Molecular Plant Advance Access published November 23, 2012

Inactivation of *OsIRX10* leads to decreased xylan content in rice culm cell walls and improved biomass saccharification

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Dear Editor,

Xylan polysaccharides constitute the major non-cellulosic components in secondary cell walls of dicots and in both primary and secondary cell walls of grasses (Scheller and Ulvskov, 2010). Xylan is composed of a linear backbone of β(1-4)-linked xylose (Xyl). In grasses, the xylan backbone is substituted with O3-linkedand O2-linkedarabinose residues and, to a lesser extent, α(1-3)-linked glucuronic acid residues. A unique feature of grass xylans is the presence of hydroxycinnamate esters (ferulic and *p*-coumaric acid residues) attached to C5 of some of the arabinosyl residues (Carpita, 1996; Scheller and Ulvskov, 2010; Vogel, 2008). Xylan contributes to the recalcitrance of plant cell walls to enzymatic degradation probably through direct interaction with cellulose microfibrils and also through formation of ferulate cross-links(Iiyama et al., 1994).In Arabidopsis, several glycosyltransferases (GTs) are required to make the xylan backbone, including IRX9/IRX9L, IRX10/IRX10L, IRX14/IRX14L, FRA8/F8H, IRX8, and PARVUS, but it is still not clear what biochemical function each of these proteins have (Liepman et al., 2010). Very recently, grass-diverged GT61 proteins were shown to be probable enzymes that decorate the xylan backbone in grasses: a putativeα-1,3-arabinosyltransferase (XAT) for the transfer of arabinofuranose residues (Anders et al., 2012), and a putative β-1,2-xylosyl transferase (*XAX1*)for the addition of xylose to arabinosyl side chains (Chiniquy et al., 2012). Besides these GT61 enzymes, no xylan biosynthetic enzymes in grasses have been studied by reverse genetics or heterologous expression.

Several GT47 genes from Arabidopsis, poplar, and tobacco (*Nicotiana plumbaginifolia*.) have been characterized as important genes involved in xylan biosynthesis (Doering et al., 2012).However, to date, no GT47 genes have been well characterized in cell wall biosynthesis of rice or other grasses. Microarray analyses indicate that all 51 rice GT47 genes are expressed (Cao et al., 2008). We selected *Os01g70200*, one of the highly expressed genes, for functional characterization. *Os01g70200* contains an open reading frame made up of four exons and encodes a protein of 417 amino acids. The deduced amino acid sequence of *Os01g70200* contains a predicted transmembrane signal anchor (amino acid residues 4-24) in the N-terminal part of the protein and an exostosin / PF03016 domain (amino acid residues from 50 to 345). *Os01g70200* belongs to a clade of six proteins in rice that are more similar to Arabidopsis IRX10

and IRX10L than to any other Arabidopsis protein, according to phylogenetic analysis (Supplemental Figure 1). *Os01g70200* is expressed in many tissues during rice development with highest expression in juvenile culms (Supplemental Figure 2). Thus, we hypothesize that *Os01g70200* is the orthologous gene in rice toArabidopsis*IRX10* and we named it *OsIRX10*.

We obtained an insertion line, RGT6229D, which contains a dissociation (Ds) transposon insertion at the *OsIRX10* locus in the cultivar Nipponbare (NPB) (Supplemental Figure 3). We confirmed that the *OsIRX10* gene is effectively "knocked out" in the RGT6229D line (Supplemental Figure 3) and renamed the homozygous mutant plants of RGT6229D as *Osirx10* whereas segregants of the original transposon mutant that lack the transposon as *OsIRX10*.

We observed no obvious morphological difference in immature plants among the mutant *Osirx10* and the wild-type genotypes, *OsIRX10* and NPB (Supplemental Figure 4A). However, in older plants, the culms supporting the inflorescence of *Osirx10* were shorter than that of the wild type (Supplemental Figure 4B and 4C). Thus, we conclude that the loss of *OsIRX10* function results in short culms and leads to a reduced plant size. In attempt to isolate the affected culm segment, we compared the length of panicle (rachis) and internodes of 20 culms collected from five *Osirx10* plants and five NPB plants and found that both the rachis and first (top) internode of the *Osirx10* tillers were significantly shorter than those from NPB ($p = 4 \times 10^{-5}$ and $p = 2 \times 10^{-6}$, respectively, Student's t-test). We found no significant difference in the length of the other internodes between *Osirx10* tillers and NPB tillers. These results demonstrate that the *OsIRX10* gene is important for culm and inflorescence growth of rice.

