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RESEARCH PAPER

# XTH acts at the microfibril–matrix interface during cell elongation

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## Abstract

**Sulphorhodamine-labelled oligosaccharides of xyloglucan are incorporated into the cell wall of *Arabidopsis* and tobacco roots, and of cultured *Nicotiana tabacum* cells by the transglucosylase (XET) action of XTHs. In the cell wall of diffusely growing cells, the subcellular pattern of XET action revealed a 'fibrillar' pattern, different from the xyloglucan localization. The fibrillar fluorescence pattern had no net orientation in spherical cultured cells. It changed to transverse to the long axis when the cells started to elongate, a feature mirroring the rearrangements of cortical microtubules and the accompanying cellulose deposition. Interference with the polymerization of microtubules and with cellulose deposition inhibited this strong and 'fibrillar'-organized XET-action, whereas interference with actin-polymerization only decreased the intensity of enzyme action. Epidermal cells of a mutant with reduced cellulose synthesis also had low XET action. Root hairs (tip-growing cells) exhibited high XET-action over all their length, but lacked the specific parallel pattern. In both diffuse- and tip-growing cell types extraction of the incorporated fluorescent xyloglucans by a xyloglucan-specific endoglucanase reduced the fluorescence, but the 'fibrillar' appearance in diffuse growing cells was not eliminated. These results show that XTHs act on the xyloglucans attached to cellulose microfibrils. After incorporation of the fluorescent oligosaccharides, the xyloglucans decorate the cellulose microfibrils and become inaccessible to hydrolytic enzymes.**

Key words: Cell wall, confocal laser scanning microscopy, expansion, growth, xyloglucan endotransglucosylase/hydrolase (XTH).

## Introduction

The primary cell walls of flowering plants consist of a framework of cellulose embedded in a matrix of hemicelluloses, pectins, and structural proteins (McNeil *et al.*, 1984; Carpita and Gibeaut, 1993; Brett and Waldron, 1996). Xyloglucan, the major hemicellulose matrix polysaccharide in the primary cell walls of dicotyledons, and xyloglucan endotransglucosylase/hydrolases (XTHs; see Rose *et al.*, 2002, for nomenclature) are thought to play an important role in the expansion and/or assembly of plant cell walls (Fry, 1995; Xu *et al.*, 1996; Nishitani, 1997; Thompson *et al.*, 1998). Xyloglucans are capable of forming tight non-covalent bonds via hydrogen-bonds with cellulose microfibrils (Valent and Albersheim, 1974; Hayashi *et al.*, 1987, 1994*a*, *b*; Hayashi, 1989) and may therefore tether adjacent microfibrils (Fry, 1989). Xyloglucan is also found to be entrapped within microfibrils, or sticking to the microfibril surface (Pauly *et al.*, 1999*a*). It

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Abbreviations: CLSM, confocal laser scanning microscopy; DCB, 2,6-dichlorobenzonitrile; LAB, latrunculin B; ORY, oryzalin; TPA, *Tetragonolobus purpureus* agglutinin; UEA I, *Ulex europaeus* agglutinin I; XET, xyloglucan endotransglucosylase (enzyme activity or action); XGO, xyloglucan oligosaccharide (general); XGO-SR, sulphorhodamine-labelled xyloglucan oligosaccharide; XTH, xyloglucan endotransglucosylase/hydrolase (protein or gene).

 

has been proposed that wall loosening could be achieved through enzymes hydrolysing portions of the xyloglucan network, thus weakening the structure surrounding and constraining the cellulose microfibrils (Hayashi, 1989; Fry, 1989; Hoson *et al.*, 1991). The hydrostatic push of the protoplasm against this weakened wall then results in creep of cellulose microfibrils. XTHs acting in XET mode cleave and rejoin xyloglucan chains or suitable xyloglucan-derived oligosaccharides (XGOs) (Baydoun and Fry, 1989; Smith and Fry, 1991; Fry *et al.*, 1992; Nishitani and Tominaga, 1992; Lorences and Fry, 1993), and are therefore the most obvious candidates for wall loosening (Fry *et al.*, 1992). Xyloglucan endotransglucosylase (XET) activity is indeed often correlated with growth rate (Fry *et al.*, 1992; Hetherington and Fry, 1993; Pritchard *et al.*, 1993; Potter and Fry, 1994; Xu *et al.*, 1995; Palmer and Davies, 1996; Antosiewicz *et al.*, 1997; Catalá *et al.*, 1997) and expression of XTH genes is especially found in elongating tissues (Ma *et al.*, 2001; Ji *et al.*, 2003). Other researchers found that measurable XET-activity also occurred in non-growing regions of plants (Potter and Fry, 1993, 1994; Pritchard *et al.*, 1993; Zurek and Clouse, 1994; Palmer and Davies, 1996; Smith *et al.*, 1996) and even during secondary cell wall formation (Bourquin *et al.*, 2002). To explain the fact that XET activity is detectable in actively elongating as well as in non-elongating cells and tissues, it could be postulated that other processes, such as peroxidase-catalysed cross-linking of wall polymers (Schopfer, 1996; Warneck *et al*., 1996; Schnabelrauch *et al*., 1996) or lignification override cell wall loosening and may be responsible for wall-tightening (Fry, 1986). Another explanation could be that one of XTHs' main functions is the restructuring (Thompson and Fry, 2001) of cell walls during and even after cell elongation. In addition, expansins are thought to act at the interface (hydrogen-bonds) between cellulose microfibrils and hemicelluloses and are therefore also obvious candidates (Cosgrove, 2000).

