Characterization of a Xyloglucan Endotransglucosylase Gene That Is Up-Regulated by Gibberellin in Rice[1][w]

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Xyloglucan endotransglucosylases/hydrolases (XTHs) that mediate cleavage and rejoining of the β (1-4)-xyloglucans of the primary cell wall are considered to play an important role in the construction and restructuring of xyloglucan cross-links. A novel rice (*Oryza sativa*) XTH-related gene, *OsXTH8*, was cloned and characterized after being identified by cDNA microarray analysis of gibberellin-induced changes in gene expression in rice seedlings. *OsXTH8* was a single copy gene; its full-length cDNA was 1,298 bp encoding a predicted protein of 290 amino acids. Phylogenetic analysis revealed that *OsXTH8* falls outside of the three established subfamilies of XTH-related genes. *OsXTH8* was preferentially expressed in rice leaf sheath in response to gibberellic acid. In situ hybridization and *OsXTH8* promoter GUS fusion analysis revealed that *OsXTH8* was highly expressed in vascular bundles of leaf sheath and young nodal roots where the cells are actively undergoing elongation and differentiation. *OsXTH8* gene expression was up-regulated by gibberellic acid and there was very little effect of other hormones. In two genetic mutants of rice with abnormal height, the expression of *OsXTH8* positively correlated with the height of the mutants. Transgenic rice expressing an RNAi construct of *OsXTH8* exhibited repressed growth. These results indicate that *OsXTH8* is differentially expressed in rice leaf sheath in relation to gibberellin and potentially involved in cell elongation processes.

The plant primary cell wall is a complex and dynamic structure that plays an important role in controlling cell shape and plant morphology as a whole. Structural modification of the cell wall is important considering regulation of cell growth and differentiation. Flowering plants have type I wall in which the principal cellulose cross-linking glycans is xyloglucan and as much as 35% of the wall mass is pectin (Carpita and Gibeaut, 1993; Cosgrove, 1997). In the type II cell wall of the grasses and cereals, the predominant glycans that cross link the cellulose microfibrils are glucuronoarabinoxylan and (1,3)(1,4)-β-D-glucan (Buckeridge et al., 2004). Type II cell walls contain a relatively low amount of xyloglucan but could nevertheless be very important (Yokoyama et al., 2004). It is considered that structural changes in these networks are regulated by enzymatic modification, and therefore wall-modifying enzymes would be expected to play an important role in regulating the plasticity of the cell walls.

A class of enzymes known as xyloglucan endotransglucosylases/hydrolases (XTHs; Yokoyama and Nishitani, 2001) catalyzes the endo cleavage of xyloglucan polymers and the subsequent transfer of the newly generated reducing ends to other polymeric or oligomeric xyloglucan molecules (Fry et al., 1992; Nishitani and Tominaga, 1992). XTH action seems to achieve regulated wall loosening during turgor-driven expansion by rearranging load bearing xyloglucan cross-links between cellulose microfibrils. XTHs are also considered to catalyze molecular grafting reactions required to integrate nascent xyloglucan polysaccharide into the existing cell wall, maintaining cell wall thickness and integrity (Rose et al., 2002).

The advent of the genome sequencing projects has revealed the presence of multigene *XTH* families in various plant species, and XTH activity has been detected in a variety of plant tissues (Nishitani, 1997; Campbell and Braam, 1999a; Rose et al., 2002). In rice (*Oryza sativa*), a family of 29 *XTH* genes has been deduced from the rice genome sequence (Yokoyama et al., 2004). For any given *XTH* gene family, typically only a few genes have been demonstrated to encode true XTHs (Schroder et al., 1998; Campbell and Braam, 1999b), but the high homology among designated *XTH* genes and the presence of conserved key motifs among them strongly suggest that they encode proteins with XTH activity. In addition to its potential ability to alter and loosen the cell wall matrix, studies have shown a strong correlation between XTH expression and activity to cell elongation zones (Nishitani and Tominaga, 1991; Xu et al., 1996; Vissenberg et al., 2000). Similarly, GA treatment, which induces the elongation of leaves

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and stems in several plant species, increases XTH activity (Potter and Fry, 1994; Smith et al., 1996). Furthermore, specific *XTH* genes have shown to be up-regulated by the growth-promoting hormones like auxin, GA, and brassinosteroid (BR; Zurek and Clouse, 1994; Xu et al., 1996; Catala et al., 1997; Schunmann et al., 1997). However, XTH activity does not always correlate with growth rate, as activity has been detected in vegetative tissues that have ceased to elongate (Smith et al., 1996; Barrachina and Lorences, 1998) and in ripening fruit (Redgwell and Fry, 1993; Maclachlan and Brady, 1994). This indicates that various types of *XTH* genes are associated with wall reorganization during cellular differentiation and fruit ripening (Arrowsmith and de Silva, 1995; Saab and Sachs, 1996; Schroder et al., 1998).

Thus, the presence of XTHs with different tissue-specific expression, hormonal regulation, and/or potentially different enzymatic properties seems to be necessary for the metabolism of xyloglucan during various stages of plant growth and development. Therefore, the characterization of individual *XTH* genes within a single species is essential to understand their specific roles. In this study, a novel *XTH* gene, named *OsXTH8*, was identified by a cDNA microarray analysis of GA-regulated genes in rice. We describe *OsXTH8* developmental and hormonal regulation and discuss its importance in growth processes of rice.

RESULTS

OsXTH8 Is a Novel XTH-Related Gene

A rice *XTH* gene named *OsXTH8* was identified by a cDNA microarray analysis of GA-regulated genes in rice. In an effort to characterize the expression of *OsXTH8* in rice, full-length *OsXTH8* cDNA was amplified by RACE PCR and sequenced. The *OsXTH8* full-length cDNA is 1,298 bp including a poly(A)$^+$ tail, and it encodes a predicted protein of 290 amino acid residues. The deduced amino acid sequence indicated the presence of a putative signal peptide rich in hydrophobic amino acids in the N-terminal region (von Heijne, 1986). The deduced amino acid sequence also showed the presence of a potential site for N-linked glycosylation (N-X-T/S; the nucleotide sequence data reported will appear in the DNA Data Bank of Japan [DDBJ] under the accession no. AB110604).

As *OsXTH* is a multigene family in rice, to examine the precise expression pattern of *OsXTH8* gene during development and in response to hormonal treatment, 3′ untranslated region (UTR) of *OsXTH8* gene was used as a specific probe. To check the specificity of 3′ UTR *OsXTH8*, genomic DNA was digested with *Eco*RI, *Xba*I, and *Xho*I, respectively. Membrane probed with the 3′ UTR region of *OsXTH8* produced a single band (Fig. 1), confirming the specificity of the 3′ UTR probe.

The deduced amino acid sequence of OsXTH8 showed sequence homology and the presence of a functional motif in comparison with other rice

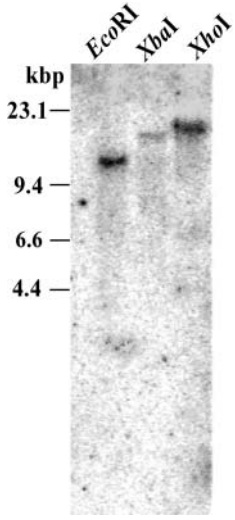

Figure 1. Genomic Southern-blot analysis of *OsXTH8*. Rice genomic DNA was digested completely with *Eco*RI, *Xba*I, and *Xho*I, respectively, separated by agarose gel electrophoresis, and then blotted onto positively charged nylon membrane. The blot was hybridized to 3′ UTR of *OsXTH8*. Molecular size markers are indicated on the left.

XTHs sequences (Fig. 2). OsXTH8 showed a sequence identity of 43%, 46%, 33%, and 38% to OsXTR1, OsXTR2, OsXTR3, and OsXTR4 (Uozu et al., 2000), respectively. XTHs contain a conserved sequence (DEIDFEFLG) that matches the bacterial endo-β-1, 3-1, 4-glucanase (Borriss et al., 1990) and has been suggested as the catalytic center for the XTH family. The corresponding sequence in OsXTH8 has two amino acid substitutions (DEIDIEFMG) compared to the consensus sequence.

