Seasonal Changes of Plasma Membrane H\(^+\)-ATPase and Endogenous Ion Current during Cambial Growth in Poplar Plants

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The plasma membrane H\(^+\)-ATPase (PM H\(^+\)-ATPase), potassium ions, and endogenous ion currents might play a fundamental role in the physiology of cambial growth. Seasonal changes of these parameters were studied in twigs of *Populus nigra* and *Populus trichocarpa*. Monoclonal and polyclonal antibodies against the PM H\(^+\)-ATPase, x-ray analysis for K\(^+\) localization and a vibrating electrode for measurement of endogenous ion currents were used as probes. In dormant plants during autumn and winter, only a slight immunoreactivity against the PM H\(^+\)-ATPase was found in cross sections and tissue homogenates, K\(^+\) was distributed evenly, and the density of endogenous current was low. In spring during cambial growth, strong immunoreactivity against a PM H\(^+\)-ATPase was observed in cambial cells and expanding xylem cells using the monoclonal antibody 46 E5 B11 F6 for fluorescence microscopy and transmission electron microscopy. At the same time, K\(^+\) accumulated in cells of the cambial region, and strong endogenous current was measured in the cambial and immature xylem zone. Addition of auxin to dormant twigs induced the formation of this PM H\(^+\)-ATPase in the dormant cambial region within a few days and an increase in density of endogenous current in shoot cuttings within a few hours. The increase in PM H\(^+\)-ATPase abundance and in current density by auxin indicates that auxin mediates a rise in number and activity of an H\(^+\)-ATPase in the plasma membrane of cambial cells and their derivatives. This PM H\(^+\)-ATPase generates the necessary H\(^+\)-gradient (proton-motive force) for the uptake of K\(^+\) and nutrients into cambial and expanding xylem cells.

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polarization and the apoplastic acidification drive the uptake of nutrients into cells via secondary active transport and cause the opening of ion channels, for instance K⁺ channels (Michelet and Boutry, 1995; Hoth et al., 1997; Maathuis et al., 1997). With respect to trees, numerous studies have shown that auxin is an important regulator of cambial growth (Savidge and Wareing, 1981; Little and Savidge, 1987; Lachaud, 1989; Little and Pharis, 1995; Ugglä et al., 1998; Sundberg et al., 2000). However, our understanding of the cellular events is still poor, and no effect of auxin on the PM H⁺-ATPase in cambial cells has been demonstrated so far.

In poplar (Populus spp.) plants, with their fast-growing trunks, shoots, and twigs, a PM H⁺-ATPase may have a vital role in the uptake of nutrients by cambial and other growing cells. Suc has been shown to be the major carbohydrate of cambial metabolism (Krabell, 2000). During the period of rapid cell divisions and cell growth in spring and summer, the demand for Suc reaches its peak, and the number and activity of a PM H⁺-ATPase might be high to energize the uptake of Suc into cells. Some support for an involvement of a PM H⁺-ATPase during cambial growth comes from investigations showing differences in electric membrane potential among the various cell types involved in assimilate flux from sieve tubes to cambial cells (van Bel and Ehlers, 2000). Moreover, transport of assimilates to the cambium has been demonstrated directly in willow (Salix viminalis), i.e. close relatives to poplar plants, using labeled sugars and microautoradiography (Fromm, 1997). Circumstantial evidence indicating the cambial cells as the major sink for nutrients is derived from the large number of plasmodesmata in tangential walls of ray cells (Sauter and Kloth, 1986).

In this study, various methods were applied to investigate seasonal changes and an assumed effect of auxin on the PM H⁺-ATPase of cambial cells: (a) Changes in potassium distribution were measured by x-ray microanalysis. (b) Specific antibodies against the plasma membrane H⁺-ATPase were used to immunolocalize the enzyme to measure its distribution and amount in thin sections of Populus trichocarpa twigs. Either the antibody was labeled with Cy3 and localized by fluorescence microscopy, or it was labeled with gold particles and visualized by transmission electron microscopy. (c) Tissue homogenates were analyzed using the western-blot technique for the presence of PM H⁺-ATPase at various times of the year and in dormant plants after the addition of auxin. (d) Endogenous ionic current as an indicator of the activity of a PM H⁺-ATPase was measured with a noninvasive, vibrating electrode near the face of stem cuttings from shoots of Populus nigra, assuming that the PM H⁺-ATPase drives H⁺ current through the plasma membrane and the adjacent apoplasm. Auxin was either applied briefly to measure short-term effects of the hormone or for several days to allow for gene activation.

