Two Divergent Xyloglucan Endotransglycosylases Exhibit Mutually Exclusive Patterns of Expression in Nasturtium

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A cDNA encoding a xyloglucan endotransglycosylase (XET) homolog was isolated from nasturtium (Tropaeolum majus) epicotyl RNA. The deduced protein encoded by the corresponding gene, termed XET1, was substantially divergent from a previously isolated nasturtium XET (NXG1) expressed in germinating seed cotyledons but was highly homologous to XET genes isolated from vegetative tissues of several distantly related species. XET1 was expressed at the level of mRNA accumulation in all vegetative tissues examined (root, epicotyl, stem, and leaf) except in germinating cotyledons. Conversely, NXG1 exhibited the opposite pattern of expression and its mRNA was detected exclusively in cotyledons. Both XET1 and NXG1 were apparently encoded by single genes. Protein extracts from epicotyls or germinating cotyledons, in which XET1 or NXG1 are specifically expressed respectively, exhibited XET activity when assayed using two different xyloglucan substrates. XET activity from epicotyl extracts used nonfucosylated seed amyloid xyloglucan or fucosylated stem xyloglucan as a substrate with equal facility, whereas XET activity from cotyledon extracts had a significantly higher activity against nonfucosylated xyloglucan. The existence in a single species of two XETs possessing divergent amino acid sequences, mutually exclusive patterns of expression, and potentially different activities against xyloglucan substrates demonstrates the existence of different classes of XET and suggests differing roles in vivo.

Amyloidal xyloglucan forms the major polysaccharide storage reserve in seeds of nasturtium (Tropaeolum majus), tamarind (Tamarindus indica), and several other dicotyledonous plant species (Reid, 1985). This type of xyloglucan, which is localized in massive thickenings on the inside of the seed cotyledon cell wall (Reis et al., 1987), is composed of a 1,4-β-D-glucan backbone with side chains of 1,6-α-xylpyranosyl residues, which are occasionally further extended by 1,2-β-galactopyranose (Le Dizet, 1972). For example, tamarind amyloidal xyloglucan has the approximate composition Glc₂Xyl₁₆Gal₂₆ (Gidley et al., 1991). However, dicotyledonous plants also have another form of polymeric xyloglucan, which is located in their primary cell walls. This structural xyloglucan differs from seed amyloidal xyloglucan principally in that approximately 60 to 80% of the α-L-fucosyl residues (McNeil et al., 1984). Thus, dicotyledonous stem xyloglucan typically has the composition Glc₂Xyl₁₆Gal₁₅Fuc (Hayashi, 1989).

Current models of the plant primary cell wall describe a network of cellulose microfibrils embedded in coextensive matrices of hemicellulosic and pectic polymers (McCann et al., 1990; Carpita and Gibeaut, 1993). In dicotyledonous plants, the principal hemicellulose is xyloglucan, which is proposed to tether and cross-link the cellulose microfibrils (Hayashi and Maclachlan, 1984; McCann et al., 1990). The action of XET catalyzes the cleavage of xyloglucan molecules and subsequent transfer of the newly generated potentially reducing ends to the nonreducing ends of other xyloglucan chains. Thus, XET activity may play a vital role in the xyloglucan metabolism of primary cell walls and has been described in a variety of lower plants and both monocotyledonous and dicotyledonous higher plant species (Fry et al., 1992).

During seed germination, amyloidal xyloglucan is subject to dramatic enzyme-mediated catabolism (Reis et al., 1987). Coincident with the rapid digestion of this transitory storage reserve is the appearance of four distinct enzyme activities: three glycosidases (Edwards et al., 1985) and a “xyloglucan-specific endoglucanase” (Edwards et al., 1986). This latter enzyme has subsequently been shown to possess XET activity (Farkas et al., 1992; Fanutti et al., 1993) but appears to be biochemically distinct from a similar enzyme found in elongating epicotyls of azuki bean (Vigna angularis) (Nishitani and Tominaga, 1992). XET activity purified from azuki bean epicotyls lacked xyloglucanase activity in the absence of added xyloglucan oligosaccharides, and could not use polymers of less than about 10 kD (equivalent to a glucan backbone degree of polymerization of approximately 32) as donor substrates (Nishitani and Tominaga, 1992). XET activity purified from germinating nasturtium cotyledons, however, exhibited xyloglucanase activity even in the absence of added xyloglucan oligosaccharides, and exogenous oligosaccharides stimulated a further reduction in average xyloglucan chain length by acting as acceptors for cleaved high-molecular-weight polymers during transglycosylation (Farkas et al., 1992).