To find the evolutionary relationships of OsXTH8 with XTHs of other plant species, a phylogenic tree was generated using full-length protein sequences. The analysis revealed that XTHs could be loosely grouped into four distinct groups (Fig. 3), as reported by other authors (Nishitani, 1997; Campbell and Braam, 1999a; Catala et al., 2001). Group 1 contains genes that share a high level of sequence identity among different species and that are expressed in young developing tissues (Catala et al., 1997; Shimizu et al., 1997; Akamatsu et al., 1999; Takano et al., 1999). Group 2 comprises *XTH* genes from several species showing diverse patterns of expression and response to hormonal or mechanical stimuli, including touch-inducible, flooding-response, BR-inducible, and fruit ripening-related *XTHs* (Catala et al., 2001). Group 3 represents a divergent group of *XTHs*, including *NXG1* from nasturtium (*Tropaeolum majus*) that can act as xyloglucan hydrolase and transglycosylase (de Silva et al., 1993). OsXTH8 has the highest homology to sequences in Group 4, consisting of well-characterized barley (*Hordeum vulgare*) genes, *HvPM2* and *HvPM5*, which are up-regulated by GA in barley leaf sheaths and leaves (Smith et al., 1996). Monocot members of group 4 revealed to have two substitutions

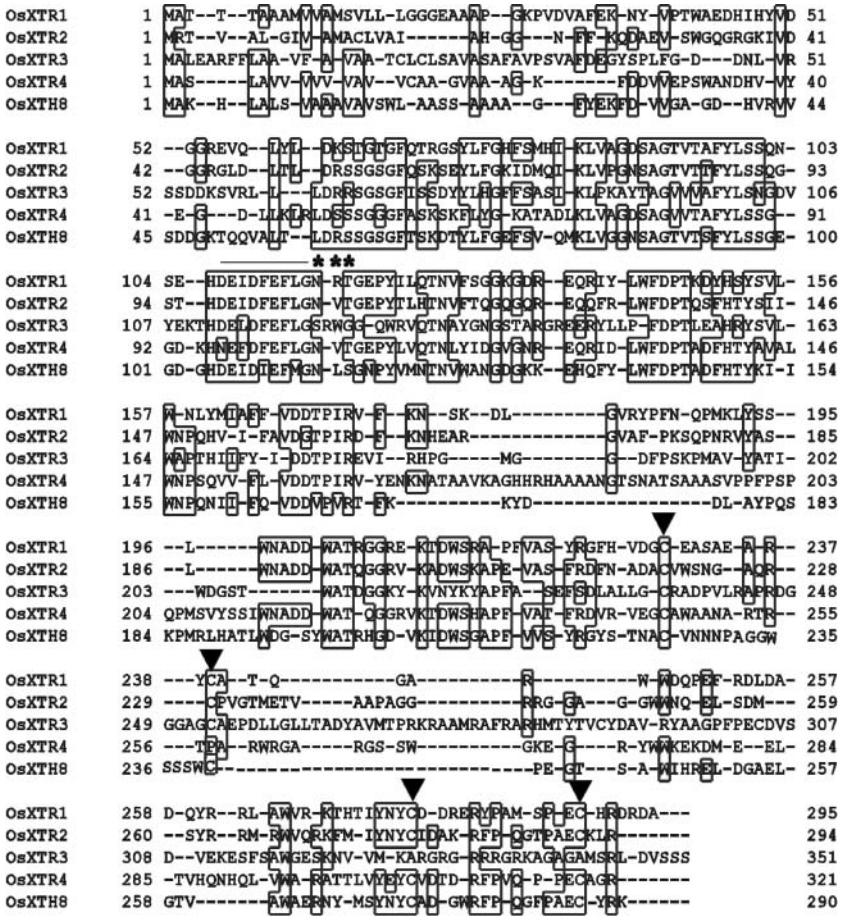

Figure 2. Amino acid sequence alignment of OsXTH8 and other members of the rice *XTH* gene family. The deduced amino acid sequence of OsXTH8 is aligned with other members of rice *XTH* gene family by Genetyx-WIN. DEIDFEFLG motif (marked by line) indicates a possible conserved catalytic region shared with the Bacillus β-glucanase. The possible N-linked glycosylation residues are indicated by asterisks. Conserved Cys residues are marked by arrowheads.

(DEIDIEFMG) compared to the consensus sequence (DEIDFEFLG). The presence of different amino acid residues in the putative catalytic region may attribute to unique enzymatic activity of OsXTH8.

Alternative Organ- and Cell Type-Expression Profiles of *OsXTH8*

It has been shown that different members of *XTH* gene family are specifically regulated by various physiological and environmental stimuli (Xu et al., 1996). In this study, *OsXTH8* was characterized for temporal and spatial expression patterns and its response to different hormones.

To examine the tissue specificity of *OsXTH8*, total RNAs from rice callus, root, leaf blade, and leaf sheath were hybridized with *OsXTH8*-specific DNA probe. A strong signal was detected in leaf sheaths but weak or no signal was observed in leaf blades, roots, and calli (Fig. 4A). These expressions were enhanced by GA_3 treatment (Fig. 4A). When 1-month-old seedlings were used to characterize the expression in leaf sheath, the expression was mainly found in the three basal parts of leaf sheath (Fig. 4B). Enhanced expression of *OsXTH8* in the third part of 1-month-old rice seedling, which corresponded to the second internode of leaf sheath, compared to two basal parts of leaf sheath showed that *OsXTH8* is differentially expressed in leaf sheath.

In situ hybridization was performed using the basal part (culm) of 2-week-old rice seedlings to learn more about the expression pattern. On hybridization with gene-specific *OsXTH8* antisense probe, the cross and longitudinal sections of culm tissue revealed an accumulation of *OsXTH8* mRNA in shoot apex meristem, vascular tissues, and young leaves (Fig. 5A). Although the expression did not seem to be delimited to specific cell types, significant hybridization was observed in large and small vascular bundles of leaf sheath and peripheral cylinder of the vascular bundles and fibers in the nodal region (Fig. 5A). No significant signal was visible when sense probe was used.

To further characterize the spatial distribution patterns of *OsXTH8* gene expression, 2,325 bp of the *OsXTH8* upstream region from the proposed first translational start site was fused to the β-glucuronidase (GUS) reporter gene. This fusion gene was introduced into rice cells, and transgenic plants were regenerated. Putative OsXTH8::GUS transgenic plants were screened by PCR. Only PCR-confirmed, transgenic lines were used for GUS staining and GUS assay. To assess whether the GUS staining patterns were consistent with the result of in situ hybridization, similar

