Studies on the Function of Arabinose Side Chain of Cell Wall Polysaccharides in the Development of Rice

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Contents

Abbreviations

ARAF : Arabinofuranosidase

ASD : Arabinofuranosidase

AXAH : Arabinoxylan arabinofuranosidase proteins

cDNA : complimentary Deoxyribonucleic Acid

DW : Distilled Water

EtOH : Ethanol

FOX : Full-length cDNA Overexpressor

IgG : Immunoglobulin G

min : minute

PCR : Polymerase Chain Reaction

PNP : p-Nitrophenol

RNA : Ribonucleic Acid

rRNA : ribosomal Ribonucleic Acid

RT-PCR : Reverse Transcription Polymerase Chain Reaction

T1 : Second Transgenic Plant

TFA : Trifluoroacetic Acid

Wt : Wild type

XLY : Xylosidase

XXT : Xylosyl Transferase

Abstract

Cellulosic biomass is available for the production of biofuel, with saccharification of the cell wall being a key process. I 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 diferuloyl cross-links and lignification. I 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. I 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 D-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 costly 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 gramineous 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 arabinose 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 arabinofuranosyl 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 thaliana* double mutant *rgp1rgp2* and transgenic UDP-arabinopyranose mutase RNAi rice plants present lethal or dwarf phenotypes [12, 13]. In this thesis, I focus on the functions of arabinose residues in arabinoxylan. I modified the arabinose content in rice using arabinofuranosidase (ARAF) overexpressor, Full-length cDNA overexpressor

(FOX) lines [14, 15]. Using the endogenous enzyme may contribute to improved public acceptance of genetically modified crops.

Beyond glycosyl composition analysis, I probed for wall modifications 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 D-Xyl (LM10 and LM11) residues. I 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 *OsARAF1* (RAP locus: *07g0686900*) and *OsARAF3* (*Os11g0131900*), 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 $\mu mol \cdot s^{-1} \cdot m^{-2}$. 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 T_3 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* (ARAF1, ARAF2, XLY1, and XLY3), and *Hordeum 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) at 30 Hz for 2 min. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and the DNase I recombinant (Roche, Basel, Switzerland) according to the manufacturers' protocols. cDNA was synthesized with ReverTra Ace® (Toyobo, Tokyo, Japan) according to the manufacturer's protocol. For the *OsARAF1*-FOX line, the quantity of transcripts was determined using primer sets of *OsARAF1*-forward (5'-CCAGAAGGGCAGTTATTG-3') and *OsARAF1*-reverse (5'-CCAGAAGGGCAGTTATTG-3'). For the *OsARAF3*-FOX line, the quantity of transcripts was determined using primer sets of *OsARAF3*-forward (5'-GCTTCTCTTCCTTCCTTCCTTGT-3') and *OsARAF3*-reverse (5'- CGATACTTGATCAAACCATACAACTCCCTT-3'). As an endogenous control, the quantity of *17S rRNA* transcript were determined using primer sets of *17S rRNA*-forward (5'-GCAAATTACCCAATCCTGAC-3') and *17S rRNA*-reverse (5'- CTATTGGAGCTGGAATTACC-3'). The amplified cDNA fragments were separated on 2% agarose gel and the bands stained with ethidium bromide.

Enzyme assay

Enzyme assay in each line was measured according to the previous study [18]. Mature leaves were frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen) at 30 Hz for 2 min. The following operations were carried out at 0–4ºC.

Ground samples were suspended in 20 mM sodium acetate buffer (pH 5.0) containing 1 M sodium chloride for 2 h. After centrifugation at 10,000 × g for 5 min, the supernatant was applied to a PD-10 column midi-Trap G-25 (GE Healthcare, Milwaukee, WI, USA) and the eluted fraction was used for the enzyme assay. The concentration of protein was determined by the method of Bradford, with bovine serum albumin as the standard [19]. Enzyme activities were determined using a reaction mixture (200 μl) consisting of protein fractions, 25 mM acetate buffer (pH 5.0), and 1 mM p-nitrophenol glycoside. After incubation at 37ºC for 2 h, the reaction was terminated by the addition of 200 mM sodium carbonate (800 μl) and monitored at 420 nm. One unit of enzyme activity is defined as 1 μmol of p-nitrophenol liberated per minute at 37ºC min^{-1}.

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) at 30 Hz for 2 min, and the resulted powder washed in 80% (v/v) 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 w/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 amylase (2 unit/ml amylase; Wako, Osaka, Japan) in 50 mM acetate buffer at 37ºC 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 121ºC for 2 h. After hydrolysis, the samples were centrifuged at $15,000 \times g$ for 5 min. The supernatant was the TFA-soluble fraction. The pellets were hydrolyzed with 72% H_2SO_4 at room temperature for 2 h and then diluted to 4% H_2SO_4 and boiled for 1 h. The H_2SO_4 solutions were neutralized with $Ba(OH)_2$. Sugar in TFA-soluble and -insoluble 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 the previous study [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 the previous study [21]. Explaining briefly, mature leaves were frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen) at 30 Hz for 2 min. 3N HCl and 0.1 ml thioglycolic 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 spectrophotomeric measurement.

