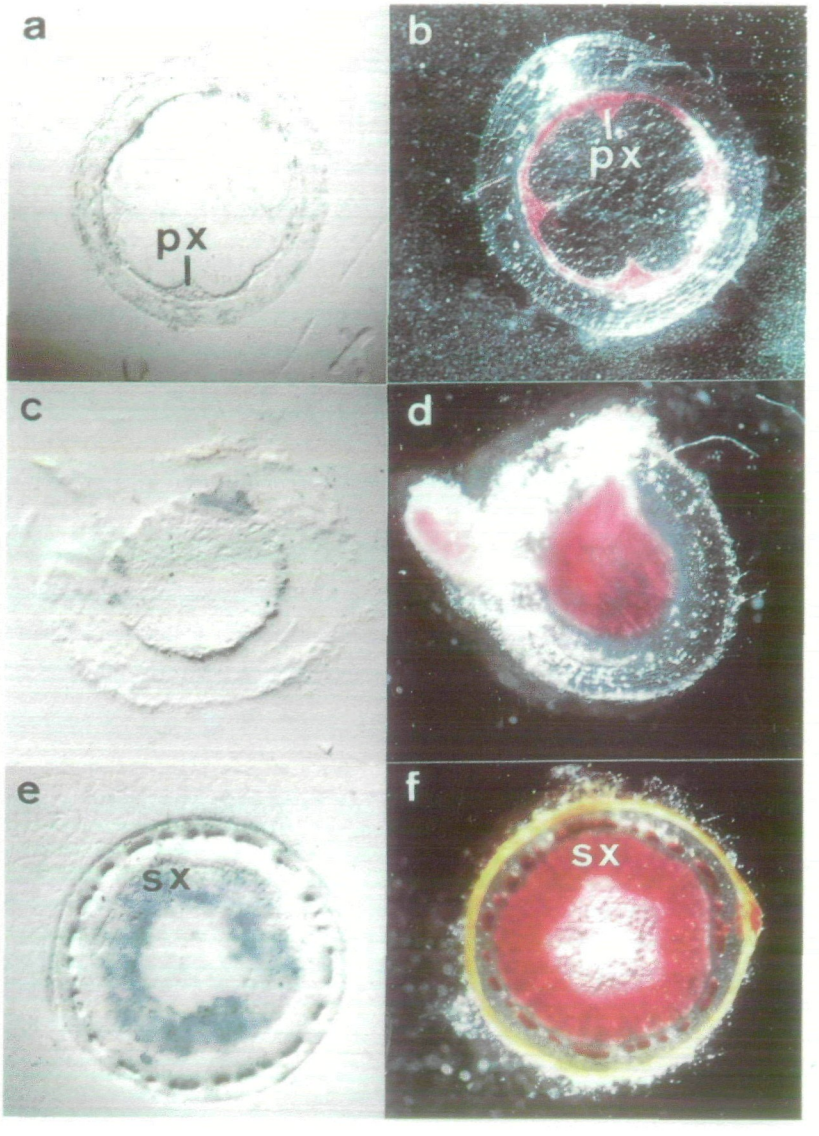

Plate 2. CAD activity and lignification. CAD activity on tissue prints of tomato stem (a), root (c), and poplar shoot (e) is represented by the blue formazan precipitate. Lignin is visualized by the phloroglucinol reaction (red) on hand-sliced tissues of tomato stem (b), root (d), and polar shoot (f). Primary xylem: px; secondary xylem: sx. Magnification: (a, b): ×20; (c–f): ×50.

very intense as more secondary xylem was laid down (Plate 3I, L).

The intensity and extent of lignification is limited in tomato as compared to poplar. Tomato internodes below the apical bud were printed and developed for CAD activity. In parallel, tissue slices from the same internodes were stained for lignin. Through image analysis, the lignified surfaces as well as the surfaces showing CAD activity were measured. Figure 1A shows the results of analysis on the first seven internodes of tomato stem. No activity or lignification was observed in the first internode below the apical bud. The kinetic of lignification in tomato shoot follows a sigmoid pattern. CAD activity, on the other hand, shows a Gauss model with the maximal activity in the 5th internode, in the middle of the sigmoid lignification curve. The kinetics of lignification and CAD activity are quite different in poplar (Fig. 1B). As in tomato stems, there is no lignification or CAD activity in the first internode below the meristem. However, CAD activity increases constantly following the expansion of lignification through the first eight internodes studied.

