

# Cloning and Expression Pattern of a Gene Encoding an $\alpha$ -Xylosidase Active against Xyloglucan Oligosaccharides from *Arabidopsis*<sup>1</sup>

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An  $\alpha$ -xylosidase active against xyloglucan oligosaccharides was purified from cabbage (*Brassica oleracea* var. capitata) leaves. Two peptide sequences were obtained from this protein, the N-terminal and an internal one, and these were used to identify an *Arabidopsis* gene coding for an  $\alpha$ -xylosidase that we propose to call *AtXYL1*. It has been mapped to a region of chromosome I between markers at 100.44 and 107.48 cM. *AtXYL1* comprised three exons and encoded a peptide that was 915 amino acids long, with a potential signal peptide of 22 amino acids and eight possible N-glycosylation sites. The protein encoded by *AtXYL1* showed the signature regions of family 31 glycosyl hydrolases, which comprises not only  $\alpha$ -xylosidases, but also  $\alpha$ -glucosidases. The  $\alpha$ -xylosidase activity is present in apoplastic extractions from *Arabidopsis* seedlings, as suggested by the deduced signal peptide. The first eight leaves from *Arabidopsis* plants were harvested to analyze  $\alpha$ -xylosidase activity and *AtXYL1* expression levels. Both increased from older to younger leaves, where xyloglucan turnover is expected to be higher. When this gene was introduced in a suitable expression vector and used to transform *Saccharomyces cerevisiae*, significantly higher  $\alpha$ -xylosidase activity was detected in the yeast cells.  $\alpha$ -Glucosidase activity was also increased in the transformed cells, although to a lesser extent. These results show that *AtXYL1* encodes for an apoplastic  $\alpha$ -xylosidase active against xyloglucan oligosaccharides that probably also has activity against *p*-nitrophenyl- $\alpha$ -D-glucoside.

Xyloglucan is the main hemicellulosic polysaccharide present in the primary cell walls of dicotyledonous plants (Fry, 1989; Hayashi, 1989). It consists of a linear (1 $\rightarrow$ 4)- $\beta$ -linked D-glucan backbone that carries  $\alpha$ -D-xylosyl,  $\beta$ -D-galactosyl-(1 $\rightarrow$ 2)- $\alpha$ -D-xylosyl and  $\alpha$ -L-fucosyl-(1 $\rightarrow$ 2)- $\beta$ -D-galactosyl-(1 $\rightarrow$ 2)- $\alpha$ -D-xylosyl side chains attached to the OH-6 of  $\beta$ -glucosyl residues. Xyloglucan is thought to be the load-bearing component in the primary cell walls because of its proposed cross-linking of the cellulose microfibrils, this cross-linking being the major factor controlling the rate of cell expansion (Fry, 1989). In addition to its structural role, xyloglucan may act as a source of signaling molecules. Oligosaccharides derived from xyloglucan have been found to be formed *in vivo* (Fry, 1986) and they have been shown to regulate auxin- (McDougall and Fry, 1988, 1990) and acid pH-induced (Lorences et al., 1990) growth.

Enzymes that modify xyloglucan oligosaccharides have been detected in plant cell walls (Fry, 1995). An  $\alpha$ -fucosidase that removes the  $\alpha$ -L-fucosyl residue from XXFG has been purified from pea epicotyls (Farkas et al., 1991; Augur et al., 1993) and has later been cloned (Augur et al., 1995). A nasturtium  $\beta$ -D-

galactosidase able to remove the terminal  $\beta$ -D-galactosyl residue from xyloglucan side-chains has also been characterized (Edwards et al., 1988). Finally, an  $\alpha$ -D-xylosidase has been purified from auxin-treated pea epicotyls (O'Neill et al., 1989), and a second one from cotyledons of nasturtium seedlings (Fanutti et al., 1991). Both enzymes showed relatively high substrate specificity. They acted on xyloglucan oligosaccharides and specifically released the unsubstituted side chain xylosyl residue attached to the backbone glucosyl residue situated farthest from the reducing end of the molecule.

It has been shown by Lorences and Fry (1993) that oligosaccharides lacking the Xyl at the non-reducing terminus are unable to act as acceptors for xyloglucan endotransglycosylase in bean and pea leaves.  $\alpha$ -Xylosidase, by removing that particular xylosyl residue, would therefore reduce the concentration of oligosaccharides available for transglycosylation reactions. Xyloglucan endotransglycosylase, when the acceptors are oligosaccharides, has the same effect on xyloglucan as endo- $\beta$ -glucanase, i.e. a reduction of the cross-linking chains. Thus,  $\alpha$ -xylosidase could be playing an important role in cell expansion (Fry, 1995). Although the action of  $\alpha$ -xylosidase on polymeric xyloglucan has not yet been proved, there is some indirect evidence that supports this possibility (Guillén et al., 1995). It should also be noted that exo- $\beta$ -glucosidase cannot act on the Glc at the non-reducing end of the oligosaccharide backbone unless

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$\alpha$ -xylosidase has previously removed the Xyl attached to it (Koyama et al., 1983).

In the present paper we report the purification of an  $\alpha$ -xylosidase from cabbage (*Brassica oleracea* var. capitata) leaves. Two peptide sequences were obtained from this protein. They were used to identify an  $\alpha$ -xylosidase coding gene from Arabidopsis that we propose to call *AtXYL1*. It was sequenced and its expression was shown to be higher in younger leaves.  $\alpha$ -Xylosidase activity displayed a similar pattern. *AtXYL1* was expressed in *Saccharomyces cerevisiae*, and a protein with  $\alpha$ -xylosidase activity was produced.

## RESULTS AND DISCUSSION

### Purification of an $\alpha$ -Xylosidase from Cabbage Leaves

The extracts obtained from cabbage leaves were able to release Xyl from xyloglucan oligosaccharides, as measured by pentose release. This  $\alpha$ -xylosidase activity was purified after four consecutive purification steps. Along this procedure the activity always appeared as a single peak. The purified extract lacked  $\beta$ -galactosidase and  $\beta$ -glucosidase activities, the only other enzymes known to act on tamarind xyloglucan oligosaccharides, thus doing away with possible interferences in  $\alpha$ -xylosidase measurements. The amount of Xyl released by the purified enzyme was never higher than the amount of Xyl linked to the terminal glucosyl residue, as expected from the previous characterizations of plant  $\alpha$ -xylosidases (O'Neill et al., 1989; Fanutti et al., 1991). The specific activity of the purified  $\alpha$ -xylosidase was 27.7 nkat mg<sup>-1</sup> (Table I). The optimum pH for this enzyme was 4.5 and its pI was 8.3, as determined by isoelectric focusing (IEF; data not shown). Three selected fractions from a Sephacryl S-100 HR column were pooled and subjected to SDS-PAGE, revealing a single band of 89 kD (Fig. 1).

