PHYSIOLOGIA PLANTARUM 117: 93–99. 2003
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ISSN 0031-9317

Cellular and subcellular localization of the lignin biosynthetic enzymes caffeic acid-*O*-methyltransferase, cinnamyl alcohol dehydrogenase and cinnamoyl-coenzyme A reductase in two monocots, sugarcane and maize

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Received 25 March 2002; revised 9 August 2002

Cinnamoyl-coenzyme A reductase (CCR, EC 1.2.1.44), cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) and caffeic *O*-methyl transferase (COMT, EC 2.1.1.68) are three enzymes of the biosynthetic pathway of lignin. This pathway has been mostly studied in dicots but is less well known in monocots. We report the histochemical localization of these enzymes in maize (*Zea mays* L.) and sugarcane (*Saccharum officinarum* L.). Antibodies directed against maize COMT and sugarcane CAD and CCR were generated. Western experiments were performed with the 3 antibodies, and immunolocalization experiments were performed with anti-CAD and anti-COMT antibodies. The three enzymes were present in actively lignifying tissues, such as maize roots and mesocotyls, and sugarcane roots and stems. At the cellular level, CAD and COMT were mainly found in xylem. No major differences could be seen in the distribution patterns of both enzymes, except that CAD was detected in the endodermis, while COMT was not. At a subcellular level, the three enzymes appeared to be mainly cytosolic.

Introduction

Lignins are phenolic polymers of the secondary cell walls of vascular plants. They are situated in supporting and conductive tissues, such as xylem or sclerenchyma, where they provide mechanical strength, impermeability and defence against wounding and infection (Lewis and Yamamoto 1990). Lignins result from the oxidative polymerization of hydroxycinnamyl alcohols, called monolignols: *p*-coumaryl, coniferyl and sinapyl alcohols. The relative abundance of these monomers in lignin depends on the plant species, tissue, developmental stage and subcellular location (Lewis and Yamamoto 1990). For instance, lignins isolated from dicotyledonous angiosperms contain mainly coniferyl alcohol and sinapyl alcohol, while monocotyledonous lignins are composed of all three monolignols. These alcohol monomers are derived from the phenylpropanoid metabolic grid (Fig. 1), through which successive reductions, hydroxylations and methylations can occur. A lot of studies have been currently dedicated to evaluate which of these reactions do occur in vivo, leading to a new scheme in dicot species (Humphreys and Chapple 2002). Whether or not this new scheme is correct for monocot species is not known.

At a cellular level, several questions also remain unanswered. It is not clear which type of cells actually synthesize monolignols. Do only lignifying cells produce the monomers ('cell autonomy concept') or do neighbouring cells produce monolignols that are transported to the lignifying cells ('cell co-operation concept')? Besides, the subcellular localization of the synthesis of the phenylpropanoid units is still unclear. Data based on radioactive

Abbreviations – 4CL, 4-coumarate CoA ligase; C3H, coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoA3H, caffeoyl CoA 3-hydroxylase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase.

labelling of cells fed with cinnamic acid precursors suggested that they were exported in ER-derived vesicles (Pickett-Heaps 1968). In agreement with such a hypothesis, the cinnamyl alcohol dehydrogenase was located both in the cytosol and membranes of ER and Golgi-derived vesicles in poplar shoots (Samaj et al. 1998), and in Zinnia mesophyl cells (Nakashima et al. 1997). Cinnamate 4-hydroxylase was associated with the ER and Golgi derived vesicles (Smith et al. 1994, Ro et al. 2001). Other enzymes have, however, been localized in the cytosol, such as an isoform of phenylalanine ammonia-lyase (Smith et al. 1994, Rasmussen and Dixon 1999), and caffeoyl CoA *O*-methyl transferase in poplar, Zinnia stems and alfalfa (Ye 1997, Kersey et al. 1999, Chen et al. 2000), and caffeic *O*-methyl transferase in alfalfa (Kersey et al. 1999).