Multiple isoforms of XTH are expressed in different organs of different plant species in response to numerous hormonal, environmental and developmental stimuli (Fry *et al.*, 1992; Xu *et al.*, 1995; Akamatsu *et al.*, 1999; Yokoyama and Nishitani, 2001*a*).

At least five useful assays of XET activity are currently available (Fry *et al.*, 1992; Nishitani, 1992; Lorences and Fry, 1993; Sulová *et al.*, 1995; Fry, 1997). In the method of Fry (1997) fluorescent acceptor substrates (sulphorhodamine conjugates of xyloglucan oligosaccharides, XGO-SRs) are used to detect the XET action of XTH. The acceptor substrate is incorporated into the cell wall yielding orange fluorescence indicative of the co-occurrence of active XTH and donor substrates. However, the assay is incapable of showing the hydrolytic action of XTH as the acceptor substrate for this enzymatic activity is water instead of the fluorescent oligosaccharide. The method already proved its applicability for 'dot-blots', tissue prints (Fry, 1997) and zymograms (Iannetta and Fry, 1999). It was shown that this method also enables XET-action to be assayed *in vivo* in whole organs and in freshly made sections (Vissenberg *et al.*, 2000*a*). It was found that high XET-action was most prominent in the cell elongation zone of all vascular plants, from the primitive plant *Selaginella* up to the most developed angiosperms (Vissenberg *et al.*, 2000*a*, 2003) and at the future site of root hair emergence (Vissenberg *et al.*, 2001).

To gain more insight into XTHs' role in expanding cell walls, their XET action was visualized at the subcellular level in single cells and in roots and the effect of several cell wall and cytoskeleton inhibitors, reduced cellulose deposition using *Arabidopsis* mutants, and the accessibility of the modified xyloglucans by a xyloglucan-specific endoglucanase, XEG were investigated (Pauly *et al.*, 1999*b*).

## Materials and methods

### *Plants and cell cultures*

Plants of *Arabidopsis thaliana* (L.) Heynh. Wild-type and *Nicotiana tabacum* L. cv. Petite Havana SR1 were grown from seed under sterile conditions on a Murashige and Skoog medium without hormones (4.7 g $l^{-1}$; Duchefa, The Netherlands), supplemented with 10 g $l^{-1}$ sucrose, and solidified with 4 g $l^{-1}$ Gelrite (Duchefa, The Netherlands), pH 5.7. The seedlings were grown at 22 °C in a 16 h photoperiod at a light intensity of 24 μmol $s^{-1}$ $m^{-2}$ (Philips tlm 65W/33), except for the *rsw*-mutant that was grown at 31 °C.

Tobacco protoplasts were isolated following the method of Stickens *et al.* (1996) by incubation of healthy leaves in 2% cellulase-R10 and 0.2% macerozyme-R10 (Yakult Honsha Co., Ltd.) dissolved in K3A culture medium (Potrykus and Shillito, 1986) for 5 h. Living protoplasts were isolated from cell debris by filtration and centrifugation (60 *g*) and immobilized on the surface of an agarose layer (K3A culture medium solidified with 1.2% agarose) in Petri dishes. They were then covered with K3A culture medium supplemented with 1 or 0.065 mg $l^{-1}$ naphthalene 1-acetic acid (NAA; Sigma) alone or 1 mg $l^{-1}$ naphthalene 1-acetic acid together with 1 mg $l^{-1}$ benzylaminopurine (BAP; Sigma). The regenerating cells were kept in culture at 22 °C in a 16 h photoperiod at a light intensity of 24 μmol $s^{-1}$ $m^{-2}$ (Philips tlm 65W/33).

Drugs that interfere with the cytoskeleton (oryzalin; Alltech Associates and latrunculin B; Calbiochem) and cell wall formation (2,6-dichlorobenzonitrile (DCB); Fluka Chemika) were dissolved in DMSO as stock solutions. Latrunculin B was used at a final concentration of 1.25 μM, oryzalin and DCB at 10 μM, all dissolved in MS before solidification and pooling of the plates or in liquid K3A culture medium for *Arabidopsis* roots and tobacco cells, respectively. Four-day-old *Arabidopsis* roots were transferred from normal MS plates to plates containing MS and the appropriate drug. For tobacco cells, the liquid K3A culture medium was replaced by one containing the different drugs.

### *Cell wall labelling*

Xyloglucan-endotransglucosylase (XET) action was assayed as described in Vissenberg *et al*. (2000*a*). In brief, regenerated tobacco cells and roots were incubated in a 6.5 μM sulphorhodamine-labelled xyloglucan oligosaccharide (XGO-SR) mixture (XLLG-SR>XXLG-SR>XXXG-SR, see Fry *et al*., 1993, for nomenclature; dissolved in K3A culture medium for cells and 25 mM MES-buffer pH 5.5 for

roots, respectively) for 1 h, followed by a 10 min wash in ethanol/formic acid/water (15:1:4, by vol.) and an incubation overnight in 5% formic acid. 6.5 μM cellotetraose-SR and cellobiose-SR-solutions were used as controls for XET-assaying followed by the same two washes as described above.

Localization of xyloglucans in the cell wall of cells and roots was done by labelling with the fucose-specific lectins *Tetragonolobus purpureus* agglutinin (TPA; Sigma) and *Ulex europaeus* agglutinin I (UEA I; Sigma) (Hoson and Masuda, 1991) after removal of the pectins and glycoproteins from the cell wall so that xyloglucan is essentially the only fucose-bearing material in the specimen. Pectic polysaccharides were extracted with 0.5% ammonium oxalate (adjusted to pH 4.0 with 0.5% oxalic acid) for 1 h at 90 °C (Asamizu *et al.*, 1984). Incubation overnight in PAW (phenol/acetic acid/water, 2:1:1, by vol.) removes the glycoproteins (Fry, 2000). Fluorescein isothiocyanate (FITC)-labelled lectins were used at 100 μg $ml^{-1}$ in 0.15 M NaCl containing 10 mM Na-phosphate buffer at pH 7.0 with or without 0.1 M fucose for 30 min in the dark followed by two washes in buffer before examination under the microscope.

