Down-regulation of OsMYB103L distinctively alters beta-1,4-glucan polymerization and cellulose microfibers assembly for enhanced biomass enzymatic saccharification in rice

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Abstract

Background: As a major component of plant cell walls, cellulose provides the most abundant biomass resource convertible for biofuels. Since cellulose crystallinity and polymerization have been characterized as two major features accounting for lignocellulose recalcitrance against biomass enzymatic saccharification, genetic engineering of cellulose biosynthesis is increasingly considered as a promising solution in bioenergy crops. Although several transcription factors have been identified to regulate cellulose biosynthesis and plant cell wall formation, much remains unknown about its potential roles for genetic improvement of lignocellulose recalcitrance.

Results: In this study, we identified a novel rice mutant (Osfc9/myb103) encoded a R2R3-MYB transcription factor, and meanwhile generated OsMYB103L-RNAi-silenced transgenic lines. We determined significantly reduced cellulose levels with other major wall polymers (hemicellulose, lignin) slightly altered in mature rice straws of the myb103 mutant and RNAi line, compared to their wild type (NPB). Notably, the rice mutant and RNAi line were of significantly reduced cellulose features (crystalline index/CrI, degree of polymerization/DP) and distinct cellulose nanofibers assembly. These alterations consequently improved lignocellulose recalcitrance for significantly enhanced biomass enzymatic saccharification by 10–28% at p < 0.01 levels (n = 3) after liquid hot water and chemical (1% H2SO4, 1% NaOH) pretreatments with mature rice straws. In addition, integrated RNA sequencing with DNA affinity purification sequencing (DAP-seq) analyses revealed that the OsMYB103L might specifically mediate cellulose biosynthesis and deposition by regulating OsCesAs and other genes associated with microfibril assembly.

Conclusions: This study has demonstrated that down-regulation of OsMYB103L could specifically improve cellulose features and cellulose nanofibers assembly to significantly enhance biomass enzymatic saccharification under green-like and mild chemical pretreatments in rice. It has not only indicated a powerful strategy for genetic modification of lignocellulose recalcitrance but also provides new insights into understanding the regulatory networks of cellulose biosynthesis in rice.
Background

Cellulose is the most abundant renewable substance convertible for biofuels and biochemicals [1, 2]. As a complicated chemical process in higher plants, cellulose biosynthesis involves in three major steps: initiation of beta-1,4-glucan chain, chain elongation and microfibril assembly [3–5]. Hence, large numbers of genes have been identified to form cellulose synthase complexes (CSCs) for cellulose biosynthesis [6, 7]. For example, CesA1, CesA3, and CesA6-like CesAs are characterized for primary wall cellulose synthesis, whereas CesA4, CesA7, and CesA8 are essential isoforms for secondary wall cellulose production in Arabidopsis [8, 9]. However, despite other genes have been also identified to associate with cellulose synthesis and assembly in plants [6, 10], much remains unknown about their regulation mechanisms.

Over the past years, various transcription factors (TFs) families have been characterized for dynamic regulations of secondary cell wall biosynthesis in plants such as VNDs (VASCULAR-RELATED NAC-DOMAIN), NSTs (NAC SECONDARY WALL THICKENING PROMOTING FACTOR1), NACs (NAM, ATAF1,2, and CUC2) and MYBs (v-myb avian myeloblastosis viral oncogene homolog) [11, 12]. Among the TFs examined, MYBs are identified with specific expressions in plant tissues, and their major targets are secondary cell wall-related genes or TFs for secondary wall thickening such as MYB46, MYB83, MYB58, MYB63 [13, 14]. Importantly, MYB103 and OsMYB103L have been examined as an R2R3-MYB transcriptional factor for regulating secondary cell wall synthesis in Arabidopsis and rice, respectively [15, 16]. In addition, OsMYB103L plays an important role in GA-regulating secondary cell wall synthesis in rice [17]. Although OsMYB103L has been reported with potential binding with OsCesAs genes of secondary cell wall [17], it has not been reported about specific regulation of any TFs for cellulose biosynthesis in rice, particularly to underlie impacts on cellulose feature and microfibrils assembly in plant cell walls.

Cellulose is composed of the beta-1,4-glucan chains that form microfibrils [4]. As a major load-bearing component of plant cell walls, cellulose is embedded with the matrix of hemicellulose and lignin for complicated wall-networks, which not only affects plant biochemical metabolism and mechanic strength, but also determines lignocellulose recalcitrance against biomass enzymatic saccharification [18–20]. Particularly, cellulose crystallinity and polymerization have been characterized as the major lignocellulose recalcitrant factors, which are accountable by measuring crystalline index (CrI) and degree of polymerization (DP) values of cellulose substrates [21–24]. More recently, it has been stated that either OsCesA site-mutation or over-production of lignocellulose-degradation enzymes could significantly reduce cellulose CrI and DP values for much enhanced biomass enzymatic saccharification [25–27]. Hence, it becomes interesting to find out a genetic approach for regulating cellulose biosynthesis towards lignocellulose recalcitrance improved in plants.