Discussion

The results obtained with tissue prints from tomato and poplar indicated that the violet formazan precipitate obtained in the experimental conditions used here, was due to coniferyl alcohol dehydrogenase activity. This can

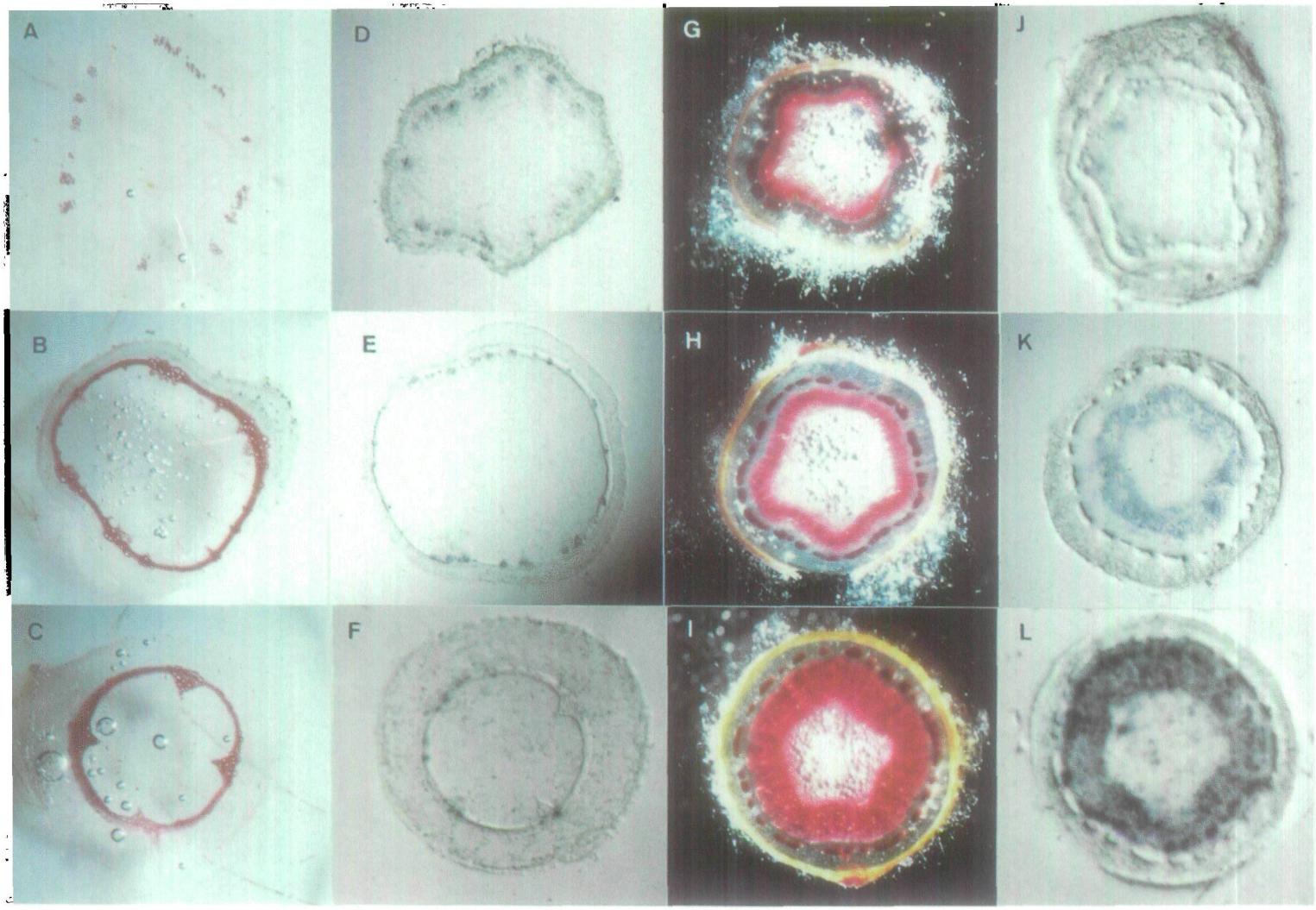

Plate 3. Lignin and CAD activity in developing tomato and poplar stems. Different internodes under the meristem of tomato (A–F) and poplar (G–L) were sectioned and printed. Lignin stain was made on the sections (A–C and G–I) and CAD activity (purple colour) was developed on the prints (D–F and J–L). Young internodes of tomato (A, D ×40) and poplar (G, J ×80); intermediate internodes of tomato (B, E ×20) and poplar (H, K ×50); old internodes of tomato (C, F ×10) and poplar (I, L ×50).

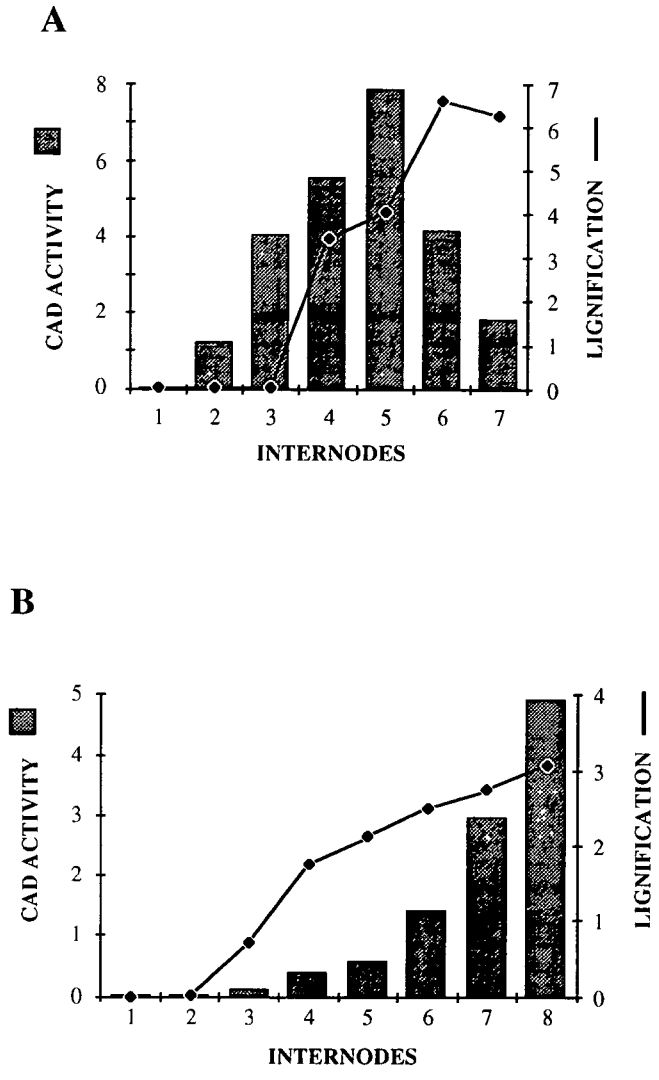

Fig. 1. Quantification of CAD activity and lignification in herbaceous and woody stems. (A) Image analysis of CAD activity ($10^{-3} \times \mu m^2$) and lignification ($10^{-5} \times \mu m^2$) in 1–7 internodes of tomato stem. (B) Image analysis of CAD activity (integrated index: surface × optical density) and lignification ($10^{-6} \times \mu m^2$) in 1–8 internodes of poplar stem In (A) the CAD activity was quantified by the total surfaces since the activity stain had the same intensity in all the prints. In (B) the variations in activity stain make it difficult to evaluate the activity by the surface. An integrated value taking the surface of the stain and the optical density was used in this case for CAD activity.

be seen by the lack of precipitate on the negative control prints (without coniferyl alcohol) compared to those that included the substrate that produced the formazan. The observation of enzyme activities with classical histochemistry often produces high background due to non-specific reactions, for example, other alcohol dehydrogenases using $NADP^+$ and endogenous substrates. On the other hand, the amount of CAD activity observed on cryosections, when endogenous substrates are present, is difficult to assess (Baudracco *et al.*, 1993). One of the advantages of the tissue printing method is that the endogenous substrate (coniferyl alcohol or aldehyde, or other alcohols) did not bind to the nitrocellulose membrane and could be washed off the prints completely. This procedure avoids the background reactions that are often observed in classical histochemical methods and provides an idea of CAD activity that is a better reflection of the total amount of CAD present. Further proof that the colour reaction was caused by CAD activity came from the inclusion of the inhibitor OH-PAS. Previous studies by Grand *et al.* (1985) have shown that OH-PAS inhibit CAD activity very specifically *in vivo* and *in vitro*.