***p*CA, FA and DFA measurement**

Cell wall was treated with 1N NaOH for 12 h, and acidified with HCl. *p*CA, FA and DFA liberated were extracted three times with diethyl ether. The extract was air-dried, then stored in the dark until determination. *p*CA, FA and DFA contents were determined using HPLC system (Shimadz) with a column Luna C18(2) column, (150×4.6 mm, 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 ml/min.

Immunohistochemistry and cellulose staining

To perform immunolocalization, leaves of the control plants, *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 TSA™ 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 1:100 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). All sections were observed using fluorescence microscopy (Leica Microsystems), with UV filter A, excitation filter BP 340–380 nm, suppression filter LP 425, and an exposure time of 190 ms.

Measurement of mechanical properties

Mature leaves of 7–8 mm width of the control plants, *OsARAF1*-FOX, and *OsARAF3*-FOX were cut 12 cm from the leaf tip and immediately used to assess mechanical properties. Both sides of a sample were adhered to the stage, with an interval of 2 cm; the stage moved to load the middle of the sample on the sensor;

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and then the change of load (break force) and extension length per leaf width were monitored using EZ Graph (Shimadzu).

Measurement of saccharification efficiency

Mature leaves were ground with a Tissue Lyser II (Qiagen) at 30 Hz for 2 min. An aliquot of 1.2 ml of 0.1 M sodium acetate buffer (pH 4.5) was added to 40 mg of the resulting powder and suspended. A sample of 60 µl was recovered and 40 µl sodium acetate buffer added at time 0. A total of 10 mg/ml Meiselase (Meiji, Tokyo, Japan) was added to the remaining sample and reacted at 45ºC with shaking at 120 rpm. After 1–24 h, 100 µl suspensions were recovered followed by centrifugation at 15,000 × g for 10 min at room temperature. Sugar content in the supernatant was determined by the phenol sulfuric acid method. The saccharification efficiency was calculated as sugar liberation (%) = µg / mg dry weight of leaves.

Results

Selection of ARAF genes from the FOX library

To select the ARAF genes of rice, I searched the Rice PIPELINE database (http://cdna01.dna.affrc.go.jp/PIPE/; [22]) and identified 16 putative ARAF genes with reference to the Carbohydrate-Active enzyme [CAZy] database (http://www.cazy.org; [23, 24]), showing that the ARAF genes are members of GH family 3 and 51. I generated a phylogenic tree using 16 rice ARAF genes, four Arabidopsis thaliana genes (*AtARAF1*: *At3g10740*, *AtARAF2*: *At5g26120*, *XYL1*: *At5g49360*, *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-xylosidase. *Arabidopsis* XLY1 and XLY3 [25, 26] have activity to hydrolyze *p*-nitrophenyl-α-L-arabinofuranoside (PNP-ARA*f*), *p*-nitrophenyl-β-D-xylopyranoside (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 1,5-α-L-arabinopentaose, sugar beet arabinan, wheat arabinoxylan, and larchwood (*Larix*) arabinogalactan, but do not release D-Xyl [28, 29]. The phylogenetic tree showed that enzymes belonging to Glycoside Hydrolases (GH) family 3 have bifunctional activity for ARAF/β-D-xylosidase and those in GH family 51 seemed to only have ARAF activity (Figure 1A). Therefore, I selected genes from GH family 51 for further study.

For a systematic approach to analyze a gain-of-function phenotype, the FOX hunting system developed by using expression libraries for full-length cDNAs (fl-cDNAs) from rice at a maximum of 28,000 fl-cDNAs clones in total, and individually overexpressed the fl-cDNAs in rice driven by the maize Ubiquitin-1 gene promoter [14, 15]. Amoung ~14,500 FOX-rice lines, I 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,respectively. I analyzed a total of 23 plants from the T1 to T3 generations of both FOX lines and they showed essentially identical results. Since *OsARAF3* has high homology to *H. vulgare* ARAF *AXAHI* and *AXAHII* [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 (stem and leaf sheath), and root of immature seedlings (14 days old) and leaf and stem of mature plants (60 days old, before heading) 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 weakly expressed in leaves, I focused on the phenotype in the leaf of FOX lines.

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 3A). Since some ARAFs have bifunctional activity for ARAF/β-D-xylosidase, I 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 3B), as predicted from the phylogenetic analysis. *OsARAF1*-FOX and *OsARAF3*-FOX lines, however, showed no visible phenotype (Figure 4).

Effects of overexpression of ARAF on cell wall composition

I determined the glycosyl composition of cell walls prepared from mature leaves of *OsARAF1*-FOX and *OsARAF3*-FOX lines. The arabinose content in the TFA-soluble fraction, which was mainly derived from the side chain of arabinoxylan, 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 arabinoxylan, 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 in the control (Figure 5A). The amount of lignin was slightly higher in *OsARAF1*-FOX and *OsARAF3*-FOX (Figure 5B). These results indicate that overexpression of *OsARAF1* and *OsARAF3* caused a decrease in arabinoxylan 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 5C).

