Increase in Cellulose Accumulation and Improvement of Saccharification by Overexpression of Arabinofuranosidase in Rice
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Minako Sumiyoshi¹*, Atsuko Nakamura¹*, Hidemitsu Nakamura²b, Makoto Hakata²b, Hiroaki Ichikawa², Hirohiko Hirochika², Tadashi Ishii¹, Shinobu Satoh¹, Hiroaki Iwai¹*

¹ Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan, ² Division of Genome and Biodiversity Research, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan

Abstract

Cellulosic biomass is available for the production of biofuel, with saccharification of the cell wall being a key process. We investigated whether alteration of arabinoxylan, a major hemicellulose in monocots, causes an increase in saccharification efficiency. Arabinoxylans have β-1,4-D-xylopyranosyl backbones and 1,3- or 1,4-α-L-arabinofuranosyl residues linked to O-2 and/or O-3 of xylopyranosyl residues as side chains. Arabinose side chains interrupt the hydrogen bond between arabinoxylan and cellulose and carry an ester-linked feruloyl substituent. Arabinose side chains are the base point for diterueloyl cross-links and lignification. We analyzed rice plants overexpressing arabinofuranosidase (ARAF) to study the role of arabinose residues in the cell wall and their effects on saccharification. Arabinose content in the cell wall of transgenic rice plants overexpressing individual ARAF full-length cDNA (OsARAF1-FOX and OsARAF3-FOX) decreased 25% and 20% compared to the control and the amount of glucose increased by 28.2% and 34.2%, respectively. We studied modifications of cell wall polysaccharides at the cellular level by comparing histochemical cellulose staining patterns and immunolocalization patterns using antibodies raised against α-(1,5)-linked l-Ara (LM6) and β-(1,4)-linked o-Xyl (LM10 and LM11) residues. However, they showed no visible phenotype. Our results suggest that the balance between arabinoxylan and cellulose might maintain the cell wall network. Moreover, ARAF overexpression in rice effectively leads to an increase in cellulose accumulation and saccharification efficiency, which can be used to produce bioethanol.

Introduction

Biofuel production from lignocellulosic biomass has attracted much attention as a method to reduce the consumption of fossil fuels, but a practical problem for use is that biofuel made from cellulosic biomass is costy because plant cell walls are very complex structures and are difficult to degrade. Therefore, for saccharification, the degradation of cell wall polysaccharides is a key process required to solve this problem.

Primary cell walls mainly consist of three polysaccharides: cellulose, hemicelluloses, and pectin. These polysaccharides determine the cell shape and mechanical strength. Lignin is a phenylpropanoid polymer deposited in the cell wall during secondary wall thickening [1]. Research on cell walls is important not only to understand mechanisms of plant growth and development but also to produce better plant material for practical use. Cell wall matrix polysaccharides generally occupy 10–30% of the dry weight of plants, and hemicellulose is responsible for cross-links between cellulose fibers. The dicot genus and monocot genus except for Commelina have type I cell walls and the hemicelluloses of members are rich in xyloglucan [2], while graminaceous monocots have type II cell walls and their hemicellulose is rich in β-1,3-1,4-glucan and arabinoxylan. Arabinoxylan occupies 20–50% of the dry mass of the cell wall in primary and secondary cell walls [3][4]. Arabinoxylans have a β-1,4-xylopyranosyl backbone and 1,3- or 1,4-α-L-arabinofuranosyl residues bonded at O-2 and/or O-3 of xylopyranosyl residues as side chains [2][4][5]. The xylan backbone has a 1,4-pyranose structure that is the same as cellulose or β-1,4-glucan, and forms strong hydrogen bonds with the surface of cellulose microfibrils. The arabinose side chain reportedly interrupts this hydrogen bond between arabinoxylan and cellulose, and the ratio of the addition of arabinoxylan into side chains to xylan backbone changes the wall mechanical properties [6]. Arabinose side chains can carry an ester-linked feruloyl substituent and these...
feruloyl groups form diferuloyl cross-links between arabinoxylans [7][8], and in secondary cell walls, feruloyl acid is bonded to lignin polymers [9]. Thus, the arabinose side chain is the base point for diferuloyl cross-links and lignification. Although arabinoxylanin residues are a quantifiably important constituent of plant primary and secondary cell walls, studies on this arabinose as a diferuloyl cross-link base point are lacking.

