Differential Expression of *AtXTH17*, *AtXTH18*, *AtXTH19* and *AtXTH20* Genes in *Arabidopsis* Roots. Physiological Roles in Specification in Cell Wall Construction

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Xyloglucan endotransglucosylase/hydrolases (XTHs) are a class of enzymes that are capable of splitting and reconnecting xyloglucan molecules, and are implicated in the construction and restructuring of the cellulose/xyloglucan framework. Thirty-three members of the XTH gene family are found in the genome of *Arabidopsis thaliana*, but their roles remain unclear. Here, we describe the tissue-specific and growth stage-dependent expression profiles of promoter::GUS fusion constructs for four *Arabidopsis* XTH genes, *AtXTH17*, *AtXTH18*, *AtXTH19* and *AtXTH20*, which are phylogenetically closely related to one another. *AtXTH17* and *AtXTH18* were expressed in all cell types in the elongating and differentiating region of the root, while *AtXTH19* was expressed in the apical dividing and elongating regions, as well as in the differentiation zone, and was up-regulated by auxin. In contrast, *AtXTH20* was expressed specifically in vascular tissues in the basal mature region of the root. This expression analysis also disclosed cis-regulatory sequences that are conserved among the four genes, and are responsible for the root-specific expression profile. These results indicate that the four XTH genes, which were generated by gene duplication, have diversified their expression profile within the root in such a way as to take responsibility for particular physiological roles in the cell wall dynamics.

**Keywords**: *Arabidopsis* — Auxin — Cell wall — Promoter — Root — XTH.

Introduction

The plant cell wall plays a critical role in the specification of cell types in plants, in that each cell is characterized by the cell wall with specific structural features and physiological functions (Martin et al. 2001). Furthermore, the cell wall undergoes construction and restructuring continuously during the developmental process (reviewed by Carpita and Gibeaut 1993, Nishitani 2002). Thus, the cell wall structure and, hence, the machinery required for cell wall metabolic processes, are intrinsic to both the cell type and its developmental stage (Carpita and Gibeaut 1993, Brett and Waldron 1996, Cosgrove 2000, Nishitani 2002). Xyloglucans occur in both dicotyledonous and monocotyledonous plants, and are found in various tissues at different developmental stages, including cell plates of dividing cells (Moore and Staehelin 1988), in the primary walls of growing cells (Nishitani and Masuda 1981) and in fully differentiated secondary walls (Bourquin et al. 2002). Xyloglucans play a central role as cross-linking glycans that tether cellulose microfibrils, thereby forming the basic framework of the cell wall. Given the ubiquitous occurrence and the principal role for xyloglucan in various types of plant cell walls, a set of genes which are involved in xyloglucan metabolism are promising molecular markers for cell wall types and, hence, cell types.

The xyloglucan endotransglucosylase/hydrolases (XTHs) are a family of enzymes that catalyze molecular grafting and/or hydrolysis of xyloglucans (Fry et al. 1992, Nishitani and Tominaga 1992, Okazawa et al. 1993, Fry 2004) and, thereby, play a principal role in the construction and restructuring of xyloglucan cross-links in the cellulose/xyloglucan framework. XTHs are typically encoded by large multigene families in flowering plants (reviewed in Nishitani 1997, Rose et al. 2002, Yokoyama and Nishitani 2001a, Yokoyama and Nishitani 2000, Yokoyma and Nishitani 2001a, Yokoyama and Nishitani 2001b, Hyodo et al. 2003, Nakamura et al. 2003, Yokoyama et al. 2004). Expression analysis of these genes has revealed that most of the family members exhibit distinct expression patterns in terms of tissue specificity, and that they respond differently to hormonal signals (Xu et al. 1995, Xu et al. 1996, Akamatsu et al. 1999, Yokoyama and Nishitani 2000, Yokoyama and Nishitani 2001a, Yokoyama and Nishitani 2001b, Hyodo et al. 2003, Nakamura et al. 2003, Yokoyama et al. 2004). The ubiquitous occurrence of xyloglucans in various cell types, and cell type-specific expression profiles of individ-

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ual XTH genes (Yokoyama and Nishitani 2001b, Rose et al. 2002) imply that a division of roles among XTH genes exists in differing physiological processes.

