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Co- and/or post-translational modifications are critical for TCH4 XET activity

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Summary

TCH4 **encodes a xyloglucan endotransglycosylase (XET) of** ***Arabidopsis thaliana*****. XETs endolytically cleave and religate xyloglucan polymers; xyloglucan is one of the primary structural components of the plant cell wall. Therefore, XET function may affect cell shape and plant morphogenesis. To gain insight into the biochemical function of TCH4, we defined structural requirements for optimal XET activity. Recombinant baculoviruses were designed to produce distinct forms of TCH4. TCH4 protein engineered to be synthesized in the cytosol and thus lack normal co- and post-translational modifications is virtually inactive. TCH4 proteins, with and without a polyhistidine tag, that harbor an intact N-terminus are directed to the secretory pathway. Thus, as predicted, the N-terminal region of TCH4 functions as a signal peptide. TCH4 is shown to have at least one disulfide bond as monitored by a mobility shift in SDS-PAGE in the presence of dithiothreitol (DTT). This disulfide bond(s) is essential for full XET activity. TCH4 is glycosylated** ***in vivo*****; glycosidases that remove N-linked glycosylation eliminated 98% of the XET activity. Thus, co- and/or post-translational modifications are critical for optimal TCH4 XET activity. Furthermore, using site-specific mutagenesis, we demonstrated that the first glutamate residue of the conserved DEIDFEFL motif (E97) is essential for activity. A change to glutamine at this position resulted in an inactive protein; a change to aspartic acid caused protein mislocalization. These data support the hypothesis that, in analogy to** ***Bacillus*** **β-glucanases, this region may be the active site of XET enzymes.**

Introduction

Current models based on biochemical and microscopic analyses propose that the plant cell wall is composed of co-extensive networks of cellulose/hemicellulose, pectins, and proteins that restrain turgor-driven cellular expansion (reviewed in Carpita and Gibeaut, 1993; McCann and Roberts, 1994). Xyloglucan, the major hemicellulose of dicotyledonous plants, can hydrogen bond to the cellulose microfibrils, potentially cross-linking adjacent microfibrils (Fry, 1989; Hayashi, 1989; McCann *et al.*, 1990; Passioura and Fry, 1992). Xyloglucan endotransglycosylases (XETs) are enzymes capable of cleaving xyloglucan polymers endolytically, and then transferring the newly generated reducing end to the free end of another xyloglucan chain (Fanutti *et al.*, 1993; Farkas *et al.*, 1992; Fry *et al.*, 1992b; Nishitani and Tominaga, 1992; Nishitani, 1995; Smith and Fry, 1991). It was hypothesized that such an enzymatic activity would be involved in loosening cell walls by altering the xyloglucan tethers holding the cellulose microfibrils in place (Albersheim, 1976; Fry *et al.*, 1992a; Hayashi, 1989; Hetherington and Fry, 1993; Hoson *et al.*, 1991; Nishitani and Tominaga, 1992).

Although it is possible that XETs are involved in cell wall loosening, evidence exists that XETs may be involved in some aspect of cell expansion, but not in the initial wall loosening. Extracts containing active XETs from cucumber hypocotyls are unable to extend isolated cucumber hypocotyls *in vitro* and are not required for the loosening of walls carried out by expansin proteins in this assay (McQueen-Mason *et al.*, 1993). In addition, although extractable XET activity levels correlate well with the region most active in cell expansion in an elongating stem, extractable XET activity remains elevated in stem regions that have ceased elongation growth (Pritchard *et al.*, 1993). We have found that the Arabidopsis *TCH4* gene encodes an XET, and analysis of *TCH4* promoter/*GUS* reporter gene expression indicates a strong correlation between gene expression and expanding tissues (Xu *et al.*, 1995). However, *TCH4* expression is also up-regulated by mechanical stimulation, a treatment that leads to an overall decrease in elongation growth of Arabidopsis (Braam and Davis, 1990). One possible explanation for this seemingly contradictory expression is that TCH4 may participate in the incorporation of nascent xyloglucan into the cell wall. The TCH4 XET could transglycosylate xyloglucan polymers secreted into the cell wall to lengthen and intercalate xyloglucan into the existing cell wall (Xu *et al.*, 1996). Such a function would be required both for the process of cell expansion and for wall reinforcement in response to mechanical strain.

Furthermore, Saab and Sachs (1996) have identified a flooding-responsive *XET* gene in maize. Their data suggest that this XET may be involved in cell wall degradation resulting in the creation of aerenchyma in flooded roots. Consistent with this idea, immunolocalization using anti-

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TCH4 antibodies indicates an abundance of XETs between rows of mesophyll cells where it is predicted air spaces will be created (Antosiewicz *et al.*, 1997). Thus, XETs may take part in both building and degrading cell walls.