Genetic modifications of the cell wall have been reported [10], and plants with decreased hemicellulose and cellulose are generally physically weak and poorly adapted to the natural environment. For example, the cell wall network containing arabinose has been studied in dicots, and the loss of arabinose was found to be critical for plant development [11]. The Arabidopsis double mutant rgl1rgl2 and transgenic UDP-arabinopyranose murase RNAi rice plants present lethal or dwarf phenotypes [12][13]. In this paper, we focus on the functions of arabinose residues in arabinoxylan. We modified the arabinose content in rice using arabinoxylaninase (ARA) overexpression, Full-length cDNA overexpressor (FOX) lines [14][15]. Using the endogenous enzyme may contribute to improved public acceptance of GM crops.

Beyond glycosyl composition analysis, we probed for wall modifications at the cellular level by comparing histochemical cellulose staining patterns and immunolocalization patterns using antibodies raised against D-(1,3)-linked D-Ara (LM6) and D-(1,4)-linked D-Xyl (LM10 and LM11) residues. We report the effect of a decrease in arabinose content by ARAF overexpression on maintenance of the cell wall network through arabinoxylan and cellulose and saccharification efficiency for production of bioethanol.

### Materials and Methods

#### Plant material and growth conditions

Rice plants of the control (Oryza sativa cv. Nipponbare) and the two FOX lines AY311 and CO035, which carry overexpression constructs for OaARAFL (RAP locus: 01g0686900) and OaARAFL (Os1g131900), respectively, were grown in soil in a greenhouse during the natural growing season. In this environment, the temperature was 20–30°C and the light value was around 1,000 μmol s⁻¹ m⁻². All plants retained normal fertility.

The presence of the constructs in genomic DNA of these lines was confirmed by PCR. All experiments were performed with the T₂ generation. Transgenic lines were selected on hygromycin-containing agar plates and tested for heritability of the expression pattern and the altered sugar trait.

#### Phylogenetic analysis

BLAST searches were conducted with amino acid sequences of selected genes, namely Oryza sativa (members of GH family 51 and 3), A. thaliana (ARAFL, ARAF2, XLY1, and XLY3), and H. vulgare (AXHAI and AXAHII). A multiple alignment was generated by the neighbor-joining method in ClustalX [16] using full-length sequences and then manually adjusted. The phylogenetic tree was visualized using TreeView [17].

#### RNA extraction and RT-PCR

Plant material was frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen, Hilden, Germany). Mature leaves of FOX lines were frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen, Hilden, Germany) at 30 Hz for 2 min, and the resulted powder washed in 80% EtOH. The supernatant was removed after centrifugation for 5 min at 15,000 × g. The pellet was washed three times with water, three times with methanol-chloroform (MC = 1:1/v/v), and three times with acetone. A drop of phenol:acetic acid:water (PAW = 2:1:1/v/v) was added to the pellet and mixed. Two drops of MC were added to the sample and washed with acetone. This process was repeated three times and the sample was then dried at room temperature for over 1 h. Starch was removed by digestion with amylose (2 unit/ml amylose; Wako, Osaka, Japan) in 50 mM acetate buffer at 37°C for 3 h. After reaction, the samples were centrifuged and the residues washed three times with water, MC, and acetone. After washing, the samples were air-dried for over 12 h. Alcohol-insoluble residues (AIRs) were used as the cell wall material. A total of 2 mg of AIR was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121°C for 2 h. After hydrolysis, the samples were centrifuged at 15,000 × g for 5 min. The supernatant was the TFA-soluble fraction. The pellets were hydrolyzed with 72% H₂SO₄ at room temperature for 2 h and then diluted to 4% H₂SO₄ and boiled for 1 h. The H₂SO₄ solutions were neutralized with Ba(OH)₂. Sugar in TFA-soluble fractions was treated with methanol:hydrogen chloride and the resulting methyl glycosides were converted into trimethylsilyl (TMS) derivatives and analyzed by gas-liquid chromatography (GC-14; SHIMADZU Kyoto, Japan). Sugar content in TFA-soluble and TFA-insoluble fractions was determined using the phenol sulfuric acid method.