Two different types of  $\alpha$ -xylosidases had been previously purified to homogeneity. Those from *Aspergillus niger* (Matsushita et al., 1985) and *Bacillus* sp. (Zong and Yasui, 1989; Zong et al., 1989) were able to hydrolyze simple xylosides such as *p*-nitrophenyl- $\alpha$ -D-xylopyranoside or isoprimeverose and they were also able to remove the terminal Xyl from xyloglucan oligosaccharides. The other type, the plant  $\alpha$ -xylo-

sidases, is comprised of the enzymes purified from pea epicotyls (O'Neill et al., 1989) and from nasturtium cotyledons (Fanutti et al., 1991). These showed higher substrate specificity. They did not hydrolyze *p*-nitrophenyl- $\alpha$ -D-xylopyranoside or isoprimeverose, but they did specifically cleave the unsubstituted side chain xylosyl residue attached to the backbone glucosyl residue situated farthest from the reducing end of the xyloglucan oligosaccharides. Both enzymes had an apparent molecular mass of 85 kD on SDS-PAGE, and the optimum pH were in the range 4.9 to 5.1. The pI was 7.35 to 7.7 for the pea enzyme and 5.0 to 7.1 for the nasturtium.

### An Arabidopsis Gene Coding for $\alpha$ -Xylosidase

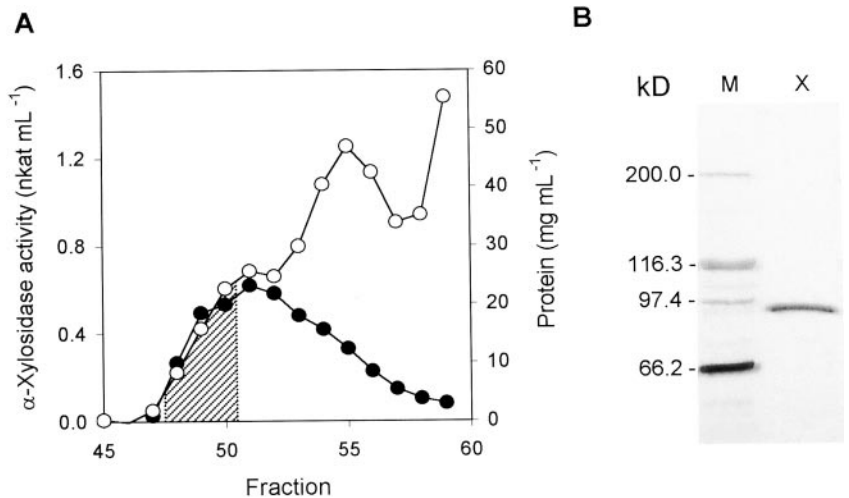
The 89-kD protein band from cabbage leaves (Fig. 1) was excised from PAGE gels and the N-terminal and an internal peptide sequences were obtained from it (Fig. 2). In the public databases, two Arabidopsis expressed sequence tags (ESTs; H8A7T7 and G10B11T7; Newman et al., 1994) were found that could be translated to protein fragments, including regions highly similar (34 out of 38 identical amino acids) to the sequences obtained from cabbage  $\alpha$ -xylosidase (Fig. 2). These ESTs had been obtained from two partial cDNA clones that were derived from the same gene, as we established by completely sequencing the longer clone (H8A7; GenBank accession no. AF087483) and partially sequencing the shorter one (G10B11). The assembled sequence was 2,917 bp long. We propose *AtXYL1* as the name for this Arabidopsis gene.

In addition, an Arabidopsis BAC end sequence (T12L6T7, accession no. AL094155) was found that showed total identity with H8A7 in a region 193 bp long. This sequence indicated that clone T12L6 from the Texas A&M University BAC library included only a fragment of *AtXYL1*. Fingerprinting data from the Genomic Sequencing Center showed that this clone most likely overlapped with two Institut für Genbiologische Forschung (Berlin) BAC clones (Mozo et al., 1998b), F2A8 and F28A15, with a Fingerprinted Contigs score value respectively of  $2 \times 10^{-17}$  and  $1 \times 10^{-13}$  (Marra et al., 1997). Both clones had been mapped by the Max Planck Institut für Molekulare Pflanzenphysiologie to a region of chro-

**Table I.** Purification of an  $\alpha$ -xylosidase from young cabbage leaves

Step	Activity	Protein	Specific Activity	Recovery	Purification Index
	<i>nkat</i>	<i>mg</i>	<i>nkat mg<sup>-1</sup></i>	<i>%</i>	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	144	300	0.48	100	1
SP-Sepharose	37.5	10.3	3.1	26	7.6
(Concanavalline-A [Con-A]) Sepharose	25.8	5.5	4.7	18	9.8
Sephacryl S-100 (high resolution [HR])	1.94	0.07	27.7	1.4	57.7

**Figure 1.** Purification of  $\alpha$ -xylosidase from young cabbage leaves. A, Selected region of gel permeation chromatography on Sephacryl S-100 HR column. Protein content (○) and  $\alpha$ -xylosidase activity (●) of each fraction were measured. B, SDS-PAGE of fractions 48 to 50 from Sephacryl S-100 HR chromatography. The selected fractions (striped area on A) were pooled, concentrated, and separated by SDS-PAGE. Left lane, Molecular markers; right lane,  $\alpha$ -xylosidase-containing fractions.



mosome I between markers pCITf117 (100.44 cM) and mi462 (107.48 cM; Mozo et al., 1998a). We screened by PCR a set of overlapping IGF-BAC clones from this region and found that F22C21 was the only one that included *AtXYL1* (Fig. 3).

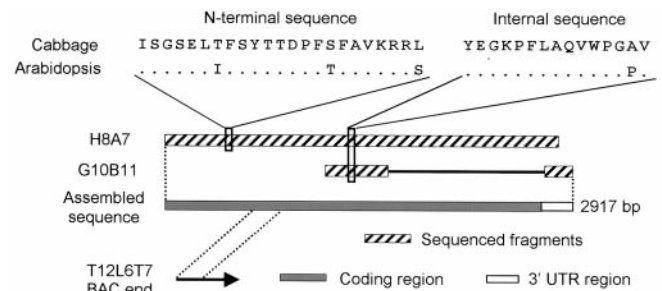
A 4,288-bp region from clone F22C21 was sequenced (Fig. 4) and submitted to GenBank (accession no. AF144078). It encompassed the entire coding sequence. According to the cDNA sequence, *AtXYL1* had two introns (91 and 622 bp long); the splice sites for both were found to be standard (data not shown). The *AtXYL1* transcription start site was located by 5'-RACE using RNA from young Arabidopsis leaves. The fragment thus amplified included a 5'-cap and overlapped with the sequence previously obtained from clone H8A7, nearly completing the known *AtXYL1* mRNA, which was now 3,037 bp long and went from the transcription start site to 18 bp downstream of a potential polyA signal in the 3'-untranslated region. A complete IGF-BAC clone (F24J5, accession no. AC008075), which included *AtXYL1*, was later sequenced by the Plant Gene Expression Center.

