Two Trichome Birefringence-Like Proteins Mediate Xylan Acetylation, Which Is Essential for Leaf Blight Resistance in Rice

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Acetylation is a ubiquitous modification on cell wall polymers, which play a structural role in plant growth and stress defenses. However, the mechanisms for how crop plants accomplish cell wall polymer O-acetylation are largely unknown. Here, we report on the isolation and characterization of two trichome birefringence-like (tbl) mutants in rice (Oryza sativa), which are affected in xylan O-acetylation. ostbl1 and ostbl2 single mutant and the tbl1 tbl2 double mutant displayed a stunted growth phenotype with varied degree of dwarfism. As shown by chemical assays, the wall acetylation level is affected in the mutants and the knock-down and overexpression transgenic plants. Furthermore, NMR spectroscopy analyses showed that all those mutants have varied decreases in xylan monoacetylation. The divergent expression levels of overexpression transgenic plants. Furthermore, NMR spectroscopy analyses showed that all those mutants have varied decreases in xylan monoacetylation. The divergent expression levels of OstBL1 and OstBL2 explained the chemotype difference and indicated that OstBL1 is a functionally dominant gene. OstBL1 was found to be Golgi-localized. The recombinant OstBL1 protein incorporates acetyl groups onto xylan. By using xylanopentose, a preferred acceptor substrate, OstBL1 can transfer up to four acetyl residues onto xylpentose, and this activity showed saturable kinetics. 2D-NMR spectroscopy showed that OstBL1 transfers acetate to both 2-O and 3-O sites of xylosyl residues. In addition, ostbl1 and tbl1 tbl2 displayed susceptibility to rice blast disease, indicating that this xylan modification is required for pathogen resistance. This study identifies the major genes responsible for xylan acetylation in rice plants.

The plant cell wall, a sophisticated multiple polymer network, encases plant cells and plays essential roles in plant growth and development, including morphogenesis, water and nutrient transduction, and protection of plants against abiotic and biotic stresses. To achieve these functions, plants often incorporate acetyl substituents into cell wall polymers (Gille and Pauly, 2012). Hence, many cell wall polymers, including pectin, several kinds of hemicellulose, and lignin, are O-acetylated (Gille and Pauly, 2012; Pawar et al., 2013). As O-acetylation impacts physicochemical properties of polymers, the abundance and position of acetyl groups on the polymers vary in different plant species and tissues and across various developmental stages. Acetyl substitution on xyloglucan (XyG), the major hemicellulose in the dicot primary wall, often occurs on side chains, specifically the galactosyl residues at O-3, O-4, or O-6 (Gille et al., 2011b). Only grass and dicotyledonous Solanaceae have an O-acetylated XyG backbone (Schultink et al., 2014; Liu et al., 2016). The acetyl moieties on the backbone glucosyl residues are at O-6 (Hoffman et al., 2005; Jia et al., 2005). Xylan is the most abundant hemicellulose in nature and has a highly acetylated backbone. The degrees of acetylation range from 0.3 to 0.6 acetate to Xyl ratio depending on the plant species (Telemann et al., 2002; Yuan et al., 2016c). Acetyl groups are attached to xylan xylopyranosyl residues at O-2 or O-3 and O-3 if O-2 contains other substituents, for example, GlcA/McGlcA/Araf (Telemann et al., 2000). In addition to these two polymers, glucomannans, pectins,
and lignin were also found to be O-acetylated at certain positions (Liners et al., 1994; Schols and Voragen, 1994; Lundqvist et al., 2002; del Río et al., 2008; Gille et al., 2011a). Although monoacetylation is the major modification form, some polymers, such as XyG, xylan, and pectins, can be substituted by two O-acetyl-groups on one sugar residue. However, the molecular mechanism for how plants process and regulate cell wall polymer O-acetylation is not well understood.