### *Fluorescence and confocal microscopy*

Fluorescence micrographs were made with a confocal laser scanning microscope (CLSM, Bio-Rad MRC 600 mounted on a Zeiss Axioskop) with a 10× (NA 0.3) dry objective, 40× (NA 0.9) water-immersion objective, or a 63× (NA 1.25) oil-immersion objective and a co-axial rotating object table using the 488 or 514 nm line of the Ar-laser depending on the fluorochrome used.

### *Solubilization of XGOs using XEG*

Xyloglucan was solubilized from the cell walls using a xyloglucan-specific endoglucanase (XEG) (Pauly *et al.*, 1999*b*) that had been purified to remove glycosidases that might otherwise degrade XGOs. Ten *Arabidopsis* roots that were labelled with the XGO-SR mixture as described above were incubated in 300 μl of 50 mM ammonium formate (pH 4.5) supplemented with excessive amounts of XEG (10 units) and incubated for 24 h at 30 °C (instead of 37 °C to prevent bacterial growth). After digestion the solubilized material was removed by centrifugation, lyophilized and the XGO-content estimated using the anthrone assay for hexoses (Dische, 1962). In addition, the xylose content of the solubilized material was determined by monosaccharide composition via derivatization of the material to their corresponding alditol acetates and subsequent analysis by gas chromatography (Albersheim *et al.*, 1967). Furthermore 1 μl of the solubilized material was subjected to matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry as described by Lerouxel *et al.* (2002). The remaining cell wall residue was subjected to further 24 h period XEG digestions. Pictures of the final remaining fluorescence in the roots were taken using the confocal microscope as indicated above.

## Results

In earlier work it was shown that XET activity and donor-substrate co-localize especially in expanding tissues (Vissenberg *et al.*, 2000*a*, 2001, 2003; Verbelen *et al.*, 2001). In the technique used, the addition of sulphorhodamine-labelled oligosaccharides of xyloglucan (XGO-SRs) gave a clear positive signal in the cell wall, whereas two compounds that are not acceptor substrates for XTHs, cellobiose-SR and cellotetraose-SR, gave no labelling at all (results not shown; see Vissenberg *et al.*, 2000*a*). The fluorescence seen after the assay is therefore indicative of co-localization of XET-active XTH and its donor-substrate. The same detection technique was used for this enzyme action as the basis for high-resolution studies on *Arabidopsis* and tobacco roots and on cultured tobacco single cells (Vissenberg *et al.*, 2000*b*). In these cells elongation can be switched on and off using simple hormone signals (Vissenberg *et al.*, 2000*b*).

In the elongation zone of roots, XET-driven incorporation of XGO-SRs into the walls of epidermis cells results in a fibrillar pattern (Fig. 1A, B). This is the case in *Arabidopsis* (Fig. 1A) as well as in tobacco (Fig. 1B). The 'fibrillar' structures have orientations varying from orthogonal to slightly oblique to the long axis of the cells. A treatment with DCB, a cellulose deposition inhibitor, for 3 h or 6 h (as shown in the picture) resulted in a strong decrease of total XET action and in the loss of the fibrillar pattern (Fig. 1C). To assess further the importance of cellulose synthesis on XET action during cell elongation, the mutant *rsw1-10*, a weak constitutive allele mutated in the gene encoding cellulose synthase catalytic subunit *CESA1*, was used (Fagard *et al.*, 2000). Figure 1D shows roots of the mutant together with Col0 wild-type after the assay for XET-action. From the figure it is clear that, in the *rsw1-10* mutant, XET action is greatly reduced in the elongation zone of the roots (upper root). Also the cells are much shorter than in wild-type plants. The wild-type lower root (Col0) has high XET action in the elongation zone (see arrow).

Cytoskeleton inhibitors had similar but specific effects on XET-action. Inhibition of microtubule-polymerization with oryzalin resulted in a slight decrease of XET action and after 6 h in the loss of the 'fibre'-like structures as well (Fig. 1E). Inhibition of microfilaments with latrunculin B for 6 h reduced the staining, but the characteristic pattern could still be observed (Fig. 1F) after changing the microscope settings (increased gain to have better contrast in the picture) compared with Fig. 1A.

Besides the XET action of XTHs, the localization of xyloglucan itself was also studied. Figure 2 shows an example of xyloglucan labelling using the fucose-binding lectin UEA I (*Ulex europaeus* agglutinin I) after removal of pectins and glycoproteins from the cell walls. It is clear that in the elongation zone of *Arabidopsis*, fluorescence appears in a dotted pattern, but not fibrillar as seen after the XET assay (Fig. 1A). Labelling with TPA (*Tetragonolobus purpureus* agglutinin, which is also specific for fucose residues) gave a similar picture (results not shown).

Besides the diffuse-growing epidermis cells, typically tip-growing structures such as root hairs are also positively stained after XET-assaying. Root hairs of both *Arabidopsis* and tobacco showed distinct cell wall fluorescence over all their length, but without a 'fibrillar' fluorescence pattern. Figure 3 shows a tobacco root hair with a homogeneous staining.