Analysis of *AtXYL1* promoter was based on F24J5 sequence and showed a TATA-like motif centered at position -30. What was more interesting was that several potential binding sites for transcription factors of the GT family were observed to be arranged in a pattern reminiscent of the one found in promoters where these transcription factors are known to be active (Fig. 5). Six SBF-1 binding sites and one GT-1 motif have been found in the *AtXYL1* promoter. Both kinds of sites are functionally related and their sequences are quite similar (Villain et al., 1994). In fact, three of the SBF-1 sites were also recognized by the software as GT-1 sites. What is more interesting is that five of the SBF-1 motifs and the GT-1 site are arranged in three regularly spaced pairs, as are the GT-1 motifs in the promoters of the *rbcS* multigene family encoding the small subunit of Rubisco (Gilmartin and Chua, 1990). These results suggest a possible regulation by light, but further studies will be necessary.

### Family 31 of Glycoside Hydrolases

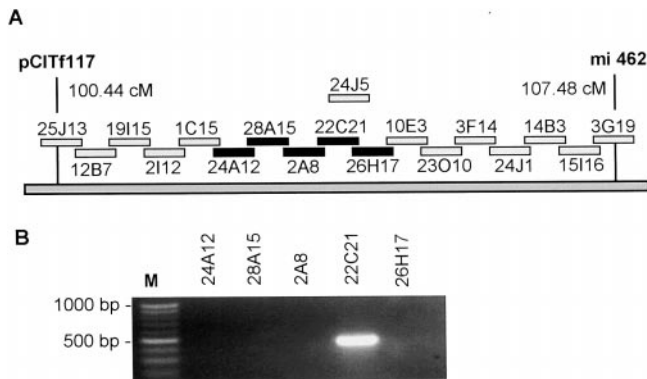
The amino acid sequence identity between the protein coded by *AtXYL1* (*AtXYL1*) and the two signature regions of family 31 glycosyl hydrolases (Henrissat, 1991; Frandsen and Svensson, 1998) showed that the  $\alpha$ -xylosidase from Arabidopsis, whose gene we had sequenced, was a member of that family (Fig. 6). More recently, an  $\alpha$ -xylosidase cDNA sequence from nasturtium cotyledons has been submitted to the public databases (accession no. AJ131520). When the translation product was compared with *AtXYL1*, it showed 75% identity over an 899-amino acids consensus length. Searching the Arabidopsis genome, a hypothetical protein (accession no. CAB82818) was found that presented an even higher identity with *AtXYL1*, 75% identity over 915 amino acids. We propose *AtXYL2* as its provisional name. Cabbage peptides showed lower similarity with *AtXYL2* than with *AtXYL1*.

*AtXYL2* gene includes two stop codons in what should be the coding sequence. This can only be solved assuming, as The Arabidopsis Genome Project has done, two introns that would create two serious gaps in the protein, 45 and 15 amino acids long, when aligned with the other plant family 31 protein sets



**Figure 2.** Diagram showing the identification of two Arabidopsis ESTs (H8A7T7 and G10B11T7) and a bacterial artificial chromosome (BAC) clone (T12L6) containing sequences highly similar to those obtained from cabbage  $\alpha$ -xylosidase. For Arabidopsis homologous sequences, dots mean conserved amino acids.





**Figure 3.** A, Tiling path of the region in chromosome I where F28A15 and F2A8 had been mapped. This diagram is based on Max Planck Institut für Molekulare Pflanzenphysiologie hybridization data. The initial F has been omitted from clone names. Five clones, including the two already mentioned, were selected for screening and are marked in black. Another clone, F24J5, has been recently sequenced by the SPP consortium (Stanford DNA sequencing and Technology Center, Plant Gene Expression Center at University of California-Berkeley, and the University of Pennsylvania). It includes the entire *AtXYL1* gene (GenBank accession no. AC008075). B, Screening of the five IGF-BAC clones by PCR in search for *AtXYL1*.

quences. The sequences of these hypothetical introns are as highly conserved with respect to *AtXYL1* as the putative coding regions. Thus, it seems likely that *AtXYL2* is really a pseudogene that has recently lost its function.

Furthermore, several ESTs from different plant species and one unidentified rice gene that presented a high similarity with *AtXYL1* were also found (Fig. 6). Most of the characterized enzymes in family 31 excise  $\alpha$ -Glc residues, although two  $\alpha$ -xylosidases from prokaryotes (accession nos. AAC62251 and AJ251975) have recently been discovered (Chaillou et al., 1998; Moracci et al., 2000). These  $\alpha$ -xylosidases are not closely related to their plant counterparts and seem to have evolved independently.

#### Arabidopsis $\alpha$ -Xylosidase Is an Apoplastic Enzyme

*AtXYL1* protein presented a potential signal peptide 22 amino acids long (Fig. 4), suggesting an apoplastic localization. Since the traditional centrifugation method to obtain the apoplastic fluid was not useful for Arabidopsis seedlings, a different experimental approach has been assayed. The seedlings were grown on water with shaking at 120 rpm to retard the development of the cuticle as described by Monroe et al. (1999) for mustard seedlings.

The apoplastic fluid was partially extracted with 1 M NaCl. When using extraction buffer at pH 4.5,  $\alpha$ -xylosidase activity detected in the apoplastic fraction accounted for 37% of the activity in the whole plant. At pH 6, just 16% of the total activity was extracted, possibly due to a higher charge in cell wall pectins. In contrast, Glc-6-P dehydrogenase (G6PDH) was not detected in any fraction when extracted at

pH 4.5 due to its instability, but when buffer at pH 6 was used for extraction, the apoplastic fraction accounted for less than 0.2% of the total G6PDH activity. This absence of G6PDH activity extracted with 1 M NaCl proved the lack of cytoplasmic contamination obtained with this mild extraction method. It follows then that at least a fraction of  $\alpha$ -xylosidase activity is located in the apoplast. The remaining activity might be inside the protoplast or, more likely, it might not have been able to diffuse into the medium due to the extremely gentle extraction procedure.

