Xyloglucan Endotransglycosylases Have a Function during the Formation of Secondary Cell Walls of Vascular Tissues

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Xyloglucan transglycosylases (XETs) have been implicated in many aspects of cell wall biosynthesis, but their function in vascular tissues, in general, and in the formation of secondary walls, in particular, is less well understood. Using an in situ XET activity assay in poplar stems, we have demonstrated XET activity in xylem and phloem fibers at the stage of secondary wall formation. Immunolocalization of fucosylated xylogucan with CCRC-M1 antibodies showed that levels of this species increased at the border between the primary and secondary wall layers at the time of secondary wall deposition. Furthermore, one of the most abundant XET isoforms in secondary vascular tissues (PttXET16A) was cloned and immunolocalized to fibers at the stage of secondary wall formation. Together, these data strongly suggest that XET has a previously unreported role in restructuring primary walls at the time when secondary wall layers are deposited, probably creating and reinforcing the connections between the primary and secondary wall layers. We also observed that xylogucan is incorporated at a high level in the inner layer of nacreous walls of mature sieve tube elements.

INTRODUCTION

Secondary walls, which are formed after cell expansion, are essential for the function of conductive and supportive tissues in terrestrial plants. In perennial plants, secondary xylem (wood) and phloem make up most of the biomass, and these plants can serve as an excellent system in which to study the development of secondary walls. Xylem cells form tripartite secondary wall structures inside the primary wall, in which successive S1, S2, and S3 layers have an alternating orientation of cellulose microfibrils and are lignified (reviewed by Funada, 2001; Mellerowicz et al., 2001). The sieve tubes in the phloem have cell walls with two or more layers, including a nacreous layer inside the primary wall, with transversely oriented cellulose microfibrils (Esau, 1969; Evert, 1977). These walls are able to withstand high turgor pressure within conductive phloem tubes, but their precise chemical nature has not been elucidated. Enzymes involved in the lignification process during vascular development have been studied in detail (Li et al., 2001; Mellerowicz et al., 2001; Humphreys and Chapple, 2002), but cell wall carbohydrate biosynthesis and the assembly of secondary walls are still relatively poorly understood, and our current understanding of secondary wall architecture has not changed much since the beginning of the 20th century (Bailey, 1954). However, the situation is changing rapidly as genes involved in these processes are being sequenced and studied (Hertzberg et al., 2001; Mellerowicz et al., 2001; Milioni et al., 2001; Whetten et al., 2001).

Xyloglucan (XG) is a primary wall hemicellulose that coats and cross-links cellulose microfibrils. Current models (Carpita and Gibeaut, 1993) assume that either breakage of the cross-links or their disconnection from the microfibrils is needed to allow the microfibrils to move apart, allowing the wall to expand. The enzyme xyloglucan endotransglycosylase (XET; EC 2.4.1.207), which can cut and rejoin XG chains, is considered a key agent regulating wall expansion and is believed to be the enzyme responsible for the incorporation of newly synthesized XG into the wall matrix (Darley et al., 2001). XET also has been implicated in the wall degradation needed for fruit ripening, the formation of lygogenous aerenchyma, abscission, and the wall strengthening that takes place during tigmomorphogenesis (reviewed by Nishitani, 1997, 1998; Campbell and Braam, 1999). XET catalyzes molecular grafting between XG molecules by endolytically cleaving the XG backbone. This is followed by the formation...
of a covalent enzyme substrate intermediate and a deglyco-
sylation step involving a XG acceptor, which releases the
enzyme and leads to the formation of a new β-1,4-glyco-
sidic bond (Fry et al., 1992; Nishitani and Tominaga, 1992;
Sulová et al., 1998, 2001). Some members of the XET family
also can use water as an acceptor, which results in XG hy-
drolysis (de Silva et al., 1993; Fanutti et al., 1993; Tabuchi et
al., 2001).

In accordance with its role in the construction and modifi-
cation of cell wall architecture, XET activity and expression
have been detected commonly in growing tissues (Fry et al.,
1992; Pritchard et al., 1993; Palmer and Davies, 1996;
Burstin, 2000; Uozu et al., 2000; Vissenberg et al., 2000,
2001). In addition, XET also is clearly present in tissues in
which expansion has ceased (Arrowsmith and de Silva,
1995; Xu et al., 1995; Palmer and Davies, 1996; Shimizu et
al., 1997; Akamatsu et al., 1999; Burstin, 2000; Catalá et al.,
2001; O’Donoghue et al., 2001). In these cases, XET is
thought to play a role in wall degradation or in poorly de-
finite XG rearrangements. Several studies have reported
XET expression in unspecifed cell types of primary vascular
tissues (Xu et al., 1995; Antosiewicz et al., 1997; Oh et al.,
1998; Vissenberg, 2000; Herbers et al., 2001) and the
internal phloem of tobacco stems (Medford et al., 1991).
These findings suggest that XET may be involved in the for-
mation of secondary walls of xylem and/or phloem cells.
However, its precise function has not been determined, and
the role of XET in the secondary vascular tissues has not
been studied previously. In this report, we present evidence
for novel functions of XET in the development of secondary
walled cells in the xylem and phloem of poplar stems.