Recent progress indicates that at least three groups of proteins, RWAs (Reduced Wall Acetylation), Trichome Birefringence-Like (TBL), and AXY9 (Altered Xyloglucan proteins), are involved in the polymer O-acetylation. RWAs consist of 10 transmembrane helices homologous to the CnCas1p from Cryptococcus (Manabe et al., 2011). Arabidopsis (Arabidopsis thaliana) possesses four FWA homologs in its genome, and mutation of FWA2 results in an overall reduction of acetylation on several polymers (Manabe et al., 2011). The quadruple mutant of all four FWA genes exhibited similar but more severe defects (Lee et al., 2011), indicating that RWAs might function as transporters by supplying acetyl-donor(s) for polysaccharide O-acetylation into the Golgi apparatus (Gille and Pauly, 2012). AXY9 is a recently reported Golgi-localized protein bearing only one transmembrane domain. The decrease in acetylation level on multiple polymers such as XyG and xylan in the axy9 mutant indicates its involvement in O-acetylation, but its precise function remains elusive (Schultink et al., 2015). TBL proteins share two conserved domains, the TBL and DUF231 domain, with Arabidopsis Trichome Birefringence protein (Potikha and Delmer, 1995; Bischoff et al., 2010). Each domain harbors one conserved motif, a Gly-Asp-Ser (GDS) and an Asp-x-x-His (DxxH), respectively (Gille and Pauly, 2012). Based on the sequence similarity, 46 TBL members have been found in Arabidopsis, potentially differing in their polymer specificity. Mutations in AXY4/TBL27 and AXY4L/TBL22 lead to compromised acetyl-XyG, providing genetic evidence for this hypothesis (Gille et al., 2011b). Furthermore, ESK1/TBL29 was identified as a xylan acetyltransferase, because the esk1 mutant displays a specific defect in 2-O- and 3-O-monocetylation on xylan (Xiong et al., 2013; Yuan et al., 2013). Several ESK1 close homologs, TBL3, TBL31, TBL32, TBL33, TBL34, and TBL35, have been characterized to be additional candidates for xylan acetylation, probably with varied activity or regiospecificity (Yuan et al., 2016a, 2016b, 2016c). Nevertheless, ESK1 is so far the only TBL being biochemically documented to be able to transfer the O-acetyl substituent to the 2-O and 3-O positions of xylopyranosyl residues in vitro (Urbanowicz et al., 2014). Biochemical activities of the other TBL proteins remain unknown.

The wide existence of acetylation modification on wall polymers indicates functional importance. However, the impetus that promotes plants to evolve this specific modification is not clear. One possibility is that this modification can enhance the interaction of the hemicelluloses with other wall polymers (Busse-Wicher et al., 2014), leading to the rigid wall conformation to facilitate relevant physiological functions. Several Arabidopsis mutants that have the compromised acetyl-xylan exhibit collapsed xylem vessels and abnormal growth status (Lee et al., 2011; Xiong et al., 2013; Schultink et al., 2015; Yuan et al., 2016c). Another potential function of polymer O-acetylation is to protect plants against invading microorganism and environmental stresses. Theesk1 (tbl29) mutant displays freezing resistance in the absence of cold acclamation (Xin and Browse, 1998). pnr5 (tbl44) exhibits tolerance to powdery mildew (Vogel et al., 2004). axy4 ( tbl27) mutant was more sensitive to aluminum treatments (Zhu et al., 2014). These data suggest that acetylation of wall polymers is vital for plant growth and adaptation to various environments.

Rice (Oryza sativa) is one of the world’s most important crops. With its grass-specific wall structure, rice is used as a model for understanding cell wall biosynthesis in monocots. The rice genome contains more TBL members than Arabidopsis. But none of rice TBL members have been characterized. It is therefore unclear what agronomic trait is correlated with polysaccharide acetylation. Here, we report on the functional characterization of two rice TBL proteins, OsTBL1 and OsTBL2, which are homologs of Arabidopsis TBL34 (Yuan et al., 2016b). We provide several lines of biochemical and genetic evidence to demonstrate that OsTBL1 and OsTBL2 are xylan acetyltransferases, which is responsible for xylan backbone monoacetylation and resistance to bacterial leaf blight disease.

RESULTS
Phylogenetic Analysis of TBL Family in Rice
Blastp and domain searches in the rice genome database (Rice Genome Annotation Project, http://rice.plantbiology.msu.edu/) were performed to systematically identify TBL members in the rice genome. As a result, 66 TBL members were found distributed over 10 chromosomes, with the highest density on chromosome 6, where 20 TBL genes are clustered. The rice TBL proteins are composed of a conserved TBL domain and a DUF231 domain with an average length of 457 amino acids, except Os04g58340, Os06g15560, and Os06g15580, which do not contain a DUF231 domain (Supplemental Table S1). Gene expression analysis showed that 61 TBLs have different expression profiles (Supplemental Table S2), indicating that they may function in a spatio-temporal manner. Based on the sequence similarity of 46 Arabidopsis and 66 rice TBLs, we constructed a phylogenetic tree using Maximum likelihood. The Arabidopsis TBLs that have been identified to affect XyG and xylan acetylation were clustered into different clades (Fig. 1; Gille et al., 2011b; Xiong et al., 2013; Yuan et al., 2016a, 2016b, 2016c). In addition, most Arabidopsis TBL proteins have at least one rice homolog (Fig. 1), indicating that gene expansion events seem to occur after the monocot-dicot divergence. Arabidopsis TBL29 is the only TBL biochemically identified as a xylan acetyltransferase (Urbanowicz et al., 2014). Rice has 15 TBL29 homologs. We sequentially
named the 66 rice TBL proteins from this clade, from OsTBL1 to OsTBL66 (Fig. 1).