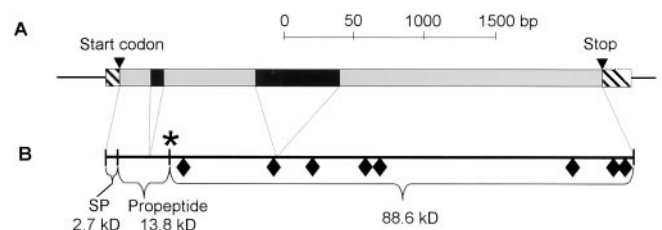
#### $\alpha$ -Xylosidase Is Developmentally Regulated

Arabidopsis plants grown for 19 d had eight or more leaves (Fig. 7). At this time there was a clear growth gradient, with younger leaves growing faster than older ones. Although the two oldest leaves had almost achieved their maximum size, the youngest pair was growing 24 times faster. The specific activity of  $\alpha$ -xylosidase showed a good positive relationship with growth rate, because it was six times higher in the fastest growing leaves than in the slowest growing ones. *AtXYL1* mRNA levels were also lower the older the leaves were. Thus, it seems apparent a positive relationship among growth,  $\alpha$ -xylosidase activity, and *AtXYL1* gene expression. This is not surprising at all since xyloglucan depolymerization increases during plant growth (Nishitani and Masuda, 1983; Hoson, 1993). This would raise the concentration of xyloglucan oligosaccharides, and  $\alpha$ -xylosidase activity should, therefore, be higher in fast-growing walls.

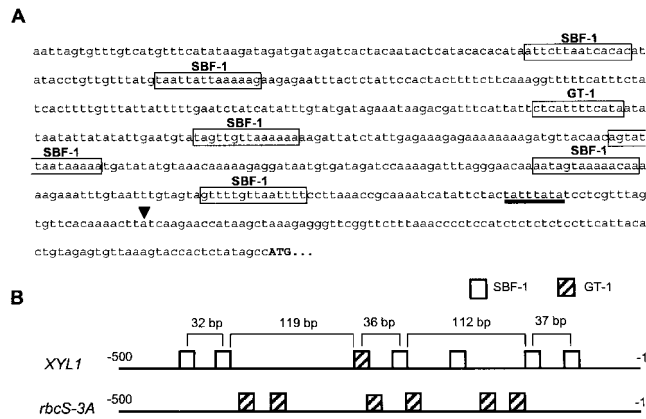
Regarding the possible expression of *AtXYL2*, it should be noted that in the public databases there are at least 56 ESTs corresponding to *AtXYL1* and none to *AtXYL2*. This would suggest that *AtXYL2* is not expressed at all or only at low levels.

#### Heterologous Expression

*AtXYL1* was used to transform *S. cerevisiae* cells and these cells were grown for 48 h in YP4 high stability expression medium.  $\alpha$ -Xylosidase activity was not



**Figure 4.** Structure of *AtXYL1* gene and its protein. A, Sequenced fragment from clone F22C21. Transcribed region is drawn as a set of boxes, stripped (5'- and 3'-untranslated regions), black (introns), or gray (coding regions). B, Protein diagram.  $\blacklozenge$ , Potential glycosylation sites. The asterisk marks the position of active cabbage  $\alpha$ -xylosidase N terminus. SP, Signal peptide.



**Figure 5.** Schematic representation of *AtXYL1* promoter. A, Sequence from -500 to the start codon. ▼, Transcription start site. A TATA-like sequence is underlined. Potential binding sites for SBF-1 and GT-1, transcription factors of the GT-family, are boxed. B, Concise view of binding sites arrangement in *AtXYL1* and *rbcS-3A* promoters.

detected in the culture medium, even though the expression plasmid included the  $\alpha$ -factor signal peptide. On the other hand, when the cells were disrupted with glass beads, low levels of  $\alpha$ -xylosidase activity were present in the extract (Fig. 8). Cells transformed with *AtXYL1* show 3.63 times the activity of cells transformed with a control plasmid that had no insert, this increase being statistically significant ( $P < 0.01$ ). Similar results were found when cells were grown in YP Expression medium (data not shown). These results support the assignation of  $\alpha$ -xylosidase activity to *AtXYL1*. It should be noted that an  $\alpha$ -glucosidase from barley that belongs to

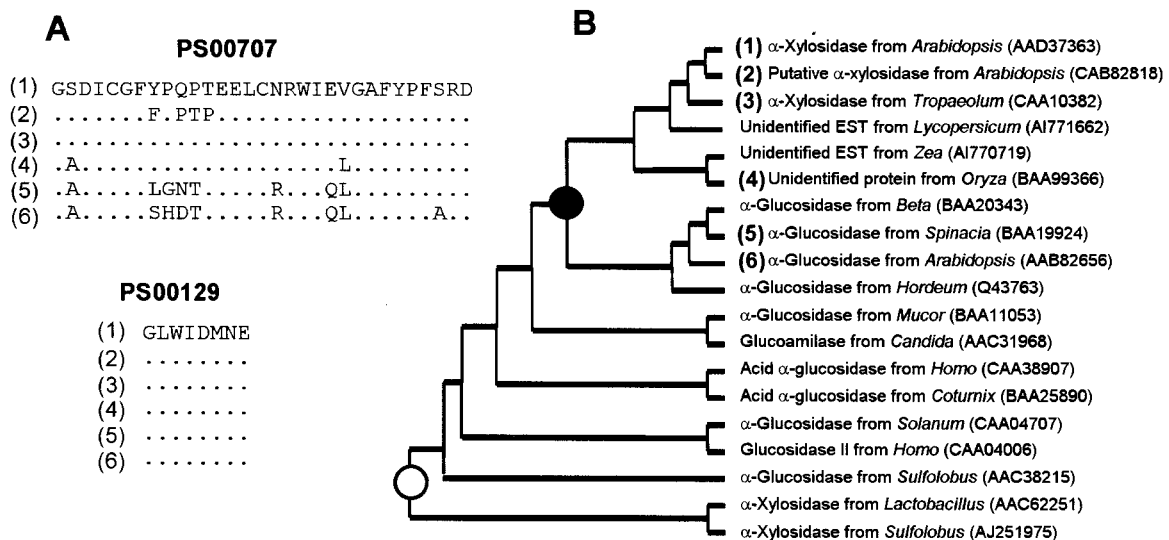
family 31 has also been heterologously expressed in yeast and its specific activity was 312 times lower than that of the native enzyme (Frandsen et al., 2000).

