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RESEARCH PAPER

Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of *Arabidopsis* and their effect on root growth and cell wall extension

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Abstract

Xyloglucan endotransglucosylase/hydrolases (XTHs) are enzymes involved in the modification of load-bearing cell wall components. They cleave xyloglucan chains and, often, re-form bonds to the non-reducing ends of available xyloglucan molecules in plant primary cell walls. The enzymic properties and effects on root growth of two *Arabidopsis thaliana* XTHs belonging to subgroup I/II, that are predominantly expressed in root hairs and in non-elongating zones of the root, were analysed here. AtXTH14 and AtXTH26 were recombinantly produced in *Pichia* and subsequently purified. Both proteins were found to exhibit xyloglucan endotransglucosylase (XET; EC 2.4.1.207) but not xyloglucan endohydrolase (XEH; EC 3.2.1.151) activity. Their endotransglucosylase activity was at least 70× greater on xyloglucan rather than on mixed-linkage β-glucan. Differences were found in pH- and temperature-dependence as well as in acceptor–substrate preferences. Furthermore, the specific activity of XET was approximately equal for the two enzymes. Removal of N-linked sugar residues by Endo H treatment reduced XET activity to 60%. Constant-load extensiometry experiments revealed that the enzymes reduce the extension in a model system of heat-inactivated isolated cell walls. When given to growing roots, either of these XTH proteins reduced cell elongation in a concentration-dependent manner and caused abnormal root hair morphology. This is the first time that recombinant and purified XTHs added to growing roots have exhibited a clear effect on cell elongation. It is proposed that these specific XTH isoenzymes play a role in strengthening the side-walls of root-hairs and cell walls in the root differentiation zone after the completion of cell expansion.

Key words: *Arabidopsis*, cell elongation, cell wall, heterologous protein production, *Pichia*, XTH.

Introduction

Growing plant cells typically possess a primary cell wall that is rich in polysaccharides and that additionally contains small amounts of structural proteins and many different enzymes. Since xyloglucans are the major hemicelluloses interconnecting adjacent cellulose microfibrils in cell walls of dicotyledonous plants such as *Arabidopsis* (Hayashi, 1989; Carpita and Gibeaut, 1993; Brett and Waldron, 1996), enzymes modifying this class of polysaccharide are considered to be very important during cell growth. Xyloglucan endotransglucosylase/hydrolases (XTHs) are such enzymes (Fry *et al.*, 1992; Nishitani and Vissenberg, 2007). They cleave donor xyloglucan chains and rejoin the newly formed ends to the non-reducing terminus of an available acceptor xyloglucan chain or oligosaccharide (= XET activity; EC 2.4.1.207) or to water (= XEH activity; EC 3.2.1.151). The former results in the grafting of different

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chains, the latter in the hydrolytic cleavage of xyloglucan (Fry *et al.*, 1992; Rose *et al.*, 2002). Comprehensive bioinformatic analysis of XTH sequences and kinetic data suggest that XEH activity evolved as a gain of function in an ancestral XET (Baumann *et al.*, 2007). To date, all putative XTH proteins (defined by sequence similarity to known XTHs) studied display XET, XEH or both activities towards xyloglucan.

Whereas many *in vitro* studies provide evidence that favours the hypothesis of XTHs mediating cell elongation, attempts to prove a relationship between XET-action and cell wall extension remained unsuccessful for a long time (McQueen-Mason *et al.*, 1993; Saladié *et al.*, 2006). It was only recently shown that a decrease in AtXTH18 mRNA abundance by RNAi resulted in some reduction of the epidermal cell length in the primary root; this was the first evidence of XET action being partially responsible for cell elongation (Osato *et al.*, 2006). In addition, high expression of a *Brassica campestris* homologue of AtXTH9 in *Arabidopsis* evoked a pronounced increase in cell expansion (Shin *et al.*, 2006), and loss of function of AtXTH21 restricted the growth of primary roots and caused an obvious dwarf phenotype in *Arabidopsis* (Liu *et al.*, 2007*a*). Using an extensiometric approach, Van Sandt *et al.* (2007*a*) have shown that recombinant XTH from *Selaginella* is capable of directly restoring extension in heat-inactivated onion epidermal cell walls. These data and reports of experiments on biocomposite materials (Chanliaud *et al.*, 2004) provide direct *in vitro* evidence for the involvement of cell wall xyloglucan-specific enzymes in mechanical changes underlying plant cell wall remodelling and growth processes.

The study of XTH proteins is complicated as they are generally encoded by large multi-gene families (33 genes in Arabidopsis, 41 in poplar, 29 in rice, 25 in tomato, at least 22 in barley, more than 58 in wheat, and at least nine in the early-diverging spore-plant *Selaginella* (Yokoyama and Nishitani, 2001; Yokoyama *et al.*, 2004; Strohmeier *et al.*, 2004; Geisler-Lee *et al.*, 2006; Saladié *et al.*, 2006; Liu *et al.*, 2007*b*, Van Sandt *et al.*, 2007*b*). Of all the 33 identified XTH genes of *Arabidopsis*, one-third occur as clusters resulting from genome duplication and gene reshuffling (Blanc *et al.*, 2000). The conservation (through evolution) of multiple genes with such a high sequence similarity suggests that they have specific expression patterns or that specific differences in enzymic activities can be expected.

In numerous previous studies, many XTH genes of several plant species were indeed found to exhibit different expression patterns in terms of organ specificity and in response to developmental and environmental stimuli. A comprehensive expression analysis of all 33 members of the *Arabidopsis* XTH gene family revealed their tissue specificity and distinct responses to hormonal stimuli. Among them, at least 10 genes were found to be predominantly expressed in roots (Yokoyama and Nishitani, 2001). By use of pXTH::GUS reporter gene constructs it was shown that expression occurred in the apical dividing zone, in all cell types of the elongating and differentiating region, as well as in the vascular tissues at the mature proximal end of the *Arabidopsis* root. Each gene showed its own distinct expression profile (Vissenberg *et al.*, 2005; Becnel *et al.*, 2006).

Using fluorescent xyloglucan oligosaccharides as exogenous acceptor substrates, Vissenberg *et al.* (2000, 2001, 2003) co-localized the donor substrate and active XTH enzymes in the elongation zone of roots, at the sites of future root hair initiation, and in the growing root hairs. At these different sites XET action exhibited different pH optima, suggesting the involvement of multiple isoforms with distinct properties (Vissenberg *et al.*, 2000, 2001). The characterization of pH- and temperature-optima of several XTHs has indeed revealed differences in catalytic properties within one species (Campbell and Braam, 1999; Steele and Fry, 2000). This suggested that it would be of interest to test the isozymes that are expressed under different physiological conditions and to determine their specific functional and enzymic characteristics. The data obtained could shed light on their potentially diverse physiological functions within the root. As the similarity between the 33 different *Arabidopsis* XTH isozymes is so high, it is not feasible to obtain purified enzymes from extracts. Therefore, the option was taken to produce the proteins recombinantly followed by purification to obtain pure enzymes.

This article reports on the differences and similarities of two *Arabidopsis* xyloglucan endotransglucosylase/hydrolases, AtXTH14 and AtXTH26, which are predominantly root-specific. They are very predominantly expressed in the root differentiation zone, and near the root/shoot junction, as well as in root hairs; they are not detectably expressed in the root elongation zone (Becnel *et al.*, 2006). They were recombinantly produced in the yeast *Pichia pastoris* and subsequently purified. The enzymic characteristics such as pH- and temperature-optimum, donor and acceptor substrate preference, optimal acceptor substrate concentration, endotransglycosylation and/or hydrolytic activity, and effect of deglycosylation on their enzymic action were tested *in vitro*. Moreover, the effect of the exogenous addition of the purified enzymes to growing plants and to isolated cell walls was studied.

