

# Radial Distribution Pattern of Pectin Methylesterases across the Cambial Region of Hybrid Aspen at Activity and Dormancy<sup>1</sup>

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Biochemical microanalysis combined with tangential cryosectioning was used to visualize the distribution of pectin methylesterases (PMEs) across the cambial region in active and dormant hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx). These novel techniques allowed us to relate activity and isoforms of PMEs to specific tissues and developmental stages of the stem to get more information on the physiological function of PMEs in cambial growth. Isoelectrofocusing analysis revealed numerous isoforms that were differentially distributed according to the tissue-type and to the cambial stage. A neutral isoform was found to be distributed ubiquitously across the stem of both active and dormant trees, which suggests that it is a housekeeping isoform involved in the maintenance of the cell wall integrity throughout the stem. In addition, two distinct isoforms having different isoelectric points were found to be related to the differentiation of cambial derivatives. A basic isoform appears to be a physiological marker of the dormant stage involved in the cessation of meristematic radial growth, whereas an acidic isoform is functionally related to the immediate expansion of the cambial daughter cells that occurs bilaterally on each side of the cambium at the active stage.

Pectin methylesterases (PME; EC 3.1.1.11) are enzymes catalyzing the demethylesterification of cell wall polygalacturonans. By generating free carboxylic groups on pectins, the PMEs affect the pH and ionic balances within the cell wall (Grignon and Sentenac, 1991) and, consequently, the activity of a wide range of hydrolytic enzymes and the interactions between structural cell wall components (Pressey, 1984; Varner and Lin, 1989). In dicotyledonous plants the PMEs are present as several isoforms, which can be distinguished by their  $M_r$ , pI, and biochemical activity (Bordenave and Goldberg, 1993). These isoforms are encoded by a family of genes (Micheli et al., 1998), some of which have been shown to be expressed constitutively throughout the plant (Gaffe et al., 1997), whereas others are differentially regulated and specifically related to the development of organs such as roots (Wen et al., 1999), flowers (Albani et al., 1991; Mu et al., 1994), or fruits (Harriman et al., 1991). At a cellular level pectin demethylesterification is thought to modulate cell expansion (Bor-

denave and Goldberg, 1994), as well as cell-to-cell cohesion (Liners et al., 1994; Wen et al., 1999). It is interesting that these observations indicate that both processes may be positively or negatively regulated by the PMEs. This raises intriguing questions about the regulation of the PME isoenzymes, their mode of action on pectins, and their biological role in cell growth and differentiation.

The vascular cambium provides an interesting model for studying pectin metabolism with regard to cell growth and differentiation. The bilateral differentiation of cambial cell derivatives to phloem on one side and xylem on the other side involves consecutive steps including phases of division, expansion, secondary wall formation, and eventually cytoplasmic autolysis of some elements such as vessels or tracheids (e.g. Chasan, 1994; Jones and Dangl, 1996; Boudet, 1998). Discrete changes within the cell wall architecture of cambial derivatives occur during the differentiation of mature xylem or phloem cells. With regard to pectin metabolism, immunohistochemical analyses on poplar stems revealed changes in the distribution of demethylesterified pectins on either side of the cambial meristem (Guglielmino et al., 1997b). Furthermore, biochemical analyses of tissue samples enriched in phloem tissue showed changes in the distribution of acidic pectins and PME isoenzymes according to the seasonal cycle (Baier et al., 1994; Guglielmino et al., 1997a).

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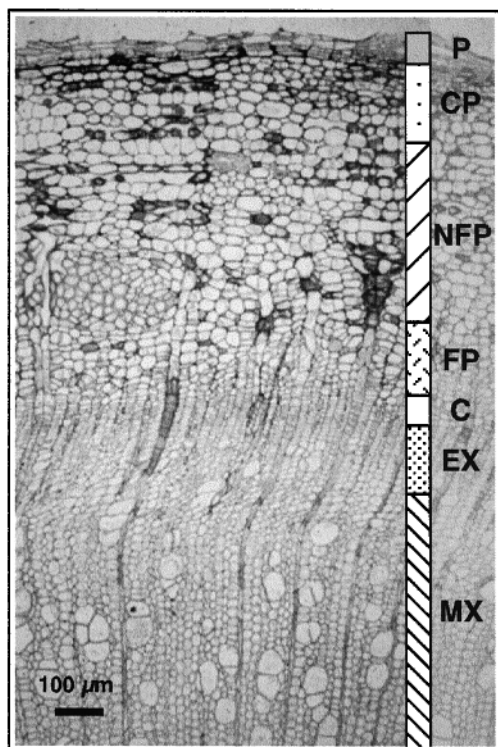
Sampling of specific tissues in different stages of xylem and phloem development for biochemical microanalysis can be obtained with high resolution by tangential cryosectioning (Uggla et al., 1996, 1998; Tuominen et al., 1997). This method was used in our study to visualize the radial distribution of the PME isoenzymes across stem tissues of the hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx) during activity and dormancy. This approach has provided accurate insights into the activity and distribution of PME isoenzymes with regard to cell type and seasonal cambial activity. The relationship between the occurrence of some newly detected PME isoenzymes and the physiology of wood formation is discussed.