No cellular localization data is available for monocot species. In order to address this issue, we studied the localization of cinnamoyl-coenzyme A reductase (CCR, EC 1.2.1.44), cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) and caffeic *O*-methyl transferase (COMT, EC 2.1.1.68) in two monocots, maize and sugarcane. Antibodies were raised against maize COMT and sugarcane CAD and CCR. Western blot and immunolocalization experiments were performed with sugarcane and maize tissues and showed that these three proteins could be present in different tissues.

Materials and methods

Plant material

Dry seeds of maize inbred line W64A were germinated in a growth chamber on wet Whatman paper under 16-h light (28°C)/8-h dark (26°C) conditions.

Sugarcane Jaronú 60-5 was micropropagated in vitro, and after a period of around 2–3 months in a root-generating medium, plants were transferred to soil in order to obtain enough plant material.

Cloning of maize COMT, sugarcane CAD and sugarcane CCR cDNAs in an expression vector

The 1.1 kb *Xho*I-*Eco*RI *COMT* fragment from maize cDNA (Collazo et al. 1992) and the 1.1 kb *Bgl*II-*Kpn*I *CAD* fragment from sugarcane cDNA (Selman-Housein et al. 1999) were cloned behind the 6×His tag linker of the pTrcHisA vector (Invitrogen, Barcelona, Spain), to obtain the plasmids pTrcHis*COMT* and pTrcHisA*CAD*, respectively. To over-express the sugarcane CCR protein, the 1.2 kb *Pvu*II-*Eco*RV fragment from the plasmid p*CCCR11* (Selman-Housein et al. 1999) was first cloned in the *Hin*cII site of pUC18 vector and the *CCR* fragment was excised from the properly orientated clone by digestion with *Bam*HI and *Hin*dIII and cloned into the vector pTrcHisB (pTrcHisB*CCR*).

Purification of recombinant proteins

All recombinant proteins were purified from *Escherichia coli* under denaturing conditions using a Ni (II)-IDA-Sepharose-4B column (The QIAexpressionist 1992; QIAGEN Inc. USA). After the elution of proteins at low pH, 20 µl of each collected fraction were analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), and fractions containing the peak of recombinant protein were pooled. The pH was adjusted to 8 and the pool was dialysed overnight against 1000 volumes of 1× PBS buffer (0.14 *M* NaCl, 2.7 m*M* KCl,