$\alpha$ -Glucosidase activity was also detected in transformed and control cells, the activity being 1.41 times higher in the former. This difference was also statistically significant ( $P < 0.01$ ). An N-terminal sequence identical to the one we obtained from cabbage  $\alpha$ -xylosidase was recently assigned to a broccoli enzyme with  $\alpha$ -glucosidase activity (Monroe et al., 1999). Our results are in agreement with the possibility of *AtXYL1* having, in addition to its  $\alpha$ -xylosidase activity, an  $\alpha$ -glucosidase activity against *p*-nitrophenyl- $\alpha$ -D-glucopyranoside. No activity against *p*-nitrophenyl- $\alpha$ -D-xyloside was detected in the transformed or the control cells.

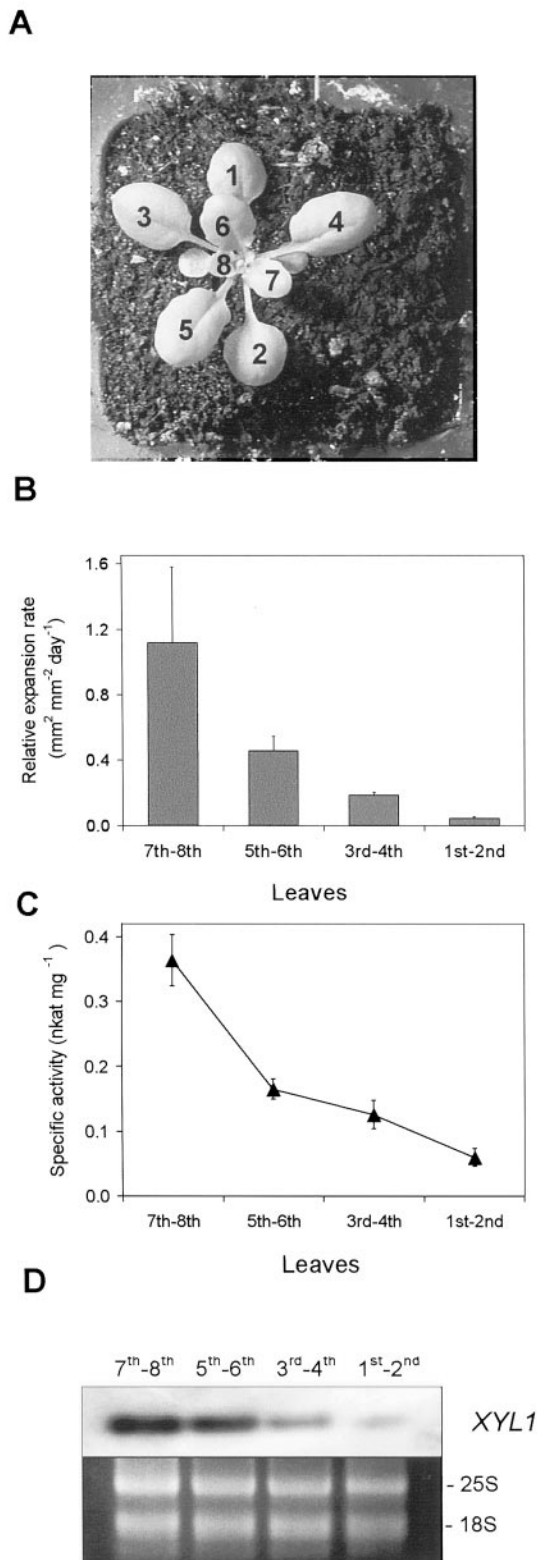
Since the recombinant protein should have incorporated a FLAG marker peptide, an ANTI-FLAG affinity gel was used to purify *AtXYL1* protein. A single band of 120 kD, corresponding to the expected  $M_r$ , was observed on SDS-PAGE gels (data not shown), but no activity could be detected, probably due to the pH 7.4 used during purification, which had been observed to inactivate cabbage  $\alpha$ -xylosidase.

**Concluding Remarks**

Our study identified an Arabidopsis  $\alpha$ -xylosidase gene (*AtXYL1*) that encodes an apoplastic enzyme able to release Xyl from xyloglucan oligosaccharides. *AtXYL1* amino acid sequence showed 75% identity with the sequence of nasturtium  $\alpha$ -xylosidase, the only other sequenced plant  $\alpha$ -xylosidase. *Aglu-2* had



**Figure 6.** Partial alignment and phylogenetic relationship among family 31  $\alpha$ -glycosyl hydrolases. A, Alignment of the family 31 PROSITE signature sequences (labeled with the PROSITE accession no.) from known and putative plant  $\alpha$ -xylosidases and two plant  $\alpha$ -glucosidases. Dots mean conserved amino acids. B, Concise cladogram for family 31 glycosyl hydrolases. ●, The probable point where plant cell wall  $\alpha$ -xylosidases started to diverge from  $\alpha$ -glucosidases. ○, The independent origin of recently discovered prokaryotic  $\alpha$ -xylosidases. Figures under brackets show sequence correspondence between A and B.



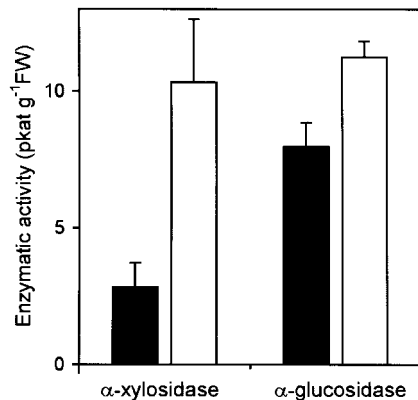
**Figure 7.** Developmental regulation of  $\alpha$ -xylosidase. A, Photograph of a typical 19-d-old Arabidopsis plant. The first eight true leaves were numbered according to their appearance order. B, Leaf relative expansion rate as function of developmental stage. The first eight leaves were sorted into four categories. Data are mean values with SD for 10 plants.

been previously proposed by Monroe's group as the name for the Arabidopsis gene we have called *AtXYL1* on the basis of its homology to the broccoli enzyme partially purified by them (Monroe et al., 1999). It is important to note that although this enzyme had  $\alpha$ -glucosidase activity against *p*-nitrophenyl- $\alpha$ -D-glucopyranoside, it was not tested for possible  $\alpha$ -xylosidase activity against xyloglucan oligosaccharides. The results from our heterologous expression experiments show that it is probable that Arabidopsis and cabbage  $\alpha$ -xylosidases have in vitro activity against certain  $\alpha$ -glucosidase substrates.  $\alpha$ -Xyl and  $\alpha$ -Glc are structurally very similar with their hydroxyl groups in the equatorial positions. *Sulfolobus sulfataricus*  $\alpha$ -xylosidase, a family 31 enzyme, has low activity against maltose and 4-*p*-nitrophenyl- $\alpha$ -glucoside (Moracci et al., 2000). However, we think *AtXYL1* is a more appropriate name, since  $\alpha$ -Glc containing polysaccharides have not been found on cell walls and xyloglucan is one of the main components of primary walls (Carpita and Gibeaut, 1993). The high similarity with nasturtium  $\alpha$ -xylosidase also supports the idea that the physiologically relevant activity of AtXYL1 protein is  $\alpha$ -xylosidase, although other possibilities cannot be ruled out at present.

