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Rapid Communication

Cinnamyl Alcohol Dehydrogenase: Identification of New Sites of Promoter Activity in Transgenic Poplar[1]

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Stem sections from poplar that were stably transformed with a eucalypt cinnamyl alcohol dehydrogenase promoter-β-glucuronidase construct were prepared by using either a technique routinely used in herbaceous species or a technique designed to take into account the particular anatomy of woody plants. Although both preparation techniques confirmed the pattern of expression previously observed (C. Feuillet, V. Lauvergeat, C. Deswarte, G. Pilate, A. Boudet and J. Grima-Pettenati [1995] Plant Mol Biol 27: 651–657), the latter technique also allowed the detection of other sites of promoter activity not revealed by the first technique. In situ hybridization confirmed the expression pattern obtained with the second sample preparation technique.

Lignin is a complex phenolic polymer that reinforces the walls of certain cells in higher plants. It is mainly found in the vascular tissues, where its hydrophobicity waterproofs the conducting cells of the xylem and its rigidity strengthens the supporting fiber cells of both the xylem and phloem. Lignin also plays an important role in protection and defense; it is constitutively present in the outer protective bark layer (periderm) of many tree species, and is synthesized in response to pathogen attack (Vance et al., 1980; Bostock and Stermer, 1989) and mechanical wounding (Hawkins and Boudet, 1996). However, in wood pulp production, lignin has to be removed by harsh chemical treatments that are costly in both financial and environmental terms (Dean and Eriksson, 1992). Much interest has therefore been generated by the recent successes in modifying and/or reducing the lignin content of plants through a genetic engineering approach (for a review, see Boudet and Grima-Pettenati, 1996).

Genetic modifications such as these have utilized an antisense strategy and concentrated on a number of enzymes involved in the biosynthesis of lignins, including CAD (EC 1.1.1.95), which catalyzes the reduction of hydroxycinnamaldehydes to give hydroxycinnamyl alcohols, or "monolignols," the monomeric precursors of lignin. However, as stated above, lignin also plays an important role in the defensive response of plants, and it is possible that plants down-regulated in CAD activity could be more susceptible to pathogen attack, even though their morphological development is apparently unaffected. Biotechnology programs concerned with modifying lignin content are then faced with two potentially contradictory scenarios, where, on the one hand, "high" levels of lignin cause problems (pulp production), and on the other hand, "low" levels could adversely affect other desirable traits such as mechanical strength and pathogen resistance. Some potential problems could be avoided, however, by targeting the antisense transgene to selected tissues through the use of tissue-specific promoters.

CAD is directly involved in lignification and has been purified from the xylem tissue of a number of tree species (Goffner et al., 1992; O'Malley et al., 1992) and the corresponding mRNA is strongly expressed in the same tissue in eucalyptus (Grima-Pettenati et al., 1993). Observations such as these naturally suggest that the CAD promoter would be a good candidate for targeted transgene expression. However, CAD activity has also been reported in apparently nonlignified tissues (O'Malley et al., 1992), and monolignols are also used for the synthesis of nonlignin products (Lewis and Yamamoto, 1990). It is possible that such polyvalency could be reflected in an expression in a number of different tissues, which would suggest that the CAD promoter may be less useful for targeting transgene expression than previously thought. The fact that CAD is also induced by fungal elicitors (Messner and Boll, 1993) and stress (Galliano et al., 1993), and that "defense lignin" is formed in response to mechanical wounding (Hawkins and Boudet, 1996) reinforce the importance of thoroughly characterizing the expression pattern of the CAD (or other) promoter before using it to target transgene expression. The analysis of poplar plants transformed with a eucalypt CAD 2 promoter-GUS construct allowed our laboratory to address such questions and to obtain, for the first time to

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Abbreviation: CAD, cinnamyl alcohol dehydrogenase.

our knowledge, preliminary data on the tissue-specific activity of this important promoter in different organs in a woody tree species (Feuillet et al., 1995). Although the use of reporter gene histochemistry to study gene expression has now become routine for many plant species, its use is still relatively uncommon in trees. In this paper we investigate the detailed cell-specific activity of the CAD 2 promoter and identify several new sites of promoter activity. We also show that a standard method of preparing sections for determining reporter gene expression patterns, commonly used with herbaceous species, is unsuitable for use in woody plants.