Mutations in OsTBL1 and OsTBL2 Disrupt MonoO-Acetylation of Xylan

To address the function of these rice TBL genes, we isolated two Tos17 insertional mutants in Os12g01560/OsTBL1 and Os11g01570/OsTBL2 (Fig. 2, A and B). Both ostbl1 and ostbl2 mutants exhibited a stunted growth habit, and ostbl1 looks severe compared to ostbl2 (Fig. 2, C and D). Sequence alignment revealed that both TBLs harbor the conserved TBL and DUF231 domains with up to 97% sequence identity (Supplemental Fig. S1). The double mutant, tbl1 tbl2, showed reduced plant height similar as ostbl1 (Fig. 2D). In addition, we generated OsTBL1 and OsTBL2 knock-down plants by artificial miRNA approach and the overexpression plants. The knock-down plants mimicked the mutant phenotypes, showing the reduced plant height, whereas the overexpression lines were morphologically indistinguishable from the wild-type plants, although quantitative real-time PCR (qRT-PCR) revealed an increased expression level of OsTBL1 and OsTBL2 in the corresponding lines (Supplemental Fig. S2).

Next, we examined structural wall attributes of wild type, mutants, and the transgenic lines. No significant reduction in neutral monosaccharide and cellulose content was found in the mutants and transgenic lines (Supplemental Table S3). We further examined the acetates bound on cell wall polymers. ostbl1 and ostbl2 contained 16% and 10% fewer acetyl esters compared to wild type, respectively, similar to decreases observed in the knock-down plants (Fig. 3A). The acetate level in the
double mutants was decreased by 20%. Although in the OsTBL1 overexpression plants an increased acetyl-content could be observed (Fig. 3A), no acetate alteration was detected in the OsTBL2 overexpression plants, which might be due to the low expression level of OsTBL2. It could be concluded that OsTBL1 and OsTBL2 are involved in wall O-acetylation. To identify the wall polymer(s) that were affected, deuterated dimethyl...
sulfoxide (DMSO-d6) dissolved cell wall residues were subjected to NMR spectroscopy. Heteronuclear single quantum coherence (HSQC) analyses showed that the signal intensity of 3-O-Ac-β-D-Xylp was significantly decreased in the mutants, whereas 2-O-Ac-β-D-Xylp was slightly altered (Fig. 3, B and C). To verify this finding, acetyl-xylans were extracted from the wild type and mutants by DMSO and their acetyl ester content was examined. The acetate content was decreased by 47% and 12% in ostbl1 and ostbl2, respectively, while a 55% reduction was found in the tbl1 tbl2 double mutant (Fig. 3D). Therefore, OsTBL1 and OsTBL2 are required for xylan monoacetylation.

In Arabidopsis, defects in xylan acetylation often cause collapsed vessels (Xiong et al., 2013; Yuan et al., 2013). We therefore examined the anatomic structure of internodes in wild type, ostbl1, ostbl2 and the double mutants. No obviously morphological changes were detected in xylem vessels (Supplemental Fig. S3). Probably due to the divergent pattern of vascular system between the monocot and dicot species, the decreased acetylation level resulted from OsTBL1 and OsTBL2 mutations does not cause collapsed vessels in rice.

**OsTBL1 Has a Major Effect Compared to OsTBL2 in Rice**

The above analyses displayed varied abnormalities in ostbl1 and ostbl2, in which ostbl1 looks more severe than ostbl2. We proposed that such phenotypic divergence may result from gene expression rather than biochemical activity, because their protein sequences are almost identical. To address this possibility, we compared the expression levels of OsTBL1 and OsTBL2 in the wild-type tissues. The transcript levels of OsTBL1 are indeed much higher than those of Ostbl2 in the rice internodes as revealed by qRT-PCR (Fig. 4A). Moreover, we examined whether there was a change in expression of OsTBL1 in a ostbl2 line and vice versa. qRT-PCR analyses showed that the expression level of OsTBL1 is unchanged in ostbl2, whereas OsTBL2 is up-regulated by 1.5-fold in ostbl2 (Fig. 4B). Considering that overexpression of OsTBL2 up to 5-fold did not show a phenotype (Fig. 3; Supplemental Fig. S2), the phenotypes observed in both mutants are mainly attributed to the gene mutation itself, respectively. To verify this conclusion at a protein level, polyclonal antibodies against OsTBL1/2 were generated. Western blotting detected only one band in the wild type and an identical but faint and wild-type-like band in ostbl1 and ostbl2, respectively (Fig. 4C). No signal was observed in the double mutant (Fig. 4C). Therefore, although this antibody was specific for OsTBL1 and OsTBL2, it could not distinguish between the two isoforms. The stronger signals observed in the ostbl2 mutant are in agreement with the higher levels of transcripts observed for OsTBL1, suggesting that the phenotypic deviations in ostbl1 and ostbl2 mutants are likely due to varied transcript and protein abundance in the mutants. Taken together, OsTBL1 has the dominant phenotypic effect.