Highly conserved cDNAs encoding XETs have been isolated from vegetative tissues of azuki bean, soybean, tomato, Arabidopsis, and wheat seedlings (Okazawa et al., 1993), whereas the XET cDNA clone obtained from nasturtium cotyledons (de Silva et al., 1993) is quite divergent and

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1 Supported in part by a grant from Zeneca Plant Sciences, Jealott’s Hill, Berkshire, UK.

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were used to identify two conserved amino acid domains for XETs raise the possibility that a single plant species that possesses both amyloid and structural xyloglucan might contain multiple, distinct XET activities, each with specific functions. This paper reports the cloning from nasturtium epicotyls of an XET with relatively low homology to nasturtium seed XET. Two distinct nasturtium XETs are described, which exhibit divergent amino acid sequences and expression patterns and, at least in crude protein preparations, apparently possess different activities against different xyloglucan substrates. These findings suggest that XET1 and NXG1 have disparate roles in vivo.

**MATERIALS AND METHODS**

**Plant Materials**

Nasturtium (*Tropaeolum majus* L. var Fiery Festival) seeds were grown in moist vermiculite for 8 d at 15°C and then transferred to 25°C. Growth was conducted in the dark for the purpose of obtaining etiolated epicotyls and in the light for all other tissues. Mature tissue (stems and mature leaves) was obtained from plants grown in a field (Davis, CA). Young leaves were categorized as those with an average diameter of 0.5 to 1.0 cm, and mature leaves were those with an average width of >5.0 cm. Root tissue was obtained from nasturtium seeds grown in moist vermiculite for 8 d at 15°C and then transferred to 25°C. The purpose of obtaining etiolated epicotyls was to prepare materials for obtaining etiolated epicotyls and in the light for all other tissues. Mature tissue was obtained from plants grown in a field (Davis, CA). Young leaves were categorized as those with an average diameter of 0.5 to 1.0 cm, and mature leaves were those with an average width of >5.0 cm. Root tissue was selected from the terminal 2 to 3 cm of young seedlings. Plant tissues were harvested at appropriate stages, immediately frozen in liquid nitrogen, and stored at −80°C.

**PCR Amplification of cDNA and Sequencing**

The nucleic acid techniques used were as described by Sambrook et al. (1989) unless specified otherwise. Amino acid alignments from five plant species (Okazawa et al., 1993) were used to identify two conserved amino acid domains for the construction of degenerate PCR primers. The 5′ primer [5′-GA(A)C(A)T(G)TA(G)A(T)C(A)T(G)T(G)C-G-3′] corresponded to amino acids 102 to 109 of *V. angularis* XET and the 3′ primer [5′-TCNGT(G)A(C)T(G)T(G)T(G)A(T)T(G)A(T)G(A)T(G)A(T)N-3′] corresponded to amino acids 267 to 274, where N = all four nucleotides. cDNA was synthesized from 2 μg of epicotyl total RNA at 37°C for 60 min using 100 pmol of random hexamers (Pharmacia), 1 mM deoxynucleoside triphosphates, 1 mM DTT, and 200 units of Moloney murine leukemia virus reverse transcriptase (BRL). PCR was carried out in 50-μL final volumes using 1 unit of AmpliTaq (Perkin-Elmer), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM deoxynucleoside triphosphates, 3 mM MgCl2, and 1 μM of the above primers with 0.5 μg of cDNA for 40 cycles (94°C for 1 min, 39°C for 1 min, 72°C for 1 min, and 72°C for 1.5 min). The resulting 521-bp DNA fragment was gel purified and cloned into pCR-Script (Stratagene) according to the manufacturer’s instructions. DNA sequence was determined with universal primers and specific internal primers synthesized by Genset (La Jolla, CA) using [35S]dATP (New England Nuclear) and the Sequenase version 2.0 sequencing kit (United States Biochemical, Cleveland, OH). Two clones were completely sequenced on both strands and found to have identical sequences.

