

RESEARCH PAPER

ZmXTH1, a new xyloglucan endotransglucosylase/hydrolase in maize, affects cell wall structure and composition in *Arabidopsis thaliana**

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Abstract

Xyloglucan endotransglucosylase/hydrolases (XTHs; EC 2.4.1.207 and/or EC 3.2.1.151) are enzymes involved in the modification of cell wall structure by cleaving and, often, also re-joining xyloglucan molecules in primary plant cell walls. Using a pool of antibodies raised against an enriched cell wall protein fraction, a new XTH cDNA in maize, *ZmXTH1*, has been isolated from a cDNA expression library obtained from the elongation zone of the maize root. The predicted protein has a putative N-terminal signal peptide and possesses the typical domains of this enzyme family, such as a catalytic domain that is homologous to that of *Bacillus macerans* β -glucanase, a putative N-glycosylation motif, and four cysteine residues in the central and C terminal regions of the *ZmXTH1* protein. Phylogenetic analysis of *ZmXTH1* reveals that it belongs to subgroup 4, so far only reported from Poaceae monocot species. *ZmXTH1* has been expressed in *Pichia pastoris* (a methylotrophic yeast) and the recombinant enzyme showed xyloglucan endotransglucosylase but not xyloglucan endohydrolase activity, representing the first enzyme belonging to subgroup 4 characterized in maize so far. Expression data indicate that *ZmXTH1* is expressed in elongating tissues,

modulated by culture conditions, and induced by gibberellins. Transient expression assays in onion cells reveal that *ZmXTH1* is directed to the cell wall, although weakly bound. Finally, *Arabidopsis thaliana* plants expressing *ZmXTH1* show slightly increased xyloglucan endohydrolase activity and alterations in the cell wall structure and composition.

Key words: Cell elongation, cell wall, plant transformation, XEH, XET, XTH, *Zea mays*.

Introduction

Modifications to plant primary cell walls are required for many processes such as plant cell expansion and growth, as well as other developmental processes that require cell wall degradation, such as fruit ripening and organ abscission (Rose and Bennet, 1999).

Xyloglucan is the major hemicellulose in the primary cell wall of non-Poaceae plants and the cellulose/xyloglucans network is probably the basic load-bearing framework in the wall. By contrast, xyloglucan is a minor component of the primary cell walls of Poaceae plants, such as rice and maize; as a consequence, other hemicelluloses such as glucuronoarabinoxylans are believed to

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play a pivotal role in the organization of the cellulose/hemicellulose network in these plants (Carpita and Gibeaut, 1993).

Modifications in the wall network are catalysed by several enzymes. Xyloglucan endotransglucosylase/hydrolases (XTHs) are involved in the reorganization of cell walls by catalysing the cleavage and often also re-joining of xyloglucan chains (Smith and Fry, 1991; Fry *et al.*, 1992; Nishitani and Tominaga, 1991, 1992) through the xyloglucan endotransglucosylase (XET) enzymatic activity (Thompson and Fry, 2001). Interestingly, an XTH from azuki bean has been reported having only the xyloglucan endohydrolase (XEH) activity (Tabuchi *et al.*, 2001; Rose *et al.*, 2002). XTHs are encoded by a multi-gene family (Campbell and Braam, 1999) with 33 genes identified in *Arabidopsis thaliana* (Yokoyama and Nishitani, 2000) and 25 in tomato (Saladié *et al.*, 2006). Genes thought to encode XTHs have also been identified and characterized in monocots such as wheat (Okazawa *et al.*, 1993; Liu *et al.*, 2007a), maize (Saab and Sachs, 1996), barley (Smith *et al.*, 1996; Schünmann *et al.*, 1997), and rice (Uozu *et al.*, 2000). Since xyloglucans are minor components in the primary cell walls of these species, it had been assumed that their XTH multigene families may be smaller than in dicots. However, this idea has been revised by the identification of 29 genes coding for putative XTHs in rice (Yokoyama *et al.*, 2004), at least 22 in barley (Strohmeier *et al.*, 2004), and more than 57 in wheat (Liu *et al.*, 2007a). Therefore, this finding opens the possibility of attributing a wide range of functions to Poaceae XTHs, as already proposed for dicot species.

The expression of XTH genes is regulated by abiotic factors, such as touch, darkness, cold-shock, and heat-shock (Xu *et al.*, 1995, 1996), and by several hormones, e.g. abscisic acid (Wu *et al.*, 1994), brassinosteroids (Zurek and Clouse, 1994), gibberellins, and auxins (e.g. Potter and Fry, 1993, 1994). Other XTHs are regulated by parasites (Divol *et al.*, 2007) and induced during arbuscular mycorrhizal symbiosis (Maldonado-Mendoza *et al.*, 2005).

Maize XET action has been detected *in vivo* (Vissenberg *et al.*, 2003) and XET activity measured *in vitro* (e.g. Pritchard *et al.*, 1993). At present only one putative XTH, namely *Zm1005*, has been reported in maize (Saab and Sachs, 1996); however, the protein encoded by this flooding-induced gene has not been tested for XET activity.

This work reports the isolation and characterization of a novel XTH in maize named *ZmXTH1*. This protein belongs to subgroup 4, shows all the domains typically found in this class of enzymes, and has XET activity when assayed *in vitro*. *ZmXTH1* is regulated by gibberellins, influenced by culture conditions, and weakly bound to the cell wall. Furthermore, *A. thaliana* plants expressing *ZmXTH1* have increased XEH activity and show

alterations in the structure and composition of primary cell walls.

Materials and methods

Plant material, growth conditions, and plant treatments

Zea mays W64A inbred line plants were grown in a greenhouse (for soil growth) or in a growth chamber in paper towel scrolls (for hydroponic culture) with a 16 h light (28 °C)/8 h dark (26 °C) photoperiod. *Arabidopsis thaliana* (ecotype Columbia) plants were grown under standard greenhouse conditions (25 °C day and 22 °C night with 50% humidity) with a 16/8 h photoperiod.

For gibberellin treatments, maize leaf segments were collected from 10-d-old plants grown in the greenhouse and floated on 10 ml of sterile water in Petri dishes containing 0, 5, or 50 µM of gibberellin (GA₃) (Sigma-Aldrich) as previously described (Jan *et al.*, 2004).

Isolation of ZmXTH1

Approximately 1 g of tissue from the root elongating zone of 9-d-old maize plantlets (from which the meristem and differentiation zone were removed) was homogenized with 10 ml of ice-cold extraction buffer (40 mM glycyl-glycine pH 7.5, 1% Triton X-100, 500 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine) and centrifuged at 1000 g for 10 min. The pellet was resuspended in 10 ml of 40 mM glycyl-glycine pH 7.5, centrifuged at 1000 g for 10 min, and washed once with sterile water. After resuspension in Laemmli's sample buffer (Laemmli, 1970), the pellet was boiled for 5 min and centrifuged for 10 min at 15 000 g. The resulting supernatant corresponds to the enriched cell wall protein fraction. Finally, 3 × 100 µg of this (enriched cell wall) protein fraction was injected into a rabbit for antibody production.

The construction of the λZAPII-cDNA library used for this work has already been published (Vignols *et al.*, 1999) and the immunoscreening was performed with the pool of antibodies raised against the maize enriched cell wall protein fraction, according to Sambrook *et al.* (1989). Clones giving a positive signal were excised *in vivo* (Sambrook *et al.*, 1989) and sequenced. One of them corresponded to a putative xyloglucan endotransglucosylase/hydrolase that was named *ZmXTH1*.

Genomic DNA and RNA gel blot analyses and RT-PCR

Genomic DNA was isolated from maize according to Dellaporta *et al.* (1983), digested with several restriction enzymes, fractionated on 0.8% agarose gel (10 µg per lane), and transferred to a nylon membrane.

Total RNA was extracted with Trizol Reagent according to the manufacturer's instructions (Invitrogen). Northern blot analysis (10 µg total RNA per lane) was performed according to standard procedures (Sambrook *et al.*, 1989).

Membranes were hybridized with [α -³²P]-labelled probe corresponding to the presumably full-length *ZmXTH1* cDNA or its 3' untranslated region (3' UTR) as a specific probe. Stringency washings were performed at 65 °C as follow: 5 min in SSC 2×/SDS 0.5%, 30 min in SSC 0.5×/SDS 0.1% twice, and 30 min in SSC 0.1×/SDS 0.1% twice.