Our previous phylogenetic study on Arabidopsis XTH genes has shown that the four members, AtXTH17, AtXTH18, AtXTH19 and AtXTH20, are phylogenetically closely related, and that they share similar nucleotide sequences in their promoter regions as well as in the coding regions (Yokoyama and Nishitani 2001b). Expression analysis of these four genes by using real-time reverse transcription–polymerase chain reaction (RT–PCR) has shown that all of these genes are expressed preferentially in roots. This raises the question as to whether each of these genes plays a particular role, or whether they are functionally redundant. To gain insight into the roles of these four genes, we have examined promoter activities of the four genes using transgenic Arabidopsis plants bearing β-glucuronidase (GUS) fusion genes. In this report, we focus on the tissue-specific expression profiles of the four genes in Arabidopsis roots. We also describe cis-regulatory regions that are conserved among the four genes, and that are responsible for gene expression in roots.

**Results**

**Tissue-specific expression profiles**

Our previous phylogenetic analysis of the Arabidopsis XTH gene family based on their genomic structures has shown that AtXTH17, AtXTH18, AtXTH19 and AtXTH20 genes were generated relatively recently by gene duplication processes, and that they are located on three different chromosomes of Arabidopsis (Arabidopsis Genome Initiative 2000, Yokoyama and Nishitani 2001b). Multiple alignment of the promoter regions of the four genes using the CLUSTAL W algorithm (http://clustalw.genome.jp) has disclosed a run of nucleotide sequences that are highly conserved among the four genes (–221 to –157 bp for AtXTH17, –226 to –162 bp for AtXTH18, –256 to –192 bp for AtXTH19, –219 to –157 bp for AtXTH20). The nucleotides that are identical among the four sequences are indicated as asterisks under the sequences. The TATA box is indicated by solid lines above the nucleotide sequences.

**Fig. 1** Comparison of AtXTH17–20 promoter sequences by multiple alignment. The alignment of AtXTH17 (~280 to –15 relative to the translation start site), AtXTH18 (~285 to –21), AtXTH19 (~300 to –21) and AtXTH20 (~221 to –24) was constructed using the CLUSTAL W algorithm. The black box indicates that nucleotide sequences are highly conserved among the four genes (~221 to ~157 bp for AtXTH17, ~226 to ~162 bp for AtXTH18, ~256 to ~192 bp for AtXTH19, ~219 to ~157 bp for AtXTH20). The nucleotides that are identical among the four sequences are indicated as asterisks under the sequences. The TATA box is indicated by solid lines above the nucleotide sequences.
Fig. 2  GUS activities of 5′-truncated XTH promoter::GUS fusion genes in shoots and roots of transgenic Arabidopsis plants. Schematic representation of the full-length (>1 kb) and 5′-truncated XTH promoter::GUS fusion genes. +1 represents the translation start site. Black boxes indicate nucleotide sequences that are highly conserved among the four genes (see text), while gray shaded boxes represent TATA boxes. (B) Expression profile of the untruncated XTH promoter::GUS fusion for the four XTH genes in 4-day-old seedlings, as visualized by GUS staining assay. (C and D) Expression of individual GUS fusion constructs in roots and shoots of transgenic Arabidopsis, respectively. The numbers of independent transgenic Arabidopsis lines for individual constructs used for the GUS assay are indicated in parentheses. The shoots and roots of 7-day-old light-grown seedlings of individual transformants were assayed separately for GUS activity. The GUS activities are expressed as nmol of 4-methylumbelliferone produced during 1 min by 1 mg of soluble protein. Note that the level of the GUS activity in roots (C) is five times larger than that in shoots (D), indicating that the four XTH genes exhibit root-preferential expression.