**OsTBL1 Is a Golgi-Localized Protein**

As OsTBL1 and OsTBL2 share almost identical protein sequence, the following study was focused on OsTBL1. The Golgi apparatus is a major organelle for matrix polysaccharide synthesis and acetylation (Gille and Pauly, 2012). To determine whether OsTBL1 is Golgi-localized, we fused OsTBL1 with a green fluorescence protein (GFP) and cotransfected this chimeric construct in Nicotiana benthamiana leaves with a mCherry-fused Golgi marker, Man49. The overlapping signals indicated that OsTBL1 is localized in the Golgi apparatus (Fig. 5, A and B). To verify this finding in planta, we performed Suc density gradient centrifugation and western blotting of fractions prepared from wild-type seedlings. The fractions labeled by anti-OsTBL1/2 antibodies were largely identified by anti-IRX14 antibodies that recognize IRX14, a Golgi-protein involved in xylan biosynthesis in rice (Chiniquy et al., 2013; Lee et al., 2014), suggesting that OsTBL1 and IRX14 are present in the same organelle (Fig. 5C). Anti-BiP and anti-PIP1s antibodies that target to the endoplasmic reticulum and plasma membrane-containing fractions, respectively, were used as the control to monitor the Suc density gradient fractionation (Fig. 5C). Moreover, to visualize such localization in the Golgi apparatus of rice cells, we performed immuno-electron microscopy in root tips. The gold particles labeled by anti-OsTBL1 antibodies

OsTBL1 Exhibits O-Acetyltransferase Activity on the Xylan Backbone

The biochemical activity of OsTBL1 was examined by expressing the protein in *Pichia pastoris* (Supplemental Fig. S4). The recombinant proteins were purified, incubated with several commercial polysaccharides, including xylan, xyloglucan, pectin, and lignin, in an attempt to determine its acceptor substrate. Acetyl groups were incorporated into xylan 5-fold higher than that into other acceptor substrates when using 3H-labeled acetyl CoA as a donor (Fig. 6A), suggesting that xylan or xylooligomers is the native acceptor substrate of OsTBL1. These biochemical data are in agreement with the observed acetate defects in the mutants. To determine what kind of xylooligomers OsTBL1 prefers, Xyl to xylohexaose (X6) were used as the acceptor substrate for enzyme activity assay. OsTBL1 could incorporate acetyl substituents onto xylotriose to X6 with the highest incorporation in xylopentaose (X5; Fig. 6B). Using X5 as the acceptor substrate we analyzed the reaction products by matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) mass spectroscopy (MS). MALDI-TOF spectra revealed that OsTBL1 can transfer up to four acetyl residues onto the X5 (Fig. 6, C and D).

Moreover, the amount of substituent on X5 reached a maximum (approximately 42%) while the reaction time was increased up to 40 h (Fig. 7A). Considering that more than 30% of X5 was acetylated by incubating with OsTBL1 for 16 h (Fig. 7B), we examined $K_m$ and $V_{max}$ of OsTBL1 under this condition. As shown in Figure 7C, the acetyltransferase activity onto X5 showed saturable kinetics, with a $K_m$ of 4.98 mM and $V_{max}$ of 0.99 μM/min, respectively. Therefore, OsTBL1 is likely a xylan acetyltransferase.

To investigate the acetylation pattern on X5, the reaction products were further subjected to MALDI-TOF/TOF tandem MS analysis. Through analysis of the generated fragment ions of monoacetylated X5, the acetyl group should locate on either internal or terminal xylosyl residues (Fig. 8A). We also detected diacetylated xylobiose and triacetylated xylotriose from the fragment ions of triacetylated X5 (Fig. 8A). As diacetylation rarely occurs on Xyl residue in rice wall, it is assumed that the acetyl groups can be transferred onto consecutive Xyl residues by OsTBL1. To determine the regiospecificity of OsTBL1 activity, we analyzed the OsTBL1 products by 2D 1H-1H COSY spectroscopy. Signals corresponding to 3-O-Ac-β-D-Xylop and 2-O-Ac-β-D-Xylop, but not 3-O,2-O-di-Ac-β-D-Xylop, were observed (Fig. 8B), demonstrating that OsTBL1 is a xylan mono-O-acetyltransferase.

