

Functional roles of rice glycosyltransferase family GT43 in xylan biosynthesis

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Keywords: rice, cell wall, GT43, glycosyltransferase, xylan

Xylan is the major hemicellulose present in both primary and secondary cell walls of rice vegetative tissues. Since xylan is one of the factors contributing to biomass recalcitrance, understanding how xylan is synthesized in rice will potentially provide tools to modify grass biomass composition better suited for biofuel production. Studies of xylan biosynthesis in *Arabidopsis* have revealed that family GT43 glycosyltransferases, which form 2 functionally nonredundant groups, IRX9/IRX9 homolog and IRX14/IRX14 homolog, are required for xylan backbone elongation. The rice genome harbors 10 genes encoding family GT43 members and it is currently unknown whether they are all involved in xylan biosynthesis. In this report, we performed biochemical analysis of xylan xylosyltransferase activity in rice stem microsomes and investigated the roles of 4 representative rice GT43 members, OsGT43A (LOC_Os05 g03174), OsGT43E (LOC_Os05 g48600), OsGT43H (LOC_Os04 g01280), and OsGT43J (LOC_Os06 g47340), in xylan biosynthesis. OsGT43 proteins were shown to be localized in the Golgi, where xylan biosynthesis occurs. Complementation analysis by expression of OsGT43s in *Arabidopsis irx9* and *irx14* mutants demonstrated that OsGT43A and OsGT43E but not OsGT43H and OsGT43J were able to rescue the mutant phenotypes conferred by the *irx9* mutation, including defective stem mechanical strength, vessel morphology, xylan content, GlcA side chains, xylan chain length, and xylosyltransferase activity. On the other hand, OsGT43J but not OsGT43A, OsGT43E, and OsGT43H restored the defective xylan phenotype in the *irx14* mutant. These results indicate that the rice GT43 family evolved to retain the involvement of 2 functionally nonredundant groups, OsGT43A and OsGT43E (IRX9 homologs) vs. OsGT43J (an IRX14 homolog), in xylan backbone biosynthesis.

Introduction

Cellulosic biomass from grasses, such as rice (*Oryza sativa*), corn (*Zea mays*), switchgrass (*Panicum virgatum*), and *Miscanthus* (*Miscanthus giganteus*), has been considered to be a promising source of renewable bioenergy for bioethanol production.¹ However, the cell wall, which is the primary constituent of cellulosic biomass, is intrinsically recalcitrant to enzymatic degradation, thus hampering the efficient utilization of cellulosic biomass for biofuel production. Lignin and xylan are the 2 major polymers in grass cell walls contributing to biomass recalcitrance, i.e., they form physical barriers to block cellulolytic enzymes from hydrolyzing cellulose into fermentable sugars.² To overcome biomass recalcitrance, it is crucial to uncover the molecular and biochemical mechanisms controlling the biosynthesis and assembly of cell wall components. The knowledge thus gained could be applied to modify cell wall composition tailored for biofuel production.

Xylan is the major crosslinking (noncovalently and covalently) hemicellulose in both primary and secondary cell walls of grasses.² It is made of a linear chain of β -1,4-linked

xylosyl residues, some of which are substituted by α -1,2/ α -1,3-linked arabinose, α -1,2-linked glucuronic acid (GlcA), α -1,2-linked 4-*O*-methylglucuronic acid (MeGlcA), and acetylated at C-2 or C-3.³ Different from the xylan in dicots, grass xylan has several unique features. In addition to arabinose as its predominant side chains, grass xylan covalently crosslinks with lignin via ferulic acid residues and has xylosyl substitution at *O*-2 of the arabinosyl side chains.⁴ Bioinformatic studies and transcriptome profiling analyses have revealed that a number of genes encoding families GT43, GT47, and GT61 glycosyltransferases and acyl-CoA transferases are implicated in xylan biosynthesis in grasses.⁵⁻⁷ Among them, one GT61 member from wheat and its rice close homologs were proven to mediate the addition of arabinosyl residues onto xylan⁸ and another GT61 member from rice is involved in the xylosyl substitution at *O*-2 of the arabinosyl side chains in xylan.⁹ Four acyl-CoA transferases from rice were proposed to be putative arabinoxylan feruloyltransferases since downregulation of their expression in transgenic rice resulted in a moderate reduction (19%) of ferulate in cell walls.¹⁰ Intriguingly, overexpression of another acyl-CoA transferase that is a close homolog of

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Submitted: 01/08/2014; Accepted: 01/09/2014; Published Online: 02/13/2014

Citation: Lee C, Teng Q, Zhong R, Yuan Y, Ye Z. Functional roles of rice glycosyltransferase family GT43 in xylan biosynthesis. *Plant Signaling & Behavior* 2014; 9:e27809; PMID: 24525904; <http://dx.doi.org/10.4161/psb.27809>

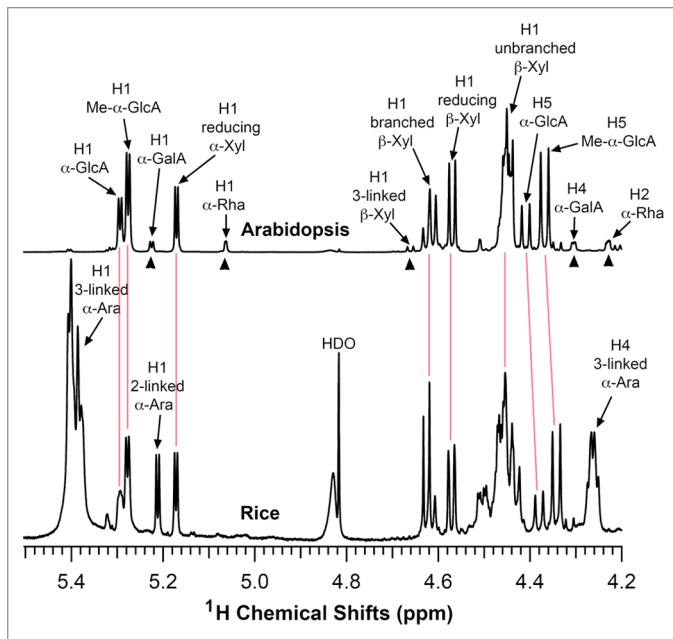


Figure 1. Structural analysis of rice xylan by NMR spectroscopy. Xylooligosaccharides generated by β -endoxyylanase digestion of alkaline-extracted xylan were subjected to ^1H -NMR analysis. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. The NMR spectrum of *Arabidopsis* xylan was included for comparison. The proton resonances shared by both *Arabidopsis* and rice xylyans are marked by vertical red lines. The resonances of H1 of α -D-GalA, H1 of α -L-Rha, H1 of 3-linked β -D-Xyl, H4 of α -D-GalA, and H2 of α -L-Rha (arrow heads) from the reducing end tetrasaccharide sequence of *Arabidopsis* xylan was not observed in rice xylan. Note the predominant resonances corresponding to 2- and 3-linked α -arabinose in rice xylan, which is absent in *Arabidopsis* xylan. HDO, hydrogen deuterium oxide.

these putative arabinoxylan feruloyltransferases in transgenic rice caused an increase in *p*-coumarate but not ferulate in cell walls, which led the authors to suggest that the protein is an arabinoxylan coumaroyltransferase.¹¹ Genes responsible for the addition and methylation of the GlcA side chains in xylan and those involved in xylan acetylation have not yet been characterized in grasses. In *Arabidopsis*, it has been demonstrated that 3 GT8 genes encode glucuronyltransferases required for GlcA substitution in xylan,¹²⁻¹⁴ 3 DUF579 domain-containing proteins are glucuronoxylan methyltransferases catalyzing the 4-*O*-methylation of the GlcA side chains,^{15,16} and 4 RWA genes and one DUF231 domain-containing protein, ESK1/TBL29, are implicated in xylan acetylation.¹⁷⁻¹⁹

The biochemical mechanism controlling xylan backbone elongation appears to be much more complicated than expected. In contrast to the biosynthesis of secondary wall cellulose in which 3 functionally nonredundant family GT2 cellulose synthases are involved,²⁰ xylan backbone biosynthesis entails glycosyltransferases from both GT43 and GT47 families.²¹⁻²⁵ It has been demonstrated that the 4 *Arabidopsis* GT43 members form 2 functionally nonredundant groups, IRX9/IRX9 homolog and IRX14/IRX14 homolog, both of which are required for xylan backbone biosynthesis.^{26,27} Biochemical analysis has

further revealed that IRX9 and IRX14 are xylosyltransferases that act cooperatively in the elongation of the xylan backbone.²⁸ Simultaneous mutations of 2 *Arabidopsis* GT47 genes, *IRX10* and *IRX10L*, also result in defects in xylan backbone elongation, indicating their essential role in xylan backbone biosynthesis.^{24,25} It is currently unknown why the elongation of β -1,4-linked xylosyl residues requires glycosyltransferases from 2 different GT families.