RESULTS

Potassium Distribution

Energy-dispersive x-ray analysis in combination with scanning electron microscopy (SEM) provides an appropriate technique for semiquantitative detecting of elements in the intact tissue on the microscopic level. Although the physical heterogeneity of the tissue matrix did not permit a comparison with a calibration standard for absolute quantification of potassium concentrations, this method revealed a strong seasonal change of the relative potassium concentration and distribution in twig tissue of P. trichocarpa. Especially the cambial region showed strong changes in potassium content reflecting its seasonal states of activity: An increased level of potassium was measured in the active cambial region in May, whereas only a low level of potassium was found in the dormant cambium and its daughter cells in January (Fig. 1). In comparison with the adjacent mature tissues of the phloem and xylem, the highest amount of potassium was found in the active cambial region as indicated by the distinct accumulation of potassium-specific x-ray signals in this part of the twigs (Fig. 2, C and D). In contrast, potassium showed an equal distribution across twigs during the time of cambial dormancy (Fig. 2, A and B).

Western-Blot Analysis

The specificity of the monoclonal antibody 46 E5 B11 F6 against a PM H⁺-ATPase of P. trichocarpa was checked by western-blot analysis of twig tissue homogenates. After electrophoresis of twig tissue homogenate collected in May after cambial reactivation, a binding of the antibody to a polypeptide in the
100-kD range was detected on the blots (Fig. 3A). A second minor band was recognized by the antibody in the 60-kD range, probably a proteolytic degradation product of the enzyme with an epitope for the antibody. In contrast, no PM H⁺-ATPase was detected by the antibody on blots that were prepared from twig tissue in November during cambial dormancy (Fig. 3A). Auxin applied for 2 d to debudded twigs (40 μM indole-3-acetic acid [IAA]-sodium salt in water) during the period of dormancy induced a new expression of PM H⁺-ATPase as indicated by immunodetectable amounts of the enzyme on the blots (Fig. 3B). A polyclonal antibody raised against a PM H⁺-ATPase from *Nicotiana plumbaginifolia* reacted with twig tissue homogenate from poplar plants similarly to the monoclonal antibody 46 E5 B11 F6 (Fig. 3C). During cambial activity a major band was detected by the polyclonal antibody in the 100-kD range and a second minor band in the 60-kD range, whereas twig tissue prepared during cambial dormancy showed only a very slight immunoreactivity in the 100-kD range.

**Immunofluorescence Microscopy**

Cross sections were cut from twigs in spring after cambial reactivation and incubated with the monoclonal antibody 46 E5 B11 F6 against PM H⁺-ATPase and a Cy3-conjugated secondary antibody. A strong specific fluorescence labeling was visible predominantly in the active cambial zone and the adjacent region of xylem cell differentiation (Fig. 4A). In the mature xylem, there was some specific labeling of ray cells that contacted vessel cells (Fig. 4B). In contrast, no specific labeling was observed in cross sections taken during cambial dormancy (Fig. 4C). After treatment of debudded twigs in December during cambial dormancy with 40 μM IAA for 4 d, specific labeling of a PM H⁺-ATPase became visible in the cambial zone and in some ray cells that contact vessels in the mature xylem (Fig. 4, D and E). Controls showed only an unspecific background labeling, either in the absence of the primary antibody or after replacement of the specific antibody with nonimmune IgG (Fig. 4F). For comparison, twig sections were also treated with the polyclonal antibody L3E against PM H⁺-
ATPase in place of the monoclonal antibody. Sections taken during cambial activity showed a fluorescence pattern different from that given with the monoclonal antibody. A distinct specific labeling occurred in cells of the inner phloem, the transition zone phloem/cambium, and the ray parenchyma, whereas no PM H$^+$/H$_{11001}$-ATPase was detected by the polyclonal antibody in the differentiating xylem (Fig. 5A). In addition, a weak specific fluorescence was visible in many cells of the cortex, indicating the presence of a small amount of PM H$^+$/H$_{11001}$-ATPase in this tissue (Fig. 5B).