To fully understand the physiological functions of XETs, it is important to elucidate the enzymatic properties of these enzymes and identify potential distinctions among different XET isozymes. In *Arabidopsis thaliana*, a gene family encoding XET-related (XTR) proteins has been identified (Xu *et al.*, 1996). This family includes *TCH4*, *Meri-5*, *EXT*, and at least seven *XTR*s (Medford *et al.*, 1991; Okazawa *et al.*, 1993; Xu *et al.*, 1996). The predicted proteins share significant sequence similarity, ranging from 37 to 87% amino acid identity. All members of the family, including XETs from other plant species (de Silva *et al.*, 1993; de Silva *et al.*, 1994; Okazawa *et al.*, 1993; Peschke and Sachs, 1994; Saab and Sachs, 1996; Zurek and Clouse, 1994), are predicted to share several conserved features: (i) an amino-terminal signal peptide that may function to target the protein to the secretory pathway; (ii) a stretch of nine amino acids that, based on similarities with *Bacillus* β-glucanases, is thought to be critical for catalytic activity; (iii) at least one N-linked glycosylation consensus motif; and (iv) either two or four cysteines in the carboxyl-terminal region that may form disulfide bonds. The impact of these different features on XET activity is currently unknown.

Recombinant TCH4 produced in *E. coli* exhibits XET activity (Purugganan *et al.*, 1997; Xu *et al.*, 1995). TCH4 activity is specific for xyloglucan and xyloglucan-derived oligosaccharides; other polysaccharides are not substrates. TCH4 is active even at temperatures as low as –5°C, with optimal activity between 12° and 18°C, and is most active between pH 6.0–6.5 (Purugganan *et al.*, 1997). Interestingly, the specific activity of bacterially produced TCH4 is quite low. The low turnover rate could be a consequence of the purification of recombinant TCH4 from inclusion bodies, requiring denaturation and renaturation, or a consequence of a carboxyl-terminal polyhistidine tag included for ease of purification by nickel affinity chromatography. An additional possibility is that co- and/or post-translational modifications that occur in plant cells but not in bacterial cells are necessary for optimal XET activity. To examine the structural requirements for optimal XET activity, we generated a number of recombinant baculoviruses designed to produce distinct forms of TCH4 and used them to infect insect cells. TCH4 protein produced in this manner is highly active; the contributions of co- and post-translational modifications, a polyhistidine tag, and the ability to form disulfide bonds are addressed. In addition, the requirement for the conserved glutamate residue in the proposed catalytic site was determined using site-directed mutagenesis.

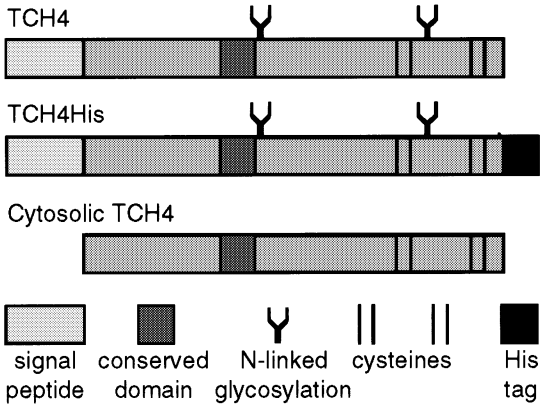


Figure 1. Schematic representation of TCH4 proteins.
Three variants of TCH4 protein were produced using recombinant baculovirus infected cells. Full-length TCH4 ('TCH4') is identical in sequence to native TCH4 protein. 'TCH4His' protein contains additional amino acids at the carboxyl-terminus, including a polyhistidine (His) tag. 'Cytosolic TCH4' was produced by replacing the putative signal peptide with a single initiatior methionine, forcing the protein to be produced in the cytosol, without co- and/or post-translational modifications of the ER/Golgi apparatus.

Results and discussion

Recombinant protein production

To characterize the effects of co- and/or post-translational modifications and a polyhistidine tag on TCH4 XET activity, we produced three variants of recombinant TCH4 protein using a baculovirus/Sf9 insect cell system. This heterologous expression system has been used previously to produce an XET from soybean (Oh *et al.*, 1998). We generated three baculovirus transfer vectors (Figure 1) containing (i) the full-length TCH4 coding region (called 'TCH4'); (ii) the same coding region with an added carboxyl-terminal polyhistidine tag ('TCH4His'); and (iii) a truncated coding region in which the signal peptide was replaced with a single initiator methionine ('cytosolic TCH4'). These transfer vectors were used to produce recombinant baculoviruses capable of producing TCH4 proteins.

TCH4 protein with the primary amino acid structure identical to the native TCH4 protein was produced by insect cells infected with recombinant TCH4 baculovirus. TCH4 was readily detected in the culture medium of infected cells by both Western blot using an anti-TCH4 antibody (Figure 2) and XET activity assays (Figure 3b); XET activity was not present in media from control cultures infected with a recombinant baculovirus producing β-glucuronidase (data not shown). The presence of TCH4 activity in the medium indicates that the TCH4 protein is secreted and therefore that the amino-terminal region can function as a signal peptide in insect cells. To determine accurately the specific activity for TCH4 produced by insect cells, we first partially purified the full-length protein. Ammonium sulfate