Fig. 3  Expression profiles of XTH promoter::GUS fusion genes in Arabidopsis roots of 7-day-old seedlings. (a) pAtXTH17::GUS, (b) pAtXTH18::GUS, (c) –329pAtXTH18::GUS, (d) –120pAtXTH18::GUS, (e) pAtXTH19::GUS, (f) –330pAtXTH19::GUS, (g) –110pAtXTH19::GUS, (h) pAtXTH20::GUS, (i) –237pAtXTH20::GUS, (j) –120pAtXTH20::GUS, (k) XET activity as disclosed by incorporation of XGO-SR in wild-type.
the root (cf. Fig. 2B–D) of 4- or 7-day-old light-grown seedlings. Relative expression levels of the four GUS fusion genes in roots are consistent with the expression level of their mRNA as studied by quantitative real-time RT–PCR (Yokoyama and Nishitani 2001b), suggesting that the expression patterns of the untruncated promoter::GUS reporter fusion genes mirror transcription patterns of the four genes in Arabidopsis seedlings.

Each of the four untruncated promoter::GUS fusion genes exhibits a characteristic expression profile along the root of 7-day-old light-grown seedlings (Fig. 3a, b, e, h, 4a, b, c, d).

AtXTH17 and AtXTH18 genes were expressed in mature or basal regions of both the main and lateral roots, but not in the tip of these roots where cell division occurs (Fig. 3a, b, 4a, b). In contrast, AtXTH19 was expressed throughout both the main root and the lateral root, with intensive expression being observed at the dividing and elongating regions (Fig. 3e, 4c). AtXTH20 exhibited a unique expression profile, in that it was expressed chiefly in the stele of mature non-elongating regions.
of both the main root and the lateral root (Fig. 3h, 4d). It should be noted that AtXTH20, like AtXTH19, is expressed in the primordium of the lateral root, but its expression ceases after the lateral root begins to grow (cf. Fig. 4c, d). Transverse sections of the main roots of 4-day-old seedlings have revealed that AtXTH17, AtXTH18 and AtXTH19 were expressed evenly in all the cell types, including the root hairs and vascular tissues (Fig. 5a, b, c, d, g, h). In contrast, AtXTH20 was expressed intensively only in the endodermis, the pericycle and the vascular tissues (Fig. 5a, b, c, d, g, h). Although a slight GUS staining is observed in cortical cells in the transverse section of the root (Fig. 5k), no staining in cortical cells is observed in the intact root under a stereomicroscope (Fig. 5l). It is possible that the slight GUS staining in the cortical cells in the section might be due to diffusion of the blue stain from the neighboring cells where GUS is expressed extensively.

The profile of the xyloglucan endotransglucosylase (XET) action of XTH along the root, as visualized by the incorporation of fluorescent xyloglucan oligomers into the cell wall, shows that the enzyme action was found throughout the root, with increased action located in the elongating region (Fig. 3k, 4e, 5m), and in the trichoblast cells at sites of future root hair emergence (Vissenberg et al. 2001). This feature has been found not only in Arabidopsis, but also in representatives of all vascular land plants, from Selaginella to the most complex angiosperms (Vissenberg et al. 2003). The fact that the XET action is found in the majority of cell types along the length of the root, and that any expression profile of the AtXTH17, AtXTH18, AtXTH19 and AtXTH20 genes in the root does not exactly represent the profile of the enzyme action, as disclosed by the fluorescent labeling, indicates that the enzyme action cannot be attributed to a single XTH protein, but to the sum of two or more XTH proteins.

Analysis of promoter regions of the four genes

Next, we examined the expression of 5’-truncated promoter::GUS fusion genes. Deletions to –329 and –330 bp in the 5’ promoter regions of AtXTH18 and AtXTH19, respectively, led to an approximately 90% reduction in their GUS activities (Fig. 2C). We did not examine expression of 5’-truncated AtXTH17 because it shares almost the same nucleotide sequence as AtXTH18, from the translation start site to –0.3 kb.