**RESULTS**

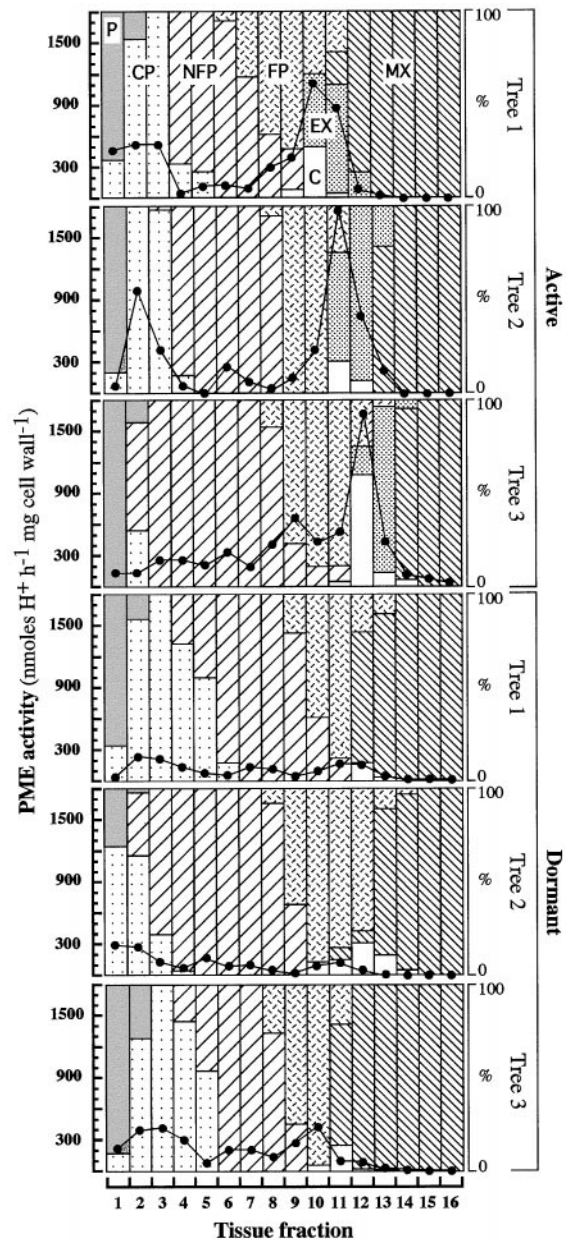
**Variation of PME Activity Across Aspen Stem with Season and Location**

*Ionic Wall-Bound PME Activity*

The activity of ionically wall-bound PMEs was investigated in stem tissues (Fig. 1) during activity and dormancy. In dormant trees the PME activity was consistently low in all tissues (Fig. 2). By contrast, the profile of PME activity across the stem during the



**Figure 1.** Anatomical appearance of the wild-type hybrid aspen tree at the active stage. The different tissue types used for PME characterization are indicated. P, Periderm; CP, cortical parenchyma; NFP, non-functional phloem; FP, functional phloem; C, cambium; EX, expanding xylem; MX, mature xylem.



**Figure 2.** Radial distribution of PME activity visualized after cell wall-bound protein extraction in 1 M NaCl in three different hybrid aspen trees during cambial activity and dormancy. Each column represents a fraction of three 25- $\mu$ m tangential sections and its relative composition (in percentage) of different tissues. PME activity for each fraction is indicated with a black point. P, Periderm; CP, cortical parenchyma; NFP, non-functional phloem; FP, functional phloem; C, cambium; EX, expanding xylem; MX, mature xylem.

active period was characterized by the presence of a minor peak related to the cortical parenchyma layers and a major peak related to the cambial meristem and its most recent derivatives. The decreasing activity observed on both sides of the active cambial zone was correlated to the progress of phloem and xylem differentiation. The activity related to maturing and mature xylem, non-functional phloem, and periderm



was found similarly low as that observed at the dormant stage. From the dormant to the active stage, PME activity increased about 1.6- and 6-fold within the cortical parenchyma and the cambial zone, respectively.

#### Soluble PME Activity

To get a more complete quantitation of PMEs across the aspen stem, soluble PME isoforms were extracted and assayed (Fig. 3). In dormant trees the activity of soluble PMEs was low across the stem. In active trees however, the highest PME activity was present in the cambial meristem and its expanding derivatives. In comparison with ionically wall-bound PMEs the activity for soluble PMEs were generally lower. It is interesting that unlike the wall-bound PMEs, significant activity from soluble PMEs was found also in maturing xylem. Moreover, no peak was observed in layers of cortical parenchyma of trees at the active stage.