Materials and methods

Construction of the expression vector

The cDNA of each of the two XTH proteins (AtXTH14: At4g25820 and AtXTH26: At4g28850) was amplified without its own predicted secretion signal sequence and ligated into a pGEM-T vector with primers including *Xho*I and *Xba*I restriction sites. The *Xho*I/*Xba*I fragment from this plasmid was ligated into the pPICZαB vector in-frame with the vector's alpha factor secretion signal sequence for production and secretion in the *Pichia pastoris* expression system (Invitrogen). This vector also provides a *c-myc* epitope and a His-tag at the C-terminus of the protein. The plasmid was sequenced with 5′ and 3′ AOX1 vector-specific primers designed to cover the whole alpha factor signal

sequence, the gene's coding region, and the C-terminal tags followed by the stop codon.

Recombinant protein production and purification

The pPICZα-XTH expression vector was linearized with *Pme*I and introduced into the *P. pastoris* strain GS115 by electroporation according to the manufacturer's protocol. Multiple-copy integrants were selected on YPDS plates (yeast/peptone/dextrose/sorbitol regeneration medium), containing elevated zeocin concentrations up to 1000 μg ml^{-1}. Colony-PCR on selected colonies confirmed the presence of the expression construct in the yeast genome.

A selected and verified colony was grown overnight in liquid buffered complex medium containing glycerol (BMGY) and 100 μg ml^{-1} zeocin at 28 °C and 250 rpm. 100 ml of BMGY medium in a 500 ml culture flask was inoculated with 80 μl of this culture and grown overnight under the same conditions until OD_{600}=4 was reached. Yeast cells were harvested by centrifugation (2000 *g* for 5 min) and diluted to OD_{600}=1 in buffered complex medium containing methanol (BMMY). Induction was performed in 1.0 l flasks containing 200 ml of culture at 28 °C and 250 rpm for 3 d with the addition of methanol to a final concentration of 1% every 24 h. At the end of the induction period, yeast cells were discarded by centrifugation (5000 *g* for 10 min) and proteins were recovered from the medium by precipitation with 40% (w/v) ammonium sulphate and centrifugation (10 000 *g* for 45 min). Pellets were redissolved in ±3 ml 50 mM MES, pH 6.0 and dialysed overnight against 4.0 l of the same buffer with 25 mM NaCl.

The protein mixture was applied to a 5 ml Hi-trap SP-Sepharose column equilibrated with the sample buffer. Unbound proteins were washed away with 30 ml of the same buffer and bound proteins were eluted with 12 ml 50 mM MES, pH 6.0, and 300 mM NaCl. Fractions containing the XTH protein were pooled and concentrated on Centricon Plus-10 columns with a membrane cut-off of 30 kDa (Millipore), yielding a final volume of ±300 μl. Samples of 50 μl were loaded on to a gel-permeation column (Superdex™ 75 HR 10/30, GE Healthcare) in an Äkta explorer chromatography system P-960. The column was equilibrated with degassed 25 mM sodium acetate, 25 mM NaCl buffer, pH 5.7, and eluted with one column-volume of the same buffer. Elution of the proteins was monitored by A_{280} measurement. All purification steps were analysed on SDS-PAGE in a Xcell™ Mini Cell system (Invitrogen) and the polypeptides were visualized with SilverQuest (Invitrogen).

Western blot

After SDS-PAGE the proteins were electrophoretically transferred on to a nitrocellulose sheet at 30 V for 1 h. For immunodetection, the blot was blocked in PBS, pH 7.2, containing 5% (w/v) skimmed milk powder for 40 min and incubated with polyclonal rabbit anti-AtXTH14 antibody (1:5000 in the same blocking solution) for 1 h. A wash in blocking solution for 45 min was followed by incubation with goat anti-rabbit peroxidase for 1 h. The blot was washed in PBS buffer for 45 min and developed with 3,3′-diaminobenzidine tetrahydrochloride (100 μM) in PBS containing 10 mM hydrogen peroxide.

To generate the anti-AtXTH14 antibody, purified AtXTH14 enzymes were sent to Invitrogen for injection in two rabbits. Bleeds were taken at the 4th, 8th, and 10th week after injection. The antibody titre of the bleeds was determined with an enzyme linked immunosorbent assay (ELISA) with peptide bound in the solid phase (1 μg/100 μl/well) on high binding 96-well plates. Sera were pooled and purified on a protein-A affinity column. Polyclonal antibody was recovered at 1 mg ml^{-1}.

XET dot-blot activity assay

The recombinant proteins were tested for xyloglucan endotransglucosylase (XET) activity with a fluorescent dot-blot assay as described by Fry (1997). In this assay the transfer of paper-bound, non-fluorescent, high-molecular-weight xyloglucan to sulphorhodamine-labelled xyloglucan oligosaccharides is monitored. The enzyme solution was spotted on to the test paper and incubated overnight at 28 °C in an aluminium foil envelope (which prevented drying). Non-reacted fluorescent oligosaccharides were washed away in a 1:1:1 by vol. formic acid:ethanol:distilled water solution for 1 h. The remaining pink fluorescence on the test paper upon UV illumination is indicative of XET activity.

Colorimetric XET assay

A colorimetric XET assay was used as developed by Sulová *et al.* (1995) and adapted by Kallas *et al.* (2005). To a solution composed of 20 μl of tamarind xyloglucan (2 mg ml^{-1}; Megazyme), 20 μl of reduced tamarind xyloglucan heptasaccharide (principally XXXGol; 0.5 mg ml^{-1}, unless otherwise stated; Megazyme) and 40 μl of a 100 mM sodium acetate buffer (pH 5.7) and 2 μl XTH enzyme (0.75 μg) was added. This mixture was incubated at 28 °C. Reactions were terminated by the addition of 40 μl 1 M HCl and divided into three replicates of 40 μl in a 96-well plate (polystyrene, clear wells in white matrix, Wallac, Finland). Afterwards, 108 μl of (20% w/v) Na_2SO_4 and 28 μl potassium tri-iodide reagent (1% KI and 0.5% I_2 in water) were added to the samples followed by a 30 min incubation in the dark at room temperature, which allowed colour development. The depolymerization of the xyloglucan donor substrate due to transglycosylation to oligosaccharide acceptors is followed colorimetrically by measuring the disappearance of the blue-green-coloured iodine:xyloglucan complex. The transglycosylase activity is calculated as the difference in absorbance at 630 nm (Elx808i, Ultra Microplate reader, Bio-tek instruments) measured along the course of the reaction in the presence and absence of XTH enzyme. All samples were performed in triplicate and reported as the difference between the mean of the sample and blank ±the standard deviations. As for both AtXTH14

and AtXTH26 no XEH activity was observed, assay reactions to account for such activity were not included and the measured activity was regarded as XET activity.

Reducing sugar assay

The BCA (bicinchoninic acid) reducing sugar assay was used for measurement of any xyloglucan endohydrolase (XEH) activity of the purified enzymes (Garcia *et al.*, 1993). 1.75 μg XTH enzyme (25 μl) or 1 μg cellulase (25 μl) from *Trichoderma viride* (Sigma) was added to a solution composed of 25 μl of tamarind xyloglucan (2 mg ml^{-1}) (Megazyme) and 20 μl of 25 mM sodium acetate, 25 mM NaCl buffer, pH 5.7. Samples were incubated at 28 °C for 0–48 h. At 0, 8, 16, 24, and 48 h, samples were withdrawn and divided into triplicates of 15 μl in a 96-well plate (polystyrene, clear wells in white matrix, Wallac, Finland). Reactions were terminated by the addition of 135 μl H_2O and 150 μl of bicinchoninic acid (BCA) solution (Sigma) containing 0.008% $CuSO_4.5H_2O$. Plates were incubated for 30 min at 80 °C, during which time Cu^{2+} was reduced to Cu^+ which forms a purple-blue complex with BCA. Samples were placed on ice and activity was measured as the increase in absorbance at 560 nm (Elx808i, Ultra Microplate reader, Bio-tek instruments).