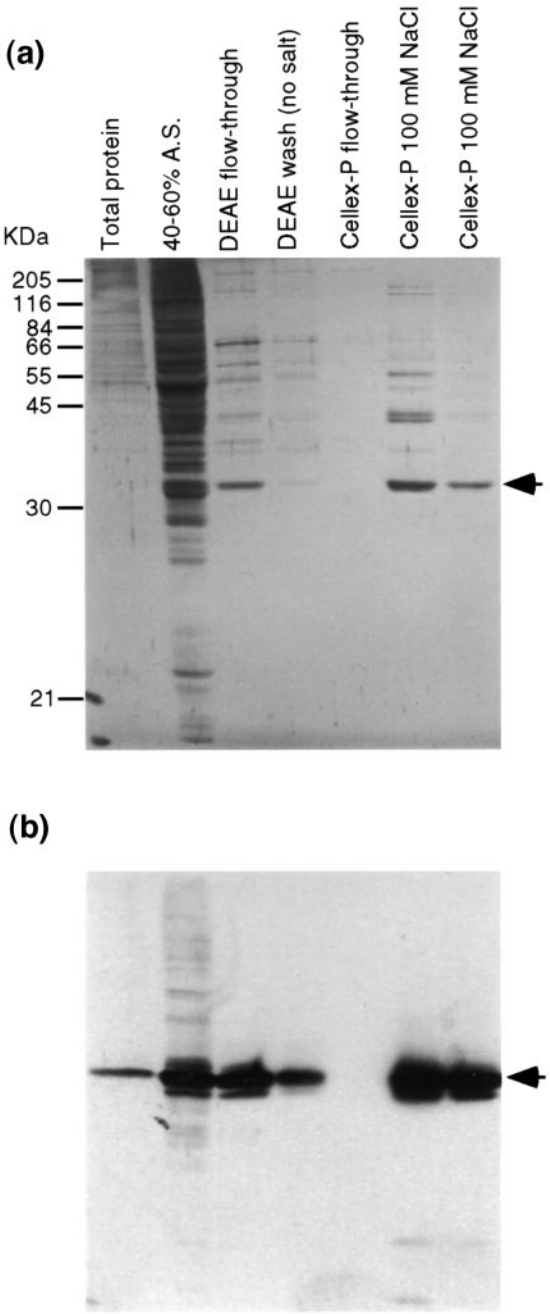
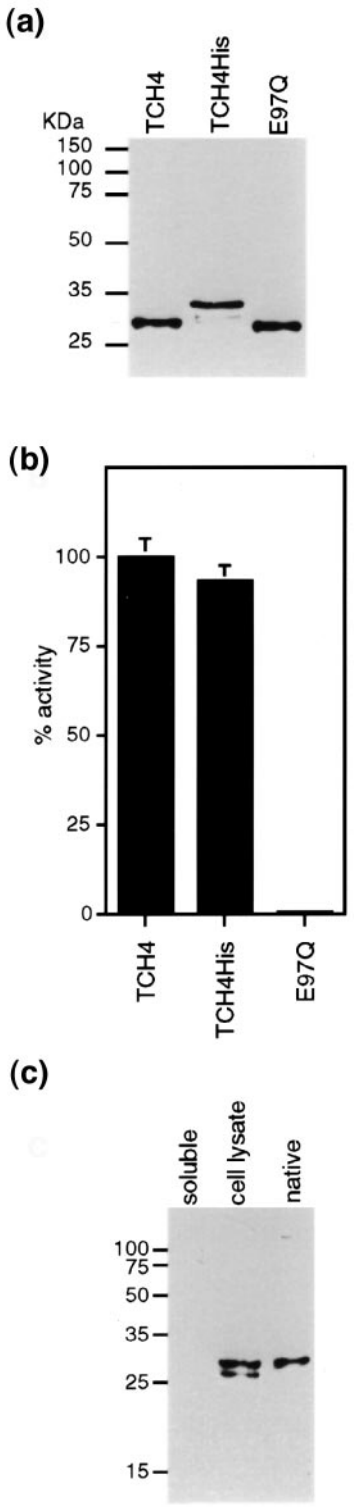


Figure 2. Purification of recombinant TCH4 from baculovirus-infected insect cell culture medium.
(a) Silver-stained gel. Relative migration of molecular weight markers is indicated at left.
(b) Western blot with anti-TCH4 IgG.
TCH4 protein was purified from insect cell culture medium using ammonium sulfate (A.S.) precipitation followed by anion and cation exchange chromatography. Equal volumes of protein from each step in the purification procedure were separated on duplicate SDS-10% acrylamide gels. One gel was silver-stained (a), whilst the other gel was used for Western blot analysis (b). The arrow indicates the band corresponding to TCH4.