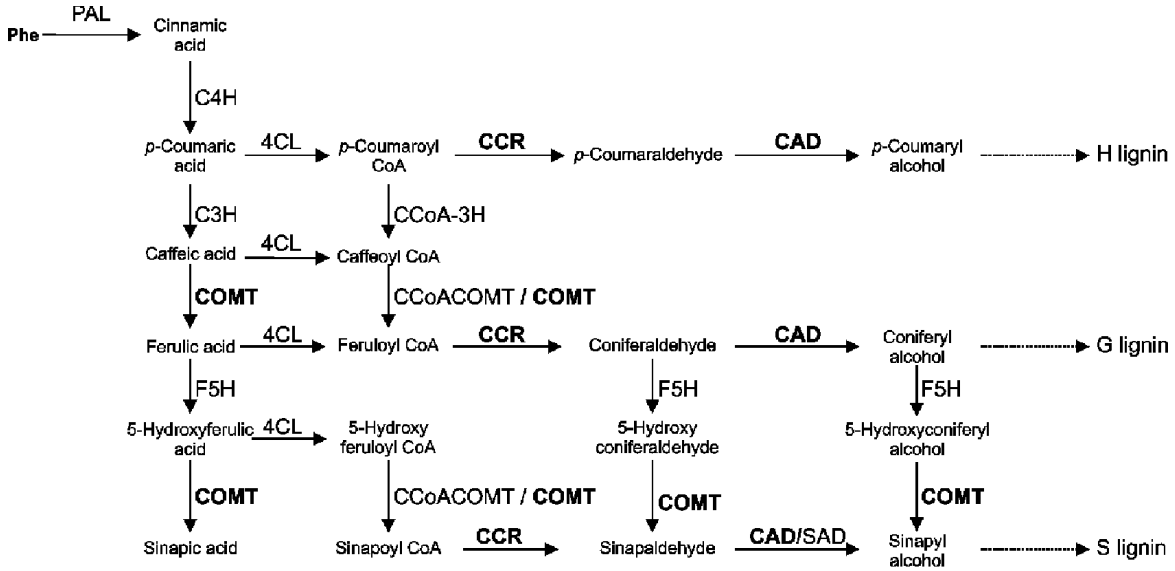

Fig. 1. Biosynthetic pathways to monolignols. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, coumarate 3-hydroxylase; 4CL, 4-coumarate CoA ligase; COMT, caffeic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase; CCoA3H, caffeoyl CoA 3-hydroxylase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase. Enzymes studied are written in bold.

1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, pH 7.4). Protein concentrations were measured according to Bradford (1976). The concentration of recombinant protein and its purity were determined by using a densitometer after the staining of polyacrylamide gels with Coomassie blue R-250 reagent.

Generation of polyclonal antibodies in rabbits

The purified recombinant proteins were used to produce polyclonal antibodies in rabbits. Three hundred μg of the purified protein in 500 μl of 1× PBS buffer were emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich Quimica S.A., Madrid, Spain) and immediately injected at multiple subcutaneous sites in rabbits. Three weeks later the animals were again subcutaneously injected with a similar antigenic emulsion. The booster injections were repeated two more times with 3-week intervals, the protein solution being emulsified with an equal volume of Freund's incomplete adjuvant. Ten ml of blood was harvested before the first injection, and 50 ml was harvested 10 days after the last booster. The antisera were collected and the antibodies were purified by ammonium sulphate fractionation followed by a DEAE-batch. Two rabbits were used for each antigen. The sera and the purified antibodies were tested against the purified recombinant protein and against plant tissues in Western blot experiments. From the two antisera, the one leading to no cross-reaction was chosen for further experiments.

Protein extraction and cell fractionation

Frozen plant tissues were ground to a fine powder. The lipids were extracted 3 times by acetone:hexane (59:41), and acetone, until the powder exhibited a pale-brown colour. The powder was then frozen in liquid nitrogen and lyophilized. The proteins of the lyophilized tissues were extracted with extraction buffer (E Buffer: 50 m*M* Tris-HCl, pH 8, 10 m*M* NaCl, 1% (w/v) SDS) to which antiproteases had been added (100 μ*M* PMSF, 10 μg ml^{-1} aprotinin, 1 mg ml^{-1} E-64, 1.46 μ*M* pepstatin), and pelleted by centrifugation (18000 *g*, 10 min, 4°C). The protein concentration was determined by the method of Bradford using a prepared reagent (Bio-Rad, Barcelona, Spain). Bovine serum albumin was used as standard.

To separate the membrane fraction from the soluble fraction, fresh tissue was ground in a mortar in a solution of 100 m*M* Tris-HCl pH 7.9, 12% (w/v) sucrose, 1 m*M* EDTA, to which antiproteases had been added (100 μ*M* PMSF, 10 μg ml^{-1} aprotinin, 1 mg ml^{-1} E-64, 1.46 μ*M* pepstatin). The mixture was centrifuged for 10 min at 4000 *g*, the supernatant was then centrifuged for 10 min at 15 000 *g*, and the resulting supernatant was then centrifuged for 1 h at 15 000 *g*. The pellet was designated as the microsomal fraction, and resuspended in E buffer. The proteins in the soluble fraction were precipitated by 15% (w/v) trichloroacetic acid (TCA) for 20 min at 4°C, and centrifuged (10 min, 15 000 *g*) and the pellet rinsed with acetone. The microsomal fraction was resuspended in the same volume of the same buffer. Both the microsomal and soluble fractions were used for Western blotting experiments.