**Race PCR**

Extensions to each side of the above PCR-amplified fragment were carried out by RACE PCR (Frohman et al., 1988). For 5′-RACE PCR, 5 μg of total RNA from nasturtium epicotyls was converted to cDNA by reverse transcriptase as described above, except that 2.5 pmol of CrpT [5′-GGCCACGGCTGACTAGTACAATGCATC(A)TGGA(T)G-3′] was used as primer. Products were diluted to 1 μL, and 5 μL were amplified by PCR as described above using the outside anchor primer CnX (5′-GGCCACGGCTGACTAGTACAATGCATCTGGA(T)G-3′) and an inside gene-specific primer corresponding to nucleotides 827 to 848, each at 0.5 μM, for 1 cycle (50°C for 2 min, 72°C for 40 min) and then for 35 cycles (94°C for 1 min, 50°C for 1 min, and 72°C for 3 min). Reaction products were diluted 20-fold and 1 μL was amplified in a second round, using inside anchor primer (5′-CGACTAGTACAATGCATC(A)TGGA(T)G-3′) and an inside gene-specific primer corresponding to nucleotides 877 to 896, except that the 2-min annealing and 40-min extension cycle was omitted. The resulting 325-bp cDNA product was gel purified and cloned into the vector pCRRII (Invitrogen, San Diego, CA). Three clones were completely sequenced on both strands, each of identical sequence and possessing the expected overlap with the original clone.

For 5′-RACE PCR, nasturtium epicotyl RNA was converted to cDNA as described above using a gene-specific primer corresponding to nucleotides 521 to 545. The resulting cDNA was tailed with d(A) at the 5′ end (Frohman et al., 1988), and products were amplified by PCR in the presence of 50 nM CrpT, 0.5 μM CnX, and 0.5 μM of the above-described gene-specific primer, as for 3′-RACE. A band of 225 bp was cloned into pCRRII, and five clones were sequenced on both strands, each having an identical sequence. The amplified cDNA contained the expected overlap with the original clone but did not extend to the putative 5′ end of the cDNA, because the primer CnX bound within the coding sequence of the gene rather than to the 5′-d(A) tail.

The 5′ end of the gene was obtained using the 5′-RACE System kit (BRL) with an outside gene-specific primer corresponding to nucleotides 319 to 330 and an inside gene-specific primer corresponding to nucleotides 307 to 327, according to the manufacturer’s instructions. The resulting 375-bp product was subcloned into pCRRII, and five clones were sequenced on both strands with no PCR errors detected.

**Sequence Alignment and Phylogenetic Analysis**

The deduced amino acid sequence of the nasturtium XET1 cDNA was aligned with one of 3 other putative XETs (Fig. 1a) using MacDNASIS Pro (version 3.5, Hitachi Software, San Bruno, CA) or with 11 full-length deduced amino acid sequences of XET homologs using Pileup (Wisconsin Package, version 8, Genetics Computer Group, Madison, WI) with a gap creation penalty of 5.0 and a gap extension penalty of 0.3 (Fig. 1b). The sequences used were from *Glycine max*, *V. angularis*, *Arabidopsis thaliana*, *L. esculentum*, and *Triticum aestivum* (Okazawa et al., 1993), tomato fruit...
TXETB1 and TXETB2 (Arrowsmith and de Silva, 1995), and nasturtium seed NXG1 (de Silva et al., 1993), as well as XET homologs from Zea mays (Saab and Sachs, 1995), Arabidopsis thaliana (Medford et al., 1991), and G. max (Zurek and Clouse, 1994). A phylogram, with NXG1 as the outgroup, was derived using PAUP software (Swofford, 1993) and bootstrap analysis (Fig. 1b). Bootstrap analysis was performed using random stepwise addition of taxa with 100 replicates and global (tree bisection and reconnection) branch swapping. Bootstrap confidence values and branch lengths (actual number of amino acid changes) are depicted above and below the lines, respectively.