Studies of xylan biosynthesis in grasses have also revealed the involvement of both GT43 and GT47 glycosyltransferases in xylan biosynthesis.^{29,30} However, analysis of the protein complex possessing xylan xylosyltransferase activity in wheat only identified one GT43 member that is a close homolog of *Arabidopsis* IRX14.²⁹ Thus, it is unclear whether the xylan biosynthesis in grasses requires 2 functionally nonredundant GT43 members as in *Arabidopsis*. Since an expansion of the GT43 family occurred in grass species such as rice, it necessitates investigation on whether there is functional diversification in the GT43 family in grasses. In this report, we studied the biochemical properties of xylan xylosyltransferase activity in rice stem microsomes and investigated the functions of rice GT43 members in xylan biosynthesis. Our results revealed both conservation and divergence in the functional roles of rice family GT43 members in xylan biosynthesis.

Results

Structure of rice xylan and biochemical properties of rice xylan xylosyltransferase activity

To investigate the biochemical mechanism controlling xylan biosynthesis in rice, we first analyzed the structure of xylan isolated from rice stems. KOH-extracted xylan was first digested with β -1,4-endoxyylanase and its structure was subsequently determined using nuclear magnetic resonance (NMR) spectroscopy (Fig. 1). Rice xylan exhibited the same resonances attributed to the backbone xylosyl residues (H1 of branched and unbranched at 4.62 and 4.46 ppm, respectively) and the GlcA side chains (H1 of GlcA and MeGlcA at 5.29 and 5.27 ppm, respectively) as *Arabidopsis* xylan. However, the resonances characteristic of the *Arabidopsis* xylan reducing end tetrasaccharide sequence, β -D-Xyl-(1 \rightarrow 3)- α -L-Rha-(1 \rightarrow 2)- α -D-GalA-(1 \rightarrow 4)-D-Xyl (H1 of 3-linked β -D-Xyl, H1 of α -L-Rha, H1 of α -D-GalA, H2 of α -L-Rha, and H4 of α -D-GalA), was not evident in rice xylan, indicating that either rice xylan does not possess the reducing end tetrasaccharide sequence or the amount is too low to be detected. The most prominent difference between rice and *Arabidopsis* xylyans is that the resonances characteristic of the arabinose side chains (H1 3-linked at 5.4 ppm, H4 3-linked at 4.26 ppm, and H1 2-linked at 5.21 ppm) were only present in rice xylan. The structural analysis demonstrated that similar to xylan in other grass species,^{31,32} xylan from rice stems consists of a linear chain of β -1,4-linked xylosyl residues, some of which are substituted with side chains of α -1,2-linked GlcA, α -1,2-linked MeGlcA, and α -1,2/1,3-linked arabinose. The ^1H -NMR spectrum shows clearly the resonances of different structural groups of rice stem

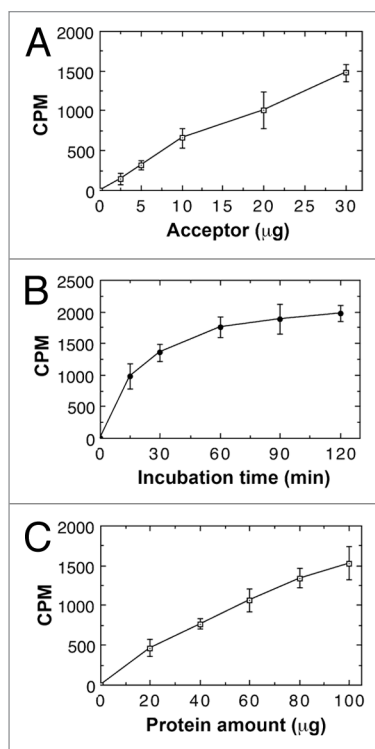


Figure 2. Biochemical properties of xylan xylosyltransferase activity in microsomes from rice stems. The xylosyltransferase activity was assayed by incubating microsomes with the UDP- ^{14}C -xylose donor and the Xyl_4 acceptor for 30 min unless otherwise indicated. The activity (CPM) was measured by the transfer of the radiolabeled xylosyl group onto the acceptor. All assays were repeated twice and the data are means \pm SE of assays from 3 independently isolated microsomes. (A) Dependence of the xylosyltransferase activity on the Xyl_4 acceptor concentration. (B) Time course of the transfer of the radiolabeled xylosyl residues onto the acceptor by the xylosyltransferase activity. (C) The xylosyltransferase activity is protein concentration-dependent.

xylan, which could be used as a reference for future study of xylan in transgenic rice plants with altered xylan structure.

We next examined the xylan xylosyltransferase activity in microsomes isolated from rice stems. It was found that rice microsomes possessed a xylosyltransferase activity capable of catalyzing the transfer of xylosyl residues from ^{14}C -labeled UDP-xylose onto the exogenous xylooligosaccharide (Xyl_n) acceptor in a concentration-dependent manner (Fig. 2A). No xylosyltransferase activity was detected in the absence of the exogenous Xyl_4 acceptor, which is different from the xylosyltransferase activity of wheat microsomes in which no exogenous acceptors are required (Zeng et al., 2010). Under the conditions used, the xylosyltransferase activity of rice microsomes was time-dependent reaching its maximum after 90 min incubation (Fig. 2B) and was also dependent on microsomal protein concentration (Fig. 2C).

The xylan xylosyltransferase activity exhibited by the rice stem microsomes were further assessed by HPLC using the fluorescent anthranilic acid (AA)-labeled xylooligomers of different lengths as acceptors (Fig. 3). The acceptors, xylooligomers, used for the assay were purchased from

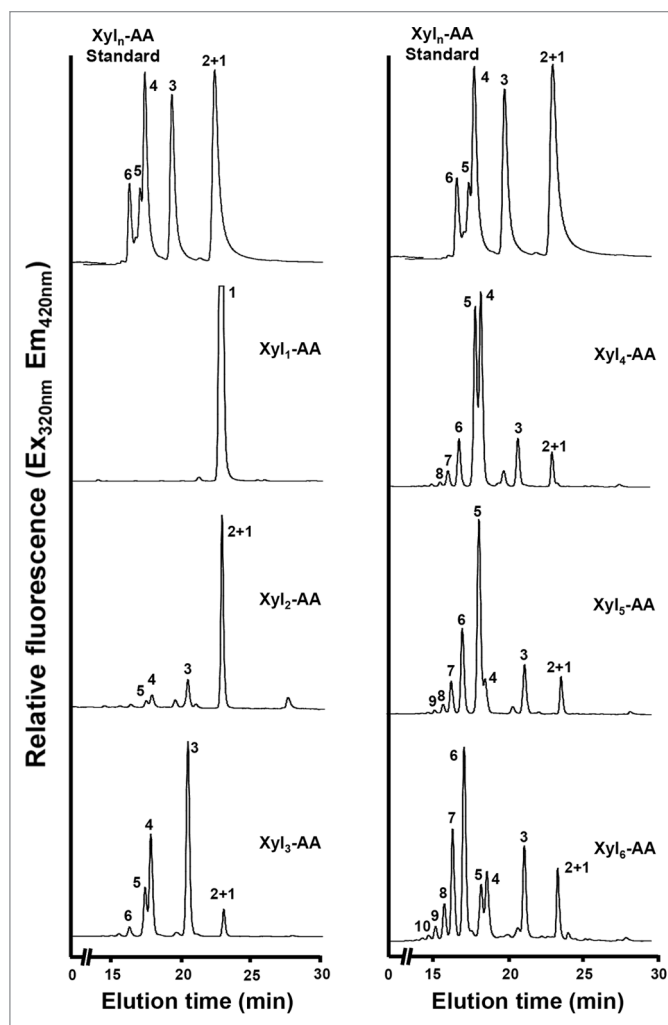


Figure 3. Xylooligomers of various lengths as acceptors for the xylosyltransferase activity of rice microsomes. The xylosyltransferase activity was assayed by incubating rice stem microsomes with UDP-xylose and the acceptor Xyl_1 -AA, Xyl_2 -AA, Xyl_3 -AA, Xyl_4 -AA, Xyl_5 -AA, or Xyl_6 -AA, and the reaction products were analyzed by reverse-phase HPLC and detected for fluorescent signals. The number at each peak denotes the number of xylosyl residues for the corresponding xylooligomer. A chromatogram of standard Xyl_6 -AA is shown at the top of each column for comparison of the xylooligomer peaks.