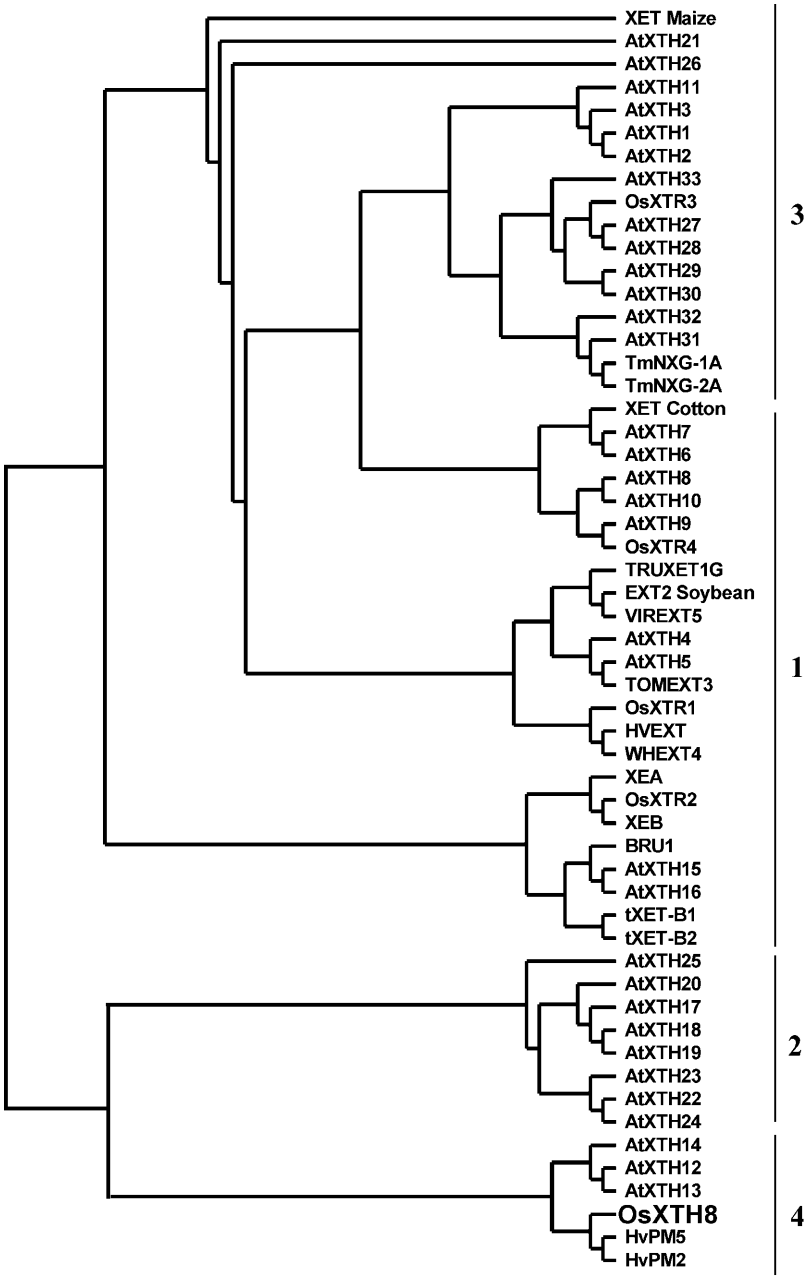

Figure 3. Phylogenic-alignment of the OsXTH8-deduced amino acid sequence with other plant XTHs. OsXTH8 was aligned with 54 full-length deduced amino acid sequences using the ClustalW and tree View software. Details and GenBank accession numbers are: AtXTH1, At4g13080; AtXTH2, At-4g13090; AtXTH3, At3g25050; AtXTH4, At2g-06850; AtXTH5, At5g13870; AtXTH6, At5g65730; AtXTH7, At4g37800; AtXTH8, At1g11545; AtXTH9, At4g03210; AtXTH10, At2g14620; AtXTH11, At3g-48580; AtXTH12, At5g57530; AtXTH13, At5g-57540; AtXTH14, At4g25820; AtXTH15, At4g14130; AtXTH16, At3g23730; AtXTH,17, At-1g65310; AtXTH18, At4g30280; AtXTH19, At30290; AtXTH20, At5g48070; AtXTH21, At2g-18800; AtXTH22, At5g57580; AtXTH23, At-4g25810; AtXTH24, At4g30270; AtXTH25, At5g57550; AtXTH26, At4g28850; AtXTH27, At2g-01850; AtXTH28, At1g14720; AtXTH29, At4g-18990; AtXTH30, At1g32170; AtXTH31, At3g44990; AtXTH32, At2g36870; AtXTH33, At-1g10550; TmNXG-1A, X68254; TmNXG-2A, X68255; TRUXET1G, L43094; VIREXT5, D16458; EXT2 (soybean), D16455; HVEXT, X91659; WHEXT4, D16457; TOMEXT3, D16456; XET (cotton), D88413; tXET-B1, X82685; tXET-B2, X82684; XEB X93175; XEA, X93174; XET (maize), U15781; HvPM2, X91660; HvPM5, X93173; and OsXTH8, AB110604. OsXTR1, OsXTR2, OsXTR3, and OsXTR4 sequences were noted down from Uozu et al. (2000).

tissue sections of leaf sheath were used. Figure 5B shows the GUS expression pattern driven by *OsXTH8* promoter in the basal part of leaf sheath and young nodal roots of 2-week-old rice seedlings. In the case of leaf sheath, strong GUS staining was observed in shoot apex meristem and vascular bundles, very much similar to the result of in situ hybridization (Fig. 5, A and B). Microscopic observation of vascular bundles revealed GUS expression in vascular bundle sheath and mesotomic sheath surrounding xylem and phloem. Stele or vascular cylinder region in young nodal roots of the coleoptile node and roots arising from nodal roots also showed GUS staining (Fig. 5B). Weak GUS staining was found in the sclerenchyma cells lining the epidermis of young leaves (Fig. 5B).

Hormonal Regulation of *OsXTH8* Expression

GA dose-dependent expression patterns of *OsXTH8* mRNA were determined in rice leaf sheaths using different concentration of GA_3 (1, 5, 10, and 50 μM). *OsXTH8* expression was found to be up-regulated with the increase in GA_3 concentration as there was no inhibitory effect of increase in GA_3 concentration up to 50 μM; however, 5 μM GA_3 induced maximum expression of *OsXTH8* (Fig. 6A). To determine temporal

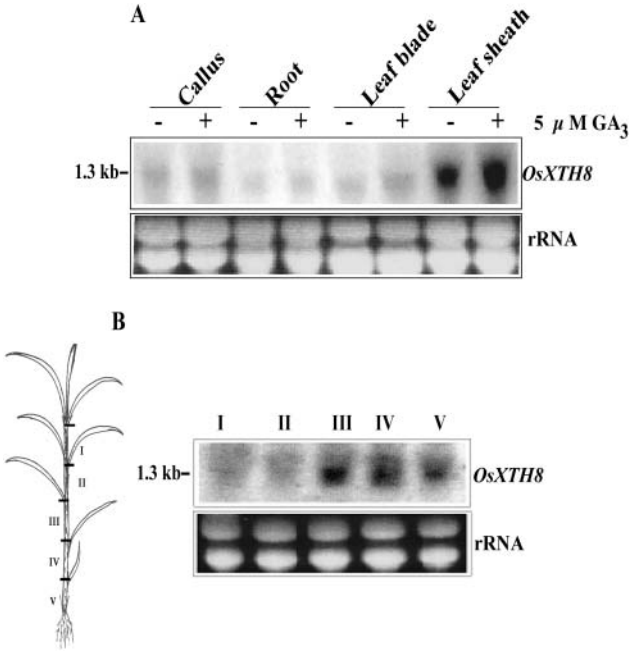

Figure 4. Tissue-specific expression of *OsXTH8*. A, Expression of *OsXTH8* in different tissues. Two-week-old seedlings were treated with 5 μM GA_3 for 24 h. For tissue-specific expression, total RNAs were extracted from roots, leaf blades, leaf sheaths, and calli. B, Expression of *OsXTH8* in five different sections of leaf sheaths using 1-month-old rice seedlings. The rice leaf sheath was divided in five sections: leafy section (I), second and third leaf section (II), first and second leaf section (III), coleoptile and first leaf section (IV), and coleoptile section (V) as shown in the figure drawing. Total RNA (20 μg each) transferred onto membrane was probed with PCR- amplified 3′ UTR of *OsXTH8* cDNA clone. rRNA stained with ethidium bromide was used as a loading control.

expression patterns of *OsXTH8* mRNA, leaf sheaths were treated for 1, 3, 6, 12, and 24 h. *OsXTH8* mRNA accumulation in leaf sheath showed that during treatment of leaf sheath fragments with GA_3, *OsXTH8* expression was up-regulated and continued to increase throughout the 24-h incubation period (Fig. 6B). As 2-week-old rice seedlings were used for time-course experiment, because of the growth of leaf sheaths, increase in *OsXTH8* expression in control could also be detected.