### Extraction and analysis of cell wall polysaccharides

Mature leaves of FOX lines were frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen, Hilden, Germany) to 0.3 mg for 2 min, and the resulted powder washed in 80% EtOH. The supernatant was removed after centrifugation for 5 min at 15,000 × g. The pellet was washed three times with water, three times with methanol-chloroform (MC = 1:1/v/v), and three times with acetone. A drop of phenol:acetic acid:water (PAW = 2:1:1/v/v) was added to the pellet and mixed. Two drops of MC were added to the sample and washed with acetone. This process was repeated three times and the sample was then dried at room temperature for over 1 h. Starch was removed by digestion with amylose (2 unit/ml amylose; Wako, Osaka, Japan) in 50 mM acetate buffer at 37°C for 3 h. After reaction, the samples were centrifuged and the residues washed three times with water, MC, and acetone. After washing, the samples were air-dried for over 12 h. Alcohol-insoluble residues (AIRs) were used as the cell wall material. A total of 2 mg of AIR was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121°C for 2 h. After hydrolysis, the samples were centrifuged at 15,000 × g for 5 min. The supernatant was the TFA-soluble fraction. The pellets were hydrolyzed with 72% H₂SO₄ at room temperature for 2 h and then diluted to 4% H₂SO₄ and boiled for 1 h. The H₂SO₄ solutions were neutralized with Ba(OH)₂. Sugar in TFA-soluble fractions was treated with methanol:hydrogen chloride and the resulting methyl glycosides were converted into trimethylsilyl (TMS) derivatives and analyzed by gas-liquid chromatography (GC-14; SHIMADZU Kyoto, Japan). Sugar content in TFA-soluble and TFA-insoluble fractions was determined using the phenol sulfuric acid method.
Cellulose analysis

Crystalline cellulose was measured according to [20]. Briefly the samples were treated with acetic and nitric acids to remove non-cellulosic polysaccharides, and the remaining pellets were hydrolyzed with 72% sulfuric acid. Glucose content in sulfuric acid was determined by phenol sulfuric acid method.

Lignin measurement

Lignin contents in each line were measured according to [21]. Explaining briefly, mature leaves were frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen, Hilden, Germany) at 30 Hz for 2 min. 3N HCl and 0.1 ml thiglycolic acid were added to 20 mg of AIR and heated at 80°C for 3 h. After centrifugation, the pellet was dissolved in 1N NaOH. The solution was submitted to spectrophotometric measurement.

pCA, FA and DFA measurement

Cell wall was treated with 1N NaOH for 12 h, and acidified with HCl. pCA, FA and DFA liberated were extracted three time with diethyl ether. The extract was air-dried, then stored in the dark until determination. pCA, FA and DFA contents were determined using HPLC system (Shimadzu, Kyoto, Japan) with a column Luna C18(2) column, (150x4.60mm, Phenomenex, USA) and monitored for UV(262 nm) and fluorescence (330 and 435 nm). The column equilibrated with 5 mM ammonium acetate (pH 4.4) containing 25% methanol was eluted by a linear gradient of methanol (25-50%, 2-10 min) and 5 mM ammonium acetate (pH 4.4) containing 50% methanol (10-20 min) at flow rate of 1.0 ml/min.

