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CEF1/OsMYB103L is involved in GA-mediated regulation of secondary wall biosynthesis in rice

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Abstract Although the main genes in rice involved in the biosynthesis of secondary wall components have been characterized, the molecular mechanism underlying coordinated regulation of genes expression is not clear. In this study, we reported a new rice variety, *cef1*, showed the culm easily fragile (CEF) without other concomitant phenotypes. The *CEF1* gene encodes a MYB family transcription factor OsMYB103L, was cloned based on map-based approach. Bioinformatics analyses indicated that CEF1 belongs to the R2R3-MYB subfamily and highly similar to *Arabidopsis* AtMYB103. Expression pattern analysis indicated that *CEF1* is mainly expressed in internodes and panicles. Biochemical assays demonstrated that OsMYB103L is a nuclear protein and shows high transcriptional activation activity at C-terminus. OsMYB103L mediates cellulose

biosynthesis and secondary walls formation mainly through directly binding the *CESA4, CESA7, CESA9* and *BC1* promoters and regulating their expression. OsMYB103L may also function as a master switch to regulate the expression of several downstream TFs, which involved in secondary cell wall biosynthesis. Furthermore, OsMYB103L physically interacts with SLENDER RICE1 (SLR1), a DELLA repressor of GA signaling, and involved in GA-mediated regulation of cellulose synthesis pathway. Our findings revealed that OsMYB103L plays an important role in GA-regulating secondary cell wall synthesis, and the manipulation of this gene provide a new strategy to help the straw decay in soil.

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Introduction

The cell wall in plant is unique. It plays critical role in plant growth and development, resistance to pathogen invasion, quality of plant-based foods and the properties of plant fibers and fuels (Keegstra 2010). However, it also brings many challenges for the plant breeders, for instance, the straw decomposition is very slow when directly decay in the fields. This is mainly due to the high cellulose content in the cell wall, which was negatively correlated with the decomposition rate (Gunnarsson and Marstorp 2002; Rahn et al. 2003). In Asia, a large number of rice straws were processed by stubble burning, which caused severe environment pollution. Therefore, it is urgent to understand the cell wall biosynthesis. The plant cell walls are usually divided into two categories: primary cell walls that surround growing cells or cells retain growing capability; and

secondary cell walls that are thickened structures containing lignin and surrounding specific cells such as vessel elements or fiber cells (Keegstra 2010). The secondary walls are typically composed of cellulose, xylan and lignin.

In the last decade, many studies have been done for secondary walls biosynthesis mainly focused on identification and functional characterization of genes involved in the biosynthesis of the secondary cell wall components. In rice, many studies have been performed to characterize the genes controlling culm mechanical strength by analysis the brittle culm mutants. The *BC7/BC11*, *BC6/BC13* and *OsCESA7* are the cellulose synthase catalytic subunit genes, involves in cellulose synthesis (Kotake et al. 2011; Song et al. 2013; Tanaka 2003; Yan et al. 2007; Zhang et al. 2009). The *BC1* gene encodes a COBRA-like protein which controls cellulose deposition of the secondary cell wall (Li 2003). The *BC3*, encoding a classical dynamic OsDRP2B essential which might function in vesicle trafficking of CESA (Hirano et al. 2010; Xiong et al. 2010). The rice *BC10* gene encodes a Golgi-located type II membrane protein that functions as a glycosyltransferase and mainly expressed in the developing sclerenchyma and vascular bundle cells (Zhou et al. 2009). The rice gene *BC15* encodes a membrane-associated chitinase-like protein that is a Golgi-localized type II membrane protein and lacks classical chitinase activity. It mediates cellulose biosynthesis and cell wall remodeling (Wu et al. 2012). Another rice gene *BC14*, which encodes a Golgi nucleotide sugar transporter that provides the glucosyl substrate for the formation of polysaccharide and thereby regulates the synthesis of cellulose (Zhang et al. 2011). The rice *BC12* gene encodes a dual-targeting kinesin-4 protein, controls cell-cycle progression and cellulose microfibril deposition (Li et al. 2011; Zhang et al. 2010b). Other genes involved in xylan and lignin biosynthesis have also been identified, the *CslF6* (Vega-Sanchez et al. 2012) and *CslD4* (Li et al. 2009a) are encode cellulose synthase-like protein, which is a glycosyltransferase family believed to be involved in the biosynthesis of cell-wall polymers. The *FC1* (Li et al. 2009b) and *OsCAD* (Hirano et al. 2012) are involved in lignin biosynthesis, loss-of-function of these genes can reduce the lignin content. Although many genes involved in secondary cell wall biosynthesis have been reported, how these genes coordinately expressed and what controls the developmental program of secondary wall biosynthesis are less well-understood.

Recently the transcriptional switch mechanism for secondary cell wall biosynthesis have been discovered in dicot species, including *Arabidopsis* (Ko et al. 2014; Kubo et al. 2005; Ohman et al. 2013; Zhang et al. 2007; Zhong et al. 2006, 2007b; Zhong and Ye 2012), and *Poplar* (McCarthy et al. 2010). Most of the transcription factors (TFs) regulating the secondary walls biosynthesis belong to two large

families: NAC and MYB. A group of the NAC family TFs function as the top master regulators to mediate secondary walls formation in fibers and/or vessels (Zhong et al. 2008). Some members of the MYB TFs family act as the second and/or the third level master regulators to control the secondary walls relevant genes expression (Zhao and Dixon 2011). Many of the MYB TFs involved in secondary walls formation can be up-regulated by *SND1* (Zhong et al. 2008). The *MYB46* and *MYB83*, expressed in both fibers and vessels, are direct targets of *SND1* and its close homologs *VND6*, *VND7*, *NST1* and *NST2* (Zhong et al. 2008). Some members of MYB TFs including *MYB20*, *MYB42*, *MYB52*, *MYB54*, *MYB103*, and *MYB63*, are also up-regulated by *MYB46* and *MYB83* (McCarthy et al. 2009; Zhong et al. 2007a). All of these NACs and MYBs collectively regulate the downstream genes expression. A few studies have been investigating the transcription switches to secondary walls formation in monocot species. To date, few TFs have been reported to be associated with secondary cell wall formation in rice (Hirano et al. 2013; Yang et al. 2014; Zhang et al. 2010c; Zhong et al. 2011). The OsMYB103L is an important TF regulates cellulose biosynthesis. Overexpression of OsMYB103L results in leaf rolling, increased cellulose content, and upregulated the expression of genes encoding cellulose synthase (CESA). Moreover, the RNA interference (RNAi)-knockdown of OsMYB103L leads to a weakened mechanical strength and decreased cellulose content in leaves (Yang et al. 2014). The OsMYB61 can directly binding the *CESAs* promoter and regulate these genes expression. Overexpression of OsMYB61 also leads leaf rolling and increased cellulose content (Huang et al. 2015). Hence finding more transcriptional switches in rice may provide valuable approach for genetically modifying grass crops for biofuel production.

In this report, we isolated a culm easily fragile (*cef1*) rice plant from Wandao60 (M1148), and developed a pair of near isogenic lines (NIL), NIL-*CEF1* and NIL-*cef1*. The NIL-*cef1* showed normal phenotype except the culm breaks easily compare to NIL-*CEF1*. We further cloned the gene responsible for the corresponding phenotype, *CEF1*. Molecular characterization of *CEF1* suggested it encodes an OsMYB103L transcription factor that regulates secondary cell wall components biosynthesis.

Materials and methods

Plant materials and growth conditions

The culm easily fragile (*cef1*) rice (*Oryza sativa* L.) was isolated from a Wandao60 (M1148), an Anhui late japonica rice variety. An F$_2$ mapping population was generated from the cross between *cef1* and a polymorphic *indica* cultivar,

NanJing11. We also developed a pair of near isogenic lines (NIL), NIL-*cef1* and NIL-*CEF1*, from the progenies of the cross between Wandao60[*cef1*] and Xiushui110. All of rice plants used in this study were grown in the experimental fields at the Institute of Technical Biology and Agriculture Engineering, Hefei Institute of Physical Science, Chinese Academy of Sciences (Hefei, China) and Sanya (Hainan province, China) in the natural growing season.

Measurement of breaking force and microscopy

Breaking force of the second internodes and flag leaves of NIL-*CEF1* and NIL-*cef1* were determined with a digital force/length tester (5848 microtester, Instron; http://www.instron.com) by cutting the tested samples into segments with equal length and width. We used the Newton (N) as the unit of mechanical force. For transmission electron microscopy, the second internodes of NIL-*cef1* and NIL-*CEF1* plants were cut with a razor and immediately placed in 70 % ethanol (V/V), 5 % acetic acid (V/V), and 3.7 % formaldehyde (V/V) for at least 2 h. Samples were dried to the critical point, sputter-coated with gold, and observed with a scanning electron microscope (S570; Hitachi, Tokyo, Japan).

Phenotypic evaluation

Several agronomic traits, including plant height, number of tillers per plant, 1000-grain weight, panicle length, setting rate and number of grains per panicle were chosen for production evaluation. The plant height was measured from the ground surface to the tallest panicle. The number of tillers per plant was the number of effective tillers with corresponding panicle has 10 or more grains. The 1000-grain weight was calculated based on 100 grains and the converted to 1000 grain weight. The panicle length was the distance from length top to the first internode top. The number of grains per panicle was the total number of grains per panicle. The setting rate was scored as the number of full grains per panicle divided by the number of grains per panicle. All the traits were analyzed at mature stage. The data from fifty plants in each NIL in 2013 and 2014 was used for analysis. The independent sample *t* test program SPSS 10.0 for Windows was used to compare the mean values.