Approximately 4 µg of total RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen), using an oligo(dT)15 primer and 2 µl of the first-strand cDNA used in subsequent PCR reactions. The gene-specific primers and the optimal number of cycles used to amplify *Zm1005* (Saab and Sachs, 1996), *ZmXTH1*, *ZmXTH2* (AY105107), *ZmXTH3* (AY106262), *ZmXTH4*

(AY103775), *ZmXTH5* (AY106776), and *ZmXTH6* (AY104207) were:

FwZm1005: 5'-ACCCGTCGCTTGGTTTTTGG-3'; *RvZm1005*: 5'-TACGATGCAGGTATACTGAG-3' (28 cycles)

FwXTH1: 5'-TAGCTGATCCGCCGTTTTTCG-3'; *RvXTH1*: 5'-GCTGCGTACATGCATCATCATCC-3' (35 cycles)

FwXTH2: 5'-TCCACCATCTGCCCTGC-3'; *RvXTH2*: 5'-GTTTATTCCTCAGTTTCTGC-3' (28 cycles)

FwXTH3: 5'-CATTGGTTTGTGTTATCCCC-3'; *RvXTH3*: 5'-TGGAATGGAAGGCAACAACATCTG-3' (28 cycles)

FwXTH4: 5'-AGGACTTGACGATTTGTAACG-3'; *RvXTH4*: 5'-GTATCAATCGATCTGCACGCG-3' (35 cycles)

FwXTH5: 5'-TAAAGGTGGCTGGATGAGCGA-3'; *RvXTH5*: 5'-CAGATGAAAGAATATCACTGCG-3' (28 cycles)

FwXTH6: 5'-TGATGATGATGCGTTGGGCCG-3'; *RvXTH6*: 5'-AGCTGCCGAAATTCGATAC-3' (28 cycles)

The primers used to amplify the maize actin (*BT016731*) were:

Fw-Actin: 5'-CCAAGCCAACAGAGAGAAA-3'; *Rv-Actin*: 5'-TGACCATCAGGCATCTCGTA-3' (31 cycles)

Production of recombinant ZmXTH1 protein in *Escherichia coli* and *Pichia pastoris*

The primers used to produce the recombinant ZmXTH1 protein in *E. coli* were: 5'-GCATATGACGGACGGACC-3' (upstream) and 5'-TAAGCTTCTAGCTGCGGCTGCAC-3' (downstream).

NdeI and *HindIII* restriction sites were used for the cloning in-frame into pET-28a(+) expression vector (Novagen). Heat-shock transformed *E. coli* (BL21 strain) were grown in 250 ml LB medium at 37 °C, and the production of the recombinant protein was induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside). The extraction and purification of the recombinant protein was performed according to the manufacturer's conditions (Novagen). Finally, the recombinant protein was renatured by performing a multistep dialysis in a buffer containing 20% glycerol, 500 mM NaCl, and 40 mM TRIS-HCl pH 7.4, with a progressive depletion of urea.

To produce recombinant XTH protein in *P. pastoris* (a methylotrophic yeast), the *ZmXTH1* cDNA was amplified by PCR using the following primers: 5'-AGAATTCACGGACGGACC-3' (upstream); 5'-ATCTAGACTAGCTGCGGCTGCAC-3' (downstream).

EcoRI and *XbaI* restriction sites were used for the in-frame cloning into pPICZαA expression vector (Invitrogen). *Pichia pastoris* X-33 competent cells were transformed by electroporation as described by Cregg and Russell (1998) and transformants were selected with zeocin (Invitrogen). Bioreactor batch cultivations (1.5 l) were carried out in a 2.0 l bench-top Biostat B bioreactor (Braun Biotech) under the following conditions: temperature 30 °C, pH 5.5, 1.5–2 l min⁻¹ air flow rate. The cultivation was started using buffered minimal glycerol medium, containing 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base (Difco), 1% (w/v) of glycerol, and 4×10⁻⁵% (w/v) biotin. After depletion of glycerol in the medium, methanol was added to a final concentration of 1% (w/v). After cultivation, the culture broth was centrifuged at 4000 g, filtered through a 0.45 μm membrane (Millipore), and concentrated 10-fold by ultrafiltration with a Pellicon® tangential flow ultrafiltration system (Millipore) equipped with a 10 kDa cut off membrane cassette, and subsequently dialysed against citrate buffer, pH 5.5, using the same system.

Enzymatic assays

The radiochemical enzymatic assay was performed according to Fry *et al.* (1992). Protein extracts from *A. thaliana* were prepared by homogenization with extraction buffer [300 mM succinate (Na⁺) pH

5.5, 10 mM CaCl₂, and 10% glycerol] and centrifuged at 13 000 g for 10 min at room temperature. The supernatant was then collected and proteins quantified by the Bradford micromethod (Bradford, 1976). Proteins were then incubated for 24 h (recombinant ZmXTH1 protein) or 15 min (*A. thaliana* crude protein extract) at room temperature in a final reaction mixture of 30 μl.

To test other donor and acceptor substrates, recombinant ZmXTH1 was incubated for 24 h at room temperature in a reaction mixture containing 0.17% (w/v) of the potential donor substrate, and 2 kBq of the ³H-labelled potential acceptor substrate, 0.33% (v/v) acetic acid, 0.33% (v/v) pyridine, and 0.17% (w/v) chlorobutanol; final pH 4.7. The reaction was stopped by addition of 10 μl of 90% formic acid. The reaction-product was spotted on to Whatman 3MM paper and chromatographed in ethyl acetate/acetic acid/water (10:5:6, by vol.) for 24 h. Once dried, the incorporated ³H was assayed by scintillation counting.

A total of 200 reactions was performed, resulting from the combination of different potential donors and acceptors (see S1 in the supplementary data at JXB online).

The XEH activity was measured by monitoring the change in viscosimetric flow time of a xyloglucan solution at 26 °C. Reaction mixtures (300 μl) contained 0.7% tamarind xyloglucan polymer, 50 mM sodium succinate pH 5.5, 5 mM sodium thiosulphate, 5 mM CaCl₂ (Fry, 1998). At each time point, the flow-time was measured three times for each sample.

Production of ZmXTH1 antibodies and western blot analysis

Four 250 μg portions of affinity-purified ZmXTH1 protein were injected into a chicken and immunoglobulins Y were recovered from egg-yolk according to Della Mea *et al.* (2004). Antibodies were aliquoted and stored at -80 °C and later immuno-affinity purified by use of HiTrap NHS-activated affinity columns (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Fresh tissues (leaf and root) were ground with extraction buffer (50 mM TRIS-HCl pH 8, 10 mM NaCl, 1% SDS, 2 mM DTT). The homogenate was centrifuged at 12 000 g for 15 min at 4 °C and the supernatant was stored at -20 °C. Proteins were then separated by 12.5% SDS-polyacrylamide gel electrophoresis. After protein migration, the gel was blotted to a nitrocellulose membrane that was incubated with ZmXTH1 antibodies (1:500 dilution) according to Sambrook *et al.* (1989). Proteins were finally detected by chemiluminescence (Jackson ImmunoResearch).

Isolation of maize apoplastic proteins

Apoplastic proteins were extracted from 3-d-old maize roots using non-disruptive methods based on vacuum infiltration of intact tissues and centrifugation at low speed (Boudart *et al.*, 2005; Zhu *et al.*, 2006). All steps were performed at 4 °C. Segments (1 cm long) from the maize root elongation zone were briefly washed in 10 mM phosphate buffer, pH 6, incubated for 5 min with 200 mM KCl, 50 mM MgCl₂, or 300 mM mannitol in the presence of a cocktail of protease inhibitors, and vacuum infiltrated for 10 min. Samples were centrifuged at 1000 g for 5 min and the apoplastic proteins were recovered from the supernatant and stored at -20 °C. The remaining roots were lightly blotted dry and homogenized in the same tube for protein extraction as described previously.

Sequence alignment and phylogenetic tree

XTH sequences were aligned using the ClustalW program (<http://www.ebi.ac.uk/clustalw/>; Higgins *et al.*, 1994). Poorly aligned positions and divergent regions were eliminated by using Gblocks 0.91b, following the given options for less stringency (Castresana, 2000). To construct the phylogenetic tree, the PHYML

(PHYlogenies by Maximum Likelihood) program (version 2.4.4) was used (Guindon and Gascuel, 2003), with the JTT model of amino acid substitution and four rate categories in the gamma distribution. The gamma distribution parameter and the proportion of invariable sites were estimated by the program.