MATERIALS AND METHODS

Transgenic poplar plants (corresponding to INRA clone 717 1B4 *Populus tremula* × *Populus alba*) containing the *Eucalyptus gunnii* Hook. CAD 2 promoter fused to the *uid a* gene (GUS) were used for characterization of CAD 2 expression patterns. Transformed plants were treated as described previously (Feuillet et al., 1995) and all experiments were performed on plants that had been grown for 3 months following acclimation. For comparisons of the different preparation techniques, stem samples were taken from the same internode.

Promoter-GUS Histochemistry

Two different sample preparation techniques were used to prepare sections for investigating the expression pattern of the CAD 2 promoter. Sample preparation technique 1 used small pieces (approximately 5 mm long) of poplar stem excised from the stem internode, prefixed under vacuum (30–45 min) in cold 0.5% paraformaldehyde in 100 mM potassium phosphate buffer, pH 7.0, rinsed twice in the same buffer, and then kept at 4°C. Transverse sections (50–100 μm) were cut by hand or on a vibratome (Bio-Rad) and incubated in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid reaction medium as described by Feuillet et al. (1995).

Sample preparation technique 2 differed fundamentally from sample preparation technique 1 by the fact that sections were prepared after the color reaction had been allowed to develop in a 2-mm-thick slice of poplar stem (in the first technique sections were cut before color development). Samples were pretreated for 30 min in cold 90% acetone to prevent any potential wound-induced induction of the CAD 2 promoter (Hemerly et al., 1993) and to facilitate substrate penetration. Samples were then rinsed twice in 100 mM potassium phosphate buffer (pH 7.0) and incubated from 3 h to overnight in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid reaction medium in darkness at 37°C until sufficient blue color had developed. Samples were then fixed in cold (4°C) 4% paraformaldehyde, 0.5% gluteraldehyde in PBS (100 mM NaCl, 10 mM PO_4), pH 7.0, for 48 h. After fixation samples were divided into two groups, depending upon whether they were going to be embedded in paraffin or sectioned by hand/vibratome. Vibratome/hand-sectioned samples were dehydrated to 90% EtOH in a graded EtOH series and left overnight to remove chlorophyll. Sections were then either cut by hand directly or on a vibratome. Samples to be embedded in paraffin were dehydrated in a graded EtOH/tertiary butyl alcohol series and then embedded following several changes of paraffin (Paraplast Plus, Sherwood Medical Co., St. Louis, MO). Semithin sections (10 μm) were then cut on a rotary microtome.

In Situ Hybridization

In situ hybridization was performed largely following the procedure of Cox and Goldberg (1988). Small pieces of tissue excised from stems were treated with cold 90% acetone for 30 min and then fixed for 48 h at 4°C in 4% paraformaldehyde, 0.5% gluteraldehyde in PBS (100 mM NaCl, 10 mM PO_4 buffer, pH 7.2). Tissue samples were then dehydrated in a graded EtOH/tertiary butyl alcohol series and embedded in paraffin (Paraplast Plus, Sherwood Medical Co.). Semithin sections (10 μm) were cut on a rotary microtome and attached to poly-L-Lys-coated slides before being deparaffined in histolemon-erba (xylene-substitute, Carlo-Erba, Milan, Italy) and rehydrated in a graded EtOH series. Slides were pretreated for 30 min with proteinase K (2 μg ml^{-1}) at 37°C in 100 mM Tris/50 mM EDTA, pH 7.5, and then rinsed in 1 × PBS. Sections were then incubated for 10 min in 0.25% acetic anhydride in freshly prepared 0.1 M triethanolamine, pH 8.0, dehydrated in a graded EtOH series, and dried under vacuum before hybridization. Sections were hybridized overnight at a temperature of 42°C or 45°C with radiolabeled (^{35}S) antisense or sense RNA probes transcribed from a 1.4-kb eucalyptus CAD 2 cDNA cloned into a pGEM (Promega) transcription vector. Hybridization buffer was 50% formamide, 10% dextran sulfate, 70 mM DTT, 1 × Denhardt's solution, 300 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA (pH 7.5), 0.5 mg ml^{-1} Poly-A, 0.15 mg ml^{-1} tRNA. Following hybridization, sections were washed in 4 × SSC, 5 mM DTT and treated with RNase A (50 μg ml^{-1}) for 30 min at 37°C. Sections were then washed under low (2 × SSC, room temperature) and high (0.1 × SSC, 60°C) stringency conditions before dehydration in a graded EtOH series and dried under vacuum. Dried slides were coated in Kodak NTB 2 nuclear track emulsion diluted 1:1 with 0.6 M ammonium acetate and exposed for 2 to 4 weeks at 4°C before developing, staining (0.02% toludine blue O), and mounting.