RESULTS

Colocalization of XET Activity and the XG Donor in
Secondary Vascular Tissues

To investigate the involvement of XET in the formation of
secondary vascular tissues, XET activity was determined in
poplar stems using the in situ assay developed by Vissenberg
et al. (2000). This assay is based on the incorporation of sul-
forhodamine-labeled xylogluco-oligosaccharides (XGO-SR)
into fresh tissue sections, which marks the location of active
XET together with the donor substrate. The cambial region
was a major site of XET activity, and in particular, the mer-
istematic cambial zone (denoted cambium) was labeled in-
tensively (Figure 1). Collenchyma tissue in the cortex also
exhibited strong label incorporation, but not as strong as
that in the cambium. Higher magnification images of the
cambium showed the label in radial (R) as well as tangential
(T) walls of both fusiform and ray cell initials (Figures 1C and
1D). In secondary phloem, XET activity was confined to the
ray and axial parenchyma (Figures 1C and 1E), whereas

sieve tube cells were labeled only during the early stages of
differentiation (Figure 1D). In addition, XET activity was
found in phloem fibers undergoing secondary wall thickening
(Figure 1E). Here, the label was associated specifically with
the thickening of the secondary layer and disappeared when
the wall was completed. Differentiating secondary
xylem cells were labeled during their radial expansion, but
surprisingly, more intense labeling was associated with xyl-
em fibers during their secondary wall thickening (Figures 1D
and 1F). The activity assay results indicated a function for
XET in several tissues and cell types of the poplar stem,
supporting the hypothesis that it has a multifunctional role in
cell wall construction. We were particularly intrigued by the
observation that secondary wall-forming xylem cells still
contain active XET capable of incorporating externally
added acceptor into the donor substrate, because other
studies have reported that the XG is localized uniquely in the
primary wall layers in mature xylem cells (Baba et al., 1994;
Stacey et al., 1995).

The unexpected pattern of XET activity in the cambial re-
gion of poplar led us to investigate the presence of XG in
these tissues. For this purpose, we used a monoclonal anti-
body (CCRC-M1) that recognizes terminal fucose-linked α(1→2)
to a galactosyl residue commonly found in dicot XG
(Puhlmann et al., 1994). Light microscopy showed a good
correlation between the cell types marked as containing XG
by the CCRC-M1 antibody (Figure 2) and the cell types that
exhibited XET activity (Figure 1). However, a major excep-
tion to this finding was the presence of CCRC-M1 label in
mature sieve tube cells (Figure 2C) that lacked XET activity
(Figure 1C). The CCRC-M1 label intensity was much higher
in developing xylem fibers than in vessels (Figure 2F).

For a more precise localization of XG, immunogold label-
ing was applied to ultrathin sections in transmission electron
microscopy. Analysis of label distribution in all xylem cells
and phloem fibers revealed the expected localization of XG
in their compound middle lamellae (primary wall layer and
middle lamella), as shown in Figures 3, 4A, and 4C. In the
cambium, the labeling was weak and equally dense in R and
T walls of fusiform initials (Figure 4B). In the radial expansion
zone of the xylem, there were always weaker CCRC-M1 sig-
nals from the developing vessels compared with fibers. This
difference also was seen by light microscopy, and it per-
sisted in the mature xylem (Figure 4C). During the early
stages of secondary wall development in the xylem fibers,
the CCRC-M1 label was particularly intense (Figures 3A to
3D). When the labeling pattern during xylem cell develop-
ment was studied semiquantitatively, a dramatic increase in
labeling was found at the stage of secondary wall deposition
(Figure 3E). Lines of deposition were apparent at the pri-
mary/secondary (S1) layer boundary, with the label present
in the primary wall (Figures 3C and 3D). Some label also was
clearly visible in the secondary wall layer and in the periph-
eral cytoplasm (Figure 3C and data not shown). However, in
fibers with an almost fully developed secondary wall layer,
this signal was absent and the label was restricted to the
Figure 1. Localization of XET Activity in Poplar Stem Tissues.

Fresh stem sections were incubated with rhodamine-labeled XG oligosaccharides. Sites of label incorporation indicate the presence of XG donor and active XET enzyme.

(A) Cross-section of the stem. Most XET activity is in the cambium, its recent derivatives, and collenchyma cells in the cortex. Activity also can be seen in developing phloem fibers.

(B) Control section heated for 10 min at 90°C before incubation with the substrate.

(C) Closeup of cambium and phloem. XET activity is seen only early during sieve tube development, whereas it is detected at all developmental stages in the phloem ray cells.

(D) High-magnification image of the cambium and its derivatives. XET activity is seen in both fusiform and ray cell initials and at the early stages of sieve tube development. In the radial expansion zone of the developing xylem, the activity clearly is decreased compared with that in the cambium. Note the separation of adjacent radial cell files caused by the differential extraction of radial walls by formic acid (arrowhead).

(E) Phloem fibers. XET activity is found in developing fibers during secondary wall deposition as well as in surrounding parenchyma cells.

(F) Differentiating xylem. Cells are labeled faintly in the radial expansion phase and strongly during the early stages of secondary wall deposition. Note that there is more activity associated with the fibers than with the vessel elements.

C, cambium; Co, cortex; Col, collenchyma; DS, developing sieve tube cells; F, fiber; Ph, phloem; PhF, phloem fibers; Ph Ra, phloem ray; R, radial wall; Ra, ray; RE, radial expansion zone; SW, secondary wall formation zone; T, tangential wall; V, vessel element; X, xylem. Bars = 100 μm in (A) and (B), 20 μm in (C) and (E), and 10 μm in (D) and (F).
Figure 2. Localization of XG in Secondary Vascular Tissues by Indirect Immunofluorescence Using the Monoclonal Antibody CCRC-M1.

Localization was performed in either resin-embedded tissue ([A] to [C]) or in nonembedded tissue ([D] to [F]).