AtXYL1 polypeptide was found to be 915 amino acids long, including a potential signal peptide of 22 amino acids (Fig. 4). Without the signal peptide AtXYL1 would be almost 100 kD in size. The maturation process for  $\alpha$ -xylosidase in Arabidopsis is presently unknown, but if it can be assumed that the N-terminal sequence of the mature Arabidopsis protein is placed in a similar position as on the purified cabbage  $\alpha$ -xylosidase, then an 11-kD pro-peptide must be cleaved during processing. This would reduce the molecular mass to 88.6 kD, which would fit our data from PAGE gels (Fig. 1). The position in the sequence where cabbage  $\alpha$ -xylosidase N terminus is located is very similar to that of 70-kD human lysosomal  $\alpha$ -glucosidase, the best known enzyme of family 31 (Wisselaar et al., 1993). It is interesting that this enzyme also loses a C-terminal fragment of more than 7.6 kD. Moreover, the presence of eight potential N-glycosylation sites in AtXYL1 sequence and the fact that cabbage  $\alpha$ -xylosidase bound to Con-A Sepharose suggests that the mature enzyme is probably glycosylated, as is also the case for lysosomal  $\alpha$ -glucosidase (Wisselaar et al., 1993). In this instance, the mass of the enzyme would be clearly increased over the 88.6 kD mentioned above, but this could be compensated if  $\alpha$ -xylosidase lost a C-terminal fragment during maturation.

C,  $\alpha$ -Xylosidase-specific activity extracted from different leaf groups. D, Analysis of *AtXYL1* expression using reverse transcriptase (RT)-PCR followed by Southern hybridization. rRNA stained with ethidium bromide was used as control.





**Figure 8.** Heterologous expression of *AtXYL1*.  $\alpha$ -Xylosidase and  $\alpha$ -glucosidase activities of extracts from *S. cerevisiae* cells transformed with *AtXYL1* (white bars) or with a control plasmid that had no insert (black bars) grown in YP4 High Stability Expression medium. Both activities are referred to the fresh weight of harvested cells.

The family 31 cladogram suggests that a gene duplication event took place in this family at an early phase of plant evolution (Fig. 6). Both known  $\alpha$ -xylosidases appear in the same branch, which includes several unidentified proteins. Since xyloglucan occurs in all higher plants (Hayashi, 1989), it seems reasonable to presume that an  $\alpha$ -xylosidase gene has always been necessary. Thus, it would not be surprising to find that all the genes in the same branch as *AtXYL1* are also  $\alpha$ -xylosidase genes.

The presence of possible light-regulated elements in *AtXYL1* promoter is also consistent with this idea of a growth-related enzyme.  $\alpha$ -Xylosidase role might be more interesting than just degrading oligosaccharides as soon as they are produced. A particular oligosaccharide concentration depends on its formation and degradation rates. As we noted before, there are several of them that show a powerful effect on growth rate (McDougall and Fry, 1988, 1990; Lorences et al., 1990), and in some cases this effect requires a Xyl residue on the non-reducing end of the oligosaccharide molecule (Lorences and Fry, 1993). For these oligosaccharides the level of  $\alpha$ -xylosidase activity would be critical. It is, therefore, likely that a more detailed regulation exists for this enzyme. Having identified an  $\alpha$ -xylosidase gene in *Arabidopsis* means that many of the genetic tools developed for this species can now be employed to better understand the regulation of a key step in xyloglucan metabolism.

## MATERIAL AND METHODS

### Plant Material

Cabbages (*Brassica oleracea* var. capitata) were purchased at a local market and were used in the same day.

For RNA and protein extraction, *Arabidopsis* ecotype Columbia was grown for 16 or 19 d at 22°C under a 16-/8-h

light/dark photoperiod. Seeds were cold treated at 4°C for 2 d and then directly sown on soil.

To separate apoplastic and cytoplasmic fractions, *Arabidopsis* seeds were superficially sterilized with 1% (w/v) NaClO for 10 min, thoroughly rinsed with sterile water, and grown for 3 d in 100-mL flasks containing 20 mL of sterile distilled water at 25°C, with orbital shaking at 120 rpm under a 16-/8-h, light/dark photoperiod.

### Enzyme and Protein Assays

Tamarind (*Tamarindus indica*) xyloglucan was digested with *Trichoderma viride* endoglucanase (Sigma, St. Louis) to obtain an oligosaccharide mixture that was employed as substrate for  $\alpha$ -xylosidase determination. This activity was determined as pentose release (Xyl being the only pentose in xyloglucan), according to the method of Roe and Rice (1948) as modified by Edwards et al. (1985). Aliquots of  $\alpha$ -xylosidase containing extracts were incubated with 400  $\mu$ g of oligosaccharides in 0.1 M Na acetate, pH 4.5, at 40°C for a variable period according to the extract activity.

The activity of extracts against *p*-nitrophenyl- $\alpha$ -D-glucopyranoside and *p*-nitrophenyl- $\alpha$ -D-xylopyranoside was also measured in 0.1 M Na acetate, pH 4.5, at 40°C, but with 2 mM substrate concentration. In the case of *p*-nitrophenyl- $\beta$ -D-galactopyranoside and *p*-nitrophenyl- $\beta$ -D-glucopyranoside, the substrate concentration was 5 mM. After incubation, the reactions were stopped by adding 200 mM Na<sub>2</sub>CO<sub>3</sub> and the A<sub>400</sub> was measured as described elsewhere.

Protein concentration was quantified using Coomassie protein assay reagent (Pierce, Rockford, IL).

### Enzyme Purification

Leaves from cabbage centers (2 kg) were cut into small pieces, frozen with liquid nitrogen, and homogenized to a fine powder with an Omnimixer (Sorvall, Newton, CT). The powder was suspended in 5 L of 50 mM sodium acetate buffer, pH 4.5, and was maintained in suspension with magnetic stirring at 4°C for 2 h. The unextracted material was collected by vacuum filtration through muslin, resuspended with the same volume of 1 M sodium acetate buffer, pH 4.5, and left overnight at 4°C with magnetic stirring. The suspension was then centrifuged at 7,000g for 40 min, and the supernatant was considered as the crude extract.