Overexpression of ARAF caused changes in localization of cell wall components

I 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

arabinoxylan epitopes ([31]; University of Georgia: http://www.ccrc.uga.edu/ ~mao/wallmab/Antibodies/antib.htm). LM11 and LM10 labeled arabinoxylan and xylan epitope, respectively [32]. When probed with LM6, *OsARAF1*-FOX and *OsARAF3*-FOX showed lower signals in all tissues (Figure 6D–F). Moreover, LM11 stained the wall of vascular bundles and the epidermis of the control, but faint signals were detected in *OsARAF1*-FOX and *OsARAF3*-FOX lines (Figure 6G–I). However, LM10 label had the same signal intensity among the control, *OsARAF1*-FOX, and *OsARAF3*-FOX lines (Figure 6J-L).

In *OsARAF1*-FOX and *OsARAF3*-FOX, a high intensity of Calcofluor staining was observed in the midrib and phloem (Figure 7D-F). Calcofluor White is reported to stain β-1, 4-glucan (cellulose), β-1, 3-glucan (callose), and mannan [33, 34]. Because callose deposites in specific process, for example, during pollen formation and in the defense response against pathogens [35, 36], and the content of mannan is low (Table 1 and Table 2). Therefore, Calcofluor White is considered to 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 I examined differences in

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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 8). These results 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 cellulosic glucose content in the cell wall of *OsARAF1*-FOX and *OsARAF3*-FOX lines implied that the efficiency of saccharification in the FOX line is higher than the control. I measured sugar liberation from *OsARAF1*-FOX and *OsARAF3*-FOX mature leaves (Figure 9A and B). The prolonged incubation led to a significantly higher saccharification rate in FOX lines than in the control, and the amount of liberated sugar after 24 h increased by 46.4% and 69.6%, respectively, compared to the control. Because the amount of starch in mature leaves accounted for only about 2% of the total sugar content (data not shown), the increase in saccharification efficiency does not appear to be influenced by starch.

Discussion

Arabinose content in the cell walls of the two ARAF-overexpressed plants decreased to 75.4% and 81.6% of that in the control, and the amount of cellulosic glucose increased to 128.2% and 134.2% of that in the control. I 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. Indeed, the use of various fungal cell wall hydrolases was previously shown to result in sterility and dwarf [37]; therefore, I used the endogenous arabinofuranosidases for overexpression. They were thought not to have bifunctional activity (Figure 3 and Figure 4). *OsARAF1*-FOX and *OsARAF3*-FOX lines showed no morphological phenotype and same improvement of mechanical strength as compared with the control (Figure 4 and 8). In addition, overexpression caused an increase in saccharification efficiency (Figure 9).

Arabinoxylans are major non-cellulosic polysaccharides in primary and secondary cell walls of graminacea plants. 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 [38]. In growing tissues, arabinoxylan content in cell walls increases slowly and arabinoxylans are highly branched with arabinosyl residues and are removed during growth [39, 40].

ARAFs are defined as enzymes that catalyze the hydrolysis of terminal, nonreducing α-L-arabinofuranoside residues. However, several enzymes in GH 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. For example, barley ARA-I, *Arabidopsis* XYL3 and ARAF1, and alfalfa MsXyl1 have bifunctional enzyme activity [25, 26, 41, 42]. However, our results showed that OsARAF1 and OsARAF3 might have only ARAF activity and no xylosidase activity (Figure 2B, C and Figure 3). And, lignin complexes with arabinoxylan are mediated by ferulic acid [9]. In *OsARAF1*-FOX and *OsARAF3*-FOX, the amounts of ferulic acid were similar to that in the control. These results suggest that OsARAF1 and OsARAF3 do not possess the ability to cleave the linkage of the arabinose residue to ferulic acid. Expression levels of *OsARAF1* and *OsARAF3* was higher in growing organs and lower in mature organs (Figure 1B). These results indicate that arabinoxylans are turned over during cell wall development in growing organs.

Arabinose residues are a quantifiably important constituent in various cell wall components, not only arabinoxylan but pectin RG-I and glycoprotein [2, 43]. Glycosyl composition analysis of cell walls and immunohistochemistry showed

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that arabinosyl residues in arabinoxylans decreased in *OsARAF1*-FOX and *OsARAF3*-FOX lines. The xylan in *OsARAF1*-FOX and *OsARAF3*-FOX also decreased in the hemicellulose fraction (Table 1). These results suggested that the xylan main chain decreases by reducing the side chain arabinose in FOX lines. On the other hand, the ratios of arabinose to xylose in *OsARAF1*-FOX and *OsARAF3*-FOX were about 1:3, the same as that in the control (Table 1). Therefore, it is strongly indicated that overexpression of *OsARAF* results in not the structure but the almost of arabinoxylan. 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 *AtARAF1* leads to increases in the secondary cell wall, especially xylan [44]. In any case, arabinofuranosidase overexpression may cause modification of the secondary cell wall.