Twig sections taken during cambial dormancy showed a reduced intensity of the fluorescence labeling, mostly restricted to ray cells crossing the cambial zone and the inner phloem (Fig. 5C). Controls showed no fluorescence, either in the absence of the primary antibody or after replacement of the specific antibody with nonimmune IgG (Fig. 5D).

**Immunogold Electron Microscopy**

Because the specific fluorescence labeling given by the monoclonal antibody appeared over all the labeled cells, immunohistochemical experiments were carried out with the transmission electron microscope for detailed localization of this PM H$^+$-ATPase. A procedure of labeling before embedding was carried out using ultrasmall gold conjugates in combination with silver enhancing. The silver-enhanced gold particles appeared predominantly along the plasma membrane of labeled cells, indicating that a large number of the PM H$^+$-ATPases was present in this cellular domain (Fig. 6, A–F). In agreement with results obtained from immunofluorescence microscopy, a specific labeling of a PM H$^+$-ATPase was found along the plasma membrane of cells of the active cambium, as well as in expanding cells of the immature xylem and in ray cells contacting vessels of the mature xylem. In cambial cells, the intensity of immunolabeling along the plasma membrane was always higher in radial sections than in cross sections because of the much better preservation of cellular structures, including membranes, in the former case (Fig. 6, A and B). Differentiating xylem cells, newly formed vessels, fiber cells, and ray cells showed labeling of a lower intensity than the cambial cells proper (Fig. 6, C–F). A very strong immunolabeling of the plasma membrane was observed in companion cells of the inner phloem in contact with the active cambium (Fig. 7C), whereas the sieve elements attached to these companion cells showed no labeling. The transmission electron microscopy results also show that a labeling of ray cells in mature xylem only occurs when these cells contact vessels (Fig. 7, A and B). A few gold particles were bound to other cell structures. The latter labeling was nonspecific, because a similar pattern was observed in control sections incubated with nonimmune IgG (Fig. 7, D and E).

**Endogenous Ionic Current during the Seasons**

The direction and density of endogenous ionic current was measured at different sites of cross sections during various times of the year. The results show that the current pattern and current density in shoot cuttings of *P. nigra* depended on the season: In dormant shoots in December, only small outward current was measured at all sites, i.e. the pith parenchyma, secondary xylem (wood), cambium/phloem zone, and cortex parenchyma (Fig. 8). In April, a period of active cambial growth, strong outward current with densities of more than 6 μA cm$^{-2}$ was measured in the cambium/phloem zone and the green cortex. Outward current was also found in the pith and the mature xylem, whereas strong inward current with densities of up to 8 μA cm$^{-2}$ was measured during cambial activity (May), respectively, cambial dormancy (November).
sured in the young xylem. A similar current pattern was measured in August, but the current direction in the cambium/phloem zone had changed to inward. The overall current density decreased from April to August and from August to December at all measurement sites.

During experiments performed in August, the ionic composition of the medium was changed to obtain information about the ions involved in the endogenous current. When the pH of the medium was increased, the current density also increased significantly at all measurement sites, and outward cur-

Figure 4. Localization of a PM H⁺-ATPase in twig tissue of *P. trichocarpa* using the monoclonal antibody 46E5B11F6. A, Section taken after cambial reactivation (April). Strong fluorescence labeling appears in the cambium zone (arrows) and the adjacent region of xylem cell differentiation. Arrowheads show newly formed vessels. B, Section taken after cambial reactivation. Fluorescence labeling of some ray cells that contact vessels in the mature xylem (arrows) can be seen. C, Section taken during cambial dormancy (December). No fluorescence labeling of a PM H⁺-ATPase is visible. D, Section taken during cambial dormancy (December) after treatment of debudded twigs with auxin for 4 d. Fluorescence labeling appears in the cambium/phloem zone (arrows). E, Fluorescence labeling of ray cells in the mature xylem after treatment of debudded twigs with auxin in December (arrow). F, Control section taken after cambial reactivation (April) incubated with nonimmune IgG instead of the monoclonal antibody against a PM H⁺-ATPase. Xy, Xylem; Ph, phloem.
rent became dominant. Figure 9 shows a typical example of current density vectors measured across the face of cuttings, first at pH 4.5 and then at pH 7.5. At both sites, i.e. the cortex parenchyma (Fig. 9A) and the cambium/phloem zone (Fig. 9B), the current density increased, and the direction changed from inward to outward in the cambium/phloem zone. No immediate changes of the current density were measured when the potassium or chloride concentration of the medium was changed (data not shown). After increasing the calcium concentration of the medium to 1.0 mM, a decrease of the outward current was observed at the cortex and a decrease of the inward current in the cambium/phloem zone.