35S::ZmXTH1–GFP expression constructs, transient onion transformation, and plasmolysis

The full *ZmXTH1* coding sequence (*ZmXTH1*Full), the same without the signal peptide (*ZmXTH1*Int), and only the *ZmXTH1* signal peptide (*ZmXTH1*sp) were cloned in pCAMBIA1302 vector under the control of a CaMV 35S promoter and fused in the 3' region with the GFP. Onion cells were bombarded at 900 psi with 5 µg of DNA plasmids for expression of the fusion, or GFP alone as a control using a biolistic PSD-1000/He particle delivery system (Bio-Rad, Hercules, CA, USA). After particle bombardment, the samples were incubated for 24–60 h at 25 °C in the dark under two different conditions: Murashige–Skoog medium (pH 5.8) or 20 mM piperazine-*N,N'*-bis (2-ethane-sulphonic acid) (PIPES)–KOH (pH 7.0). Confocal laser scanning microscopy was performed with a Leica TCS SP microscope (Heidelberg, Germany) fitted with spectrophotometers for emission band wavelength selection. When indicated, cells were plasmolysed in 0.8 M mannitol for 15 min.

35S::ZmXTH1 expression construct and A. thaliana transformation

The cDNA sequence of *ZmXTH1* was placed in the sense orientation between the double CaMV 35S promoter sequence and the pA35S transcription terminator using the vector described by Reichel *et al.* (1996). This construct was cloned into the pCAMBIA1300 vector and transferred into *A. tumefaciens* (C58C1 strain) by heat shock. *Arabidopsis thaliana* plants were transformed by floral dip (Clough and Bent, 1998).

Transmission electron microscopy

Hand-cut transverse sections of the basal region of stems of 5-week-old wild-type (Col 0) and 35S::*ZmXTH1* *A. thaliana* plants were processed for transmission electron microscopy as previously described (Day *et al.*, 2005). Wall polysaccharides of ultra-thin transverse sections (50 nm) were PATAg (periodic acid–thio carbohydrazide silver proteinate) stained, according to the method adjusted by Ruel *et al.* (1977). Observations were performed at 80 kV with a Philips CM 200 cryo-electron microscope.

Cell wall polysaccharide determination

Dried plant material (1 g) was added to Poly-Prep tubes and extracted with 10 vols of 70% EtOH for 5 d at room temperature with wheel-shaking, then washed six times with 70% EtOH, six times with acetone, and air-dried to obtain the alcohol-insoluble residue. The alcohol-insoluble residue was then de-starched, treated with acidified phenol, and washed with organic solvents to obtain the cell wall residue as described by Encina *et al.* (2002).

Neutral sugar analysis was performed as described by Albersheim *et al.* (1967). Dried cell walls were hydrolysed with 2 M TFA (trifluoroacetic acid) for 1 h at 121 °C and the resulting sugars were derivatized to alditol acetates and analysed by gas chromatography on a Supelco SP-2330 column. Uronic acid contents were determined by the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973), with galacturonic acid as a standard. Cellulose was quantified in crude cell walls by the Updegraff method (Updegraff, 1969) with the hydrolytic conditions described by

Saeman *et al.* (1963) and quantification of the glucose released by the anthrone method (Dische, 1962) with glucose as a standard.

Results

ZmXTH1 belongs to the subgroup 4 of the xyloglucan endotransglucosylase/hydrolase family: structure and comparison

The immuno-screening of a maize root elongation zone cDNA expression library allowed the isolation of several cDNA clones coding for different types of cell wall proteins. Among them was one corresponding to a new XTH enzyme. The phylogenetic analyses performed with full-length XTH from dicot and monocot species indicate that *ZmXTH1* belongs to subgroup 4, which comprises only acidic XTHs from Poaceae species (Fig. 1).

In addition to the already described *Zm1005* (Saab and Sachs, 1996) belonging to subgroup 2, six other maize XTHs have been identified in EST databases. Three of them (*ZmXTH2*, *ZmXTH3*, and *ZmXTH7*) belong to subgroup 4, two (*ZmXTH4* and *ZmXTH5*) belong to subgroup 2, whilst *ZmXTH6* (corresponding to AY104207; Zhu *et al.*, 2006) belongs to subgroup 1.

ZmXTH1 has a putative N-terminal signal peptide, a catalytic domain homologous to that of *B. macerans* β-glucanase (Borris *et al.*, 1990), a putative *N*-glycosylation motif (NASG) immediately following the catalytic domain, and four cysteine residues in the central and C terminal regions (Fig. 2). In addition, the PFAM (Protein Families databases of Alignments and hidden Markov models) prediction program indicates that *ZmXTH1* has a central domain typically attributed to family 16 of the glycosyl-hydrolase enzymes and a C-terminal domain typically associated with XTH enzymes.

Interestingly, all the maize XTHs belonging to subgroup 4 present two amino acid substitutions within the catalytic domain: one phenylalanine and the leucine (with respect to the *Bacillus* domain) are replaced by two methionines (Fig. 2). In addition, the first of the two strongly conserved tyrosines required for substrate recognition is replaced by a histidine and, of the three amino acids involved in the binding to the xyloglucan substrate, only the tryptophan is conserved (Fig. 2).

ZmXTH1 has XET activity when expressed in Pichia pastoris

The cDNA coding for *ZmXTH1* was cloned and expressed in *P. pastoris* and *E. coli* (Fig. 3). The XET activity detected in concentrated, cleared culture supernatants from *P. pastoris* expressing *ZmXTH1* was very low and no XET activity was detected when *ZmXTH1* was produced in *E. coli*, although the presence of the recombinant XTH1 protein was confirmed by western blot analysis (Fig. 3B).

