CEF3 is involved in membrane trafficking and essential for secondary cell wall biosynthesis and its mutation enhanced biomass enzymatic saccharification in rice

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Abstract

Background: As one of the most important staple food crops, rice produces large of agronomic biomass residues that contain lots of secondary cell walls (SCWs). Membrane trafficking plays key roles in SCWs biosynthesis, but information association membrane trafficking and SCWs formation in plants is limited.

Results: In this study, we report the function characterization of a rice mutant, culm easily fragile 3 (cef3), that exhibits growth retardation and fragile culm phenotype with significantly altered cell wall composition and reduced secondary cell wall thickness. Map-based cloning revealed that CEF3 encodes a homologous protein of Arabidopsis STOMATAL CYTOKINESIS DEFECTIVE2 (SCD2). The saccharification assays revealed that CEF3 mutation can improve biomass enzymatic saccharification. Expression pattern analysis indicated that CEF3 is ubiquitously expressed in many organs at different developmental stages. Subcellular localization revealed that CEF3 is a Golgi-localized protein. The FM4-64 uptake assay revealed CEF3 is involved in endocytosis. Furthermore, mutation of CEF3 not only affected cellulose synthesis-related genes expression, but also altered the abundance of cellulose synthase catalytic subunit 9 (OsCESA9) in the PM and in the endomembrane systems.

Conclusions: This study has demonstrated that CEF3 participates in the membrane trafficking that is essential for normal cellulose and other polysaccharides biosynthesis of the secondary cell wall, thereby manipulation of CEF3 could alter cellulose content and enhance biomass enzymatic saccharification in rice plants. Therefore, the study of the function of CEF3 can provide a strategy for genetic modification of SCWs in bioenergy crops.

Keywords: Secondary cell wall (SCW), Rice, Cellulose biosynthesis, Biomass, Saccharification, membrane trafficking, Map-based cloning

Background

The plant cell wall is a basic cellular structure that not only plays important role in plant growth and development, but also represents one of the most plentiful natural resources on earth [1]. Plants produce two typical types of cell walls, the primary cell walls (PCW) surrounding in all cells and the secondary cell walls (SCW) which are rigid and thickened structures in specific cell
types [1]. The secondary cell walls not only provide mechanical strength for plant body, but also greatly contribute to the bulk of renewable plant biomass [2]. Rice is one of the most important staple crops for feeding half of population in the world [3]. Rice plants mechanical strength directly associate with lodging and ultimate yield. In addition, rice straw is a very important raw material for the production of bioenergy and bio-based products [4]. However, due to the lignocellulose recalcitrance of the secondary cell wall, the utilization of rice straw for bio-energy is very limited [5, 6]. Hence, to understand the mechanism of SCWs biosynthesis can provide a strategy for improving rice lodging resistance and/or manipulating plant biomass production. The SCWs are typically composed of cellulose, hemicellulose and lignin [7].

In the past decades, many studies for secondary cell walls biosynthesis have been reported in both dicot and monocot plants [8]. In current understanding, the cellulose is synthesized at the plasma membrane (PM) by the cellulose synthase complex (CSC) containing at least three different cellulose synthases, which are assembled into CSCs in either the endoplasmic reticulum (ER) or the Golgi apparatus and trafficked by vesicles to the plasma membrane (PM) [9]. Nevertheless, the hemicellulose and lignin are produced in the Golgi apparatus, and are then transported to the walls through secretory pathways [10]. Therefore, vesicular trafficking of required proteins and SCW materials from the Golgi apparatus to the PM or the extracellular matrix plays a very important role for SCW biosynthesis. Decadal researches have discovered several factors involved in vesicular trafficking of proteins and polysaccharides in the cell wall [11–13]. Live-cell imaging of fluorescently tagged CESAs has provided direct evidence of CSC trafficking [14]. The CSCs have been detected in the Golgi apparatus, and the glycosyltransferases STELLO (STL1 and STL2) are involved in regulating CSCs assembly in the Golgi [15]. CSCs are then trafficked to the PM through the trans-Golgi network/early endosome (TGN/EE) compartment [16]. Membrane trafficking depends on intermediate compartments and a large number of proteins for ensuring accuracy and directionality of each transport route [12].