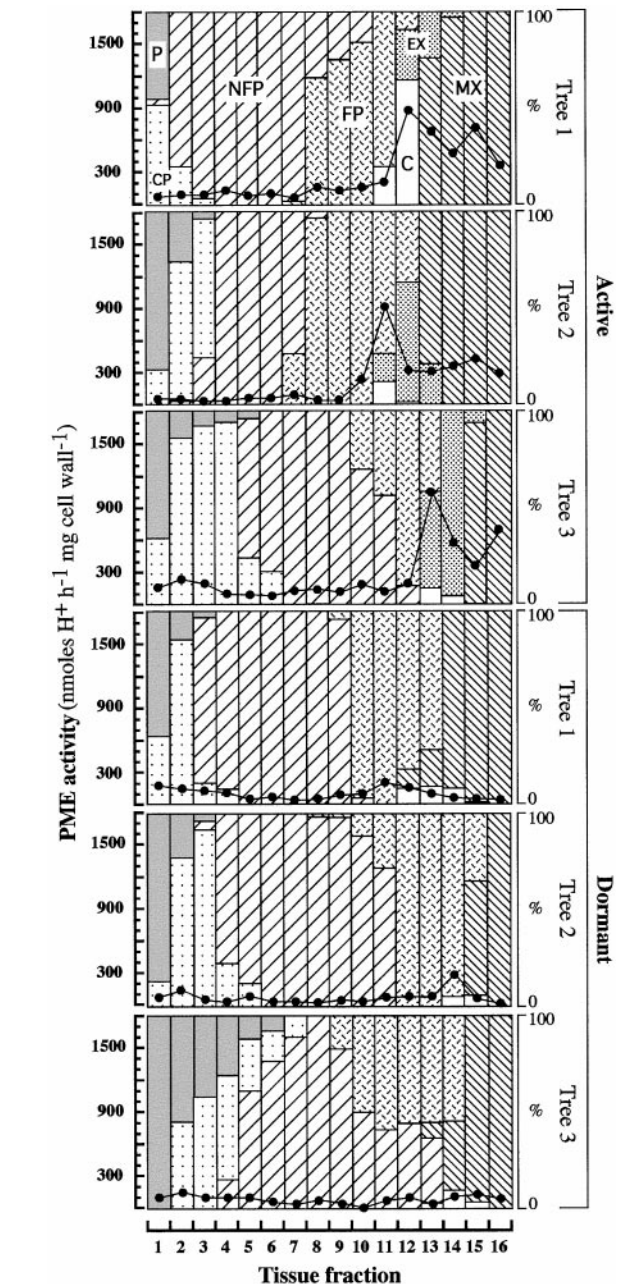
#### Radial Distribution of the PME Isoforms Across the Aspen Stem

##### Ionically Wall-Bound PMEs

To relate the radial profile of PME activity in active and dormant trees to the presence of different isoforms, the ionically wall-bound PMEs were fractionated by isoelectrofocusing (IEF) and detected on the gel using the zymogram technique (Fig. 4). At the active stage the pattern of the PMEs across the stem comprised a number of isoforms distinguishable by their acidic (A2), neutral (N1–N3), and basic (B2–B4) apparent pI. The N3, B2, and B3 isoforms with respective pI of 7.6, 8.1, and 8.5 occurred in all tissues of the stem with the exception of the mature xylem. The amount of these isoforms slightly declined in the region corresponding to the non-functional phloem. The cortical parenchyma comprised three additional isoforms with discrete intensities showing pIs about 7.0 (N1), 7.2 (N2), and 9.3 (B4). After gel filtration and zymogram analysis the apparent molecular masses of the three main isoforms A2, N3, and B4 were estimated to be 38, 35, and 15 kD, respectively.

One of the most striking observations made on stems at the active stage related to the occurrence of an acidic PME isoform (A2) with an apparent pI of 5.6. It is interesting that this isoform was confined to fractions including the cambium and the young phloem and xylem derivatives. Moreover, this isoform was undetectable across the stem at the dormant stage.