Immunohistochemistry and cellulose staining

To perform immunolocalization, leaves of the control, OsARAF1-FOX, and OsARAF3-FOX lines at the same developmental stage were fixed with 4% paraformaldehyde, 0.25% glutaraldehyde, and 0.05 M phosphate buffer (pH 7.5) and embedded in 5% agar. Sections of 30 μm were cut with a microtome (VT1200S; Leica Microsystems, Nussloch, Germany). The TSATM Kit no. 12, with HRP-goat anti-rabbit IgG and Alexa Fluor 488 tyramide (Molecular Probes/Invitrogen, Eugene, OR, USA), was used according to the manufacturer’s protocol. The primary antibodies LM6, LM10, and LM11 (PlantProbes, Leeds, UK) were used at a 1:30 dilution. HRP conjugate working solution was used at a 1:10 dilution. A negative control experiment was performed without the primary antibody. Cellulose was stained with 0.01% calcofluor white (Fluorescent Brightener 28; Sigma-Aldrich, St. Louis, MO, USA) and identified 16 putative ARAF genes with reference to the Carbohydrate-Active enzyme [CAZy] database [http://www.cazy.org; [22]], showing that the ARAF genes are members of GH family 3 and 51. We generated a phylogenetic tree using 16 rice ARAF genes, four Arabidopsis thaliana genes (AtARAF1; At3g10740, AtARAF2: At5g26120, XYL1: At1g49360, XYL3: At5g09730), and two Hordeum vulgare genes (AXHAI and AXAHII). Several enzymes of the GH family 3 and 51 have been reported to have bifunctional activity for ARAF/β-d-xilosidase. Arabidopsis XYL1 and XYL3 [25][26] have activity to hydrolyze p-nitrophenyl-β-L-arabinofuranoside (PNP-ARAf), p-nitrophenyl-β-d-xylpyranoside (PNP-Xyl), oat spelt xylan, rye arabinoxylan, wheat arabinoxylan, and oligo-arabinoxylan [25]. ASD1 and ASD2 have only ARAF activity [27]. The barley AXAH-I and AXAH-II have ARAF activity to release arabinose from 1,3-β-arabinofuranose, sugar beet arabinoxylan, wheat arabinoxylan, and larchwood (Larix) arabinogalactan, but do not release d-Xyl [28][29]. The phylogenetic tree showed that enzymes belonging to GH family 3 have bifunctional activity for ARAF/β-d-xilosidase and those in GH family 51 seemed to only have ARAF activity (Figure 1A). Therefore, we selected genes from GH family 51 for further study.

For a systematic approach to analyze a gain-of-function phenotype, [14][15] developed the FOX hunting system by using expression libraries for full-length cDNAs (fl-cDNAs) from rice at a maximum of 28,000 fl-cDNAs cloned in total, and individually overexpressed the fl-cDNAs in rice driven by the maize Ubiquitin-1 gene promoter. Among ~14,500 FOX-rice lines, we identified two lines overexpressing fl-cDNAs for OsARAF1 (Accession no. AK064838, Rap-Id:Os07g0686900), and OsARAF3 (AK065240, Os11g0131900) in GH family 51 and named them the OsARAF1-FOX and OsARAF3-FOX lines. We analyzed a total of 23 plants from the T1 to T3 generations; they showed essentially identical results. Since OsARAF3 has high homology to H. vulgare ARAF AXAXI and AXAXII [23], OsARAF3 was expected to cleave various arabinofuranosyl side chains, e.g., arabinoxylan and rhamnogalacturonan-I (RG-I).

The expression levels and patterns in OsARAF1 and OsARAF3 of leaf, stem, and root of immature seedlings (14 days old) and leaf and stem of mature plants (60 days old) were analyzed by reverse transcription (RT)-PCR expression analysis (Figure 1B). OsARAF1 was expressed strongly in 14- and 60-day-old stems but was not detectable in 14- and 60-day-old leaves. OsARAF3 was expressed weakly in 14- and 60-day-old leaves. These results are consistent with data retrieved from the RiceXPro [http://ricexpro.dna.affrc.go.jp; [30]]. Because OsARAF1 and OsARAF3 are not expressed or only expressed weakly in leaves, we focused on their effects on leaves.
OsARAF1-FOX and OsARAF3-FOX lines were ARAF overexpressors

RT-PCR analysis showed that OsARAF1 and OsARAF3 transcripts were elevated in OsARAF1-FOX and OsARAF3-FOX, respectively (Figure 2A). ARAF activity in mature leaves, assayed using p-nitrophenyl arabinofuranoside as substrate, increased 1.7-fold in OsARAF1-FOX and OsARAF3-FOX compared to the control (Figure 2B and Figure S1A). Since some ARAFs have bifunctional activity for ARAF/b-D-xylosidase, we measured xylosidase activity using p-nitrophenyl xyloside as substrate. The xylosidase activities in OsARAF1-FOX and OsARAF3-FOX lines were almost the same as in the control (Figure 2C and Figure S1B), as predicted from the phylogenetic analysis.