Cell wall composition analysis

The second internodes of NIL-*CEF1*, NIL-*cef1* and complementary transgenic plants (NIL-*cef1*[*pCEF1::CEF1*]) at mature stage were used to prepare alcohol-insoluble residues (AIRs) of the cell walls. De-starched AIRs were produced as previously described (Xiong et al. 2010). The AIRs were hydrolyzed in 67 % v/v H_2SO_4 for 1 h at room temperature, and then in 2M H_2SO_4 at 121 °C for 1 h. The alditol acetate derivatives were prepared from AIRs and then determined by GC–MS. The crystalline cellulose was measured with a modified Updegraff method, as previously described (Updegraff 1969).

Map-based cloning

The *cef1* locus was mapped and cloned using 2307 mutant F_2 plants, which were selected from the population of Wandao60[*cef1*] and Nanjing11. Molecular markers distributed throughout the whole rice genome were used for *cef1* locus rough mapping. Molecular markers for fine mapping were developed to narrow down the mutated locus to a 102-kb region on chromosome 8. The corresponding DNA fragments in this mapping region were amplified from mutants and wild type plants using LA-Taq (TaKaRa, http://www.takara-bio.com) and sequenced using an Applied Biosystems 3730 sequencer (ABI, http://www.appliedbiosystems.com). For complementation of *cef1* mutant, the full-length *CEF1* coding sequence, a 2.5-kb fragment of upstream sequence from ATG and 0.2-kb fragment of downstream sequence from TAA were inserted into binary vector pCAMBIA2300 between the EcoRI and BamHI sites to generate the construct *pCEF1::CEF1*, which was introduced into the NIL-*cef1* by the *Agrobacterium*-mediated transformation procedure as described previously (Raineri et al. 1990).

Bioinformatics analysis of *OsCEF1*

Domain prediction for OsCEF1 was performed using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1). A search for R2R3-MYB-related family members in rice and Arabidopsis was performed using the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree of R2R3-MYB-related proteins in Arabidopsis and rice was generated using MAGE4 (Tamura et al. 2007). The number of bootstrap replicates was 1000.

Transactivation analysis in yeast cells and rice protoplasts

A yeast strain (AH109) containing the HIS3 and ADE2 reporter genes was used to analyst transactivation of OsMYB103L and truncation forms as described previously (Wang et al. 2012). The full length coding sequence of *OsMYB103L* and sequences encoding N-terminal and C-terminal truncation products were amplified and cloned into *pGBKT7* and fused with the GAL4 DNA-binding domain to construct the necessary serial vectors, which

were then transformed into the AH109 yeast strain. The empty *pGBKT7* (BD) and fusing the GAL4 vectors were used as negative and positive controls, respectively. The transactivation activity of these proteins were evaluated according to the growth on SD/-Trp (synthetic defined, Tryptophan dropout) and SD/-Trp, His, Ade (synthetic defined, Tryptophan, Histidine, Adenine dropout).

The transactivation activity was also examined in the rice protoplast system. To construct the effecter vectors, the full length coding sequence of OsMYB103L and sequences encoding N-terminal and C-terminal truncation products were amplified and fused with GAL4 binding domain (GAL4BD). The empty GAL4BD and fused with VP16 were used as negative and positive controls, respectively. For the reporter construct, the *pUC19* containing the firefly luciferase (LUC) reporter gene driven by the minimal TATA box of the 35S promoter plus five GAL4 binding elements was used. A *pTRL* plasmid that contained a CaMV (Cauliflower mosaic virus) 35S promoter and *Renilla* LUC, was used as an internal control. The *pTRL*, effector and reporter were simultaneously transformed into the rice protoplast system, then kept in dark for 16 h. The LUC activity was measured as described previously (Ohta et al. 2000).

Subcellular localization of OsMYB103L

To observe the OsMYB103L subcellular localization, GFP fused to the C-terminus of OsMYB103L and inserted into the pCAMBIA2300 between the EcoRI and BamHI sites. Rice protoplasts expressing GFP positive OsMYB103L were observed using confocal laser scanning microscope (Leica TCS SP5). The cell nucleus was stained by DAPI dye.

Expression analysis

Total RNA was extracted from various rice tissues using TRIzol reagent (Invitrogen), as described previously (Wadsworth et al. 1988). The complementary DNA (cDNA) was synthesized from total RNA using a reverse transcriptional kit (TransGen, http://www.transgen.com. cn/). Quantitative RT-PCR was performed using relevant primers and qRT-PCR kit (TransGen, http://www.transgen. com.cn/) on a quantitative 7500 PCR system (ABI). All assays were repeated at least three times, the *Actin* gene was used as an internal control.

Yeast one-hybrid assay

The *MYB103L* encoding sequence fused with activation domain (B42AD) to construct effector (*pB42AD-MYB103L*) (Fig. 7a), The 2 kb fragment of upstream sequence from start codon of *BC1*, *CESA4*, *CESA7*, *CESA9*

were amplified and inserted into *pLacZi* to construct the reporters (*pLacZi-BC1*, *pLacZi-CESA4*, *pLacZi-CESA7*, *pLacZi-CESA9*) (Fig. 7a). The effectors and reporters were cotransformed into the yeast strain EGY48. The transformants were grown on synthetic dropout plates without tryptophan or uracil containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for color development. The empty *pB42AD* and *pLacZi* were used as negative control.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed as described previously (Liu et al. 2013b). The full length cDNA for *SLR1* was amplified and cloned into *pGBKT7* vector (Clontech), and the full length *OsMYB103L* cDNA sequence was inserted into the *pGADT7* vector (Clontech). The vectors were transformed into yeast strain AH109. The resulting transformants were grown on a synthetic dropout (SD) medium (Difco Yeast Nitrogen Base without amino acid; BD) lacking tryptophan and leucine and also spotted on an SD medium lacking tryptophan, leucine, histidine and adenine.

BiFC assay

The BiFC assay was performed as described previously (Bracha-Drori et al. 2004). The full length CDS sequence of OsMYB103L was amplified and inserted into the pSY735 vector, the full length of SLR1 CDS sequence was amplified and cloned into the pSY736 vector. Arabidopsis protoplasts were prepared, transfected and visualized as described elsewhere (Sun et al. 2014).

Coimmunoprecipitation assay

Coimmunoprecipitation assay was performed as described elsewhere (Liu et al. 2013b). The SLR1-Flag and MYB103L-HA or BC1-HA simultaneously expressing in WT Arabidopsis protoplasts. Anti-Flag (Tiangen) immunoprecipitation was carried out as described previously (Zhang et al. 2010a), and the amount of MYB103L-HA or BC-HA in the immune precipitates was analyzed by anti-HA immunoblot.

Results

Identification and map-based cloning of *CEF1*

Wandao60^{cef1} showed the culm easily fragile, we found the Wandao60^{cef1} internodes were smashed by rice combine harvester and evenly distribute into the field (Fig. 1b, d, e), compare with other varieties (Fig. 1a, c, e). To find
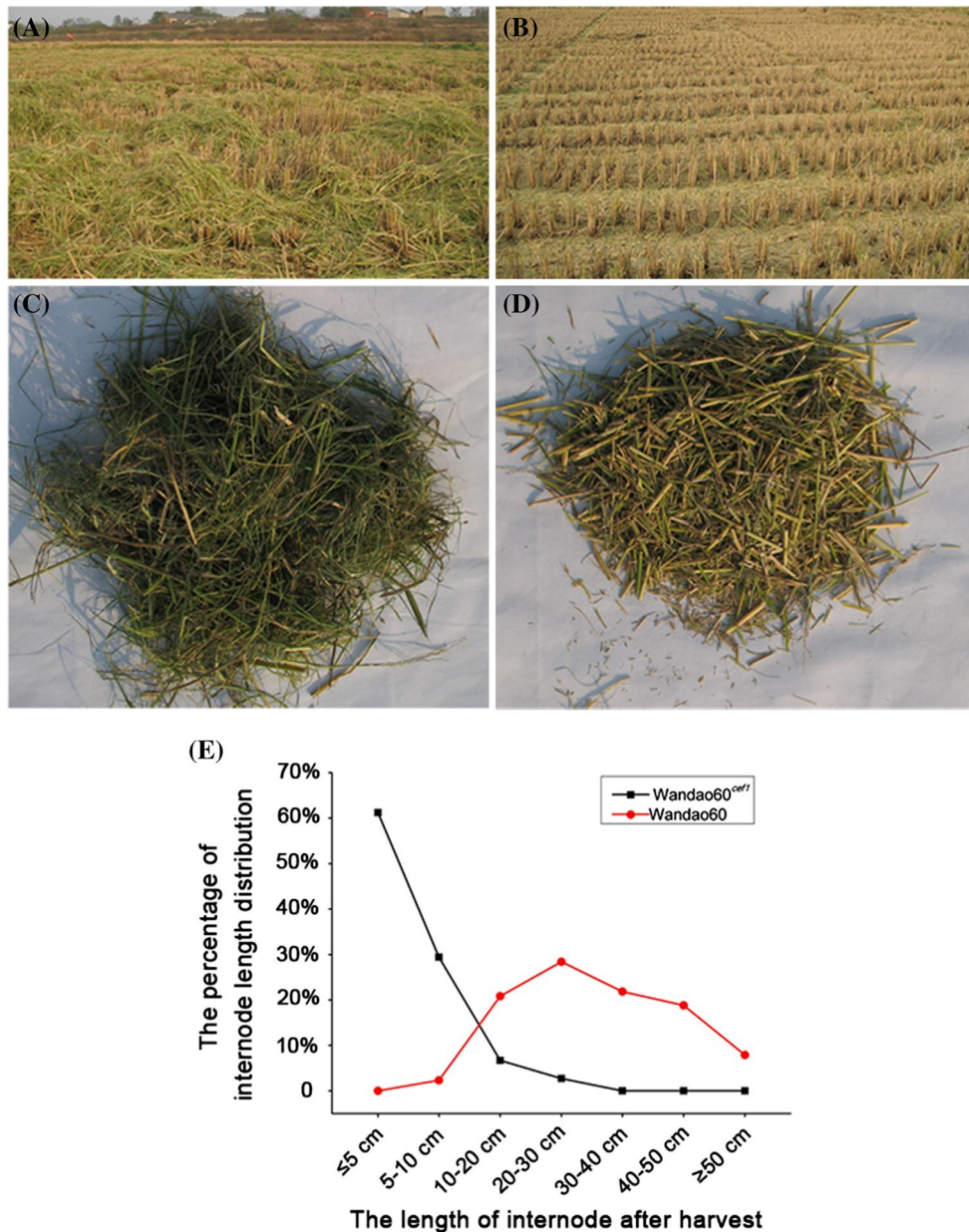
Fig. 1 *cef1* plants easily smash by rice combine. **a** The normal plants plot after harvest. **b** The Wandao60*cef1* plants plot after harvest. **c** The normal plants culms after harvest. **d** The Wandao60*cef1* culms after harvest. **e** The length of plants culms distribution after harvest