Because it is well known that plant hormones are involved in cambial growth and differentiation of trees, we investigated the effects of two prominent phytohormones, i.e. IAA and abscisic acid (ABA), on the endogenous ionic current of poplar shoots. The effect of 30 μM IAA was measured in December when only small endogenous current was present. In accord with the IAA activation of a PM H⁺-ATPase shown with immunoblot and immunofluorescence (compare with Figs. 3 and 4), IAA caused an increase of current density at all measurement sites already 2 h after application (Fig. 10A). The mean current density increased by 60% from 1.7 to 2.8 μA cm⁻².

The formation of latewood at the end of summer has been attributed to an increase in the level of ABA in the cambial zone (Jenkins and Shephard, 1974; Wodzicki and Wodzicki, 1980). ABA was also shown to have a strong inhibitory effect on wood growth (Fromm, 1997) and magnitude of endogenous current of willow roots (Fromm et al., 1997). During the time of cambial activity and high-current densities in spring, application of 30 μM ABA to the medium caused the density of outward current to decrease by almost 50% within 2 h, i.e. from 6.2 to 3.2 μA cm⁻² (Fig. 10B). These results suggest an opposing role of both hormones in the regulation of endogenous current and activity of the PM H⁺-ATPase activity in poplar plants.
Figure 6. Cellular fine localization of a PM H\(^+\)-ATPase in the active cambium zone and the region of xylem cell differentiation using the monoclonal antibody 46 E5 B11 F6. Samples were taken in May. A and B, Radial section of an active cambial cell with strong labeling along the plasma membrane (arrows). C and D, Cross section of newly formed xylem cells. Labeling of the plasma membrane appears in a ray cell and a fiber cell (arrows). E and F, Radial section of an enlarging vessel cell with labeling of the plasma membrane (arrows). V, Vacuole; CW, cell wall; VC, vessel; RC, ray cell; FC, fiber cell.
Figure 7. Cellular fine localization of a PM H⁺-ATPase in the inner phloem and in ray cells of mature xylem. Samples were taken in May. 
A and B, Cross section of mature xylem. Labeling appears along the plasma membrane of a ray cell that contacts a mature vessel cell (arrows). C, Cross section of a sieve element/companion cell complex of the inner phloem. Strong labeling occurs along the plasma membrane of the companion cell. D and E, Controls. Cross sections of a ray cell and a cambial cell. VC, vessel; RC, ray cell; CA, cambial cell; SE, sieve element; CC, companion cell.
discriminates between different PM H$^+/H_1$-ATPase isoforms in a cell-specific manner (Langhans et al., 2001). The complete lack of specific labeling for a PM H$^+/H_1$-ATPase in pith cells remains an open question, because this tissue drove ionic currents during periods of high-growth activity. Besides the occurrence of further PM H$^+/H_1$-ATPase isoforms, an alternative explanation for the outward current at the pith might be an efflux of K$^+$ and Na$^+$ or an influx of Cl$^-$ ions. K$^+$ and Na$^+$, however, are very unlikely to carry the outward current. A massive and prolonged leakage of K$^+$ from the small number of injured cells at the cutting plane does not seem feasible, and a Na$^+$/K$^+$ ATPase that would transport Na$^+$ to the medium is absent in plant cells. Net Cl$^-$ uptake, on the other hand, might be a real possibility for the outward current. Such uptake has been reported from bean leaves during photosynthesis (Shabala and Newman, 1999), and pith cells showed a distinct fluorescence of chlorophyll when twig sections from poplar plants were inspected with the fluorescence microscope.

During all seasons, the cortex parenchyma, the mature xylem, and the pith parenchyma drove current from the protoplasts to the apoplast. The cambial zone showed outward current in the spring, but inward current in the late summer. Young wood cells were a current sink during the entire growing season. The current densities were highest in spring, declined in late summer, and became rather small in winter when the plants were dormant.