Viscometric XEH assay

100 μl NaOAc buffer, or 100 μl AtXTH14 in NaOAc buffer, or 100 μl AtXTH26 in NaOAc or 100 μl cellulase of T. viride in NaOAc buffer was added to a 200 μl mixture of 1% tamarind xyloglucan. All enzymes reached a final concentration of 1 ng μl^{-1}. The viscosity of the mixtures was measured after 0, 2, 8, and 24 h of incubation and was seen as a function of their flow rate through 200 μl pipettes.

Deglycosylation

A range of 0–2000 U of endoglycosidase H (New England Biolabs) was added to purified XTH enzyme and incubated at 37 °C for 2 h. One part of each sample was used for analysis on SDS-PAGE and the other part was used in a colorimetric XET assay. A control sample was tested without XTH enzyme and with different amounts of endoglycosidase H, but no significant reaction could be observed.

Radioactive XET assay

With the exceptions mentioned in the next paragraph, the method for radiochemical XET assays was routinely as follows (based on Fry *et al.*, 1992). Reactions were performed in mixtures containing final concentrations of 100 m*M* acetate buffer (Na^+ salt, pH 5.7), 10 mM $CaCl_2$, 0.3% w/v tamarind xyloglucan, 0.65 μM [^{3}H]XLLGol (specific activity 51 MBq μmol^{-1}, thus 0.033 kBq μl^{-1}) and either 0.027 ng μl^{-1} purified AtXTH14 or 0.109 ng μl^{-1} purified AtXTH26. Both enzymes were previously dissolved in 1% BSA. The reaction mixture was incubated for 2 h at 20 °C. The reaction was stopped by addition of 20 μl 50% formic acid. Reaction mixtures were then spotted on to Whatman 3MM paper, air-dried and washed under running tap water overnight. The papers were redried and scintillation-counted in 2 ml of Wallac 'OptiScint' scintillant. Blanks were measured identically except that the XTH enzyme was omitted from the 1% BSA solution.

Departures from the routine sample work-up were as follows: (a) When the donor substrate was water-soluble cellulose acetate (WSCA), which may not hydrogen-bond to filter paper, the reaction was stopped by exposing the reaction mixture to ammonia overnight to convert the WSCA to insoluble cellulose. (b) When the donor substrate was carboxymethylcellulose (CMC), hydroxyethylcellulose or methylcellulose, the reaction products were subjected to paper chromatography in ethyl acetate/acetic acid/water (10:5:6 by vol.) instead of washing in tap-water. (c) When the acceptor substrate was [^{3}H]cellohexaitol, the reaction-products were not applied to paper (because cellohexaitol is difficult to wash off paper); instead, the high-molecular-weight products were precipitated with 70% ethanol and repeatedly washed in 70% ethanol; the final polysaccharide pellet was redissolved in water and assayed for radioactivity. (d) In the experiment to measure the pH optimum, the buffer (final concentration 100 mM) was citrate (Na^+ salt, pH 3.5–6.5) or HEPES (Na^+ salt, pH 7.0–8.0) instead of acetate. (e) In the experiment to measure the temperature optimum, the reaction mixtures were incubated at 0–37 °C instead of 20 °C.

For investigation of the donor-substrate preferences of the XTHs, various water-soluble β-glucans were used in place of tamarind xyloglucan: CMC [Na^+ salt, low viscosity; degree of substitution (DS) 0.65–0.85 carboxymethyl groups per glucose residue], methylcellulose (DS 1.5–1.9 methyl groups per glucose residue) and barley mixed-linkage (1→3,1→4)-β-glucan (MLG) were from Sigma Chemical Co.; hydroxyethylcellulose (HEC; DS 0.9–1.0 hydroxyethyl groups per glucose residue) was from Fluka; and water-soluble cellulose acetate (WSCA; DS 0.5–1.0 acetyl groups per glucose residue) was prepared as described by Fry *et al.* (2008). For investigation of the acceptor-substrate preferences of the XTHs, various ^{3}H-labelled oligosaccharides were used in place of [^{3}H]XLLGol, and the [^{3}H]XLLGol, were prepared as described by Hetherington and Fry (1993) or Steele and Fry (2000).

Since the concentration of the acceptor substrate used in these assays (usually 0.65 μM XXXGol) was well below the K_m of XTHs (typically 30–300 μM; discussed by Fry *et al.*, 2008), it is impossible to quote enzyme activities in katals. Instead, it is most valid to report *relative* incorporation rates [(Bq incorporated into polymeric product) (Bq oligosaccharide supplied)$^{-1}$ (reaction time)$^{-1}$] (Fry *et al.*, 1992), and this has been done in Fig. 4 where diverse substrates are compared. However, to facilitate comparisons of assays conducted at various temperatures and pH values, these relative activities have been reported as a percentage of the maximal relative activity measured within the experiment in

question assay, i.e. as 'relative %' (see Fig. 3). All assays were performed in triplicate and are reported as (mean minus enzyme-free blank) ±SD.

Exogenous addition of XTH enzyme to Arabidopsis *seedlings*

Seeds were surface-sterilized in 6% sodium hypochlorite for 10 min and washed three times with sterile water, incubated for 2 d in liquid MS medium (Murashige and Skoog medium including vitamins, pH 5.7, from Duchefa, The Netherlands;) at 4 °C and grown for 4 d in growth chamber conditions (22 °C, 60% RH, 16/8 h light/dark cycle). 4-d-old seedlings were transferred to fresh MS medium with pure XTH enzyme added to a final concentration of 50 ng μl^{-1} or the same amount of heat-inactivated (5 min at 80 °C) pure XTH enzyme as a control. Inactivation of the enzymes was confirmed by an XET dot-blot assay. Petri dishes were subsequently incubated in the growth chamber under the same conditions for two more days and pictures were taken with an Olympus C-5050 ZOOM digital camera.

For detailed microscopic pictures and movies the 4-d-old seedlings were transferred into a 2:1 mixture of liquid MS medium with pure XTH enzyme dissolved at 45 ng μl^{-1} and MS medium with agar at 40 °C. Seedlings were covered with a microscope cover slide and after partial coagulation of the medium, plates were placed vertically in the growth chamber under the same conditions as mentioned above for 2 d for confocal pictures or followed immediately in a horizontal position under the light microscope for the generation of movies. Here, pictures were taken every 15 min during a period of 2.5 h. The individual pictures were used to generate time-lapse movies. Controls were prepared identically except that the XTH enzyme solution was replaced with sodium acetate buffer obtained from the last purification column.

Plant material and sample preparation for extensiometry

Onion (*Allium cepa* L. cv. Sturon) bulbs were collected in a field at the beginning of June at the stage of active growth in girth. These bulbs had an average diameter of 19 mm at the moment of harvest. Strips of epidermis, 20–25 mm long and 4 mm wide, were peeled from the equatorial part of the adaxial side of the 4th (from the outside) live scale transversely to the axis of the bulb. The epidermal strips were immediately frozen in liquid nitrogen and stored at –20 °C.

Denaturation of the endogenous protein activity was achieved by heat-inactivation of the tissue by a 15 s incubation in boiling water.

Constant-load extensiometry

In vitro extension of onion epidermal cell walls was measured with a custom-built constant-load extensiometer as described in Suslov and Verbelen (2006).

A 5 mm segment of an epidermal strip was secured between the clamps and preincubated in the experimental buffer/enzyme solution for 5 min. The buffer used was 25 mM sodium acetate (pH 5.7) containing 25 mM sodium chloride. AtXTH26 (or AtXTH14), when present, were dissolved in that buffer at a final concentration of 10 or 100 ng μl^{-1}.

The wall extension was induced by a 10 g load application and expressed as the amount of the time-dependent deformation (creep) from 10 min to 30 min after loading (Van Sandt *et al.*, 2007*a*).