out the genetic basis of the *cef1* phenotype and isolate the causative gene, we crossed the Wandao60*cef1* with an *indica* cultivar, Nanjing11 to generate genetic analysis and mapping population. All of the F$_1$ plants showed the normal phenotype. Within the F$_2$ population, 256 and 84 individual plants showed normal and brittle culm phenotype, respectively. This results was consistent with the Mendelian segregation ratio of 3:1 ($\chi^2 = 0.004 < \chi^2_{0.05} = 3.84$) (Table S1). The result suggested that the culm easily fragile phenotype is controlled by a single recessive gene. We try to isolate the *CEF1* by map-based cloning method. After

screening 108 pleomorphic simple sequence repeat (SSR) markers that distribute evenly among the 12 rice chromosomes, the *cef1* locus was mapped between markers N1-1 and HSR4 on chromosome 8 (Fig. 2a), and further narrowed to an approximate 102-kb region between markers N1-4 and N3-9 (Fig. 2b), which contains 15 putative open-reading frames (ORFs) (Fig. 2c). Then we sequenced all of the candidate genes and a 13-bp deletion was found in LOC_Os08g05520 (Fig. 2d). The mutated site was confirmed by amplifying the DNA fragment covering the deletion site (Fig. 2e). This deletion occurred in the first exon
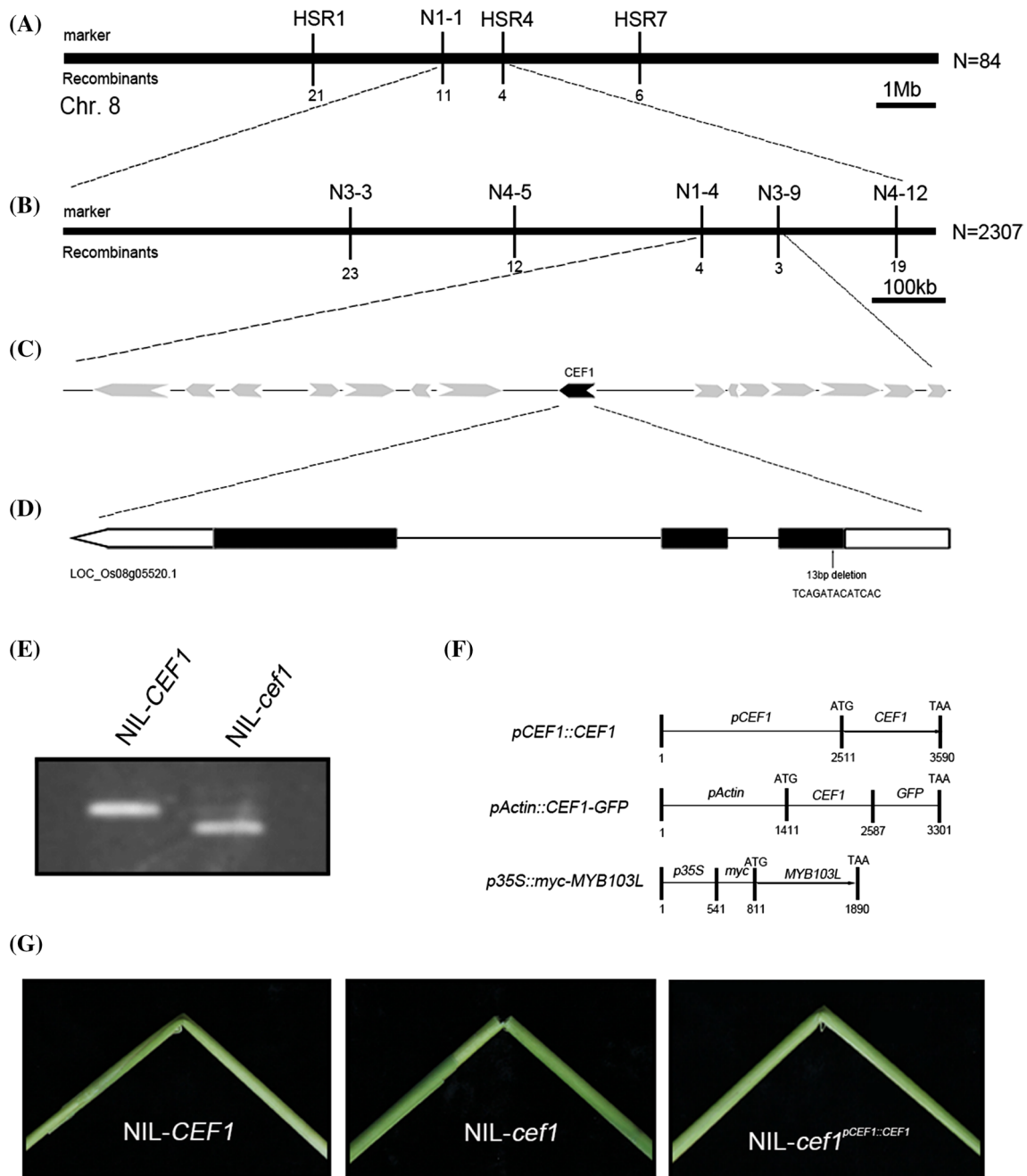
Fig. 2 Map-based cloning of the *CEF1* gene. **a** Rough mapping of *CEF1* gene. The gene was mapped to the region between markers N1-1 and HSR4 on chromosome 8. **b** Fine mapping of *CEF1* gene. The *CEF1* locus was narrowed to an approximately 102 kb region between markers N1-4 and N3-9. *Vertical lines* represent the positions of molecular markers and the number of recombinants. **c** 15 predicted ORFs within the fine mapping region. **d** Genomic structure of the ORF, resulting a frame-shift and premature translational product of 56 amino acids (Fig. 2d). Therefore, *cef1* is very likely to be a loss-of-function mutation. To further confirm that LOC_Os08g05520 corresponds to the *cef1* locus, a 3.6-kb DNA fragment containing the 2.5-kb

of *CEF1*, *boxes* indicate exons and the mutation site is in the first exon (13-bp deletion). **e** The amplified DNA fragments that cover the mutation site to confirm the genotypes of NIL-*CEF1* and NIL-*cef1*. **f** Constructs for complementation and biochemical assays. **g** Folding the internodes of rice plants to show the rescued mechanical property in the complemented plants

putative promoter and the coding region was cloned vector *pCAMBIA2300* to generate the plasmid *pCEF1::CEF1* (Fig. 2f), which was introduced into the NIL-*cef1* plants by Agrobacterium tumefaciens-mediated transformation. The transgenic NIL-*cef1^{pCEF1::CEF1}* plants complete rescued the

Table 1 Cell wall composition analysis of internodes of NIL-*CEF1* and NIL-*cef1* plants

Sample	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Cellulose
NIL-*CEF1*	1.93 ± 0.02	0.86 ± 0.01	22.39 ± 0.38	161.84 ± 4.42	1.40 ± 0.01	11.47 ± 0.13	70.16 ± 0.58	536.17 ± 4.44
NIL-*cef1*	2.30 ± 0.20	0.85 ± 0.03	35.32 ± 0.99	275.42 ± 5.67	1.39 ± 0.02	14.00 ± 0.80	73.39 ± 2.93	384.59 ± 6.23
NIL-*cef1*$^{pCEF1::CEF1}$	1.96 ± 0.01	0.86 ± 0.01	21.89 ± 0.24	171.45 ± 1.49	1.40 ± 0.01	11.35 ± 0.26	70.89 ± 1.18	522.73 ± 3.31

The results are mean ± SE of five independent assays. Each wall component was calculated as mg g^{-1} of alcohol-insoluble cell-wall residue

culm easily fragile and the cell wall composition phenotypes (Fig. 2g; Table 1). Therefore the LOC_Os08g05520 was the *CEF1* gene responsible for the mutant phenotypes described above.