Cultured cells of tobacco derived from mesophyll protoplasts offer the opportunity to study on a single cell level

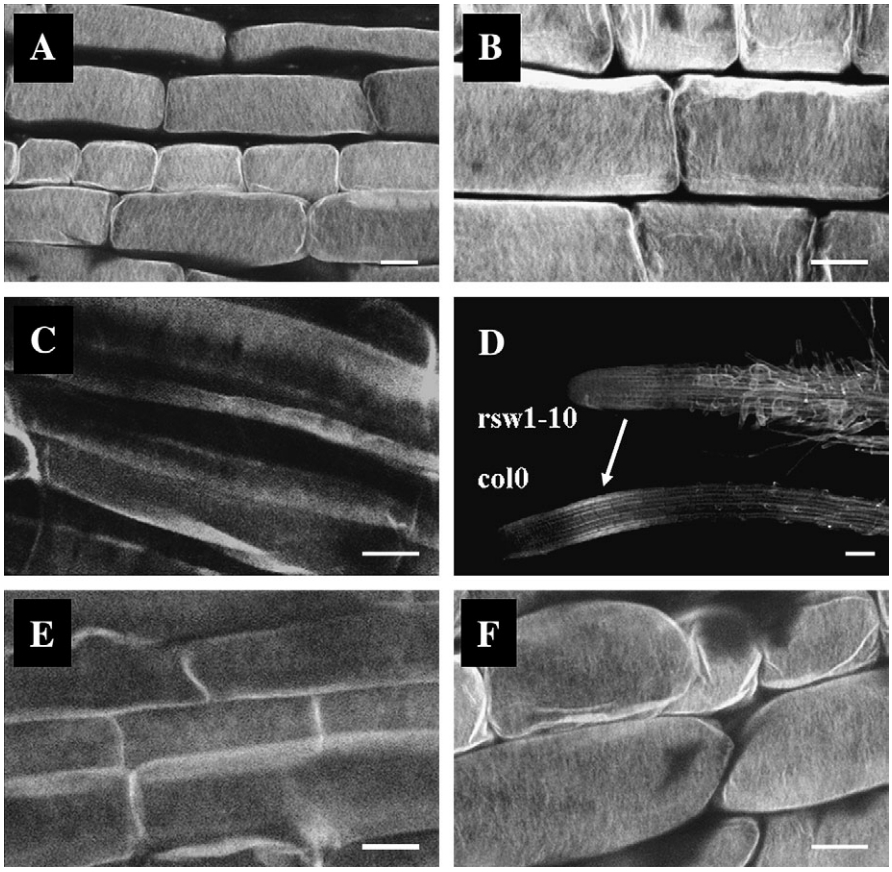


**Fig. 1.** Pattern of XET action of XTH on its endogenous donor substrate in the epidermis of roots of 4-d-old *Arabidopsis* and tobacco. (a) *Arabidopsis* and (b) tobacco root show a fibrillar, more or less parallel staining pattern orthogonal to the long axis of the cell. (c) Treatment of *Arabidopsis* with DCB leads to the loss of a fibre-like staining pattern. (d) In the elongation zone the *Arabidopsis* mutant in the *rsw* allele (upper root) shows a clear decrease in fluorescence caused by XET action compared with the wild type (lower root, see arrow). (e) Disruption of the microtubules of *Arabidopsis* with oryzalin for 6 h resulted in a decrease in XET action and the loss of fibre-like structures. (f) Disruption of the F-actin of *Arabidopsis* with latrunculin B only decreased XET action, but did not interfere with the fibrillar staining pattern. Bars are 10 μm, except in (d) where the bar is 100 μm.

the major developmental events that occur in a plant: division, isotropic expansion, and anisotropic elongation. Elongation growth is, however, much slower than that of cells in an intact root. In these cells, XTH was active in a similar way as in root epidermis cells. Figure 4A represents the fluorescence pattern after incorporation of XGO-SRs into the cell wall of a young, spherical cell that just started cell wall formation. In the Confocal Laser Scanning Microscope (CLSM) picture, an organized 'fibrillar' fluorescence pattern can be detected with no net orientation. The white spots seen in this and in the following pictures are caused by chloroplast autofluorescence. Upon addition of NAA the cells start to elongate. In the same time the fluorescence pattern reorganizes and becomes more or less parallel and orthogonal to the long axis of the elongating cells (Fig. 4B). This feature is very clear in older well-elongated cells (Fig. 4C). Culture in a suboptimal NAA concentration makes the cells expand isotropically, the volume increases but the cell keeps a spherical shape. Figure 4D represents the fluorescence pattern after XET assaying in such a spherical cell. The patterning is one of arranged fibrillar structures with no net orientation. When, besides auxins, cytokinins are also administered, the cells divide (Vissenberg *et al.*, 2000*b*). In these cycling cells the incorporation of XGO-SRs fails to exhibit a fibrillar pattern (Fig. 4E). By contrast, blurry patches of fluorescence can be found (see striped arrows); further inspection confirmed that they are caused by retracted cytoplasm. The newly formed cell wall between the two cells exhibits a higher fluorescence than the other parts of the cell wall (see full-line arrows).