Results

Recombinant production of XTH proteins and purification

To enable comparison of the enzymic characteristics of AtXTH14 and AtXTH26, both enzymes were recombinantly produced using the *Pichia pastoris* heterologous expression system. In the 40% (w/v) ammonium sulphate-precipitate of culture medium of methanol-induced yeast containing the pPiczαB-AtXTH14 and pPiczαB-AtXTH26 expression vector, XET activity was detected with the fluorescence dot-blot activity assay. This proves that active enzymes were produced. No XET activity was detected in the medium of induced empty vector-containing yeast (data not shown). The ammonium sulphate-precipitated samples of empty vector and XTH-expressing yeast are shown in Fig. 1A and B, lanes 2 and 3, respectively. AtXTH14- and AtXTH26-containing samples were desalted and subjected to cation-exchange chromatography. This resulted in a significant purification of both proteins (Fig. 1A, B, lane 4). As small amounts of contaminating proteins were still present on the silver-stained protein gel, the semi-purified proteins were subjected to gel-permeation chromatography, yielding highly purified AtXTH14 and AtXTH26 proteins, respectively (Fig. 1A, B, lane 5). All detectable contaminants were excluded after this extra step. During the purification procedure, the protein content and the XET activity of the enzymes was monitored with Western blotting (for AtXTH14, Fig. 1C) and the XET dot-blot assay (for AtXTH26, Fig. 1D), respectively. Identical amounts of proteins to run the gels (in Fig. 1A, B) were used to perform the Western blot and dot-blot assay. Both proteins were abundantly produced and successfully purified in an active form. In the iodine-based colorimetric assay, both proteins were found to exhibit similar XET activities at equal concentrations (data not shown).

BCA assay for endohydrolase activity

As the fluorescent dot-blot activity assay detects only xyloglucan endotransglucosylase (XET) (EC 2.4.207) activity and not xyloglucan endohydrolase (XEH) (EC.3.2.1.151) activity, both proteins were tested for their potential to catalyse xyloglucan hydrolysis with the BCA reducing sugar assay. This assay measures the concentration of reducing ends, which would increase upon hydrolysis of xyloglucan. No net formation of reducing ends by purified AtXTH14 and AtXTH26 could be detected. This is in contrast with

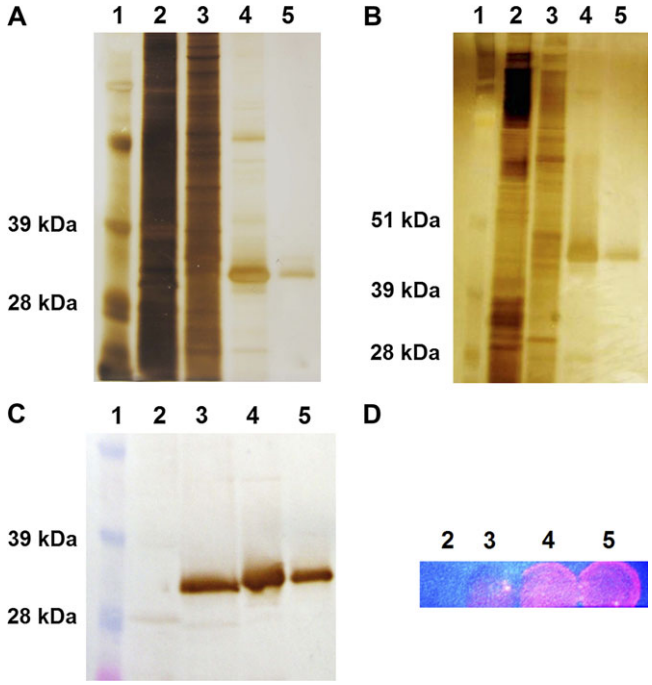

Fig. 1. Production and purification of recombinant AtXTH14 and AtXTH26. (A, B) Silver-stained protein gel from different purification steps of AtXTH14 and AtXTH26, respectively. From left to right: (1) molecular weight marker, (2) 40% (w/v) ammonium sulphate precipitate of the medium of an induced *Pichia* culture transformed with an empty pPicZαB vector as a control (redissolved in buffer and dialysed), (3) 40% ammonium sulphate precipitation of induced pPicZαB-XTH culture, (4) semi-purified XTH enzyme after cation-exchange chromatography, (5) purified XTH enzyme after gel-permeation chromatography. (C) Analysis of AtXTH14 purification as performed with Western blot with anti-XTH14. The numbers correspond to the lanes in (A). (D) Detection of AtXTH26 XET activity using the XET dot blot activity assay during the purification protocol. The numbers correspond to the lanes in (B).

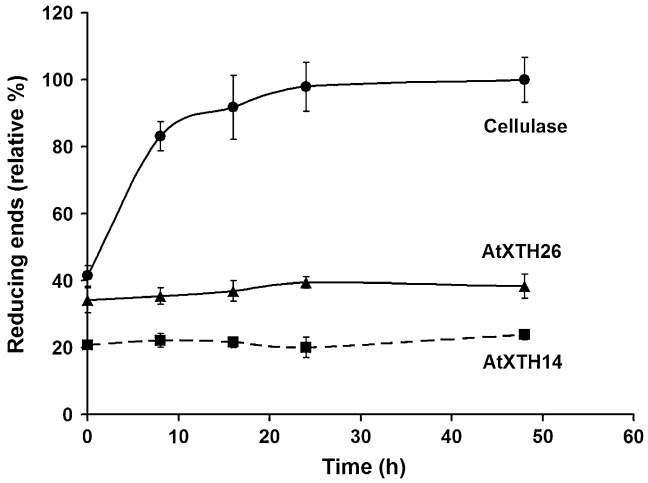

Fig. 2. Test for xyloglucan endohydrolase (XEH) activity of purified AtXTH14 and AtXTH26. Tamarind xyloglucan was incubated with endo-(1→4)-β-glucanase (cellulase), AtXTH14 or AtXTH26 for 0–48 h, and the production of reducing termini was monitored by the BCA assay.

the control enzyme cellulase, a β-(1-4)-endoglucanase, which hydrolyses xyloglucan, seen as an increase in the concentration of reducing ends (Fig. 2). These data were confirmed by a viscometric test on xyloglucan in the presence of these three enzymes (data not shown). As both recombinant enzymes display no detectable hydrolytic activity, several tests were set up for characterization of the transglucosylating activities of AtXTH14 and AtXTH26.

Temperature dependence

Purified AtXTH14 and AtXTH26 proteins were assayed for XET activity with the radiometric method at various temperatures. AtXTH14 shows high activity at 37 °C, and at least 80% of this activity is maintained down to 20 °C. Even at 4 °C the protein still displays more than 60% of its activity at 37 °C. The activity, however, abruptly decreases to about 30% at 0 °C (Fig. 3A).

AtXTH26 seems to be more temperature-sensitive. Beneath 37 °C its activity shows a much steeper decline than

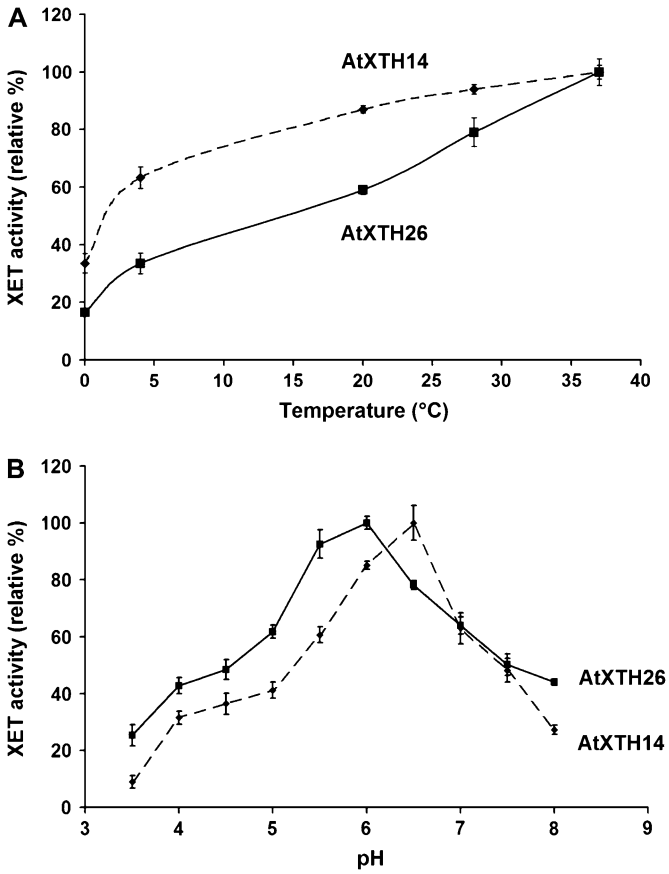

Fig. 3. Temperature (A) and pH (B) profile of XET activity of purified AtXTH14 and AtXTH26, studied with the radioactive XET assay. Data are presented as a percentage of the maximal activity measured in the assay and activity is expressed as relative % activity. Background binding of the oligosaccharide to Whatman 3MM paper was subtracted. Each assay was performed in triplicate and standard deviations are shown.