Phenotypic characterization of NIL-*CEF1* and NIL-*cef1*

We also developed a pair of near isogenic lines, NIL-*cef1*, which carries an approximately 1.2-Mb segment including the Wandao60 *cef1* allele in the Xiushui110 background, and its matching line, NIL-*CEF1*, carries the homologous segment from Xiushui110. Compare with the NIL-*CEF1*, the plant height of *NIL-cef1* slightly decreased (Fig. 3a, h). The internodes of the NIL-*cef1* plants were easily broken and the forces applied to break NIL-*cef1* internodes were reduced to approximately 35 % compared with those of the NIL-*CEF1* (Fig. 3b, c). Apart from the plant height and the break force, other traits, viz. the number of tillers per plant (Fig. 3i), 1000-grain weight (Fig. 3j), panicle length (Fig. 3k), seed setting rate (Fig. 3m) and number of grains per panicle (Fig. 3l) showed no significant difference in the two NILs.

To understand the underlying cause of the observed brittleness, we used scanning electron microscope (SEM) to observe the second internodes cross-sections of NIL-*CEF1* and NIL-*cef1* plants. The observations revealed that the sclerenchyma cell wall of NIL-*cef1* was extremely thinned (Fig. 3e, g), compare with the NIL-*CEF1* (Fig. 3d, f). On the other hand, no significant difference in parenchyma cell wall was observed (Fig. 3d, e) between the pair of NIL. These results demonstrated the culm easily fragile phenotype in NIL-*cef1* was due to the defect in thickening in sclerenchyma cell wall.

To figure out whether the thinner parenchyma cell walls and the reduced mechanical strength in *cef1* plants was combined with altering cell wall composition, we next examined the cell wall composition in the second internodes of NIL-*CEF1*, NIL-*cef1* and NIL-*cef1*$^{pCEF1::CEF1}$ plants at mature stage. As shown in Table 1, the cellulose content was dramatically decreased by approximately 28 % in NIL-*cef1* relative to NIL-*CEF1* plants, whereas the contents of Ara and Xyl, the two major sugars of arabinoxylan, were substantially increased by approximately 37 and

41 %, respectively (Table 1). The level of glucose was increased by approximately 18 % (Table 1). The contents of other neutral sugars altered only slightly between NIL-*cef1* and NIL-*CEF1* samples.

Taken together, the *cef1* defects in mechanical strength was correlated with the thinned parenchyma cell walls and lower cellulose content in the mutant.

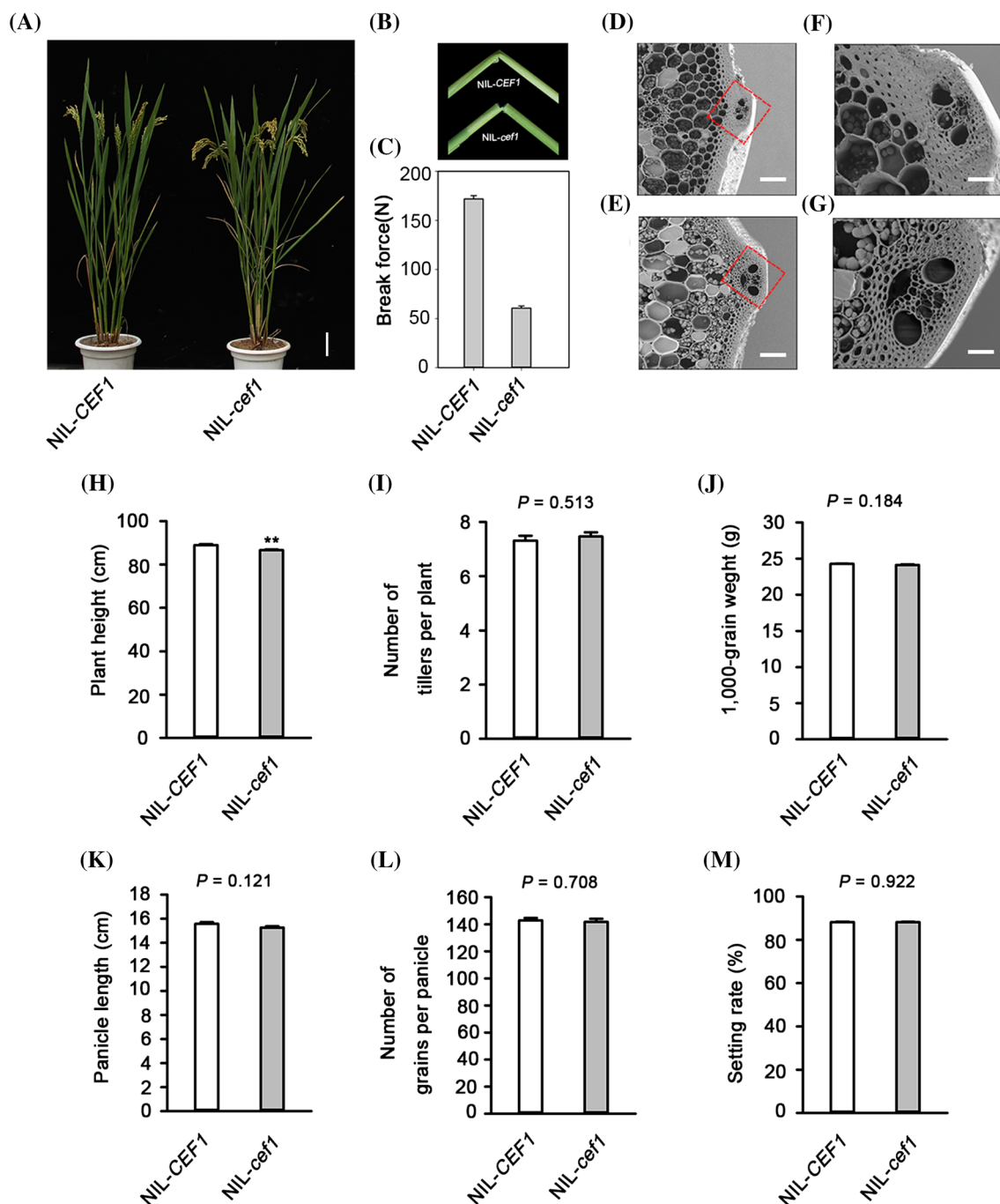## *CEF1* is mainly expressed in internodes and panicles

To examine expression pattern of *CEF1*, we used quantitative real-time (qRT)-PCR to analyze the expression level of *CEF1* in various organs from NIL-*CEF1* plants. The results suggested that *CEF1* is ubiquitously expressed in all organs while it was predominantly expressed in internodes and panicles. However, the expression level of *CEF1* is relatively low in leaves, sheaths and roots, during heading stage and seedling stage (Fig. 4). The expression pattern was consistent with the culm easily fragile phenotype of the Wandao60^{cef1}.

CEF1 encodes a R2R3-MYB family transcription factor

The coding sequence of the *CEF1* is 1080 bp in length and encodes a protein of 359 amino acid residues with a predicted molecular mass of approximately 40 kDa. A search in the National Center for Biotechnology Information (NCBI) Conserved Domains database and the Simple Modular Architecture Research Tool (SMART) to analysis CEF1 showed that it belongs to R2R3-MYB family member and has two MYB DNA binding domains at N-terminus, and four low complexity domains at C-terminus (Fig. S1A).

A BLASTP search for CEF1 homologs in *Oryza sativa* (*japonica*) and *Arabidopsis thaliana* genomes identified 17 and 25 closer homologs, respectively. Phylogenetic analysis revealed that the closest homolog of CEF1 is an Arabidopsis protein, AtMYB103 (At1g63910) (Fig. S1B). They are highly conserved in the R2- and R3-MYB DNA-binding domains, shown by protein sequence alignment.