(60%) was used to precipitate TCH4 from the culture medium. After dialysis, the TCH4-containing fraction was separated on DEAE-cellulose. The flow-through containing TCH4 was separated on a Cellex-P column, and TCH4 was eluted with 100 mM NaCl. SDS-PAGE analysis of the purification process followed by silver staining revealed a major band of approximately 32 kDa (Figure 2a). Western blot analysis of an equivalent gel using an anti-TCH4 antibody verified that the predominant band is recombinant TCH4 (Figure 3b). The specific activity of the substantially purified full-length TCH4 protein is approximately 150 $BqKBq^{-1}\,h^{-1}\mu g^{-1}$, compared with 8 $BqKBq^{-1}\,h^{-1}\mu g^{-1}$ for the bacterial protein (Purugganan *et al.*, 1997). Because TCH4 is only one of many XETs and XET-related proteins in Arabidopsis, it is not possible to compare directly the activities of the recombinant TCH4 protein with purified native TCH4. However, by purification of XET activities from cell wall fractions by Concanavalin-A and DEAE-cellulose chromatographies, we were able to obtain fractions highly enriched in XET activity. Comparable quantities of TCH4-antigenic material from Arabidopsis and recombinant TCH4 protein from baculovirus-infected cells as determined by Western analysis yielded similar levels of activities (with a range of 20% difference; data not shown). Therefore, it is likely that the recombinant TCH4 protein displays a comparable level of activity to that of native XETs from plants. One possible explanation for the 20-fold difference in activity between the bacterial and baculoviral proteins is the presence of a polyhistidine tag on the carboxyl terminus of the bacterially produced protein. To exclude this possibility, we produced a polyhistidine-tagged TCH4 (TCH4His, Figure 1) and compared it with full-length TCH4. Equivalent amounts of Concanavalin-A purified TCH4 and TCH4His, as determined by Western blot (Figure 3a), were assayed for XET activity. The TCH4His protein possesses approximately 93% of the full-length TCH4 activity (Figure 3b). Therefore, the histidine tag does not significantly affect TCH4 enzymatic activity.

Using the polymerase chain reaction, we replaced the nucleotides encoding the proposed signal peptide region (amino acids 1–21, Xu *et al.*, 1995) with a single initiator methionine codon such that TCH4 protein would be synthesized in the cytosol of insect cells (cytosolic TCH4, Figure 1). The resulting TCH4 protein did accumulate in the insect cell cytosol, as demonstrated by Western blot analysis of the soluble fraction of insect cell lysate (data not shown). Remarkably, the TCH4 protein produced in the cytosol had no detectable XET activity. The fact that the XET produced in bacteria had some detectable activity, whereas TCH4 produced in the insect cell cytosol had no detectable activity, is explained by the limits of detection used in the two different studies. Bacterially produced TCH4 protein was assayed at higher concentrations of purified protein (two- to 10-fold more) and radiolabeled oligosaccharide

(two- to threefold higher), for much longer periods of time, 4–8 h instead of 1–2 h. We conclude from these data that co- and/or post-translational modifications typical of eukaryotes must be critical for optimal XET activity.



Disulfide bond analysis

Disulfide bond formation and reshuffling between cysteine residues is one important co- and post-translational protein modification that contributes to the stability of a properly folded protein (Huppa and Ploegh, 1998). We tested whether TCH4 protein produced in baculovirus-infected cells has disulfide bonds and whether this modification is necessary for full TCH4 activity. Frequently, when a protein contains an intramolecular disulfide bond, its denatured form is slightly more compact than its reduced, denatured form. Upon separation on an SDS-polyacrylamide gel, the non-reduced protein would migrate slightly faster than the reduced form (Creighton, 1989). We reduced the full-length TCH4 protein with dithiothreitol (DTT) and examined whether the reduced protein demonstrated a change in mobility relative to the non-reduced protein. Indeed, as seen in Figure 4(a), TCH4 protein treated with DTT (lane ' + ') migrated slower than untreated protein. Although the migration difference was slight, the clear reproducibility of this behavior is evidence for the presence of at least one disulfide bond in TCH4 protein secreted by insect cells. The presence of this disulfide bond(s) is probably important for full XET activity (Figure 4b); increasing concentrations of DTT resulted in decreasing TCH4 activity. A similar decrease in enzymatic activity is found when TCH4 is assayed in the presence of 2-mercaptoethanol or glutathione (data not shown). Disulfide bond(s) may be important for stabilizing the most active conformation of the enzyme.

Glycosylation and its effect on XET activity

Lectin affinity chromatography using Con A-Sepharose has been a key step in purifying XET activities from different plant extracts (Edwards *et al.*, 1986; Nishitani and Tominaga, 1992; Schröder *et al.*, 1998), indicating that at least some XETs are glycosylated. As we have shown previously (Figure 3a), full-length TCH4 protein binds to

Figure 3. Direct comparison of enzymatic activities of the TCH4 variant proteins.
(a) TCH4, TCH4His, and TCH4(E97Q) proteins were separated by SDS-PAGE and analyzed by Western blot with anti-TCH4 IgG to identify equivalent amounts of each protein. Relative migration of molecular weight markers is indicated on the left.
(b) Equivalent amounts of TCH4, TCH4His and TCH4(E97Q) proteins were tested for XET activity. Activity was measured as Bq of ^{3}H-xyloglucan polymer created per KBq of ^{3}H-XLLGol oligosaccharide added to the reactions. This activity was normalized to that of full-length TCH4 and expressed as a percentage of total activity. All reactions were performed in triplicate and reported as the mean ± standard deviation.
(c) Detection of TCH4(E97D) in the insoluble cell pellet. Proteins from the soluble fraction of freeze/thaw-treated cells (soluble) or from the cell pellet boiled in SDS were separated by SDS-PAGE and analyzed by Western blot with anti-TCH4 IgG. TCH4 (equivalent to (a) lane 1) was included for size comparison. Relative migration of molecular weight markers is indicated at left.