Neither *OsARAF1*-FOX nor *OsARAF3*-FOX affected plant growth (Figure 4). The increase in cellulose may compensate for the reduction in arabinoxylan (Table 1, Table 2 and Figure 7). Additionally, amount of crystalic cellulose in *OsARAF3*-FOX increased compared to the control (Figure 5A). Staining with Calcofluor White increased in the midrib and phloem of FOX lines, which could be associated with the increased mechanical strength (Figure 7D–F and Figure 8). These results support a hypothesis that rice maintains plant body strength via a balance between cellulose and hemicellulose. In fact, *OsARAF1*-FOX and

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OsARAF3-FOX lines showed slight increase of mechanical strength compared to the control (Figure 8). In dicots, xyloglucan is hydrogen-bonded to cellulose microfibrils. In xylogulcanase-overexpressed poplar, the amount of cellulose increased and the basal region of the stem was prevented from bending upward [45]. Because the FOX lines used in this study showed a lower arabinoxylan content, it was expected to have a similar phenotype to the transgenic popular; however, no influence on growth occurred (Figure 4). Unlike dicots, rice has interspersed vascular bundles, which contributes less to 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 9A and B). In industrial processes, plant biomass is treated with hemicellulase and cellulase because cellulose covers the cell wall matrix. I expected that the increased saccharification efficiency of FOX lines would depend on decreases in hemicellulose and substituted xylan [46]. The saccharification efficiency after 24 h reaction increased in FOX lines (Figure 9A), 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. Because the hydrolysis of β-1,3-1,4-glucan is accelerated during growth, the β-1,3-1,4-glucan level is low in mature leaves [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 via formation of diferulic bond [9]. In this study, the decrease in arabinoxylan had little effect on the assembly of cell wall network 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.

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Tables and Figures

Table 1 Monosaccharide compositions of TFA-soluble fractions.

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".

	control	*OsARAF1*-FOX	*OsARAF3*-FOX
Ara	41.9 ± 2.6 [a]	31.6 ± 5.6 [b]	33.8 ± 6.6 [b]
Rha	2.7± 1.4 [a]	2.5 ± 0.9 [a]	2.5 ± 0.5 [a]
Fuc	N.D.	N.D.	N.D.
Xyl	153.2 ± 7.4 [b]	131.6 ± 15.9 [a]	135.6 ± 11.8 [a]
Man	11.2 ± 4.3 [a]	9.8 ± 3.4 [a]	14.9 ± 4.2 [a]
Gal	11.8 ± 4.3 [a]	11.4 ± 3.4 [a]	12.9 ± 4.2 [a]
Glc	128.3 ± 15.2 [a]	65.1 ± 29.9 [b]	90.0± 18.4 [ab]
GalA	2.3 ± 1.0 [a]	1.4 ± 1.5 a	2.2 ± 1.0 [a]
GlcA	7.3 ± 1.9 [a]	5.8 ± 2.6 [a]	9.1 ± 3.0 [a]
total	351.7 ± 125 [a]	283.6 ± 28.3 [b]	323.7 ± 18.5 [ab]

Table 2 Monosaccharide compositions of TFA-insoluble fractions.

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".

	control	*OsARAF1*-FOX	*OsARAF3*-FOX
Ara	N.D.	N.D.	N.D.
Rha	N.D.	N.D.	N.D.
Fuc	N.D.	N.D.	N.D.
Xyl	N.D.	N.D.	N.D.
Man	27.8 ± 5.8 [a]	28.6 ± 13.0 [a]	36.4 ± 17.4 [a]
Gal	2.2 ± 2.5 [a]	4.3 ± 11.4 [a]	2.4 ± 3.5 [a]
Glc	370.3 ± 28.1 [a]	474.6 ± 40.0 [b]	497.0 ± 32.4 [b]
GalA	N.D.	N.D.	N.D.
GlcA	N.D.	N.D.	N.D.
total	400.3 ± 31.3 [a]	508.5 ± 36.8 [b]	535.7 ± 32.1 [b]

Figure 1 Characteristics of *Oryza sativa* ARAF1 and ARAF3.

(A) Phylogenetic tree of putative ARAFs and xylosidases (members of the GH families 51 and 3) in *Oryza sativa*, *Arabidopsis thaliana* (AtARAF1: At3g10740, AtARAF2: At5g26120, XLY1: At5g49360, XYL3: At5g09730) and *Hordeum vulgare* (AXHAI and AXAHII). Phylogenetic trees were constructed by the neighbor-joining method in ClustalX.

(B) The expression patterns of *OsARAF1* and *OsARAF3*. RT-PCR analysis was performed using total RNA isolated from different organs of 14-day-old seedlings and 60-day-old mature plants. The numbers in parentheses indicate the numbers of PCR cycles. These experiments were performed at least twice with similar results.

A

Os11g0297800
Os11g0291000   Os11g0297300   GH family 3
Os02g0752200   XYL1(At5g49360)
Os04g0530700   Os11g0673200
Os01g0296700   XYL3(At5g09730)
                                      Os04g0640700

                                          Os03g0749100
                                          Os02g0131400
                                          Os03g0749500
AtARAF2   Os03g0749300 Os01g0771900
(At5g26120)

AtARAF1
(At3g10740)

OsARAF4
OsARAF3
AXAHII
AXAHI   OsARAF1   0.1
OsARAF5 OsARAF2

GH family 51

B

	14 days			60 days	
	Root	Stem	Leaf	Stem	Leaf
OsARAF1 (25)					
OsARAF3 (25)					
17S rRNA (20)					