DNA Isolation and Analysis

Genomic DNA was isolated from young nasturtium leaves using the method described by Sambrook et al. (1989), digested with the indicated restriction enzyme, fractionated on 0.8% (w/v) agarose gels, and transferred to Hybond-N membrane (Amersham). The blots were hybridized with probes derived from templates of either the 521-bp nasturtium XET1 PCR product described above or a 732-bp fragment corresponding to nucleotides 497 to 1229 of the nasturtium seed NXG1 cDNA (a generous gift of Dr. Jacqueline de Silva, Unilever Research Laboratories, Bedford, UK) (de Silva et al., 1993). Both templates were radiolabeled by random hexamer priming using [α-32P]dATP (3000 Ci mmol⁻¹, New England Nuclear) and Klenow DNA polymerase (United States Biochemical). Labeled probes were separated from unincorporated nucleotides by centrifugation through spin columns of Sephadex G-50 and centrifugation at 10,000 g for 1 h. The suspensions were centrifuged at 19,000 g for 30 min. Supernatants were filtered through Miracloth (Calbiochem), made to 90% saturation with (NH₄)₂SO₄, and stirred at 1°C for 1 h. The suspensions were centrifuged at 10,000 g for 20 min, the supernatants were decanted, and the pellets were resuspended in a minimum volume of buffer A. The resuspended solutions were dialyzed (molecular mass cutoff 12-14 kD) overnight against buffer A except that the NaCl concentration was reduced to 0.1 M, and then clarified by centrifugation at 25,000 g for 20 min. The supernatants were dialyzed (two changes) with buffer A without NaCl, centrifuged at 25,000 g for 20 min, and filtered through Miracloth, and the final supernatants were frozen at −80°C for use in subsequent enzyme assays.

The protein content of the extracts was determined using a Bio-Rad Protein Assay kit with BSA as a standard.

XET Activity Assays

The radiolabeled acceptor substrate used in the assays was the xyloglucan nonasaccharide [3H]XLLG (nomenclature after Fry et al., 1993), derived from nasturtium seeds and custom titrated (Amersham International, Little Chalfont, UK) to a specific activity of 103 MBq mg⁻¹. The donor xyloglucans in the transglycosylase reaction were either from tamarind cotyledons, isolated by the method of Rao (1959), or from pea stems (the latter a generous gift of Prof. Takahisa Hayashi, Kyoto University, Kyoto, Japan).

Transglycosylase activity was assayed based on the method of Fry et al. (1992). Reactions were initiated by the addition of 1 volume of protein extract to 3 volumes of assay buffer, giving final concentrations of 0.2% (w/v) xyloglucan, 0.36 MBq [3H]XLLG, 150 mM Na+—Mes, pH 5.8, 0.66 mM DTT, and 6.6 mM CaCl₂. Protein extracts were diluted to a point at which enzyme concentration was proportional to the initial reaction rate and was therefore the limiting factor in the reaction. Aliquots of 40 μL were removed at the indicated times, and reactions were stopped by mixing with 100 μL of 20% (w/v) formic acid. Activity was assayed using liquid scintillation spectroscopy by determining incorporation of labeled oligosaccharides into high-molecular-weight xyloglucan capable of binding to 3MM paper (Whatman).

RESULTS

Cloning of a cDNA Encoding Nasturtium XET1

A full-length cDNA encoding an XET homolog was derived from nasturtium epicotyl mRNA with PCR techniques, using degenerate primers designed to two amino acids.
acid domains conserved between XET sequences from five plant species (Okazawa et al., 1993). The original 521-bp clone was extended at the 3' and 5' termini to yield a 1202-bp clone, designated *Tropaeolum majus* XET1 (*TmXET1*). The cDNA clone of this gene encoded 293 deduced amino acids, together with 103 bp of the 5' flanking sequence and 220 bp of the 3' flanking sequence. A putative polyadenylation signal (AATAAA) was located 16 bp before the poly(A) tail.

The deduced amino acid sequence from the *TmXET1* gene was aligned with those of three other putative XETs exhibiting differing degrees of homology (Fig. 1a). *TmXET1* showed only 38% amino acid identity with *TmNXG1*, an XET from nasturtium seed cotyledons (de Silva et al., 1993). However, alignment with the highly conserved deduced amino acid sequence of *LeEXT* (Okazawa et al., 1993) from tomato showed 70% amino acid identity, whereas the more divergent tomato XET gene *LeTXETB2* (Arrowsmith and de Silva, 1995) showed an intermediate amino acid identity of 51%. These three sequences were selected as representatives of different phylogenetic branches of putative XET proteins (see Fig. 1b).

A phylogram generated from the derived amino acid alignment of 12 XET genes revealed three distinct clades (Fig. 1b). One branch contained the divergent XET expressed in the seed cotyledons of certain species, such as nasturtium. A second clade contained XETs originally derived from vegetative tissues, which were highly conserved between genetically diverse species such as wheat, tomato, and nasturtium. A third clade contained a protein sequence (tomato TXETB2) confirmed by heterologous expression to exhibit XET activity and a related sequence, TXETB1 (Arrowsmith and de Silva, 1995), as well as three genes: *GmBRUl* (Zurek and Clouse, 1994), *Atmeri-5* (Medford et al., 1991), and *Zmwusl* (Saab and Sachs, 1995), possessing considerable amino acid sequence similarities to XETs.