The CESAs are known to localize in two types of compartments, the vacuolar-type H^+–ATPase subunit a1 (VHA-a1)/SYP61 compartment and the microtubule-associated cellulose synthase compartment (MASC) [17]. Although the membrane trafficking mechanism of enzymes involved in cell wall biosynthesis is relatively clear, little is known about the membrane trafficking of polysaccharide. This is partially attributed to technical challenges in biochemically determining polysaccharide cargo in specific vesicles [11]. Recent research revealed SYP61 vesicles are involved in the transport and deposition of structural polysaccharides and glycoproteins [11].

Clathrin-coated vesicle (CCV) is surrounded by clathrin lattice formed at PM and TGN/EEs in plant cells and plays important roles in vesicular trafficking [18]. The clathrin-mediated trafficking pathways are regulated by trafficking factors including Rab and ADP-ribosylation factor (ARF)-related small GTPases [19, 20], and soluble N-ethylmaleimide-sensitive factor adaptor protein receptors (SNARE) proteins [21]. Arabidopsis stomatal cytokinesis defective1 (scd1) and scd2 mutants exhibit dwarfism and have defects in cell division and expansion phenotypes, which are similar to exocyst mutants [22, 23]. SCD1 and SCD2 together constitute the SCD complex that cooperates with members of the exocyst complex and RabE1 GTPases to mediate post-Golgi trafficking to the PM [22]. In addition, dynamin-related proteins (DRPs) are essential for CCV formation through scission of the budding vesicle from the PM [24]. In rice, BC3, encoding a OsDRP2B participates in the endocytic pathway, probably as well as in post-Golgi membrane trafficking. Mutation of BC3/OsDRP2B disturbs the membrane trafficking that is essential for normal cellulose biosynthesis of the secondary cell wall, thereby leading to inferior mechanical properties in rice plants [13]. Clathrin-mediated vesicle trafficking is a well-studied transport pathway in plant growth and development [25–28], but how it regulates SCW biosynthesis remains elusive.

In this study, we identified a novel rice *culm easily fragile* 3 (*cef3*) mutant, that exhibits growth retardation and fragile culm phenotype with significantly altered cell wall composition and reduced secondary wall thickness. Map-based cloning revealed that *CEF3* encodes a homologous protein of Arabidopsis STOMATAL CYTOKINESIS DEFECTIVE2 (SCD2). We further revealed mutation of *CEF3* not only affected cellulose synthesis and hemicellulose synthesis-related genes expression, but also altered the abundance of cellulose synthase catalytic subunit 9 (*OsCESA9*) in the PM and in the endomembrane systems. The saccharification assays revealed that CEF3 mutation can improve biomass enzymatic saccharification. Based on these findings, we can conclude that CEF3 participates in the membrane trafficking that is essential for normal cellulose and other polysaccharides biosynthesis of the secondary cell wall, thereby manipulation of *CEF3* could alter cellulose content and enhance biomass enzymatic saccharification in rice plants.

**Results**

The *cef3* mutant results in altered mechanical strength and growth status

The *culm easily fragile* 3 (*cef3*) mutant was obtained from the *japonica* cultivar, Xiushui63 by treatment with
a heavy ion beam. Compared to wild type (WT), we observed that the cef3 mutant had obviously reduced plant height, due to small panicle and shorten internodes (Fig. 1A, B, C, H). In addition, the cef3 mutant exhibited easily broken culms but not leaves (Fig. 1D, E). Unlike most other brittle culm mutants previously reported [4, 29–36], the cef3 mutant showed no easily broken phenotype at seedling stage (Additional file 1: Figure S1). To accurately describe the broken phenotype, we quantitatively compared the extension forces of the second internodes and the flag leaves in the WT and cef3 plants. The results indicated the force applied to break cef3 internodes was reduced by more than 40% compared with that of the wild type, but no significant difference in leaves (Fig. 1F, G).

Reduced thickness of the sclerenchyma cell walls is the main reason why rice plants shown brittle culm phenotype. Therefore, we used scanning electron microscope (SEM) to observe the second internodes cross sections of WT and cef3 plants. The observations revealed that the sclerenchyma cell wall of cef3 was extremely thinned (Fig. 2B, D, F, G), compare with the WT (Fig. 2A, C, E, G).