40
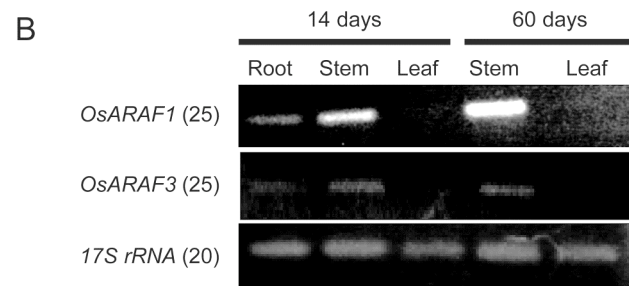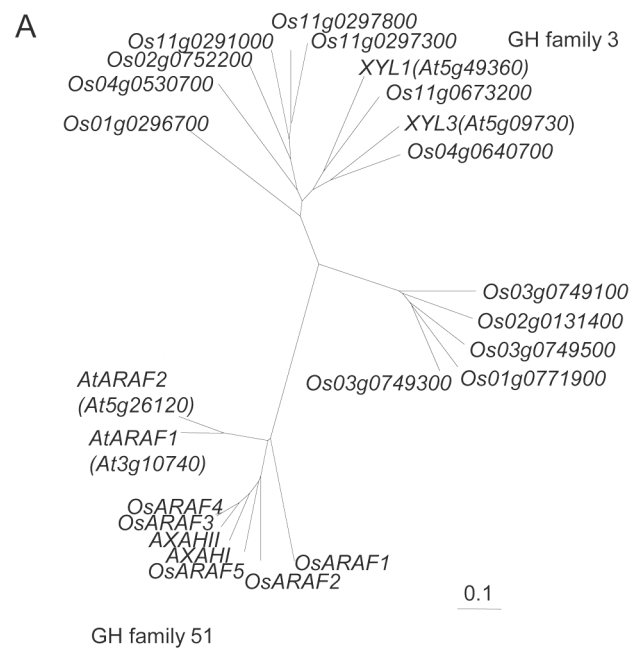
Figure 2 Characteristics of the *OsARAF1*-FOX and *OsARAF3*-FOX lines.

(A) RT-PCR analysis of transcripts in mature leaves from the control, *OsARAF1*-FOX and *OsARAF3*-FOX lines. 17S Ribosomal RNA-specific primers were used as controls. The numbers in parentheses indicate the numbers of PCR cycles. These experiments were performed at least twice with similar results.

(B) Relative ARAF activities in *OsARAF1*-FOX and *OsARAF3*-FOX leaves determined using 4-nitrophenyl-α-L-arabinofuranide as a substrate. Activity is expressed as a ratio of the activity in each FOX line to that in the control leaves. Error bars indicate the SD (n = 3). Letters in each panel indicate significant differences at $P < 0.05$ (Tukey's test). Black, white and gray columns indicate the control, *OsARAF1*-FOX and *OsARAF3*-FOX lines, respectively.

(C) Relative xylosidase activities in *OsARAF1*-FOX and *OsARAF3*-FOX leaves determined using 4-nitrophenyl-β-D-xylopyranoside as a substrate. Activity is expressed as a ratio of the activity in each FOX line to that in the control leaves. Error bars indicate the SD (n =3). Letters in each panel indicate significant differences at $P < 0.05$ (Tukey's test). Black, white and gray columns indicate the control, *OsARAF1*-FOX and *OsARAF3*-FOX lines, respectively.

A

OsARAF1 (25)   OsARAF1-FOX   control

OsARAF3 (25)   OsARAF3-FOX   control

17S rRNA (20)   17S rRNA (20)

B

C
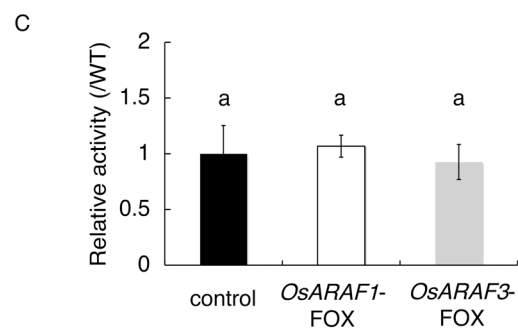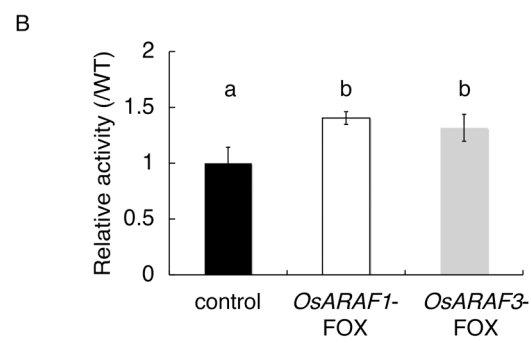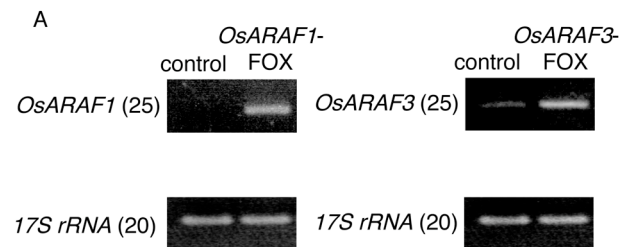
Figure 3 Enzyme activity in the control, *OsARAF1*-FOX and *OsARAF3*-FOX.

(A) ARAF activity using 4-nitrophenyl-α-L-arabinofuranide as substrate in the control, *OsARAF1*-FOX and *OsARAF3*-FOX leaves.