Western analysis

Proteins were separated on a 12% SDS-PAGE and electrotransferred onto nitrocellulose membrane (Schleicher & Schuell, Barcelona, Spain). The membrane was incubated in the blocking buffer (5% non-fat milk in PBS) overnight at 4°C, and then incubated for 4 h at room temperature with anti-CAD, anti-CCR or anti-COMT IgG (diluted 1/750, 1/400 and 1/500, respectively). After the membrane was washed, it was incubated with peroxidase-conjugated goat antirabbit secondary antibodies (dilution 1:15000 in PBS) for 1 h at room temperature. Signals were detected with chemiluminescent reaction reagents using the ECL kit (Amersham Biosciences Europe GmbH, Barcelona, Spain) according to the manufacturer's protocol.

Phloroglucinol staining

A sliding microtome was used to cut sections of 250 μm from maize and sugarcane roots and maize mesocotyl. Sections were stained in 1% (w/v) phloroglucinol and 70% (v/v) ethanol (5 min). Excess phloroglucinol was removed and replaced with 18% HCl.

Immunolocalization of CAD, COMT and CCR by light microscopy

Small pieces of tissues were cut in a drop of 80:3.5:5 (v/v/v) ethanol:formaldehyde:glacial acetic acid, and immediately transferred to a vial containing the same fixative solution and vacuum infiltrated for 10 min. After 1 h at room temperature the fixative solution was changed and the vials were left at 4°C for 1 week. The samples were then rinsed in 70% ethanol, and dehydrated in ethanol (80%, 90% and 100%) and tert-butanol (25%, 50% in ethanol, 100%) series. The samples were then embedded in paraffin at 60°C. Eight μm sections were cut with a microtome and placed on slides coated with 50 μg ml^{-1} poly D-lysine. Slides were de-paraffinized by 10 min incubation in xylene, and the fixed sections were rehydrated by incubation in a progressive series of ethanol (100%, 90%, 70%, 50% and 30%) and water. In order to deactivate endogenous peroxidase activities, the slides were first incubated for 3 h in 3% H_2O_2 in PBS, then rinsed for 30 min in PBS, and incubated for 4 h in bovine serum albumin (BSA) 2% (w/v in (PBS). The sections were then incubated overnight with the primary IgG diluted 1/75 in 2% BSA in PBS. The staining was performed using the Vectastain ABC Kit, according to the manufacturer's instructions (Vector laboratories, USA). The peroxidase substrate used was 1% (w/v) diaminobenzidine tetrahydrochloride (DAB), which pro-

duces a reddish brown precipitate in the presence of active enzyme.

Localization of CAD, COMT and CCR by immunoelectron microscopy

Small pieces of tissue (1 mm^3) were fixed overnight in 4% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.2, at 4°C, after a few minutes of vacuum infiltration. Tissues were then washed (4 × 10 min) in 0.1 *M* phosphate buffer, pH 7.2, at 4°C, and incubated in 0.15 *M* NH_4Cl, 0.1 *M* phosphate buffer, pH 7.2 for 5 min and 30 min. Tissues were washed (4 × 5 min and 2 × 10 min) in 0.1 *M* phosphate buffer, pH 7.2, at 4°C. After dehydration through a gradient series of ethanol, sections were infiltrated with Lowicryl K4M embedding medium. Ultrathin sections were prepared for immunogold electron microscopy. After incubation with 1% (w/v) BSA, 20 m*M* glycine in 0.1 *M* PBS, pH 7.2, sections were incubated with the IgG diluted 1/75 in 1% (w/v) BSA, 0.1 *M* PBS, pH 7.2, for 2 h at room temperature. Sections were then washed in 1% (w/v) BSA, 0.1 *M* PBS, pH 7.2 (4 × 5 min) and incubated for 1 h at room temperature with 15-nm colloidal gold conjugated goat antirabbit antibody diluted 60 times in 0.1 *M* PBS, pH 7.2. Sections were then washed in 0.1 *M* PBS, pH 7.2 (3 × 5 min) and water (4 × 5 min). The tissue samples were analysed with a transmission electron microscope.