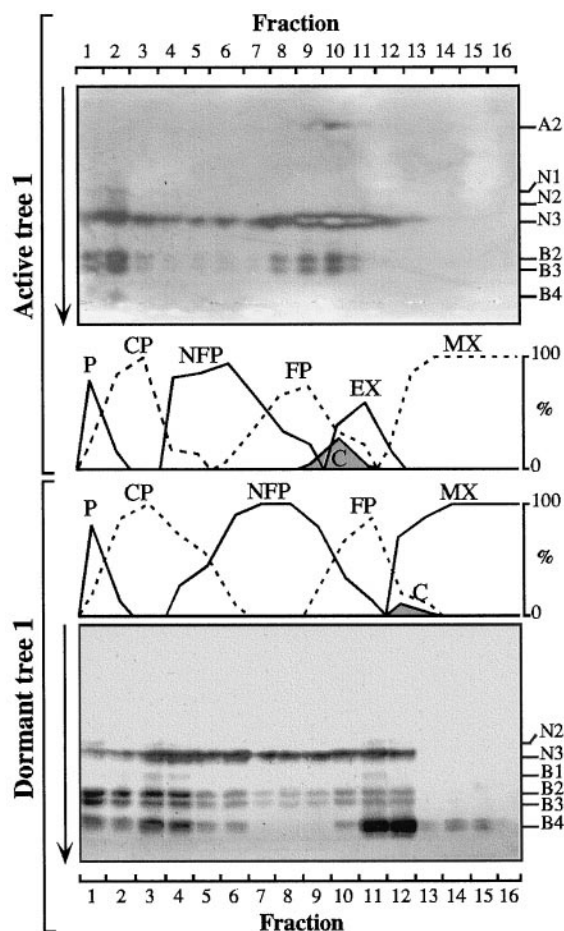
In parallel to the disappearance of the A2 isoform, the establishment of the dormant state is characterized by the increase of the B4 isoform throughout the stem tissues except in fractions enriched in non-functional phloem. Although it was barely detectable at the active stage, the B4 isoform displayed almost



**Figure 3.** Radial distribution of PME activity visualized after soluble protein elution in three different hybrid aspen trees during cambial activity and dormancy. Each column represents a fraction of three 25- $\mu\text{m}$  tangential sections and its relative composition (in percentage) of different tissues. PME activity for each fraction is indicated with a black point. P, Periderm; CP, cortical parenchyma; NFP, non-functional phloem; FP, functional phloem; C, cambium; EX, expanding xylem; MX, mature xylem.

the highest activity among the PMEs at the dormant stage, particularly in fractions including the cambial zone and the functional phloem.

Some other qualitative changes discriminate between the dormant and the active stages, such as the appearance of a slightly basic isoform (B1) of pI 7.7 discretely associated with the cortical parenchyma



**Figure 4.** IEF of wall-bound proteins extracted from 75- $\mu$ m aspen stem fractions. Cell walls were isolated from stem tissues during cambial activity and dormancy. Excepted for the fractions marked with an asterisk, the PME activities were calibrated onto 0.1 and 0.6 mg of cell walls for active and dormant trees, respectively. PME activities were revealed by the acrylamide-pectin sandwich technique. Isoform names are indicated on the right of gels. Percentage of tissues contained in each fraction is represented at the center of the figure. Cambium is shaded in gray. P, Periderm; CP, cortical parenchyma; NFP, non-functional phloem; FP, functional phloem; C, cambium; EX, expanding xylem; MX, mature xylem. Arrow indicates the gradient from acidic to alkaline pH.

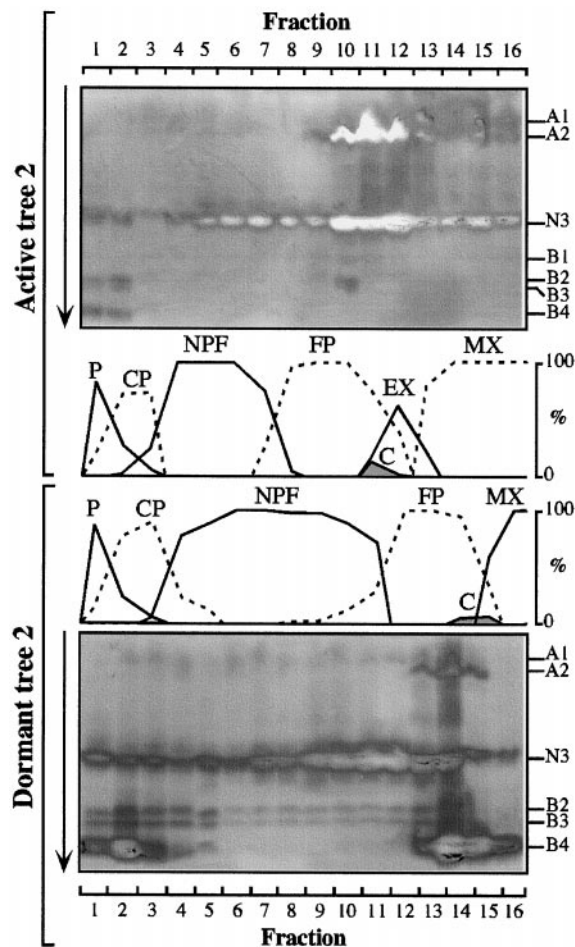
and the functional phloem at the dormant stage. With respect to the neutral PMEs the N1 isoform disappeared, whereas the N2 isoform occurred in the periderm, the cortical parenchyma, and the functional phloem of stem at the dormant stage.