*ostbl1* and *ostbl2* Mutants Are Susceptible to Blight Disease

Polysaccharide O-acetylation can impact the ability of plants resistant to biotic and abiotic stresses (Diener et al., 2016). To determine whether OsTBL1 is involved in the resistance of *O. sativa* to both bacterial and fungal diseases, we examined the disease resistance of *ostbl1* and *ostbl2* mutants. The mutants showed higher susceptibility to *X. oryzae* pv. *oryzae* and *Fusarium graminearum* on rice seedlings (Fig. 9). This result indicates that OsTBL1 is involved in the resistance to fungal diseases and that OsTBL1 may function in the biosynthesis of resistance-related polysaccharides.
and Ausubel, 2005; Xin et al., 2007; Manabe et al., 2011). Online expression data suggested that OsTBL1 is coexpressed with multiple disease resistance response genes such as LOC_Os03g28190/Os03g0400200 (Buell et al., 2005; Supplemental Fig. S5), indicating that OsTBL1 may be induced by pathogens. Leaf blight is a worldwide rice disease and causes devastative yield losses (Zhang and Wang, 2013; Liu et al., 2014). To examine whether OsTBL1 and OsTBL2 contribute to rice leaf blight disease resistance, two blight pathogen strains, Pxo145 and Pxo61, were inoculated onto the leaves of wild-type and mutant plants. The lesion length in the leaves of ostbl1 and tbl1 tbl2 was 2- and 3-fold longer than those of wild-type plants, whereas the lesion length in ostbl2 remained identical to that of the wild type (Fig. 9). Therefore, OsTBL1 is essential for the resistance to leaf blight disease. Mutation in OsTBL1 alters the wall structure that may cause susceptibility to this disease.

DISCUSSION

The Rice Genome Harbors More TBL Members Than Arabidopsis

O-acetyl substitution is a common modification of cell wall polymers, which is important for polysaccharide network stability and recalcitrance. Three groups of proteins have been identified to be involved in O-acetylation of cell wall polymers (Gille et al., 2011b; Manabe et al., 2011; Schultink et al., 2015), in which TBLs constitute the largest gene family. Forty-six TBL members are present in the Arabidopsis genome (Gille and Pauly, 2012), but only a few of those TBLs have been characterized (Gille et al., 2011b; Xiong et al., 2013; Yuan et al., 2013, 2016a, 2016c). Sequence analyses identified 66 TBL members in rice (Fig. 1). We therefore proposed that rice may share a conserved mechanism in wall polymer
O-acetylation. However, to date none of the rice TBLs have been functionally characterized. Here, through isolation and characterization of ostbl1 and ostbl2 mutants, we found that mutations in both TBLs result in a decrease in xylan monoacetylation (Fig. 3), providing the first evidence to our knowledge that rice TBLs are involved in cell wall acetylation. ostbl1 and ostbl2 mutants exhibited minor but different alterations in plant height and acetyl-xylan content (Figs. 2 and 3). Expression analyses at transcriptional and translational levels in wild type and mutants revealed that OsTBL1 seems to play a dominant role in xylan O-acetylation, as it is more abundant than OsTBL2 (Fig. 4). However, the minor phenotypes shown in both TBL mutants suggested that additional TBLs may participate in xylan acetylation in rice.

OsTBL1 Represents a Xylan O-Acetyltransferase

To date, only TBL29 has been shown to have xylan O-acetyltransferase activity (Urbanowicz et al., 2014). Several TBL29 homologs were also identified to function in xylan O-acetylation based on the characterization of mutant chemotypes (Yuan et al., 2016a, 2016b, 2016c). The members clustered in this clade are likely to have a similar function with varied activity or regio-specificity. Therefore, identifying the enzyme activity of TBLs is crucial for understanding their functions. Fifteen rice TBLs are grouped in this clade, and OsTBL1 and OsTBL2 are orthologs of Arabidopsis TBL34 and TBL35 (Fig. 1), which were shown to affect xylan acetylation without enzymatic characterization (Yuan et al., 2016b). OsTBL1 and OsTBL2 have canonical TBL structures, including a TM domain at the N terminus and a TBL domain and a DUF231 domain containing the GDS and DxxH motifs, respectively (Bischoff et al., 2010; Gille and Pauly, 2012; Supplemental Fig. S1). Here, we provide multiple lines of biochemical and genetic evidence to corroborate that OsTBL1 is a xylan O-acetyltransferase.