One of the significant features of MYB family members is nuclear localization. To determine the subcellular localization of CEF1/MYB103L, a green fluorescent protein (GFP) tagged CEF1/MYB103L protein was generated by

(A)

NIL-*CEF1*   NIL-*cef1*

(B)

(C)

(D) **(F)**

(E) **(G)**

(H)

(I) P = 0.513

(J) P = 0.184

(K) P = 0.121

**(L)** P = 0.708

(M) P = 0.922

Fig. 3 Comparison of the phenotypic characterization between the NIL-*CEF1* and NIL-*cef1*. **a** Three-month-old plant of NIL-*CEF1* and NIL-*cef1*. Bar = 8 cm. **b** the *cef1* culms are easily broken. **c** Triple measurements of the break force of culms. **d–g** Scanning electron micrographs of the sclerenchyma cell walls of the NIL-*CEF1* (**d**) and NIL-*cef1* (**e**). **f**, **g** are enlargements of the *red boxed areas* in **d** and **e**, respectively. Bars = 25 μm (**d**, **e**) and 5 μm in (**f**, **g**). H-M, Contrasting grain yield of NIL-*CEF1* and NIL-*cef1*. **h** Plant height. **i** Tiller number. **j** 1000-grain weight. **k** Panicle length. **l** Number of grains of panicle. **m** Seed setting rate. All phenotypic data measured from plants grown with 20 × 20 cm spacing in paddies under normal cultivation conditions. Error bars represent SE (n = 50)

fusing GFP to the C-terminus of CEF1/MYB103L in a construct *pActin::CEF1-GFP* which was driven by Rice Actin promoter (Fig. 2f). The localization of GFP positive CEF1/MYB103L was observed using confocal laser scanning microscope. Our results indicated that the MYB103L-GFP fusion protein was located predominantly in nucleus (Fig. S1C).
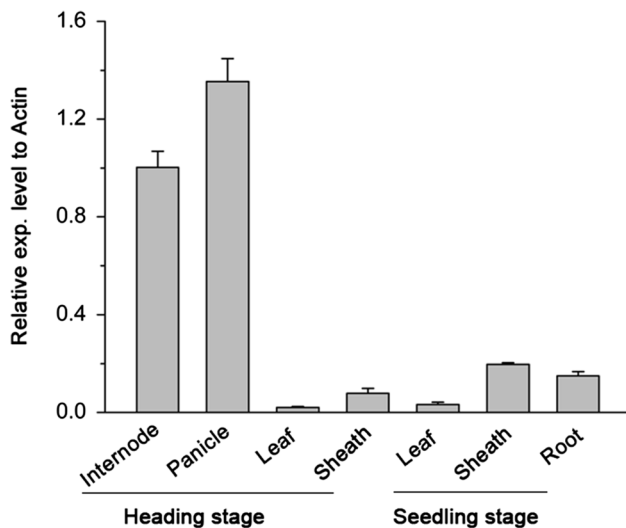
Fig. 4 Expression pattern of *CEF1/MYB103L*. qRT-PCR analysis of *CEF1* expression in various rice organs and different developmental stage, using the Actin1 gene as an internal control

C-terminal domains have strong transcriptional activation activity

To investigate the potential transcriptional activity of MYB103L, we used a yeast assay system to investigate full length MYB103L, N-terminal and C-terminal truncations. The growth of transformants containing *pBD-MYB103L* on selective medium (SD/-Trp) and (SD/-Trp, His, Ade) indicated that the MYB103L protein has transcriptional activity (Fig. S2A). To elucidate which domain of MYB103L has transcriptional activity, a series of N-terminal and C-terminal truncations (Fig. 5a, left panel) were made and cloned into the pGBKT7. The results indicated that C-terminal domains have the transcriptional activity (Fig. 5a, right panel).

We also used the dual-luciferase reporter (DLR) assay system in the rice protoplast to test the transcriptional activation of full length MYB103L and different truncations (Fig. 5a, left panel). The constructs were illustrated in Fig. S2B. Compared with the GAL4-BD negative control, the OsMYB103L-M4 (1-280Aa), OsMYB103L-M5 (281-359Aa), OsMYB103L-M6 (251-359Aa), OsMYB103L-M7 (201-359Aa), OsMYB103-M8L (119-359Aa), OsMYB103L-M9 (1-323Aa), OsMYB103L-M10 (deletion at 251-280Aa), OsMYB103L-M11 (deletion at 281-323Aa) strongly activated the reporter gene, while the OsMYB103L-M1 (1-118Aa), OsMYB103L-M2 (1-200Aa), OsMYB103L-M3 (1-250Aa), OsMYB103L-M12 (deletion at 251-323Aa) did not activate the *LUC* gene (Fig. 5b). These results suggested that the two C-terminal domains (251-280Aa and 281-323Aa) of OsMYB103L have strong activation ability. The proteins containing 281-323Aa (OsMYB103L-M5~M10) have higher activation ability than those only containing 251-280Aa (OsMYB103L-M4, M11).

Expression of *BC1*, *OsCESA4*, *OsCESA7* and *OsCESA9* are down-regulated in NIL-*cef1*

The lower cellulose content in NIL-*cef1* was the main cause of the culm easily fragile phenotype, therefore we measured cellulose synthesis relative genes expression level in NIL-*CEF1*, NIL-*cef1* and NIL-*cef1*[p35S::myc-MYB103L] transgenic plants. qRT-PCR analysis showed that the *BC1*, *OsCESA4, OsCESA7, OsCESA9* genes were down-regulated more than twofold in NIL-*cef1*, comparing to that of NIL-*CEF1* (Figs. 6, S3A). Interestingly, these genes were up-regulated by several fold in overexpression (OX) transgenic plants (NIL-*cef1*[p35S::myc-MYB103L]) and the increased expression levels were coordinate with that of *MYB103L* (Figs. 6, S3B, S4). The results suggested that these genes were downstream of *MYB103L*.

Transcription of *BC1*, *CESA4*, *CESA7* and *CESA9* is directly activated by MYB103L

To address whether MYB103L can directly activate transcriptions of *BC1* and three *CESA4, 7, 9* genes, we used yeast one-hybrid system to verify. The yeast one-hybrid assay showed that the *pB42AD-MYB103L* strongly activated the *LacZ*, which were driven by *BC1* or three *CESA* genes promoters while the pB42AD without *MYB103L* did not activate the *LacZ* expression or to a lesser extent (Fig. 7b).

We also used the dual-luciferase reporter (DLR) assay system in rice protoplasts. The CaMV 35S promoter drive *MYB103L* as an effector plasmid (Fig. 7c) and the firefly luciferase reporter gene was driven by *BC1* and three *CESA* genes promoter (2 kb) as a reporter plasmid (Fig. 7c). The effector plasmid without *MYB103L* was used as negative control. The *Renilla LUC* driven by the 35S promoter was used as an internal control. These plasmids were coexpressed in rice protoplasts. Transactivation activity analysis showed that the luciferase activity in the protoplasts coexpressing an effector containing MYB103L and a reporter containing the *BC1* and three *CESA* genes promoter driving luciferase was significant higher than the negative control (Fig. 7d). The results indicated MYB103L can directly activate transcription of *BC1*, *CESA4, 7, 9*.

MYB103L can regulate expression of other transcription factors

Hierarchical TFs involved in secondary wall biosynthesis exist in *Arabidopsis thaliana*. Therefore we tested if
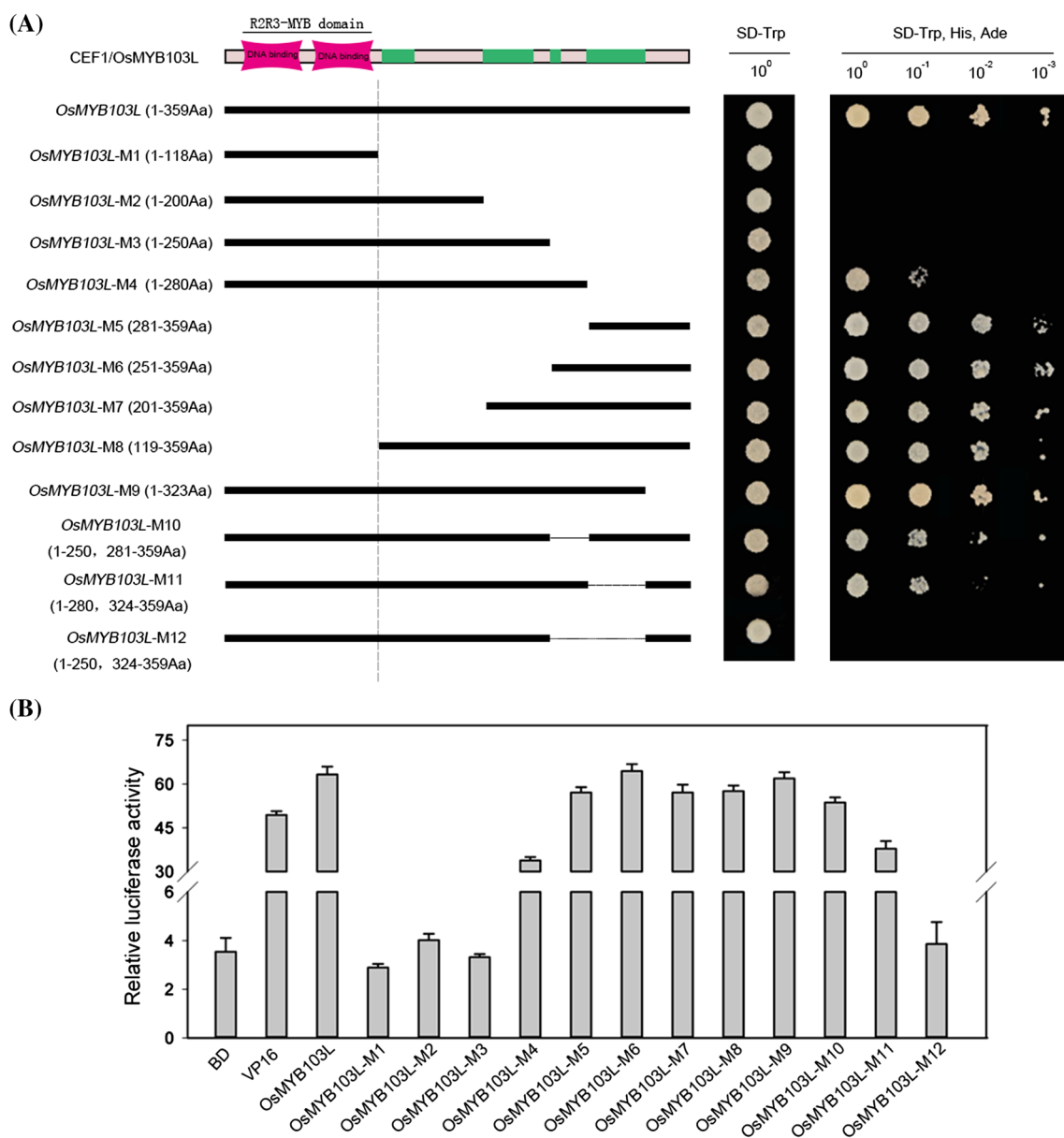
Fig. 5 Transcriptional activation of CEF1/MYB103L. **a** Transactivation activity of MYB103L in a yeast assay. Schematic representation (*left panel*) and transactivation activity in a yeast assay (*right panel*) of truncated *OsMYB103L*, *OsMYB103L*-M1 to *OsMYB103L*-M12 represent constructs with different truncated versions of *OsMYB103L*. *Vertical broken lines* from the left-hand side to the right-hand side indicate positions of DNA-binding domain and activation domain. The *middle panel* and the *right panel* indicate yeast growth on SD-Trp and SD-Trp, His, Ade medium, respectively. **b** Transcriptional activation ability of OsMYB103L and OsMYB103L-M1 to OsMYB103L-M12 as revealed by relative LUC activity