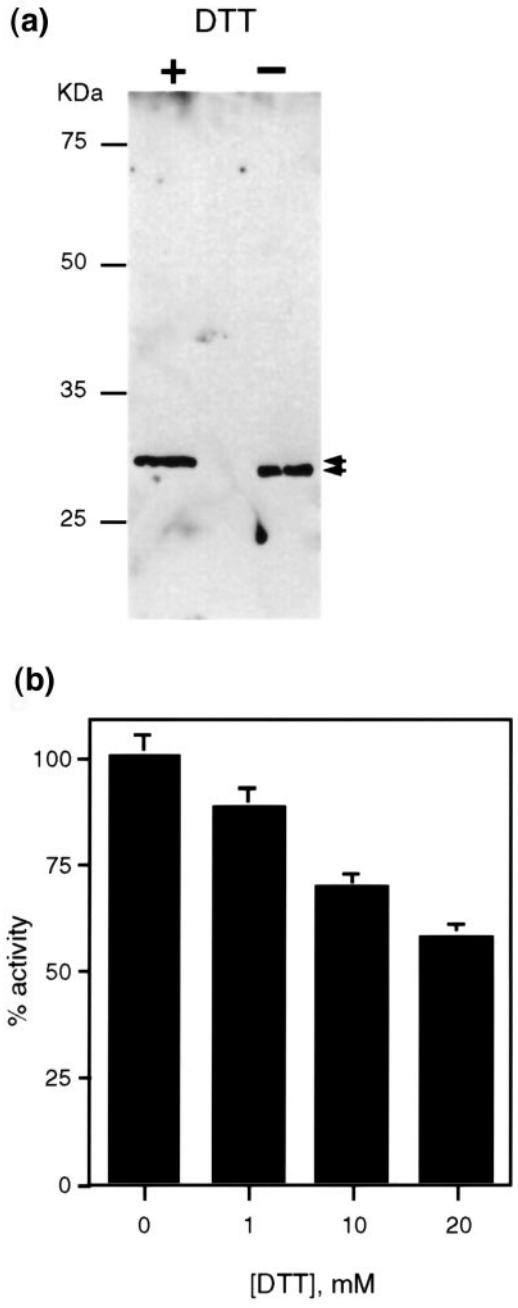


Figure 4. Effects of DTT on TCH4 mobility and activity.
(a) Purified TCH4 protein was denatured by boiling in 1× Laemmli's buffer in the presence (+) or absence (–) of 100 mM DTT, separated by SDS-PAGE and analyzed by Western blot with anti-TCH4 IgG. Arrows indicate relative positions of the two TCH4 bands. Relative migration of molecular weight markers is indicated on the left.
(b) TCH4 XET activity was measured in the presence of 0, 1, 10, and 20 mM DTT. Activity was measured as Bq of ^{3}H-xyloglucan created per KBq of ^{3}H-XLLGol oligosaccharide added to the reactions. This activity was normalized to that of maximum TCH4 activity and expressed as a percentage of total activity. All reactions were performed in triplicate and reported as the mean ± standard deviation.

Con A-Sepharose, indicating that the protein is glycosylated by insect cells. To test whether N-linked glycosylation is important for TCH4 XET activity, we treated TCH4 protein using two enzymes, Endoglycosidase H (Endo H) and Peptide:N-glycosidase F (PNGase F). Endo H cleaves between the two core N-acetylglucosamine residues of N-linked glycosylation, leaving one N-acetylglucosamine residue attached to the N residue of the protein, while PNGase F removes all sugar subunits from the protein and converted the N to an aspartic acid (D). Glycosidase and mock-treated proteins were separated by SDS-PAGE and analyzed by Western blot (Figure 5a). TCH4 treated with either glycosidase migrated farther than mock-treated samples, confirming that both Endo H and PNGase F remove carbohydrate side chains from TCH4. Removal of all but one N-acetylglucosamine with Endo H reduced TCH4 XET activity by 48% (Figure 5b). Strikingly, removal

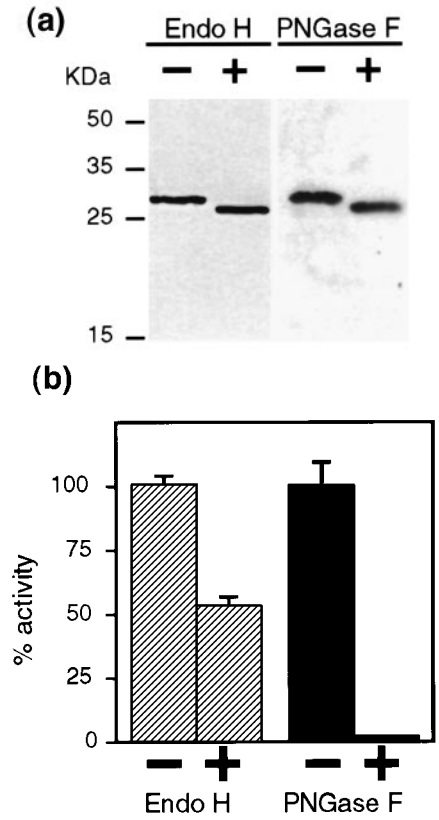


Figure 5. Glycosidase treatment of TCH4 reduces XET activity.
TCH4 was incubated for 2 h at 37°C in the presence (+) or absence (–) of the glycosidase enzymes Endo H and PNGase F.
(a) Aliquots of each reaction were analyzed by Western blot wtih anti-TCH4 IgG to verify the removal of the carbohydrate side chains. Relative migration of molecular weight markers is indicated at left.
(b) Mock-and glycosidase-treated TCH4 were assayed for XET activity. Activity was measured as Bq of ^{3}H-xyloglucan created per KBq of ^{3}H-XLLGol oligosaccharide added to the reactions. This activity was normalized to that of mock-treated TCH4 and expressed as a percentage of total activity. All reactions were performed in triplicate and reported as the mean ± standard deviation.