It has been shown that *XTH* genes are regulated by various hormones. For example, BRU1, a soybean *XTH*, is regulated by BR (Zurek and Clouse, 1994), and TCH4, an Arabidopsis (*Arabidopsis thaliana*) *XTH* gene, is up-regulated by auxin and BR (Xu et al., 1995). To characterize hormonal regulation of *OsXTH8* expression, the effect of several plant hormones was examined on *OsXTH8* mRNA abundance (Fig. 6C). When leaf sheaths were treated with GA_3, brassinolide (BL), 6-bezyladenine (BA), indole-3-acetic acid (IAA), and abscisic acid (ABA), GA_3 up-regulated the expression of *OsXTH8* and there was very little effect of other hormones. Uniconazole, which is a potent GA biosynthesis inhibitor, had an inhibitory effect on the *OsXTH8* mRNA accumulation (Fig. 6C).

To understand the physiological functions of *OsXTH8*, its expression in rice mutants with abnormal heights was investigated. Tanginbozu is a GA-deficient semidwarf mutant and a single recessive gene controls the semidwarfism of Tanginbozu. Mutation in Tanginbozu blocks the three oxidative steps whereby *ent*-kaurene is converted to *ent*-kaurenoic acid resulting in less accumulation of active GA (Ogawa et al., 1996). Northern-blot analysis showed that the level of *OsXTH8* mRNA in the mutant was lower than that in its wild-type cv Ginbozu (Fig. 6D). The expression of *OsXTH8* in the mutant was induced to exceed wild-type level following treatment with GA_3 for 24 h. *Slender rice1* (*slr1*), a GA-insensitive mutant, shows a constitutive GA-response phenotype (Itoh et al., 2002). Stem of the *slr1* mutant grows 2 to 3 times more than the stem of wild-type cv Nipponbare. Northern-blot analysis confirmed that the level of *OsXTH8* expression was higher in *slr1* mutant than that of its wild type (Fig. 6D).

To analyze whether the 2,325-bp 5′-promoter region of the *OsXTH8* locus is sufficient for the induction of its expression, an independent transgenic line (Fig. 7B), which was transformed with 2,325 bp of *OsXTH8* promoter fused to the GUS reporter gene, was treated with 5 μM GA_3. It was observed that GA_3 treatment enhanced the expression of the GUS reporter gene compared to untreated (mock) *OsXTH8*::*GUS* transgenic rice (Fig. 7B). This indicated that the 2,325-bp promoter region of *OsXTH8* was sufficient for hormone-induced *OsXTH8* expression. This observation was also confirmed by GUS assay using methylumbelliferylglucuronide as a substrate (Fig. 7C).

RNAi *OsXTH8* Transgenic Plants Exhibited Altered Development

To assess the effects of loss of function of *OsXTH8* on rice growth and development, a 360-bp 3′ UTR fragment of *OsXTH8* was cloned into the pIG121-Hm vector in both sense and antisense orientations (Fig. 8A) for subsequent generation of rice RNAi transgenic plants. RNAi *OsXTH8* was introduced into rice using Agrobacterium-mediated transformation. Rice plants transformed with only pIG121-Hm vector were used as control. At regeneration stage, the regeneration efficiency of the RNAi *OsXTH8* transformed callus was only about 37% when compared to control transformed callus. After transformation, 29 transformants were generated, out of which 13 plants appeared like wild type and 16 exhibited altered vegetative growth. RNAi transformed plants showed various degrees of repressed growth and were 20% to 50% shorter than the control plants when they reached maturity (Fig. 8B). Three different RNAi transgenic lines (T_1-1, T_1-3, and T_1-5) were selected for further analysis. RNAi transgenic line T_1-1 appeared like control in phenotype, while the other two transgenic lines T_1-3 and T_1-5 had

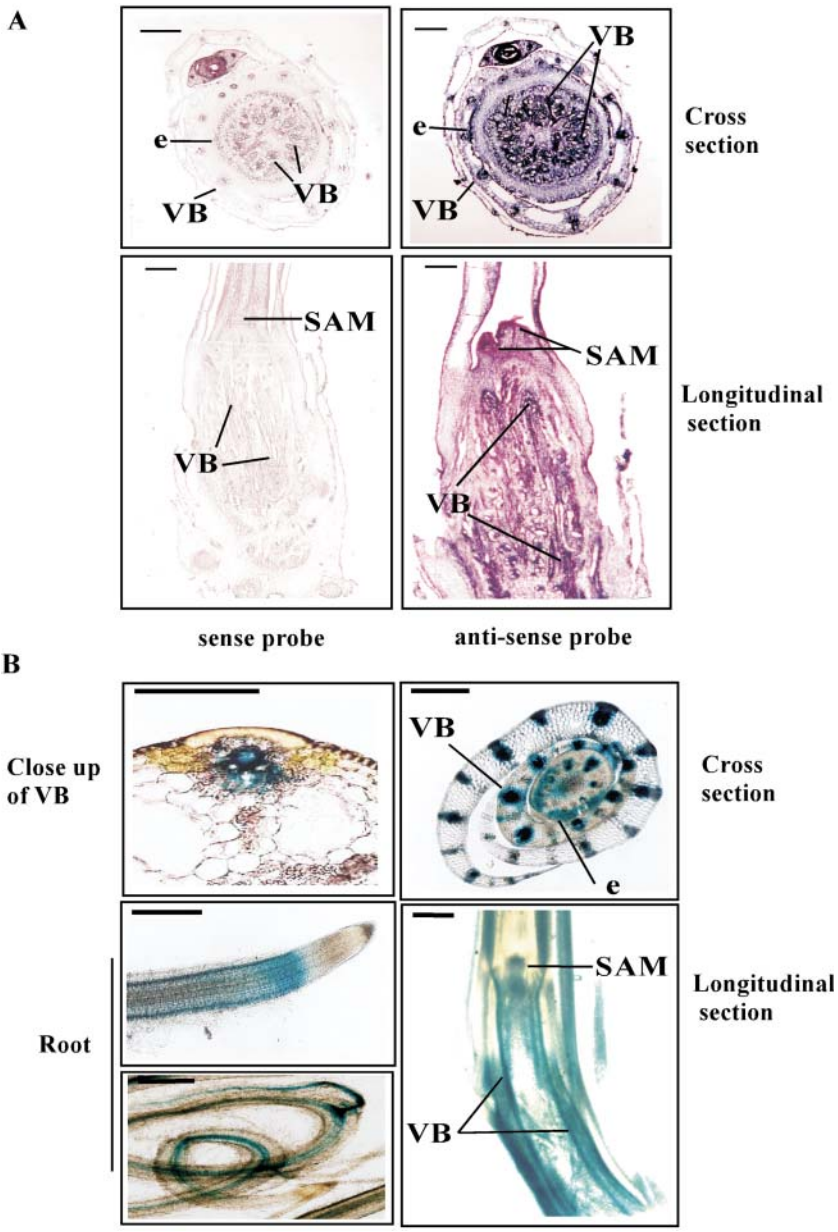

Figure 5. In situ hybridization of *OsXTH8* and GUS expression driven by *OsXTH8* promoter in different tissues of rice. A, In situ localization of *OsXTH8* mRNA. Sections of rice leaf sheaths were hybridized with Dig-labeled antisense RNA prepared from *OsXTH8* expressed sequence tag (A, right). Dig-labeled sense RNA was used as a negative control (A, left). B, GUS expression in different tissues of transgenic rice carrying *OsXTH8*::*GUS* construct. Cross-section and longitudinal section of leaf sheath and intact roots were used for histochemical localization of GUS activity. VB, Vascular bundle; e, peripheral cylinder of vascular tissues; SAM, shoot apical meristem. Bar represents 500 μm.

almost a 40% to 50% reduced height when compared to the control (Fig. 8E). To determine whether a relationship existed between the Os*XTH8* RNA levels and the phenotype, RNA was extracted from the three transgenic lines to analyze their transcript levels compared with that in the control. The results are shown in Figure 8D, with ethidium bromide-stained rRNA to show that equal amounts of RNA were used. Two lines, T_1-3 and T_1-5, in which *OsXTH8* transcript accumulated in very little amount, had a 40% and 50% reduction in height when compared to the control. Transgenic line T_1-1 that appeared like control in phenotype had slightly reduced *OsXTH8* mRNA accumulation. To assess whether the phenotype of the RNAi transgenic plants was caused by the knockout of *OsXTH8* or other *OsXTHs* genes were also affected, these lines were tested for the expression of *OsXTR1*, *OsXTR2*, *OsXTR3*, and *OsXTR4*. It is clear from Figure 8D that there was no significant reduction in the transcript level of *OsXTH* genes tested. The internodes of RNAi *OsXTH8* transgenic elongated almost normally (T in Fig. 8C), but three internodes (II, III, and IV) seemed to be shorter than the control plants. The apparent repressed growth of transgenic rice was attributed to the reduced elongation of these three internodes.