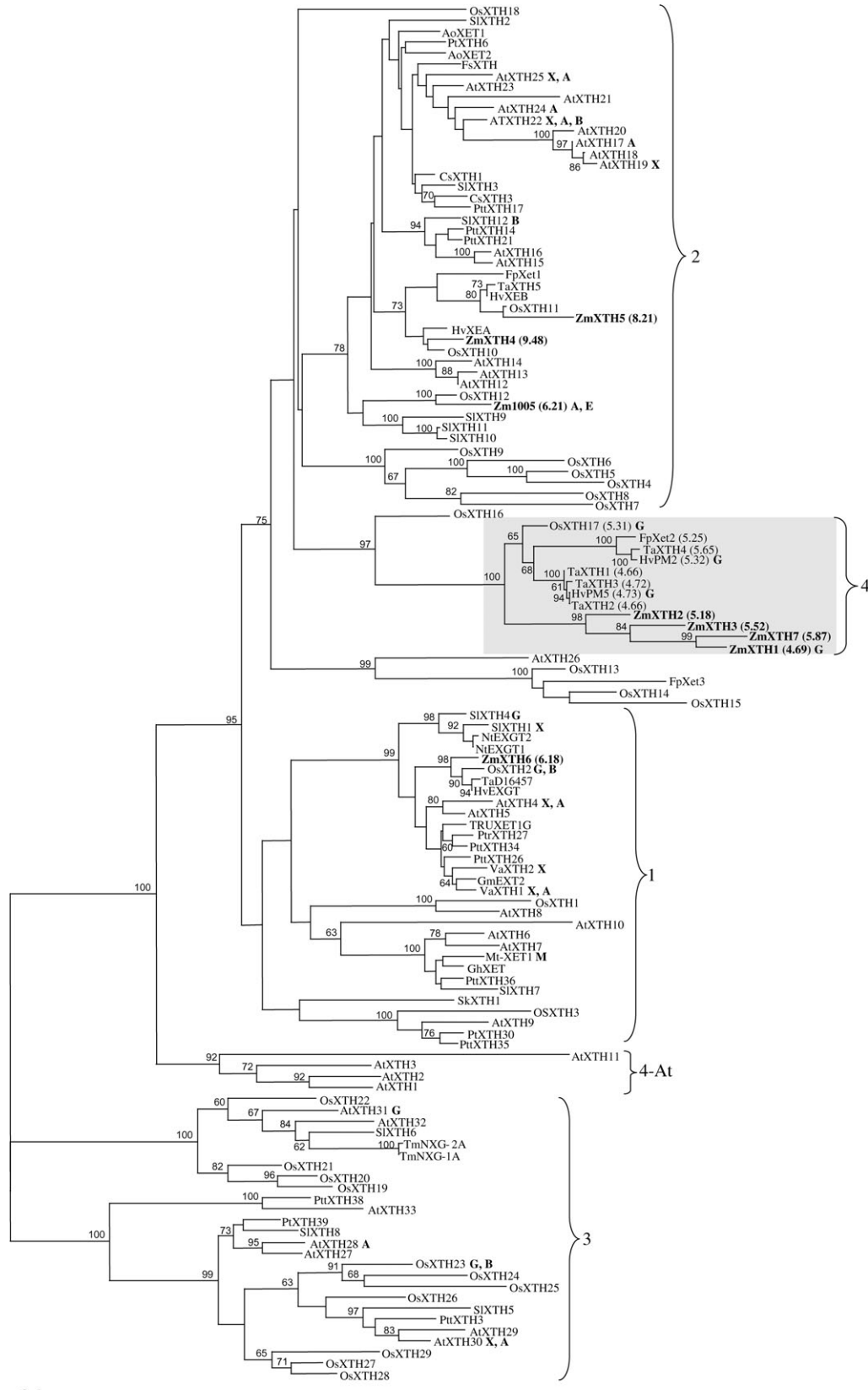


Fig. 1. Phylogenetic tree of ZmXTH1 and other XTH proteins from dicot and monocot species, and *Selaginella kraussiana*. Numbers within the phylogenetic tree refer to branches supported at a bootstrap proportion $\geq 60\%$. 4-At subgroup refers to Saladié *et al.* (2006). Numbers in brackets refer to the isoelectric point of the proteins. XTHs regulated by gibberellins, G; brassinosteroids, B; ethylene, E; abiotic stresses, A; auxins, X; induced in mycorrhizae, M. Accession numbers are given in S2 in the Supplementary data at JXB online.

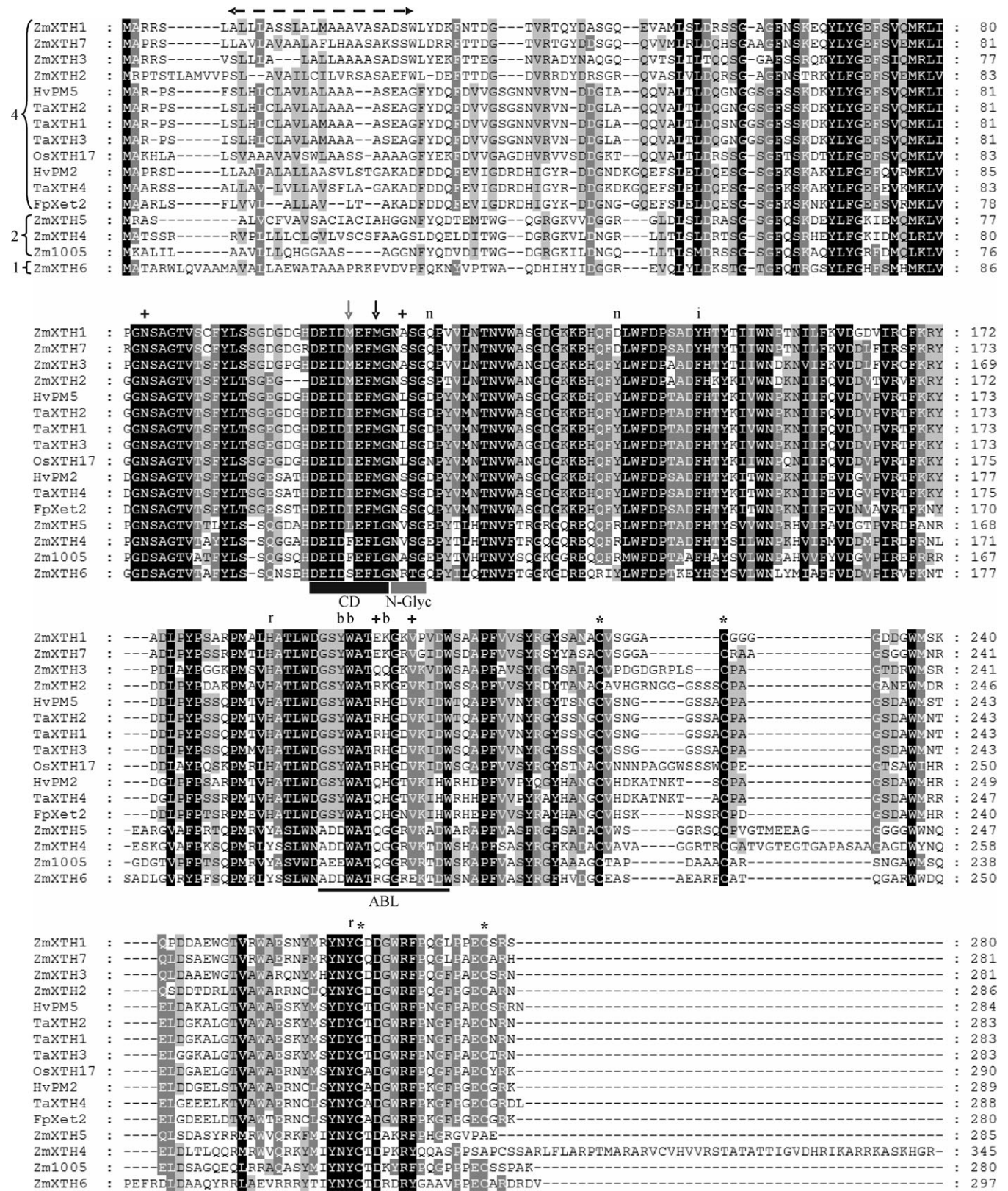


Fig. 2. Protein sequence alignment of ZmXTH1 with other monocot XTHs belonging to subgroup 4 and other maize XTH enzymes. Dark boxes refer to amino acid identity, grey boxes to amino acid homology. The signal peptide of ZmXTH1 is marked by a broken arrow. The thick black bar shows the XTH catalytic domain (CD). The methionine residues of the CD are indicated by a black and a grey arrow. The putative N-glycosylation domain (N-Glyc) is indicated by a grey bar. The thin bar shows the acceptor binding loop (ABL). 'i' refers to the amino acid residue possibly

Over 200 other potential donor/acceptor-substrate permutations (see Materials and methods) were also tested. No permutation revealed transglycosylation more rapidly than in the standard one (tamarind xyloglucan + [³H]XXXGol; data not shown). For the first time, a novel potential donor substrate relevant to poalean xyloglucan, namely water-soluble cellulose acetate, was included. This polysaccharide may model specific domains of poalean xyloglucans since these possess *O*-acetyl groups in place of some of the α -D-xylose residues at position 6 of the β -glucan backbone (Gibeaut *et al.*, 2005). However, water-soluble cellulose acetate was also a poor substrate for *ZmXTH1* (data not shown). These results suggest that XET activity is the only endotransglycosylase activity shown by *ZmXTH1*.

Another enzymatic activity described for some XTHs is the xyloglucan endohydrolase (XEH). Cleared culture supernatants from *P. pastoris* expressing *ZmXTH1* were tested for depolymerizing xyloglucan activity in a viscometric assay. The results obtained indicated that *ZmXTH1* does not present XEH activity when expressed in *P. pastoris* (data not shown).

The maize genome contains at least three XTH genes belonging to subgroup 4

It is well reported that *XTHs* constitute multigene families. However, the precise number of *XTH* genes in maize is still unknown. Southern blot analysis was performed to identify whether other *XTHs* belonging to subgroup 4 exist in the maize genome. Results (Fig. 4A) agree with EST databases and suggest the presence of at least two other genes encoding *ZmXTH1*-related proteins. Finally, the 3'-UTR region of the *ZmXTH1* cDNA was shown to be a specific probe for expression studies of the *ZmXTH1* gene (Fig. 4B).

Expression analysis of the maize XTH genes

In addition to *ZmXTH1* and *Zm1005*, six other *XTH* genes were identified in maize EST databases (Fig. 2). Therefore, a time-course of the expression pattern of these *XTHs* was analysed in maize plants (Fig. 5A).