Figure 8. OsTBL1 mediates xylan mono-O-acetylation. A, MALDI-MS/MS spectra of monoacetylated X5 (m/z 743.20 [M+Na]+, top) and triacetylated X5 (m/z 827.23 [M+Na]+, bottom) generated by incubating with OsTBL1. B, COSY spectra of acetylated X5 in the absence (Mock) and the presence of OsTBL1. The COSY experiment was carried out for two replicates.

Figure 9. OsTBL1 and OsTBL2 affect defense against leaf blight disease. A to C, The phenotypes of leaves from wild types, ostbl1, ostbl2, tbl1 tbl2, and tbl1 tbl2 inoculated with water (Control, A) or the pathogen strains Pxo61 (B) and Pxo145 (C). The lesion lengths were measured after 2-week inoculation. D, Statistics analysis of lesion length shown in A to C. Bars = 1 cm. Error bars indicate SD of the mean of n = 5 leaves. *P < 0.01 by Student’s t test.
Firstly, OsTBL1 is located in the Golgi apparatus (Fig. 5). Secondly, the purified recombinant OsTBL1 displayed acetyl transferase activity on xylan in vitro (Fig. 6). Thirdly, when using acetyl-CoA as the donor, OsTBL1 transferred up to four acetyl residues onto X5 and this activity showed saturable kinetics (Fig. 7). Fourthly, COSY analysis showed that OsTBL1 can transfer acetyl groups to the 2-O and 3-O position of xylosyl residues, resulting in mono-O-acetylated xylooligosaccharides (Fig. 8). Most importantly, all of these biochemical findings were corroborated by the corresponding mutant analyses, in which the ostbl1, ostbl2, and double mutants had an overall reduction in 2-O and 3-O monoacetylation of xylan (Fig. 3). Since spontaneous migration of acetyl residues occurs between different positions of a glycosyl-residue in vitro and in vivo (Gille and Pauly, 2012; Yuan et al., 2016a, 2016c), the observed acetylation in plants might not reflect the direct effect of enzyme activity. This might be the reason why the observed decrease in 2-O monoacetylated xylosyl residues is not as significant as at the 3-O position in ostbl1 and ostbl2 and the double mutants (Fig. 3), which is similar to the results found for the Arabidopsis tbl3 tbl31 mutants (Yuan et al., 2016c). For this reason, we cannot conclude that OsTBL1 is specifically required for xylan 2-O or 3-O monoacetylation, unless real-time acetyl transfer monitoring is implemented. Moreover, MALDI-TOF/TOF tandem MS analysis revealed that OsTBL1 can transfer acetyl groups to the wild type (Supplemental Fig. S7), suggesting that a sugar yields were not different between the mutants and the wild type (Supplemental Fig. S7), suggesting that a 10% to 20% acetate decrease in lignocellulosic material of these mutants is not sufficient for an improvement for saccharification conversion rate in rice.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

The rice (*Oryza sativa*) ostbl1 and ostbl2 mutants were ordered from the Rice Tos17 Insertion Mutant consortium (http://tos.nias.affrc.go.jp). The Tos17 insertion sites were confirmed by sequencing (Supplemental Table S1). The tbl1 tbl2 double mutant was generated by crossing ostbl1 and ostbl2 single mutants during the growing season. To generate the overexpression transgenic plants, the full coding sequences of OsTBL1 and OsTBL2 were amplified and inserted into the pCAMBIA1300 vector between the CaMV 35S promoter and NOS terminator. We also generated the OsTBL1 and OsTBL2 knock-down plants by artificial miRNA approach. The targeted fragments were amplified (Supplemental Table S4) and cloned into the pCAMBIA1300 vector containing Osu-miRNAAS28. The resulting constructs were transformed into Nipponbare, a wild-type variety. The wild type, mutants, and transgenic plants used in this research were grown in the experimental fields at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China) and at Linshei, Hainan Province, during the natural growing season.

**Bioinformatic Analyses**

Rice TBLs were identified based on the annotation of the rice genome database (Rice Genome Annotation Project, http://rice.plantbiology.msu.edu/). An unrooted tree of the TBLs in rice and Arabidopsis (*Arabidopsis thaliana*) was generated using Maximum Likelihood with the MEGA6 software (Tamura et al., 2013) with 1,000 bootstrap replications. The spatio-temporal expression profiles of OsTBL1 and OsTBL3 were analyzed based on the expression data in RiceXPro database (http://ricexpro.dna.affrc.go.jp/). Alignment of OsTBL1 and OsTBL2 proteins was performed using ClustalX. Coexpression analysis of OsTBL1 was conducted by using the online data from the RiceFREND database (http://ricefrend.dna.affrc.go.jp/).