*Soluble PMEs*

The pattern of the soluble PMEs was investigated across the aspen stem, as for the ionically wall-bound PMEs (Fig. 5). The most striking observation from the analysis of active trees concerned the high abundance of the acidic A2 isoform when compared with the ionically wall-bound proteins. As in the previous observations the tissue specificity of A2 toward the

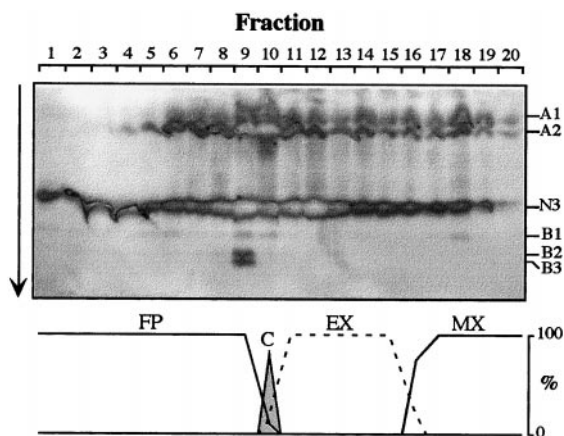
cambial zone and the immediately adjacent tissues is again confirmed. It is interesting that traces of A2 extended from the young xylem derivatives to the maturing xylem, whereas it was clearly absent from older tissues of the phloem. Moreover, the A2 isoform in the soluble fraction was present also within the cambial region of the dormant stem, but at a remarkably lower level. Taking into account the amount of cell wall used for calibration, the relative abundance of A2 within the cambial zone is about 20-fold higher at the active stage than at the dormant stage.

Analysis of the soluble proteins also revealed a novel acidic PME isoform, named A1, showing a pI of 5.2. In the dormant tissues A1 was continuously distributed from the cortical parenchyma to the cam-



**Figure 5.** IEF of soluble proteins eluted from 75- $\mu$ m aspen stem fractions during cambial activity and dormancy. PME activities were calibrated onto 0.27 and 0.83 mg of cell walls for active and dormant trees, respectively. PME activities were revealed by the acrylamide-pectin sandwich technique. Isoform names are indicated on the right of gels. Percentage of tissues contained in each fraction is represented at the center of the figure. Cambium is shaded in gray. P, Periderm; CP, cortical parenchyma; NFP, non-functional phloem; FP, functional phloem; C, cambium; EX, expanding xylem; MX, mature xylem. Arrow indicates the gradient from acidic to alkaline pH.





**Figure 6.** IEF of soluble proteins eluted from 25- $\mu$ m aspen stem fractions during cambial activity. PME activities were calibrated onto 0.06 mg of cell walls. PME activities were revealed by the acrylamide-pectin sandwich technique. Isoform names are indicated on the right of gels. Percentage of tissues contained in each fraction is represented at the center of the figure. Cambium is shaded in gray. FP, Functional phloem; C, cambium; EX, expanding xylem; MX, mature xylem. Arrow indicates the gradient from acidic to alkaline pH.

bial region at the dormant stage, whereas it was found from the cambial region to the mature xylem at the active stage.

For the neutral and basic isoforms, the patterns were qualitatively quite similar at the active stage to those observed for the ionically wall-bound proteins (Fig. 4). At the dormant stage slight changes resulting from the different protein extraction procedures were noticeable. One of these changes concerned the relative abundance of the N3 and the B4 isoforms with regard to the couple of isoforms B2 and B3 (Fig. 5). Another change concerned the ionically wall-bound B1 isoform that was found associated to the cortical parenchyma and the functional phloem and that is undetectable among the soluble proteins.

To increase resolution of the distribution of acidic PMEs across the cambial region of active trees, analysis was performed in each 25- $\mu$ m tangential cryosection (Fig. 6). For this purpose soluble proteins were extracted because it increased dramatically the recovery of the acidic PMEs. The results enable more accurate assignment of the peak activity of the A2 isoform to the cambial meristem and its recent derivatives. Moreover, the asymmetrical distribution of A2 on both sides of the cambium was visualized. Unlike the phloem layers, the maturing xylem derivatives retained a substantial amount of this isoform. In other respects the xylem tissues were also distinguishable by the presence of the other acidic A1 isoform. Although A1 behaves like the A2 isoform during the active stage, the distribution of both of these acidic PMEs differs slightly at the dormant stage, as shown previously (Fig. 5).