DISCUSSION

The existence of a family of 29 *XTH* genes in rice suggests that individual *XTHs* may exhibit distinct patterns of expression in terms of tissue specificity and

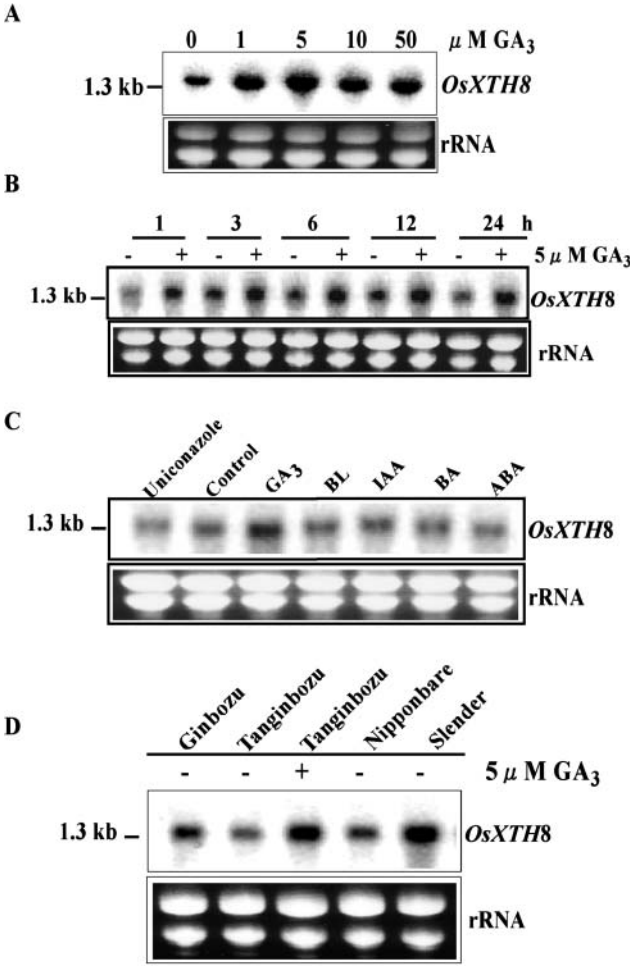

Figure 6. Hormonal regulation of *OsXTH8* expression. A, Dose-dependent effect of GA_3. Rice leaf sheaths were treated with 0, 1, 5, 10, and 50 μM GA_3 for 24 h. B, Time-course changes in the expression of *OsXTH8*. Rice leaf sheaths were treated with 5 μM GA_3 for 1, 3, 6, 12, and 24 h for time-course experiment. C, Effects of different phytohormones on the expression of *OsXTH8*. Rice leaf sheaths were treated with 1 μM BL, 10 μM uniconazole, and 5 μM each GA_3, IAA, BA, and ABA for 24 h. D, *OsXTH8* expression in different GA mutants exhibiting abnormal heights. Ginbozu and Nipponbare are the wild type of Tanginbozu and *Slr1* mutant, respectively. Total RNA (20 μg each) was extracted from leaf sheaths of 2-week-old seedlings and probed with 3′ UTR of *OsXTH8*. Experiments were replicated three times.

responses to hormonal and environmental stimuli. The individual XTH enzymes encoded by the *XTH* gene family are thought to have varying kinetic properties as well as different catalytic functions, including transferase and hydrolase activities. Therefore, it is predicted that combinatorial expression of multiple *XTHs* is critical for a broad spectrum of plant developmental processes (Rose et al., 2002). In this study, we characterized the new *XTH*-related gene, named *OsXTH8*, which is highly expressed in rice leaf sheath and is up-regulated by GA_3. Southern-blot analysis and database search revealed that *OsXTH8* is a novel gene. To study the functional role of *OsXTH8* in rice, the enzymatic activity profile of OsXTH8 must be examined.

The deduced amino acid sequence of OsXTH8 shares high homology, and many conserved sequences, with other XTH family members. OsXTH8 also has amino acid residues peculiar to monocotyledons within the presumed catalytic region. Most XTH family members in rice and other plants have the conserved amino acid sequence DEIDFEFLG (Fig. 2), whereas the corresponding sequence in OsXTH8 is DEIDIEFMG. This conserved sequence is also found in the catalytic region of bacterial endo-β-1, 3-1-4-glucanases (Borriss et al., 1990). Site-directed mutagenesis of *Bacillus licheniformis* endo-β-1, 3-1, 4-glucanases indicated that the second Glu (E) is critical for catalytic activity, while other studies demonstrated the importance of the first Glu (E) and the second Asp (D) (Planas et al., 1992; Juncosa et al., 1994). The conservation of these critical amino acids in the catalytic domain of OsXTH8 argues that its enzymatic activity will act on xyloglucan-β-1, 4-glycosyl linkages in plants.

Most sequence differences among XTH proteins are found in the carboxyl terminal and, based on the relatedness of carboxyl-terminal regions, the XTHs from many plant species can be loosely organized into four groups (Campbell and Braam, 1999a). The three main subfamilies (1, 2, and 3 in Fig. 3) are found in a wide range of flowering plants, including both dicots and monocots. The diversification of the structure of *XTH* genes among these three subfamilies may reflect a unique functional assignment for each subfamily that is essential throughout flowering plants. An obvious consideration is that this phylogenetic divergence reflects the evolution of XTH subgroups with different biochemical mechanisms of action, such as transglucosylation versus hydrolysis (Rose et al., 2002). The *OsXTH8* gene isolated in this study grouped into the fourth subfamily (Fig. 3), suggesting that it may have some peculiar functional features. Other related *XTH* genes that do not fit within the three main subfamilies have been reported in barley (Schunmann et al., 1997) and rice (Uozu et al., 2000) and show a unique expression pattern during leaf sheath and stem development.

An insight into the physiological role of XTHs can be gained from their enzymatic activity, the expression pattern of *XTH* genes, and the activity of promoter-GUS fusions for individual genes at various developmental stages in various parts of plants. *OsXTH8* is expressed in rice leaf sheath, and, less abundantly, in root, leaf blade, and callus (Fig. 4). Similarly, *OsXTR1*, *OsXTR2*, *OsXTR3*, and *OsXTR4* were shown to express mainly in rice culm (stem), and their expression patterns in culm seem to overlap with that of *OsXTH8*. *OsXTR1* is expressed in both the elongation and division zones of internodes, with a higher level of expression in the former. In contrast, *OsXTR4* is expressed in the division zone of internodes and nodes. *OsXTR2* is expressed in most organs, with the exception of well-developed leaf blades and roots. *OsXTR3*