Results

Characterization of the antibodies

The maize *COMT*, and sugarcane *CAD* and *CCR* cDNAs were over-expressed in *E. coli* under the *trc* promoter, and the recombinant proteins were purified by immobilized metal affinity chromatography under denaturing conditions using a Ni (II)-IDA-Sepharose-4B column. The purified recombinant proteins were used to produce polyclonal antibodies in rabbits and the specificity of antibodies were checked by Western blotting (Fig. 2). With the three antibodies, bands were detected in sugarcane stem and roots and maize mesocotyl and roots. No band was detected in sugarcane and maize leaves. Anti-COMT, anti-CAD and anti-CCR antibodies seemed specific, each revealing only one band of 38 kDa, 45 kDa and 40 kDa, respectively. It can be noticed that with the anti-CAD antibody, in the maize root, a band of 27 kDa was also detected. This is probably due to a proteolytic form of CAD. Indeed, depending on the extraction procedures, the relative intensity of the 45 kDa band to the 27 kDa band changed. Besides, when a 10% acrylamide gel was used, an additional band of about 18 kDa was detected. These results demonstrated that the antibodies were specific, and could be used for the localization of the enzymes.

Tissue immunolocalization of CAD, COMT and CCR

At the cellular level, CAD, COMT and CCR were immunolocalized with light microscopy. In addition, to compare the expression patterns with the lignification process, cells were stained by phloroglucinol.

No clear localization could be obtained with anti-CCR antibodies (result not shown). This must be due to the loss of antigenic motif in CCR as a consequence of the fixation treatment with ethanol:formaldehyde:glacial acetic acid.

In maize mesocotyl, which is the first internodal part of the shoot, or in maize roots, no specific labelling was seen using preimmune sera (Fig. 3A,C). In mesocotyl, CAD and COMT labelling was only detected in the vascular area of the organ, more precisely in the cells surrounding the vessels (Fig. 3E,F,I,J). In maize roots, CAD and COMT were detected in cells surrounding the metaxylem vessels and cells of the protoxylem pole (Fig. 3G,H,K,L). There was no labelling in the phloem islands situated between the protoxylem tissues with any of the two antibodies (Fig. 3H,L). The endodermal layer was only labelled with anti-CAD antibody, while the exodermis, which is the external cell layer of roots, beneath the epidermis that is no longer present, was labelled with both antibodies. (Fig. 3G,K). At the stage of maize development used for these experiments, the roots were weakly lignified (Fig. 3D), and only early metaxylem vessels appear to be lightly stained by phloroglucinol.

In sugarcane, no labelling was seen using preimmune sera either in stem or in roots (Fig. 4A,C). In sugarcane stem, a highly vascularized organ (Fig. 4B), labelling was only found in vascular bundles; more precisely in schlerenchyma cells and cells surrounding the metaxylem vessels (Fig. 4E,F,I,J). In sugarcane roots, COMT and CAD were detected in cells surrounding the metaxylem vessels (Fig. 4H,L). There was no labelling in the phloem islands situated between the protoxylem tissues.