OsARAF1-FOX and OsARAF3-FOX lines, however, showed no visible phenotype (Figure 3). Vegetative parameters, plant height, stem diameter, and number of tillers, and reproductive parameters, fertility rate, number of rachis-branches, and spike length, were unchanged in OsARAF1-FOX and OsARAF3-FOX lines compared to the control (data not shown). Two FOX lines did not show high lodging.

Effects of overexpression of ARAF on cell wall composition

We determined the glycosyl composition of cell walls prepared from mature leaves of OsARAF1 and OsARAF3 lines. The
arabinose content in the TFA-soluble fraction, which was mainly derived from the side chain of arabinosylxylan, in OsARAF1-FOX and OsARAF3-FOX lines decreased to 75.4% and 81.6% of that in the control, respectively (Table 1). The xylose content, which was derived from the main chain of arabinose, also decreased in both lines. The glycosyl residue composition of cell walls isolated from leaves of the control and FOX lines was determined to establish whether overexpression of arabinofuranosidase affected the cell wall polymer content.

The glucose content in the TFA-insoluble fraction in OsARAF1-FOX and OsARAF3-FOX increased to 128.2% and 134.2% that of the control, respectively (Table 2). The cellulose content was determined using the acetic/nitric acid method. The analysis revealed that the amount of sugar in the acetic/nitric acid-insoluble fraction of OsARAF3-FOX was about 120.0% that of the control (Figure 4A). The amount of lignin was slightly higher in OsARAF1-FOX and OsARAF3-FOX (Figure 4B). These results indicate that overexpression of OsARAF1 and OsARAF3 caused a decrease in arabinosylxylan and increases in cellulose and lignin. However, the other cell wall sugars and phenolics were almost the same among the control, OsARAF1-FOX, and OsARAF3-FOX lines (Table 1, Table 2 and Figure 4C).

Overexpression of ARAF caused changes in localization of cell wall components

We further determined the distribution of cell wall sugars using monoclonal antibodies against cell wall polysaccharide epitopes and Calcofluor White. The monoclonal antibody LM6 labels arabinan and arabinose side chains in arabinosylxylan epitopes ([31]; University of Georgia: http://www.ccrce.uga.edu/mao/wallmab/ Antibodies/antib.htm). LM11 labeled the arabinosylxylan and LM10 labeled the xylan epitope [32]. When probed with LM6, OsARAF1 and OsARAF3 had fewer signals in all tissues (Figure 5D–F). Moreover, LM11 stained the wall of vascular bundles and the epidermis of the control, but few signals were detected in OsARAF1 and OsARAF3 lines (Figure 5G–I). However, LM10 label had the same signal intensity among the control, OsARAF1-FOX, and OsARAF3-FOX lines (Figure 5J–L).

In OsARAF1 and OsARAF3, a high intensity of Calcofluor staining was observed in the midrib and phloem (Figure 6D–F). Calcofluor White is reported to stain β-1, 4-glucan (cellulose), β-1, 3-glucan (callose), and mannan [33][34]. Because callose is present in specific cell wall components [35], and the resulting glycosyl composition showed very little mannan (Table 1 and Table 2), Calcofluor White should stain cellulose.

Overexpression of ARAF affects the improvement of mechanical strength

Glycosyl composition analysis and histochemical observation showed that overexpression of ARAF caused a decrease in arabinose and xylose content and an increase in cellulosic glucose in the cell wall. The altered cell wall composition may influence plant mechanical properties, and thus we examined differences in mechanical properties between the control, OsARAF1-FOX, and OsARAF3-FOX leaves using the creep meter test. The breaking force and extension of leaves was similar among the control, OsARAF1-FOX, and OsARAF3-FOX. The extension length was slightly increased in OsARAF3-FOX (Figure 7). These results

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Table 1. Monosaccharide compositions of TFA-soluble fractions.