(B) Xylosidase activity using 4-nitrophenyl-β-D-xylopyranoside as substrate in the control, *OsARAF1*-FOX and *OsARAF3*-FOX leaves.

Black, white, and gray synbis indicate the control, *OsARAF1*-FOX, and *OsARAF3*-FOX lines, respectively.

A

B

44
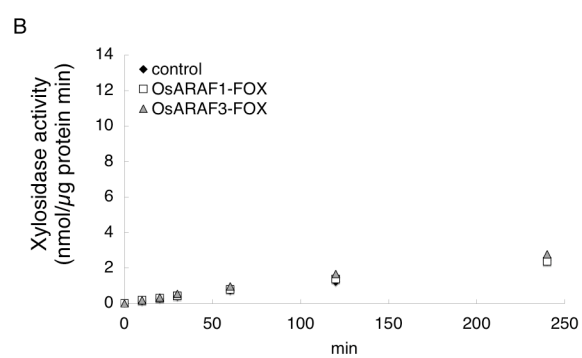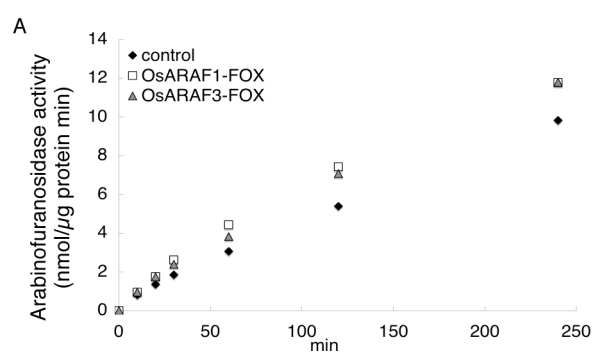
Figure 4 The phenotypes of the control (A), *OsARAF1*-FOX (B) and *OsARAF3*-FOX (C) in 60-day-old plant after sowing.

The growth levels of FOX lines were very similar to those of the control, and all FOX lines had normal fertility. Bar = 10 cm.

control   *OsARAF1*-FOX   *OsARAF3*-FOX
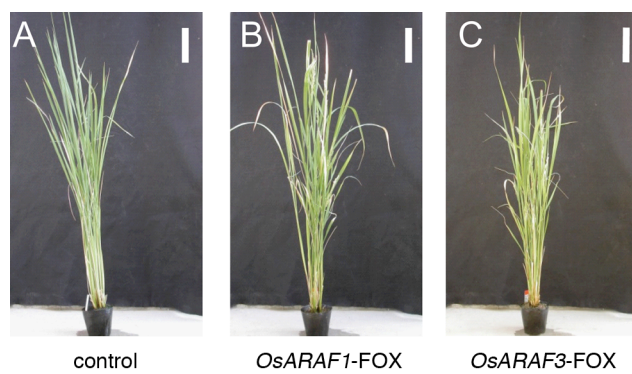
Figure 5 Cell wall component analysis of the control, *OsARAF1*-FOX and *OsARAF3*-FOX.

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).
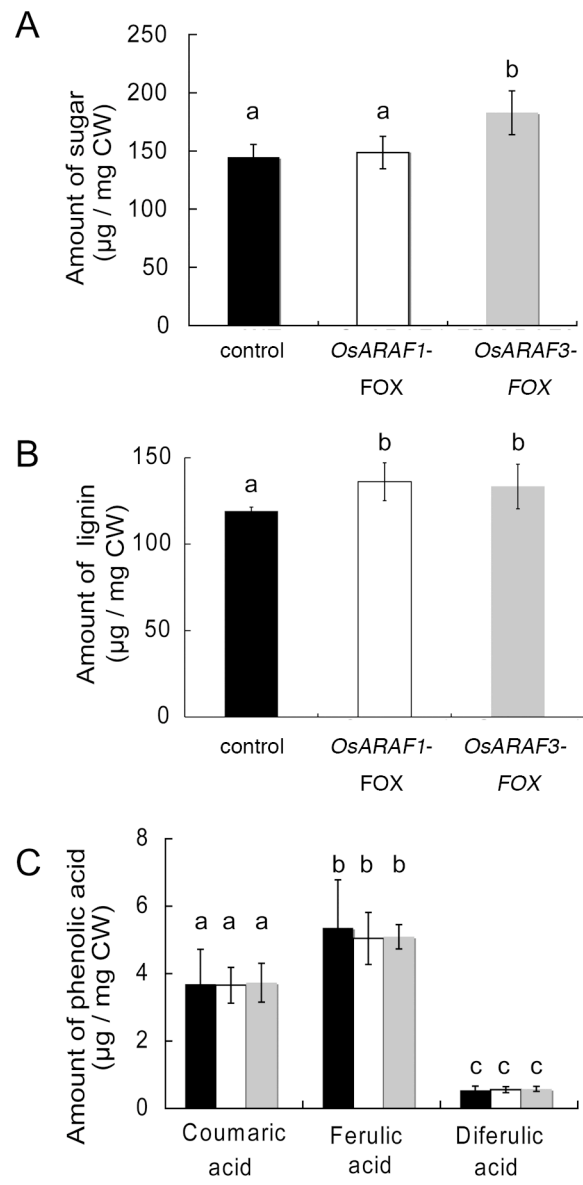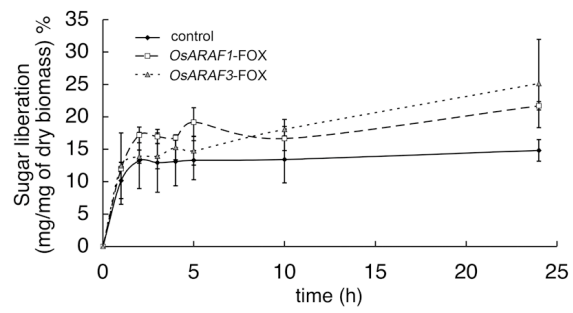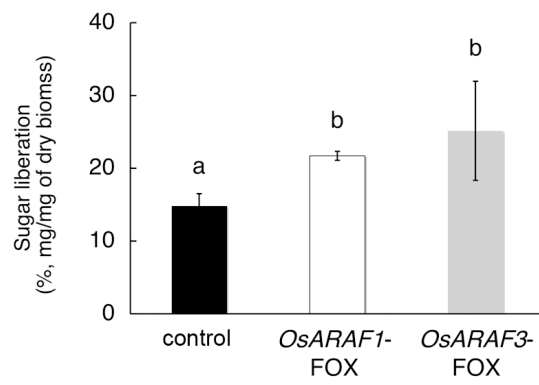
Figure 6 Immunofluorescent labeling of the control and FOX lines with arabinoxylan related antiibodies.