Whereas deletion of the AtXTH18 promoter to –329 bp led to a drastic reduction in the GUS expression levels, the deletion did not affect the pattern of GUS staining along the root (cf. Fig. 3b, c). Further deletions of the 5’ promoter regions to –120 bp clearly nullified their activities (Fig. 2C, 3c, d). These results indicate that the promoter region between –329 and –120 bp contains the nucleotide sequence that is necessary for specification of gene expression in the root, while the upstream region between –1,460 and –330 bp is capable of enhancing the expression in the root.

The truncation of the 5’ promoter regions of AtXTH19 caused not only a reduction in the GUS expression level (Fig. 2C), but also an apparent change in the GUS staining profiles along the root (Fig. 3e, f). Whereas the expression of –330pAtXTH19::GUS was still observed in the elongating and differentiating regions of the root, it was no longer observed in the apical dividing region (Fig. 3e, f). Further truncation to –110 bp led to a complete loss of expression (Fig. 2C, 3f, g). This indicates that the conserved sequence located between –330 and –110 bp is required for expression of the AtXTH19 gene in the elongating and differentiating region of the root, whereas the region between –1,096 and –330 bp contains the sequence which is necessary for expression in the apical region of the root.
In contrast, the −237pAtXTH20::GUS fusion gene was no longer expressed in the root, despite the fact that this construct contained the conserved sequence (Fig. 2C, 3h, i, j).

Responses to signals

In the −956 to −951 bp region upstream of the AtXTH19 gene, the sequence TGTCTC was found. To examine the responsiveness of the promoter region to indole-3-acetic acid (IAA), we applied 0.1 nM IAA to 5-day-old transgenic plants, and incubated them for an additional 2 days. The IAA treatment increased 3-fold the expression of the AtXTH19::GUS fusion gene, which contained the TGTCTC sequence (Fig. 6), but no prominent enhancement by the hormone application was observed for other promoter::GUS fusion genes. Thus, AtXTH19 is clearly distinct from other members, in terms of the response to auxin in the root.

Finally, we examined the effect of light conditions on the expression of the four genes. Seedlings were grown in the dark or in the light for 7 days, and the roots were subjected to quantitative GUS expression analysis. The data show that expression of AtXTH17, AtXTH18 and AtXTH19 was greatly increased in the dark, whereas the expression of AtXTH20 was reduced in the dark as compared with in the light. A typical result is shown in Fig. 7. Thus, the four genes responded differently to hormones and light conditions and exhibited differential tissue expression specificities.

Discussion

Root tissues, such as the epidermis, cortex and vascular tissues, are all arranged in a well-described radial pattern. Cells in individual root tissues are arrayed longitudinally along the root axis, in order of their developmental stage from the apical meristem to the basal mature region. Based on the developmental stages, the longitudinal axis of the root is broadly classified into three zones: the division zone; the elongation zone; and the differentiation zone (Rost and Bryant 1996, Costa and Dolan 2000). Individual cell types at different developmental zones display distinct structural features of the cell wall, characteristic of both its developmental stage and cell type. The present study has shown that the four AtXTH genes exhibit characteristic expression profiles in terms of the developmental stage and the tissue type of the root. AtXTH17 and AtXTH18 are expressed in all tissue types in the elongation and differentiation zone, while AtXTH19 expression is found in all tissue types throughout the whole root. In contrast, AtXTH20 is expressed in the vascular tissues in the basal region of the differentiation zone.