Rice is a major food crop all over the world and provides large amounts of lignocellulose-based straw for biofuel production [26, 28, 29]. In this study, we identified a novel rice mutant with deletion in the binding motif of OsMYB103L, and then generated RNAi-silenced OsMYB103L transgenic lines to explore OsMYB103L regulation roles in cellulose biosynthesis and plant cell wall formation. Using both genetic mutant and transgenic lines, we determined specifically reduced cellulose levels in plant cell walls with little alteration of other major wall polymer (hemicellulose, lignin) contents in rice mature straws. Furthermore, this study detected significantly reduced cellulose CrI and DP values with distinct nanofibers assembly, leading to remarkably enhanced biomass enzymatic saccharification examined in the mutant and RNAi transgenic lines. By performing multi-omics analyses, RNA-seq and DAP-seq assays along with gene co-expression profiling, this work found out that the OsMYB103L may predominately regulate cellulose biosynthesis in rice, providing a powerful strategy for genetic modification of plant cell walls with little impact on plant growth and biomass yield in bioenergy crops.

Results

Identification of novel rice OsMYB103L mutant and RNAi lines

Using our previously established T-DNA mutagenesis pool of the rice variety (Nipponbare), this study screened out a fragile-culm mutant, termed fragile culm 9 (Osfc9). By means of total 1050 F2 mutant plants generated from a cross between Osfc9 and an indica cultivar MH63, a classic map-based genetic approach was applied to clone the Osfc9 gene (Fig. 1). The Osfc9 gene was preliminarily

Keywords: Cellulose synthesis, Cellulose polymerization, Microfibril assembly, OsMYBs, Multi-omics, Biomass saccharification, Fragile culm, Rice
identified between RM22342 and RM22392 on chromosome 8 by using 145 polymorphic SSR markers (Fig. 1A). We further used other three known SSR markers (RM5647, RM22358, RM4955), and five designed primers (SSR1-SSR5) to map the gene between SSR2 and RM4955 in a 46-kb genomic region, which contained four open reading frames. Among the CDS (Coding DNA Sequence) of all candidate genes, only one gene was identified with 18-base deletion in LOC_Os08g05520, a R2R3-MYB like transcriptional factor termed as OsMYB103L. We then identified a single-base mutation from A (adenine) to C (cytosine) in the end of the second intron, which resulted in an error in the recognition of intron splicing for the deletion of 18-base in CDS sequence. However, further analysis showed that the deletion of 18-base in CDS did not significantly alter the transcript level of LOC_Os08g05520 (Fig. 2C), suggesting that missense or termination mutation of OsMYB103L should not occur in the mutant.

Meanwhile, this study observed that the myb103 mutant had a normal plant growth with similar grain yield to the wild type (WT) (Fig. 2A; Additional file 1: Fig. S1D), but it was of obvious fragile-culm phenotype and remarkably reduced plant height which should be the major cause accounting for significantly decreased lodging index examined in the mutant (Fig. 2B; Additional file 1: Fig. S1B, C). To confirm the fragile-culm phenotype, we performed genetic complemental experiment by expressing a 2851-bp full genomic sequence of LOC_Os08g05520 driven with its upstream 2 kb sequence as promoter in the myb103 mutant. Notably, the fragile-culm phenotype of the mutant was almost completely restored in the complementation line (Fig. 2A, B).

Furthermore, this study generated OsMYB103L RNAi-silencing transgenic lines and examined two independent lines with significantly reduced OsMYB103L expression levels by 46% and 41%, compared to WT (Fig. 2F). As a further comparison with the myb103 mutant, two RNAi lines showed a highly similarity in the fragile-culm phenotype and other major traits examined (Fig. 2D, E; Additional file 1: Fig. S1), suggesting that both mutant and transgenic lines could be applicable to investigate OsMYB103L function in rice.

Reduced cellulose production in myb103 mutant and RNAi line
As the myb103 mutant and RNAi transgenic lines were of fragile-culm phenotypes, this study examined their cell wall structures and major wall polymer contents. Using the β-glucuronidase (GUS) reporter gene driven by OsMYB103L putative promoter, we first observed OsMYB103L special expression in vascular bundles...
**Fig. 2** Phenotype observation of myb103 mutant and OsMYB103L-RNAi transgenic lines. A Images of WT (NPB, wild type), myb103 (Osfc9 mutant) and its complementary line. B Stem brittleness of WT, myb103 and its complementary line. C Gene expression analysis of OsMYB103L between WT and myb103. D Images of WT and two OsMYB103L-RNAi transgenic lines (RNAi-1 and RNAi-2). E Stem brittleness of WT and two OsMYB103L RNAi transgenic lines (RNAi-1 and RNAi-2). F Gene expression analysis of OsMYB103L in WT, and two OsMYB103L RNAi transgenic lines (RNAi-1 and RNAi-2). **As significant differences of two OsMYB103L-RNAi transgenic plants compared with WT at p < 0.01 (n = 3) with decreased (−) percentage, respectively**

**Fig. 3** Morphological observation of vascular bundles in stem tissues of myb103 mutant and RNAi transgenic line. A Examination of GUS activity by expressing proOsMYB103L::GUS. B View of vascular bundles development and distribution; the stem slices obtained with 100 μm thickness, and imaged using a Nikon Eclipse Ni-U microscope under bright field. C S4B cellulose staining; the stem slices obtained with 100 μm thickness, stained with S4B (Direct Red 23, Sigma), and imaged using a Nikon Eclipse Ni-U microscope under 530–560 nm excitation and 620–660 nm emission
Then, we found obviously altered vascular bundles distribution in stem tissues of the myb103 mutant and RNAi transgenic line, compared to the WT (Fig. 3B, C). For instance, both myb103 mutant and RNAi line showed an increase in the number of vascular bundles, but had a reduction in the area of single vascular bundle (Fig. 3C). In addition, the distribution of vascular bundles in the epidermis was altered by moving into inside cells in the mutant and transgenic line (Fig. 3B). The alteration of vascular bundles distribution in stem tissues may be one factor accounting for their reduced mechanical strength and culm brittleness.