We sectioned the first internode of the culms of NPB, *Osirx10*, and *OsIRX10* homozygous segregants for observation by electron and light microscopy. Both primary and secondary cell walls in the *Osirx10* mutant have a reduction in thickness compared to wild type (25% and 87% reduction, respectively; Figure 1A, Supplemental Figure 5). We also found that the average size of vascular bundles was significantly smaller in the *Osirx10* mutant (Figure 1A and Supplemental Figure 6). These results demonstrate that the development of both the primary and secondary cell walls are altered in the *Osirx10* mutant and that *OsIRX10* is required for secondary cell wall biosynthesis.

We observed approximately a 10% reduction in xylose content in the de-starched AIR and the supernatant resulting from 1 M KOH treatment in the *Osirx10* mutant compared with wild type (Figure 1B). Despite the absence of a change in the length of the internodes distal from the

panicle, we also found a quantitatively similar reduction in the xylose content of AIR prepared from the lower portion of the mutant culm (~20 %) (Supplemental Figure 7). In addition, we observed a small but significant increase in the arabinose content of the AIR in the 1 M KOH fraction (Figure 1B). As the glucose (essentially representing mixed linkage glucan) and other sugar components do not also increase in the mutant relative to wild type, the increase in arabinose content is not merely a consequence of the increase in percentage of AIR that is arabinose. Although the mutant has thinner secondary walls, we did not detect any significant change in lignin content as measured by the acetyl bromide method (Supplemental Table 1).

The height of the transformed *Osirx10* plants carrying the transgene *OsIRX10* was fully restored to that of the wild type (Supplemental Figure 8) and the cell wall monosaccharide composition was reverted to the wild type profile in the transformants (Figure 1C). These results confirmed that the short stature and reduction in xylose content in the *Osirx10* mutant was due to loss of *OsIRX10* function.

The CoMPP analysis showed that LM10, a monoclonal antibody against (1,4)-β-D-xylan, gave a weaker signal on AIR from the mutant suggesting that the *Osirx10* mutant had a smaller amount of xylan compared to the wild type (Supplemental Table 1). To support this conclusion, we compared equivalent transverse sections of NPB and *Osirx10* culm internodes labeled with antibodies against xylan (LM10 and LM11), or stained with Calcofluor (stains β-glucan). Both antibodies directed to xylan showed a significantly reduced occurrence of their epitope in *Osirx10,* while β-glucan polysaccharides did not show significant difference between the *Osirx10* mutant and the wild type NPB (Figure 1D). There were no significant differences in cellulose content between mutant and wild type (Supplemental Table 1).These results demonstrate that, in the *Osirx10* mutant, the decrease in xylose content is due to a reduced amount of xylan in the cell walls.

Most of the xylan in the Arabidopsis *irx10/irx10L* double mutant is much smaller than in the wild type plant with the largest peak of the distribution shifted from near 150 kDa to <12kDa (Wu et al., 2009). Our analysis by size exclusion chromatography revealed that rice culm xylan has a molecular weight >25kDa and in contrast to the Arabidopsis mutants, the distribution did not change significantly in the *Osirx10* mutant compared to the wild type (Supplemental Figure 9).

We subjected destarched AIR samples to hot water pretreatment and cellulolytic enzyme

treatment. We found that the differences in the amount of released free sugars between the *Osirx10* mutant and the wild type are highly significant at both 2 and 24 hours post saccharification (Figure 1E).These results indicate that the*Osirx10* mutant has high saccharification efficiency compared to wild type.

We determined the subcellular localization of OsIRX10 with C-terminally fused yellow fluorescent protein (YFP) and found that the OsIRX10-YFP signal coincided with the GmMan1-CFP signal, the α-mannosidase I cis-Golgi marker from soybean (*Glycine max*) (Figure 1F). This result demonstrates that OsIRX10-YFP is Golgi localized. The Golgi localization of OsIRX10 is consistent with the current understanding of xylan biosynthesis that xylan is synthesized in the Golgi.