Also, in the tobacco cells, inhibitors of cellulose deposition and of the cytoskeleton interfered with the 'fibrillar' staining pattern. The cellulose deposition inhibitor DCB (10 μM) drastically decreased the incorporation of XGO-SRs into the cell wall and the result is a very faint fluorescence (Fig. 4F, mean fluorescence intensity of the box is 225 where 0 is white, 255 is black). After 21 h of oryzalin (10 μM) or latrunculin B (1.25 μM) treatment, XET action decreases, but 'fibrillar' parallel fluorescence patterns can still be found (results not shown). Staining for actin or microtubules was negative indicating the effectiveness of

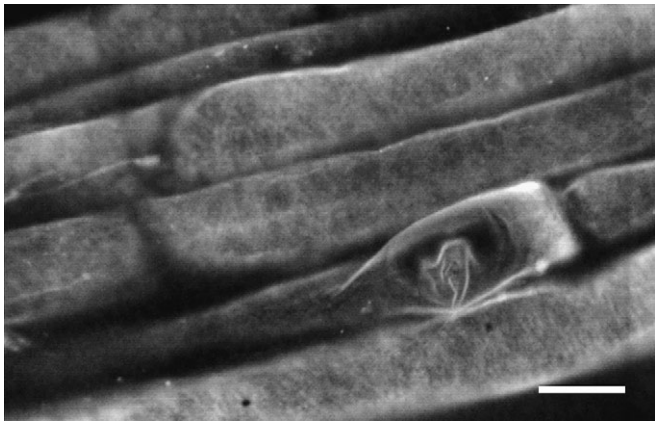

**Fig. 2.** Localization of xyloglucan using lectins. Xyloglucan staining using the fucose-binding lectin UEA I after the removal of pectins and glycoproteins from the cell wall, results in a dotted fluorescence pattern in the epidermis of the elongation zone of a 4-d-old *Arabidopsis* root. Bar is 10 μm.

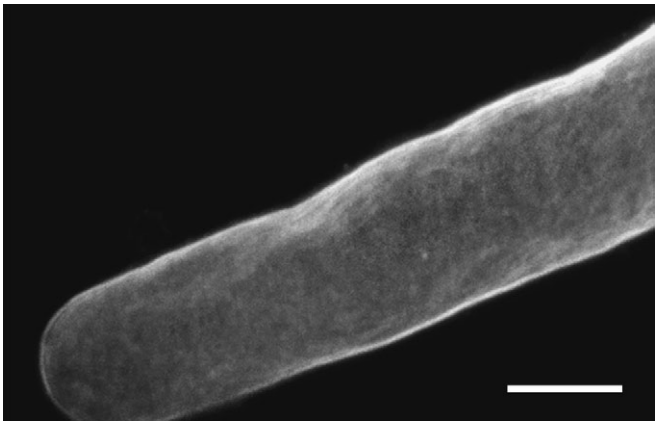

**Fig. 3.** Pattern of XET action of XTH on its endogenous donor substrate in a tobacco root hair. The root hair shows homogenous XET action all over the length of the cell. Bar is 10 μm.

both inhibitors (results not shown). After 45 h of treatment with oryzalin the intensity of staining (mean intensity is 175) is clearly lower than in a control cell (mean intensity is 95) and the 'fibre'-like pattern is lost (Fig. 4G). After 45 h of treatment with latrunculin B the intensity of staining (mean intensity is 221) is drastically lower than in a control cell, but closer examination still reveals some parallel 'fibres' (Fig. 4H).

To assess the accessibility of the newly incorporated xyloglucan oligosaccharides for xyloglucan modifying enzymes, *Arabidopsis* roots were first assayed for XET action and then they were incubated with xyloglucan-specific endoglucanase (XEG) for subsequent periods of 24 h. The specificity of the enzyme was confirmed by MALDI-TOF MS that only xyloglucan oligosaccharides were released by XEG from the ethanol/formic acid-washed cell walls (results not shown). No ions representing XGO-SRs were present in the mass spectrum suggesting a much larger abundance of non-labelled XGOs present in those walls. Using the anthrone assay the amount of xyloglucans released from the walls by XEG was determined (expressed as absorbance at 620 nm) in these 24 h periods (Fig. 5), as well as by buffer only (as control for XEG activity). The amount of enzyme-extractable xyloglucans decreases only slowly over time and, based on the amount of released XG, even after 144 h, it can be expected that not all xyloglucan had been removed. This could be mainly due to hindered diffusion of the enzyme into the intact root tissue. The reduction in XEG-released xyloglucan, but probably not the complete removal of xyloglucan was confirmed by monosaccharide composition of the XEG extracts. Microscopic analysis of the residual fluorescence gave a very specific result. Figure 6A shows as a reference the XTH-driven fluorescence in an untreated root. The fluorescence intensity was strongly decreased in the roots treated with XEG for 24 h (Fig. 6B) up to 144 h (Fig. 6C), by contrast with labelled roots treated with buffer only (data not shown), where no change in fluorescence occurred. There was no obvious difference in fluorescence between 24 h and 144 h XEG-treated roots, although more xyloglucan was extracted in these periods. The zone of highest fluorescence intensity, that labels the early elongation zone (Fig. 6A), however, remained clearly visible, even after 144 h of treatment (Fig. 6C). A close-up of this remaining XET-incorporated fluorescence shows that the 'fibrillar' pattern as described in Fig. 1A is still present, although the fluorescence is clearly less. The root treated with the digestion buffer (without XEG) had a similar remaining fluorescence as the control root (results not shown).

## Discussion

The precise roles of XTHs in cell growth are still under debate. *Arabidopsis* has 33 different XTH genes (Yokoyama and Nishitani, 2001*a*). It can thus be expected that the different gene products are active in different aspects of cell wall metabolism. Besides the expression analysis of the different XTH genes, the localization of the enzyme action could greatly increase insights into the precise function of these enzymes.