Under transmission electron microscopy (TEM), we observed significantly reduced cell wall thickness in myb103 mutant and RNAi-1 transgenic line at $p < 0.05$ and 0.01 level, compared to the WT (Fig. 4A, Additional file 1: Fig. S2). Accordingly, this study detected significantly reduced cellulose levels by 13% and 19% in mature straws of the myb103 mutant and RNAi-1 transgenic line, respectively (Fig. 4B), consistent with the Pontamine fast scarlet 4B (S4B) staining of cellulose that showed relatively weaker intensity in the stem tissues of both mutant and transgenic line than that of the WT examined (Fig. 3C). By comparison, other two major wall polymers (hemicellulose, lignin) were not significantly altered in both mutant and transgenic line at $p > 0.05$, compared to WT (Fig. 4B), which was supported by observation of the FTIR spectroscopy profiling that did not show much difference in the characteristic peaks for interlinkages among hemicellulose and lignin such as at 1727, 1605, 1511, 1051, and 754 cm$^{-1}$ in all samples examined [30, 31] (Additional file 1: Fig. S3). Hence, the results suggested that down-regulation of OsMYB103L may mainly lead to reduced cellulose biosynthesis for relatively thinner cell walls in both myb103 mutant and RNAi-1 transgenic line.

### Altered cellulose features and microfibril assembly in myb103 mutant and RNAi line

With respect to the myb103 mutant and RNAi transgenic line that were of specifically reduced cellulose deposition, this study detected two major cellulose features (CrI, DP) in the mature rice straws (Fig. 5). Compared to the WT, the myb103 mutant and RNAi transgenic line, respectively, showed the decreased cellulose CrI values by 12 and 16% (Fig. 5A), whereas their cellulose DP values were significantly reduced by 11 and 23% at $p < 0.05$ and 0.01 level (Fig. 5B). Furthermore, under atomic force microscopy (AFM), we observed cellulose microfibers assembly in parenchyma-type secondary cell walls of rice stem tissue (Fig. 5C). As a comparison, the WT exhibited a normal cellulose microfibrils orientation in a common direction as a poly-lamellate structure, while both myb103 mutant and RNAi transgenic line showed a fibril-broken and disordered pattern with a rough surface for cellulose nanofibers accumulation, consistent with the previous findings that low-DP cellulose deposition leads to cellulose nanofibers assembly [25, 32]. Hence, the results suggested that down-regulations of OsMYB103L should affect cellulose biosynthesis for significantly reduced cellulose level and features and altered cellulose microfibril assembly.

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**Fig. 4** Measurements of cell wall thickness and cell wall composition in myb103 mutant and RNAi transgenic line. **A** Observation of cell wall thickness by transmission electron microscopy. **B** Cell wall composition analysis. **As significant differences between myb103 and RNAi-1 with WT at $p < 0.01$ ($n = 3$). SCW, secondary cell wall. PCW primary cell wall.
Enhanced biomass enzymatic saccharification in \textit{myb}103 mutant and RNAi line

Because cellulose features distinctively affect biomass enzymatic saccharification, this study measured hexose yields (\% cellulose) released from enzymatic hydrolysis of pretreated rice straws in the \textit{myb}103 mutant and RNAi transgenic line (Fig. 6). Without any pretreatment, both \textit{myb}103 mutant and RNAi transgenic line showed significantly higher hexose yields released from enzymatic hydrolysis of raw materials.
than those of the WT at $p < 0.01$ level with increased rates of 19% and 31% (Fig. 6A). Using mild chemical (1% $\text{H}_2\text{SO}_4$, 1% $\text{NaOH}$) and liquid hot water pretreatments as previously established [24, 26, 33], we further examined significantly enhanced hexose yields by 10%-28% released from enzymatic hydrolysis of pretreated straw residues in the mutant and transgenic line, compared to the WT (Fig. 6B–D), indicating a consistently enhanced biomass enzymatic saccharification in the $\text{myb103}$ mutant and $\text{OsMYB103L}$-RNAi transgenic line under relatively cost-effective pretreatments.

Biochemical identification of the OsMYB103L motif for regulating $\text{OsCesAs}$ expression

To test $\text{OsMYB103L}$ regulation on cellulose biosynthesis, we performed DAP-seq analysis by using a high-throughput TF binding site discovery method [34, 35]. As a result, the characteristic proteins of OsMYB103L and mutated OsFC9 in vitro were detected by means of wheat germ system (Fig. 7A). Further DAP-seq analysis identified nearly 20,000 peaks across the whole genome of OsMYB103L, whereas less than 10 peaks were found in the mutated OsFC9 protein (Fig. 7B), suggesting that the $\text{myb103}$ mutation should not affect its protein production, but may result in the significant loss of binding ability with down-stream DNA sites. As the single-base mutation caused 6 amino acid deletion in R3 binding motif of the $\text{myb103}$, we predicted the three-dimensional structure of the $\text{myb103}$ using the SWISS-MODEL (https://swissmodel.expasy.org/) [36]. Compared to the standard OsMYB103L protein structure, the mutated OsFC9 protein was of a lost $\alpha$-helix region (Fig. 7C). Since the $\alpha$-helix region plays an important role for DNA binding [37], the results suggested that six amino acids deletion in the R3 motif of OsMYB103L protein should significantly affect down-stream gene expression.