AtXTH14, losing about half its activity when cooled to 20 °C. AtXTH26 is clearly less cold-tolerant than AtXTH14.

pH dependence

The activity of both proteins shows a bell-shaped pH profile with an optimum at pH 6.0 and 6.5 for AtXTH26 and AtXTH14, respectively (Fig. 3B). Both enzymes show a fast and symmetrical decrease in activity on both sides of their optima followed by an acidic shoulder between pH 4.0 and 5.0 for AtXTH14 and between pH 4.0 and 4.5 for AtXTH26. At pH 4.0, AtXTH14 retains about 30% and AtXTH26 even 40% of its maximal activity. Both enzymes can therefore be regarded as rather acid-tolerant and acting over a broad pH range.

Substrate specificity

To study the substrate specificity of both enzymes, their ability to catalyse the transfer of different donor substrate polysaccharides to [^{3}H]XXXGol, a xyloglucan-derived oligosaccharide, was tested. AtXTH14 used donor substrates in order of preference xyloglucan>>water-soluble cellulose acetate (WSCA)>hydroxyethylcellulose (HEC)>mixed-linkage β-glucan (MLG)>carboxymethylcellulose (CMC)>methylcellulose (MC); thus, all non-xyloglucan substrates showed very low activity rates in comparison to xyloglucan (Fig. 4A). AtXTH26 was able to use WSCA at a low rate but showed no activity with any of the other non-xyloglucan substrates tested (Fig. 4B). The extremely low activity of XTHs extracted from angiosperms (including the MLG-rich Poales) on MLG as donor substrate has been reported before (Hrmová *et al.*, 2007; Fry *et al.*, 2008; Genovesi *et al.*, 2008). This is in contrast to extracts from *Equisetum*, which possess an endotransglucosylase that specifically attacks MLG and grafts a portion of this polysaccharide on to xyloglucan oligosaccharides as acceptor substrates (Fry *et al.*, 2008). Both of the AtXTH enzymes studied here, therefore, show a very high preference for xyloglucan as donor substrate, yet do not work exclusively on xyloglucan.

As the composition of xyloglucans may be quite heterogeneous, both enzymes' acceptor substrate preferences were tested on a selection of oligosaccharidyl-[^{3}H]alditols with various side chains and with backbones differing in the number of glucose units. Xyloglucan was successfully grafted on to all tested oligosaccharides by AtXTH14 and AtXTH26, but with a clear preference for the octasaccharide, XXLGol (Fig. 4C, D, respectively). XXXGol is also a good substrate for both enzymes, but a clear difference in preference towards XLLGol is detected. Whereas XLLGol is the second best (tested) substrate for AtXTH14, it seems a rather poor substrate for AtXTH26, which prefers the fucosylated form of XXLGol, i.e. the nonasaccharide XXFGol. By comparison, it is clear that cellohexaitol is not a preferred substrate for either protein.

Calculation of the specific activity of each XTH was done to reveal the magnitude of their enzymic activities. From the pH studies (Fig. 3A), it is clear that, at pH 5.7, AtXTH26 performs at at least 95% of its maximal activity, whereas AtXTH14 acts at only 60%. Furthermore, the protein

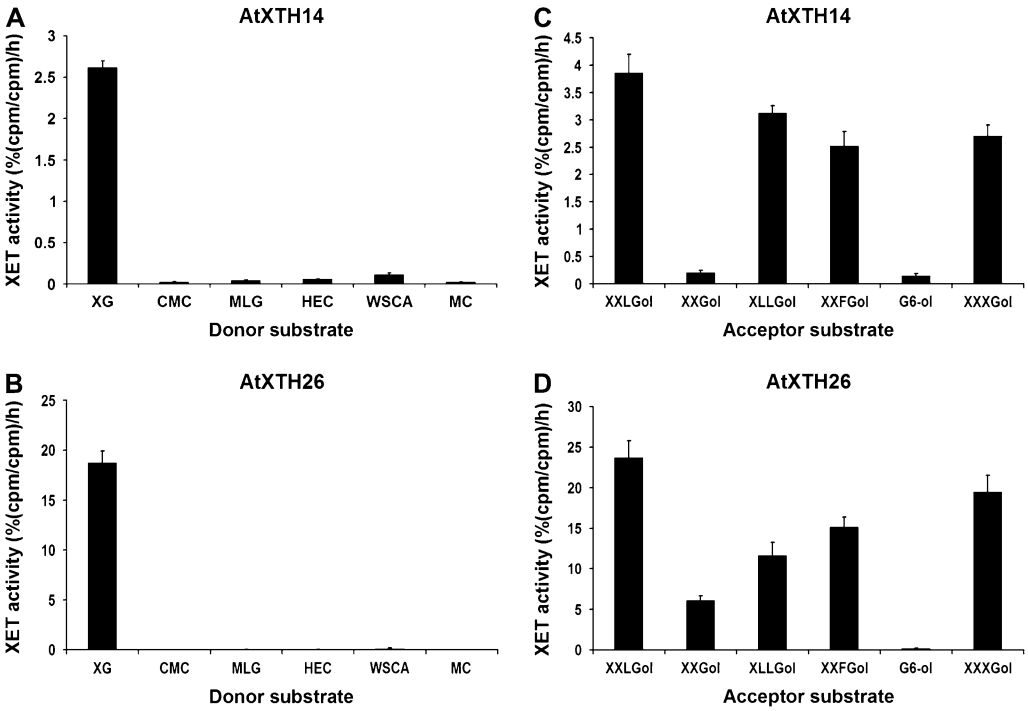

Fig. 4. Donor and acceptor substrate preference of purified AtXTH14 and AtXTH26, studied with the radioactive XET assay. Data are presented as a percentage of the total activity of the labelled acceptor substrate and expressed per hour of incubation time. Background binding of the oligosaccharide to Whatman 3MM paper was subtracted. In the case of [^{3}H]cellohexaitol, ethanol washing of precipitated products was used instead of water-washing of filter paper. Each assay was performed in triplicate and standard deviations are shown.

concentration of AtXTH14 used in these experiments was 4.04 times lower than that of AtXTH26. For a valid comparison with the AtXTH26 data in Fig. 4B, the counts found for AtXTH14 in Fig. 4A should therefore be multiplied by 1.58 (to deal with pH differences) and by 4.04 (to account for the concentration difference). Multiplying the values of AtXTH14 by 6.4 indeed approaches the values found for AtXTH26. This can be done for the values in Fig. 4C and D as well, revealing that the two enzymes have a more or less equal maximal specific activity towards xyloglucan.

Effect of deglycosylation

In silico analysis (http://www.cbs.dtu.dk/services/NetNGlyc-1.0) revealed one potential site for N-linked glycosylation for AtXTH14, and four for AtXTH26 (data not shown). To determine the presence of N-linked glycans and their importance for enzyme activity, the removal of these glycans was combined with a 3 h colorimetric activity assay and SDS-PAGE analysis. Both enzymes were treated for 2 h with different activities of endoglycosidase H, which cleaves within the chitobiose core of high-mannose and some hybrid oligosaccharides in N-linked glycoproteins. XET activity was measured together with the glycosylation state of the enzyme, which was visualized on a silver-stained protein gel. Both untreated enzymes were clearly glycosylated as a decrease in molecular weight can be followed on gel (Fig. 5A, B). In Fig. 5B several bands corresponding to proteins of different mass can be seen, suggesting that several of the potential glycosylation sites in AtXTH26 are effectively N-glycosylated. With the lower amounts of Endo H (0–200 U), XET activities fluctuate, but when an increasing amount is used (500–2000 U) proteins shift towards lower glycosylation states and their XET activity retains a stable 60–70% of the activity of the fully glycosylated proteins (Fig. 5C).