The cef3 defects in mechanical strength and wall structure suggested that the cell wall composition in the mutant plants may be altered. Therefore, we examined the cell wall composition in the second internodes of the WT and cef3 plants at the mature stage. As shown in Table 1, the cellulose content was significantly deceased in cef3 internodes, whereas the neutral sugars content derived from non-cellulosic polysaccharides were generally increased in cef3 samples, except for the xylose (Xyl), which is the major sugar of hemicellulose. We also detected the hemicellulose content was significantly decreased in cef3 internodes, but the lignin content has no significant change, compared to WT (Fig. 2H).

Taken together, the cef3 defects in mechanical strength was correlated with the thinned sclerenchyma cell walls and complicated alterations in wall composition.

Map-based cloning of CEF3
To understand the molecular basis of the above phenotypes, a map-based cloning approach was performed to isolate the CEF3 gene. We crossed the mutant with 93–11, a wild type polymorphic indica variety, to generate a F2 mapping population. The cef3 locus was located between molecular markers ASR41 and ASR43 on
chromosome 1, and further narrowed within an approximately 52-kb region between markers A6 and A11 (Fig. 3A). The 52-kb region contains 7 putative open-reading frames (ORFs) (Fig. 3B). Then, we sequenced all of the ORFs and a 5-bp deletion was found in LOC_Os01g70320 (Fig. 3B, C). This deletion occurred in the fourth exon of the ORF, resulting a frame-shift and premature translational product of 235 amino acids (Fig. 3B and Additional file 2: Figure S2). Therefore, cef3 is very likely to be a loss-of-function mutation. To confirm that LOC_Os01g70320 corresponds to the cef3 locus, a 4.2-kb DNA fragment containing the 2.5-kb putative promoter and the coding region was cloned vector pCAMBIA2300 to generate the plasmid pCEF3::CEF3 (pCEF3F), which was introduced into the cef3 plants by Agrobacterium tumefaciens-mediated transformation. The transgenic cef3:pCEF3::CEF3 plants completely rescued the dwarfism (Additional file 3: Figure S3), fragile culm phenotype and the altered cell wall composition (Fig. 3D and Table 1).

To further verify the function of CEF3, the CRISPR/Cas9 approach was performed to generate other mutant alleles of CEF3 in wuyunjing7 (W7) background. We designed a sequence-specific single guide RNA (sgRNA) target site (TCAAAACCACGACGACATTTG), which in the second exon of CEF3. Three transgene-free homozygous knockout lines with different genotypes,
cef3-c1, cef3-c2 and cef3-c3 were obtained (Fig. 4A). Protein sequence alignments of the three homozygous mutants and the wild type protein revealed that cef3-c1, cef3-c2 and cef3-c3 showed coding frame shifts and premature translational stops (Additional file 4: Figure S4). All of these mutants exhibited dramatically dwarfism (Fig. 4B; Additional file 6: Figure S6A, C) and culms easily broken phenotype (Fig. 4C; Additional file 6: Figure S6B, D). We also found that the cell wall compositions were altered in cef3-c1 plants, compare with its wild type (Additional file 9: Table S1). Therefore, the LOC_Os01g70320 was the CEF3 gene responsible for the mutant phenotypes described above.

The cef3 mutant enhances biomass saccharification
In recent years, rice straw has been highlighted as an important material for biofuel production, but the lignocellulose recalcitrance determined by high cellulose content and crystallinity directly lead to costly biomass processing. As previously reported, the lower cellulose content rice mutants can improve enzymatic saccharification [29, 31, 32]. To detected whether the CEF3 mutation also enhances biomass saccharification. We examined the saccharification efficiency of lignocellulosic material derived from WT and cef3 plants. The sugar yields were significantly higher in cef3 than that of WT (Fig. 5). To further prove CEF3 mutation can improve enzymatic saccharification, we also examined the saccharification efficiency of lignocellulosic material derived from cef3-c1 plants generated by CRISPR-Cas9 system. The sugar yields were also significantly higher in cef3-c1 than that of WT (Additional file 5: Figure S5). These results suggest that the CEF3 mutation can enhance biomass enzymatic saccharification.