In earlier work (Vissenberg *et al.*, 2000*a*, 2001, 2003; Verbelen *et al.*, 2001) it was demonstrated that *in vivo* the XET action of XTH on endogenous donor substrates is most prominent in elongating cells. It is now reported that at the subcellular level, XET action is localized in either a 'fibrillar' or a uniform pattern in different cell walls. The fibrillar pattern is clearly associated with the presence of cellulose microfibrils. The uniform pattern, by contrast, could also be the result of different XTH isoenzymes having a different function from the ones causing the fibrillar staining pattern. The XET action in root hair side walls is of the latter kind. It points to a role in the integration

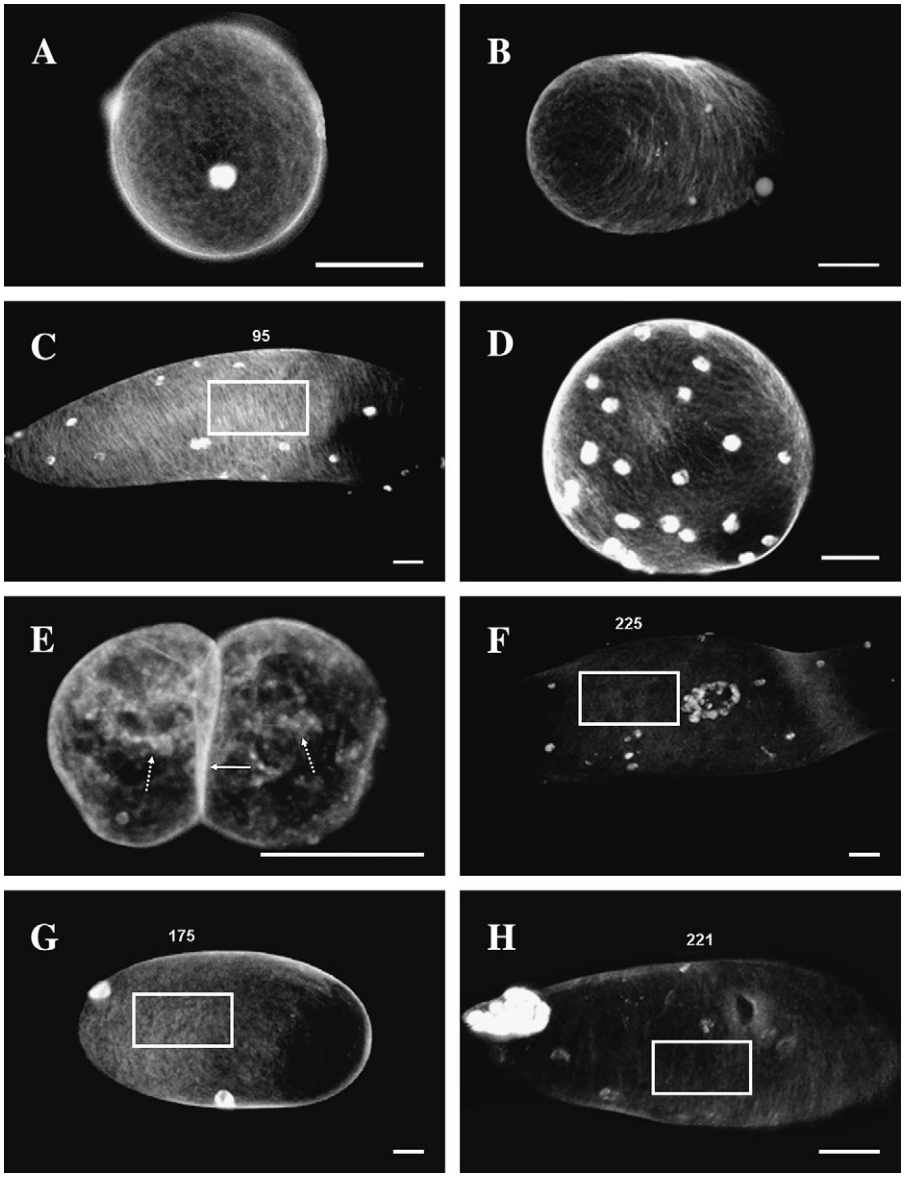


**Fig. 4.** Pattern of XET action of XTH on its endogenous donor substrate in *Nicotiana tabacum* (cv. SR-1) regenerated mesophyll cells in culture. (a) Fluorescence exhibits a 'fibrillar' pattern with no net orientation in a young, protoplast-derived, spherical cell. (b) At the onset of elongation, the rearrangement from a pattern with no net orientation to a more parallel pattern can be observed. (c) In a well-elongated cell a parallel arrangement orthogonal to the long axis of the cell is very obvious. The mean fluorescence intensity of the box is given as 95 where 0 represents white and 255 black. (d) A cell cultured in a suboptimal concentration of NAA expands, but keeps a spherical shape and shows a fibrillar pattern with no net orientation in the wall. (e) In a dividing cell, no fibrillar pattern is visible at all, but fluorescence can be seen in the newly formed cell wall (see full-line arrow). Blurry patches of retracted cytoplasm show autofluorescence (see striped arrows). (f) An elongated cell treated with DCB shows a dramatic reduction of XET action, as also seen from mean intensity value of 221. (g) Oryzalin treatment previous to the XET-assay also resulted in a decrease of action (mean intensity value is 175) and the loss of fibre-like structures. (h) Actin-depolymerization by latrunculin B decreased XET action (mean intensity value is 221) but did not eliminate the fibrillar pattern. Bars are 25 μm.

of newly deposited xyloglucans in non-expanding cell walls. Emons and Wolters-Arts (1983) describe that, in the subapical and basal part of *Equisetum* root hairs, many subsequent layers of microfibrils are deposited (see Fig. 6 of their article).