Among the 20,000 peaks examined, this study attempted to identify binding motif and potential target genes, and about 27.42% of the OsMYB103L-binding sites were found in the promoter region (Fig. 7D). From promoter binding sites, the motif NACCCANN showed the most significance (E-value 2.2e-1710), and the motif ACCAAAAC (SMRE3) was detectable in the promoters of OsCesA4, OsCesA7 and OsCesA9 (Fig. 7E), which were also examined with enriched peaks from DAP-seq analysis (Additional file 1: Fig. S4). In addition, we observed

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**Fig. 7** Biochemical identification of OsMYB103L binding sites. **A** Protein expression of HALO-tagged OsMYB103L and OsFC9 in vitro. **B** Peak analysis of OsMYB103L and OsFC9 by DAP-seq. **C** Protein structure prediction between OsMYB103L and OsFC9. **D** Distribution of combined peaks of OsMYB103L by DAP-seq. **E** Motif analysis of combined peaks of OsMYB103L with the most significant E-value. **F** Gene expression analysis of OsCESA4, OsCESA7 and OsCESA9 in WT, $\text{myb103}$ and RNAi-1. * and ** as significant differences between $\text{myb103}$ and RNAi-1 with WT at $p < 0.05$ and 0.01 ($n = 3$), respectively.
OsMYB103L localization in nucleus from transient transformation experiment, and detected its transactivation (Additional file 1: Fig. S5). Taken together, this study demonstrated that the OsMYB103L could act as an active transcription factor for regulating genes involved in cellulose biosynthesis.

Genome-wide analysis of OsMYB103L regulation on cellulose deposition

To understand how OsMYB103L regulation consequently affects cellulose microfibril assembly, this study performed transcriptome analysis of myb103 mutant (Fig. 8). As a result, total 1652 genes were significantly up-regulated, whereas 2048 genes were significantly down-regulated with slightly altered expressions of 23,678 genes, compared to the WT (Fig. 8A, Additional file 4: Table S3). Integrating the differentially expressed genes (DEGs) of the myb103 mutant with ones of the OsMYB103L-overexpressed transgenic line as previously reported, total 478 overlapped genes were identified (Fig. 8B). Using DAP-seq analysis, we further identified 149 overlapped genes that were significantly regulated (Additional file 5: Table S4). Based on the AgriGO v2.0 analysis of 149 genes [38], two GO terms were sorted out with significantly enriched such as microtubule-based process and microtubule-based movement (Fig. 8C), which has been characterized to involve in cellulose synthesis and deposition [4].

By means of the microarray datasets of 149 genes from CREP, this study finally screened 11 genes, which

![Fig. 8](multi-omics_analysis_of_osmyb103l_regulation_on_cellulose_synthesis_and_assembly.png)

**Fig. 8** Multi-omics analysis of OsMYB103L regulation on cellulose synthesis and assembly. A Volcano analysis of significantly expressed genes of myb103 compared with WT by RNA-seq. B The number of genes overlapped by significantly expressed genes in myb103 mutant and OsMYB103L-overexpression plant, and targeted genes of OsMYB103L by DAP-seq. C The most significant terms of overlapped genes by GO analysis. D Co-expression analysis of 11 overlapped genes in two different stem tissues. Stem 1, stem at 5 days before heading. Stem 2, stem at heading stage.
were highly expressed in the secondary cell walls of rice stem tissues (Fig. 8D). Among the 11 genes, we identified 3 genes directly participated to catalyze cellulose biosynthesis such as OsCESA4 (LOC_Os01g54620), OsCESA7 (LOC_Os10g32980) and CSLA11 (LOC_Os08g33740), whereas other 8 genes (LOC_Os03g30250, LOC_Os10g36880, LOC_Os03g56260, LOC_Os08g43400, LOC_Os01g43580, LOC_Os01g59150, LOC_Os03g13460, LOC_Os03g50520) should involve in in cellulose deposition and microfibril assembly (Table 1). Notably, those 11 genes were significantly down-graded in the myb103 mutant, but 8 of 11 genes (11 genes were significantly down-graded in the myb103 mutant; Table 1). However, this mutation causes 6-amino acid deletion in the R3 binding domain of OsMYB103L and significantly alters the three-dimensional structure of OsMYB103L protein, which should be the major factor affecting recognition of downstream gene sites (Fig. 1B; Fig. 7). In the previous study, OsMYB103L was identified from a culm easily fragile 1 (cef1) mutant, and its mutation occurred in the first exon of the CDS sequence with a 13-bp deletion, resulting in a frame-shift and premature translational product of 56 amino acids [17]. Despite of different mutation sites in OsMYB103L, both Osfc9 and cef1 mutants exhibited significantly decreased stem strength (breaking force) in rice. However, unlike other brittle-culm mutants as previously identified in rice [39, 40], the Osfc9 mutant has showed relatively slight effect on plant growth with similar agronomic traits to the WT (Fig. 2; Additional file 1: Fig. S1). Hence, the novel allele mutation in R3 domain of OsMYB103L provides a genetic strategy for plant cell wall modification in bioenergy crops.