**Chemical Assays**

The second internodes from mature wild type, mutants, and transgenic plants were collected for the preparation of wall materials. Neutral monosaccharide composition was determined as described previously (Zhang et al., 2012). The cellulose content was determined as described previously (Updegraff, 1969). For the extraction of acetyl-xylan, the destarched wall residues was depectinated using 1% ammonium oxalate and then delignified with 11% peracetic acid at 85°C. Acetyl-xylan was extracted twice with DMSO at 70°C and precipitated with the ethanol:methanol:water (7:2:1) for 3 d at 4°C. The pellets were rinsed with anhydrous ethanol four times and collected by centrifugation. The acetate content was analyzed using the Megazyme Acetic Acid Assay Kit (K-ACET, Megazyme) as described previously (Gille et al., 2011b). The experiments were carried out with at least three biological replicates.

**Western Blotting**

Young internodes from mutants and wild-type plants were ground in liquid nitrogen and homogenized in extraction buffer (25 mM Tris-HCl, pH 7.5, 0.25 mM Suc, 2 mM EDTA, 2 mM DTT, 15 mM β-mercaptoethanol, 10% glycerol, protease inhibitor cocktail [Roche]). After centrifugation at 10,000 g for 20 min at 4°C, the supernatant was centrifuged at 100,000 g for 2 h at 4°C to obtain a microsomal pellet. Twenty micrograms of microsomal protein was separated by SDS-PAGE gel electrophoresis and transferred to a nitrocellulose filter membrane. The proteins were probed with primary antibodies (1:1,000) and the secondary HRP conjugated antibody (1:5,000; Sigma). The signals were developed with the ECL plus western Blotting Detection System kit (GE Healthcare).

**The Functions of O-Acetylated Xylan in Rice**

Xylan is a major hemicellulose in plant cell wall and O-acetylation is one of the most important modifications on this polymer. The biological functions of O-acetyl-xylan are still under investigation. The phenotypes of several tbl mutants and transgenic plants include collapsed xylem vessels, stunted growth, pathogen defense, and freezing tolerance, providing genetic clues for the roles of O-acetyl-substituents in plant growth and development (Vogel et al., 2004; Xin et al., 2007; Lee et al., 2011; Manabe et al., 2011; Xiong et al., 2013; Yuan et al., 2016c). However, the function of O-acetylation of wall polymers in crops is still unclear. Rice ostbl1 and ostbl2 and the tbl1 tbl2 double mutants that have reduced level of acetyl-xylan exhibit reduced growth phenotypes similar to those observed in Arabidopsis (Fig. 2). In addition, these mutants showed susceptibility to leaf blight (Fig. 9), a devastating disease in rice production.

O-acetylation of wall polymers also represents a major obstacle to the economic conversion of lignocellulosics to biofuel (Loqué et al., 2015). Here, we examined the saccharification efficiency of lignocellulosic material derived from ostbl1, ostbl2, and the tbl1 tbl2 double mutants. The sugar yields were not different between the mutants and the wild type (Supplemental Fig. S7), suggesting that a 10% to 20% acetate decrease in lignocellulosic material of these mutants is not sufficient for an improvement for saccharification conversion rate in rice.
For Suc density gradient centrifugation, wild-type seedlings were homogenized in extraction buffer (250 mM sorbitol, 50 mM Tris-acetate, pH 7.5, 1 mM ethylene glycol-bis-(2-aminoethyl)ether)-N, N’, N’-tetracetic acid, 2 mM DTT, 1% protease inhibitor [Roche], 2% [w/v] polyvinylpyrrolidone, and 4 mM EDTA). The samples were then centrifuged at 10,000 g to remove tissue debris. The supernatant was centrifuged at 100,000 g for 2 h. The pellets were suspended in a microscope (Hitachi HT7700) equipped with a CCD camera (Gatan 832).

acetate (70% ethanol), the images were acquired with a transmission electron microscope (Hitachi HT7700) equipped with a CCD camera (Gatan 832).