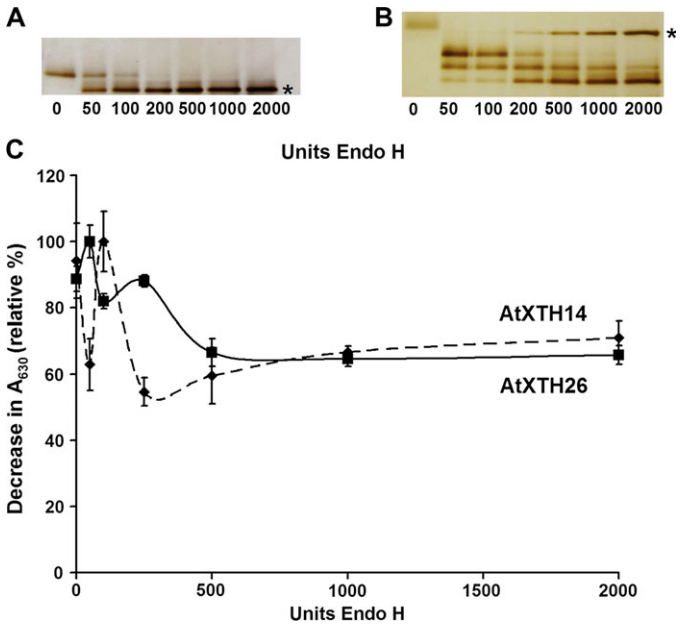

Fig. 5. Deglycosylation of purified AtXTH14 (A) and AtXTH26 (B) visualized on silver-stained protein gel. XET activity of the same samples was measured with a 3 h colorimetric activity assay (C). The fast-migrating band in (A) is the Endo H enzyme itself (*), the slow-migrating protein in (B) is the dimer of Endo H (*). Although both monomer and dimer were present on both gels, only one of them is present per gel because of image cropping.

Addition of purified XTH enzyme to Arabidopsis

In addition to an *in vitro* analysis of the XTH enzymes their effect on the walls of living plant cells was tested by exogenous addition of purified proteins to growing *Arabidopsis* seedlings. 4-d-old seedlings were transferred to fresh liquid MS medium with pure XTH enzyme added to a final concentration of 10, 20 or 40 ng μl^{-1} (Fig. 6). The phenotypic effect was compared with seedlings transferred to medium with 50 ng μl^{-1} of heat-inactivated enzyme (Fig. 6A, E). Seedlings were grown for two more days in these conditions and pictures were taken. Both proteins slowed down growth in a concentration-dependent way, seen as the difference in root length compared with the control roots. From the pictures it is also clear that AtXTH26 had a more dramatic effect than AtXTH14. The growth of seedlings incubated with 40 ng μl^{-1} active AtXTH26 ceased almost completely.

A more detailed microscopical study revealed that the seedlings incubated in buffer showed normal root growth with normal root hair formation (Fig. 6I). Seedlings incubated with AtXTH14 (30 ng μl^{-1}) showed an almost absent elongation zone and very short cells in the differentiation zone, which results in root hairs stacked very close together (Fig. 6J). Cell elongation was thus clearly impaired. The growth of initiated root hairs was affected as well. They seemed to elongate slower than in normal conditions and, in many instances, the root hairs were swollen at the base (see arrows). Seedlings incubated with AtXTH26 (30 ng μl^{-1}) showed very little or no elongation, root hair formation had ceased completely or had been limited to bulging in a few less severe cases. Cortical cells of the root had a swollen or irregular appearance (Fig. 6K).

These results were confirmed and complemented by a kinematic analysis of root growth with the XTH incubation occurring under a microscope. Images were taken at 15 min intervals for 2.5 h. Comparison was made with seedlings incubated with buffer alone. The time-lapse movies resulting from the individual pictures clearly demonstrate the effect of the addition of the XTH proteins (http://webh01.ua.ac.be/fymo/movies/Root+buffer.gif; http://webh01.ua.ac.be/fymo/movies/Root+AtXTH14.gif; http://webh01.ua.ac.be/fymo/movies/Root+AtXTH26.gif). Compared with the control plants, elongation of the roots and root hairs in AtXTH14-treated plants occurs much slower and in the AtXTH26-treated plants elongation was nearly completely inhibited.

Constant-load extensiometry on isolated heat-inactivated cell walls

As the former experiments suggest an impact of the added enzymes at the level of the cell wall, the effect of XTHs on

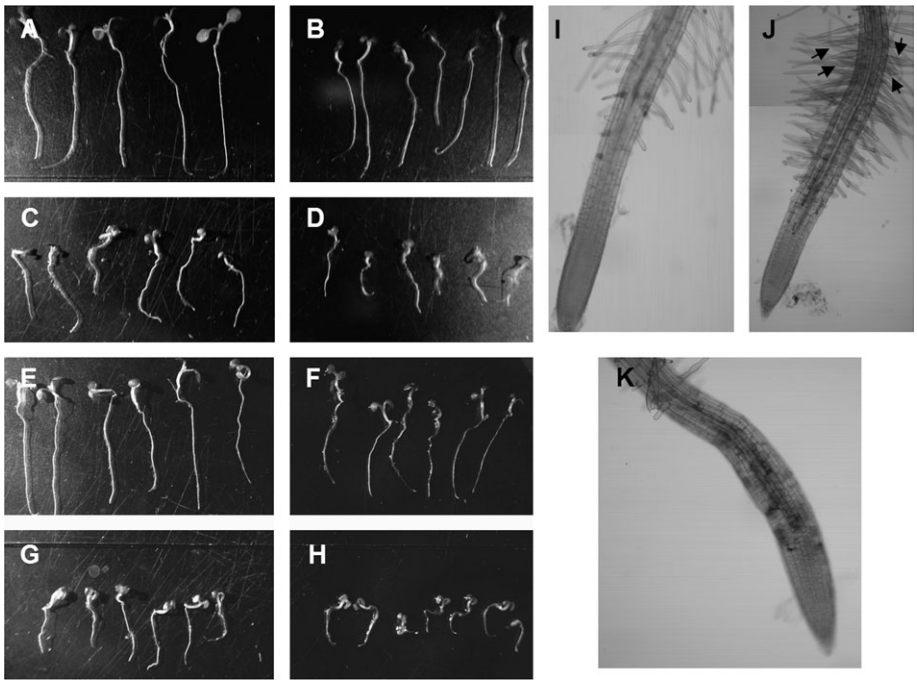

Fig. 6. General picture of *Arabidopsis* seedlings incubated for 2 d with heat-inactivated (A, E) or active purified AtXTH14 (B–D) or AtXTH26 (F–H) at concentrations of 10 ng μl^{-1} (B, F), 20 ng μl^{-1} (C, G), and 40 ng μl^{-1}(D, H). Transmission confocal pictures were made of *Arabidopsis* seedlings incubated for 2 d with buffer (I), with 30 ng μl^{-1} AtXTH14 (J) or with 30 ng μl^{-1} AtXTH26 (K).

the *in vitro* extension of isolated cell walls was studied with the adaxial onion bulb epidermis as a model (Suslov and Verbelen, 2006; Van Sandt *et al.*, 2007*a*). This model has a net longitudinal cellulose microfibril orientation in the cell wall with respect to the bulb axis. The adaxial epidermis extension *in vitro* at neutral pH and its stimulation in a pH 4.5 buffer were similar to those reported earlier for the epidermis of a different onion cultivar (Van Sandt *et al.*, 2007*a*) (data not shown). Wall extension was induced by a 10 g load application and is expressed as the amount of the time-dependent deformation (creep) occurring from 10–30 min after loading (Van Sandt *et al.*, 2007*a*).