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Control</th>
<th>OsARAF1-FOX</th>
<th>OsARAF3-FOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara</td>
<td>419±2.6 a</td>
<td>316±5.6 b</td>
<td>338±6.6 b</td>
</tr>
<tr>
<td>Rha</td>
<td>2.7±1.4 a</td>
<td>2.5±0.9 a</td>
<td>2.5±0.5 a</td>
</tr>
<tr>
<td>Fuc</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Xyl</td>
<td>153.2±7.4 b</td>
<td>131.6±15.9 a</td>
<td>135.6±11.8 a</td>
</tr>
<tr>
<td>Man</td>
<td>11.2±4.3 a</td>
<td>9.8±3.4 a</td>
<td>14.9±4.2 a</td>
</tr>
<tr>
<td>Gal</td>
<td>11.8±4.3 a</td>
<td>11.4±3.4 g</td>
<td>12.9±4.2 a</td>
</tr>
<tr>
<td>Glc</td>
<td>128.3±15.2 a</td>
<td>65.1±29.9 b</td>
<td>90.0±18.4 b</td>
</tr>
<tr>
<td>GalA</td>
<td>2.3±1.0 a</td>
<td>1.4±1.5 a</td>
<td>2.2±1.0 a</td>
</tr>
<tr>
<td>GlcA</td>
<td>7.3±1.9 a</td>
<td>5.8±2.6 a</td>
<td>9.1±3.0 a</td>
</tr>
<tr>
<td>Total</td>
<td>351.7±125 a</td>
<td>283.6±28.3 b</td>
<td>323.7±18.5 ab</td>
</tr>
</tbody>
</table>

The monosaccharide compositions of alcohol-insoluble residues (AIRs) in mature leaves of the control, OsARAF1-FOX and OsARAF3-FOX were determined by GC. The values are means ± SD (n = 12). Different letters within the same column indicate significant differences among means (P<0.05) as determined by Tukey’s test. N.D. means “not detected”.

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Table 2. Monosaccharide compositions of TFA-insoluble fractions.

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Control</th>
<th>OsARAF1-FOX</th>
<th>OsARAF3-FOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rha</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Fuc</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Xyl</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Man</td>
<td>27.8±5.8 a</td>
<td>28.6±13.0 a</td>
<td>36.4±17.4 a</td>
</tr>
<tr>
<td>Gal</td>
<td>2.2±2.5 a</td>
<td>4.3±11.4 a</td>
<td>2.4±3.5 a</td>
</tr>
<tr>
<td>Glc</td>
<td>370.3±28.1 a</td>
<td>474.6±40.0 b</td>
<td>497.0±32.4 b</td>
</tr>
<tr>
<td>GalA</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>GlcA</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Total</td>
<td>400.3±31.3 a</td>
<td>508.5±36.8 b</td>
<td>535.7±32.1 b</td>
</tr>
</tbody>
</table>

The monosaccharide compositions of alcohol-insoluble residues (AIRs) in mature leaves of the control, OsARAF1-FOX and OsARAF3-FOX were determined by GC. The values are means ± SD (n = 12). Different letters within the same column indicate significant differences among means (P<0.05) as determined by Tukey’s test. N.D. means “not detected”.

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Figure 3. The phenotypes of the control (A), OsARAF1-FOX (B) and OsARAF3-FOX (C) in 60-day-old plant after sowing. The growth levels of FOX lines were very similar to those of the control, and all FOX lines had normal fertility. Bar = 10 cm. doi:10.1371/journal.pone.0078269.g003
indicate that overexpression of ARAF may affect the improvement of mechanical properties of the leaf.

**Overexpression of ARAF led to an increase in saccharification efficiency**

The increase in cellulose glucose content in the cell wall of OsARAF1-FOX and OsARAF3-FOX lines implied that FOX lines produce more glucose in saccharification compared to the control. We measured sugar liberation from OsARAF1-FOX and OsARAF3-FOX mature leaves (Figure 8A and B). The rates of saccharification were almost the same for 1 h in the control, but

![Figure 4. Cell wall component analysis of the control, OsARAF1-FOX and OsARAF3-FOX.](image)

Figure 4. Cell wall component analysis of the control, OsARAF1-FOX and OsARAF3-FOX. The amounts of sugar in the acetic/nitric acid-insoluble fraction (A) and the amounts of lignin (B) and phenolic acids released by mild alkaline hydrolysis (C) from AIRs in the control, OsARAF1-FOX and OsARAF3-FOX lines are shown. Black, white and gray symbols indicate the control, OsARAF1-FOX and OsARAF3-FOX, respectively. Error bars indicate the SD (n = 4). Different letters in each panel indicate significant differences at P < 0.05 (Tukey’s test).