In conclusion, we demonstrated that the rice locus *Os01g70200*, which we named *OsIRX10*, encodes a putative GT with a similar but not identical function to that of Arabidopsis *IRX10* and *IRX10L*. Although the rice *Osirx10* mutant shows reduced stature, thinner cell walls and reduced xylan content, in contrast to the results with the Arabidopsis *irx10*/*irx10L* mutant, we find that the size of the remaining xylan polymer is unchanged in the mutant relative to the wild type. The difference in the change in xylan polymer phenotype between the rice and Arabidopsis mutants suggests that the *IRX10* gene may have a different biosynthetic mechanism in grass species.The *Osirx10* mutant displays higher enzymatic cell wall saccharification efficiency indicating that cellulose is more accessible in the mutant, which demonstrates that manipulating the xylan content in grass cell walls can facilitate sugar extraction for biofuel production.

Acknowledgements

We thank Dr. Venkatesan Sundaresan from University of California at Davis (UCD) for providing rice mutant, *RGT6229D*; Dr. Caiyin Jiang, Brian Conlin and Jorge Avila from UCD for technical assistance. These studies were supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy, Sichuan “Hundred Talents Plan” funding and Sichuan Agricultural University “High Talents” start-up funding in China to X.C., Villum-Kann Rasmussen grant to the Pro-Active Plant Centre (www.proactiveplants.life.ku.dk), the Villum Foundation’s Young Investigator Program to J.H. and the National Science Foundation EPSCOR grant EPS-0814361 to L. B. and

F.L.

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Figure Legends

Figure 1. Inactivation of OsIRX10 leads to decreased xylan content in rice culm cell walls and improved biomass saccharification.

(A) Microscopy analyses on the culm section of NPB, the mutant *Osirx10* and *OsIRX10*. Representative micrographs of primary cell walls from sclerenchyma cells of NPB, *Osirx10* and *OsIRX10* under transmission electron microscope (12000X) (top panel, Scale bar: 1 μm). The views of primary cell wall thickness are marked with dark squares and their views with low amplification (4X) are shown in the middle panel. Representative micrographs of vascular bundle of NPB, *Osirx10* and *OsIRX10* under light microscope (400X). Vascular bundle size (tangential (indicated with "T") and radial (indicated with "R") cross-section) is marked with lines. Scale bar: 25 μm.

(B) Monosaccharide composition of alcohol-insoluble residues (AIR) and cell-wall fractions of the wild type and mutant. AIR was prepared from the first internode (top) plus the panicle culm and destarched. Composition of non-cellulosic polysaccharides in the destarched AIR, cell-wall fractions sequentially extracted with 1 M KOH and 4 M KOH and the residue after extraction was determined after TFA hydrolysis. Error bars indicate standard errors (SE, n = 3). "*" indicates the difference is significant ($P < 0.01$, Student's t-test).

(C) Monosaccharide composition of cell walls after complementation. Destarched AIR was prepared from first internode (top) plus the panicle culm. Monosaccharide composition was determined after TFA hydrolysis for transgenic plants carrying *OsIRX10* in the *Osirx10* genetic background as indicated with "*D6*", the mutant *Osirx10* and the wild-type NPB. Error bars indicate SE (n=4), "*" indicates the difference is significant ($P < 0.01$, One-way ANOVA analysis).

(D) Immunodetection and staining of transverse sections of *Osirx10* and NPB culm internodes. Representative micrographs of equivalent sections of mature culm internodes immunolabelled with antibodies directed to xylan (LM10 and LM11), mixed linkage-glucan (MLG) and stained with Calcofluor (β-glucan staining). Arrows highlight the detection of xylan epitopes in the secondary cell walls of vascular bundles and sclerenchyma. Scale bar: 50 μm.

(E) Saccharification of *Osirx10* and NPB cell walls. Destarched AIR from the culms of *Osirx10* and NPB plants were used for analysis. The data were collected from four independent samples of *Osirx10* and NPB plants. Error bars indicate standard deviations. "**" indicates the difference

is highly significant ($P < 0.001$, Student's t-test).

(F) Sub-cellular localization of OsIRX10. Confocal imagies of onion epidermal cells expressing OsIRX10-YFP, α-mannosidase I cis-Golgi marker tagged with CFP (GmMan1-CFP), and emerged YFP and CFP. Scale bar: 20 μm.

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