RESULTS

Measurements in longitudinal sections of poplar stems showed that fusiform initials in the poplar vascular cambium were 300 to 400 μm long, whereas elongating fibers in the phloem were often longer than 1 mm. These results suggest to us that if samples were prepared using a common technique (sample preparation technique 1) in which 50- to 100-μm sections are cut before color development, elongated vessels/fibers would be cut in several places, resulting in the formation of "open tubes" and the loss of cytoplasmic contents. The initial prefixation treatment is most likely insufficient to affect this process, and under such conditions little or no GUS activity will be

detected in these "empty" cells. To investigate this hypothesis we decided to use sample preparation technique 2, which consists of color development in thick (approximately 2 mm) stem slices before fixation and sectioning. Sections from poplar that were stably transformed with a CAD 2-GUS construct were produced using both preparation techniques and the gene expression patterns were compared.

Both sample preparation technique 1 (Fig. 1a) and technique 2 (Fig. 1b) indicated that the CAD promoter was mainly active in the vicinity of "lignifying" cells in the bark (phloem fiber cells, sclerids, and periderm) and in the ray cells of the xylem. In addition, the second technique (Fig. 1b) revealed that the promoter was also very active in the vascular cambium/differentiating xylem. Examination of sections prepared with the first preparation protocol at higher magnification (Fig. 1c) clearly indicates that the blue coloration is limited to the ray parenchyma cells. In contrast, however, sections prepared using the second protocol (Fig. 1d) show that strong promoter activity can be detected in all cells of the vascular cambium/differentiating xylem, as well as in the rays and more mature xylem cells.

Identical differences in expression patterns were obtained when a number of different transformants were examined, suggesting that the observed differences are related to the preparation protocol used and not to transformation events and/or environmental factors. Nevertheless, to be sure that the differences in the expression pattern of the CAD 2 promoter observed with the two preparation techniques were not artifactual, we used in situ hybridization to localize CAD 2 mRNA and to confirm the CAD 2 expression pattern. Although high background proved to be a problem, hybridization of poplar stem sections with ^{35}S-labeled CAD 2 antisense probe (Fig. 1e) revealed that CAD 2 mRNA could be localized to the vascular cambium/differentiating xylem and ray cells. Whereas heavy concentrations of silver grains could also be observed over xylem axial parenchyma, suggesting that CAD 2 mRNA is also present in these cells, little or no GUS activity was observed in such cells and further investigation is necessary to see whether CAD 2 is expressed in axial as well as radial parenchyma. Control sections incubated with the sense probe (data not shown) showed no specific hybridization.

Differences in the expression pattern of samples prepared using the two different protocols were also observed in secondary phloem fibers. Although both techniques indicated CAD 2 promoter activity in parenchymatous cells surrounding groups of phloem fibers, the second technique also showed that GUS activity could be observed in the lumens of individual fiber cells (Fig. 1f). The latter pattern of expression was again confirmed by in situ hybridization (Fig. 1g). The higher resolution obtained by embedding material and cutting semithin sections also enabled the detection of CAD 2 promoter activity in other specific cell types. Figure 1f shows that the CAD 2 promoter is active in "chambered crystalliferous cells" (Trockenbrodt, 1995), which are closely associated with phloem fibers. Isolated crystal-containing cells (druses) expressing GUS activity could also sometimes be observed in the outer cortex (data not shown). Histochemical tests to determine whether these isolated cells were lignified proved inconclusive. Figure 1b shows that the CAD 2 promoter is active in the parenchyma tissue associated with the large metaxylem vessels of the primary xylem; promoter activity was also observed in pith tissue close to the primary xylem. Figure 1, d and f, suggests that the promoter is active in nonlignified phloem companion cells, and Figure 1h shows that there is also a strong activity associated with the phellogen and phelloderm layers of the periderm.

DISCUSSION

Measurements of the length of axially elongated cells in poplar stems led us to hypothesize that a standard method commonly used in preparing sections for reporter gene histochemical studies in herbaceous species might be unsuitable for similar studies in woody plants. This hypothesis was validated by the differences in the pattern of CAD 2 promoter activity obtained when the two different sample preparation techniques were used. In situ hybridization largely confirmed the expression pattern obtained with the second preparation protocol. This is an important observation because the current growth in the number of available promoters and transformable tree species implies that reporter gene histochemistry will become a widespread tool for investigating patterns of gene expression in woody plants. Our results indicate that the anatomy of trees must be taken into account when analyzing gene expression patterns with such a technique.