doi:10.1371/journal.pone.0078269.g004

![Figure 5. Immunofluorescent labeling of the control and FOX lines with arabinoxylan related antibodies.](image)

Figure 5. Immunofluorescent labeling of the control and FOX lines with arabinoxylan related antibodies. Immunohistochemistry on sections of agar-embedded mature leaves of the control (D, G, J and M), OsARAF1-FOX (E, H, K and N) and OsARAF3-FOX (F, I, L and O) line were observed. The sections were labeled with the monoclonal antibodies LM6 (D–F), LM11 (G–I) and LM10 (J–L). Sections were observed under bright-field illumination (A–C). The micrographs (M–O) show the negative control performed without the first antibody step. All experiments were performed at least twice with similar results. Bars = 100 μm.

doi:10.1371/journal.pone.0078269.g005

![Figure 6. Calcofluor white staining for cellulose of mature leaves of the control and FOX lines.](image)

Figure 6. Calcofluor white staining for cellulose of mature leaves of the control and FOX lines. The section of agar-embedded mature leaves of the control (D), OsARAF1-FOX (E) and OsARAF3-FOX (F). Sections were observed under bright-field illumination (A–C). The micrographs (G–I) show negative controls performed without calcofluor white. Bars = 500 μm. All experiments were performed at least twice with similar results.

doi:10.1371/journal.pone.0078269.g006
Discussion

We analyzed ARAF-overexpressed rice plants. Arabinose content in the cell walls of the two ARAF-overexpressed plants decreased to 75% and 80% of that in the control, and the amount of cellulose glucose increased by 128.2% and 134.2% of that of control. We did not use fungal ARAF for which the exact activity against multiple substrates is known because overexpression of fungal ARAF might have resulted in lethality due to xylan degradation and inhibition of cell wall synthesis. The use of fungal cell wall hydrolases was previously shown to result in a low yield [36]; therefore, we used the endogenous arabinofuranosidases. OsARAF1-FOX and OsARAF3-FOX lines showed no morphological phenotype and some improvement of mechanical strength as compared to the control (Figure 3 and 7). In addition, overexpression caused an increase in saccharification efficiency (Figure 8).

Arabinoxylans are major non-cellulosic polysaccharides in primary and secondary walls of grass cell walls. They are hydrogen-bonded to themselves and cellulose microfibrils to form cellulose–hemicellulose networks. Ferulic acids are ester-linked to arabinose side chains of arabinoxylan to form diferulate cross-linked arabinoxylan. In monocots, most arabinose residues exist as side chains of arabinoxylans. In secondary cell walls, ester-linked ferulic acid is incorporated into lignin to form an arabinoxylan–lignin complex [9]. Hence, arabinose residues are thought to be the base point for cross-linking between arabinoxylan and lignin [37]. In growing tissues, arabinoxylan content in cell walls increases slowly and arabinose residues are highly branched with arabinosyl residues and are removed during growth [38][39].

ARAFs are defined as enzymes that catalyze the hydrolysis of terminal, nonreducing α-L-arabinofuranoside residues. However, several enzymes in family 51 are capable of hydrolyzing both L-Ara and D-Xyl from a variety of substrates in vitro, and therefore may be considered as bifunctional ARAF/β-D-xylosidase enzymes.

![Figure 7. Mechanical properties of leaves of the control, OsARAF1-FOX and OsARAF3-FOX lines. The break force is expressed per leaf width. The horizontal axis shows the extension length. Black, white and gray symbols indicate the control, OsARAF1-FOX and OsARAF3-FOX, respectively. Error bars indicate the SD (n = 8). doi:10.1371/journal.pone.0078269.g007](Image 58x579 to 288x730)

![Figure 8. Saccharification of the control OsARAF1-FOX and OsARAF3-FOX lines in dry leaves. Time-dependent saccharification (A) and saccharification efficiency after 24 h (B) were measured. Error bars indicate the SD (n = 3). Black, white and gray columns indicate the control, OsARAF1-FOX and OsARAF3-FOX, respectively. Different letters in the same column indicate significant differences at P < 0.05 (Tukey’s test). doi:10.1371/journal.pone.0078269.g008](Image 315x422 to 553x730)
Overexpression of Arabinofuranosidase in Rice

arabinoxylan in the FOX lines as compared to the control. The amounts of arabinose side chains may influence the xylan backbone extension, and arabinose residues may be important in the formation of the cell wall network. On the other hand, overexpression of OsARAF1 leads to increases in the secondary cell wall, especially xylan [45]. In any case, arabinofuranosidase overexpression may cause modification of the secondary cell wall.