In plants, hundreds of TFs have been identified to involve in plant growth and development, and the NACs, VNDs, NSTs and MYBs are particularly characterized for dynamic regulations of plant cell wall biosynthesis [11, 12]. However, little is yet reported about TF special regulation for cellulose biosynthesis. Although OsMYB103L has been examined as the R2R3-MYB transcriptional factor regulating impacting cell wall synthesis and enabled to bind with the promoters of CesAs genes [16, 17], it remains to explore if it is specific for regulating cellulose

### Table 1 Candidate genes regulated by OsMYB103L for cellulose synthesis and assembly

| Biological processes | Genes | Putative function | log2(fold_change) in myb103 mutant vs WT | log2(fold_change) in OsMYB103L overexpression line vs WT |
|----------------------|-------|------------------|----------------------------------------|--------------------------------------------------------|
| Cellulose synthesis  | LOC_Os01g54620 | CESA4—cellulose synthase, expressed | −1.62 down | 3.59 up |
|                      | LOC_Os10g32980 | CESA7—cellulose synthase, expressed | −2.01 down | 1.70 up |
|                      | LOC_Os08g33740 | CSLA11—cellulose synthase-like family A, expressed | −2.55 down | −4.25 down |
| Cellulose deposition  | LOC_Os03g30250 | BC1, COBRA-like protein precursor, putative, expressed | −2.49 down | 2.39 up |
|                      | LOC_Os10g36880 | Kinesin motor domain-containing protein, putative, expressed | −1.67 down | 1.54 up |
|                      | LOC_Os03g56260 | Kinesin motor domain-containing protein, putative, expressed | −1.56 down | −2.74 down |
|                      | LOC_Os08g43400 | Kinesin motor domain-containing protein, expressed | −1.30 down | −2.64 down |
|                      | LOC_Os01g43580 | Kinesin motor domain-containing protein, putative, expressed | −1.05 down | 1.46 up |
|                      | LOC_Os01g59150 | Tubulin/FtsZ domain-containing protein, putative, expressed | −1.42 down | 3.36 up |
|                      | LOC_Os03g13460 | Microtubule-associated protein, putative, expressed | −1.34 down | 2.38 up |
|                      | LOC_Os03g50520 | Dynamin family protein, putative, expressed | −1.18 down | 1.44 up |
biosynthesis and how cellulose assembly is consequently mediated. As the down-regulation of OsMYB103L could predominately lead to remarkable reductions of cellulose levels and features without significant impacts on other two major wall polymers (hemicellulose, lignin) in the myb103 mutant and RNAi transgenic line (Fig. 4B, Fig. 5A, B), this study has thus provided solid evidences about the OsMYB103L special regulation on cellulose biosynthesis. Notably, due to a reduced DP of beta-1,4-glucans, this work has unexpectedly observed distinct cellulose nanofibers assembly in the myb103 mutant and RNAi transgenic line (Fig. 5), which should further confirm the finding of OsMYB103L specific regulation on cellulose biosynthesis in rice.

Since multi-omics analyses have become a powerful tool for gene functional study [41], combined with transcriptome assay and DAP-seq in the myb103 mutant and MYB103L-overexpressed transgenic line, this study has sorted out several well-known genes related to cellulose deposition and assembly in plant cell walls (Table 1). For instances, the BC1 gene encoded a COBRA-like protein has been proved to control cellulose deposition of secondary cell walls [42], whereas other genes are characterized to mediate beta-1,4-glucans orientation and assembly such as kinesin motor domain, dynamin-related proteins and microtubule-associated protein [43, 44]. In addition, we analyzed the cis-acting elements in the promoters of these 11 candidate genes, and found most of these genes contained the reported cis-acting elements related to cell wall synthesis regulated by OsMYBs such as AC-II and SMRE1 (Additional file 6: Table S5). Therefore, this study has further revealed that the OsMYB103L could not only regulate cellulose biosynthesis, but also consequently mediate cellulose microfibril assembly, interpreting why the down-regulation of OsMYB103L could lead to a broken and disordered cellulose microfibrers assembly in the myb103 and RNAi transgenic line as described above (Fig. 5B).

Cellulose is an important loading-bearing skeletal component to maintain plant mechanical strength, and it also provides the most abundant biomass resource convertible for biofuels and biomaterials. However, the natural lignocellulose recalcitrance basically determines a costly biomass enzymatic saccharification, which is unacceptable for biofuel production at large scale [1, 20]. To reduce recalcitrance, genetic modification of plant cell walls has been considered as a promising solution [39, 45]. Because cellulose features (CrI, DP) are the major factors of lignocellulosic recalcitrance [21–24], the myb103 and OsMYB103L-RNAi transgenic line that are of much reduced cellulose CrI and DP values in mature straws (Fig. 5), should enable to provide recalcitrance-improved lignocellulose substrates for significantly enhanced biomass enzymatic saccharification examined in this study (Fig. 6). In addition, due to the length-reduced cellulose nanofibers accumulation, the myb103 mutant and RNAi line should be applicable to generate high-value and low-cost nanomaterials and nanocarbons. Hence, genetic engineering of transcriptional factors should offer a potential approach for plant cell wall modification in bioenergy crop breeding.