Megazyme and they are free from contamination as verified by HPLC. No xylosyl transfer was detected when the monomer AA-Xyl was used as the acceptor and a low level of xylosyl transfer was observed when AA- Xyl_2 was used. Up to 4 xylosyl residues were able to be efficiently transferred onto the acceptors ranging from Xyl_3 to Xyl_6 by the xylosyltransferase activity of rice microsomes. No addition of xylosyl residues onto the xylooligomer acceptors was observed when UDP-xylose was absent (Fig. 4A). As the elongation of xylooligomers depends on the presence of the xylosyl donor, UDP-xylose, and the reducing end of the xylooligomers is blocked by anthranilic acid, it is unlikely that this observed addition of xylosyl residues onto the xylooligomer acceptors could be mediated by putative xylan transglycosylase activities. It should be pointed out that

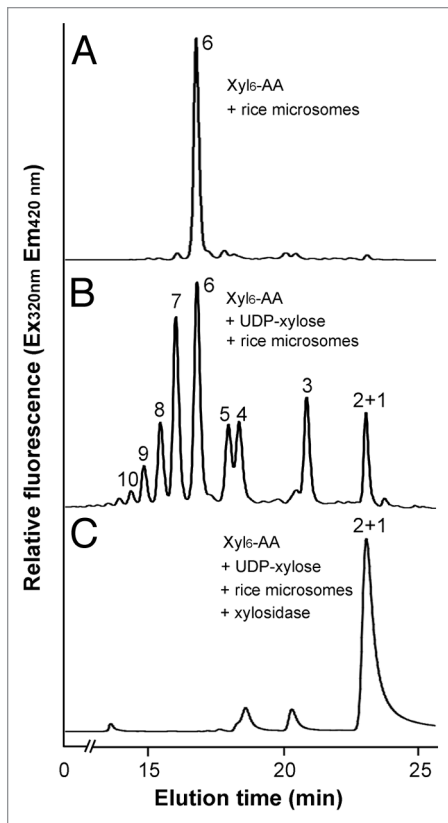


Figure 4. Digestion of the xylosyltransferase-catalyzed reaction products by β -1,4-xylosidase. Microsomes were incubated with the fluorescent Xyl₆-AA acceptor and UDP-xylose. The reaction products were digested with β -1,4-xylosidase and then analyzed by reverse-phase HPLC. (A) The control reaction without UDP-xylose showing the Xyl₆-AA acceptor peak. (B) Rice microsomes possess the xylosyltransferase activity capable of adding xylosyl residues onto the Xyl₆-AA acceptor peak. (C) β -1,4-Xylosidase digestion of the reaction products from (B) results in their degradation into Xyl₁-AA and Xyl₂-AA, indicating that the reaction products are β -1,4-linked. The number at each peak denotes the number of xylosyl residues for the corresponding xylooligomer.

although the rice microsomes exhibit a xylosyltransferase activity that is able to successively transfer up to 4 xylosyl residues onto the xylooligomer acceptors (Fig. 3), prolonged incubation of the reactions did not generate reaction products with a longer chain length. One possible explanation is that long xylooligosaccharides without GlcA side chains and acetyl modifications are insoluble as shown in the alkaline-extracted xylan from the *gux1 gux2* double mutant,¹² and thus they could not be produced. The other possible explanation is that a xylosidase activity present in rice microsomes degrades reaction products (see below), which prevents the accumulation of xylooligosaccharides with a longer chain length. The data shown here are in agreement with an early report showing that wheat microsomes possess a xylosyltransferase activity that adds up to 2 xylosyl residues onto the xylooligomer acceptor.³³

It was of note that the reaction products not only consisted of xylooligomers longer than the acceptor but also contained shorter xylooligomers (Fig. 3), indicating that in addition to the xylosyltransferase activity catalyzing the addition of xylosyl

residues, the rice microsomes contain a xylosidase-like activity that removes xylosyl residues from the acceptors. It remains to be investigated what physiological roles this xylosidase-like activity may play during xylan biosynthesis in rice. Little xylosidase-like activity was observed in xylan xylosyltransferase activity assays with microsomes from *Arabidopsis* and poplar.^{22,34}

The reaction products, xylooligosaccharides, generated by rice xylosyltransferase activity were further verified for their sugar linkage by digestion with β -(1,4)-xylosidase (Fig. 4). HPLC analysis of digested products showed that treatment of the microsome-catalyzed reaction products (Fig. 4B) with β -(1,4)-xylosidase led to their hydrolysis into xylose or xylobiose (Fig. 4C), demonstrating that the reaction products are made of β -1,4-linked xylosyl residues.

Family GT43 glycosyltransferases in rice

In *Arabidopsis*, all 4 members of the GT43 family are xylosyltransferases involved in xylan backbone elongation.^{21-23,26-28} There exist 10 GT43 genes in the rice genome and phylogenetic analysis showed that there is a significant expansion of OsGT43 members that are closely grouped together with *Arabidopsis* IRX9 and I9H/IRX9L (Fig. 5A). All the rice GT43 genes have a similar exon-intron organization, i.e., they contain 3 exons and 2 introns (Fig. 5B). The expression of all these rice GT43 genes, except *OsGT43C* and *OsGT43D*, was evident in shoots and roots (Fig. 5C). We selected 4 representative members, OsGT43A, OsGT43E, OsGT43H, and OsGT43J, which are positioned at different nodes in the GT43 phylogenetic tree, for further functional analysis. Among them, OsGT43A and OsGT43E are phylogenetically closer to IRX9, OsGT43H closer to I9H/IRX9L, and OsGT43J closer to IRX14.

To investigate whether rice GT43 proteins are involved in xylan biosynthesis, we first examined their subcellular localization. The OsGT43A/E/H/J proteins were predicted to be type II membrane proteins with one transmembrane helix at the N-terminus (Fig. 6A) and when expressed in *Arabidopsis* protoplasts, the yellow fluorescence protein (YFP)-tagged OsGT43 proteins were co-localized with the cyan fluorescence protein (CFP)-tagged FRAGILE FIBER8 (FRA8) (Fig. 6D-S), a GT47 glycosyltransferase known to be localized in the Golgi.³⁵ The control protoplasts expressing YFP alone exhibited signals throughout the cytoplasm (Fig. 6B, C). These results demonstrate that the OsGT43 proteins are localized in Golgi, where xylan biosynthesis occurs.