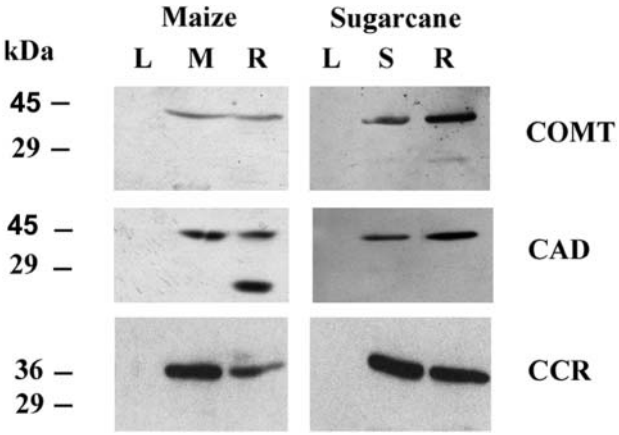

Fig. 2. Immunodetection of COMT, CAD and CCR (diluted 1/750, 1/400 and 1/500, respectively) in different tissues from sugarcane and maize by Western blot analysis. L, leaves; M, mesocotyl; R, root; S, Spindle stem. One hundred and twenty μg of proteins were loaded in each lane.

Subcellular localization of CAD, CCR and COMT

The subcellular localization of the enzymes was first investigated by cell-fractionation. Microsomal and soluble fractions from maize roots and maize mesocotyl were analysed by Western blot (Fig. 5). In maize roots, as well as in maize mesocotyl, CAD, COMT and CCR were only detected in the soluble fraction. An antibody directed against a protein of the plasma membrane, TM20 (Stiefel et al. 1999), was used as a control of protein preparation integrity. It reacted only with the microsomal fraction (data not shown). This subcellular localization was further confirmed by immunocytochemistry with parenchyma cells surrounding maize root vessels. Once again, no clear localization could be obtained with anti-CCR antibody. Anti-CAD and anti-COMT antibodies appeared to label mostly the cytosolic compartment. No specific labelling of membrane structures such as endoplasmic reticulum or Golgi complex could be revealed (Fig. 6).

Discussion

Organ specific and cell specific localization

Using antibodies raised against recombinant proteins, Western blot and inmunocytochemical experiments demonstrated that CAD, CCR and COMT were present in roots, stem or mesocotyl, which are organs involved in active lignification. No signal was found in leaves of young maize seedlings, which are not heavily lignified. Even in older leaves, such as those used for sugarcane Western blot analysis, enzymes involved in lignification are not likely to be abundant, when compared to the proteins involved in carbon metabolismFig. 6. The relative abundance of the three studied enzymes in maize and sugarcane is in agreement with previous gene expression data (Collazo et al. 1992, Selman-Housein et al. 1999).

At the cellular level, no major differences could be found between the expression patterns of CAD and COMT. In mesocotyl and stems, they were expressed in

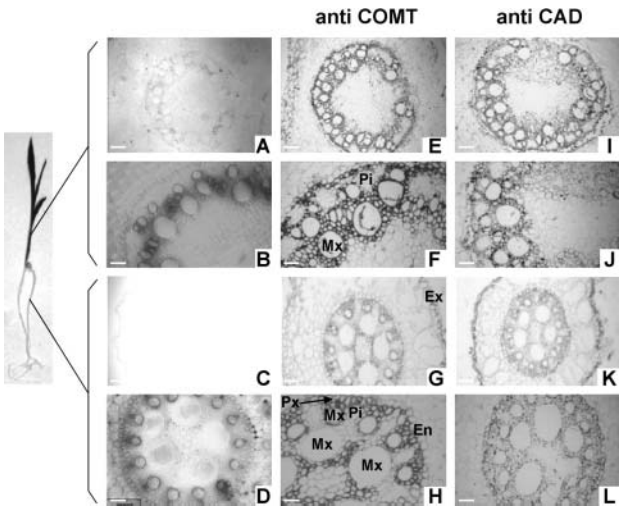

Fig. 3. Immunolocalization of CAD and COMT in maize mesocotyl and roots. Mesocotyl (A) and roots (C) incubated with the preimmune serum; mesocotyl (B) and roots (D) stained with phloroglucinol; mesocotyl (E, F) and roots (G, H) incubated with the antibody directed against COMT; mesocotyl (I, J) and roots (K, L) incubated with the antibody directed against CAD. Scale bars represent 50 μm for rows 1 and 3 and 25 μm for 2 and 4. Endodermis, En; Exodermis, Ex; Metaxylem, Mx; Protoxylem pole, Px; Phloem island, Pi.