Hierarchical TFs regulating secondary wall biosynthesis exist in rice and if MYB103L can regulate expression of other TFs. We selected a number of TFs which are coexpressed with *MYB103L* and tested expression level of these TFs in NIL-*CEF1*, NIL-*cef1* and NIL-*cef1*[p35S::myc-MYB103L] overexpression transgenic plants. The qRT-PCR assay showed that the expression of *OsSND2* (LOC_Os05g48850), *OsMYB42/85* (LOC_Os09g36250) and *OsMYB52/54* (LOC_Os03g51110) were down-regulated in NIL-*cef1* plants and up-regulated in the transgenic overexpression lines (Fig. S4) with increased expression level of *MYB103L* (Fig. 8). The results suggested these TFs may be downstream of *MYB103L*. To address whether MYB103L can directly regulate expression of *SND2*, *MYB42/85* and *MYB52/54*, we also used yeast one-hybrid system to verify. The yeast one-hybrid assay showed that the *pB42AD-MYB103L* cannot activate the *LacZ* expression (Fig. S5), so these TFs are regulated indirectly by MYB103L.
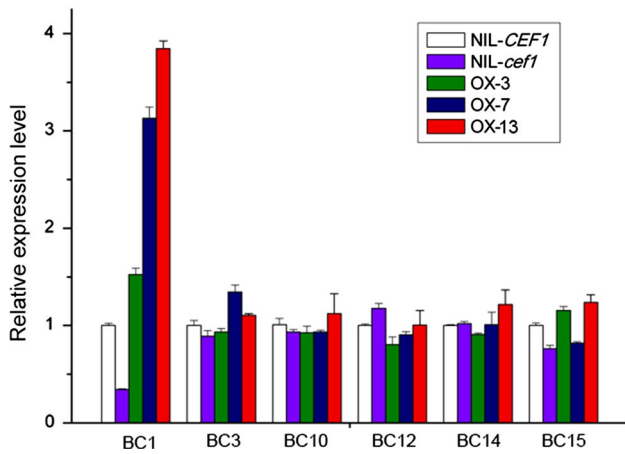
Fig. 6 Expression of BC genes. Error bars indicate the SE of three biological repeats. The Actin1 gene was used as an internal control

SLR1 interacts with MYB103L for secondary wall formation

As previously reported, gibberellin (GA) promotes cellulose biosynthesis. It functions through relieving the interaction between SLENDER RICE1 (SLR1), a DELLA repressor of GA signaling, and NACs, the crucial transcription factors for secondary wall formation. The SLR1-NACs interaction can effectively block the NACs-MYB-CESAs

hierarchical regulation pathway (Huang et al. 2015). Therefore we were interested to test if the exogenous GA treatment can rescue the *cef1* plants brittle culm phenotype. We treated the NIL-*CEF1* and NIL-*cef1* plants with exogenous GA and examined CESAs and BC1 expression in the shoots. The *CESAs* and *BC1* expression of NIL-*CEF1* was dramatically upregulated by GA treatment, comparing to the NIL-*CEF1* without GA treated. More interestingly, the *CESAs* and *BC1* expression of NIL-*cef1* was only slightly upregulated (Fig. 9). Furthermore, the exogenous GA treatment cannot rescue the brittle culm phenotype (not shown). Hence the expression of MYB103L was required for the GA regulated cellulose biosynthesis pathway.

SLR1 is a key repressor to mediate the downstream responses through protein interactions. To address whether MYB103L can interact with SLR1, we used yeast two-hybrid (Y2H) system to verify the physical interaction of MYB103L and SLR1 in vitro (Fig. 10a). Bimolecular fluorescence complementation (BiFC) analysis in *Arabidopsis* revealed a direct interaction between MYB103L and SLR1 in the nuclei of living plant cells (Fig. 10c). Using MYB103L-HA or BC1-HA and SLR1-Flag transient coexpression in *Arabidopsis* protoplasts, we also confirmed the physical interaction between MYB103L and SLR1 by coimmunoprecipitation in vivo (Fig. 10b).

To further understand the mechanism how the MYB103L-SLR1 complex regulate CESAs and BC1 gene
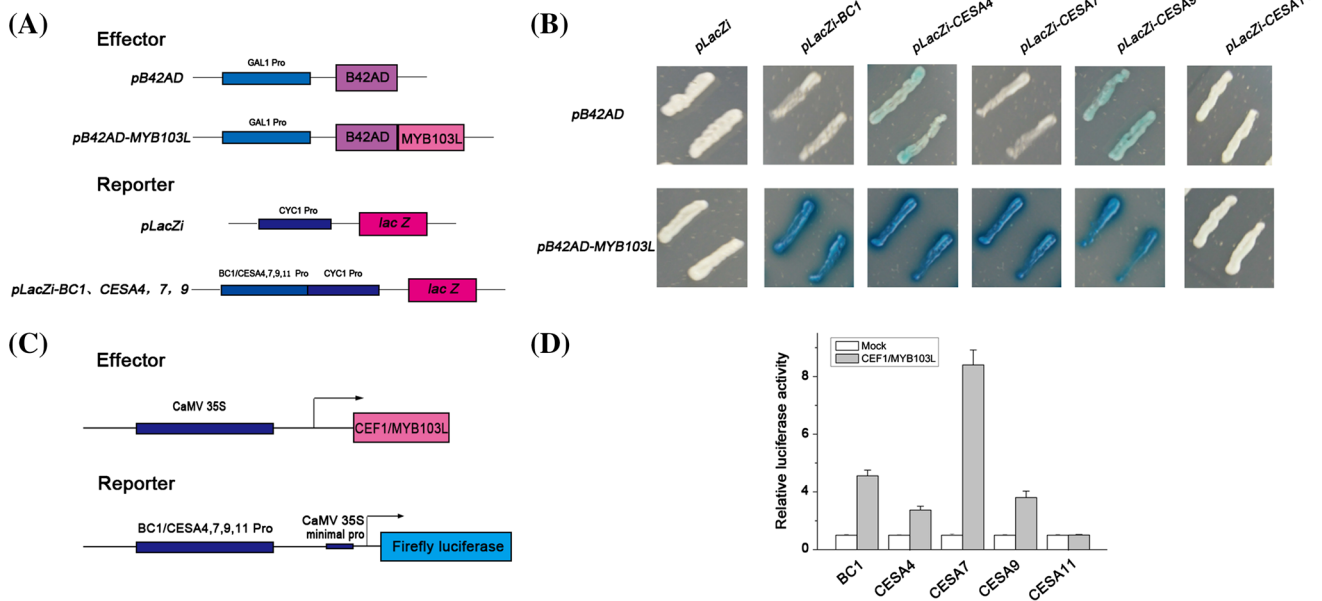
Fig. 7 CEF1/MYB103L directly regulate *BC1, CESA4, CESA7, CESA9* expression. **a** Diagram of the effector construct and the reporter constructs used in **b**. **b** Yeast one-hybrid assay showing the activity of LacZ reporters driven by *BC1*, *CESA4*, *CESA7*, *CESA9* promoters and activated by activation domain (AD) fusion effectors. The empty *pB42AD* and *pLacZi* were used as negative control. **c** Diagram of the effector construct and the reporter constructs used in **d**. **d** Luciferase activities in protoplasts contransfected with the constructs shown in effectors and reporters. The transactivation activity was monitored by assaying the luciferase activities, with the activity in protoplasts transfected with an empty effector construct defined as 1. Error bars, SE of three biological replicates
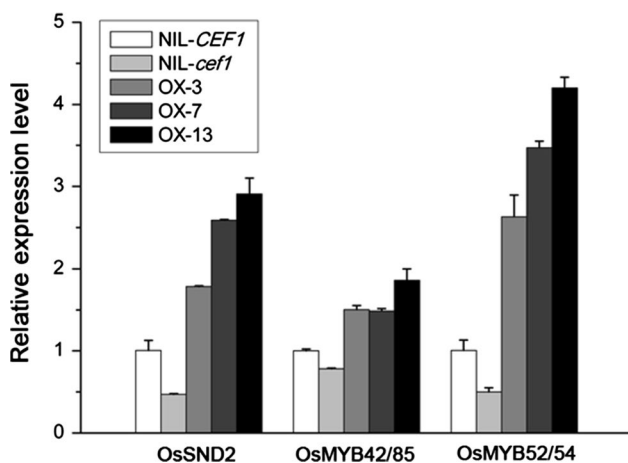
Fig. 8 Expression of other secondary wall related TFs. qRT-PCR assay of the expressions of *OsSND2*, *OsMYB42/85* and *OsMYB52/54* in NIL-*CEF1*, NIL-*cef1* and NIL-*cef1*$^{p35S::myc-MYB103L}$ transgenic plants. The Actin1 was used as an internal control. Error bars, SE of three biological replicates
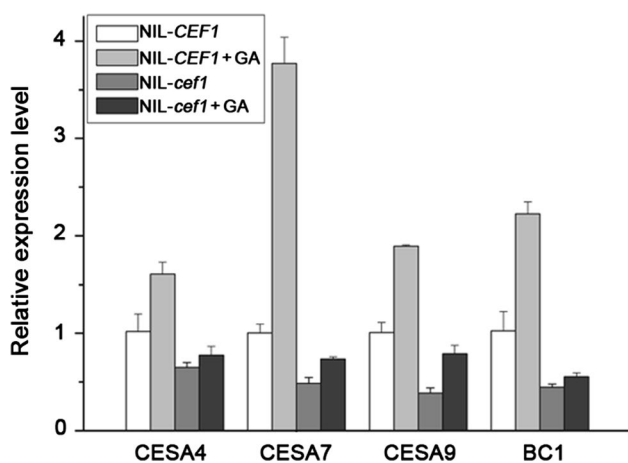
Fig. 9 Expression of secondary wall genes in the control (−GA) and 6 h GA treated (+GA) in NIL-*CEF1* and NIL-*cef1* plants. The Actin1 was used as an internal control. Error bars, SE of three biological replicates