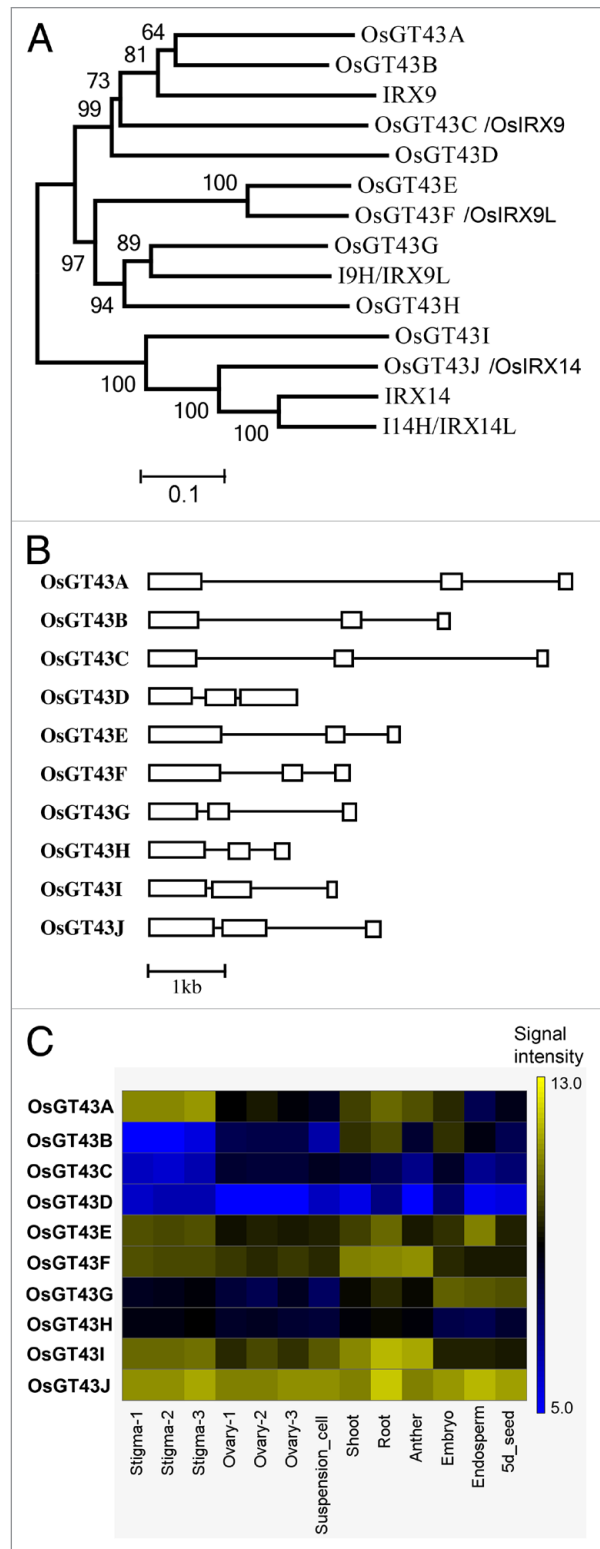
OsGT43A and OsGT43E but not OsGT43H and OsGT43J are functional orthologs of *Arabidopsis* IRX9

Arabidopsis GT43 members form 2 functionally non-redundant groups, IRX9/IRX9 homolog and IRX14/IRX14 homolog, both of which are required for xylan biosynthesis.^{26,27} To investigate the functional roles of OsGT43 members in xylan biosynthesis, we examined the ability of the 4 representative OsGT43 genes, *OsGT43A*, *OsGT43E*, *OsGT43H*, and *OsGT43J*, to complement the xylan defects conferred by the *irx9* and *irx14* mutations in *Arabidopsis*. The full-length *OsGT43* cDNAs driven by the cauliflower mosaic virus (CaMV) 35S promoter were transformed into the *irx9* and *irx14* mutants. The transformation was performed independently twice

Figure 5. Phylogenetic analysis of rice and *Arabidopsis* family GT43 proteins (A), exon-intron organization (B) and expression patterns (C) of OsGT43 genes. (A) The GT43 amino acid sequences were aligned using ClustalW and their phylogenetic relationship was analyzed using the neighbor-joining method in MEGA5.2 (Tamura et al., 2011). Bootstrap values resulted from 1,000 replicates are shown at the nodes. (B) Open boxes and lines denote exons and introns, respectively. The bar scale shows the number of nucleotides. (C) The expression of OsGT43 genes in various tissues shown as the heat map was from Li et al.⁵⁰ Shoot and root tissues are from 2-week-old shoots and roots of rice (*Oryza sativa* Japonica Nipponbare).

and more than 64 independent transgenic lines for each construct were analyzed. The expression of *OsGT43* genes in 8 representative transgenic lines for each construct was confirmed by reverse transcription PCR analysis of *OsGT43* transcripts (Fig. 7A). The *irx9* mutation led to a reduction in stem mechanical strength down to 15 to 20% of that of the wild type due to a defect in secondary wall thickening (Fig. 7B). Examination of transgenic *irx9* lines expressing *OsGT43* genes revealed that *OsGT43A* and *OsGT43E* were able to restore the stem mechanical strength to the wild-type level (Fig. 7B). In contrast, expression of *OsGT43H* and *OsGT43J* in the *irx9* mutant did not complement the stem mechanical strength. Consistent with the stem mechanical strength data, expression of *OsGT43A* and *OsGT43E* but not *OsGT43H* and *OsGT43J* rescued the collapsed vessel phenotypes caused by the *irx9* mutation (Fig. 7C to N).

To determine whether the restored stem strength and vessel morphology were accompanied with the complementation of xylan defects, we examined xylan content and structure in *irx9* expressing *OsGT43* genes. Mature inflorescence stems of 10-wk-old plants from 8 independent transgenic lines were pooled for extraction of cell walls. Sugar composition analysis of cell walls from inflorescence stems revealed that although the xylose content in *irx9* was only 52% of that of the wild type, expression of *OsGT43A* and *OsGT43E* restored the xylose content to about 80% of the wild-type level. In contrast, no restoration of xylose level was seen in the *irx9* lines expressing *OsGT43H* and *OsGT43J* (Table 1). Similarly, the *irx9* mutation caused a reduction of cellulose and lignin down to about 70% of the wild type, whereas expression of *OsGT43A* and *OsGT43E* but not *OsGT43H* and *OsGT43J* restored the levels of cellulose and lignin close to the wild-type level (Fig. 8). To analyze xylan structure, xylan was isolated from inflorescence stems and digested with endoxylanase to generate xylooligosaccharides, which were subsequently subjected to matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). The *irx9* mutation caused a loss of GlcA side chains in xylan as evidenced by the absence of the signal peak corresponding to the GlcA-substituted Xyl₄ (*m/z* 745) compared with the wild type (Fig. 9A, B). A partial restoration of the signal peak for the GlcA-substituted Xyl₄ was seen in the *irx9* lines expressing *OsGT43A* (Fig. 9C) and *OsGT43E* (Fig. 9D) but not in those expressing *OsGT43H* (Fig. 9E) and *OsGT43J* (Fig. 9F). The restoration of the GlcA side chains in the *irx9* lines complemented with *OsGT43A*



and *OsGT43E* was further verified by ¹H nuclear magnetic resonance (NMR) spectroscopy (Fig. 10). It was shown that although the resonance peak characteristic of ¹H GlcA at 5.31 ppm was absent in *irx9* compared with the wild type, it was evident in *irx9* complemented with *OsGT43A* and *OsGT43E*. In addition to the loss of GlcA side chains, the *irx9* mutation caused a defect in xylan chain length that was