The observation made by Feuillet et al. (1995) that the CAD 2 promoter was apparently not active in lignifying cells (vessels and fibers) but was instead active in adjacent parenchyma cells led them to suggest that the lignification of a cell's wall occurred through a process of "cell cooperation." In this process the monolignols necessary for lignification of a cell are not produced in that cell, but in associated parenchyma cells, and then exported to the cell undergoing lignification. Our results strongly suggest that the CAD 2 promoter is, in fact, also active in cells undergoing lignification and that the lignin monomers necessary for the synthesis of the lignin polymer are produced in both types of cells. Convincing evidence for the latter hypothesis is provided by the work of Leinhos and Savidge (1993), who localized coniferin (glycosylated form of the monolignol coniferyl alcohol) to both large and small protoplasts isolated from differentiating xylem of *Pinus banksiana* and *Pinus strobus*. This result suggests that fusiform initials (giving rise to large protoplasts) as well as ray initials (small protoplasts) are capable of storing and, presumably, of synthesizing monolignols. Although studies (Shufflebottom et al., 1993; Subramaniam et al., 1993; Smith et al., 1994; Ye et al., 1994) on the expression patterns of other genes involved in monolignol synthesis have permitted the expression to be localized to the differentiating xylem, they have not made the distinction between cells undergoing lignification and associated parenchyma cells. More detailed analyses are necessary to see whether such genes exhibit a similar expression pattern to CAD 2, but recent results (Lacombe et al., 1997) suggest that another key