The proteins encoded by the four genes in this study belong to the class II subfamily in the XTH protein family. Members of this subfamily have been shown to exhibit exclusively NTE activity. Given that the four AtXTH genes show a striking resemblance to each other, and that they share the same catalytic activity, they are redundant in terms of enzymatic action. Then, the physiological significance of the gene duplication is that it leads to an increased variation in the expression profiles of individual cells of the root. Alternatively, as the proteins are not identical, one could envisage that the four enzymes have differing enzymatic characteristics, such as subtle differences in substrate affinities, pH dependencies and mode of catalytic action (Campbell and Braam 1999a, Campbell and Braam 1999b). If they differ in their enzymatic actions, the gene duplication leads to increased variation both in enzymatic functions and in expression profiles. To address this issue, further analysis of their enzymatic action in vivo is necessary.

Comparison of the GUS expression profile among the pAtXTH18::GUS, −329pAtXTH18::GUS and −120pAtXTH18::GUS fusion genes has suggested that an enhancer region is present between −1,460 and −329 bp upstream of the translation start site of the AtXTH18 gene. Deletion of AtXTH18 to −329 bp led to a drastic reduction in the GUS activity without causing any change in the expression region along the root. At present, no well-documented sequences potentially involved in the enhancer activity or root-specific expression is found in the upstream region of AtXTH18.

In contrast, the deletion of the AtXTH19 promoter to −330 bp not only led to a reduction in the GUS expression level, but it also caused a drastic alteration in the expression profile along the root. The AtXTH19 promoter contains the TGTCTC sequence that is implicated in ARF-mediated regulation of transcription by auxin signaling (Liu et al. 1994, Ulmasov et al. 1997). The application of 0.1 nM IAA caused a 3-fold up-regulation of the AtXTH19::GUS gene in the root, whereas the hormone did not affect expression of the −330pAtXTH19::GUS gene, in which the TGTCTC sequence was deleted. It is therefore quite likely that the TGTCTC sequence functions as the cis-acting element necessary to confer auxin responsiveness to the AtXTH19 gene. The expression of −330pAtXTH19::GUS was only observed in the basal region of the root, but not in the apical region. Thus, it is probable that the expression of AtXTH19 in the apical region of the root might be related to its responsiveness to auxin signaling, although this has not yet been demonstrated, and further dissection of the promoter region is needed. Auxin is involved in various aspects of growth and differentiation of roots, such as cell elongation, differentiation of vascular tissues, formation of lateral root primordial and root hair formation (Pitts et al. 1998, Mattsson et al. 1999, Sabatini et al. 1999, Himanen et al. 2002). Thus, the AtXTH19 gene, which is expressed in all cell types in the root, may be involved in various aspects of the auxin-regulated cell wall dynamics. Apart from the TGTCTC element, no well-documented element has been found in the region between −1,096 and −330 bp of the AtXTH19 promoter.

During the evolution of the AtXTH genes, the root-preferential gene expression pattern has been conserved among the four genes. This implies that the nucleotide sequences conserved among their promoter regions is necessary for
this expression in roots. The GUS expression profiles of –329\textit{AtXTH18}::GUS and –330\textit{AtXTH19}::GUS suggest that the cis-acting region responsible for the root-preferential expression resides in the promoter regions between approximately –0.3 and –0.1 kb, at least in \textit{AtXTH18} and \textit{AtXTH19}. In contrast, \textit{pAtXTH20}::GUS exhibits an expression profile that is different from the other three genes, in that it is expressed specifically in vascular tissues. In addition, –237\textit{pAtXTH20}::GUS, which also possesses the conserved sequence, is not expressed in the root at all. This means that the sequences thought to be conserved among the four genes have diversified in terms of their cis-activities. In fact, \textit{AtXTH20} is less homologous to \textit{AtXTH18} and \textit{AtXTH19} than \textit{AtXTH18} is to \textit{AtXTH19}. Our previous phylogenetic study suggested that \textit{AtXTH20} was generated by duplication of the ancestor of the \textit{AtXTH17}, \textit{AtXTH18} and \textit{AtXTH19} genes, before the three genes diversified (Yokoyama and Nishitani 2001b). Thus, it is likely that the promoter function of \textit{AtXTH20} has changed, and the sequence found between –219 and –157 bp of \textit{AtXTH20} is no longer sufficient in directing expression of this gene in the root. However, we cannot exclude the possibility that the sequence, while not sufficient in itself to direct the root-specific expression, may still play a role in this process. The well-documented cis-element potentially involved in the peculiar pattern of \textit{AtXTH20} expression in root is not found between –219 and –157 bp of the gene, nor are found any putative cis-regulatory sequences that might direct the root-specific expression of the gene outside this conserved region of the gene. Whereas detailed promoter dissection for the \textit{AthH20} gene is not carried out in the present study and needs further investigation, it is clear that \textit{pAtXTH20}::GUS is expressed preferentially in cells with secondary cell walls, including xylem and phloem. Bourquin et al. (2002) have demonstrated the presence of an \textit{XTH} member that is expressed specifically in cells where secondary walls are being constructed and modified. This line of evidence strongly suggests the possible involvement of \textit{AtXTH20} in the secondary wall construction in the vascular tissues of the \textit{Arabidopsis} root.