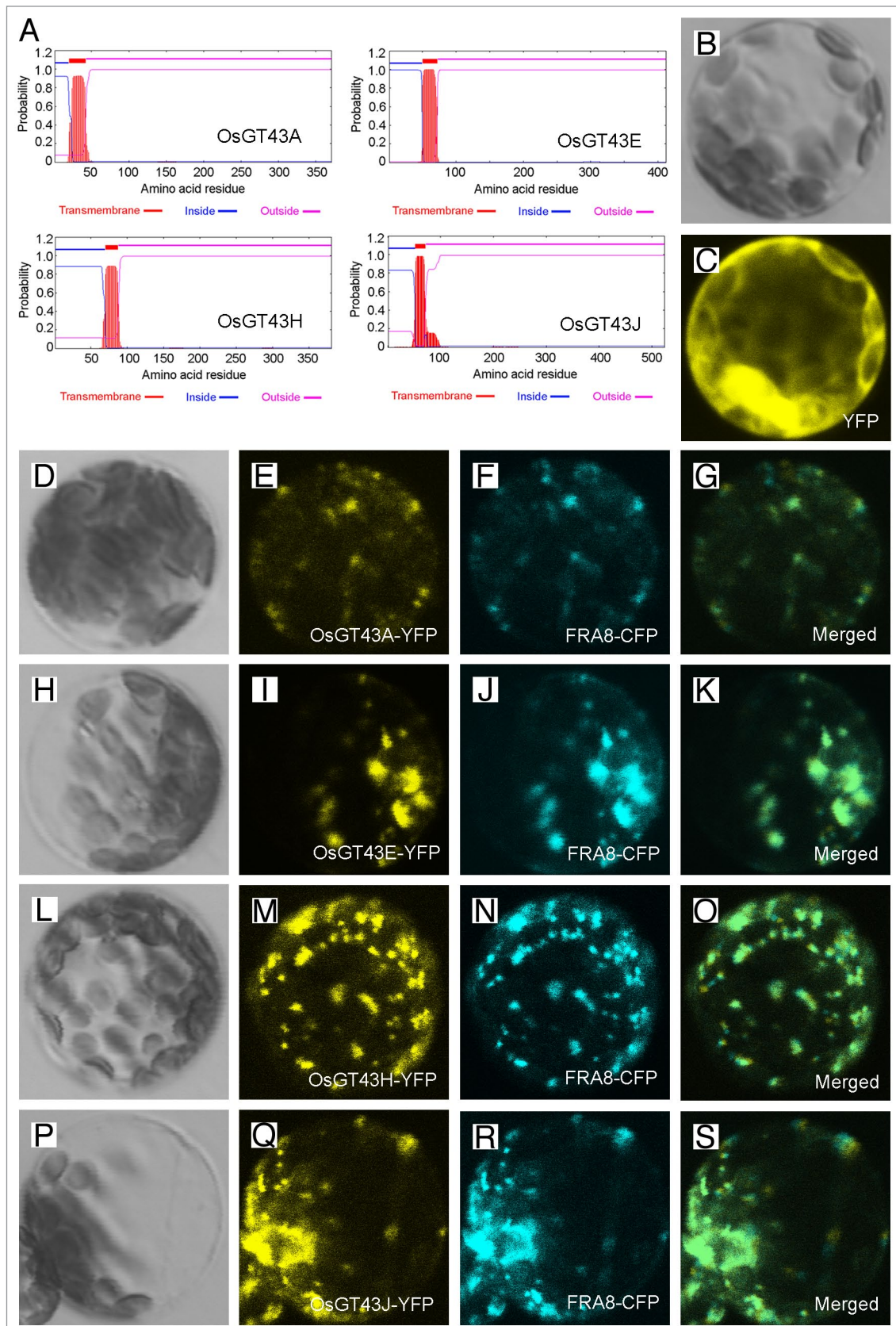


Figure 6. For figure legend, see page 7.

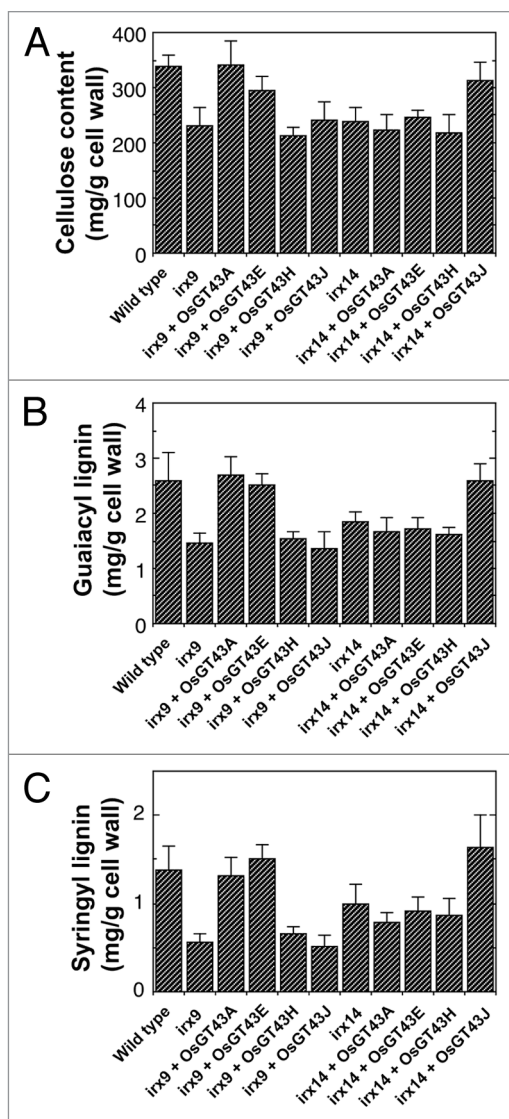


Figure 8. Measurement of cellulose and lignin amounts in *irx9* and *irx14* expressing *OsGT43* genes. Cell walls were isolated from pooled mature inflorescence stems of 8 independent transgenic lines for each construct and used for measurement of the amounts of cellulose (A), guaiacyl lignin (B) and syringyl lignin (C). The data are mean \pm SE of 2 separate assays. Note that expression of *OsGT43A* and *OsGT43E* in *irx9* restored cellulose, guaiacyl lignin and syringyl lignin to the wild-type level and that expression of *OsGT43J* in *irx14* restored cellulose, guaiacyl lignin and syringyl lignin to the wild-type level.

shortened from an average of 93 in the wild type to 22 (Table 2), thereby resulting in a relative increase in the abundance of NMR signal peaks attributed to the xylan reducing end sequence, β -D-Xyl-(1 \rightarrow 3)- α -L-Rha-(1 \rightarrow 2)- α -D-GalA-(1 \rightarrow 4)-D-Xyl (Fig. 10). Expression of *OsGT43A* and *OsGT43E* in *irx9* led to a partial restoration of the xylan chain length (Table 2) and concomitantly a significant reduction in the relative abundance of the xylan reducing end sequence (Fig. 10).

Consistent with the restored xylan content and structure, expression of *OsGT43A* and *OsGT43E* also rescued the defective

Table 1. Cell wall composition analysis of the *irx9* and *irx14* plants expressing rice *GT43* genes

Sample	Xylose	Glucose	Mannose	Galactose	Arabinose	Rhamnose
Wild type	115.3 \pm 0.4	413.1 \pm 5.3	21.0 \pm 0.4	16.8 \pm 0.4	15.1 \pm 0.7	13.0 \pm 2.8
<i>irx9</i>	60.8 \pm 0.3	337.9 \pm 0.4	39.0 \pm 0.1	38.4 \pm 1.3	28.6 \pm 0.2	16.4 \pm 0.6
<i>irx9</i> + <i>OsGT43A</i>	92.0 \pm 4.0	394.0 \pm 7.0	19.8 \pm 1.6	13.2 \pm 2.3	12.6 \pm 2.7	10.9 \pm 1.5
<i>irx9</i> + <i>OsGT43E</i>	89.1 \pm 4.3	451.3 \pm 4.0	22.9 \pm 1.1	14.7 \pm 1.0	14.2 \pm 1.3	11.8 \pm 0.4
<i>irx9</i> + <i>OsGT43H</i>	66.5 \pm 2.0	330.4 \pm 3.0	30.0 \pm 1.2	31.3 \pm 0.9	22.3 \pm 2.2	12.8 \pm 0.3
<i>irx9</i> + <i>OsGT43J</i>	60.5 \pm 1.6	338.3 \pm 6.8	33.6 \pm 1.9	24.1 \pm 1.7	14.6 \pm 1.9	11.2 \pm 1.5
<i>irx14</i>	52.5 \pm 5.0	335.0 \pm 8.4	26.2 \pm 0.7	31.6 \pm 2.9	14.4 \pm 1.5	14.4 \pm 0.9
<i>irx14</i> + <i>OsGT43A</i>	58.2 \pm 0.6	330.9 \pm 6.3	28.3 \pm 1.1	20.8 \pm 1.5	13.3 \pm 2.2	10.6 \pm 1.1
<i>irx14</i> + <i>OsGT43E</i>	53.6 \pm 1.9	329.5 \pm 17.7	26.1 \pm 1.4	19.2 \pm 1.2	12.1 \pm 1.3	10.0 \pm 0.2
<i>irx14</i> + <i>OsGT43H</i>	57.3 \pm 3.1	321.1 \pm 9.7	24.8 \pm 2.1	25.3 \pm 0.6	11.0 \pm 0.7	11.7 \pm 1.8
<i>irx14</i> + <i>OsGT43J</i>	110.3 \pm 3.2	395.3 \pm 7.7	18.4 \pm 0.9	13.7 \pm 1.8	11.4 \pm 2.1	10.4 \pm 0.5

The wall residues for cell wall composition analysis were prepared from a pool of mature inflorescence stems of 8 independent transgenic lines for each construct. The data are means (mg/g dry cell wall) \pm SE of 2 technical replicates.

xylosyltransferase activity conferred by the *irx9* mutation (Fig. 11A). While the xylan xylosyltransferase activity in *irx9* microsomes was only 9% of the wild-type level, the activity in *OsGT43A*- and *OsGT43E*-complemented lines was restored to 52% and 78%, respectively, of the wild-type level. In contrast, expression of *OsGT43H* and *OsGT43J* did not result in any significant changes in the xylosyltransferase activity. Taken together, these results demonstrate that *OsGT43A* and *OsGT43E* are functional orthologs of *IRX9* involved in the biosynthesis of the xylan backbone.