## DISCUSSION

Quantitative and qualitative analysis of PMEs has been undertaken in the stem of hybrid aspen to investigate the control of pectin demethylesterification during growth and differentiation of cambial derivatives. To visualize the radial distribution of PMEs across developing cambial tissues we used microanalysis of PMEs in consecutive tangential cryosections. This novel approach allowed the analysis of higher plant PME isoforms in specific and defined tissues. To gain insight on the overall isoforms present within stem tissues, both soluble and wall-bound PMEs were extracted.

Major differences in active and dormant tissues were found in PME activity as well as in the radial pattern of the PME isoforms across the stem of hybrid aspen trees. These changes are fine-tuned according to cell types along the radial axis of the stem. The most striking observation concerns the burst of PME activity detected within the cambial zone of the stem at beginning of the active stage. This event reflects that PMEs represent a particular cell wall function that is associated to the resumption of cambial activity during spring. To extend our investigations, the PME isoform pattern of each tissue fraction was obtained using IEF. Among the numerous isoforms identified, several PMEs (an acidic, A2; a neutral, N3; and a basic, B4) were strikingly distinguishable from the others according to their respective pI, their tissue specificity, and their relationship with regard to the seasonal cycle.

In view of the diversity of the PME isoforms found across the stem of the hybrid aspen, many questions arise about their respective biological function with regard to growth and differentiation. It has been postulated that PMEs modulate cell wall metabolism by several mechanisms that base their action patterns on pectins (Catoire et al., 1998). On one hand, PMEs are thought to act randomly on pectins by releasing protons that may stimulate the activity of cell wall loosening hydrolases (Nari et al., 1986; Moustacis et al., 1991). On the other hand, blockwise de-methylesterification of the pectins by PMEs may generate junction zones via calcium ions, which contribute to the gelification of the pectins and the stiffening of the wall (Fry, 1986). This will alter the sensitivity of polysaccharides to the action of several hydrolases (e.g. Fisher and Bennett, 1991) and expansins (Carpita et al., 1996). These different functions of PMEs are likely to exert diametrically opposite effects on cell wall extensibility, porosity, and cell-to-cell adhesion, and suggest therefore a multidimensional biological role of PMEs in plant development.

It has been proposed that basic PME isoforms catalyze demethylesterification of pectins linearly along the chain of the molecule giving rise to blocks of carboxyl groups, whereas acidic isoforms cause a random cleavage of methylesterified carboxyl groups (Markovic and Kohn, 1984). However, some recent

studies have questioned this. It has been shown that the apoplastic pH strongly affects the action pattern of some basic and neutral PME isoforms and therefore the efficiency of the demethylesterification process (Catoire et al., 1998). Moreover, the accumulation of an acidic isoform in the non-growing basal part of the mung bean hypocotyl throws into doubt the proposed role in promoting cell elongation (Bordenave and Goldberg, 1994). A molecular study on pea root development contradicts the idea that basic isoforms act processively along the polygalacturonan chain to generate pectinate gel, which may account for stiffening and cell-to-cell adhesion (Wen et al., 1999). The *rcpme1* gene described in this study encodes a PME isoform with a basic pI as deduced from sequence analysis, which displays functional features that are ascribable to acidic isoforms. The *rcpme1* gene product is suggested to play a role in the solubilization of the wall through pH-mediated changes in the activity of cell wall hydrolases, leading to root cap cell separation. Taken together it is clear that the biological functions assigned to different PME isoforms on the bases of in vitro kinetics or features such as pI must be regarded with caution.

Among the PME isoforms detected across the stem of the hybrid aspen, the basic isoform B4 exhibited characteristics (apparent pI of 9.31 and molecular mass around 15 kD) close to those of basic PMEs with low molecular mass, originating from flax calli (Gaffe et al., 1992), mung bean hypocotyl (Bordenave and Goldberg, 1993), or tomato (Pressey and Avants, 1972). The B4 appears to be a physiological marker of the dormant stage. Because B4 is distributed only in tissues exhibiting meristematic activities during the active period, i.e. the vascular cambium and the external cortex including the cork cambium, a good correlation can be made between the presence of this isoform and the cessation of radial growth at the dormant stage. One can hypothesize that action of B4 on pectins disrupts some rheological properties of the cell wall that are required for growth during the active period.

Unlike the B4 isoform, the neutral N3 isoform is distributed throughout all stem tissues and does not show any significant variation between active and dormant tissues. Similar ubiquitous isoforms have been described in higher plant species, but they mostly correspond to some alkaline isoforms as observed in tomato (Gaffe et al., 1994), flax (Gaffe et al., 1992), and Arabidopsis (Richard et al., 1994). The only exceptions are the neutral PMEs found along the mung bean hypocotyl (Bordenave and Goldberg, 1994). The physiological role of such ubiquitous isoforms is unknown. However, un-methylesterified pectin epitopes within the middle lamella surrounding intercellular spaces have been observed by immunocytochemistry in all higher plant cell types so far studied (Knox et al., 1990; Knox, 1992; Liners and Van Cutsem, 1992; Schindler et al., 1995). These data

support the hypothesis that housekeeping PME isoforms may be involved in the formation, the maintenance, or the function of the intercellular spaces throughout the plant.