(A) Overview of the XG localization pattern in the stem. XG is detected in cell walls of the cambial cells, developing xylem, and phloem. It also is seen in the phloem rays, developing phloem fibers, and the cortex.

(B) Control section incubated with CCRC-M1 antibodies saturated with Rubus XG.

(C) Closeup of the cambium and phloem. XG labeling is particularly strong in the walls of the sieve tube elements.

(D) Closeup of the cambium and its recent derivatives. XG is detected in cambial cells and in developing phloem sieve tubes. Note the reduction of labeling in the radial expansion zone.

(E) Phloem fibers. Note the presence of the label in fibers in which secondary wall is being deposited.

(F) Developing secondary xylem. Note the strong labeling of walls and cytoplasm in fibers undergoing secondary wall thickening. Less label is detected in the radial expansion zone and in developing vessel elements.

All labels are as in Figure 1. Bars = 50 μm ([A] and [B]) and 20 μm ([C] to [F]).
compound middle lamella (Figure 3D). Thus, the pattern of CCRC-M1 labeling and the transient presence of XG in secondary wall layers showed that most XG deposition in the primary wall layer takes place when the secondary wall is being formed. Interestingly, the XG distribution pattern found in the phloem sieve tube cells was completely different from that found in xylem cells. In these cells, the intense labeling was concentrated in the inner wall layer (Figure 4D). Companion cells and phloem parenchyma cells did not have such an XG-rich inner zone.

Cloning of PttXET16A, a Member of XET Subfamily I

Consistent with the idea that XET has many different functions in poplar wood, the poplar EST database currently contains 12 different XET-like sequences obtained from cDNA libraries of wood-forming tissues (Sterky et al., 1998) (http://www.biochem.kth.se/PopulusDB/ and unpublished data). We focused initially on the most abundant XET clone in the library from the cambial region (Sterky et al., 1998). Full-length cDNA sequencing placed the enzyme in the GH16 glycosyl hydrolase/transglycosylase family (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Following the recommendations of Henrissat et al. (1998) for naming polysaccharide-hydrolyzing enzymes, the gene was named PttXET16A and the protein was named PttXET16A. The sequence contained a putative signal peptide (amino acids 1 to 22), the conserved DEIDFEFLG domain corresponding to the active site of enzymes of the GH16 family (Campbell and Braam, 1999), a putative N-glycosylation site, and four conserved Cys residues (Figure 5). Sequence comparisons placed the PttXET16A gene in subfamily I of the XET genes (Figure 6). The closest relatives of PttXET16A are XTH4 and XTH5 of Arabidopsis (Yokoyama and Nishitani, 2001a), NTXET1 of tobacco, and VaEXT of adzuki bean.

PttXET16A Is Expressed in Secondary Vascular Tissues and in the Root

A probe was designed to investigate the expression of PttXET16A in different poplar tissues by RNA gel blot analysis, and its specificity was confirmed by testing it on PttXET16A and two other closely related XET genes that also are present in the cambial region, PttXET16B and PttXET16C (our unpublished data) (Figure 7B). As expected, PttXET16A was highly upregulated in the mature stem,

![Figure 3](image-url)
where it was expressed both in the phloem/cambium fraction and in the xylem fraction containing primarily secondary wall-forming cells (Figure 7A). The gene also was expressed in root tips and young roots, in developing leaves (transiently), and in the apical bud (at low levels).

Localization of the PttXET16A protein in the secondary vascular tissues was performed using polyclonal antibodies that reacted with SDS-denatured recombinant PttXET16A produced in *Escherichia coli* (data not shown). Proteins extracted with low-salinity buffer from both phloem/cambium and xylem fractions gave one distinct band of the correct molecular mass in protein gel blot analysis (Figure 8), whereas no signal was observed with the preimmune serum (data not shown). However, because most XET proteins have approximately the same molecular mass, it was not possible to determine how many different XET isoenzymes the antibody reacted with. A strong signal was obtained in both phloem/cambium and xylem fractions, indicating the presence of XET protein in both primary and secondary walled cells.

To determine the cellular localization of XET, poplar stem sections were immunolabeled with anti-PttXET16A antibodies. Saturation of the antibodies with recombinant PttXET16A protein before the immunolocalization confirmed the specificity of the signal in all but ray cells, which were labeled faintly by the saturated antibodies (Figures 9A and 9B). Therefore, the ray signal was considered partially non-specific. In the secondary phloem, the PttXET16A protein was detected in the walls of sieve tubes (Figure 9C). There also was vivid labeling in the innermost secondary wall lay-

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**Figure 4.** Transmission Electron Microscopy Immunolocalization of XG in Cambial Region Cells Using the Monoclonal Antibody CCRC-M1.

(A) Phloem fibers depositing secondary wall layers. Note the accumulation of label in the primary wall layer.
(B) Cambial fusiform initials. Note the presence of label in both radial and tangential walls.
(C) Late secondary wall deposition stage in xylem fibers and vessels. Note that the XG label is associated with fibers but not with the vessel element.
(D) Comparison of the nacreous sieve tube wall with companion cell and parenchyma cell walls. XG is abundant in the inner nacreous layer, CC, companion cell; F, fiber; PP, phloem parenchyma; R, radial wall; ST, sieve tube; T, tangential wall; V, vessel. Bars = 2 μm.
ers of developing phloem fibers (Figure 9E). In the cambium, the signal was found in both fusiform and ray initials, but it was weaker compared with the phloem signal (Figure 9C). When nonembedded material was examined, the signal was seen clearly in the cytoplasm of cambial cells (Figure 9D). Expanding xylem cells had less label compared with the cambium, and expanding vessel elements had less label than expanding fibers. In accordance with the XET activity assay, the fiber signal increased during secondary wall deposition (Figures 9A, 9D, and 9F), and it was located primarily in the cytoplasm. At later stages of secondary wall formation, no signal was detected.