OsGT43J* but not *OsGT43A*, *OsGT43E*, and *OsGT43H* is a functional ortholog of *Arabidopsis* *IRX14

Examination of transgenic *irx14* plants expressing the 4 representative *OsGT43* genes by comprehensive histological, biochemical, and structural analyses revealed that only *OsGT43J* complemented the *irx14* mutant phenotypes, including the restoration of stem mechanical strength, vessel morphology, xylan content, cellulose and lignin levels, GlcA side chains, xylan chain length, and xylosyltransferase activity (Figs. 7–11; Tables 1 and 2). Neither *OsGT43A* and *OsGT43E* nor *OsGT43H* was able to rescue the *irx14* mutant phenotypes. These results demonstrate that *OsGT43J* is a functional ortholog of *IRX14*. Together with the complementation analysis in *irx9*, these results indicate that the rice *GT43* family retained 2 functionally non-redundant groups of *GT43* members. However, the functions of some of the rice *GT43* members, such as *OsGT43H*, may have diverged from those of *Arabidopsis*.

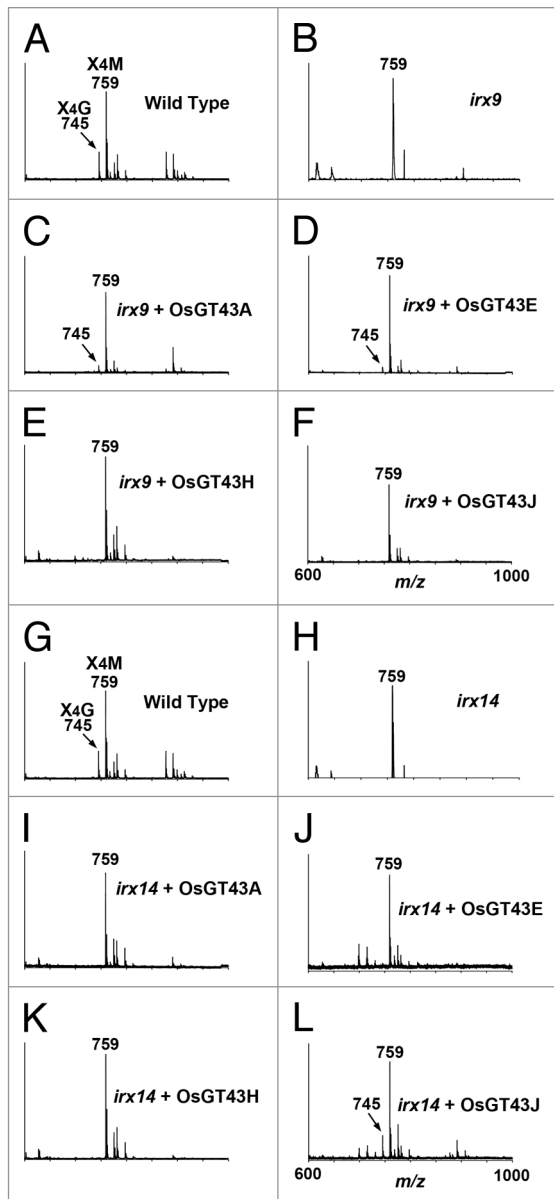


Figure 9. MALDI-TOF mass spectra of xylooligosaccharides generated by xylanase digestion of xylans from the wild type, *irx9*, *irx14*, and the mutants expressing OsGT43 genes. The ions at *m/z* 745 and 759 in the wild type (A and G) correspond to xylotetrasaccharides bearing a GlcA residue (X_4G) or a methylated GlcA residue (X_4M). (A) to (F) The missing ion at *m/z* 745 corresponding to X_4G in *irx9* (B) was partially restored by expression of OsGT43A (C) and OsGT43E (D) but not OsGT43H (E) and OsGT43J (F). (G) to (L) The missing ion at *m/z* 745 corresponding to X_4G in *irx14* (H) was restored by expression of OsGT43J (L) but not OsGT43A (I), OsGT43E (J), and OsGT43H (K).

Discussion

There exist 4 GT43 members in *Arabidopsis*, all of which have been demonstrated to be involved in xylan backbone biosynthesis. Previous genetic and biochemical analyses have shown that they form 2 functionally nonredundant groups, IRX9/IRX9 homolog and IRX14/IRX14 homolog, which act cooperatively in the elongation of the xylan backbone.^{21-23,26-28}

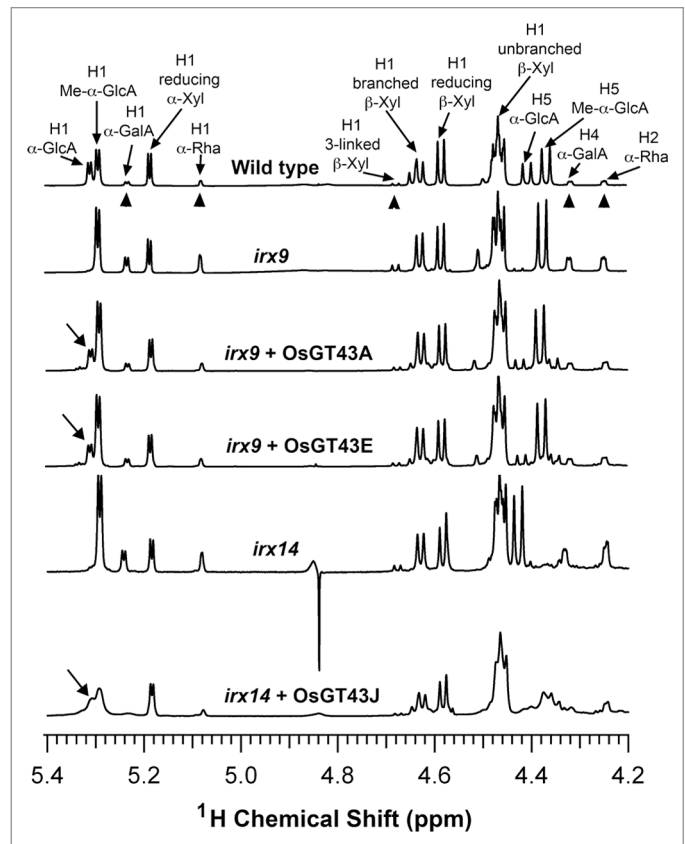


Figure 10. 1H -NMR spectra of xylooligosaccharides generated by xylanase digestion of xylans from the wild type, *irx9*, *irx14*, and the mutants expressing OsGT43 genes. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. Note the restoration of the resonance of H1 of α -GlcA in *irx9* complemented with OsGT43A and OsGT43E and in *irx14* complemented with OsGT43J. The resonances of H1 of α -D-GalA, H1 of α -L-Rha, H1 of 3-linked β -D-Xyl, H4 of α -D-GalA, and H2 of α -L-Rha (arrow heads) are from the GX reducing end tetrasaccharide sequence. Note the relatively elevated resonance intensities of the GX reducing end tetrasaccharide sequence in *irx9* and *irx14* compared with the wild type and the reduction in the intensity of these resonances in *irx9* complemented with OsGT43A and OsGT43E and in *irx14* complemented with OsGT43J.

The grass species such as rice apparently evolved to have an expansion of the GT43 family. Since expansion of a gene family is often accompanied by functional diversification³⁶ and glycosyltransferase families are classified based on their amino acid sequence similarity rather than on their uses of common acceptors and/or donors,³⁷ one could not simply assume that all rice GT43 genes perform the same functions as those in *Arabidopsis*. Furthermore, it is currently unknown whether grass species evolved to retain 2 functionally nonredundant groups of GT43 genes for xylan biosynthesis because an early study of wheat protein complexes possessing xylan xylosyltransferase activity only identified an IRX14 homolog.²⁹ Therefore, it is critical to investigate whether the involvement of 2 functionally nonredundant groups of GT43 genes in xylan biosynthesis is evolutionarily conserved between dicots and grasses.