of all sugar residues with PNGase F eliminated 98% of TCH4 activity. This decrease in TCH4 activity could reflect the replacement of N with D instead of the absence of sugar residues. However, because treatment with Endo H also decreases TCH4 function, the sugar residues most likely play an important role in VET activity. Consistent with these results, glycosidase treatment of partially purified heterogeneous XETs from Arabidopsis also substantially reduces enzyme activity (approximately 80%; data not shown). However, the 37°C incubation required for glycosidase activity results in significant degradation of total proteins (as determined by colloidal gold staining of protein blots) and of the XETs (as indicated by the anti-TCH4 western analysis). Therefore, the requirement for glycosylation on the heterogeneous population of XETs in plant tissues is difficult to assess unequivocally. The 50% residual activity of the Endo H-treated TCH4 is most likely a consequence of stabilizing effects of the one N-acetylglucosamine residue remaining after treatment with Endo H. One glycosylation site of TCH4 is immediately adjacent to the proposed catalytic domain, DEIDFEFLGN. Removal of the carbohydrate side chain may result in a destabilization of ligand binding and thus a concomitant reduction in enzymatic activity.

Site-directed mutagenesis

XETs and XET-related proteins share significant amino acid sequence similarity to the β-1,3–1,4-glucanases of several species of *Bacillus*. One highly conserved motif between these two groups of proteins is the amino acid sequence DEIDFEFLG (Borriss *et al.*, 1990). Active-site directed inhibitors and site-directed mutagenesis provide strong evidence that this motif may encode the active site of these β-glucanases, with the first glutamate (E) residue critical for activity (Høj *et al.*, 1992; Keitel *et al.*, 1993; Planas *et al.*, 1992). These results have led several groups to suggest that the DEIDFEFLG motif may be critical for XET activity as well (de Silva *et al.*, 1993; Nishitani, 1995; Nishitani, 1997; Okazawa *et al.*, 1993; Xu *et al.*, 1996).

To test this hypothesis, we used site-directed mutagenesis to convert the codon of the first conserved E (E97) to the codon for either aspartate (E97D) or glutamine (E97Q). TCH4(E97D) protein was not detectable in the cell culture medium or the soluble fraction released by repetitive freeze/thaw treatment of the cell pellet by either XET assay or Western blot with an anti-TCH4 antibody (data not shown). The mutant protein was detected by Western blotting only when the cell pellet was boiled in the presence of SDS (Figure 3c). TCH4(E97D) migrated as two closely spaced bands, suggesting that the mutant protein had not been completely processed. These data suggest that TCH4(E97D) protein cannot be secreted, perhaps as a result of improper processing or misfolding, and likely remains trapped in the ER or another intracellular organelle. In contrast, the TCH4(E97Q) protein was secreted from cells, and we partially purified the protein using Con A-Sepharose chromatography. The mutant protein was readily detected in Western blots (Figure 3a). However, when equivalent amounts of TCH4 and TCH4(E97Q) were assayed for XET activity under identical conditions, TCH4(E97Q) showed less than 2% of the wild-type level of activity. Thus, E97 is critical for enzymatic activity and may constitute one of the catalytic residues of the active site. It is also possible that mutation of this residue eliminates activity simply by disrupting the proper tertiary structure of the protein. Overall, however, these data support the hypothesis that this region may be conserved with glucanases because it forms the active site of XETs (de Silva *et al.*, 1993; Nishitani, 1995; Nishitani, 1997; Okazawa *et al.*, 1993; Xu *et al.*, 1996).

Conclusions

Current research is just beginning to elucidate the molecular mechanisms responsible for cell wall biogenesis and modification and cell expansion. XETs can modify xyloglucan, one of the primary load-bearing components of the cell wall; therefore XETs may be critical for these processes. Analyses of how XET structure influences function will enable researchers to compare and contrast different members of the XET family, permitting the attribution of particular physiological functions to specific XETs. Determination of the roles for co- and post-translational modifications of the TCH4 XET is the first step towards this goal.