DISCUSSION

The Cambial Region as a Model for Studying the Differentiation of Secondary Walled Cells

The vascular cambium is a meristem with a highly ordered pattern of cell division in which the resulting secondary xylem and phloem cells are laid down in radial files (Mellerowicz et al., 2001). The meristematic cells, and their derivatives on both sides of the cambium, undergo radial expansion in which R walls are extended and T walls are not (Figure 10). In addition, both xylem and phloem fibers elongate by intrusive tip growth. When the final radial dimensions are attained, all xylem cells, phloem fibers, and sieve tube elements in the phloem undergo substantial wall thickening. Developing xylem constitutes a particularly rich source of cells undergoing quasisynchronous secondary wall deposition, and most of them lignify and undergo programmed cell death. The easily recognized developmental phases of the cambial region make this an ideal system in which to study questions specifically related to the sequence of primary and secondary cell wall differentiation (Hertzberg et al., 2001), giving it similar value to that of the developmental gradients in elongating roots and monocot leaves for studying the development of primary walled cells.

The Cambial Region Is the Major Site of XET Activity in the Stem

The results of this study show that the cambial region is the center of XET activity in parts of the stem that exhibit secondary growth. Both transcript and immunolocalization analyses suggest that PttXET16A is one of the major isoenzymes, but probably not the only one, responsible for this activity. Nine genes that encode different isoenzymes of XET were highly expressed in the roots of 4-week-old Arabidopsis plants studied by Yokoyama and Nishitani (2001a), which typically have a vascular cambium and secondary xylem (Chaffey et al., 2002). Interestingly, one of the genes was XTH5 (also known as EXGT-A4 or XTR12) (Akamatsu et al., 1999), which is the closest Arabidopsis homolog of PttXET16A (Figure 6). Another closely related gene, NtXET1 of tobacco, was found to be highly expressed in leaf vascular bundles (Herbers et al., 2001). Other XET genes expressed in vascular tissues include soybean BRU1 (Oh et al., 1998), Arabidopsis TCH4 (Xu et al., 1995; Antosiewicz et al., 1997), and meri-5 (Medford et al., 1991) from subfamily 2 of XET enzymes (Campbell and Braam, 1999). Thus, it is likely that orthologs of these XET genes also contribute to the high enzymatic activity observed in the poplar vascular cambium region.

Novel Role of XET during Secondary Wall Deposition

In light of the established role of XET in the biogenesis of primary walls and wall loosening, the finding of major significance in this work was the presence of XET activity in several cell types that were in the process of secondary wall formation. These include xylem fibers, phloem fibers, and

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```plaintext
maaaypwtlf lgmlvmvsrg mgAALRKPVD VAFGRNYVP T WAFDHKYYFN GGEIQPLHLD
KYGTVGFOASK GSYLFGHFSM QMKLVPGDSA GTVTAFLYLSQ QNSEHEIDFD EFLGQRTGQ
YIQDNVFTG KGGKOREQTY LWFPDKKTHF YYSVLWNNMYM IFVFLVDVPI RVFKNCQDLG
VKFPFNQPMK IYSSLNWADD WATRGGLEKT DWSKAPFIAIS YRSFHIIGCE ASVEAKFCAT
QGRAMWDOQE FQODLAFQYR RLSWVRKQKTY IYNYCTORSR YPSMPPCKR DRDI
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**Figure 5.** Structure of PttXET16A Coding Sequence.
xylem ray cells (Figure 1). CCRC-M1–reacting epitopes also were found in the same cell types (Figure 2), confirming the presence of fucosylated XG in secondary walled cells. The distribution of XG, as demonstrated by transmission electron microscopy, showed that it was present mainly in the compound middle lamella, confirming earlier reports in *Zinnia* tracheary elements (Stacey et al., 1995), pine tracheids, and poplar wood fibers (Baba et al., 1994). One possible explanation for the presence of XET activity during the early stages of secondary wall formation and the localization of XG in the primary wall layer is that all XG is deposited during the primary wall stage but the enzyme is still being secreted redundantly during later developmental phases. However, evidently, XG was localized in the cytoplasm and within the secondary wall layer during secondary wall formation, so it must be secreted during this stage (Figures 2E and 2F). Moreover, the label increased substantially specifically during early secondary wall deposition of xylem fibers and accumulated at the primary/secondary (S1) layer junction (Figure 3). This finding shows that newly produced XG is transported through the secondary wall layers and incorporated into the primary wall layer and at the primary and secondary wall junction.

It is unlikely that the increase in CCRC-M1 signal in sec-

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**Figure 6.** Phylogenetic Tree of XET Genes.