Based on our previous data on the expression levels of \textit{Arabidopsis} XTH genes obtained by quantitative RT–PCR procedures and those obtained by Campbell and Braam (1999a), we have proposed the hypothesis that each member of the XTH gene family is regulated specifically by different sets of plant hormones, and is involved in certain specific processes in a specific tissue, at specific stages of development (Yokoyama and Nishitani 2001b, Nishitani 2002). The present study, in which we focused on the expression profiles of the \textit{XTH} gene family encoding enzymes implicated in the construction and modification of the cell wall, provides evidence in support of this hypothesis. Recently, Bimbaum et al. (2003) have determined cell types using DNA microarray technology and cell sorting procedures, and depicted the gene expression map for \textit{Arabidopsis}. Their analysis has disclosed sets of regulatory genes expressed coordinately in each cell type, at each developmental stage of the root. This evidence leads us to assume that a master gene, specific for each cell type, controls a set of enzymes required for certain types of cell wall structure and, thereby, defines the cell wall type, and hence cell type, during the process of plant development. It is not known which types of transcriptional regulatory systems govern these enzymes. The nucleotide sequence well conserved among \textit{AtXTH17}, \textit{AtXTH18} and \textit{AtXTH19} will be a promising probe to gain insight into the regulatory system by which root cell walls are constructed and modified during the root development and maturation processes.

**Materials and Methods**

**Plant materials**

Seeds of \textit{A. thaliana} (L.) Heynh. ecotype Columbia and its transgenic plants were sown and grown on rock wool moistened with MGRl medium (Tsukaya et al. 1991), or sterilized, germinated and grown on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3% sucrose at 22°C under continuous illumination at 100 µmol m\(^{-2}\) s\(^{-1}\).

**Hormone treatment**

For the examination of IAA action on the expression of the four \textit{XTH} promoter::GUS fusion genes, seedlings were grown on vertically oriented solid MS medium for 5 days, then transferred to a new solid medium with or without 0.1 mM IAA, and were grown for an additional 2 days. GUS activity in the roots of the 7-day-old seedling was measured. Mean values are shown with the SDs as horizontal lines (see Fig. 6).

**Promoter::GUS fusion constructs**

Genomic fragments of the promoter regions of the four \textit{AtXTH} genes were amplified from genomic DNA of \textit{A. thaliana} Columbia using the following forward and reverse PCR primer sets for: \textit{AtXTH17} (1,014 bp), 5’-CATAAGCTTTAATGTGTTTGGTAGACAGAAATAA-3’ and 5’-TTGGAAGCATGCTGTTCCTCTTGA-3’; for \textit{AtXTH18} (1,460), 5’-GATGCTGATGACGGTGGCGTTCCTTTTATGTGTA-3’ and 5’-ACTGGATCCATTTTATTTTATTTGGAAGGTTTGA-3’; for \textit{AtXTH19} (237), 5’-GGATGCTGATGACAGAATGACATCTTCTCTCCTTCCATCTTCGGTGGG-3’ and 5’-TTTATTAGATCTCTCTAGGAGTTTTGA-3’; for \textit{AtXTH20} (1,096), 5’-TATTGCTTTATTTTGGAAAGGAGCGGCT-3’ and 5’-GATGCTGATGACAGAATGACATCTTCTCTCCTTCCATCTTCGGTGGG-3’. Each of these primers was designed to contain one restriction site (HindIII, SalI or BamHI), which is underlined. The number in parentheses after the gene name denotes the numbers of nucleotide pairs of the genomic fragments used.