ZmXTH6 mRNA is detected in both leaves and roots, although its expression in leaves decreases in 20-d-old plants. *Zm1005*, *ZmXTH4*, and *ZmXTH5* are expressed preferentially in leaves. Indeed, their expression is complementary as *ZmXTH5* is only detected in 3-d-old plants, *ZmXTH4* in 3- and 10-d-old plants, and *Zm1005* in 10- and 20-d-old plants.

With respect to the maize *XTH* genes belonging to subgroup 4, *ZmXTH1* is preferentially expressed in roots,

whereas in leaves its mRNA accumulates mainly at 10 d. The expression of *ZmXTH2* is predominant in roots, while in leaves its mRNA accumulation is limited to 3-d-old plants. *ZmXTH3* is detected in all stages analysed, in both leaves and roots, while several attempts to amplify *ZmXTH7* were unsuccessful.

The expression pattern of *ZmXTH1* was also analysed by northern and western blots (Fig. 5B). In accordance with the RT-PCR data, *ZmXTH1* is preferentially expressed in roots, whereas in leaves its mRNA accumulates mainly at 10 d. These findings were confirmed also at the protein level.

To study the expression pattern of *ZmXTH1* better, northern blot analyses using different parts of 6-d-old hydroponically grown seedlings were performed. Although *ZmXTH1* mRNA is detected in all the aerial regions of the plant, it preferentially accumulates in leaves while in the roots *ZmXTH1* transcript is only detected in the 'young root' region (10–20 mm from root apex; Fig. 6A).

Since the expression of *XTH* genes is influenced by several factors, experiments were performed to assess whether *ZmXTH1* is regulated by different stresses (salt, osmotic, wounding, and MeJa). The results indicate that *ZmXTH1* gene expression is not significantly regulated by these stresses (data not shown). By contrast, *ZmXTH1* gene expression is regulated by gibberellins, as it happens for other *XTHs* belonging to subgroup 4 (Jan *et al.*, 2004) (Fig. 6B). However, although *ZmXTH2* and *ZmXTH3* also belong to subgroup 4, their gene expression is not regulated by gibberellins (Fig. 6C).

In addition, to investigate better whether the different culture conditions influence the expression of *ZmXTH1*, a time-course of the leaf-to-root biomass ratio of hydroponic versus soil conditions was performed. Soil-grown plants show a general reduction of their total leaf-to-root biomass ratio compared with plants grown in paper scrolls (Fig. 6D). *ZmXTH1* mRNA accumulates predominantly in roots of soil-grown plants, while in hydroponic conditions it mainly accumulates in leaves (Fig. 6D). This result is in line with the previously observed leaf-to-root biomass ratio of hydroponic versus soil-grown plants.

ZmXTH1 is detected in apoplastic fluids at low salt concentration and is weakly bound to the cell wall

In previous western blot analyses (Fig. 5B), *ZmXTH1* was detected in soluble cellular fractions, suggesting that it is weakly bound to the cell wall. To investigate this hypothesis further, non-disruptive methods at low salt concentration (KCl and MgCl₂) to extract loosely wall-bound

interacting with the catalytic site; '+' refers to amino acids possibly involved in the formation of salt bridges (*ZmXTH6*); 'n' refers to amino acids possibly interacting with the *N*-glycan; 'r' and 'b' represents amino acid residues possibly involved in the recognition and binding of the xyloglucan substrates, respectively. Asterisks refer to the four conserved cysteine residues. Numbers on the left side of the figure refer to the subgroup of each protein.

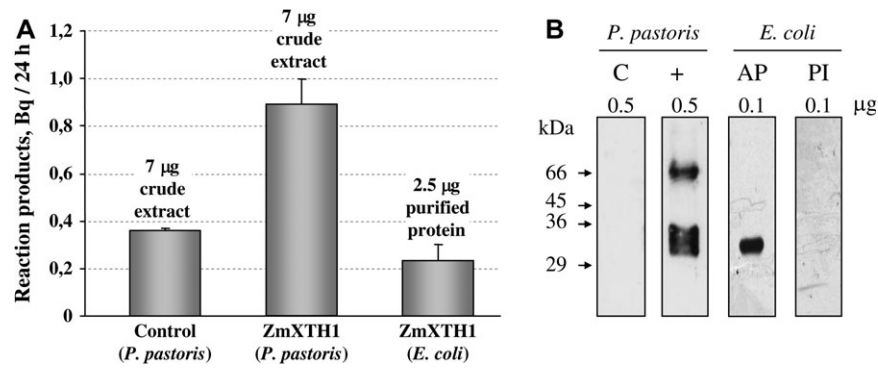


Fig. 3. (A) *In vitro* XET assay of ZmXTH1 produced in *P. pastoris* and *E. coli*. Each value represents the mean of three independent assays \pm SD. (B) Immuno-detection of ZmXTH1. C, Crude protein extract from a control strain of *P. pastoris*; +, crude protein extract from a *P. pastoris* strain expressing ZmXTH1; AP, affinity-purified ZmXTH1 produced in *E. coli*; PI, affinity-purified ZmXTH1 incubated with the pre-immune serum.

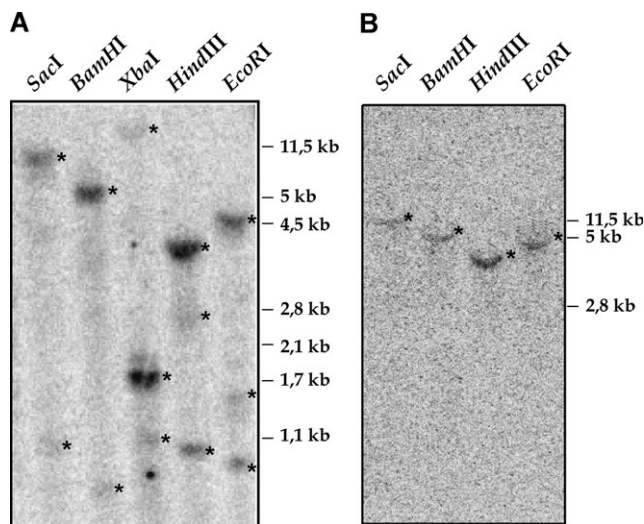


Fig. 4. Maize genomic DNA Southern blot analysis: (A) 10 µg of maize genomic DNA were hybridized with the presumably full-length ZmXTH1 cDNA; (B) 10 µg of genomic maize DNA were hybridized with the 3' UTR of the ZmXTH1 cDNA. Markers are shown on the right side of the figures. Asterisks indicate the position of the bands.

proteins in the apoplastic fluids were used. The western blot analyses indicate that, at least in part, ZmXTH1 can be detected in these fractions and also in apoplastic fluids obtained by vacuum infiltration in 300 mM mannitol without salt, which is expected to contain acidic proteins with no ionic interaction with the cell wall (Fig. 6E). These findings reinforce the idea of a very weak or no interaction of ZmXTH1 with cell wall components.

To elucidate the subcellular localization of ZmXTH1, three different constructs fused to GFP (ZmXTH1Full-GFP, ZmXTH1sp-GFP, and ZmXTH1Int-GFP) (Fig. 7A) were produced to transform onion cells. After 24 h of incubation of transformed onion cells under routine experimental conditions (pH 5.8), ZmXTH1 is detected in the secretory pathway (Fig. 7B, panel 1). In fact, projections of continuous sections of the onion cells by

confocal laser scanning microscopy show that the GFP fluorescence is found in structures similar to the cortical network of endoplasmic reticulum. Similar results are obtained when the ZmXTH1 signal peptide is fused to GFP (Fig. 7B, panel 4). This result confirms the role of this signal peptide for the correct entry of ZmXTH1 in the secretory pathway. In line with these results, the truncated ZmXTH1 that lacks the signal peptide is not localized in this secretory pathway (Fig. 7B, panel 9) and has a similar behaviour to GFP control (panel 10). However, both the full ZmXTH1 and the signal peptide constructs are not detected in the wall of plasmolysed cells (Fig. 7B, panels 2 and 5) analogously to the truncated ZmXTH1 protein and GFP control (data not shown).