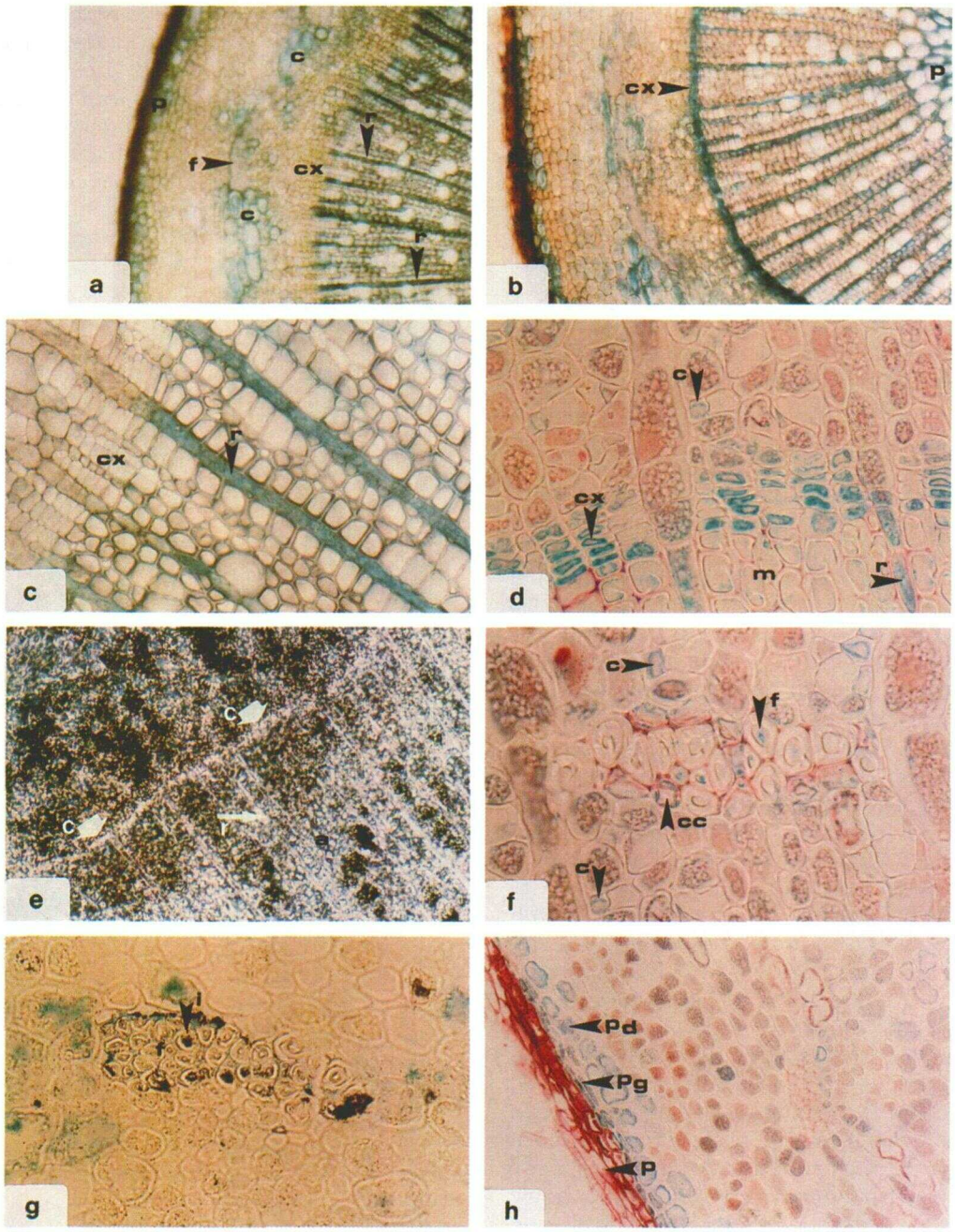

Figure 1. a, CAD 2 promoter-GUS histochemistry, transverse section of poplar stem. Sample preparation technique 1 (PT1), hand section, ×100; GUS activity (blue coloration) is present in xylem rays (r), parenchyma cells (c), surrounding groups of phloem fibers (f), and cells interior to periderm (p). No coloration can be seen in the vascular cambium/differentiating xylem zone (cx). b, Same as in (a), but using sample preparation technique 2 (PT2), vibratome section, ×100; GUS expression pattern similar to (a) above plus strong activity in vascular cambium/differentiating xylem zone (cx) and primary xylem/pith zone (p). c, Same as in (a), vibratome section, ×400; GUS activity restricted to rays (r), no activity in vascular cambium/differentiating xylem (cx). d, Same as in (b), paraffin-embedded semithin (10 μm) section counterstained with Safranin O, ×400; GUS activity present in cells of vascular cambium/differentiating xylem (cx) and xylem ray cells (r). Faint activity can also be detected in remains of cytoplasm in more mature xylem cells (m) undergoing autolysis and lignification, as well as in phloem companion cells (c). e, In situ hybridization with CAD 2 antisense probe, ×200, dark-field optics; above background signal (silver grains) can be seen in vascular cambium/differentiating xylem (c), ray cells (r), and axial parenchyma cells (a). f, CAD 2 promoter-GUS histochemistry (PT2, paraffin), ×400; GUS activity present in lumens of phloem fiber cells (f), chambered crystalliferous cells (cc) surrounding phloem fibers, and companion cells (c). g, In situ hybridization with CAD 2 antisense probe, ×400, bright-field microscopy; dark points indicate presence of CAD 2 mRNA inside (i) and around phloem fiber cells (f). h, CAD 2 promoter-GUS histochemistry (PT2, paraffin), ×200; GUS activity present in phellogen (pg) and phelloderm (pd) layers of periderm. P, Phellem.

enzyme of the lignin biosynthetic pathway, cinnamoyl coenzyme-A reductase, is also expressed in both lignifying cells and ray parenchyma cells in the vascular cambium/differentiating xylem zone of poplar stems.

Our results also clearly show that the CAD 2 promoter is active in other cells and tissues apart from the xylem. This observation is important for a number of reasons. First, it indicates that this promoter would not make an ideal candidate for the specific targeting of transgene expression to xylem tissue only. Second, it provides us with further information concerning the biological role of the CAD enzyme. The close spatial association between promoter activity and lignified tissues clearly confirms the involvement of the CAD 2 enzyme in the lignification process. The strong promoter activity in the periderm confirms previous studies (Hawkins and Boudet, 1994) of CAD 2 activity in such tissues and is presumably associated with the formation of lignified (and possibly suberized) cells in the phellem. Such observations suggest an important role for this enzyme in the generation of protective dermal layers in woody plants. The observed promoter activity in the primary xylem/pith zone is probably linked to the continued lignification of the metaxylem vessels and the formation of sclerenchyma, which can develop in the pith of older stems and branches. Whereas CAD 2 promoter activity could to some extent be predicted in the above tissues, its activity in phloem companion cells was unexpected, because such cells are apparently not lignified. It is possible that CAD activity in such cells is related to the production of monolignols for "non-lignin" products such as lignans (Lewis and Yamamoto, 1990). Certainly, the detection of CAD activity in the apparently nonlignified megagametophytes of loblolly pine (O'Malley et al., 1992) indicates that CAD activity is not necessarily spatially associated with lignification.

In conclusion, our results have revealed new sites of CAD 2 expression and contribute to our understanding of the process of lignification while raising interesting questions concerning the other biological roles of this important enzyme. In addition, we have demonstrated the importance of using a sample preparation technique for reporter-gene histochemistry adapted to the anatomy of the plant in question.

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