expression. We used the dual-luciferase reporter (DLR) assay system in *Arabidopsis* protoplasts. These effectors and reporters plasmids were coexpressed in *Arabidopsis* protoplasts (Fig. 10a). Transactivation analysis showed that luciferase activity in the cells coexpressing reporters containing the CESAs or BC1 promoter driven luciferase and an effector containing MYB103L was significantly repressed by the additional coexpression of SLR1. This repression was relieved by the exogenous GA treatment (Fig. 11b), suggesting that SLR1 inhibits the MYB103L regulating CESAs and BC1 expression. Thus GA promoting cellulose biosynthesis not only through disrupting the

interaction between SLR1 and NACs, but also through the relieving of the SLR1/MYB103L complex.

Discussion

In this study, we found a new rice variety (Wandao60) that showed its culm easily fragile in breeding, its straws were smashed by rice combine and evenly distribute into the field and not affect its lodging, although its break force reduced to approximately 35 % compared with that of the NIL-*CEF1*. We studied the phenotype of the *cef1* plants, *CEF1* gene mapping and its expression pattern. We confirmed *CEF1* gene lead to the phenotype of *cef1* through functional complementation assay. We also further investigated CEF1 protein function and downstream genes of CEF1.

CEF1 is a member of the MYB family

In plants, the MYB proteins are the one of the largest of transcription factor families (Riechmann 2000; Romero et al. 1998). They can be classified into four subfamilies based on the sequence and number of adjacent repeats in the MYB domain (R1, R2R3, 3R, 4R) (Dubos et al. 2010; Jin and Martin 1999). Among them, R2R3-MYB subfamily is the largest and plays important roles in processes including plant development, secondary metabolism, and stress responses (Stracke et al. 2001). In rice, at least 155 MYB genes distribute into different chromosomes and R2R3-MYB accounts for approximately 56.77 % of all MYB genes (Katiyar et al. 2012). *CEF1* encodes a putative MYB-like DNA-binding domain containing protein. Phylogenetic analysis indicates that *AtMYB103* is the most closely homolog of *CEF1* (Fig. S1B). AtMYB103 is a typical R2R3-MYB protein (Ohman et al. 2013; Zhong et al. 2008). It is similar to AtMYB103, CEF1 has two DNA-binding domain at N-terminus (Fig. S1A), belongs to the R2R3-MYB subfamilies, and located in the nucleus (Fig. S1C). In R2R3-MYB subfamilies, highly conserved at MYB domains, while the C-terminal regions are high variable (Stracke et al. 2001). Go analysis of MYB proteins suggested that 98.7 % OsMYB involved in transcription activation (Katiyar et al. 2012). Transactivation analysis indicated that CEF1/OsMYB103L has strong transcriptional activation activity at C-terminal domains (Fig. 5).

### CEF1/OsMYB103L plays an important role in secondary cell wall synthesis

Secondary cell walls providing the mechanical support and protection to the plant body, are mainly composed of cellulose, xylan and lignin. The complicated assembly causes
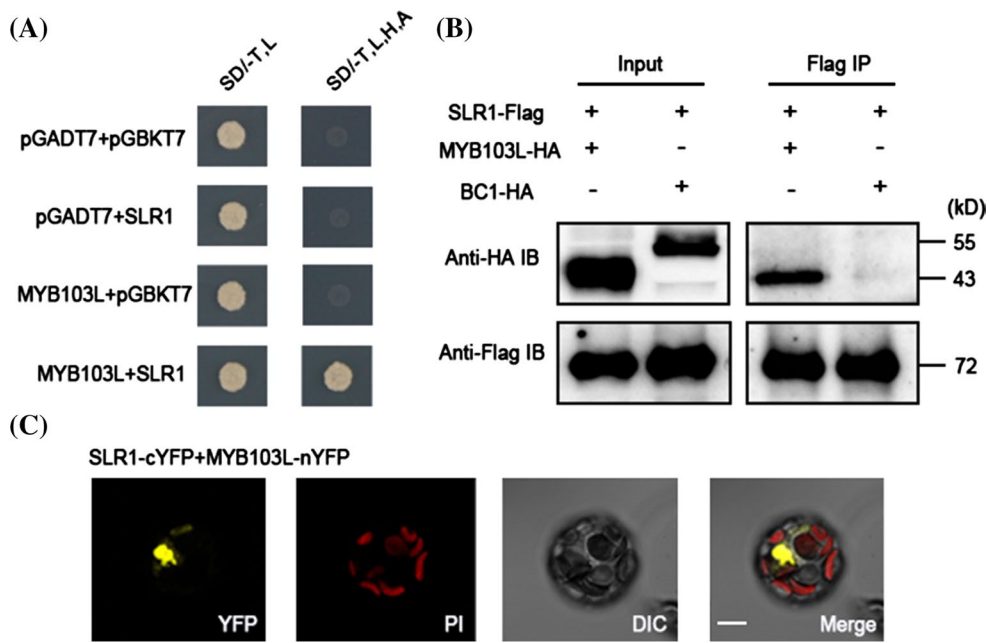
Fig. 10 MYB103L interacts with the SLR1. **a** MYB103L interacts with SLR1 in yeast. MYB103L, a prey plasmid containing full length cDNA of OsMYB103L; SLR1, a bait plasmid containing full length cDNA of SLR1; pGADT7, empty prey plasmid; pGBKT7, empty bait plasmid; SD/-T, L, SD medium lacking tryptophan and leucine; SD/-T, L,H, A, SD medium lacking tryptophan, leucine, histidine, and adenine; **b** MYB103L interacts with SLR1 in Arabidopsis protoplasts. MYB103L-HA and BC1-HA were expressed in WT Arabidopsis protoplasts, along with SLR1-Flag, immunoprecipitated with anti-Flag antibody, and the bound protein was detected by immunoblot with the indicated antibodies. **c** BiFC analysis of the interaction between MYB103L and SLR1 in Arabidopsis protoplasts
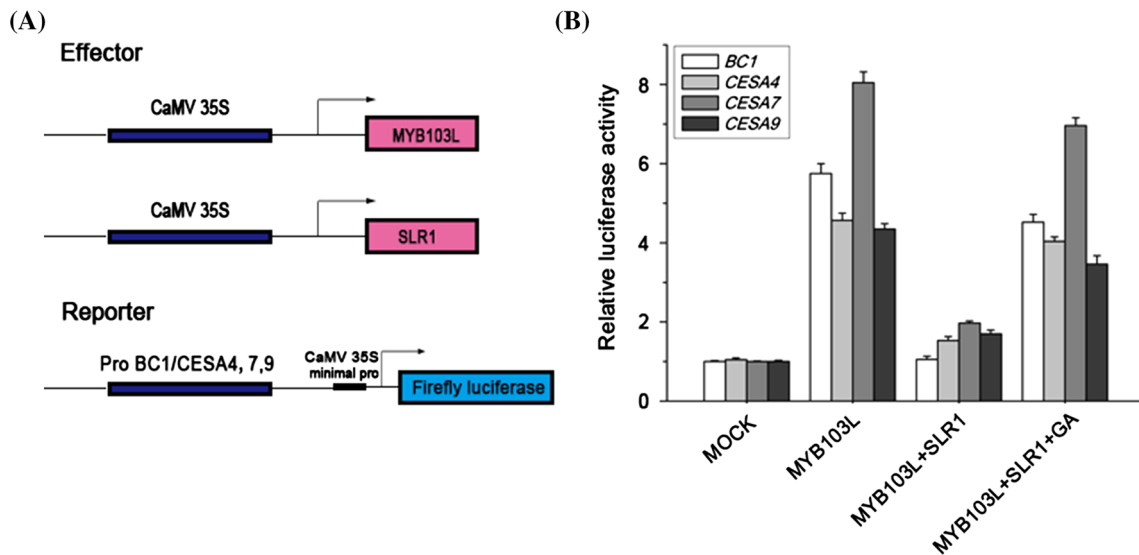
Fig. 11 SLR1-MYB103L Interaction regulates CESAs and BC1 expression. **a** Diagrams of the effector and reporter constructs used in **b**. **b** Luciferase activities in protoplasts cotransfected with the reporter and different combinations of effectors. The transactivation activity was monitored by assaying the luciferase activities, with the activity in protoplasts transfected with an empty effector construct defined as 1. Error bars indicate the SE of three biological repeats

problems for the straw disposal. Therefore, understanding the formation of secondary cell walls is critical for the straw disposal. In the past decade, many studies were focus on the biosynthesis of cell walls, however up to date only three rice TFs have been reported to be associated with secondary cell walls formation (Hirano et al. 2013; Yang et al.