In order to eliminate activities of endogenous wall proteins that could mask effects of AtXTH14 and AtXTH26 on the wall extension, the onion epidermis was heat-inactivated. The walls heat-inactivated in boiling water for 15 s showed significantly higher extension at pH 5.7 compared to the native walls (Fig. 7). Interestingly the same heat-inactivated epidermis was much less extensible than the native walls at pH 4.5 (data not shown), in line with the previous data (Van Sandt *et al.*, 2007*a*).

The addition of purified AtXTH26 to the heat-inactivated cell walls caused a concentration-dependent decrease in the wall extension at pH 5.7 restoring the greater part of the wall tightness lost during heat-inactivation (Fig. 7). AtXTH26 (100 ng μl^{-1}) heat-inactivated for 10 min at 90 °C did not change the wall extension (data not shown). The inhibitory effect of AtXTH14 (100 ng μl^{-1}) on onion epidermis extension *in vitro* was lower that that of AtXTH26 (100 ng μl^{-1}) (data not shown), which is consistent with their effects on *Arabidopsis* root growth.

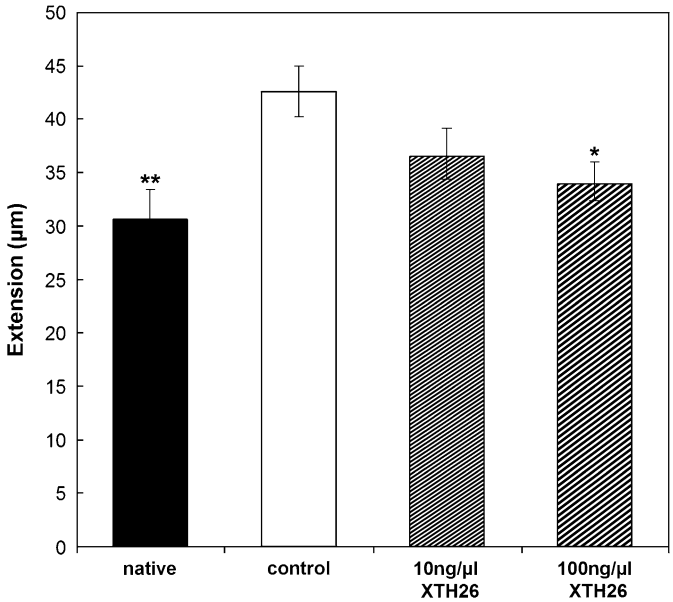

Fig. 7. Effect of AtXTH26 on the *in vitro* extension of isolated onion epidermal cell walls. Creep was measured from 10–30 min after load application and is expressed in µm. Heat-inactivation of the wall proteins caused an increase in extensibility, and this increase was diminished in a concentration-dependent way by addition of AtXTH26. * , ** Differences from the control (heat-inactivated sample) at P <0.05 and P <0.01, respectively (Newman–Keuls test).

Discussion

In an expression analysis study of all 33 members of the *Arabidopsis* XTH gene family, at least 10 genes were found

to exhibit predominantly root-specific expression (Yokoyama and Nishitani, 2001; Becnel *et al.*, 2006). Two of them, AtXTH26 and AtXTH14, are highly expressed in root hair cells. To gain insight into the potential differential roles of these two enzymes, AtXTH14 and AtXTH26 were produced in the heterologous expression system of *Pichia pastoris*. To rule out interference by other components, both enzymes were purified and subsequently subjected to identical *in vitro* and *in vivo* tests.

By definition, XTHs exhibit endotransglucosylase (XET) and/or endohydrolase (XEH) activity on xyloglucans. Based on structural features of the genes, the XTH family is divided into three classes (Yokoyama and Nishitani, 2001; Rose *et al.*, 2002). As both AtXTH14 and AtXTH26 are members of class II, the presence of XET and the absence of XEH activities correlate well with their predicted activities based on the structural features of their genes.

Analysis of the activity–temperature profiles of AtXTH14 and AtXTH26 revealed a broad activity range. Whereas AtXTH14 showed high activity levels all over the range, AtXTH26 showed a clear preference for higher temperatures. Both enzymes show about a 20% decline in activity between 4 °C and 0 °C, so neither of them is extremely cold-tolerant. These broad-range temperature profiles have been seen in most XTH enzymes analysed so far and confirm the idea that broad temperature-tolerance can be expected in enzymes that play an important role during the growth of the plant. Indeed, the data on XTH enzymes all show optimum temperatures ranging from 18 °C to 37 °C, although extremes have been reported for heat-tolerant (55 °C) as well as cold-tolerant (–5 °C) enzymes (Campbell and Braam, 1999; Steele and Fry, 2000; Kallas *et al.*, 2005; Van Sandt *et al.*, 2006).

Whether the difference in cold-tolerance between AtXTH14 and AtXTH26 points to a different physiological role is rather unlikely as a soil temperature of 37 °C seems quite uncommon in the moderate climate where the plants normally grow. Moreover, the genes of both enzymes are expressed at 22 °C in *Arabidopsis* roots (Becnel *et al.*, 2006) and the exogenous addition of AtXTH26 to *Arabidopsis* seedlings growing at 22 °C had a severe effect on the root system. Furthermore, the expression analysis of AtXTH26 revealed that besides a dominant root tissue specificity, the second-highest expression was located in the siliques (Yokoyama and Nishitani, 2001). Therefore it seems more likely that an optimal activity of the AtXTH26 enzyme might not be necessary or favourable within the roots and that the preference for higher temperatures may be a quality linked to the enzyme's activity in aerial plant parts.

Analysis of the activity–pH profiles of both enzymes showed rather sharp pH optima around pH 6.0 for AtXTH26 and pH 6.5 for AtXTH14, which is quite common for XTH enzymes (Purugganan *et al.*, 1997; Campbell and Braam, 1999; Steele and Fry, 2000) and reflects the pH of the cell wall (pH 5.5–6.0). In addition to their narrow optimum, both enzymes are rather tolerant to extreme pH values with AtXTH26 showing a higher tolerance than AtXTH14. Their acid tolerance might be an advantage during the pH drop that accompanies root hair initiation (Bibikova *et al.*, 1998).

Tests on the substrate preferences of both enzymes revealed a clear preference for xyloglucan compared with all tested non-xyloglucan water-soluble β-glucans. Both enzymes showed a greater tolerance towards differences in the side-chains of the acceptor substrate oligosaccharides, but with a clear preference for xyloglucan-derived oligosaccharides and XXLGol in particular. They differ, however, in their preference towards XLLGol. Whereas XLLGol is the second-best acceptor substrate for AtXTH14, it is a rather poor substrate for AtXTH26, which seems to prefer the fucosylated form of XXLGol, i.e. XXFGol, over the di-galactosylated form, XLLGol. Whether this has a physiological relevance remains to be elucidated as little is known about the variety of xyloglucan side chains within cell walls of roots and root hairs. In a previous study, Campbell and Braam (1999) pointed towards differences in efficiency between fucosylated and non-fucosylated donor substrates. Probably the presence of fucose-residues alters the enzyme's capacity to bind the substrate and therefore the presence/absence of fucose-containing substrate can control XTH activity. It is assumed that the major natural acceptor substrate enabling XET action *in vivo* is the non-reducing terminus of the polysaccharide (xyloglucan), free xyloglucan oligosaccharides being present in the apoplast *in vivo* at relatively low concentrations (Fry, 1986; McDougall and Fry, 1991). However, nothing is known about the specific oligosaccharide repeat-units located at the non-reducing terminus of root xyloglucans, i.e. the site that acts as acceptor substrate.