### DNA Gel Blot Analysis

Southern blots of nasturtium genomic DNA were hybridized with partial-length clones derived from *XET1* and *NXG1* cDNAs, isolated from epicotyls and cotyledons, respectively (Fig. 2). The *XET1* probe hybridized to a single band in each of three restriction digests, suggesting the presence of a single gene. The *NXG1* probe also hybridized to single bands, confirming the result of de Silva et al. (1993) that this gene is present as a single copy. *XET1* and *NXG1* hybridized to different bands on the DNA gel blots, indicating that they occur at different loci in nasturtium.

### Tissue-Specific Expression of *XET1* and *NXG1* Genes in Nasturtium

A cDNA fragment derived from *XET1* was used to probe a northern blot containing nasturtium RNA from various tissues (Fig. 3). Hybridization occurred to a single band of approximately 1.4 kb. High levels of *XET1* mRNA were detected in actively growing tissues, such as young epicotyls and roots, with lower levels in stems and in young and mature leaves. Phosphorimager analysis showed that rela...
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XET1  NXG1

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Figure 2. DNA gel blot analysis of nasturtium XET1 and NXG1 genes. Genomic DNA (20 μg/lane) was digested to completion with the indicated restriction enzymes, and the DNA gel blots were hybridized with radiolabeled cDNA probes corresponding to a 521-bp fragment of XET1 or to a 732-bp fragment of NXG1 (de Silva et al., 1993). The blots were hybridized in 6× SSPE at 37°C and washed at moderate stringency (0.5× SSC at 55°C).

The same northern blot was stripped and reprobed with a 732-bp fragment derived from NXG1 cDNA (Fig. 3), detecting a single band of approximately 1.5 kb (de Silva et al., 1993). NXG1 mRNA was detected only in RNA derived from cotyledons, even after prolonged exposure of the blot (data not shown). This indicates that XET1 and NXG1 show mutually exclusive tissue-specific patterns of mRNA accumulation in nasturtium.

XET Activity Assays with Epicotyl and Seed Enzyme Preparations

Protein was extracted from nasturtium epicotyls and germinating cotyledons, and XET activity was assayed by measuring the incorporation of radiolabeled xyloglucan oligosaccharide acceptors into two different xyloglucan donor substrates. The donor substrates used were nonfucosylated amyloid xyloglucan purified from tamarind seeds and fucosylated dicycledonous primary cell-wall xyloglucan obtained from pea seedlings. Protein extracts from epicotyl tissue exhibited XET activity at high levels, and labeled acceptor oligosaccharides were incorporated into high-molecular-weight products using donor xyloglucan from either source with equal facility (Fig. 4a). In contrast, XET activity in protein extracts from germinating cotyledons was considerably greater when nonfucosylated amyloid xyloglucan was used as the donor substrate rather than fucosylated xyloglucan (Fig. 4b).

Figure 4. Time course assays of XET activity in protein extracts from elongating nasturtium epicotyls (a) or germinating nasturtium seeds (b) using amyloid (□) or fucosylated stem (●) xyloglucan as donor substrate. Activity was determined by measuring the incorporation of [3H]XLLG oligosaccharides into a high-molecular-weight xyloglucan polymer. Each time point represents the mean ± se of triplicate assays (n = 6).

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DISCUSSION

Reports describing xyloglucan-specific transglycosylases have used the names XET (Fry et al., 1992; de Silva et al., 1993; Fanutti et al., 1993) and endo-xyloglucan transferase (EXT) (Nishitani and Tominaga, 1992; Okazawa et al., 1993). Since the designation EXT has already been used to denote genes encoding the cell-wall glycoprotein extensin (Commission on Plant Gene Nomenclature, 1994), we have adopted the abbreviation XET to avoid ambiguity. This paper describes the isolation of a cDNA encoding an XET homolog (XET1) from epicotyls of nasturtium. The first XET cloned from this species, NXG1, was isolated from cDNA derived from germinating cotyledons (de Silva et al., 1993). XET1 shares a very low amino acid sequence identity (38%) with NXG1, but is closely related (70–83% amino acid identity) to XET genes from seedlings of other species, including the monocotyledonous species wheat and the dicotyledonous species azuki bean, soybean, tomato, and Arabidopsis (Fig. 1). The presence of at least two divergent XET genes in nasturtium and in tomato (Okazawa et al., 1993; Arrowsmith and de Silva, 1995) shows that different XET genes are expressed within a single species. In nasturtium it would appear that both XET1 and NXG1 are present as single genes (Fig. 2).