Conclusions

Using a novel rice Osfc9/myb103 mutant and OsMYB103L-RNAi transgenic line, this study examined that down-regulations of OsMYB103L could significantly reduce cellulose crystallinity and polymerization without significant impacts in other major wall polymers (hemicellulose, lignin), which thereby resulted in distinct cellulose nanofiber assembly in plant cell walls. These consequently improved lignocellulose recalcitrance for much enhanced biomass enzymatic saccharification under green-like and cost-effective chemical pretreatments. By integrating RNA-seq analyses with DAP-seq assays, this work attempted to demonstrate that OsMYB103L may act as an active transcription factor to specifically mediate cellulose biosynthesis and deposition by regulating OsCesAs genes and other genes associated with microfibril orientation and assembly. Hence, this study has provided a powerful strategy for genetic modification of plant cell walls in bioenergy crops.

Methods

Selection of rice genetic mutant (Osfc9/myb103)

Rice (Oryza sativa) fragile culm 9 (Osfc9) mutant, termed as myb103, was selected from a T-DNA mutagenesis pool of the japonica variety Nipponbare in 2008. The homozygous plants of myb103 were grown in the experimental field of Huazhong Agricultural University from 2011 to 2019 (Wuhan, China). To identify the gene of Osfc9 mutant, a F2 mapping population was generated by crossing Osfc9 with MH63 (a wild-type polymorphic indica variety). Map-based cloning approach was used for gene identification of Osfc9, based on 1050 F2 mutant plants with public SSR (Simple Sequence Repeats) molecular markers. Using 145 polymorphic markers, the Osfc9 gene was preliminarily localized between markers RM22342 and RM22392 on chromosome 8 (Fig. 1). We further used markers (RM5647, RM22358, RM4955) and primers SSR1–SSR5 (Additional file 2: Table S1) to finally map the gene between SSR2 and RM4955 in a 46-kb genomic region, which contained four open reading frames. These four genes were cloned by amplifying the CDS sequences using KOD-Plus (TOYOBO, Japan), and only one gene, LOC_Os08g05520 named OsMYB103L, was found with 18-base deletion.
For genetic complementation of Osfc9 mutant, a 2851-bp full genomic sequence of LOC_Os08g05520 driven by its upstream 2 kb sequence as promoter was cloned into the binary vector pC1300T. This complementary vector was introduced into Agrobacterium tumefaciens strain EHA105 and transformed into the Osfc9 mutant. Positive T₀ transgenic lines were selected using hygromycin (Hyg) as plant selection marker.

**Generation of RNAi-silenced OsMYB103L transgenic lines**

According to the full-length CDS of OsMYB103L, the 395-bp 3’ fragment was cloned into pTCK303 vector using RNAi-F and RNAi-R primers (Additional file 2: Table S1). The RNA interference (RNAi) vector of OsMYB103L was introduced into Agrobacterium tumefaciens strain EHA105 and transformed into Nipponbare rice. Positive T₀ transgenic lines were selected using Hyg as plant selection marker, and segregating T₁ plants were screened by PCR and verified by quantitative real-time PCR (qRT-PCR).

**Total RNA isolation and qRT-PCR analysis**

Rice samples were collected from second internodes of stem tissues at heading stage. Total RNAs were extracted using Trizol reagent (Invitrogen) and reverse-transcribed into cDNA with the GoScript™ Reverse Transcription System (Promega). qRT-PCR was independently performed by using the SYBR Green PCR Master Mixture (ZF101, ZOMANBIO) with three biological replicates as described [46].

**Measurements of stem strength and agronomic traits**

Rice stem strength and lodging index were measured as described previously [25, 29]. The breaking resistance of rice third internode was detected using a prostrate tester (DIK7401, Japan) with the distance between fulcra of the tester at 5 cm. Fresh weight (W) of the upper portion of the rice plant was measured including panicle and the tester at 5 cm. 1000-grain weight was evaluated according to the growth on SD/-Trp, -His, -Ade (synthetic defined, tryptophan, histidine, adenine dropout).

**GUS staining analysis**

The promoter of OsMYB103L (about 2.2 kb upstream of ATG) was amplified from the rice genomic DNA (Nipponbare) using the primers GUS-F and GUS-R (Additional file 6: Table S1). The DNA fragment was then cloned into the pC1301gT vector resulting in fusion of the promoter and the β-glucuronidase reporter gene. The proOsMYB103L:GUS construct was transformed into wild type (WT) rice cultivar Nipponbare. Three independent transgenic lines were obtained after screening using Hyg marker. GUS staining of stem tissue was performed as previously described [47].

**Subcellular localization and transactivation assay**

For observation of OsMYB103L subcellular localization, the full-length OsMYB103L sequence was cloned into pC1300-EGFP to create 35S: OsMYB103L-EGFP vector using primers (EGFP-F and EGFP-R) listed in Additional file 2: Table S1. The construct was transformed into Agrobacterium strain GV3101 and infiltrated into tobacco leaves for confocal microscopy. The subcellular localization of fused enhanced green fluorescent protein (EGFP) was visualized using a confocal laser scanning microscope (Leica SP5 CLSM) as previously described [48].