Neither OsARAF1-FOX nor OsARAF3-FOX affected plant growth (Figure 3). The increase in cellulose may compensate for the reduction in arabinoxylan (Table 1, Table 2 and Figure 6). Additionally, amount of crystallic cellulose in OsARAF3-FOX increased compared to the control (Figure 4A). Staining with Calcoflower White increased in the midrib and phloem of FOX lines, which is associated with the increased mechanical strength (Figure 6D-F and Figure 7). From these results, it appears that rice might maintain plant body strength by maintaining a balance between cellulose and hemicellulose. In fact, OsARAF1-FOX and OsARAF3-FOX lines showed slightly increase of mechanical strength compared to the control (Figure 7). In dicots, xyloglucan is hydrogen-bonded to cellulose microfibrils. In xyloglucanase-overexpressed poplar, the amount of cellulose increased [44] and led to the generation of abnormal tensile stress. In this study, ARAF-overexpressed rice had a lower arabinoxylan content, and so it was expected to have a similar phenotype; however, no influence on growth occurred (Figure 3). Unlike dicots, rice has interspersed vascular bundles, which have less influence on retaining the strength of the plant body, so an increase of cellulose may have a positive effect on growth.

OsARAF1-FOX and OsARAF3-FOX lines had increased saccharification efficiency (Figure 8A and B). In industrial processes, plant biomass is treated with hemicellulose and cellulase because cellulase covers the cell wall matrix. We expected that the increased saccharification efficiency of FOX lines would depend on decreases in hemicellulose and substituted xylan [45]. The saccharification efficiency after 24 h reaction increased in FOX lines (Figure 8A), which may have been caused not only by an increase in accessibility of the cellulose due to a decrease in hemicelluloses but also an increase in cellulose content. In cell walls, glucose is contained in cellulose and β-1,3-1,4-glucan. The hydrolysis of β-1,3-1,4-glucan is accelerated by growth regulators; in mature leaves, the β-1,3-1,4-glucan level is low [2]. The amount of lignin increased slightly in OsARAF1-FOX and OsARAF3-FOX lines, but this didn’t influence saccharification efficiency. Lignin complexes with arabinoxylan and is mediated by ferulic acid [9]. In this study, the decrease in arabinoxylan had little effect on cell wall network assembly and growth. The small change in cell wall components led to higher cellulose content in the plant but had no effect on growth. And, in previous studies, few examples of increases in cellulose content in cell walls were reported. Hence, our report provides an important contribution to the study of cell wall biosynthesis and future technology. The modification cell walls in rice through ARAF overexpression improves saccharification efficiency, and these FOX rice lines could prove useful for producing cellulosic bioethanol.

Supporting Information

Figure S1 Enzyme activity in WT, OsARAF1-FOX and OsARAF3-FOX. (A) ARAF activity using 4-nitrophenyl-β-D-araabinofuranide as substrate in WT, OsARAF1-FOX and OsARAF3-FOX leaves. (B) Xylosidase activity using 4-nitrophenyl-β-D-xylopyranoside as substrate in WT, OsARAF1-FOX and OsARAF3-FOX leaves. Black, white, and gray columns indicate the WT, OsARAF1-FOX, and OsARAF3-FOX lines, respectively. Black, white, and gray columns indicate WT, OsARAF1-FOX, and OsARAF3-FOX, respectively. (PDF)

Author Contributions

Conceived and designed the experiments: H. Iwai. Performed the experiments: MS AN TI SS H. Iwai. Contributed reagents/materials/analysis tools: HN MH H. Ichikawa HH. Wrote the paper: MS H. Iwai.

References


Author Contributions

Conceived and designed the experiments: H. Iwai. Performed the experiments: MS AN. Analyzed the data: MS AN TI SS H. Iwai. Contributed reagents/materials/analysis tools: HN MH H. Ichikawa HH. Wrote the paper: MS H. Iwai.