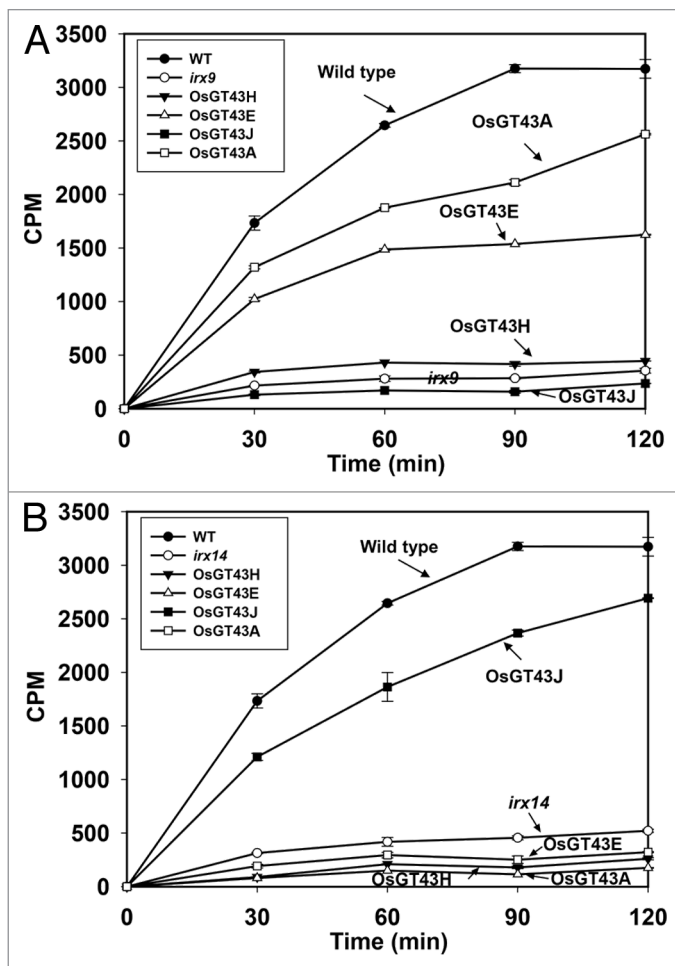


Figure 11. Time course of the xylosyltransferase activity in the microsomes from the wild type, *irx9*, *irx14*, and the mutants expressing OsGT43 genes. Microsomes were isolated from stems of 8 independent transgenic lines for each construct. The isolated microsomes were incubated with radiolabeled UDP-xylose and the Xyl₄ acceptor, and the xylosyltransferase activity (CPM) was measured by the amount of radiolabeled xylosyl residues transferred onto the acceptor. The data are mean ± SE of 3 technical replicates. (A) Restoration of the xylosyltransferase activity in *irx9* by expression of OsGT43A and OsGT43E but not OsGT43H and OsGT43J. (B) Restoration of the xylosyltransferase activity in *irx14* by expression of OsGT43J but not OsGT43A, OsGT43E and OsGT43H.

In this report, we studied the functions of rice GT43 members through complementation analysis in *Arabidopsis irx9* and *irx14* mutants. Phylogenetic analysis indicates that rice GT43 members are more diverged than those from *Arabidopsis*. In particular, there are 8 IRX9 homologs in rice, whereas only 2 members, IRX9 and IRX9 homolog, exist in *Arabidopsis*. Therefore, we selected 4 representative OsGT43 genes to carry out reciprocal complementation analysis in the *Arabidopsis* mutants. It was found that OsGT43A and OsGT43E, which are phylogenetically more related to IRX9, rescued the mutant phenotypes of *irx9* but were unable to complement those of *irx14*. By contrast, OsGT43J, which is grouped closer to IRX14, rescued *irx14* mutant phenotypes but was unable to complement those of *irx9*. These results demonstrate that OsGT43A and

Table 2. Relative abundance of the reducing end sequence and the DP of xylan in the *irx9* and *irx14* plants expressing rice GT43 genes

Sample	Frequency of occurrence of GlcA side chains ^a	Relative abundance of the reducing end sequence ^b	Average
Wild type	11.00%	100%	93
<i>irx9</i>	11.60%	415%	22
<i>irx9</i> + OsGT43A	12.10%	172%	54
<i>irx9</i> + OsGT43E	12.60%	161%	58
<i>irx14</i>	11.20%	391%	24
<i>irx14</i> + OsGT43J	10.00%	184%	51

^aThe frequency of occurrence of the MeGlcA side chains was calculated from the ratios of the NMR resonance of the branched β-Xyl to that of total β-Xyl.

^bThe relative abundance of the reducing end tetrasaccharide sequence was determined by the ratios of the NMR resonance of the reducing end sequence to that of the branched β-Xyl, and the abundance of the reducing end tetrasaccharide sequence in the wild type was taken as 100%.

^cDegree of polymerization (DP) of xylan was calculated according to Pena et al.²³

OsGT43E are orthologs of IRX9, and OsGT43J is an ortholog of IRX14. This finding provides the first line of genetic evidence indicating that rice evolved to retain 2 functionally nonredundant groups of GT43 genes involved in xylan backbone biosynthesis. The involvement of 2 functionally nonredundant groups of GT43 genes in xylan backbone biosynthesis is likely conserved in other grass species. A recent report showed that RNA interference suppression of an IRX9 homolog in wheat results in a decrease in arabinoxylan content.³⁸ Together with an early report of the presence of an IRX14 homolog in wheat protein complexes possessing xylan xylosyltransferase activity,²⁹ these reports indicate that homologs of both IRX9 and IRX14 are likely involved in xylan biosynthesis in wheat though it was still unknown whether wheat homologs of IRX9 and IRX14 are functionally nonredundant. Our finding further supports the hypothesis that 2 functionally nonredundant groups of GT43 xylosyltransferases are needed to overcome the steric problem for the transfer of xylosyl residues onto the growing xylan chain because one xylosyl residue is flipped nearly 180° relative to its neighboring residue.²⁶

In addition to the functional orthologs of IRX9 and IRX14, some rice GT43 members may have divergent functions. OsGT43H, which is phylogenetically close to I9H/IRX9L, could not complement the mutant phenotypes of either *irx9* or *irx14*. One possibility is that OsGT43H might have evolved to have catalytic functions different from IRX9 and IRX14. It is common that glycosyltransferase members in the same GT families may perform different catalytic functions. For example, FRA8 and IRX10, both of which are phylogenetically closely related members of GT47 family, are involved in the biosynthesis of the xylan reducing end sequence and the xylan backbone, respectively.^{23,24} The other possibility is that although it could not complement *irx9* and *irx14* mutants due to its sequence divergence from the *Arabidopsis* GT43 protein, OsGT43H is still part of the xylan synthase complex in rice. It remains to be investigated whether the other *OsGT43* member, OsGT43G,

which is phylogenetically closely related to OsGT43H, is able to rescue the mutant phenotypes of *irx9* or *irx14*.

It is interesting to note that although expression of *OsGT43A* and *OsGT43B* in *irx9* and expression of *OsGT43J* in *irx14* almost fully restored the stem strength, vessel morphology and xylose content to the wild-type levels, the chain length of xylan in the complemented plants is only restored to about 60% of that of the wild type. These results indicate that plants could tolerate a significant variation of xylan chain length and xylan with a 60% of wild-type xylan chain length is sufficient to accommodate the normal assembly of cell walls.

During the course of our investigation of the functions of rice GT43 proteins, a study reported that 2 other OsGT43 members, *OsGT43C/OsIRX9* and *OsGT43F/OsIRX9L*, complemented the *irx9* mutant phenotypes and *OsGT43J/OsIRX14* complemented the *irx14* mutant phenotypes.³⁹ However, it was stated that the defective xylan chain length in *irx14*, which is the primary defect of the mutant, was not complemented by examination of one presumably complemented transgenic line expressing *OsGT43J/OsIRX14*. Thus, the results from Chiniquy et al.³⁹ could not conclude that *OsGT43J/OsIRX14* functionally complements the xylan defect exhibited by *irx14*. Our results demonstrated that *OsGT43J/OsIRX14* was able to rescue the mutant phenotypes of *irx14* including the defective xylan chain length (Fig. 10; Table 2), which unequivocally establishes that *OsGT43J* is a functional ortholog of *IRX14*.

In summary, we show that rice stem microsomes possess both a xylan xylosyltransferase activity and a xylosidase-like activity, and the rice family GT43 retains 2 functionally non-redundant groups involved in xylan backbone biosynthesis. Although the xylan structure in rice differs from those in dicots by having no apparent reducing end sequence, abundant arabinosyl side chains, and ferulate as xylan cross-linking molecules, it appears that the biosynthetic machinery responsible for the xylan backbone elongation is conserved between rice and dicots, both of which involve 2 functionally nonredundant groups of GT43 members. Further investigation of the biochemical mechanism controlling xylan biosynthesis in rice could provide knowledge basis for genetic modification of grass crops, such as switchgrass and *Miscanthus*, better suited for biofuel production.