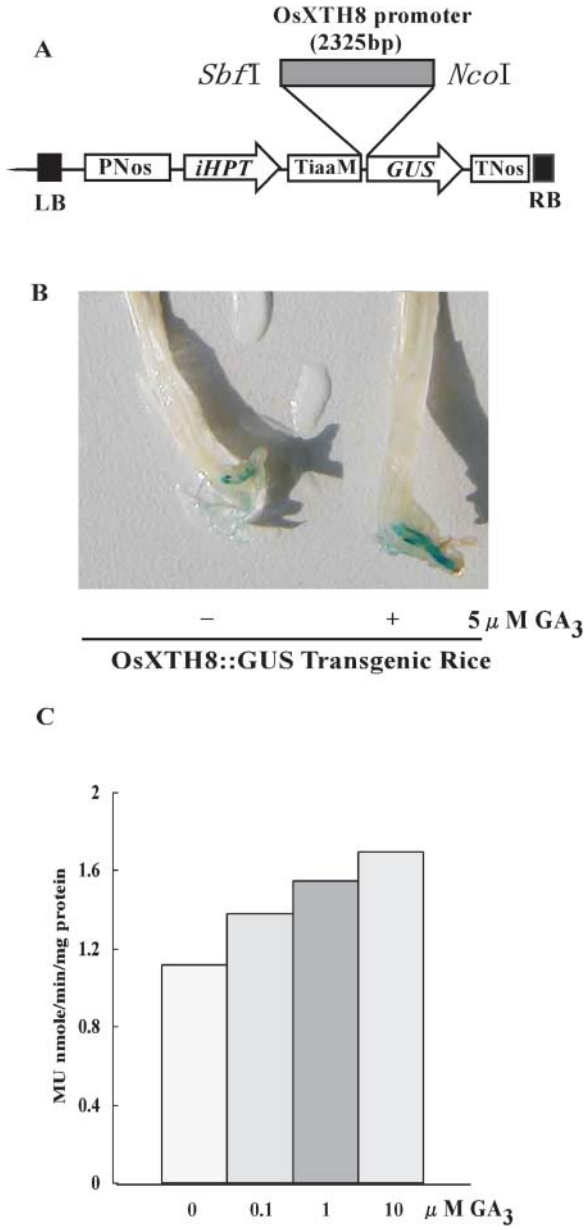

Figure 7. GUS activity in *OsXTH8*::*GUS* transgenic rice seedlings in response to GA treatment. A, Binary vector pSMAHdN627 harboring GUS gene under the control of the 2,325-bp promoter region of *OsXTH8*. B, Histochemical localization of GUS activity in transgenic rice treated with or without GA_3. C, Ten-day-old seedlings were treated with 0, 0.1, 1, and 10 μM GA_3 for 24 h. GUS activity was measured by fluorescent method as described in "Methods and Materials." Values are mean of triplicate experiment.

expression showed a strict organ specificity in elongating stems, being notably higher in the internode elongation zone (Uozu et al., 2000). *OsXTH8* may play a cooperative role with the above-mentioned genes and others that are expressed in overlapping regions. Further detailed expression studies, as well as biochemical analysis, will provide insight into the physiological function of *OsXTH8*. mRNA localization by in situ hybridization and histochemical localization of GUS activity in *OsXTH8*::*GUS* transgenics indicated that *OsXTH8* was expressed in growing regions such as shoot apical meristem, vascular bundles of leaf sheath, and young crown roots developing from nodes (Fig. 5); thus, *OsXTH8* exhibited a unique expression pattern in terms of organ and stage specificity during leaf sheath elongation and young nodal root development. The expression of *OsXTH8* in the shoot apex meristem, vascular bundles in leaf sheath, and young nodal roots supports its role in cell wall modification processes during active growth.

OsXTH8 expression increased in a dose- and time-dependent manner with GA_3 treatment. A significant increase in *OsXTH8* mRNA was observed in leaf sheath segments or intact plants with an increase in GA_3 concentration, and there was no inhibitory effect associated with further increase in GA_3 concentration up to 50 μM (Fig. 6A). *OsXTH8* also showed a temporal increase in transcript levels when leaf sheaths were treated with GA_3. Relative to controls, the earliest increase in *OsXTH8* mRNA level and leaf sheath growth was detectable after 1 h of incubation with GA_3, and it continued to increase up to 24 h of incubation (Fig. 6B). Transcriptional up-regulation of *XTH* gene expression by GA has been demonstrated in several instances (Uozu et al., 2000), but the mechanism has not yet been established. GAs regulate tissue elongation in several plants, and an effect of GA on wall extensibility, including promotion of wall loosening reactions, has been proposed (Cosgrove and Sovonick-Dunford, 1989). It has been reported that GA has a large effect on internodal elongation, especially activating cell division and cell elongation (Kamijima, 1981). Furthermore, it has been proposed that XTH activity and mRNA levels of *XTH*-related genes are regulated by GA to induce leaf elongation (Smith et al., 1996; Schunmann et al., 1997). In agreement with the above reports, GA also induced the expression of *OsXTH8* and acted as a candidate marker of rice leaf sheath growth. This suggests that *OsXTH8* acts to alter the structure of cell wall in response to this hormone.

Tanginbozu is a GA-deficient semidwarf mutant of rice, the phenotype of which is controlled by a single recessive gene. The mutation in Tanginbozu blocks the three oxidative steps whereby *ent*-kaurene is converted to *ent*-kaurenoic acid, resulting in less accumulation of active GA (Ogawa et al., 1996). The level of *OsXTH8* mRNA in Tanginbozu indicated that reduced accumulation of active GA was accompanied by preferential suppression of *OsXTH8* expression, and *OsXTH8* expression increased when exogenous GA_3 was applied to the semidwarf mutant (Fig. 6D). This result confirms the up-regulation of *OsXTH8* by GA and the correlation of this gene's expression with leaf sheath elongation. The tall mutant *Slr*1 is thought to have a defect in a suppressive gene in the GA signal