Combining the data from PM H$^+/H_1$-ATPase localization and current measurements, there are several indicators that a major component of the endogenous current is H$^+$ ions: (a) The high concentration of PM H$^+/H_1$-ATPases, particular in the cambial zone and the differentiating xylem, during cambial growth correlates positively with outward current during the same time. The slight amount of immunodetectable PM H$^+/H_1$-ATPases in dormant plants conversely correlates with low-current densities. (b) The current density increased with increasing pH and addition of auxin. A higher pH on the outside of cells favors H$^+$

**DISCUSSION**

In this study, the PM H$^+/H_1$-ATPase, K$^+$-ion content and endogenous ionic currents were investigated in poplar plants using specific antibodies, x-ray analysis, and a noninvasive vibrating probe. A PM H$^+/H_1$-ATPase was visualized by the monoclonal antibody 46 E5 B11 F6 in a very sensitive manner in physiologically active poplar plants during cambial growth, but not in dormant ones. In contrast to herbaceous species, this PM H$^+/H_1$-ATPase was localized in the cambium zone, in differentiating xylem cells and in ray cells surrounding vessels in the mature xylem. Auxin applied to dormant plants induced the formation of this PM H$^+/H_1$-ATPase in the cambial region as well as in ray cells. Experiments carried out additionally with the electron microscope showed that the PM H$^+/H_1$-ATPase was localized in the plasma membrane of the labeled cells. No immunoreactivity was obtained with this antibody in the cortex parenchyma and in pith cells. This result came as a surprise because these tissues produced ionic currents with similar direction and density as the cambial region during periods of high-growth activity. Using a polyclonal antibody, further PM H$^+/H_1$-ATPases were localized in the inner phloem and in cells of the cortex parenchyma. Surprisingly, no immunoreactivity was found using the polyclonal antibody in the differentiating xylem and in pith cells. Only a slight labeling was observed during cambial dormancy.

Taken together, a clear seasonal variation in the amount of the PM H$^+/H_1$-ATPase was shown in poplar twigs using different specific antibodies and correlated to cambial growth activity. Similar to this observation, a seasonal variation of different PM H$^+/H_1$-ATPase transcripts was demonstrated recently in the bud tissue of peach trees (Gevaudant et al., 2001). The different labeling pattern given by monoclonal and polyclonal antibodies indicated that different isoforms of the enzyme are present in poplar tissues, of which one group is mainly found in the cambial tissue and the differentiating xylem. Molecular studies have shown that in plants, the PM H$^+/H_1$-ATPase is encoded by a multigene family, and PM H$^+/H_1$-ATPase isoforms exhibit a distinct pattern of tissue-specific expression (compare with Palmgren, 1998; Palmgren, 2001). For castor bean (Ricinus communis) plants, it was recently demonstrated that the monoclonal antibody 46 E5 B11 F6 discriminates between different PM H$^+/H_1$-ATPase isoforms in a cell-specific manner (Langhans et al., 2001). The complete lack of specific labeling for a PM H$^+/H_1$-ATPase in pith cells remains an open question, because this tissue drove ionic currents during periods of high-growth activity. Besides the occurrence of further PM H$^+/H_1$-ATPase isoforms, an alternative explanation for the outward current at the pith might be an efflux of K$^+$ and Na$^+$ or an influx of Cl$^-$ ions. K$^+$ and Na$^+$, however, are very unlikely to carry the outward current. A massive and prolonged leakage of K$^+$ from the small number of injured cells at the cutting plane does not seem feasible, and a Na$^+$/K$^+$ ATPase that would transport Na$^+$ to the medium is absent in plant cells. Net Cl$^-$ uptake, on the other hand, might be a real possibility for the outward current. Such uptake has been reported from bean leaves during photosynthesis (Shabala and Newman, 1999), and pith cells showed a distinct fluorescence of chlorophyll when twig sections from poplar plants were inspected with the fluorescence microscope.

**Figure 8.** Pattern and densities (±se) of endogenous ionic current of different tissues across sections of vertically oriented shoot cuttings from *P. nigra* measured in April, August, and December. Current densities were measured in more than 10 different shoots collected from the trunks or roots of *P. nigra* trees.
export by the PM H\textsuperscript{+}-ATPase (Takeuchi et al., 1985; Takeshige et al., 1986), and auxin is known to stimulate H\textsuperscript{+} excretion (Talbott et al., 1988; Lüthen et al., 1990; Hager et al., 1991). (c) The current density decreased with increasing Ca\textsuperscript{2+} concentration and addition of ABA. Both compounds are known to turn down the activity of the PM H\textsuperscript{+}-ATPase (Beffagna et al., 1995; Goh et al., 1996; Lino et al., 1998).