Relationships among Arabidopsis XET genes, poplar *PttXET16A*, and its relatives in other species: *NtXET1* (tobacco), *BRU1* (soybean), and *VaEXT* (adzuki bean). Arabidopsis genes were named according to Yokoyama and Nishitani (2001a). The scale bar represents 0.1 substitutions per site. 1, 2, and 3 represent subfamily number.
ondary wall-forming fibers resulted from unmasking of the CCRC-M1 epitope, because accumulation of the XG at the primary/S1 layer junction (Figure 3) also can be seen on the micrographs of poplar fibers and pine tracheids presented by Baba et al. (1994), who used a different antibody to detect XG. Collectively, these observations demonstrate a novel function for XET in the biogenesis of secondary cell walls. We propose that the increase in CCRC-M1 signal observed during secondary wall formation is a result of XG secretion and transport to the primary wall layer, where it is incorporated, thus creating reinforcing links between the primary and secondary walls. A recent report of a net increase in XG polymerization in cotton fibers after the cessation of elongation (Tokumoto et al., 2002) also suggests the role of XET in wall restructuring during secondary wall deposition. The importance of this wall restructuring for the function of xylem fibers has to be evaluated in mutant plants. However, given that the cellulose microfibrils have different angles in primary and secondary wall layers, we postulate that such a reinforcing action would help to bind these wall layers together. The localization of both PttXET16A protein (Figures 8 and 9) and mRNA (Figure 7) in secondary wall-forming cells indicates that this XET isoenzyme is involved in the process.

**XG, the Major Component of Sieve Tube Walls**

In addition to the apparent role for XET in the formation of secondary walled xylem and phloem fibers, the enzyme appears to be of importance in the formation of sieve tubes. Sieve tube walls have a thick inner nacreous layer that has been suggested by several authors to be cellulose rich and pectin poor (Esau, 1969; Evert, 1977; Schlag and Gal, 1996). We have demonstrated the presence of XG in the nacreous sieve tube walls by immunolabeling with CCRC-M1 monoclonal antibody (Figures 2C and 4D) and by colocalization of PttXET16A protein (Figure 9C). Evidence also has been obtained in a study by Hogetsu (1990) for the presence of hemicelluloses in the nacreous layer, in which wheat germ agglutinin, a lectin that reacted with hemicellulose extracted from cell walls, labeled phloem sieve tube cells in pea. For the following two reasons, we propose that XG in the sieve tubes forms a distinct association with the nacreous wall framework, possibly forming a mucilage layer lining the...
Figure 9. Immunolocalization of PtXET16A in Secondary Vascular Tissues of Poplar.

Localization was performed in either resin-embedded tissue (A) to (C), (E), and (F) or in nonembedded tissue (D).

(A) Overview of the localization pattern. The strongest signals are found in the phloem and rays, and weaker signals are present in the cambium, developing xylem, phloem fibers, and cortex.

(B) Negative control. Antibodies were saturated with the recombinant protein before labeling.

(C) Closeup of the phloem and cambium. The most intense label is found in the sieve tubes.

(D) Cambial region. High-intensity label is found in nonembedded material in the walls and cytoplasm of cambial fusiform and ray initials, developing phloem sieve tubes, and xylem fibers. Less PtXET16A label is detected in the radial expansion zone and developing vessel elements.

(E) Phloem fibers. Label is evident in cells undergoing secondary wall thickening.

(F) Secondary xylem at the final differentiation stages. Signal is seen in the cytoplasm fibers and ray cells in the early stages of secondary wall formation.

All labels are as in Figure 1. Bars = 50 μm in (A) and (B), 20 μm in (C) and (E), and 10 μm in (D) and (F).
tubes. First, the XG label accumulated in the innermost wall layer and at the boundary between the plasmalemma and the wall (Figure 4D). This localization differs from the localization seen in any other cell type. Second, XGO-SR was not incorporated in the mature sieve tubes (Figure 1C), in spite of the presence of PttXET16A protein in their walls (Figure 9C). This labeling discrepancy can be explained by the incorporation of the XGO-SR label into a fraction that was unstable during formic acid washes, as would be expected for the mucilage layer. However, other interpretations of the findings described above are possible, so the nature and functions of the XG in sieve tubes need to be investigated further.

### Role of XET in Primary Walled Dividing and Expanding Cambial Derivatives

The intense XET activity and the presence of PttXET16A observed in the cambial meristem (Figures 1D and 2D) are attributed to XET activity in cell plate formation and wall biogenesis. A homolog of \( XTH4 \) (\( EXGT-A1 \)) of Arabidopsis and \( VaEXT \) of adzuki bean has been implicated in incorporating XG into growing cell plates in tobacco BY2 cells (Yokoyama and Nishitani, 2001b). A similar function can be ascribed to the PttXET16A protein in the cambial zone, because young T walls of the poplar cambium contain XG (our unpublished data) and PttXET16A is very similar to both \( XTH4 \) and \( VaEXT \) (Figures 6 and 9). The meristematic cells also actively deposit XG in the R walls, which must stretch without thinning between cell division cycles (Figure 10) (Mellerowitz et al., 2001).

Both XET activity and PttXET16A labeling were weak in the radial expansion zone (Figures 1D, 1F, and 9D). This finding was unexpected because this zone is the site where the most intense wall-loosening activity takes place, and XET has been considered an important agent for cell wall loosening. Its ability to cut and repair XG cross-links in the cellulose-XG framework of the wall was thought to allow the microfibrils to move apart in a controlled manner (Fry et al., 1992). However, in a recent article, Takeda et al. (2002) suggested that XG metabolism providing XG donors of different length, rather than XET availability controls cell expansion. The potential importance of XG donor and acceptor concentrations, accessibility, or composition in regulating differential loosening of walls also has been discussed (Campbell and Braam, 1999). Moreover, applied XET did not induce wall extension in heat-killed cucumber (McQueen-Mason et al., 1993). Thus, it has been proposed that XET functions primarily in wall biogenesis rather than in wall loosening during the expansion process (Nishitani, 1997, 1998; Campbell and Braam, 1999).