Experimental procedures

Polymerase chain reaction (PCR) and construction of baculovirus expression vectors

Three different forms of recombinant TCH4 were produced using the baculovirus/Sf9 insect cell system (Figure 1). To create a histidine-tagged TCH4, the DNA region encoding the entire TCH4 protein sequence (including the putative signal peptide) was generated by polymerase chain reaction (PCR) using the primers TCH4–5-Bam (5′-GCG GGA TCC ATG GCG ATC ACT-3′, the underlined sequence corresponds to nucleotides 1–12 of the *TCH4* cDNA sequence, Genbank Accession No. AF051338) and TCH4–3-Sac (5′-GTC GAG CTC AAT GCA GCT AAG C-3′, the underlined sequence is complementary to nucleotides 842–853) with the *TCH4* cDNA clone, cT4IIλYES, as template. The TCH4–3-Sac primer eliminates the stop codon, allowing in-frame ligation to the carboxyl-terminal polyhistidine tag encoded by the vector pET21 (Novagen, Madison, WI, USA). Using standard molecular biology techniques (Sambrook *et al.*, 1989), the PCR product was digested with *Bam*HI and *Sac*I (sites introduced with the PCR primers at the 5′ and 3′ ends, respectively), and the product was cloned into pET21 to generate a fusion protein with six histidine residues at the carboxyl-terminus. The resulting plasmid, pMP6, was

sequenced to verify that the correct sequence was amplified. The *Bam*HI/*Pst*I fragment of pMP6 was cloned into the same sites of pVL1392 (Pharmingen, San Diego, CA, USA) to generate the baculovirus transfer vector pVL-TCH4His.

To produce TCH4 protein identical to the native protein, a *Bam*HI/*Xho*I fragment of pMP6 containing a 5′ portion of the *TCH4* coding region (nucleotides 1–551) was subcloned into the same sites of pBluescript II KS (+) (Stratagene, La Jolla, CA, USA). A *Xho*I fragment of cT4IIλYES, containing the 3′ end of the *TCH4* cDNA (nucleotides 552–1016), was cloned into the *Xho*I site to recreate the entire coding region with the native stop codon (plasmid pPHC1). Proper orientation of the *Xho*I fragment was determined by PCR using the pair of primers listed above. The *TCH4* coding region was then cloned as a *Bam*HI/*Bsp*120I fragment into the *Bam*HI and *Eag*I sites of pVL1393 (Pharmingen) to create the transfer vector pBAC-TCH4.

Cytosolic production of TCH4 protein was engineered by replacing the coding region for the putative signal peptide (nucleotides 1–63, amino acids 1–21) with a methionine start codon using the primers BACT4 (5′-GGA TCC AAA AAT GAA TTT CCA AAG AGA CGT TGA G-3′, the underlined sequence corresponds to nucleotides 64–84) and TCH4–3-*Sac* for PCR amplification. The resultant PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol, to create pPHC2. This truncated *TCH4* cDNA did not contain a stop codon. To regenerate the proper *TCH4* stop codon, the *Xba*I/*Bsp*120I fragment of pPHC1 was subcloned into the same sites of pPHC2, creating pPHC3. The *Bam*HI/*Bsp*120I fragment of pPHC3 was then subcloned into pBACgus-1 (Novagen) to create the transfer vector pBAC-cytoTCH4. The *TCH4* coding region of pBAC-cytoTCH4 was sequenced to verify that no errors were introduced during PCR amplification and subsequent cloning steps.

Site-directed mutagenesis was performed on plasmid pPHC1 using the Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. The E at position 97 (from the initiator methionine) was mutated to a D and a Q. The native *TCH4* sequence, complementary to nucleotides 274–305, is 5′-AAC TCG AAA TC**A** AT**C** T**C**G TCC CAT GTT GTT CC −3′; the bolded sequences indicate the mutated nucleotides. The mutagenic oligonucleotide primers were E97D (5′-AAC TCG AAA TCG ATG T***C***G TCC CAT GTT GTT CC −3′) and E97Q (5′- AAC TCG AAA TCG AT*C* T**G**G TCC CAT GTT GTT CC −3′). Both mutagenic primers also included a silent mutation to introduce a *Cla*I restriction site to facilitate the screening of mutagenized *TCH4* cDNAs. The selection primer used was Kpn I-*Bgl*II (5′-GGG AAC AAA AGC TGA GAT CTG GGC CCC CTC GAG TCG −3′; this selection primer mutates the *Kpn*I site of Bluescript II KS (+) to a *Bgl*II site). Elimination of the *Kpn*I site during mutagenesis is used to screen for potential site-directed mutants. The mutated cDNAs were sequenced to verify that no other mutations were inadvertently introduced during the procedure. The *TCH4(E97D)* and *TCH4(E97Q)* cDNAs were cloned as *Bam*HI/Bsp 120I fragments into the *Bam*HI and *Eag*I sites of pBACgus-1 (Novagen) to create the transfer vectors pBAC-TCH4(E97D) and pBAC-TCH4(E97Q).

Recombinant protein production and purification

Recombinant baculoviruses were produced by co-transfection of BacVector-2000 linearized baculoviral genomic DNA (Novagen) with the transfer vectors pVL-TCH4His, pBAC-TCH4, and pBAC-cytoTCH4 by co-transfection (following the manufacturer's protocol). After 4 days, the insect cell culture medium was separated from the cells and saved as a source of recombinant virus. Sf9 insect cells cultured in SF900 II serum-free medium (Life Sciences, Bethesda, MD, USA) were seeded in tissue culture flasks at a density of 5×10^5 cells ml^{-1}. After allowing the cells to adhere to the flask for 1 hour, the appropriate recombinant virus was added to the cells at approximately a 1:200–1:400 dilution. Three days after infection, the Sf9 cells and cell culture medium were separated by low-speed centrifugation (500 ***g***) in a table-top centrifuge. The cell culture medium was used both as a source of amplified virus for subsequent infections, and as starting material for protein purification and XET assays.