Could it be that the PM H\textsuperscript{+}-ATPases, the endogenous current, and auxin have a role in growth and differentiation of the cambial region? We think they have. During growth and differentiation of cambial cells, K\textsuperscript{+} ions are taken up, the surface area of the cells expands, and cell wall polysaccharides/proteins are secreted (Savidge, 1996). In spring, high concentrations of endogenous auxin are present in young shoots and twigs (Little and Savidge, 1987), and a promotion of cambial activity by auxin that was applied to debudded stems has been demonstrated (Wareing et al., 1964). This auxin was transported to the cambium and its young derivatives (Lachaud and Bonnemain, 1984; Tuominen et al., 1997; Uggla et al., 1998). Auxin has also been shown to mediate an increase in H\textsuperscript{+}-ATPase transcripts in the plasma membrane of maize coleoptiles (Hager et al., 1991; Frias et al., 1996), and small "auxin up RNA’s" and "auxin genes" have been found during cell elongation (Abel et al., 1994; Abel and Theologis, 1996). Similar effects of auxin are likely to occur in the cambial region of poplar plants because the number of PM H\textsuperscript{+}-ATPases and the ionic current density increased with the addition of auxin. The increase in current density indicates more H\textsuperscript{+} ions in the apoplast and hyperpolarization of the membrane potential in cells of the cambial zone. An elevated proton gradient and a higher membrane voltage favor the uptake of sugars via symport with protons into the rapidly growing cambial cells and an influx of K\textsuperscript{+} ions due to opening of ion channels in the plasma membrane of expanding young vessel cells (Briskin and Gawienowski, 1996). We were recently able to demonstrate that the formation of fewer and larger vacuoles during cambial reactivation is caused by increased K\textsuperscript{+} uptake, probably modulated by the

Figure 9. Typical examples of the changes in current density and direction after increasing the pH of the medium from pH 4.5 to 7.5. A, Endogenous current at the cortex parenchyma of a poplar shoot cutting. B, Endogenous current at the cambium/phloem zone. Both examples were measured in August in shoots from *P. nigra* trees.

Figure 10. Typical examples of short-term effects of IAA and ABA on the endogenous ionic current of shoot cuttings from *P. nigra*. A, IAA (30 \(\mu\)M) was added for 2 h to the artificial pond water (APW) bathing a dormant shoot cutting in December. B, ABA (30 \(\mu\)M) was added for 2 h to the APW bath of a shoot cutting during high cambial activity in April. Absolute current densities are given and projected onto the cross sections of the shoots as columns of inward or outward current.
activity of a PM H\(^+\)-ATPase (Arend and Fromm, 2000). Thus, the auxin-induced up-regulation and activation of a PM H\(^+\)-ATPase in poplar plant cells seems to be a prerequisite for nutrient uptake and growth of cambial and young xylem cells. This is similar to roots, where inward H\(^+\)-current is correlated with nutrient uptake and plasmatic growth at the root apex, and outward current with uptake of minerals and cell enlargement in the elongation zone (Weisenseel et al., 1992). A change in current direction during the season, as for instance measured in the cambial zone, may, therefore, be an indicator of a shift in the mode of growth. ABA, in contrast to auxin, strongly reduced the endogenous current of poplar plants. This correlates positively with the strong inhibitory effect of ABA on wood growth (Fromm, 1997). In late summer, the formation of late-wood has also been attributed to an increase in the level of endogenous ABA in the cambial zone, effecting cessation of cambial activity (Jenkins and Shephard, 1974; Wodzicki and Wodzicki, 1980). Our present results indicate a role for auxin in hardwood formation of poplar trees via activation of a PM H\(^+\)-ATPase in the cambial region.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Poplar (Populus nigra var. italica L. and Populus trichocarpa Torr. et Gray.) plants were selected for the experiments because of the well-known advantages of the genus Populus as a model for hardwood secondary vascular growth (Chaffey, 1999). For electron microscopic investigations (SEM-energy-dispersive x-ray analysis), immunoblot analysis, and immunocyto-chemical procedures, twig tissue samples were collected from mature poplar (P. trichocarpa) trees grown in a forest near the campus of the Technical University of Munich. For measurements of endogenous current, stem cuttings with a length of approximately 50 mm and a diameter of 5 to 7 mm were taken from 1- to 2-year-old upright shoots sprouting from the trunk or roots of poplar trees (P. nigra) growing on the campus of the University of Karlsruhe.