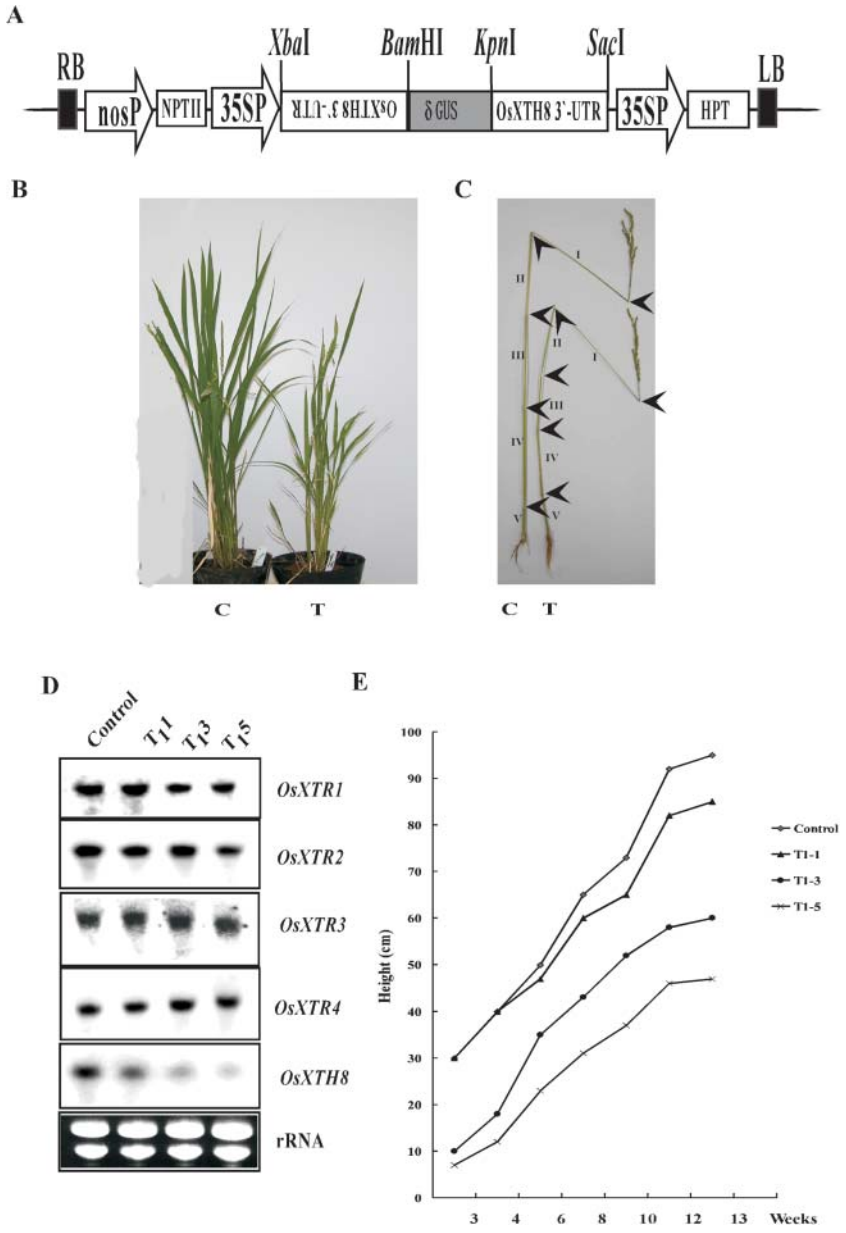

Figure 8. Phenotypes of transgenic rice constitutively overexpressing RNAi *OsXTH8*. A, A binary vector pIG121-Hm harboring RNAi *OsXTH8* under the control of CaMV 35S promoter. B, Transgenic rice 2 months after transferred to soil in the isolating green house. C, Elongation of the upper five internodes of the control (C) and RNAi *OsXTH8* transgenic rice (T). D, Expression levels of *OsXTR1*, *OsXTR2*, *OsXTR3*, *OsXTR4*, and *OsXTH8* mRNA in RNAi *OsXTH8* transgenic lines. E, Growth curves of the three RNAi *OsXTH8* transgenic plants and a vector control transgenic plant.

transduction pathway, which causes a constitutive GA response without application of exogenous GA (Itoh et al., 2002). This finding that *OsXTH8* expression is up-regulated in *slr1* mutant compared to the wild-type Nipponbare further supports the regulation of *OsXTH8* by GA.

GUS activity and histochemical localization of GUS activity showed that a 2,325-bp fragment 5′ upstream of the *OsXTH8*-coding region was sufficient to drive and up-regulate GUS reporter gene expression in response to GA_3 (Fig. 7, B and C). Computer analysis using the PLACE signal scan program, a database of plant cis-acting regulatory DNA elements (Higo et al., 1999), revealed many potential cis-elements in the 2,325-bp sequence of *OsXTH8*. In the putative promoter region of *OsXTH8*, three elements of a GA-response complex were found, including a pyrimidine box CCTTTT (Skriver et al., 1991), a GA-response element (GARE) TAACGTAG (Gubler and Jacobsen, 1992), and a CAACTC regulatory element (CARE) CAACTCAA (Sutoh and Yamauchi, 2003), at 228, 154, and 311 bp upstream of the translation initiation site, respectively. It will be interesting to dissect further the *OsXTH8* promoter region to find out whether it contains other known or novel cis regulatory elements that function to confer responsiveness to different stimuli or hormones. Although many auxin-regulated genes have been shown to be transcriptionally controlled, regulation of *BRU1* by BRs is thought to occur posttranscriptionally (Zurek and Clouse, 1994; Xu et al., 1995). It is possible

that *OsXTH8* is regulated both transcriptionally and posttranscriptionally, depending on the type of hormone or stimulus. Identification of trans-acting factors will help elucidate the signal transduction pathways by which environmental stimuli or different hormones lead to regulation of *OsXTH8*.

One way to examine the role of *XTHs* in plant growth and development is to increase or decrease endogenous *XTHs*' mRNA content by applying transgenic methodology. RNAi *OsXTH8* expressed under the control of constitutive cauliflower mosaic virus (CaMV) 35S promoter produced plants with altered growth (Fig. 8B). The repression of growth in a subpopulation of RNAi transgenic rice was attributable to the reduced second, third, and fourth internodal elongation (Fig. 8C) and is consistent with the observation that in 1-month-old wild-type rice seedlings, *OsXTH8* mRNA accumulation was only observed in the first three parts of the leaf sheath (Fig. 4B), which at this stage of development correspond to the third and fourth internode. As *OsXTH8* is a member of a multigene family, it is possible that silencing of *OsXTH8* could affect the expression of other *OsXTH* genes. For this reason, we selected four *OsXTR* genes, where *OsXTR1*, *OsXTR2*, and *OsXTR4* are fairly closely related genes and *OsXTR3* is distantly related gene to *OsXTH8* (Yokoyama et al., 2004). As the expressions of these genes were not affected by expression of RNAi *OsXTH8* (Fig. 8D), it could be argued that the silencing of OsXTH8 was quite specific.

Whether the repressed internode elongation in the transgenic plants is because of some discernible alteration in cell expansion or development awaits further investigation. Sakamoto et al. (2003) modified the level of GA by overproduction of a GA catabolic enzyme, GA 2-oxidase, and the first (uppermost) internode was more severely affected than the other internodes. In contrast, all internodes were severely reduced in severe transformants. Transgenic Arabidopsis plants with altered expression of *AtXTH24* showed developmental defects (Verica and Medford, 1997), but one cannot conclude specifically that these defects were due to the alterations in *AtXTH24* expression. Transgenic tobacco plants with reduced XET activity were shown to accumulate xyloglucan with a M_r at least 20% greater than that of wild-type plants (Herbers et al., 2001). The consequences of these alterations for wall properties and plant fitness are not yet clear (Rose et al., 2002). In spite of the low amount of xyloglucan in rice and the possibility that other wall loosening agents also mediate growth, our results support that *XTHs* have a role in cell wall modification and growth promoting functions in rice.

In conclusion, this study shows that *OsXTH8* is differentially expressed in rice leaf sheath in relation to GA. Based on the specific accumulation of *OsXTH8* transcripts in young rice leaf sheath and the altered phenotype of RNAi *OsXTH8*, it is suggested that *OsXTH8* is involved in cell wall modification processes during rice growth and development.

MATERIALS AND METHODS

Plant Materials and Treatments

Wild-type rice (*Oryza sativa* L. cv Nipponbare or cv Ginbozu) and two rice mutants, Tanginbozu and *Slr1*, were grown under white fluorescent light (about 200 μmol m^{-2} s^{-1}, 12-h light period/d) at 25°C and 75% relative humidity in a growth chamber. Leaf sheath segments of 2-week-old seedlings were floated on 10 mL of distilled water in 60 mm × 15 mm petri dishes containing BL (Fuji Chemical, Toyama, Japan), IAA, GA_3, ABA, and BA uniconazole (Wako Pure Chemical, Osaka) for various times.

cDNA Microarray Analysis

A cDNA microarray containing 4,000 cDNA clones randomly selected from the rice cDNA library was used. The mRNAs for microarray probes preparation were purified with an Oligotex-dT-30 mRNA purification kit (Takara, Kyoto) according to the manufacturer's instructions. mRNA samples (1 μg) prepared from leaf sheath treated with 5 μM GA_3 for 24 h or water as a control, were reverse transcribed in 20-μL volume containing 1 mM Cy3 or Cy5 dCTP (Amersham Biosciences, Piscataway, NJ), anchored oligo$(dT)_{25}$, random nonamer, dithiothreitol, dNTPs, and SuperScript II (Invitrogen, Carlsbad, CA). After incubation at 42°C for 2 h, the reaction was stopped, and RNA was degraded by first heating at 94°C for 3 min and then treated with NaOH at 37°C for 15 min. Fluorescently labeled probes were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Probe hybridization and scanning of the hybridized microarray slide were done according to the method of Yazaki et al. (2000). Hybridization was done twice and data were analyzed with Array Gauge version 1.21 (Fujifilm, Tokyo). Only those clones that showed the same changes in two experiments were selected as the candidates.

cDNA Cloning and Sequencing

RACE-PCR was used to clone the full-length sequence of *OsXTH8* using Gene Racer kit (Invitrogen). Oligo(dT) was used for first strand synthesis. PCR was performed by using Gene Racer 5′ Primer and a gene-specific primer (5′-CACACCGCCCAACTGTGCAAGATGAACT-3′). The PCR product was purified and cloned into pCR 4Blunt-TOPO vector (Zero Blunt TOPO PCR Cloning Kit for Sequencing; Invitrogen). Sequencing of the full-length cDNA was accomplished for both strands using dye-labeled terminations (PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster City, CA) and an automated DNA sequencer (model 373A; Applied Biosystems). DNA sequence was analyzed with Genetyx-WIN Version 5.1 (Software Development, Tokyo).