To determine the preferred acceptor substrate of OsTBL1, we incubated 0.25 mM xylooligosaccharides, such as Xyl (Sigma), xylose, xylitol, xylotetraose, X3, or X6 from Megazyme, with 10 μg of purified recombinant OsTBL1 in the above reaction buffer for 40 h. The resulting acetylated products were examined by LC-QTOF-MS. To analyze the acetylated X5 by MALDI-TOF MS, 3 μg of purified recombinant OsTBL1 protein was incubated with 0.25 mM X5 and 1 mM acetyl-CoA in sodium phosphate buffer (50 mM, pH 6.8) for 16 h. The reaction products were mixed with a matrix solution (10 mg/mL 2,5-dihydroxybenzoic acid) and spotted on the target plate with 0.5 μL of 5-chloro-2-mercaptobenzothiazole (10 mg/mL). The positive ion spectra were recorded on an ABI 4700 Proteomics Analyzer (Applied Biosystems) using a 200-Hz frequency of Nd:YAG laser operating at a wavelength of 355 nm. Averages of 2,500 shots were used to obtain the MS spectra in a reflector mode. Cellulohexaose was included as an internal standard for quantification. To examine the OsTBL1 activity in a time-dependent manner, the reactions were performed as MALDI-TOF MS analysis except incubation for different time (0 to 80 h). The ratio of acetylated X5 to total X5 added in each reaction was measured. To determine the kinetics of OsTBL1 the purified protein (3 μg) was incubated with X5 in the presence of 1 mM acetyl-CoA for 16 h. The reaction was performed and analyzed with a MALDI-TOF mass spectrometer to quantify the acetyl residues on X5. The steady-state parameters K_m and V_max were determined by Michaelis-Menten plots using nonlinear curve-fitting in Origin 9.1 (OriginLab). For MALDI-TOF/TOF tandem mass spectrometry analysis, collision-induced dissociation mass spectra were obtained on an ABI 4800 Proteomics Analyzer (Applied Biosystems). The parent ion was collision-induced dissociation cell under high collision energy (1,000 V). The experiments were carried out with three biological replicates.

NMR Analysis
NMR characterization of cell wall residues from the wild type and mutants was performed as described previously (Cheng et al., 2013). Twenty mg of cell wall residue was milled with a planetary ball mill PM 200 (Retsch) and dissolved in 0.6 ml of DMDSO-d6 (99.9%; Sigma) supplemented with 1-Ethyl-3-methylimidazolium acetate, a kind gift of Prof. Suojiang Zhang, Institute of Molecular Engineering, AS. The samples were subjected to NMR analyses.

5H-NMR and HSQC NMR spectra were acquired at 298 K on an Agilent DD2 600 MHz NMR spectrometer equipped with a gradient 5-mm HCN triple resonance cold probe. The Agilent standard pulse sequence gHSQCAD was used to determine the one-bond 13C-1H correlation in wall residues. All the 1H-13C HSQC spectra were collected using a spectrum width of 10 ppm in the F2 (1H) dimension and 200 ppm in F1 (13C) dimension. 2048 × 512 (F2 × F1) complex data points were collected with the receiver gain set to 30, and 64 transients were acquired with an interscan delay (d1) of 1 s. The spectra were calibrated by the DMSO solvent peak (dC 39.5 ppm and dH 2.49 ppm). The NMR data processing and analysis were conducted using the MestReNova 10.0.2 software.

To examine the regiospecificity of OsTBL1, 0.5 mg X5 and 600 μg OsTBL1 recombinant protein was incubated in potassium bicarbonate buffer (50 mM, pH 6.8) containing acetyl-CoA (1 mM). After 40 h of incubation at 25°C, the reactions were stopped and freeze-dried. The products were dissolved in D_2O and loaded on an Agilent DD2 600 MHz NMR spectrometer. The Agilent standard pulse sequence gCOSY was used to determine the 1H-1H correlation.

Inoculation of Leaf Blight
To determine the resistance to leaf blight disease, three to five fully expanded leaves of 3-month-old wild-type, tbl1 tbl2, and the tbl1 tbl2 plants were inoculated with water or Philippine Xanthomonas oryzae pv. oryzae (Xoo) races Pxo61 and Pxo145 according to the leaf-clipping method (Kauffman et al., 1973). The lesion length was measured 2 weeks after inoculation (Zhong et al., 2003).

Saccharification Assay
One milligram destarched wall material was suspended in water and boiled for 1 h to inactive endogenous enzymes. After cooling down, the digested material was performed with an enzyme mixture containing 2 μL Cell2 enzyme (Novozymes) in 50 mM citrate buffer (pH 6.2) at 50°C with shaking at 220 rpm for 3 and 24 h. The released sugars in the supernatant were measured by reading the A_540 on an ELISA reader (Tecan) as described previously (Huang et al., 2015).
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