Very little is known about how these findings and hypotheses may apply to the situation in planta. From our results, it is evident that XET activity and PttXET16A labeling did not correlate with the differential expansion patterns observed in developing xylem (i.e., the preferred stretching of R walls compared with T walls) and the preferred radial expansion of vessel elements compared with fibers. On the contrary, we found less XET activity and less XG in developing vessel elements than in fibers (Figures 1F, 2F, 4C, and data not shown). Thus, it is clear from this and other studies that the XET found in expanding cells should be interpreted as playing a role not only in wall loosening but also in wall biogenesis and strengthening. In support of this hypothesis, observations showing that the XG cross-links were formed after elongation in epidermal pea cells (Fujino et al., 2000) suggest that the creation of reinforcing cross-links may play an important role in preventing further cell expansion. A similar situation might apply to the radially expanding cambial derivatives, in which the low XET activity and XG abundance in expanding vessels may reflect the low number of XET-generated XG cross-links and explain the higher extensibility of vessel elements compared with fibers.

### Conclusions

By visualizing fucosylated XG and XET activity in cambial derivatives of poplar, we have demonstrated that XET is active in several types of secondary xylem and phloem cells during secondary wall deposition. XG was deposited with two contrasting patterns in different cell types. In cells that form typical secondary wall layers differing in the inclination of their cellulose microfibrils (all secondary xylem cells and phloem fibers), the XG was deposited into the compound middle lamella and at the primary/S1 layer junction. We postulate that XG forms reinforcing cross-links between microfibrils of the primary and S1 wall layers. In the sieve tube elements, XG was deposited at the inner (nacreous) wall layer, where it was very abundant. Colocalization of
PttXET16A, a member of XET subfamily 1, in the secondary walled cells during XG deposition suggests the enzyme’s involvement in the incorporation of XG in secondary vascular tissues.

METHODS

Plant Material

Hybrid aspen (Populus tremula × P. tremuloides), subsequently referred to as poplar, was grown in a greenhouse with an 18-h photoperiod, a temperature regime of 22/17°C (day/night), and a RH of at least 70%. Natural daylight was supplemented with light from HQI-TS 400W/DH metal halogen lamps (Osram, Munich, Germany). Plants were watered daily and fertilized once per week with a 1:100 dilution of SUPERBA S (Hydro Supra AB, Landskrona, Sweden).

Xyloglucan Transglycosylase Activity in Situ

Substrate xyloglucan (XG) oligosaccharides (XGOs) were prepared by digestion of tamarind seed XG (Megazyme, Bray, Ireland) with XG-specific Trichoderma reesei cellulase (Fluka, Buchs, Switzerland) (Sulová et al., 1995). Analysis by high-performance anion-exchange chromatography-pulsed-amperometric detection indicated that the product was a mixture of the XGOs XXXG, XLXG, XLLG, and XLXLG (Vincken et al., 1995), using the nomenclature of Fry et al. (1993), in the ratio 15:7:32:46. The total amount of other XGOs was estimated to be <5%. The mixture of XGOs was converted to sulfurhexadimate conjugates (XGO-SR) according to the method of Fry (1997). Lissamine rhodamine B sulfonyl chloride was obtained from Molecular Probes (Leiden, The Netherlands) as a mixture of isomeric 2- and 4-sulfanyl chlorides/sulfonates. The fluorescence spectra of the product showed absorbance at 570 nm and emission at 586 nm.

To localize the xyloglucan transglycosylase (XET) activity in wood-forming tissues, fresh free-hand sections of poplar stems were incubated with 6.5 μM XGO-SR in 25 mM Mes buffer, pH 5.5, in the dark at room temperature as described by Vissenberg et al. (2000). Incubations for 15 min to 2 h were followed by extensive washes in ethidium:formic acid:water (15:1:4 [v/v]) and then in 5% formic acid to remove all unincorporated XGO-SR. Heat-denatured sections (10 min at 90°C) and sections incubated in the presence of unlabeled XGO competitor (1 mM) were used as negative controls for the reaction. The sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined by confocal laser scanning microscopy using a Zeiss LSM 510 instrument (Jena, Germany) with a 568-nm argon-krypton laser. The signal was detected between 585 and 615 nm and superimposed onto the transmitted light image.

cDNA Cloning, Sequencing, and Phylogenetic Analysis

Full-length sequencing of a XET clone from a poplar cDNA library (Sterky et al., 1998) was performed using the Big Dye Terminator Cycle Sequencing kit and an ABI 377 sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). Clustal W was used to align the deduced protein sequence. The phylogenetic tree was obtained by the neighbor-joining method using 1000 bootstrap replicates and was visualized using TreeView-PPC (version 1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/treview.html). The sequences used for the alignment and their respective Arabidopsis Genome Initiative identification numbers are as follows: XTH1 (At4g13080), XTH2 (At4g13090), XTH3 (At3g25050), XTH4 (At2g06850), XTH5 (At5g13870), XTH6 (At5g05730), XTH7 (At4g37800), XTH8 (At1g11545), XTH9 (At4g03210), XTH10 (At2g14620), XTH11 (At3g45850), XTH12 (At5g57530), XTH13 (At5g57540), XTH14 (At4g25820), XTH15 (At4g14130), XTH16 (At3g23730), XTH17 (At1g65310), XTH18 (At4g30280), XTH19 (At4g30290), XTH20 (At5g48070), XTH21 (At2g18800), XTH22 (At5g57560), XTH23 (At4g25810), XTH24 (At4g30270), XTH25 (At5g57550), XTH26 (At4g28850), XTH27 (At4g01850), XTH28 (At1g14720), XTH29 (At4g18990), XTH30 (At1g32170), XTH31 (At3g44990), XTH32 (At2g36870), and XTH35 (At1g10550).