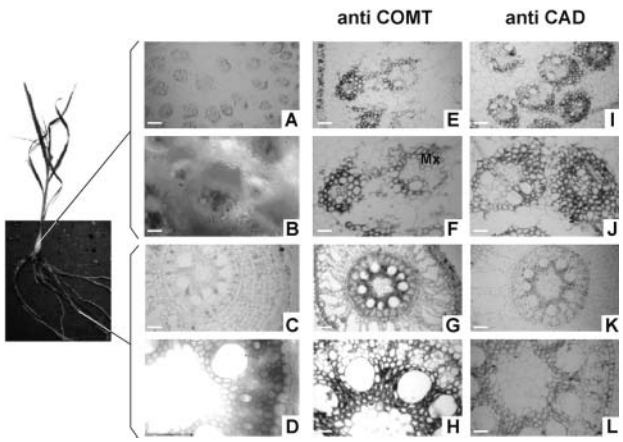

Fig. 4. Immunolocalization of CAD and COMT in sugarcane stem and roots. Stem (A) and roots (C) incubated with the preimmune serum; stem (B) and roots (D) stained with phloroglucinol; stem (E, F) and roots (G, H) incubated with the antibody directed against COMT; stem (I, J) and roots (K, L) incubated with the antibody directed against CAD. Scale bars represent 50 μm for rows 1 and 3 and 25 μm for 2 and 4. Metaxylem, Mx

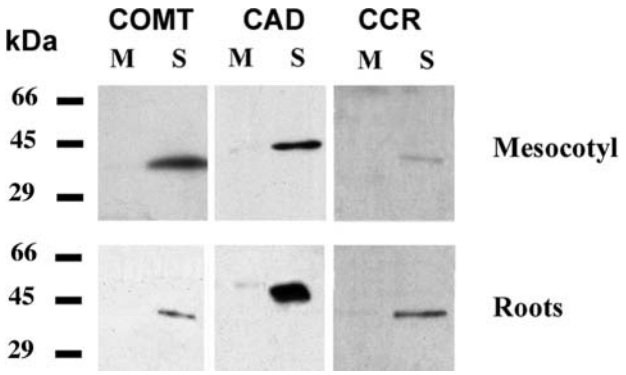

Fig. 5. Subcellular immunolocalization of CAD, COMT and CCR in maize mesocotyl and roots by Western blotting. M, microsomal fraction; S, soluble fraction.

the cell layer surrounding xylem vessels and in sclerenchyma cells. In roots, both proteins were found in the exodermis and in cells surrounding xylem vessels. The only difference in the expression patterns appeared in maize root endodermis, where lignification and suberization occur. In this cell layer no COMT could be detected, even though CAD was present. An isoform of COMT however, has been shown to be present in maize root endodermis and endodermis (Held et al. 1993). Cells in the exodermis and endodermis are known to synthesize suberin, a complex polymer of aliphatic and phenylpropanoid units, whose aromatic subunits are *p*-coumaryl alcohol and coniferyl alcohol (Kolattukudy 2001). Thus, the presence of CAD and COMT in the exodermis and endodermis is in agreement with their role in the lignification process.