SEM and X-Ray Microanalysis

Small sections of twig tissue were cut with a razor blade and immediately shock-frozen in liquid isopentane at its melting point. After freeze-drying, the samples were coated with chromium and examined in a scanning electron microscope (AMR 1200, Leitz, Wetzlar, Germany) fitted with a KEVEX 4000 x-ray analyzer. Element-specific x-ray spectra were obtained from a reduced scan raster area at 1,000× magnification. Relative potassium concentrations were expressed as peak to background ratio from 10 relative to 2000. Thus, the auxin-induced up-regulation and activation of a PM H\(^+\)-ATPase in poplar plant cells was tested by western-blot analysis (see below). For comparison, the polyclonal antibody LE3 raised against a polypeptide corresponding to the PMA 2 isogene (plasma membrane H\(^+\)-ATPase 2) of Nicotiana plumbaginifolia was used for western blotting and immunolocalization (Morosomme et al., 1998).

SDS-Page and Immunoblot Analysis

Poplar twigs were collected in spring during cambial activity and in autumn during cambial dormancy. From each sample, 0.5 g of fresh tissue was homogenized in an ice-cooled mortar with 5 mL of 10 mm Tris-HCl, 5 mm EDTA, and 1% (v/v) plant protase inhibitor cocktail (Sigma, St. Louis), pH 6.8, and filtered through a layer of gauze. The filtrate was centrifuged at 20,000 g for 10 min at 4°C. The resulting pellet was resuspended in 0.5 mL of solubilization buffer containing 50 mm Tris-HCl, 2% (w/v) SDS, and 3% (v/v) ß-mercaptoethanol, pH 6.8. After incubation for 1 h at 25°C, the solution was centrifuged for 1 min at 8,000 g, and a sample of the supernatant was loaded without heating onto an 8% (v/v) SDS-polyacrylamide minigel. Protein standards of known M\(_t\) (prestained broad range, Bio-Rad, Hercules, CA) were run on the same gel. Proteins were transferred to a polyvinylidene difluoride membrane using a semidry transfer unit (TRANS-BLOT SD, Bio-Rad). The membrane was washed in Tris-buffered saline (TBS), blocked for 2 h with 1% (w/v) bovine serum albumin (BSA) in TBS, incubated for 1 h with the monoclonal mouse antibody 46 E5 B11 F6, diluted 1:500 or with the polyclonal rabbit antibody LE3, and diluted 1:1,000 both in PBS containing 0.5% (w/v) BSA. After washing in TBS containing 0.05% (v/v) Tween 20, the membrane was incubated for 1 h with 1-nm gold-conjugated goat anti-mouse antibody or anti-rabbit antibody (British Biocell International, Cardiff, UK), diluted 1:400 in TBS containing 1% (w/v) BSA. The labeling of the membrane was developed with a silver enhancing Kit (British Biocell International) according to the manufacturer's instructions.

Fluorescence Microscopy Immunolabeling

Samples of 10 to 20 mm in length were cut from 1- or 2-year-old twigs and fixed with 3% (w/v) formaldehyde (freshly prepared from paraformaldehyde) and 3 mm EDTA in 50 mm PIPES, pH 6.8, for 1 h at 4°C temperature. After washing in PIPES solution, cross sections of 50 μm thickness were cut with a microtome and rinsed in PBS, pH 7.2. To reduce unspecific labeling, the sections were blocked with 100 mm glycine in PBS-T (PBS containing 0.2% (w/v) Tween 20 and 3% (w/v) BSA in PBS-T, both for 30 min. For immunolocalization, sections were incubated for 2 h at 37°C with the monoclonal mouse antibody 46 E5 B11 F6 diluted 1:500 or with the polyclonal rabbit antibody LE3 diluted 1:1,000, both in PBS containing 0.5% (w/v) BSA. After washing in PBS, the sections were incubated for 1 h at 37°C with Cy3 labeled anti-mouse antibody, respectively, anti-rabbit antibody (Cy3 conjugates, Dianova, Hamburg, Germany) diluted 1:250 in PBS containing 0.5% (w/v) BSA. After washing in PBS, the sections were incubated for 1 h at 37°C with Cy3 labeled anti-mouse antibody, respectively, anti-rabbit antibody (Cy3 conjugates, Dianova, Hamburg, Germany) diluted 1:250 in PBS containing 0.5% (w/v) BSA. After the incubation period, the sections were rinsed in PBS-T and then viewed using an axiophot microscope (Zeiss, Jena, Germany) equipped with the filter combination 456-nm exciter and 590-nm emitter. Photographs were taken with Kodak Ektachrome 320 T film (Eastman Kodak, Rochester, NY). Incubations in medium containing Cy3-conjugated secondary antibody without primary antibody or in medium with mouse nonimmune IgG diluted 1:200 instead of primary antibody served as controls.