In addition to the PME isoforms discussed above the acidic isoform A2 arouses a particular interest because it was shown to be confined within the cambial meristem and the young developing derivatives of the active trees. A large part of the high activity of PMEs in the cambial tissues of the active trees can be accounted to the A2 isoform. It is likely that A2 remains weakly adsorbed in muro onto the cell wall components because the soluble protein extraction procedure increases its recovery dramatically. Unlike the neutral or the alkaline PMEs, acidic isoforms have been only rarely reported in higher plant species. Acidic PMEs are barely detected among proteins extracted from isolated cell wall (Komae et al., 1990; Richard et al., 1994; Guglielmino et al., 1997a), whereas they are abundant among proteins eluted directly from tissues (Lin et al., 1989) or from infiltrated organs (Bordenave and Goldberg, 1994). These observations together with the present data suggest that some failure to detect acidic isoforms in higher plants may be related to the experimental conditions used for protein extraction.

The detailed distribution pattern of the isoform A2 across the cambial region of active hybrid aspen trees raises fundamental questions about its function in cambial growth. Optimization of the histobiochemical analysis to the level of a single-tissue cryosection reveals an asymmetric allocation of A2 on both sides of the cambial meristem. The distribution of A2 coincides with the expanding zones where xylem and phloem cells acquire their function. This suggests that A2 is involved in the differentiation of cambial daughter cells into the xylem as well as the phloem derivatives. According to this idea the isoform A2 would be functionally related to the immediate expansion of the cambial daughter cells that occurs bilaterally on each side of the cambium. Although not yet demonstrated in planta, the possible functional relationship of the acidic PMEs to cell expansion has been stated already (Nari et al., 1986; Moustakas et al., 1991). It has been postulated that such isoforms modulate the activity of several cell wall-loosening hydrolases through changes in the apoplastic pH. However, because anionic isoforms are known to be freely diffusible throughout the apoplasm (Bordenave and Goldberg, 1994), the possibility that A2 would diffuse laterally from the cambium toward the phloem derivatives without contributing to the differentiation of this cell type cannot be excluded. If so, a preferential role of the isoform A2 in early stage of xylem differentiation is addressed.

In summary we have defined the distribution pattern of the PME isoforms across extraxylary tissues of the hybrid aspen stem in dormant and active trees. A

number of isoforms with differential expression pattern and function in cambial growth has been identified. In particular the isoforms A2 and B4 are related to the active and dormant cambial meristem, respectively, and deserves future molecular and functional characterization.

## MATERIALS AND METHODS

### Plant Material

A clonal material of hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx) was grown in a greenhouse under a photoperiod of 18 h. Plants were exposed to natural light supplemented with artificial light (HQI-TS 400 W/DH metal halogen lamps, Osram, Haninge, Sweden) and to a temperature of 22°C/15°C (day/night). The plants were grown in 5-L pots with fertilized peat, watered daily, and fertilized weekly with a 1:100 dilution of SUPERBA S (HYDRO SUPRA AB, Landskrona, Sweden). To obtain dormant trees some plants were placed in an unheated growth room in the end of July and exposed to natural day lengths. Trees 1.5 to 3 m tall were harvested at the active or dormant stage of the cambial growth.

### Sample Preparation and Anatomical Characterization

The main stem (1–2 cm in diameter) was cut transversally in segments 1.5 cm thick that were quickly frozen in liquid nitrogen and kept in –80°C. The frozen segments were further trimmed to produce blocks (3 mm tangentially × 15 mm vertically × 10 mm radially) comprising mature xylem and all the extraxylary tissues. The blocks were cut in serial 25- $\mu$ m tangential sections from the periderm to the mature xylem as described by Uggla et al. (1996). Sections were made at –20°C using an Open Top cryostat (Bright Instrument Company, Hundigton, UK) equipped with a steel knife. For radial localization of the tangential sections, transverse sections of the specimen were hand-cut with a razor blade after each third tangential section and examined under a light microscope. The tissue type and developmental stage were defined according to the following anatomical criteria: The transverse sections of the whole radial block included the periderm consisting of rectangular flattened brown cells at the outside of the stem; the cortical parenchyma made of large cells arranged in staggered rows inside the periderm; the functional phloem corresponding to the part of the phloem arranged in orderly radial files; the non-functional phloem corresponding to the compressed cells found between the functional phloem and the cortical parenchyma; the cambial zone consisting of dividing cells; the expanding xylem defined by the presence of primary-walled xylem; and the maturing and mature xylem recognizable by the presence of secondary walls (Fig. 1). The percentage of each tissue type was determined by computer-assisted image analysis (NIH Image 1.54, Wayne Rasband, National Institutes of Health, Bethesda, MD).