As GFP fluorescence is pH-dependent and can decrease at low pH (Scott *et al.*, 1999; Chen *et al.*, 2007), experiments were repeated at pH 7. Under these conditions, and after 24 h of incubation, the full ZmXTH1 protein and the signal peptide were detected, both in the protoplast and in cell walls of plasmolysed cells (Fig. 7B, panels 3 and 6) and, after 60 h of incubation, nearly all the GFP fluorescence is detected in cell walls (Fig. 7B, panel 7). Moreover, after plasmolysis the GFP fluorescence expands throughout the apoplastic region (Fig. 7B, panel 8), suggesting that ZmXTH1 is a weakly cell wall-bound protein.

Arabidopsis plants expressing ZmXTH1 have increased XEH activity and show alterations in cell wall structure and composition

The ZmXTH1 cDNA was placed under the control of the 2X35S CaMV gene promoter and *A. thaliana* transgenic plants were generated. The expression of ZmXTH1 was confirmed by northern and western blot analyses (Fig. 8A) and the XET and XEH activities were measured. Although several transgenic lines were analysed, the results indicate that these plants do not display an increase in total XET activity (Fig. 8B), while a small increase in total XEH activity was detected (Fig. 8C).

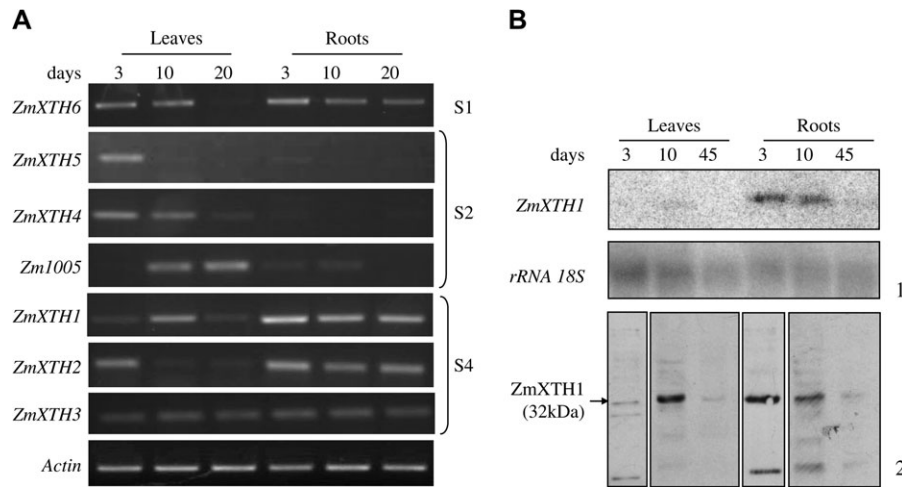


Fig. 5. (A) Time-course of maize *XTH* gene expression. S1, S2, and S4 refer to *XTH* subgroups. Plants were harvested at 3, 10, and 20 d and transcript accumulation was analysed by RT-PCR. (B) 1, Time-course of *ZmXTH1* gene expression in maize leaves. Maize plants grown in soil were harvested at 3, 10, and 45 d and transcript accumulation was analysed by northern blot. The 18S rRNA was used as loading control. 2, Immuno-detection of *ZmXTH1*. 30 µg of proteins from roots and leaves of the same plants were analysed by western blot.

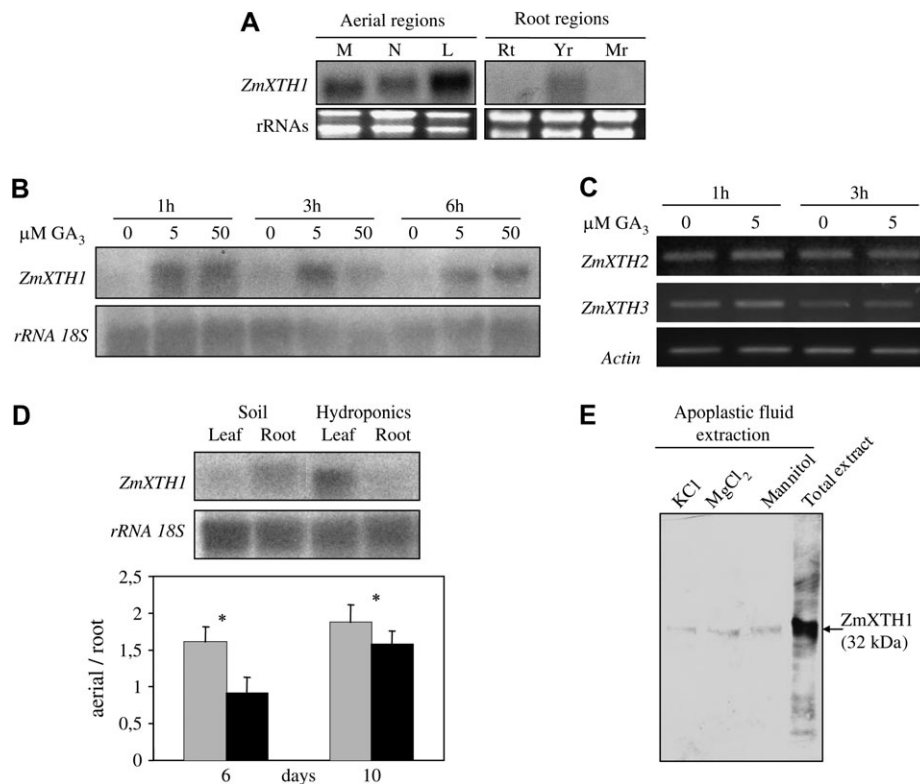


Fig. 6. (A) Northern blot analysis of *ZmXTH1* expression in different maize plant tissues. M, mesocotyl; N, coleoptilar node; L, leaves; Rt, root tip (5 mm of the root apex including the root cap); Yr, young root (10–20 mm from root apex); Mr, mature root (30–40 mm from root apex) from 6-d-old plantlets. Ethidium bromide staining of rRNAs was used as loading control. (B) Effect of gibberellins on *ZmXTH1* expression analysed by northern blot. Leaf segments of 10-d-old maize plants grown in soil were incubated with 5 µM or 50 µM of GA₃ for 1, 3, or 6 h. The 18S rRNA was used as loading control. (C) Effect of gibberellins on *ZmXTH2* and *ZmXTH3* expression analysed by RT-PCR. Leaf segments of 10-d-old maize plants grown in soil and incubated with 5 µM of GA₃ for 1 h and 3 h. (D) *ZmXTH1* expression in different culture conditions. Northern blot analysis was carried out on leaves and roots of the 10-d-old maize plantlets grown in soil or hydroponically. The 18S rRNA was used as loading control. Comparison of the aerial to root biomass ratio of hydroponics (grey columns) versus soil (black columns) growth conditions: maize plantlets were grown for 10 d and total fresh weight of the aerial and root parts was determined at 6 d and 10 d. Samples constituted seven plants each and data are expressed as mean values ±SD. *, *P* < 0.05. (E) Determination of *ZmXTH1* by western blot analysis in different apoplastic fluids. Total extract refers to the proteins extracted from maize roots that were not removed with mannitol.