In the study of cell elongation, the most interesting pattern is clearly the fibrillar pattern. The possibility was ruled out that this typical pattern of XET action is simply the result of the localization of the xyloglucans themselves. The fucose-binding lectins are probably able to bind the fucoses from all xyloglucans present in the wall except those that are embedded within the microfibrils; this is in contrast to XTHs. The lectin-binding observations show that the fibrillar patterns of XET action do not arise simply as an artefact — such as a kind of 'negative staining' whereby the non-staining bulk of a microfibril creates the

illusion of a fibrillar pattern within the rest of the wall matrix. Then it was demonstrated that the presence of cellulose is needed to alter the fibrillar pattern of XET action. The nearly absent incorporation of XGO-SRs in the walls of cells or roots treated with DCB or in a low-cellulose mutant of *Arabidopsis* is a clear indication that XET action decreases in a cellulose-dependent way. All this is in accordance with the theory that xyloglucans largely bind to cellulose microfibrils (Valent and Albersheim, 1974; Hayashi *et al.*, 1987, 1994*a*, *b*; Hayashi, 1989; Pauly *et al.*, 1999*a*). Shedletzky *et al.* (1990) showed that tomato cells, adapted to growth on DCB, secrete their newly synthesized xyloglucans into the medium as they lack the cellulose with which to interact. It thus seems that certain XTH isoenzymes utilize xyloglucan–cellulose complexes as their preferred substrate. However, it can not be ruled out that XTH gene expression is reduced in the absence of cellulose synthesis by the kind of negative feedback mechanism previously described for the *shrunken* gene in maize (Maas *et al.*, 1990). The reduction in cellulose synthese by DCB could lead to the accumulation of UDP-glucose in the cytoplasm, which serves as a feedback mechanism for the down-regulation of the expression of certain genes. Takeda *et al.* (2000) describe a decrease in XTH mRNA levels in suspension-cultured plant cells upon the addition of DCB within 1 h. An equally important observation was that the fibrillar XET action coincides with the orientation of the cellulose microfibrils. Since microtubules are thought to act as guides for oriented cellulose deposition (Giddings and Staehelin, 1991; Burk and Ye, 2002), drugs were used that interfered with the microtubules to see the effect on XET action. The 'fibrillar' staining pattern disappears after destruction of the cortical microtubules, but only after prolonged treatment. Sugimoto *et al.* (2003) recently reported that disruption of cortical microtubules in the *Arabidopsis* root (by drugs or in mutant plants affected in

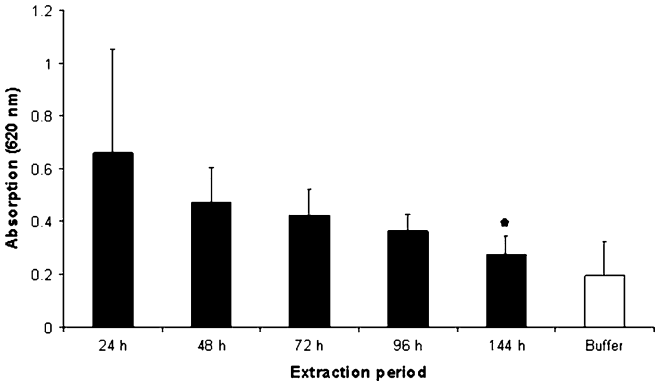


**Fig. 5.** Amount of xyloglucan extracted by a xyloglucan-specific endoglucanase (XEG). The figure shows the amount of xyloglucan XEG-extracted from 10 4-d-old *Arabidopsis* seedlings in periods of 24 h as estimated by the anthrone assay and expressed as the absorbance at 620 nm (different repeats were done and the average is shown). During subsequent 24 h extractions the amount of released xyloglucan decreases and reaches control (buffer) levels after 144 h (asterisk means there is no significant difference from the control), indicating that the maximal amount of xyloglucans had been released.

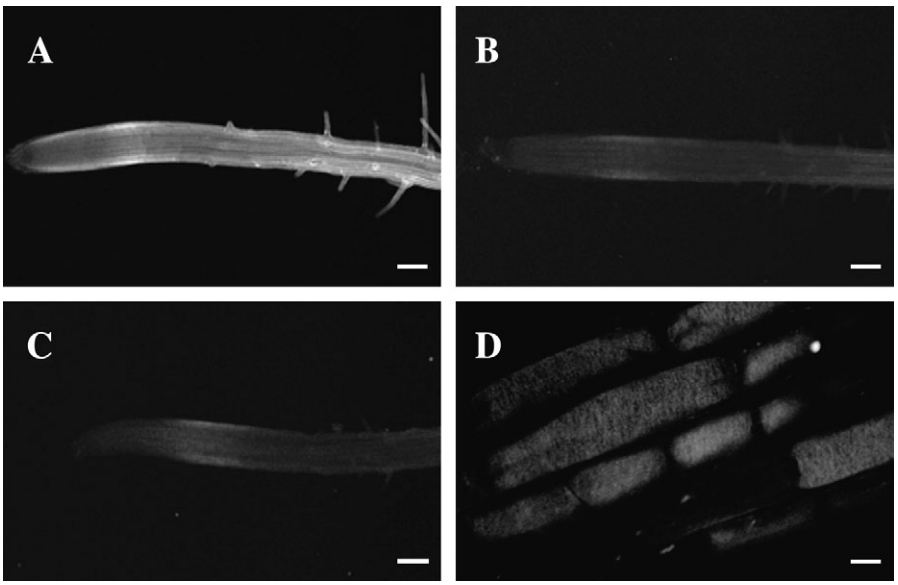


**Fig. 6.** Pattern of fluorescent reaction products remaining wall-bound in *Arabidopsis* roots assayed for the XET action of XTH on endogenous donor substrate and then digested with XEG. (a) A control left in the second wash solution (5% formic acid) during the complete time of the experiment (144 h), shows a normal pattern of transglucosylation products. (b) An XET-assayed root extracted with XEG for 24 h shows a reduction in XET-incorporated fluorescence compared with the control root. (c) A root extracted with XEG for 6×24 h (144 h) shows a reduction in XET-incorporated fluorescence compared with the control root, but not compared with the root extracted for 24 h. (d) A close-up of the remaining XET-incorporated fluorescence after 144 h XEG-extraction shows that the 'fibrillar' staining pattern is still present, although the fluorescence is much lower than in the control root (Fig. 1A). Bars are 100 μm, except in (d) where it is 10 μm.