Localization of CAD activity correlates to lignification in both tomato and poplar stems. The degree by which xylem cells became lignified as the tomato stem matured was significantly less than in poplar stem. This is due to the small amount of secondary growth that occurs in tomato shoots. CAD activity was found on the protoxylem of younger internodes and on restricted areas of developing xylem in older ones. The location of CAD in tomato root was limited to the differentiating xylem on the periphery of the lignified region.

In the woody stems of poplar, the formation of secondary xylem is a prominent feature that causes the stem to increase in girth. Another characteristic of poplar is the large amount of lignification that is associated with primary and secondary growth which provides the plant with enough rigidity to support its growth. Phloroglucinol staining for lignin indicated that most lignin was deposited on the secondary xylem where CAD activity was very intense. The more the stem matured and secondary growth in poplar stems increased, the more intense the activity of CAD observed. This was surprising since the secondary xylem is formed mostly by dead cells in which the cell wall is the only part of the cell in the tissue. It seems reasonable to suppose that CAD activity was present in living cells where $NADP^+$ and the substrate were provided by the metabolism. When the CAD promoter drove a GUS cDNA in transgenic poplar, the gene was strongly expressed in xylem ray cells (Feuillet *et al.*, 1995). This fully agrees with the localization of CAD with antibodies raised against the *Eucalyptus* enzyme (Goffner *et al.*, 1992) and with the strong CAD activity found through tissue prints in the secondary xylem of poplar stem. The possibility cannot be excluded that other isoforms of CAD could be present in the vessel elements giving a uniform staining through the secondary xylem. However, there is support for the idea that monolignols are exported from their site of synthesis in the parenchyma cells towards the site of assembly in the vessel elements (Feuillet *et al.*, 1995). The tissue prints obtained from the secondary xylem in these experiments, however, did not give enough anatomical details to discriminate within the different cell types.

A discrepancy is observed when the kinetics of both lignin deposition and CAD activity are compared in

tomato and poplar stems (Fig. 1). Surprisingly, CAD activity drops after the fifth internode in tomato whereas the lignin deposition still increases. A possible explanation of this observation is that lignin deposition results from a cumulative process and does not reflect a dynamic situation at a specific time. The results obtained would mean that the flux of monolignol synthesis is not maximum in the older internodes.

An image of the tissue distribution of different molecules is obtained by pressing the cut surface of a tissue section on to a suitable membrane or film and developing the print with appropriate reagents (Varner and Ye, 1994). Tissue printing allows the detection of molecules that can be transferred and can bind to the membrane. Insoluble materials or molecules not retained by the membrane cannot be localized by this technique. On the other hand, no restrictions have been found concerning differences in cell type (Reid *et al.*, 1992). One of the limitations found with tissue prints is the identification of the cell type in which a particular molecule is localized, when the cell type is restricted to one or two layers of cells. This has been observed with the expression of extensin mRNA in the cambium layer of soybean stem (Ye and Varner, 1991) and in this report with the parenchyma ray cells from the secondary xylem. Tissue printing has not been widely used for enzyme activity (Reid *et al.*, 1992) and experience has shown that this simple technique can be extremely helpful and specific for CAD activity. Even if tissue prints do not provide anatomical details, it gives good information on the localization of particular molecules at tissue level (not always at cell type level) on a whole section or organ. The combination of classical histochemistry, tissue printing and image analysis proved to be particularly useful for the study of enzyme activity in relation to lignin synthesis in plant development. It was possible to show that tomato and poplar had different patterns of CAD activity correlated to different anatomical characteristics of lignified tissues. Seasonal changes in the activity of enzymes involved in lignification in woody species (Bugos *et al.*, 1991) could easily be studied by this technique.

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