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promoter fragments, thus amplified, were cloned in-frame upstream of the GUS gene in the respective restriction sites of pBI101 (Clontech, Palo Alto, CA, U.S.A.) to generate 10 GUS fusion gene constructs (Jefferson et al. 1987), designated pAtXTH17::GUS, pAtXTH18::GUS, pAtXTH19::GUS, pAtXTH20::GUS, pAtXTH21::GUS, pAtXTH22::GUS and pAtXTH23::GUS. Each of these GUS fusion genes was introduced into A. thaliana ecotype Columbia via the Agrobacterium tumefaciens C58 strain, using the floral-dip transformation technique (Clough and Bent 1998). At least 10 independent homozygous transformant lines for each of these constructs were selected, based on the resistance to 50 μg ml⁻¹ kanamycin and/or PCR amplification of insertion sequences.

GUS assay

For GUS enzymatic activity analysis, 7-day-old light-grown seedlings for each independent line of the 10 individual GUS fusion genes were divided into shoots and roots, and were assayed separately for their GUS activities using 4-methylumbelliferyl β-D-glucuronide as the substrate, according to Jefferson et al. (1987). Briefly, roots or shoots of transgenic plants were homogenized in GUS extraction buffer (50 mM NaH₂PO₄ pH 7.0, 10 mM EDTA, 0.1% Triton-X100, 0.1% Sarcosyl, 10 mM β-mercaptoethanol), followed by centrifugation at 10,000g for 5 min. After the protein content of the supernatant was quantified, a portion of the supernatant fraction was mixed with the reaction buffer containing 1 mM MUG extraction buffer (GUS extraction buffer, 1 mM 4-methylumbelliferyl β-D-glucuronide (Nakalai Tesque, Kyoto Japan) with a reaction volume of 250 μl. The reaction mix was incubated at 37°C, and 50 μl aliquots were removed at zero time, and subsequently at 30 and 60 min. The reaction was terminated with the addition of 1.0 ml of 0.2 M Na₂CO₃. Fluorescence intensities were measured by a spectrofluorometer (RF-1500, Shimadzu, Japan) set at 365 nm (excitation) and 455 nm (emission). The protein concentration was estimated by the Bradford method (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The specific activity of the GUS enzyme in extracts was calculated as nmol of 4-methylumbelliferone produced per min per mg of total protein. To determine the GUS activity in extracts of different organs, transgenic seeds were sown in vertically oriented MS plates and grown at 22°C for 7 days. To determine the GUS activity in the dark, seedlings were induced to germinate in the GUS staining solution (0.1 M Na₂CO₃) set at 365 nm (excitation) and 455 nm (emission). The fluorescence pictures were taken using a Nikon DVM1200 digital camera.

Assay for XET action

XET action was assayed, as described by Vissenberg et al. (2000). Arabidopsis roots were incubated in a 6.5 μM sulfurfodamine-labeled xyloglucan oligosaccharide (XGO-SR) mixture [XLGSR-XXLGSR-XXXSR, see Fry et al. (1993) for nomenclature] dissolved in 25 mM MES buffer pH 5.5 for 1 h, followed by a 10 min wash in ethanol/formic acid/water [15:1:4 (by vol.)], and an incubation overnight in 5% formic acid. Fluorescent pictures were taken with a Zeiss Axioskop equipped with a Nikon DVM1200 digital camera under 514 nm excitation light.

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