The crude extract was fractionated by ammonium sulfate precipitation. The protein precipitated between 40% and 80% of saturation was dissolved in 20 mM imidazole-HCl buffer, pH 6.5, containing 50 mM NaCl and dialyzed against the same buffer. Afterward, the extract was chromatographed on an SP-Sepharose (Amersham Pharmacia Biotech AB, Uppsala) column (30  $\times$  2.5 cm). It was eluted in 20 mM imidazole-HCl buffer, pH 6.5, containing a linear gradient of NaCl (50 mM to 1 M), and 4.5-mL fractions were collected. The active fractions were pooled and brought to 90% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.

In preparation for affinity chromatography, the precipitate was dissolved in binding buffer (20 mM sodium acetate buffer, pH 5.0, containing 200 mM NaCl, 1 mM CaCl<sub>2</sub>, and 1 mM Mn<sub>2</sub>Cl) and then was dialyzed against the same buffer. The partially purified extract was then applied to a Con-A-Sepharose (Amersham Pharmacia Biotech) column (3 × 1.5 cm) and was washed with binding buffer. Retained proteins were then eluted with 80 mM methyl-*O*-D-glucopyranoside, dissolved in the same buffer, and immediately brought to 90% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.

The precipitate was dissolved in 20 mM sodium acetate buffer, pH 4.5, containing 200 mM NaCl and was then dialyzed against the same buffer. The protein extract was applied on a Sephacryl S-100-HR (Sigma) column (100 × 1 cm) and 1.5-mL fractions were collected. All the purification procedures were performed at 4°C.

### SDS-PAGE

Gel permeation selected fractions containing  $\alpha$ -xylosidase activity and 70  $\mu$ g of total protein was pooled and divided in two similar aliquots. These were then concentrated with a Centricon-10 (Millipore Corporation, Bedford, MA), vacuum dried, and analyzed by SDS-PAGE on two 7.5% (w/v) gels using Mini-Protean II Electrophoresis Cell (Bio-Rad, Hercules, CA), following the manufacturer directions. After a brief staining with Coomassie Blue, the only visible band was sliced from both gels. Eurosequence (Groningen, Netherlands) performed N-terminal sequence with one band and tryptic peptide sequencing with the other.

Analytical electrophoresis was done as described above, except for staining where Silver Stain Kit, Protein (Amersham Pharmacia Biotech) was used. Silver Stain SDS-PAGE standards, high range (Bio-Rad), were employed to determine apparent molecular weights.

### IEF

IEF was conducted using a 110-mL focusing column (AMPHOLINE 8100, Amersham Pharmacia Biotech) according to the manufacturer specifications. AMPHOLINE ampholites (Amersham Pharmacia Biotech) were employed to create the pH gradient (3.5–10). After 24 h at 500 V, 5-mL fractions were collected.

### Genetic Material

Genomic DNA clones from IGF-BAC library (Mozo et al., 1998b) and cDNA clones from Kieber and Ecker Columbia library (Kieber et al., 1993) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus).

### PCR Screening

BACs suspected to include *AtXYL1* were purified from *Escherichia coli* cultures (500 mL) using Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany). Primers SCREEN1 (5'-TTCTCACCCACCCAATCCTA-3') and SCREEN2 (5'-

GATAAGTTCGGATCCGGAGA-3') were employed for PCR in the following conditions: 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min.

### Nucleotide Sequencing

DNA was sequenced using T7 Sequenase Quick-denature Plasmid Sequencing Kit (Amersham Pharmacia Biotech). Gels were run in Sequi-Gen GT Sequencing Cell (Bio-Rad).

### RACE

Enhanced Avian Reverse Transcriptase (Sigma) was employed according to the manufacturer instructions to produce single-stranded cDNA from 1.7  $\mu$ g of RNA extracted from the leaves of 16-d-old Arabidopsis plants. RACE1 primer (5'-CTACATTGATGAAATCCTAAAG-3'), annealing to the region encompassing exons 2 and 3 in *AtXYL1* RNA, was employed. A 5'-end polyA tail was then incorporated to the cDNA using Terminal Deoxynucleotidyl Transferase (Amersham Pharmacia Biotech). Double-stranded cDNA was subsequently produced with RACE2 primer (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT-3') and Accu *Taq* LA DNA Polymerase (Sigma) in the following conditions: 94°C for 3 min, 30°C for 30 s, and 45°C for 5 min. RACE3 (5'-GACTCGAGTCGACATCGA-3') and a second gene-specific primer, SCREEN2, were then added to the reaction mixture and 40 cycles of PCR amplification were performed (94°C for 1 min, 50°C for 1 min, and 68°C for 1 min). A clear band (approximately 550 bp) was observed in agarose electrophoresis, purified with Wizard PCR preps (Promega, Madison, WI), reamplified, and then finally sequenced.

### Computer Analysis

Family 31 cladogram was based on multiple alignments done with CLUSTAL W (1.5). Trees were then drawn with Windows Easy Tree v 1.32 (Joaquin Dopazo, Glaxo Wellcome, Spain) using the Neighbor Joining Method. EST placement was based in a separate tree that only included data from the region where they all overlap. Complete sequences were used to place the other branches. Similarity searches were done with the National Center for Biotechnology Information BLAST 2.0 set of programs (Altschul et al., 1997). Promoter analysis was done with MatInspector V 2.2 (Genomatix Software, Munich) and PatSearch 1.1 using the TRANSFAC 4.0 plant matrices and the TRANSFAC 3.5 sites (Wingender et al., 2000).

### Apoplasmic Fraction Extraction

The apoplasmic fluid from Arabidopsis seedlings was obtained as described by Monroe et al. (1999). Seedlings, grown on water for 3 d, were transferred to 10 mL of 0.1 M sodium acetate buffer, pH 4.5, containing 1 M NaCl, and



were shaken for 6 h at 120 rpm. The saline extract was considered as the apoplastic fraction.

Seedlings were then homogenized in 5 mL of 0.1 M sodium acetate buffer, pH 4.5, containing 1 M NaCl. The homogenate was shaken for 2 h and was centrifuged at 15,000g for 20 min. The supernatant was considered as the cytoplasmic fraction. Both extracts were concentrated with a Centricon-10 (Millipore) to approximately 200  $\mu$ L and were then diluted to 2 mL with 100 mM sodium acetate, pH 4.5. This procedure was repeated twice. The extract was concentrated and  $\alpha$ -xylosidase activity was then measured.