E97D mutant and cytosolic TCH4 proteins were prepared from pBAC-TCH4(E97D) and pBAC-cytoTCH4 infected cells, respectively, as a crude cell extract. Cells collected from 5 ml of culture were resuspended in 300 µl of 1× phosphate-buffered saline, pH 7.3 (137 mM NaCl, 2.7 mM KCl, 5.3 mM Na_2HPO_4, 1.7 mM KH_2PO_4). The cells were lysed by freezing in a dry ice/ethanol bath for 5 min and thawing at room temperature. After two additional freeze-thaw cycles, lysed cells were centrifuged at 14 000 ***g*** for 10 min, 4°C. The supernatants were used for enzyme assays and Western blot analysis.

Full-length TCH4 protein was purified from 100 ml of cell culture medium of pBAC-TCH4-infected insect cells. The protein from a 40–60% ammonium sulfate cut of the culture medium was resuspended in 3 ml of 20 mM Tris–HCl, pH 7.0 and dialyzed against 4 l of this buffer for 2 h. The dialysis was repeated twice against fresh buffer. The protein sample was loaded onto a 1 ml DE52 column (Whatman, Maidstone, UK) equilibrated with 20 mM Tris–HCl and washed with 3 ml of the same buffer. The column flow-through and washes containing TCH4 protein were loaded onto a 500-µl Cellex-P column (BioRad, Richmond, CA, USA) equilibrated with 20 mM Tris–HCl, pH 7.0 and washed with 2 ml of the same buffer. TCH4 protein was eluted from the column by applying two 1 ml aliquots of 100 mM NaCl, 20 mM Tris–HCl, pH 7.0. The eluted protein was stored in small aliquots at –20°C.

To directly compare the enzymatic activity levels of full-length, polyhistidine-tagged, and E97Q mutant TCH4 proteins, all three proteins were partially purified under identical conditions (Freeze, 1995). 30 ml of cell culture medium from the appropriately infected cells was dialysed three times for 4 hours against 4 l of Con A column buffer (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 10 mM Tris–HCl, pH 7.5) at 4°C. The proteins were loaded onto a 500-µl Concanavalin A-Sepharose CL-4B column (Sigma, St. Louis, MO, USA) equilibrated in Con A buffer. After washing the column with 5 ml of Con A buffer, all bound proteins were eluted with 0.5 α-methyl-D-mannoside (Sigma) in Con A buffer.

Protein concentrations were determined using the NanoOrange protein quantitation kit (Molecular Probes, Eugene, OR, USA).

Glycosidase-treatment, SDS-PAGE, and Western blot analysis

For treatment with Endo H (New England Biolabs, Beverly, MA, USA), approximately 500 ng of purified TCH4 diluted into 50 mM sodium citrate, pH 5.5, was incubated with or without Endo H at 37°C for 2 h. For treatment with PNGase F (New England Biolabs), approximately 500 ng purified TCH4 diluted into 50 mM sodium phosphate, pH 7.5, was incubated with or without PNGase F at 37°C for 2 h. After digestion, 100 ng aliquots of TCH4 from each treatment were subsequently analyzed by Western blotting with anti-TCH4 IgG as described previously (Antosiewicz *et al.*, 1997) and enzyme activity assays, as described below.

Disulfide bond analysis

Purified TCH4 protein in 1× Laemmli's buffer (2% SDS, 10% glycerol, 60 mM Tris–HCl, pH 6.8, 0.001% Bromophenol Blue), with or without 100 mM DTT, was denatured by boiling for 3 min and electrophoresed on an SDS-12% polyacrylamide gel using a Hoeffer Tall Mighty Small electrophoresis system. The proteins were then analyzed by Western blotting as previously described (Antosiewicz *et al.*, 1997). The effects of DTT on TCH4 enzymatic activity were determined by performing XET assays, as described below, in the presence of the specified amounts of DTT.

XET activity assays

XET assays were performed in 20 μl reaction volumes containing 200 mM succinate, pH 6.0, 0.1% tamarind xyloglucan, 1.6 KBq [^{3}H]XLLGol (a xyloglucan fragment consisting of four β-1,4-linked glucose residues substituted at O-6 with side chains of α-D-xylose or α-D-xylose with β-D-galactose at O-2; see Fry *et al.*, 1993; for a precise explanation of the nomenclature), and 175 ng of purified TCH4 protein. The reaction mixture was incubated for 1–2 h at room temperature (25°C), then stopped by the addition of 70 μl of 30% formic acid. The reactions were spotted onto 4 by 5 cm pieces of 3 MM Whatman paper, allowed to air dry, and washed in running tap water for 2 h. The pieces of Whatman paper were baked to dryness at 200°C for approximately 15 min. The reactions were scintillation counted in 2 ml of Econosafe scintillant (Research Products International Corp., Mount Prospect, Il). All samples were performed in triplicate and reported as the mean ± standard deviation. Each experiment was reproduced at least twice with similar results.

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