### Protein Extraction

Cell wall proteins were extracted from one or three consecutive 25- $\mu$ m tangential sections according to the experiment being carried out. The small amount of plant material available in the sections required some modification of the methods conventionally used for cell wall protein analysis (Goldberg et al., 1986). Tissue samples were ground very finely in 0.1% (v/v) Triton X-100 and 1 mM phosphate buffer (0.2 M  $\text{PO}_4\text{H}_2\text{K}$ , 0.2 M  $\text{PO}_4\text{HNa}_2$ , pH 6.0) using a 0.1-mL micropotter (Bioblock Scientific, Illkirch, France), and the homogenates were centrifuged for 5 min at 3,000 rpm. Supernatants were discarded and the pellets were washed three times in double-distilled water with intermittent low-speed centrifugation. The pellets, which consisted of insoluble cell walls, were resuspended in 1 M NaCl and then incubated for 1 h on ice to extract ionically bound cell wall proteins. Finally, the suspension was centrifuged for 15 min at 3,000 rpm to separate the pelleted cell walls from the eluted proteins, which were dialyzed, and then used for biochemical analysis.

An alternative method of protein extraction was used for the biochemical analysis of soluble PME isoenzymes, which might be expected to be washed out under the procedure described previously. Samples were ground very finely in 0.1% (v/v) Triton X-100, 1 mM phosphate buffer (0.2 M  $\text{PO}_4\text{H}_2\text{K}$ , 0.2 M  $\text{PO}_4\text{HNa}_2$ , pH 6.0), and 0.05% (v/v) of protease inhibitor cocktails suitable for plant extraction (Sigma, St. Louis). The homogenates were centrifuged for 5 min at 3,000 rpm and proteins of the supernatant were used directly, without dialysis, for biochemical analysis. All steps were conducted at 4°C.

### Quantification of Cell Wall Amount

Because of the small sample size, the dry weight of cell wall was determined using a method based on turbidimetry. The pelleted cell walls were resuspended in 0.1% melted-agarose (Molecular Biology grade, Appligene, France) to prevent sedimentation of the cell wall particles. The turbidimetry of the homogenate was measured at 600 nm using a spectrophotometer (Shimadzu, Kyoto). The absorbance was converted to an equivalent of dry weight by reference to a standard curve made from a large-scale preparation of cell walls isolated from hybrid aspen stem. The curve indicated that 1 unit of  $A_{600}$  corresponds to 1.15 mg of dried cell walls (data not shown).

### Microassay of PME Activity

PME activity was measured spectrophotometrically (Shimadzu) at 525 nm using a pectin-methyl red solution (pH 6.1) as substrate, as described by Richard et al. (1994). A calibration curve was obtained by adding from 1 to 100 nEq  $\text{H}^+$  to 1 mL of substrate solution.

### IEF

Cell wall proteins were fractionated by IEF on ultrathin polyacrylamide slab gels containing 10% (v/v) ampholines



(pH range from 3–10; Pharmacia, Uppsala). Protein samples were calibrated according to a fixed amount of cell wall from which proteins were eluted. Active PME were revealed on the gel under the conditions described by Bertheau et al. (1984) in which agar-pectin was replaced by acrylamide-pectin (0.2 M NaCl, 87 mM Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O, 6 mM citrate, 0.5% [w/v] apple pectine, 40% [v/v] acrylamide, 0.75% [w/v] persulfate, and 0.075% [v/v] TEMED [(N,N,N',N'-tetramethylethylenediamine), pH 7.0] to enhance resolution. Apparent pI was determined by reference to pI markers (Bio-Rad, Hercules, CA).

### Molecular Mass Determination

The molecular mass of PME isoenzymes was determined in cambial region tissues (10 g fresh weight). The tissues were obtained by peeling the bark and scraping the exposed surfaces with a scalpel. Sample included the cambial meristem, radially expanding xylem elements, some xylem elements undergoing secondary wall thickening, and functional and non-functional phloem. The sample was ground in liquid nitrogen and then homogenized in 0.1% [v/v] Triton X-100 and 1 M NaCl as described by Goldberg et al. (1986). Proteins were precipitated in 80% saturated ammonium sulfate and resuspended in a buffer made of 0.1% [v/v] Triton X-100 and 0.2 M NaCl. They were loaded onto a Sephacryl 200 column equilibrated with the same buffer and calibrated using proteins of known molecular mass (Sigma). The PME activity was measured spectrophotometrically at 525 nm as previously described. Fractions including PME activity were analyzed by IEF as described above.

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