2014; Zhong et al. 2011). We reported in this study that CEF1 is a TF regulating secondary cell walls formation. Loss-of-function mutation in *CEF1* can reduce cellulose content (Table 1) and decrease the thickness of sclerenchyma cell walls (Fig. 3d, g). Even the *cef1* plant exhibits easily broken in clum, it is different from other brittle mutants reported previously. The *cef1* has no morphological abnormalities such as dwarfism, low fertility and withering of leaf apex. Hence the discovery of CEF1 gene may have great contribution to genetic modification of rice.

In rice, three synthase (CESA4, CESA7, CESA9) are necessary for cellulose production in the secondary cell walls (Tanaka 2003). Mutation of these *CESA* genes can induce brittle culm phenotype and decrease the cellulose content (Kotake et al. 2011; Song et al. 2013; Tanaka 2003; Zhang et al. 2009). CEF1/OsMYB103L as a transcription factor can regulate expression of these genes (Fig. 7b, d). The *BC1* is also involved in cellulose synthesis, and plays an important role in the biosynthesis of the secondary cell walls of mechanical tissues (Li 2003; Liu et al. 2013a). We also demonstrated that CEF1/OsMYB103L regulates BC1 expression (Fig. 7b, d). *AtMYB46*, a master switch for secondary cell wall formation in Arabidopsis, can function as a direct regulator of all three secondary wall-associated cellulose synthase genes: *AtCESA4*, *AtCESA7* and *AtCESA8* (Kim et al. 2013). Dominant repression or RNA interference inhibition of *AtMYB46* resulted in thinner secondary cell walls and a concomitant collapsed vessel phenotype (Zhong et al. 2007a). Mutation of the any of the *AtCESA4/IRX5*, *AtCESA7/IRX3* and *AtCESA8/IRX1* also caused severe reduction in cellulose content, secondary cell walls thickness and a collapsed vessel phenotype (Taylor et al. 1999, 2000, 2003). Consistent with these findings in Arabidopsis, CEF1/OsMYB103L regulated the cellulose content through mediating the expression of cellulose synthases. Therefore mutation of *CEF1/OsMYB103L* resulted in the brittle clum phenotype.

In the *cef1* plants, in addition to decreased cellulose content, other cell wall components were altered. The content of arabinose and xylose, the two major sugars of arabinoxylan, were substantially increased by approximately 37 and 41 %, respectively (Table 1). Many brittle culm mutants, such as *bc1* (Li 2003), *bc11* (Zhang et al. 2009), *bc13* (Song et al. 2013) and *bc15* (Wu et al. 2012), also showed the decreased cellulose content and increased content of arabinose and xylose, corresponding with their wild type controls. It may have a feedback mechanism to compensate the decreased cellulose to maintain whole plant carbon balance in these brittle culm mutants.

The *cef1* is a tissue specific brittle culm plant

The *cef1* internodes are easily broken, but compare with other rice brittle culm mutants, the leaves are not fragile.

The specific expression pattern of CEF1 in internodes and panicles (Fig. 4) was important to this phenotype in culm and panicle. This unique expression pattern have also been reported in Arabidopsis. In Arabidopsis the NAC and MYB family transcription factors regulate secondary cell wall biosynthesis in various secondary wall-forming cell types. The *SND1*, *NST1* and *NST2* are expressed specifically in fibers (Zhong et al. 2006, 2008). *VND6* and *VND7* are specially expressed in metaxylem and protoxylam, respectively. The *MYB46* and *MYB83* are expressed in both vessels and fibers (McCarthy et al. 2009; Zhong et al. 2007a). Dominant repression of these genes result in severe decreased in secondary cell wall thickening in corresponding cells and tissues.

Overexpression of *OsMYB103L* resulted in leaf rolling phenotype in rice (Yang et al. 2014), it may be caused by ectopic deposition of secondary cell walls in leaves. In Arabidopsis, overexpression of *MYB46* also caused the leaf rolling phenotype (Kim et al. 2013). Overexpression of SND1 also exhibited a prominent visual phenotype that shows small rosette size and stunted growth of leaves with severely upward-curling blades, it mainly caused by massive deposition of secondary walls in cells that are normally nonsclerenchymatous (Zhong et al. 2006). Due to the expression of *CEF1* in specific tissue, the *cef1* plants result in brittle phenotype in internodes, but not in leaves. Similar to these reports, the overexpression lead to *CEF1/OsMYB103L* high expressed in all organs and caused the leaf rolling phenotype (Yang et al. 2014).

Hierarchical TFs involved in secondary wall biosynthesis may exist in rice

In Arabidopsis, the coordinate activation of secondary cell wall biosynthesis genes is controlled by a transcriptional network. In this transcriptional network, the secondary wall NAC families (SWNs) are the top-level master switches that can activate a battery of downstream transcription factors, including *SND2*, *SND3*, *MYB20*, *MYB42*, *MYB46*, *MYB83*, *MYB103* and *KNAT7* (Zhong et al. 2008). The *MYB46* acts as the secondary-level master switch to regulate downstream transcription factors and the expression of the biosynthesis genes for cellulose, xylan and lignin (Zhong et al. 2007a). In rice, hierarchical TFs involved in secondary wall biosynthesis have been reported is rarely (Huang et al. 2015). In this study, we find some secondary wall-associated TFs, such as *OsSND2*, *OsMYB42/85* and *OsMYB52/54* are downstream of *CEF1/OsMYB103L*. We propose that *CEF1/OsMYB103L* may act as a master switch to regulate these TFs. Further illustration of the transcriptional network for secondary wall biosynthesis can help to depict the hierarchy of TFs in rice.

GA regulates cellulose biosynthesis also via SLR1-MYB103L-CESAs pathway

GA is an important hormone for plant growth and development, it mainly promotes the degradation of DELLAs and consequently activate the downstream responsive processes (Fu 2002). Previous study shows that GA regulates cellulose biosynthesis via SLR1-NAC Signaling Cascade (Huang et al. 2015). In the presence of GA, the GA-induced degradation of SLR1 released the suppression of MYB103L, which further upregulated the *CESAs* and *BC1* expression (Fig. 11b). Comparing to the significant increase of *CESAs* and *BC1* genes in NIL-*CEF1,* only slight increase of these genes were found in the NIL-cef1 plants (Fig. 9). Although the SLR1-NACs-MYB-CESAs signaling cascade has been reported in rice (Huang et al. 2015), the SLR1-NACs-MYB and SLR1-MYB103L may be together involved in GA regulates secondary cell wall synthesis. The *OsMYB103L* also is downstream of NAC family TFs (Huang et al. 2015). Hence, GA may regulates *MYB103L* expression via SLR1-NACs signaling cascade. Interestingly, the loss-of-function of MYB103L impaired regulation of *CESAs* and *BC1* genes by GA (Fig. 9), GA regulates cellulose biosynthesis via SLR1-NACs signaling cascade may dependent on MYB103L. Therefore the MYB103L is a central factor in GA regulates cellulose biosynthesis pathway.

The *cef1* has a potential prospect for residue management

The processing of rice straw has been a challenge for breeders because the rice straws cannot decompose in short-term. Straw burning is preferred by farmers, as it is economic and convenient, but it causes environmental problems. The high cellulose content of cell wall of rice straw was the major cause of slow decomposition (Tian et al. 1992). The brittle stem rice mutants have faster residue decomposition due to the lower cellulose content and finer breakage during threshing (Cabiles et al. 2008; Johnson et al. 2006). However, these mutants have concomitant phenotypes such as dwarfism, low fertility and withering of leaf apex, made them inadequate for breeding. The *cef1* plants were different from other brittle culm mutants, since it has no morphological abnormalities. Under field conditions, the grain yield of NIL-*cef1* and NIL-*CEF1* plants was indistinguishable (Fig. 3h–m). Hence Wandao60*cef1* is an ideal material to resolve the straw processing problem, and the mutation site of *CEF1/OsMYB103L* can also be introduced to other high-yielding varieties.

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