XET1 was expressed at the level of mRNA accumulation in all vegetative tissues examined (epicotyl, stem, root, and leaf), except in germinating cotyledons (Fig. 3). In contrast, NXG1 showed an opposite expression pattern, with high levels of mRNA in germinating cotyledons, but was not detected in other tissues examined. Thus, these two nasturtium XET genes show a mutually exclusive pattern of mRNA accumulation.

XET1 is expressed predominantly in vegetative tissues, where its substrate is presumably fucosylated cell-wall structural xyloglucan. This gene and highly homologous XETs from other species (as depicted in Fig. 1b) may therefore play a role in cell-wall xyloglucan metabolism, such as the incorporation of newly synthesized xyloglucan into the expanding primary cell wall or the modification of xyloglucan polymers forming cross-links between cellulose microfibrils. In contrast, the expression pattern of NXG1 in nasturtium suggests that its main physiological function is the mobilization of nonfucosylated xyloglucan seed storage reserves. Examination of crude protein preparations from epicotyl tissue, in which XET1 is expressed, and from germinating cotyledon tissue, in which NXG1 is expressed, suggests that the XET enzymes present in each tissue may have different activities against different xyloglucan substrates (Fig. 4). Epicotyl protein extracts did not distinguish between fucosylated and nonfucosylated xyloglucan donor substrates (Fig. 4a) in an in vitro XET assay. However, protein preparations from cotyledons, in which NXG1 is expressed, showed a markedly higher XET activity versus nonfucosylated amylopectin xyloglucan, its presumed in vivo substrate, than for fucosylated structural xyloglucan (Fig. 4b). Studies using purified nasturtium cotyledon XET showed that the enzyme was substantially more active as a xyloglucanase against nonfucosylated seed xyloglucan than against fucosylated stem xyloglucan (Edwards et al., 1986). The Xyl-Gal-Fuc side chain of primary cell-wall xyloglucan is thought to wrap around the glucan backbone (Levy et al., 1991), and this may cause a degree of steric hindrance interfering with the binding of NXG1, but not of XET1, to primary cell-wall xyloglucan. It should be noted that the data of Figure 4 represent XET activities assayed in vitro using crude protein extracts and that the potential presence of additional xyloglucan-modifying enzymes in the extracts necessitates that the findings be interpreted cautiously.

The differences in both protein sequence (Fig. 1a) and enzyme activity between NXG1 and other XETs suggest that they have evolved to perform distinct physiological functions. NXG1 has been purified to homogeneity (Edwards et al., 1986) and is a specialized enzyme that in vivo predominantly exhibits xyloglucanase activity (Edwards et al., 1985, 1986). In contrast, XETs purified from Vigna (Nishitani and Tominaga, 1992) or overexpressed from tomato (Arrowsmith and de Silva, 1995) act as transglycosylases and lack detectable glycanase activity. NXG1 thus appears to belong to a distinct class of XETs, which could perhaps be termed class I. The existence of more than one putative class of XET is also indicated phylogenetically in the tree shown in Figure 1b and in two different phylogenetic trees derived from 10 sequences (de Silva et al., 1994; Nishitani, 1995). However, further analyses of XET sequence information, expression patterns, and enzymatic activities are required to elucidate additional XET classes.

ACKNOWLEDGMENTS

We thank Dr. Jacqueline de Silva (Unilever Research Laboratories, Bedford, UK) and Prof. Takahisa Hayashi (Kyoto University, Kyoto, Japan) for their generous gifts of nasturtium partial-length NXG1 cDNA and pea stem xyloglucan, respectively. We also thank Drs. John Labavitch, Carl Greve, and Don Nevins for providing laboratory facilities and useful discussions; Dr. Christine Smart for assistance with phylogenetic analysis; and Dr. Gordon Maclachlan for valuable advice.

Received June 30, 1995; accepted November 1, 1995.

The GenBank accession number for the sequence reported in this article is L43094.

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