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–l) 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.
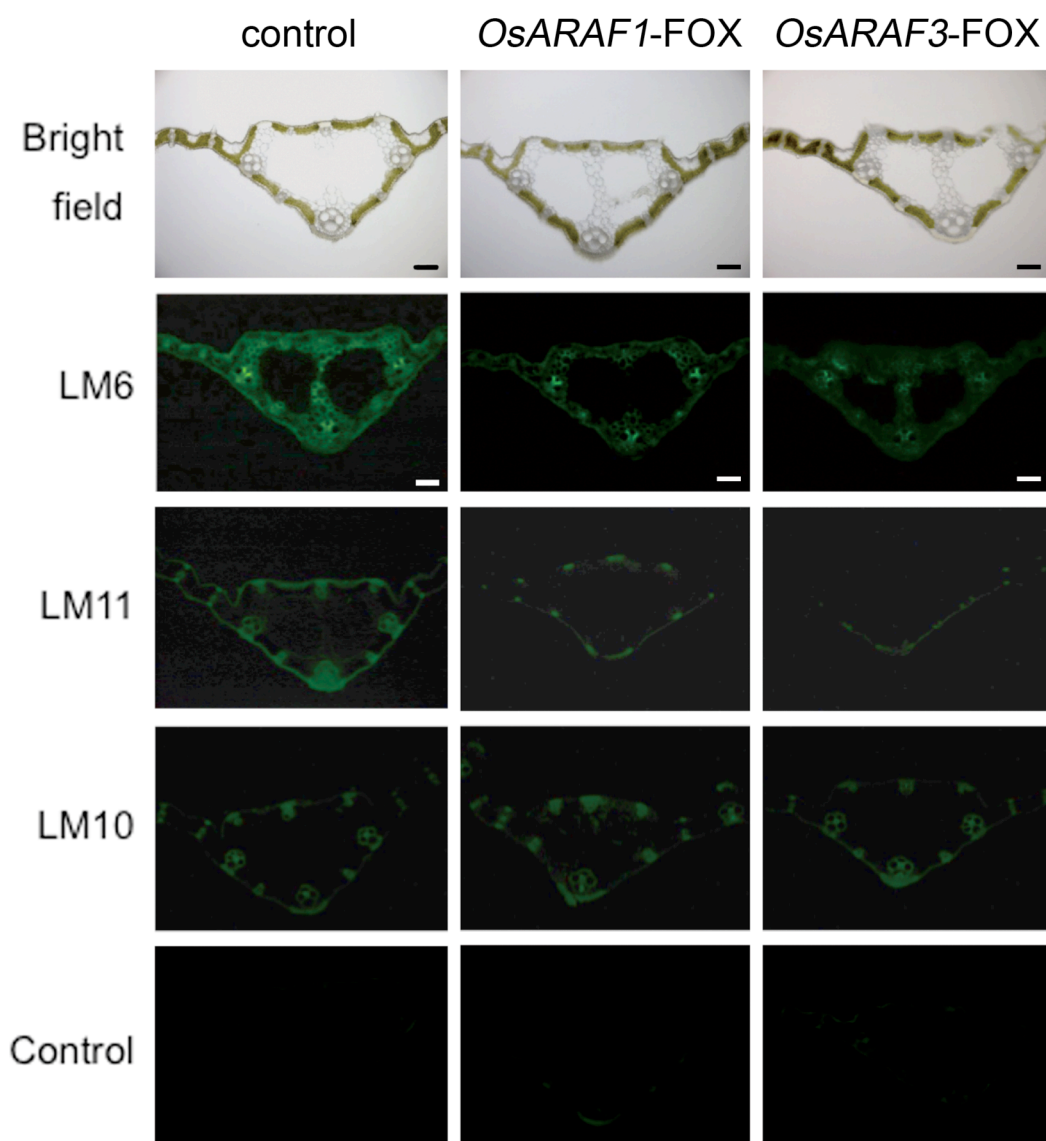
Figure 7 Calcofluor white staining for cellulose of mature leaves of the control and FOX lines.