Genomic Southern Hybridization

Rice genomic DNA was digested with *Eco*RI, *Xba*I, and *Xho*I, respectively, separated by 0.8% agarose gel electrophoresis, and then transferred onto Hybond+ membranes under alkaline conditions. A 3′ UTR of *OsXTH8*, which was PCR amplified using gene-specific primers, purified from agarose gel (QIAEXII Gel Extraction Kit; Qiagen), and radiolabeled using [alpha-^{32}P] dCTP (Amersham Biosciences) random prime labeling system (Rediprime II; Amersham Biosciences), was used as probe for Southern-blot analysis. Hybridization was performed at 42°C in an ultrasensitive hybridization buffer (ULTRAhyb, Ambion, Austin, TX) overnight. The blot was washed twice first in 2× SSC, 0.1% × SDS at 42°C for 5 min and in 0.1× SSC, 0.1% SDS at 68°C for 15 min. It was finally analyzed by the phosphor image program with the Typhoon 8600k variable imager (Amersham Biosciences).

RNA Extraction and Northern-Blot Analysis

Tissue samples were quick-frozen in liquid nitrogen. Samples (0.5 g) were ground to powder by mortar and pestle, and total RNAs were isolated according to the procedure of Chomczynski and Sacchi (1987). For northern-blot analysis, 20 μg of total RNA was separated on 1.2% agarose containing 6% formaldehyde and transferred on to a Hybond-N^+ nylon membrane (Amer-

sham Biosciences). Loading of equal amounts of total RNA for northern blots was determined by visualization of ethidium bromide-stained rRNA bands. Probe for northern blots was the 3′ UTR of *OsXTH8*, which was cut from pBluescript SK+ or SK− plasmids, purified from agarose gels (QIAEXII Gel Extraction kit; Qiagen), and radio labeled using [alpha-^{32}P]dCTP (Amersham Biosciences) random prime labeling system (Rediprime II; Amersham Biosciences). Hybridization was performed at 42°C in the ultrasensitive hybridization buffer (ULTRAhyb) overnight. The blots were washed twice first in 2× SSC, 0.1% × SDS at 42°C for 5 min and in 0.1× SSC, 0.1% SDS at 68°C for 15 min. They were finally detected with x-ray film (Kodak, Rochester, NY) or analyzed by the phosphor image program with the Typhoon 8600k variable imager (Amersham Biosciences).

Promoter Analysis and GUS Localization

To amplify *OsXTH8* promoter fragment, rice genomic DNA was extracted from 1-week-old seedlings, grown on Murashige and Skoog medium (Murashige and Skoog, 1962) using DNeasy Plant Mini kit (Qiagen). The expected *OsXTH8* promoter fragment was amplified using primer pairs of 5′-ATGCCCTGCAGGGAGGGAGTAGTAGCTAGCTTGAG-3′ (5′ side, *Sbf*I site is underlined in the adaptor sequence) and 5′-TTCGCCATGGCTACTGTACTTGCTTG-3′ (3′-side, *Nco*I site is underlined in the adopter sequence). The 3′-side primer was designed in a way that it gave the initiation codon of *OsXTH8* on cutting with *Nco*I restriction enzyme. The *OsXTH8* promoter fragment was amplified using KOD plus (Toyobo, Osaka) using the PCR conditions: 94°C for 2 min (1 cycle), 94°C for 15 s, 63°C for 30 s, 68°C for 2 min (30 cycles), and 68°C for 7 min (1 cycle). The amplified fragment was gel purified using Wizard SV gel and PCR clean up system (Promega, Madison, WI). The purified product was incubated with dATP and Ex Taq (Takara) at 72°C for 5 min and purified again using Wizard SV gel and PCR clean up system. The purified fragment was cloned into pGEM-T easy of pGEM-T easy vector system (Promega). *OsXTH8* promoter fragment was confirmed by sequencing using ABI 3100. *OsXTH8* promoter fragment was released by digesting with *Sbf*I and *Nco*I and cloned into a binary vector pSMAHdN627 (H. Nakamura, unpublished data; Fig. 7A). The resulting plasmid carrying *OsXTH8* promter::GUS fusion was transformed into rice via Agrobacterium-mediated transformation (Tanaka et al., 2001).

GUS assay was conducted according to Jefferson (1987). For histochemical analysis, leaf, stem, and root segments were sectioned into 30-μm-thick pieces by a microslicer and incubated in 50 mM sodium phosphate buffer, pH 7.2, containing 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Wako Pure Chemical) and 5% methanol at 37°C for 2 to 24 h. The reaction was stopped by adding ethanol.

GUS activity was measured using the fluorogenic substrate 4-methylumbelliferylglucuronide (Sigma, St. Louis) and measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan). Each assay was carried out with crude extracts containing 5 μg proteins. The relative GUS activity was calculated as the mean of triplicate experiment.

In Situ Hybridization

Nodal part tissues taken from 2-week-old seedlings were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde under vacuum. Fixed samples were then dehydrated through a graded ethanol series followed by a t-butanol series, and finally embedded in paraplast. Microtome sections (10 μm thick) were mounted on silicon-coated glass slides (Matsunami, Hamamatsu, Japan). Paraplast was removed through a graded ethanol series. Probes for in situ hybridization were labeled with digoxigenin11-UTP (Roche Diagnostics, Manheim, Germany). *OsXTH8* expressed sequence tag pBluescript SK plasmid was either treated with *Xho*I and transcribed with T7 RNA polymerase (Stratagene, La Jolla, CA; antisense probe) or digested with *Eco*RI and transcribed with T3 RNA polymerase (Stratagene; sense probe). Immunological detection was done with an anti-digoxygenin-AP conjugate and 4-nitrobluetetrazolium (Roche Diagnostics; Kouchi and Hata, 1993).

Construction of RNAi *OsXTH8* Transgenic Rice

For construction of RNAi transgenic plants, *OsXTH8* cDNA in the pBluescript SK+ vector was amplified by PCR in both sense and antisense directions. Sense RNAi fragment was amplified using primer pairs of 5′-GGGGTACCTTTTGAACTCGATCGATTCAAA-3′ (5′ side, *Kpn*I site underlined as a linker) and 5′-GCGAGCTCTGTTCATACCTGAGAGCATAAG-3′ (3′-side, *Sca*I site underlined as a linker). Anti-sense RNAi fragment was amplified using primer pairs of 5′-CGTCTAGATGTTCATACCTGAGAGCATAAG-3′(5′ side, *Xba*I site underlined as a linker) CGGGATCCTTTTGAACTCGATCGATTCAAA-3′ (3′ side, *Bam*HI site underlined as a linker). The resulting PCR fragments were ligated between the CaMV 35S promoter and nopaline synthase terminator in the binary vector pIG121-Hm (Ohta et al., 1990) in a position sandwiching 700 bp of partial GUS coding region (Fig. 8A). The pIG121-Hm/RNAi *OsXTH8* construct was confirmed by restriction mapping and sequencing. The pIG121-Hm/RNAi *OsXTH8* plasmid and the control vector pIG121-Hm were then transferred into *Agrobacterium tumefaciens* strain EHA 105 (Hood et al., 1986) and transformed into rice as described (Toki, 1997). Transgenic plants were selected on medium containing hygromycin. Hyygromycin-resistant plants were transferred to soil and grown to maturity at 30°C in 16-h light/8-h dark cycle in closed greenhouse.

Total RNAs were extracted from leaves of 2-month-old control and transgenic plants and subjected to northern-blot analysis. The membrane was hybridized with the same probe and condition as described in the previous northern-blot analysis section.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AB110604.

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