RNA Gel Blot Analysis

Various poplar tissues and organs, including apical buds, internodes 2 to 5, leaves 1 to 5, internodes 6 and 7, leaves 6 and 7 (all counted from the top), mature stems (separated into periderm/cortex, phloem/cambium, and differentiating xylem), young roots, and root tips (including the differentiation zone up to the first lateral roots), were frozen in liquid N and ground to a fine powder. The identity of tissues extracted from the mature stem was verified by microscopic observations. Total RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA) either directly from the ground plant material or from crude RNA preparation obtained by the hot hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO) extraction procedure (Chang et al., 1993). Ten micrograms of RNA from each tissue sample was separated on a formaldehyde gel and blotted onto a nylon membrane. The loadings were similar according to the signals from ethidium bromide-stained gels. PttXET16A-specific DNA probe was prepared by random priming (Strip-EZ DNA; Ambion, Austin, TX) from the 3’ region of ~300 bp. Hybridization was performed in Church buffer (Church and Gilbert, 1984) overnight at 42°C. The filters were washed as recommended by Ambion and exposed to a phosphor screen in a phosphorimager (GS 525; Bio-Rad, Hercules, CA) for 26 h. Probe specificity was tested further in a dot-blot assay that included full-length cDNAs of three closely related cambial-region XET genes.

Production of PttXET16A Recombinant Protein

The Escherichia coli expression vector pAff8c (Larsson et al., 1996), containing the serum albumin binding region from streptococcal protein G, was used for the production of PttXET16A protein for immunization of rabbit and chicken. Two gene-specific primers were used to amplify the predicted coding sequence of the mature PttXET16A protein by PCR using pfu polymerase (Stratagene, La Jolla, CA). The forward primer was designed to anneal to the predicted signal peptide cleavage site and contained an introduced BamHI site (5’-GGGAGATCCGCTGCTGAGGAGGATGATATCCACTGCATGAG-3’); the reverse primer annealed to the stop codon region and contained an introduced HindIII site (5’-GGGAGGAACTTTATATGATCGTCCCCTGGACTGATC-3’). The BamHI-HindIII-digested PCR-amplified fragment and pAff8c were ligated and transformed into E. coli strain BL21(DE3) (Invitrogen, Madison, WI). The cells harboring the
PtXET16A construct were grown in Luria-Bertani medium supplemented with 50 μg/mL kanamycin until the OD600 reached ~1.0. Iso-propyl-β-D-thiogalactopyranoside then was added to a final concentration of 1 mM, and the culture was incubated for another 3.5 h. The cells were harvested by centrifugation at 5000 g for 10 min, resuspended in lysis buffer (50 mM NaH2PO4, 10 mM Tris-HCl, 6 M guanidine-HCl, and 100 mM NaCl, pH 8.0), and then stored at −20°C. The cells were thawed and sonicated to release the intracellular proteins. β-Mercaptoethanol was added to a concentration of 7.5 mM. Cellular debris was removed by centrifugation at 25,000 g for 10 min, and the supernatant, containing the denatured and solubilized proteins, was filtered through a 12-μm syringe filter (Sartorius AG, Göttingen, Germany) before purification by the immobilized metal ion affinity chromatography procedure. The immobilized metal ion affinity chromatography purification of the His6-tagged proteins was performed using a Talon column (Clontech Laboratories, Palo Alto, CA) according to the manufacturer’s protocol. Antibody production was performed at Agrisera (Vännäs, Sweden) by injecting the purified recombinant PtXET16A protein into rabbit and chicken.

**Protein Gel Blot Analysis**

Buffer-soluble proteins were extracted with ice-cold 50 mM Na phosphate buffer, pH 7.2, containing 2 mM EDTA, 4% polyvinylpyrrolidone (M, of 360,000), and 1 mM DTT from the tissues ground in liquid nitrogen. Samples were stirred for 30 min at 4°C and then centrifuged at 15,000 g for 10 min to remove cellular debris. Polyvinylpyrrolidone was removed from the supernatant by precipitation with ammonium sulfate at 20% saturation followed by centrifugation as described above, and the soluble proteins were precipitated with ammonium sulfate at 80% saturation. They were dissolved in 10 mM Tris-HCl and 1 mM EDTA buffer, pH 8.0, and desalted using a PD10 column (Pharmacia, Uppsala, Sweden). The proteins were quantified using the Coomassie Plus Protein Assay (Pierce Chemical Co.). Thirty micrograms of protein was denatured, reduced, separated on a NuPAGE polyacrylamide gel (Novex, San Diego, CA), and electroblotted onto a polyvinylidene difluoride membrane (Pall, Portsmouth, UK) according to the manufacturer’s instructions.

Membranes were blocked in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% low-fat dried milk powder, and 0.05% Tween 20, washed in a washing buffer (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl), and incubated with a 1:1000 dilution of rabbit PtXET16A antibodies in an antibody diluent (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% milk powder, and 0.25% Triton X-100). After 1 h of incubation, membranes were washed as described above and incubated with secondary antibodies. Secondary anti-rabbit antibody conjugated to peroxidase (Sigma) were diluted to 1:20,000 in the antibody diluent. After 1 h of incubation, the membranes were washed as described above. Peroxidase activity was detected in situ using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK) followed by a 10-min exposure to x-ray film.