A yeast strain (AH109) containing HIS3 and ADE2 reporter genes was used to analyze transactivation of OsMYB103L as previously described [17]. The full-length coding sequence of OsMYB103L was amplified using BD-F and BD-R primers (Additional file 6: Table S1), cloned into pGBK7T7 by fusion with the GAL4 DNA-binding domain, and then transformed into the AH109. The empty pGBK7T7 (BD) was used as negative control. The transactivation activity of these proteins was evaluated according to the growth on SD/-Trp, -His, -Ade (synthetic defined, tryptophan, histidine, adenine dropout).

**Observation of plant cell walls and cellulose microfibrils**

Cellulose staining by S4B was performed as previously described with little modifications [49]. The rice second internodes of stem (0.5 cm sections above the node) at the heading stage were sectioned with 100 μm thickness using a microtome (VT1000S, Leica). The sections were stained with 0.01% S4B in 50 mM NaCl for 15 min, and washed three times using phosphate buffered saline (PBS) buffer. The stained sections were observed using a Nikon Eclipse Ni-U microscope under 530–560 nm excitation and 620–660 nm emission and bright field to analyze cellulose distribution and cell morphology.

TEM was used to observe cell wall structures in the middle 0.5-cm sections from the third leaf veins of three-leaf-old rice plants as previously described [25, 29]. The samples were post-fixed in 2% (w/v) OsO₄ for 1 h after extensively washing in the PBS buffer and embedded with Super Kit (Sigma), cut with an Ultracut E ultramicrotome (Leica) and picked up on Formvar-coated copper grids. After post-staining with uranyl acetate and lead citrate, the specimens were viewed under a Hitachi
H7650 (Hitachi Ltd., Tokyo, Japan) transmission electron microscope.

AFM was applied to observe cellulose microfibrils as described previously [32]. The basal region of the second internodes of rice plants at heading stage were sectioned with 100 μm thickness, and then incubated with 8% acidic chlorite (1 mM HCl added to 1 g sodium chlorite) at 50 °C for 48 h. After washed with distilled water five times, the sections were used for AFM (CSPM5500) observation.

Detection of cellulose features (DP, CrI)
The DP and CrI of cellulose substrates were, respectively, detected as previously described [24, 50, 51]. For cellulose DP assay, the crude cellulose samples were prepared by extracting raw biomass materials with 4 M KOH (containing sodium borohydride at 1.0 mg mL⁻¹) and 8% (w/v) NaClO₂, and all experiments were conducted in independent triplicate. For cellulose CrI assay, the X-ray diffraction method was performed using a Rigaku D/MAX instrument (Ultima III, Japan). Technical standard errors of the CrI method were measured as ±0.05–0.15 using five representative samples in independent triplicate.

Fourier transform infrared spectroscopy scanning
A PerkinElmer spectrophotometer (NEXUS 470, Thermo Fisher Scientific, Waltham, MA, USA) was applied to scan biomass samples and the he Fourier transform infrared (FTIR) spectra were recorded in absorption mode over 32 scans at a resolution of 4 cm⁻¹ in the range of 4000 to 400 cm⁻¹ as previously described [24, 50].

Plant cell wall fractionation and determination
Plant cell wall fractionation procedure was applied to extract wall polymers as previously described [24, 29]. The well-mixed biomass powders were ground with potassium phosphate buffer (pH 7.0), followed with chloroform–methanol (1:1, v/v), DMSO–water (9:1, v/v) and 0.5% (w/v) ammonium oxalate to consequently remove soluble sugars and protein, lipid, starch and pectin. The remaining residue was extracted with 4 M KOH containing 1.0 mg mL⁻¹ sodium borohydride, yielding KOH-extractable hemicelluloses. The residue was finally added with H₂SO₄ (67%, v/v) to completely dissolve cellulose and non-KOH-extractable hemicelluloses. Cellulose content was calculated by determining the hexoses using the anthrone/H₂SO₄ method. Total hemicelluloses were calculated by determining the hexoses and pentoses using the orcinol/HCl method. Lignin content was measured by the two-step acid hydrolysis method according to the NREL’s laboratory analytical protocol (LAP). All experimental analyses were completed in independent triplicate.

Biomass pretreatment and enzymatic hydrolysis
Three biomass pretreatments and sequential enzymatic hydrolysis were performed as described previously [24, 26, 33] with minor modifications. For acid pretreatment, the well-mixed biomass powder (0.300 g) was added to 6 mL 1% H₂SO₄ (v/v), heated at 121 °C for 20 min in an autoclave (0.15 MPa), and then shaken under 150 rpm at 50 °C for 2 h. For alkali pretreatment, the biomass power was added into 6 mL 1% NaOH (w/v), and incubated at 50 °C under 150 rpm shaking for 2 h. For liquid hot water pretreatment, the well-mixed biomass powder was added into well-sealed stainless steel capsules at 12.5% solid loading, and heated at 200 °C under 15 rpm shaking for 30 min.

The pretreated biomass residues were washed with distilled water five times, and then washed once with 10 mL of mixed-cellulase reaction buffer. The washed residues were incubated with 6 mL (2 g L⁻¹) of mixed cellulases (containing cellulases at 10.60 FPU g⁻¹ biomass and xylanase at 6.72 U g⁻¹ biomass from Imperial Jade Bio-technology Co., Ltd) at 5% solid loading, and shaken under 150 rpm at 50 °C for 48 h, while 1% Tween-80 was co-supplied. After the samples were centrifuged at 3000 g for 5 min, the supernatants were collected for hexoses and pentose assay. All experiments were carried out in independent triplicate.