As in most class I/II XTHs, a potential N-linked glycosylation site follows the catalytic site of AtXTH14 and AtXTH26. While there is one only site for AtXTH14, AtXTH26 possesses five additional possible N-linked glycosylation sites as recognized by the NXS/T amino acid sequence. Multiple glycosylation states of AtXTH26 are indeed obvious as differences in mobility on a protein gel when increasing amounts of EndoH are used. Both proteins retain 60–70% of their activity after deglycosylation. An earlier deglycosylation test on AtXTH14 reported no loss of activity (Campbell and Braam, 1999). This test was performed with PNGase F on extracts from insect cell cultures where AtXTH14 comprised only half of the total protein amount. A potential small stabilizing effect on the XTH protein by other components in the extract can therefore not be excluded since other enzymes were reported to lose XET activity totally when glycosylation was absent (Campbell and Braam, 1999).

The elucidation of the three-dimensional structure of PttXTH34 revealed certain amino acid interactions which stabilize its active enzyme conformation. These interactions are thought to be crucial for stability when the interactions between other amino acids and the N-glycan are disrupted by the removal of the N-glycan (Johansson *et al.*, 2004). AtXTH14 and AtXTH26 do not have the crucial amino acids which are conserved and interact in PttXTH34. For AtXTH14, alternative electrostatic interactions were

proposed which could account for the stabilization and positioning of the acceptor-binding loop (Kallas *et al.*, 2005). This is an arginine at the X position of the N-linked glycosylation site near the catalytic site (–DEIDFEFLG-NRTGHPYTIHTN–) and there is another arginine and a glutamic acid on the acceptor-binding loop which could form salt bridges with each other as well as with the arginine near the catalytic cleft (–YSSLWEADDWATEG-GRVKIDW–). For AtXTH26, these alternative amino acid interactions cannot account for its stability as the interacting arginine near the catalytic cleft is replaced by a non-polar alanine side chain (–DEIDFEFLGNATGQ-PYTIHTN–) and the glutamic acid of the acceptor-binding loop is replaced by an uncharged glutamine side chain (–FASLWNAEDWATQGGRVKTNW–). AtXTH26, however, possesses six possible N-linked glycosylation sites which might provoke slight differences in protein folding so that other amino acids than the ones reported in PttXTH34 could become candidates for electrostatic interactions.

In addition to the biochemical analysis of the XTH enzymes, their effect on living plants was tested by exogenous addition of purified proteins to growing *Arabidopsis* seedlings. Both enzymes have an inhibitory effect on root elongation and act very quickly as demonstrated by the kinematic analysis (see movies). AtXTH26 almost abolishes and AtXTH14 greatly impairs elongation, both of the cells in the elongation zone and of the root hairs developing from the trichoblasts. It is, however, clear that this kind of experiment takes neither the enzyme's normal localization nor its physiologically relevant concentration into account. Both enzymes are expressed in growing root hairs together with several other XTH enzymes but not in the most active part of the elongation zone of roots (Becnel *et al.*, 2006). It is therefore more likely that their inhibitory effect on elongation points to a role in strengthening of growing cell walls. This hypothesis agrees well with the down-regulation of AtXTH14 transcripts just before the onset of rapid cell elongation as seen in an expression analysis experiment on *Arabidopsis* hypocotyls (X Pelletier *et al.*, unpublished results). The idea of wall strengthening by these XTHs found confirmation in extensiometry experiments where increasing amounts of the enzyme reduced the *in vitro* extension of heat-inactivated cell walls. Related to this, a strengthening role for XET activity in the generation of tensile stress during fibre maturation in tension wood has been proposed (Mellerowicz *et al.*, 2008), and a strengthening role for a novel heterotransglucosylase activity was proposed in mature *Equisetum* stems (Fry *et al.*, 2008).

The broadening of the bases of older root hairs, seen after 2 d of treatment, cannot be explained by a role in wall strengthening. The root hair mutant *rhd1* and double mutant *xt1*/*xt2* show similar broadenings of their root hair bases (Schiefelbein and Somerville, 1990; Cavalier *et al.*, 2008). In these mutants, xyloglucan synthesis has been drastically impaired as XT1 and XT2 are xylosyl transferase isoforms and RHD1 is specifically required for the galactosylation of xyloglucan (Siefert *et al.*, 2002). It is therefore possible that the prolonged treatment with the XTH enzymes results in wall loosening due to disruption of the existing xyloglucan network in older root hairs causing the bulging base appearance. Indeed, such broadenings never occurred in the first few hours of treatment when a clear enzyme effect on general cell elongation was obvious (see movies). Why in our study and in the *xt1*/*xt2* double mutant (Cavalier *et al.*, 2008), the broadening was not seen all over the root hair length, remains unclear.

The influence of AtXTH14 on root growth and root hair morphology suggests that the same enzyme can induce wall tightening or wall loosening depending on the cell type. It probably participates in different processes having opposite effects on cell extension. XTH-induced growth may result from incremental wall extension during xyloglucan cleavage and rejoining by the enzyme (Thompson and Fry, 2001). At the same time XTH-mediated incorporation of newly secreted xyloglucans into the cell wall may reinforce the wall thus contributing in some situations to the wall tightening and growth inhibition (Antosiewicz *et al.*, 1997). In addition, slow XTH effects on growth may be caused by its involvement in cellulose synthesis and deposition (Liu *et al.*, 2007*a*; Genovesi *et al.*, 2008). It was shown before that suppression of cellulose synthesis in *Arabidopsis* hypocotyls inhibits the subsequent rapid growth of that organ (Refrégier *et al.*, 2004) and in roots, root hair base broadenings were seen in the cellulose synthase mutant plants of *procuste 1* (Singh *et al.*, 2008).

The extensiometry experiments showed that the effect of heat-inactivation on cell walls depends on the pH at which the walls are subsequently extended. In a buffer at pH 5.7 the inactivated walls were more extensible than the native walls (Fig. 7) while at the more acidic pH 4.5 heat-inactivated walls demonstrated lower extension compared to the native control. Apparently boiling in water inactivates both cell wall loosening and cell wall tightening proteins but they have different pH optima. Acidic pH could be optimal for the former so the wall extension at pH 4.5 is mostly controlled by cell wall loosening proteins. As a result, heat-inactivation leads to the drop of the wall extension at acidic pH. On the other hand, the contribution of cell wall tightening proteins in the control of wall extension might be prevailing at pH 5.7. It is their inactivation after boiling that defines the increase in the wall extension at pH 5.7 (Fig. 7).

The extensiometric data support the role for AtXTH14 and AtXTH26 in wall strengthening as these enzymes added to heat-inactivated cell walls significantly reduced their extension *in vitro* (Fig. 7). Their strengthening effect could be mediated by the incorporation of unbound xyloglucans into the existing cellulose/xyloglucan network. The fraction of these unbound xyloglucans that were secreted to the cell wall just before the moment of the epidermis freeze-killing might be very small. Due to that reason the enzyme's inhibitory effect on the extension of onion cell walls *in vitro* is relatively small compared to their effect on the growth of *Arabidopsis* roots where unbound xyloglucans are continuously secreted from live cells. It is interesting that another XTH, SkXTH1 from the lycopodiophyte *Selaginella*

kraussiana, stimulated cell wall extension *in vitro* in the same onion epidermis model that was used here (Van Sandt *et al.*, 2007*a*). Thus the mechanisms mediating XTH-induced cell wall loosening and tightening can work in the same plant tissue. It is a really intriguing topic for future research to learn what makes XTH function as a cell wall loosening factor in one situation and as a cell wall tightening factor in another situation.

Conclusion

AtXTH14 and AtXTH26 were recombinantly produced in *Pichia pastoris* and purified in an active form. Both enzymes exclusively exhibit XET activity with a clear preference towards xyloglucan as donor substrate, but with differences in their substrate specificity, pH and temperature profiles, and acceptor concentrations. Both enzymes contain N-linked sugars, the removal of which reduces the activity to 60%. AtXTH14 and AtXTH26 both have a negative effect on the extensibility of cell walls, and on the elongation of root cells. *In vivo* they inhibit the outgrowth of root hairs. It is suggested that these two particular XTH isoenzymes, normally expressed in non-expanding root tissues, act predominantly to strengthen or 'tighten' cell walls—unlike another XTH isoenzyme tested, which was shown to loosen cell walls (Van Sandt *et al.*, 2007*a*).

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