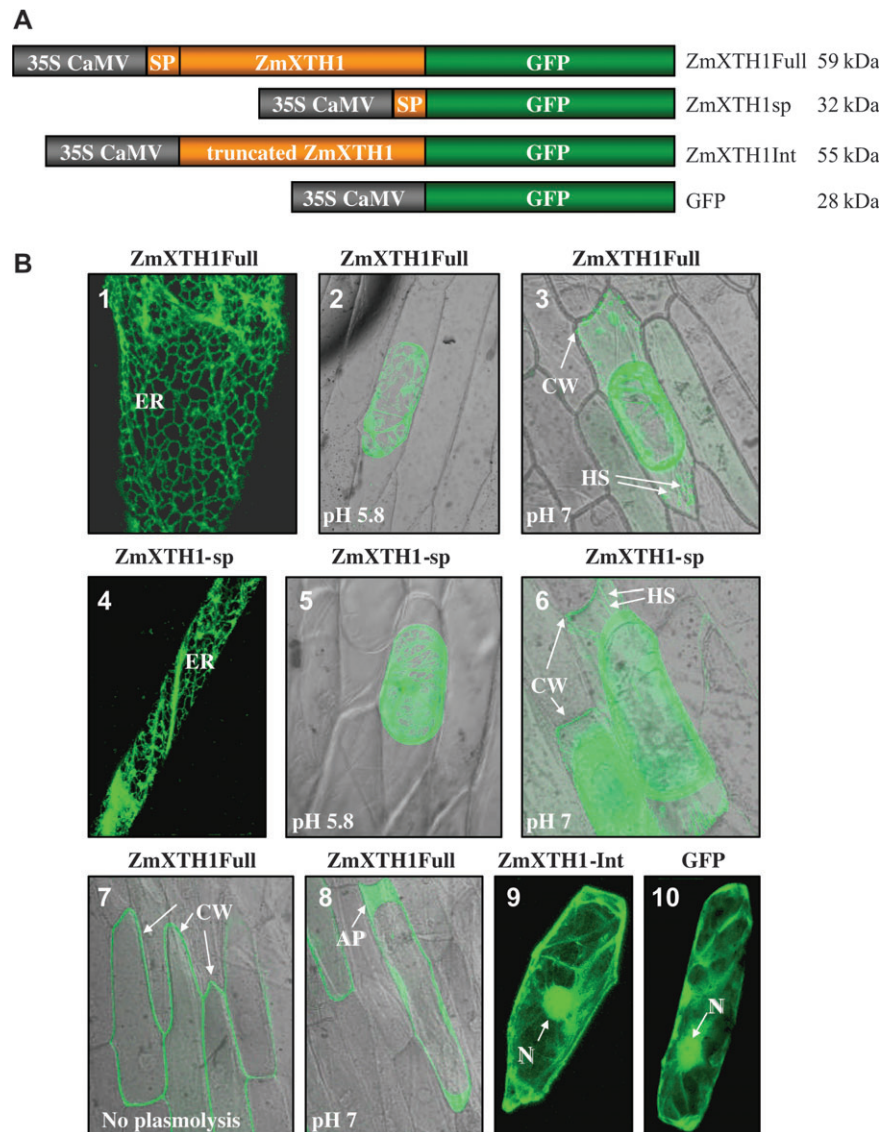


Fig. 7. Subcellular localization of ZmXTH1 by transient expression in onion cells. (A) Schematic representation of ZmXTH1 constructs fused to GFP. SP, Signal peptide. (B) Panels 1, 4, 9, and 10, projections of continuous sections of the onion cells by confocal laser scanning microscopy; panels 2, 3, 5, 6, and 8, transmission and fluorescence images of plasmolysed cells; panel 7, transmission and fluorescence images from a single optical section of non-plasmolysed cells. ER, Endoplasmic reticulum; CW, cell wall; HS, Hechtian strands; AP, apoplast; N, nucleus.

At a macroscopic level, the transgenic plants do not show significant alterations in plant height and development with respect to the wild-type plants. Nevertheless, the results obtained analysing these plants by transmission electron microscopy reveal that ZmXTH1 affects *A. thaliana* cell walls (Fig. 9A). The middle lamella region between parenchyma cells appears wider and less compact, thus resulting in a widening of the double wall between cells and the collapse of the walls.

The overall monosaccharide composition of the cell walls of the *Arabidopsis* stems was determined. The monosaccharide yield hardly varied between wild-type and transgenic cell walls (Fig. 9B). Matrix polysacchar-

ides were mainly composed of xylose and uronic acids and no significant differences in cell wall sugar composition were found between wild-type and transgenic plants. Interestingly, transgenic cell walls showed 20% less cellulose than wild-type.

Discussion

This paper reports the isolation and characterization of ZmXTH1. The precise number of *XTH* genes in maize is still unknown; however, based on EST databases six other *XTH*s were identified (ZmXTH2–7), in addition to ZmXTH1 and Zm1005 (Saab and Sachs, 1996).

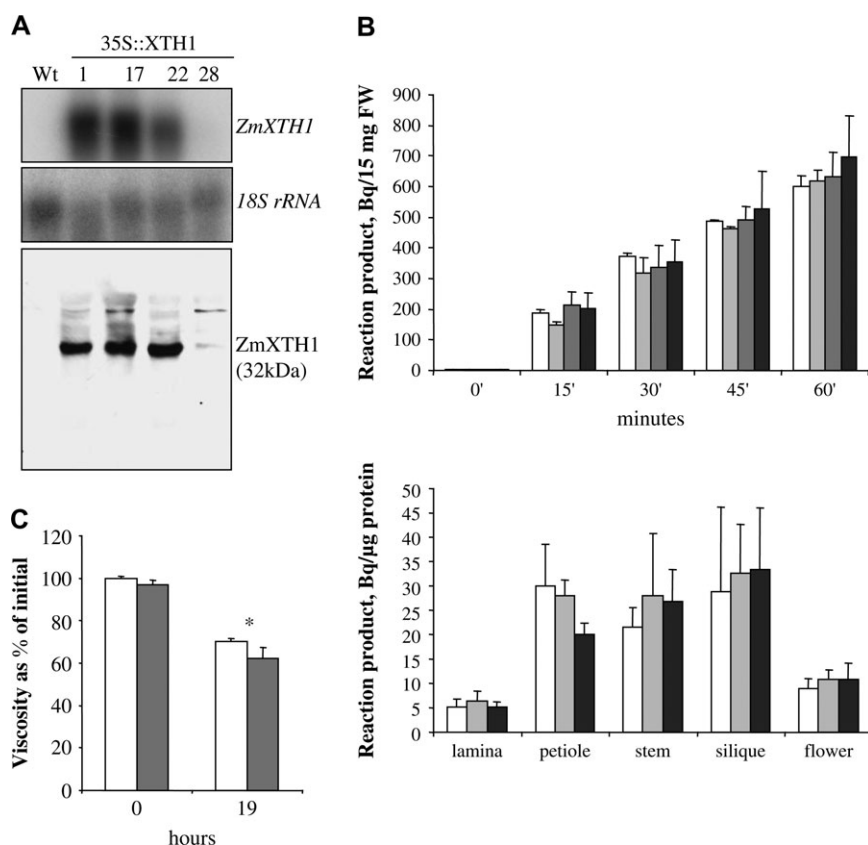


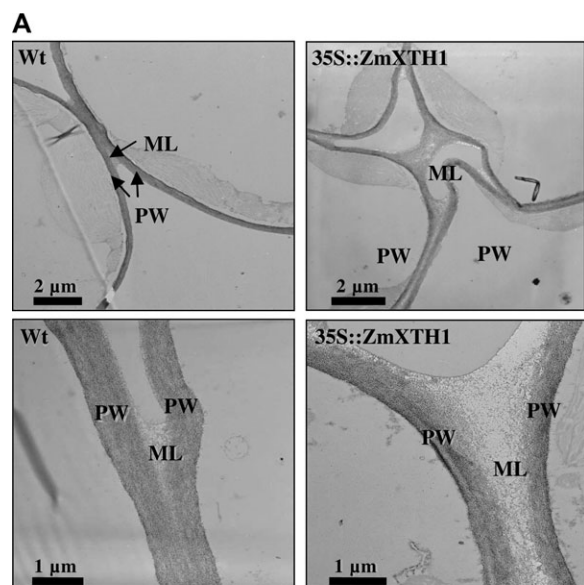
Fig. 8. Expression of *ZmXTH1* in *A. thaliana*. (A) Northern and western blot analyses of wild-type (Wt) and several transgenic lines (1, 17, 22, and 28). (B) *In vitro* XET assays. Top histogram: kinetic XET assay. White columns correspond to *A. thaliana* wild type; light grey, dark grey, and black columns refer to the *A. thaliana* transgenic lines 1, 17, and 22, respectively. Bottom histogram: XET assays performed on extracts of different plant regions; white columns correspond to *A. thaliana* wild-type; grey and black columns refer to the *A. thaliana* transgenic lines 17 and 22, respectively. Each assay was repeated three times and the results are expressed as the mean value \pm SD. (C) *In vitro* XEH assay. White columns correspond to *A. thaliana* wild-type, while the grey columns refer to the *A. thaliana* transgenic line 17. Each assay was repeated five times and results are expressed as the mean value \pm SD. The asterisk indicates significant differences by Student's *t*-test ($P < 0.05$).

The phylogenetic analysis indicates that ZmXTH1, ZmXTH2, ZmXTH3, and ZmXTH7 belong to subgroup 4, a subgroup that contains only acidic Poaceae XTHs. All the maize proteins belonging to this subgroup have two substitutions within the catalytic domain; the presence of two methionines replacing a phenylalanine and a leucine, respectively. Modifications in these two amino acids have already been described for other XTH proteins belonging to subgroup 4, but in all these cases, the phenylalanine is replaced by an isoleucine (Jan *et al.*, 2004; Liu *et al.*, 2007a). Therefore, the substitution of the phenylalanine by a methionine is a typical feature of subgroup 4 maize XTHs.