DAP-seq assay
The DNA affinity purification sequencing (DAP-seq) experiments and data analysis were conducted as previously described with minor modification [35]. For experiments, DNA was first extracted from young leaf tissue of rice cultivar *Nipponbare*, and fragmented by Bioruptor Plus to 250 bp. After selecting targeted fragments by magnetic beads (KAPA Pure Beads, KK8002), the fragmented DNA was successively performed with end repair, A-tailing and adapter ligation steps. The ampDAP-Seq DNA library was prepared by PCR amplification using KAPA HiFi HotStart ReadyMix with unique index primers, and selectively recovered as different DNA libraries. The full-lengths of *OsMYB103L* and *Osfc9* were, respectively, cloned to the HALO-tagged vector (pFN19K HaloTag® T7 SP6 Flexi® Vector) using DAP-F and DAP-R primers (Additional file 2: Table S1). HALO-tagged *OsMYB103L* and *OsFC9* were expressed in vitro in a wheat germ system, and HALO-tagged GFP was expressed as control. The protein expression was detected by Western blot using HALO-Tag antibody. HALO-OsMYB103L and OsFC9 were immobilized on Magne HALO-Tag beads, and incubated with the DNA
library. After bead washing, DNA was eluted and amplified with corresponding indexed primers. The final products were selected from 250 to 500 bp, and sent to ANOROAD company for ChIP-seq sequencing.

For DAP-seq analysis, sequencing fastq files were trimmed using trimmomatic (v0.32) [52]. Trimmed reads were mapped to the Oriza sativa IRGSP reference genome using bowtie2 (v2.2.2) [53]. The mapped reads were filtered for unique mapping reads and reads containing MAPQ30 using samtools (v0.1.13) [54] to restrict reads aligned to multiple positions in the genome. MAPQ-filtered reads were used for peak calling using macs2 (v2.1.1) [55]. The predicted motif fasta sequence files were generated by extracting 50 bp upstream and downstream of the peak summit. All motifs were determined using meme-chip (v5.0.5) [56]. The target gene identification was defined as the closest gene containing a peak within 1 kb upstream and downstream and got by getfasta utilities of bedtools (v2.25.0) [57].

**RNA-seq assay**

Total RNA was isolated using the Trizol Reagent (Invitrogen Life Technologies). Sequencing libraries were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in an Illumina proprietary fragmentation buffer. First strand cDNA was synthesized using random oligonucleotides and SuperScript II, and second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and the enzymes were removed. After adenylation of the 3’ ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. DNA fragments with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 15 cycle PCR reaction. Products were purified (AMPure XP system) and quantified using the Agilent high sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). The sequencing library was then sequenced on a Hiseq platform (Illumina) by Shanghai Personal Biotechnology Co. Ltd. For data analysis, genes with fold change ≥ 2.0 and significance level p ≤ 0.05 were differentially expressed. DEGs were annotated against the GO (Gene Ontology). Each sample (mutant, wild type) was analyzed in biological triplicate.

**Gene co-expression analysis and other bioinformatics**

Gene co-expression analysis has been conducted as previously described with modifications [58]. The Affymetrix Rice GeneChip Genome Array microarray datasets of candidate genes were obtained from CREP (Collections of Rice Expression Profiling, http://crep.ncpgr.cn) from an indica variety (Zhenshan 97).

The predictions of the three-dimensional structure of OsMYB103L and mutational protein OsFC9 were conducted using the SWISS-MODEL (https://swissmodel.expasy.org/). The volcano plot was drawn by GraphPad Prism 9.0.0. The vein plot was online completed by DeepVein (http://www.deepvein.com/). GO analysis was performed using online agriGO database v2.0 [38]. The gene co-expression heatmap was constructed by using heatmap of R documentation.

**Data statistical analysis**

The SPSS statistical software was used for data analysis. Student’s t test was used for comparison analysis. Significance was measured at the levels of p < 0.05 and p < 0.01. All experiments were conducted with at least three independent replicates.

**Abbreviations**

AFM: Atomic force microscopy; Crl: Crystalline index; DAP-seq: DNA affinity purification sequencing; DP: Degree of polymerization; FTIR: Fourier transform infrared; GO: Gene Ontology; GUS: β-Glucuronidase; RNAi: RNA interference; S4B: Pontamine fast scarlet 4B; TEM: Transmission electron microscopy.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13068-021-02093-8.
Additional file 6: Table S5. Analysis of AC elements and SMREs in the promoters of candidate genes regulated by OsMYB103L.

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Authors’ contributions
LW completed most of the experiments and drafted the manuscript. MZ identified mutant and completed major genetics experiments. RZ observed mutant and completed major genetics experiments. HY, JL and YMW collected samples and participated partial chemical analyses. ZH, YTW and LW discussed the experiments and analyzed the data. LP supervised the experiments, analyzed the data and revised the manuscript. TX co-supervised experiments, analyzed the data and finalized the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets (including the RNA-seq of Osfic9 mutant and WT, the DAP-seq of OsMYB103L and Osfic9) were submitted to the SRA (Sequence Read Archive) repository (PRJNA743986). All data generated or analyzed during this study are included in this published article and its additional files.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors have no conflict of interest to declare.

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