microtubule organization) does not interfere with correct parallel cellulose microfibril alignment for 8 h. Verbelen *et al.* (2005) also concluded that in the *Arabidopsis* root the elongation rate of cells devoid of microtubules was affected only after oryzalin treatment for longer than 3 h, whereas DCB had a quick and drastic effect. Disruption of actin, which has less influence on the ordered deposition of cellulose microfibrils, did not change the 'fibrillar' pattern, but led to a decrease in XET action, probably due to interference with vesicle delivery at the cell membrane (Foissner *et al.*, 1996).

It should be noted that the elongation rate of the cultured tobacco cells is much slower than that of cells in the elongation zone of the *Arabidopsis* root epidermis. This explains why the effects of oryzalin and DCB on tobacco cells were only seen after longer treatments than in *Arabidopsis*.

Cultured cells kept in division have a pattern of XET action clearly different from non-dividing cells, as indicated by the blurry, non-fibrillar fluorescence pattern. These cells always remain close to a meristematic condition; they never grow beyond the volume of the mother cell and certainly do not elongate and remain more or less spherical. It was previously shown that XET-active XTH was localized in the new cell plate (Yokoyama and Nishitani, 2001*b*). It is shown here that its action is also localized to the newly formed cell wall, confirming these results.

While the authors are aware that *Arabidopsis* and tobacco contain different types of xyloglucans, namely poly-XXXG and poly-XXGG, respectively (Sims *et al.*, 1996; Vincken *et al.*, 1997), this is not thought to interfere with the XET assay (Truelsen *et al.*, 1999).

Pauly *et al*. (1999*a*) describe three different xyloglucan domains in the cell wall. Xyloglucans can be entrapped within cellulose microfibrils, stick to the surface of microfibrils, or form tethers between adjacent microfibrils. A fourth domain could be attributed to the xyloglucans that are not attached to the cell wall at all and that are water-extractable, a fifth domain could be the loose ends. The fibrillar pattern described here could arise from the decoration of cellulose microfibrils by transglucosylation of the fluorescent oligosaccharides to xyloglucan that extends from the microfibril's surface (Fig. 7). This could result in trimming of the tethering xyloglucans. It was reported that a gradual, but quite extensive trimming of non-cellulose-bound domains of wall-bound xyloglucans occurs during normal growth of *Spinacia*, *Rosa*, *Populus*, and maize cells (McDougall and Fry, 1991; Thompson and Fry, 1997; Kakegawa *et al*., 2000; Kerr and Fry, 2003). In any case, the fluorescently modified xyloglucans that extend from the microfibril surface, i.e. the loose ends that are not directly hydrogen-bonded, are only partially accessible to XEG. The fluorescence intensity decreases during XEG treatment. However, the remaining fluorescence exhibits also some 'fibrillar' structure. This indicates either that part of the modified xyloglucans is probably converted from an enzyme-accessible to an enzyme-inaccessible domain or that XEG has less access to the xyloglucans in close vicinity of microfibrils than does XTH. The data suggest that some XTH isoenzymes prefer to act on xyloglucan-cellulose complexes (possibly at sites 1 and 2 in Fig. 7) and have a role in the wall-loosening process that is necessary for cell elongation. This fact is strengthened by the absence of fibrillar XET action in dividing cultured cells and in the non-elongating side-walls of the root hairs. These walls, in fact, don't elongate in the sense of the walls of epidermis cells in the *Arabidopsis* elongation zone.

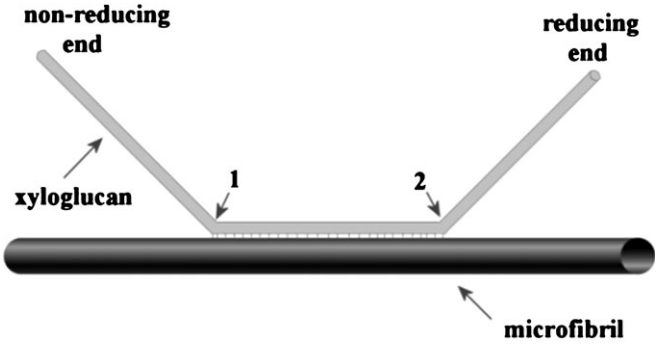


**Fig. 7.** Schematic representation of the location of XET action in the cell wall. XTH probably works on the xyloglucans that extend from the cellulose micofibril's surface, at locations indicated as 1 and 2. By working at these specific points, XTH could trim the xyloglucans, which is important in the cell wall loosening process necessary for cell growth.

This study led to the conclusion that the high XET action (fibrillar and uniform) found in elongating cells could be involved in the incorporation of newly synthesized xyloglucan molecules into the cell wall and/or the tethering of newly deposited microfibrils by xyloglucans and/or restructuring of the existing cellulose/xyloglucan network. It furthermore suggests that some XTH isoenzymes use cellulose-xyloglucan complexes as substrates, and that they gradually trim the xyloglucans that extend from the microfibril surface, a feature that should favour wall loosening.

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