Materials and Methods

Cell wall preparation

Rice (*Oryza sativa* Japonica Teipei 309) stems and *Arabidopsis* (*Arabidopsis thaliana*) inflorescence stems were used for cell wall isolation according to Zhong et al.³⁵ Cell walls were extracted for xylan with 1N KOH, and the solubilized xylan was digested with β -xylanase M6 (Megazyme) to generate xylooligosaccharide for matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and nuclear magnetic resonance (NMR) spectroscopy.

¹H-NMR spectroscopy

NMR spectra of rice and *Arabidopsis* xylooligosaccharides were acquired at 20 °C on a 600 MHz spectrometer (599.7

MHz, ¹H) using a 3 mm cryogenic triple resonance probe.⁴⁰ All NMR samples were prepared with 100% D₂O. For all experiments, 128 transients were collected using a spectral width of 6,000 Hz and an acquisition time of 5 s. The residual water resonance was suppressed by a 1 s presaturation pulse at a field strength of 40 Hz. All spectra were processed with 0.2-Hz apodization followed by zero-filling to 128 k points. The ¹H NMR assignments were done by comparing them to the NMR spectral data for xylan structure.^{23,31,32,35}

Assay of xylosyltransferase activity

Microsomes were isolated from the stems of rice and *Arabidopsis* following the procedure of Kuroyama and Tsumuray³³ and stored at -80 °C until used. For assay of the xylosyltransferase activity using the radiolabeling method, microsomes (100 μ g) were incubated with the reaction mixture containing 50 mM HEPES-KOH, pH 6.8, 5 mM MnCl₂, 1 mM dithiothritol, 0.5% Triton X-100, 0.1 mM cold UDP-Xyl (CarboSource Service), 0.2 μ g/ μ l Xyl₄ (Megazyme) and UDP-[¹⁴C]Xyl (0.1 μ Ci; American Radiolabeled Chemical) at 21 °C for 30 min. The reaction products were separated from UDP-[¹⁴C]Xyl by paper chromatography according to Ishikawa et al.⁴¹ and counted for the amount of radioactivity with a PerkinElmer scintillation counter. For assay of the xylosyltransferase activity using fluorescent anthranilic acid (AA)-labeled xylooligomer acceptors, microsomes (100 μ g) were incubated with the reaction mixture containing 50 mM HEPES-KOH, pH 6.8, 5 mM MnCl₂, 1 mM DTT, 0.5% Triton X-100, 0.1 mM cold UDP-Xyl, 0.5 mM Xyl_n-AA at 21 °C for 30 min. The reaction products were analyzed by reversed-phase HPLC analysis. HPLC was performed with a PerkinElmer series 200 LC system and a PerkinElmer series 200a fluorescence detector (Ex_{320nm}, Em_{420nm}). The incorporated Xyl_n-AA products were separated using a Kinetex C18 (2.6 μ m) column (100 mm long, 4.6 mm i.d.) (Phenomenex) according to Lee et al.²²

Xylosidase digestion of xylooligomers

The rice microsome-catalyzed reaction products using the Xyl₆-AA acceptor were incubated with β -(1,4)-xylosidase (0.005 U; Sigma) for 1 h at 37 °C. The reaction was terminated with 0.1 M acetic acid and subjected to reverse-phase HPLC analysis.

Subcellular localization

The subcellular localization of *OsGT43* proteins was performed by co-transfecting yellow fluorescent protein (YFP)-tagged fusion proteins and the cyan fluorescent protein (CFP)-tagged Golgi marker (FRA8) into *Arabidopsis* protoplasts.⁴² The full-length *OsGT43* cDNAs were fused in frame with the *YFP* cDNA and ligated between the CaMV 35S promoter and the nopaline synthase terminator in pBI221 (Clontech). After transfection, protoplasts were incubated for 16 h and fluorescence signals in transfected protoplasts were visualized using a Leica TCs SP2 spectral confocal microscope (Leica Microsystems).

Complementation of the *irx9* and *irx14* mutants

The full-length *OsGT43* cDNAs driven by the CaMV 35S promoter were cloned into the pGPTV binary vector containing

hygromycin resistant selection marker gene⁴³ to create *OsGT43* expression constructs, and the cloned *OsGT43* cDNA sequences in the expression constructs were verified by sequencing. The *OsGT43* expression constructs were introduced into the *Arabidopsis irx9* or *irx14* mutant by the agrobacterium-mediated transformation. Transgenic plants were selected on hygromycin, and the expression of *OsGT43* genes in rosette leaves of transgenic plants was verified using reverse-transcription PCR. Inflorescence stems from the first generation of complemented transgenic plants were used for morphological and chemical analyses of phenotypes. Basal parts of the main inflorescence of 10-wk-old plants were measured for the breaking force using a digital force/length tester.⁴⁴ The breaking force was calculated as the force needed to break apart a stem segment. Basal parts of the main inflorescence of 10-wk-old plants were also fixed for light microscopy.⁴⁵ For each construct, at least 64 transgenic plants were generated and examined.

Cell wall analysis

Cell wall sugars (as alditol acetates) were assayed following the procedure described by Hoebler et al.⁴⁶ The alditol acetates of the hydrolyzed cell wall sugars were analyzed on a PerkinElmer Clarus 500 gas-liquid chromatograph instrument equipped with a 30 min x 0.25 mm (i.d.) silica capillary column DB 225 (Alltech Assoc.). Cellulose content was assayed with the anthrone reagent according to Updegraff.⁴⁷ Lignin composition was determined as described.^{48,49} Cell walls were hydrolyzed in 4 N NaOH and the released lignin monomers were extracted into diethylether and vacuum dried. The residue was dissolved in pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide and analyzed for phenolics by gas chromatography. Phenolic compounds were identified by comparison of their mass spectra with those of the authentic compounds. Guaiacyl lignin unit is the sum of vanillin, acetovanillin, and vanillic acid. Syringyl lignin unit is the sum of syringaldehyde, acetosyringaldehyde, and syringic acid.

MALDI-TOF MS

Xylooligosaccharides released from xylanase digestion of KOH-extracted xylan were analyzed using a MALDI-TOF mass

spectrometer operated in the positive-ion mode with an accelerating voltage of 30 kV, an extractor voltage of 9 kV, and a source pressure of approximately 8×10^{-7} torr. The aqueous sample was mixed (1:1, v/v) with the MALDI matrix (0.2 M 2,5-dihydroxybenzoic acid and 0.06 M 1-hydroxyisoquinoline in 50% acetonitrile) and dried on the stainless steel target plate. Spectra are the average of 100 laser shots.

Accession numbers

The locus identifiers for the rice GT43 genes are OsGT43A (LOC_Os05 g03174; Os05 g0123100), OsGT43B (LOC_Os03 g17850; Os03 g0287800), OsGT43C/OsIRX9 (LOC_Os07 g49370; Os07 g0694400), OsGT43D (LOC_Os01 g06450; Os01 g0157700), OsGT43E (LOC_Os05 g48600; Os05 g0559600), OsGT43F/OsIRX9L (LOC_01 g48440; Os01 g0675500), OsGT43G (LOC_Os10 g13810; Os10 g0205300), OsGT43H (LOC_Os04 g01280; Os04 g0103100), OsGT43I (LOC_Os04 g55670; Os04 g0650300), and OsGT43J/OsIRX14 (LOC_Os06 g47340; Os06 g0687900). The *Arabidopsis* Genome Initiative locus identifiers for the *Arabidopsis* GT43 genes are IRX9 (At2g37090), I9H/IRX9L (At1g27600), IRX14 (At4g36890), and I14H/IRX14L (At5g67230). The GenBank accession numbers of the rice GT43 genes are KJ206898 for OsGT43A, KJ206899 for OsGT43B, KJ206900 for OsGT43C, KJ206901 for OsGT43D, KJ206902 for OsGT43E, KJ206903 for OsGT43F, KJ206904 for OsGT43G, KJ206905 for OsGT43H, KJ206906 for OsGT43I, and KJ206907 for OsGT43J.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was funded by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the US Department of Energy (DE-FG02-03ER15415).

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