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.

	control	*OsARAF1*-FOX	*OsARAF3*-FOX
Bright field	A	B	C
Calcofluor white	D	E	F
Control	G	H	I
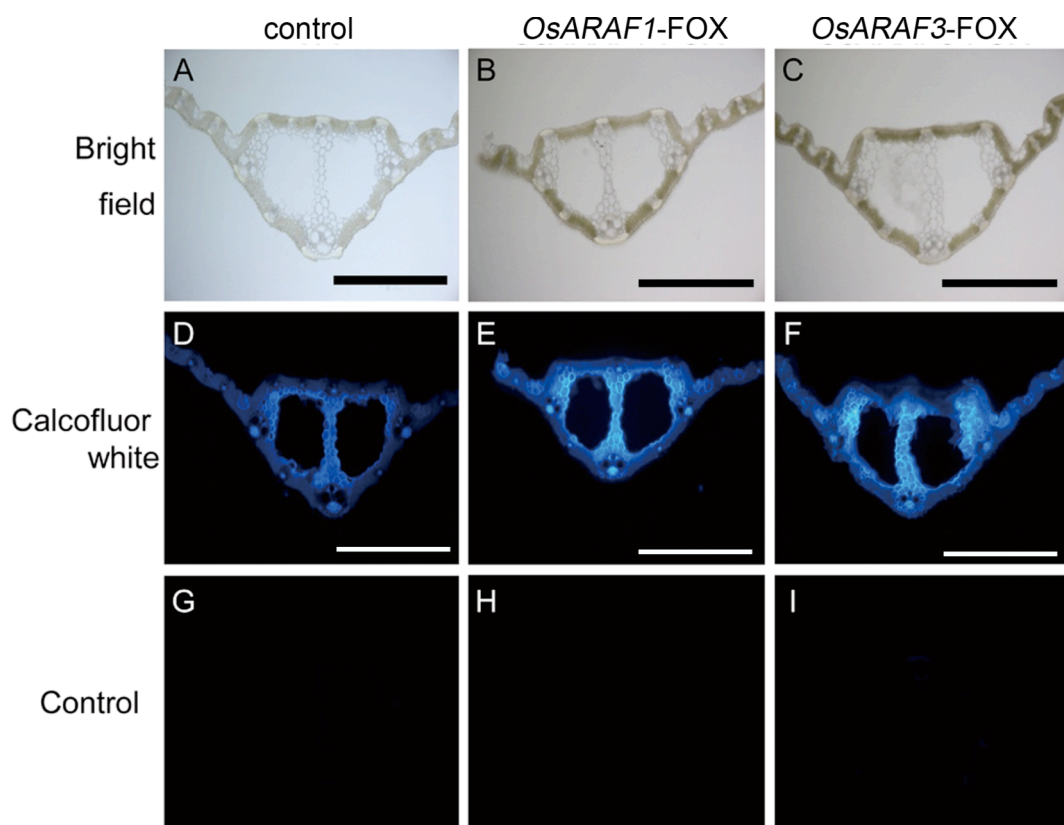
Figure 8 Mechanical properties of leaves of the control, *OsARAF1*-FOX and *OsARAF3*-FOX lines.

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).
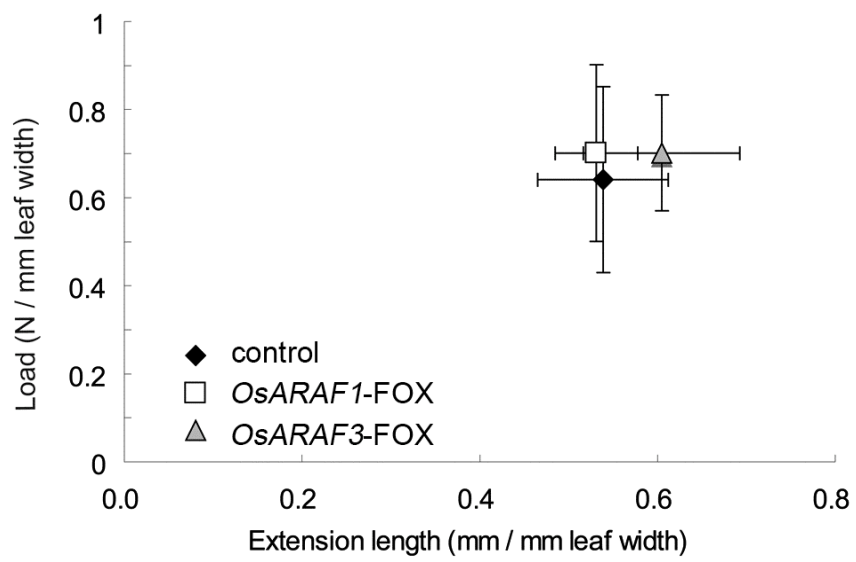
Figure 9 Saccharification of the control *OsARAF1*-FOX and *OsARAF3*-FOX lines in dry leaves.

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).

A

B