In addition to the modifications within the catalytic domain, the maize proteins also have other substitutions in amino acids involved either in recognition and binding of the substrate (Kallas *et al.*, 2005; Van Sandt *et al.*, 2006). Finally, none of the maize XTHs except ZmXTH6 contains the amino acids proposed to play a role in the stabilization of the protein through salt bridges (Kallas *et al.*, 2005; Van Sandt *et al.*, 2006).

On the other hand, Zm1005, ZmXTH4, and ZmXTH5 belong to subgroup 2 while ZmXTH6 belongs to subgroup 1. Taking into account that rice, barley, *A. thaliana*, and tomato have 29, 22, 33, and 25 XTH enzymes, respectively, the existence of other XTHs in maize can be proposed.

Previous studies have shown that some *A. thaliana* XTH display tissue-specific expression profiles indicative of a possible evolutionary specialization (Vissenberg *et al.*, 2005). Furthermore, XTH enzymes are thought to have different transferase and hydrolase activities and their combinatorial effect is critical for a broad spectrum of plant developmental processes (Rose *et al.*, 2002). The present observations about the expression of *ZmXTH4*, *ZmXTH5*, and *Zm1005* are in line with these observations and led to the hypothesis that these maize genes could act at different developmental stages. In this regard, the maize XTHs belonging to subgroup 4, *ZmXTH1* and *ZmXTH2* are preferentially expressed in roots, whereas *ZmXTH3* is systemically expressed, thus indicating little or no functional redundancy.



B

	μg/mgDW	
	Wt	35S::ZmXTH1
Rhamnose	2.9 ± 0.9	2.3 ± 0.8
Fucose	0.2 ± 0.1	0.2 ± 0.1
Arabinose	3.9 ± 1.3	4.1 ± 0.6
Xylose	274.5 ± 3.5	274.2 ± 2.7
Mannose	2.2 ± 0.5	2.2 ± 0.7
Galactose	3.0 ± 0.8	3.3 ± 0.6
Glucose	4.4 ± 1.5	4.5 ± 1.3
Uronic acids	171.6 ± 4.6	179.1 ± 23.5
Cellulose *	441.6 ± 20.5	348.4 ± 15.3

Fig. 9. (A) Transmission electron microscopy of stalks of wild-type and *35S::ZmXTH1 A. thaliana* plants (line 17). ML, Middle lamella; PW, primary wall. (B) Sugar analyses of cell walls from stems of wild-type and *35S::ZmXTH1 A. thaliana* plants. Data were expressed as mean value of six independent assays ±SD. The asterisk indicates significant differences by Student's *t*-test ($P < 0.05$).

The enzymatic assays indicated that ZmXTH1 possesses XET activity when expressed in *P. pastoris* in an extremely sensitive radiochemical assay (Fry *et al.*, 1992) and xyloglucan is the only substrate used by ZmXTH1, as no endotransglycosylase activity was detected in enzymatic assays with different acceptor and donor substrates. On the other hand, the absence of XET activity in the affinity-purified ZmXTH1 expressed in *E. coli* could be due to the fact that this prokaryotic system does not produce glycosylated proteins that could be critical for the enzymatic activity and/or to an incorrect folding of the purified protein, as it has to be purified under denaturing conditions.

ZmXTH1 is mainly expressed in tissues characterized by active cell expansion, in agreement with a possible role of this enzyme in the cell wall loosening processes. Previous works reported that XTHs of subgroup 4 are regulated by gibberellins (Jan *et al.*, 2004) but only *ZmXTH1* is

regulated by this hormone (but not *ZmXTH2* and *ZmXTH3*), suggesting once more a role in cell wall modifications during plant growth.

In addition, *ZmXTH1* gene expression varies according to the culture conditions. Soil-grown plant roots have a higher *ZmXTH1* gene expression level than plant roots grown in paper scrolls. These results can be related to previous studies that describe an increase of XET activity in maize roots grown in water-limited conditions (Wu *et al.*, 1994). Soil-grown maize plants showed a significant reduction in the leaf-to-root biomass ratio compared with hydroponically grown plantlets. This can be explained by the reduced oxygen availability, which reduces root growth in paper scrolls compared with soil (Drew, 1997; Verslues *et al.*, 1998).

Cell wall proteins have been classified in different groups according to their interactions with the cell wall, showing that weakly wall-bound proteins have acidic isoelectric points (Jamet *et al.*, 2006). In that context, the acidic ZmXTH1 was also detected in apoplastic fractions obtained with mannitol without salt and in apoplastic fractions with low salt concentration, suggesting that this enzyme is weakly bound to the cell wall.

ZmXTH1 was detected both in the secretory pathway and the cell wall. The same localization pattern is observed when only the ZmXTH1 signal peptide is fused to GFP, indicating that the signal peptide is functional and directs the protein to the secretory pathway. Although the functionality of a XTH signal peptide for directing the protein to the secretory pathway has been observed (Yokoyama and Nishitani, 2001), as far as is known this is the first work in which GFP fluorescence is detected in cell walls using an XTH signal peptide fused to GFP. The fact that GFP fluorescence expands through the apoplastic region when transformed onion cells are plasmolysed suggests once more that ZmXTH1 is weakly associated with the cell wall.

Arabidopsis thaliana transgenic plants present slightly increased levels of hydrolase activity and this effect could be caused directly by ZmXTH1 or indirectly by the induction of the endogenous hydrolases and/or endo-β-1-4-glucanases. Although ZmXTH1 shows XET activity in an *in vitro* assay, *A. thaliana* plants expressing *ZmXTH1* do not show an increased XET activity. This effect could be due to a feeble XET activity of ZmXTH1 masked by the endogenous XET enzymes. It should be noted that ZmXTH1 (like all the maize subgroup 4 XTHs identified so far) does not have the canonical catalytic domain as it possesses an additional methionine residue that could account for a feeble XET activity. Another possible explanation relies on the observation that XTH enzymes exhibiting predominantly XEH activity can perform xyloglucan endotransglycosylation at elevated concentration of acceptor substrates (Baumann *et al.*, 2007). This supports the idea that ZmXTH1 could act as an XEH enzyme in

maize. In addition, the expression of genes coding for xyloglucanases in expanding tissues is compatible with a potential role in xyloglucan degradation allowing rapid wall extension (Baumann *et al.*, 2007), and this agrees with the ZmXTH1 expression pattern in maize.

Transgenic plants display alterations in primary cell walls: the middle lamella region appears wider and less compact, resulting in a widening of the wall between cells and the collapse of the walls. In addition, stems from *A. thaliana* plants expressing ZmXTH1 had a 20% reduction of the cellulose content with no variation in the composition of matrix polysaccharides. This reduction has already been observed in a XTH *A. thaliana* mutant (Liu *et al.*, 2007b) and also in the case of an Azuki bean XEH (Kaku *et al.*, 2002). The down-regulation of an expansin, another enzyme involved in cellulose–xyloglucan cross-links, also leads to a reduction of the cellulose content (Zenoni *et al.*, 2004).

In all these cases it was suggested that modifications of one of the complex components of the cell wall can have some effects on the others. Thus an alteration of the cellulose–xyloglucan cross-links could alter the stability of the cellulose microfibrils.

Therefore, the cell wall morphology of transgenic plants expressing ZmXTH1, with a wider and less compact middle lamella, suggests an altered architecture of the cell wall, in which polymer cross-links could be modified due to the increased levels of hydrolase activity.

Despite the reduction in cellulose content, the *A. thaliana* plants expressing ZmXTH1 do not show phenotypic alterations at a macroscopic level. This feature is in line with previous works showing that the total cellulose content in potato can be reduced down to the 40% level without affecting normal plant development (Oomen *et al.*, 2004).

In summary, the expression of ZmXTH1 in growing tissues and its induction by gibberellins, together with the phenotype observed in transgenic plants, suggests a role for this enzyme in cell wall modification processes in maize.

Supplementary data

S1 refers to the XET activity assays performed with a total of 200 reactions resulting from the combination of different potential donors and acceptors. S2 reports the accession numbers of the XTH proteins used to generate the phylogenetic tree of figure 1.

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