Considering expression at the xylem level, our observations suggest that the monolignols necessary for lignin synthesis are produced both within the cells that are going to be lignified, and in associated parenchyma cells. This is in agreement with results published for enzymes of the lignin biosynthetic pathway in dicot species (Feuillet et al. 1995, Lacombe et al. 1997, Kersey et al. 1999),

Subcellular immunolocalization

Fractionation experiments showed that CAD, COMT and CCR were present in the soluble fraction, and not in the microsomal fraction. By immunolocalization techniques, CAD and COMT were found to be distributed in the cytoplasm. These results are in agreement with subcellular localization predictions made with Psort (http://psort.nibb.ac.jp) using the translated sequences of the maize *COMT* and sugarcane *CAD* and *CCR* cDNAs. The localization of COMT in the cytosol is also in agreement with the localization of this enzyme in dicot species (Kersey et al. 1999). Dicot CAD has been found to be located in the cytosol and in the membrane of the endoplasmic reticulum (Nakashima et al. 1997, Samaj et al. 1998). We have not observed localization of CAD with membrane structures.

While PAL (Smith et al. 1994, Rasmussen and Dixon 1999), CAD, CCR and COMT (this article) are cytosolic, other enzymes involved in lignin biosynthetic pathway, such as cinnamate 4-hydroxylase (C4H), appear to be anchored to the external surface of the ER membrane (Chapple 1998). However, all these proteins could be assembled in supramolecular structures, as suggested by Stafford (1974) for the enzymes of the general phenylpropanoid, flavonoid and lignin pathways. Recent evidence shows that it is indeed the case for the general phenylpropanoid and flavonoid pathways (Rasmussen and Dixon 1999, Winkel-Shirley 1999). The general phenylpropanoid pathway is a common pathway leading to flavonoid and lignin pathways. It is thus possible that the enzymes of the lignin pathway are also associated in a complex on the cytosolic face of the ER, all enzymes

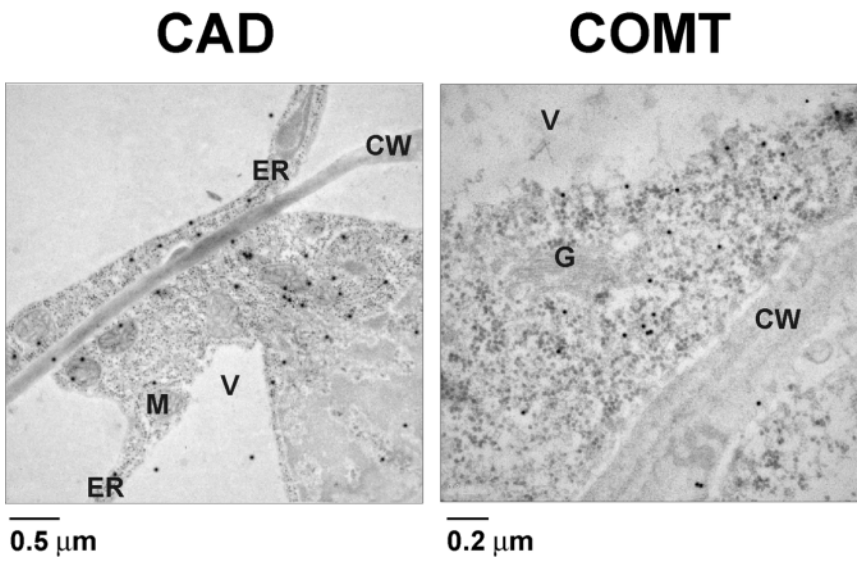

Fig. 6. Subcellular localization of CAD and COMT in maize mesocotyl and roots by electron microscopy. Scale bars represent 0.5 μm for CAD, and 0.2 μm for COMT. ER, endoplasmic reticulum; V, vacuole; CW, cell wall; M, mitochondria; G, Golgi apparatus.

being held together by weak forces. Anti-CAD, anti-COMT, and anti-CCR polyclonal antibodies could be appropriate tools to investigate whether such an additional level of regulation does exist in monolignol biosynthesis in monocots.

Acknowledgements – This work has been partially supported by the EEC INCO project No. ERB3514PL962073, and by Plan Nacional de Investigación Científica y Técnica (grand Bio97-0729).

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Edited by C. Larsson