The absence of cytoplasmic contamination was assessed according to Sánchez et al. (1997) by measuring G6PDH. To measure this activity, extracts were obtained as stated above except for the extraction buffer that was 0.1 M MES [2-(*N*-morpholino)-ethanesulfonic acid]-HCl, pH 6, containing 1 M NaCl. G6PDH activity was determined measuring the absorption increase at 340 nm, in a reaction mixture (1 mL) containing 100  $\mu$ L protein extract, 1 mM Glc-6-P, 0.2 mM NADP<sup>+</sup>, 1 mM MgCl<sub>2</sub>, and 100 mM Tricine [N-[2-hydroxy-1,1-Bis(hydroxymethyl)ethyl]glycine]-NaOH, pH 8.0 (Takahama, 1993).  $\alpha$ -Xylosidase activity was also determined in these extracts by hydrolysis of xyloglucan oligosaccharides, as described earlier.

### RNA and Protein Extraction in Arabidopsis

The first eight true leaves of 19-d-old plants were harvested, sorted into four groups according to their appearance order, and immediately frozen in liquid nitrogen. For each pair of leaves, protein was extracted from 200 mg of tissue (100 mg for 7th–8th leaves) in 800  $\mu$ L of 1 M sodium acetate, pH 4.5, with the addition of 4  $\mu$ L of 100 mM phenylmethylsulfonyl fluoride stock solution. After 2.5 h of shaking at 4°C, the homogenate was centrifuged twice at 10,000g for 10 min. In each case the supernatant was collected. The extract was then concentrated with a Centricon-10 (Millipore) to approximately 100  $\mu$ L and was diluted to 2 mL with 100 mM sodium acetate, pH 4.5. This procedure was carried out three times. After a last concentration step the volume was adjusted to 400  $\mu$ L with the same buffer.

RNA was extracted from 200 mg of each category of leaves (60 mg for 7th–8th leaves) with Rneasy Plant Mini Kit (Qiagen, Valencia, CA) and was quantified through A<sub>260</sub>.

### RT-PCR

Enhanced Avian RT-PCR Kit (Sigma) was employed as recommended by the manufacturer to perform one-step RT-PCR. RACE1 primer was chosen to avoid amplifying genomic DNA, and RT1 (5'-TCCATCGAAGAATCTCCCGA-3') was the second primer. The expected product was 782 bp long. For each leaf category, a reaction was set up that included 500 ng of RNA, 50 pmol of each primer, and 2.75 mM MgCl<sub>2</sub> in a total volume of 50  $\mu$ L. After 45 min at 45°C, PCR tubes were heated at 94°C for 3 min, followed by 12 cycles of 94°C for 1 min, 50°C for 1 min, and 68°C for 1 min, followed by 68°C for 4 min.

RACE1 and RT1 primers show 20% and 14% mismatch when aligned with *AtXYL2*. To unequivocally identify the amplified mRNA, a longer amplification (40 cycles) was performed in similar conditions with RNA from the youngest leaves. The resulting band was purified and identified as an *AtXYL1* amplification product by restriction mapping.

### Southern Hybridization

A digoxigenin-labeled probe was produced with digoxigenin-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Mannheim, Germany) using as template a 593-bp DNA fragment amplified by PCR from clone H8A7, using primers RACE1 and HYB1 (5'-GAACAACCACCACAAGTCGG-3').

RT-PCR products from 12 cycles of reactions (15  $\mu$ L) were electrophoresed in a 0.9% (w/v) agarose gel containing ethidium bromide. After the gel was photographed, it was blotted to a positively charged nylon membrane (Roche) using VacuGene XL Vacuum blotting System (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Probe detection was done with DIG-High Prime DNA Labeling and Detection Starter Kit II. In brief, after blocking the membrane, hybridization was carried out overnight and then membranes were washed, blocked, incubated with anti-DIG-AP antibodies, washed again, and finally incubated with disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo{3.3.1.1<sup>3,7</sup>}decan]4-yl)-Star chemiluminescent reagent.

### Heterologous Expression

FLAG Yeast N-Terminal Expression System (Sigma) was employed as suggested by the manufacturer. *AtXYL1* coding region, excluding the potential signal peptide, was amplified by PCR using H8A7 clone as a template and EXP-1 (5'-GAATTCTACAAAACCATCGGCAAAGGCTA-3') and EXP-2 (5'-GCGGCCGCTTAATTGATACCCATTTTCAGGA-3') as primers. These primers include restriction sites for *EcoRI* and *NotI*, respectively. PCR conditions were as follows: 94°C for 2 min followed by 25 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 2 min, followed by 72°C for 5 min. Ex-*Taq* polymerase (Takara, Shiga, Japan) was used, according to the manufacturer's recommendations. The reaction included 25 pmol of each primer and 50 ng of template.

A unique PCR product of correct size was observed on agarose electrophoresis, purified, and ligated to YEFLAG expression Vector, using T4DNA ligase (Promega). XL1-Blue *E. coli* was transformed with the recombinant plasmid (Sambrook et al., 1989). BJ3505 *Saccharomyces cerevisiae* was then transformed with plasmid purified from *E. coli* as indicated by the Expression System manufacturer.

Fresh saturated cultures of two independently transformed colonies were used to inoculate 500-mL flasks containing 100 mL of YP4 high stability expression medium (1% [w/v] Glc, 3% [w/v] glycerol, 1% [w/v] yeast extract,

8% [w/v] peptone, and 20 mM CaCl<sub>2</sub>) or YP expression medium (1% [w/v] Glc, 3% [w/v] glycerol, 1% [w/v] yeast extract, and 2% [w/v] peptone). Cultures were grown for 48 or 96 h, respectively, at 30°C and 200 rpm. Cells were harvested by centrifugation (4,500g for 10 min), disrupted with glass beads, and extracted in 5 mL of 1 M sodium acetate buffer, pH 4.5, for 2 h. The extracts were brought to 90% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the precipitate were resuspended in 0.1 M sodium acetate buffer, pH 4.5. Control transformations were performed as indicated by the manufacturer and cells were grown and treated as above.

Purification of the expressed protein was performed as indicated by the manufacturer using a 1-mL column packed with ANTI-FLAG M1 Affinity Gel. The extract containing  $\alpha$ -xylosidase activity was filtered, concentrated, and diluted with water repeatedly and then finally diluted with buffer to achieve a final concentration of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 10 mM CaCl<sub>2</sub>. This sample was loaded in the affinity column and after several washes with the same buffer, the retained protein was eluted in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 2 mM EDTA.

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