Electron Microscopy Immunolabeling

Small cuttings from poplar twigs were collected in spring after cambial reactivation and fixed for 1 h at room temperature with 3% (w/v) formaldehyde (freshly prepared from paraformaldehyde) in PBS, pH 7.2, containing 1% (w/v) saponin for permeabilization of cells. After washing in PBS-T, unspecific binding sites were blocked for 30 min with 100 mm glycine and for another 30 min with 5% (v/v) goat normal serum and 1% (w/v) BSA, both in PBS-T. For immunolocalization, samples were incubated overnight at 4°C with the antibody 46 E5 B11 F6 diluted 1:500 in PBS containing 0.5% (w/v) BSA, then washed in PBS-T and incubated for 2 h at room temperature with 1 nm gold-conjugated goat anti-mouse antibody (British Biocell International) diluted 1:400 in PBS containing 0.5% (w/v) BSA. Controls were incubated with nonimmune mouse IgG in place of the specific primary antibody. After washing in PBS-T, the sections were post-fixed for 30 min with 2% (v/v) glutaraldehyde in PBS and washed in distilled water. For visualizing the 1-nm gold particles at the electron microscope level, silver Enhanced Kit (British Biocell International) according to the manufacturer's instructions.
enhancement was carried out with the silver-enhancing kit from British Biocell International according to the manufacturer’s instructions before the samples were dehydrated and embedded in Spurr’s epoxy resin. This unusual procedure greatly improved the detection of a PM H+-ATPase. Ultrathin sections from the peripheral tissue layers were cut with a diamond knife on an ultramicrotome (LBK, Uppsala), transferred onto Formvar-coated copper grids, and stained with lead citrate. Sections were examined using a Zeiss EM 10 transmission electron microscope operated at 80 kV.

Measurement of Endogenous Ionic Currents

Stem cuttings of approximately 50 mm in length from shoots were trimmed at their apical surface to an angle of 30° and fastened vertically into small petri dishes with a front window of cover glass and a downward extending tube of Plexiglas. The dishes were filled with 25 mL of APW containing 1.0 mM NaCl, 0.1 mM KCl, 0.1 mM CaCl2, and 1.0 mM MES; adjusted to pH 6.0 with Tris. The dishes were suspended into the stage of an inverted microscope and viewed from the front with a horizontal stereoscope (Leitz). The cuttings were left to settle and recover from handling for 30 to 60 min before the measurements. Endogenous current was measured with a three-dimensional vibrating-probe set-up as described by Weiseenseel et al. (1992). In brief, the three-dimensional probe measures the total current density and its spatial components, i.e. it measures current density vectors. For the measurements, a 50–tilted, insulated metal electrode (SS300005A, Micro Probe, Clarksburg, MD) with a 30-μm spherical tip of platinum black was vibrated lengthwise and approximately 200 μm above the green cortex parenchyma, the cambial zone, young and mature xylem cells, and the pith parenchyma. Three consecutive measurements were carried out at each site by rotating the probe, which was mounted to the bottom of a horizontally revolving stage, into three positions separated by 30°, respectively. The probe was vibrated with a frequency of 317 Hz, an amplitude of 35 μm, and a duration of 20 s in each position. At sites with an electric potential difference, originating from ionic current flow through the aqueous medium, this voltage was picked up by the electrode and transformed into an AC voltage by the vibration. The latter was measured using a lock-in amplifier (S210, PerkinElmer Life Sciences, Boston). The three values of voltage measured at each site were then used to calculate the local current density vector with the aid of a custom-made algorithm. Current densities may be translated into ion fluxes by equating 1 μA cm−2 with 10 pmol cm−2 s−1 of monovalent ions.

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