**Immunolocalization**

**Light Microscopy**

Tissues prepared in two different ways were used in immunolocalization experiments: butyl-methylmethacrylate resin-embedded, semi-thin sectioned material (Chaffey et al., 1997), in which fine tissue structure is preserved; and manually cut sections without any embedding, in which epitope preservation is better but structure is less well preserved.

The resin-embedded material was prefixed with 100 μM m-maleimidobenzoyl N-hydroxysuccinimide ester (Sigma) in 25 mM Pipes buffer (Sigma), pH 6.9. It then was fixed in 3.7% formaldehyde with 0.2% (v/v) glutaraldehyde in 25 mM Pipes buffer, pH 6.9, embedded in a butyl-methylmethacrylate resin mixture, polymerized under UV light, and sectioned at 7 μm. After resin removal with acetone, sections were incubated in a blocking solution containing 5% skim milk powder in PBS containing 137 mM NaCl, 2.7 mM KCl, 2 mM Na2HPO4, 2 mM KH2PO4, pH 7.2 to 7.4, and 1% Tween 20 for ~45 min before application of the primary antibody. Sections were incubated in primary antibody solution for 2 h at room temperature or overnight at 4°C before washing in 0.1% Tween 20 in PBS and application of the secondary antibody conjugated to fluorescein isothiocyanate (FITC) (see below). Sections then were incubated for 1 h at room temperature, washed extensively as described above, and stained with 0.01% toluidine blue O for 1 min to minimize tissue autofluorescence. The sections were mounted in Vectashield (Vector Laboratories) and examined by confocal laser scanning microscopy using the Zeiss LSM 510 instrument, with 488- and 568-nm argon-krypton lasers, generating the FITC signal (detected at 505 to 550 nm) and the autofluorescence signal (detected at >585 nm). These signals were detected in separate channels.

In the second method of tissue preparation, fresh material was sectioned with a razor blade and fixed in 4% paraformaldehyde in 50 mM Pipes buffer, pH 7, containing 5 mM MgSO4 and 5 mM EGTA for 30 min. After fixation, the material was treated basically as the methylmethacrylate-embedded material, except that autofluorescence quenching was not needed. Because the autofluorescence of the walls was low, the FITC signal was projected on a transmitted light image for anatomical detail.

**Antibodies**

Polyclonal antibodies raised against recombinant protein PtXET16A in chicken and rabbit were used to localize PtXET16A. Both the chicken and rabbit antibodies produced the same staining pattern, but the chicken antibody gave a stronger signal, especially compared with the signal obtained with the preimmune antibody. Therefore, the chicken antibody was used for more detailed immunolocalization studies. Secondary antibodies were anti-chicken FITC conjugates (Sigma) or anti-rabbit FITC conjugates (Jackson Immunoresearch Laboratories, West Grove, PA), as appropriate, both at a dilution of 1:100.

To localize XG in sections, the monoclonal antibody CCRC-M1 (Puhlmann et al., 1994) was used at a dilution of 1:10, and the secondary antibody was FITC conjugated with anti-mouse antibody (Sigma) at a dilution of 1:100.

**Controls**

Control and experimental preparations were processed in parallel and viewed at the same confocal laser scanning microscopy settings. To test for tissue autofluorescence, material was processed without the secondary antibodies. To visualize possible binding of secondary antibodies to poplar tissues, sections were incubated
only in secondary antibodies. To account for possible signals caused by antibodies reacting with other antigens, the sections were incubated with preimmune chicken and rabbit antibodies. These three controls all gave some background signals that were considered nonspecific (data not shown). Finally, primary antibodies saturated with the antigen at 10 nmol/mL were used to determine if the signal from experimental sections was attributable to the antibodies reacting with the target antigen or some other antibodies. To ensure that the saturation of anti-PttXET16A antibodies was specific to PttXET16A protein and not to the saturation of antibodies against the His tag present in the recombinant protein, a nonrelated protein with a His tag was used at the same concentration. This protein was not able to block the signal (data not shown).

Transmission Electron Microscopy

Small blocks (L, 5 mm; T, 2 mm; R, 3 mm) including cambium and developing xylem and phloem were taken from the 30th internode and fixed in 4% paraformaldehyde in Pipes buffer, pH 7.2. These blocks were dehydrated in a graded ethanol (30, 50, 75, 90, 95, 100, and 100%) series and embedded in LR White resin (TAAB, Berkshire, UK). Transverse sections (80 to 100 nm thick) were cut with an ultramicrotome (MT-7000; RMC, Tucson, AZ) and transferred to 200-mesh nickel grids (TAAB) coated with formvar. Sections were blocked with blocking solution (6% BSA, 0.1% fish skin gelatin, 5% goat serum, and 0.05 M Gly in PBS) for 1 h at room temperature and incubated in CCRC-M1 antibody solution diluted 10-fold for 2 h at room temperature or overnight at 4°C. They were washed with PBS containing 2% Tween 20 three times for 10 min each and incubated with 12-nm colloidal gold–conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories). They were washed as described above and refixed with 2% glutaraldehyde for 5 min, washed with distilled water several times, and observed with a Zeiss EM 109 transmission electron microscope (Leo Electron Microscopy, Oberkochen, Germany).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The GenBank nucleotide accession number for PttXET16A is AF515607. The GenBank accession numbers of XETs from other plants are as follows: NtXET1 (*Nicotiana tabacum*), D86730; BRU1 (*Glycine max*), L22162; and VaEXT (*Vigna angularis*), D16458.

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Xyloglucan Endotransglycosylases Have a Function during the Formation of Secondary Cell Walls of Vascular Tissues

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