CEF3 is ubiquitously expressed in many organs at different developmental stages
To characterize the spatial and temporal expression profile of CEF3, we performed quantitative real-time (qRT)-PCR to analyze the expression level of CEF3 in various tissues at different developmental stages. The results suggested that CEF3 is ubiquitously expressed in all organs,
with relatively high levels in roots of different developmental stages and 15–20 cm length panicles. However, the expression level of \( \text{CEF3} \) is relatively low in leaves at any developmental stages (Fig. 6A). This expression pattern fits with the pleiotropy phenotypes (dwarfism, culm brittleness, small panicle etc.) of \( \text{cef3} \).

CEF3 is a plant-specific protein homologous to AtSCD2

The coding sequence of the \( \text{CEF3} \) is 1713 bp in length and encodes a protein of 570 amino acid residues with a predicted molecular mass of approximately 63 kDa. A BLASTP search for CEF3 homologs in Oryza sativa (japonica) and Arabidopsis thaliana genomes identified 2 and 5 closer homologs, respectively (Additional file 7: Figure S7A). Phylogenetic analysis revealed that CEF3 is a homolog of AtSCD2 (At3g48860), which functionally interacts with subunits of the exocyst complex for regulating proper membrane trafficking. CEF3 and AtSCD2 shared three conserved coiled-coil (CC) domains in the mid-region and a SCD2 domain with unknown function at the C terminus (Additional file 7: Figure S7B).

The subcellular distribution of proteins often provides important clues for understanding their cellular functions. To explore the subcellular localization of CEF3, we fused CEF3 with a green fluorescence protein (GFP) and cotransfected this chimeric construct in \( \text{Nicotiana benthamiana} \) leaves with a mCherry fused Golgi marker, Man49. The overlapping signals indicated that CEF3 is a Golgi-localized protein (Fig. 6B).

As previously reported [23], AtSCD2 is required for post-Golgi and/or endocytic trafficking and given that CEF3 is a Golgi-localized protein, we hypothesized that it might also has a function in membrane trafficking. To test this hypothesis, we used confocal laser scanning...
microscope to monitor the internalization levels of FM4-64 in root cells of WT and cef3 seedlings. The results indicated that accumulation of FM4-64 in the cef3 mutant was lower than in the WT (Fig. 7A, B). Quantification of FM4-64 internalization assay revealed that the relative internalization of FM4-64 was significantly decreased in cef3 mutant roots compared with its WT (Fig. 7C). All above results indicated that CEF3 participated in membrane trafficking, similar with its homolog of AtSCD2.

**CEF3 regulates secondary cell wall-related gene expression and OsCESA9 abundance at the PM**

To explain the reason why cef3 mutant exhibits brittle culm phenotype and alters cell wall composition. We first detected the secondary cell wall-related genes expression in the second internodes of WT and cef3 plants. qRT-PCR analysis showed that the cellulose biosynthesis genes, such as OsCESA4, OsCESA7, OsCESA9 and BC12, xylan biosynthesis genes, such as OsCSLF6, OsIRX10, OsGT61-1, OsIRX8L and OsIRX14 were down-regulated in the cef3 mutant, compared with WT (Fig. 8A, B). There is no significant difference in lignin biosynthesis genes expression between WT and cef3 plants (Fig. 8C).

Cellulose is synthesized by plasma membrane-localized cellulose synthase complexes (CSCs), which are assembled in either the endoplasmic reticulum (ER) or the Golgi apparatus and trafficked by vesicles to the plasma membrane (PM) [9]. To determine whether the CSCs trafficking are affected in cef3 mutant, we examined the distribution and/or abundance of OsCESA9 between
the PM and endomembrane systems in the second internodes of WT and cef3 plants by western blotting. OsCESA9 is one isoform of the CSCs involved in cellulose biosynthesis of secondary cell walls. Given that there is no good endogenous OsCESA9 antibody in our lab, we first generated the transgenic plants that overexpress OsCESA9-Flag fusion protein in the cef3 mutant background, and then cross into WT background. We further used anti-Flag antibodies to examine the subcellular distribution of OsCESA9-Flag, the western blot results showed that the level of OsCESA9-Flag has no significant difference in total membrane, but it was lower in the PEG (PM) and higher in the DEX (endomembrane) fractions (Fig. 9), respectively, compared with WT. Thus, CEF3, a protein involved in membrane trafficking, directly or indirectly affects SCW-related genes expression and CSCs abundance at the PM.

Discussion
Secondary cell walls play a critical role in plant growth and development, and they also contain high amounts of lignocellulose, a key feedstock for the production of bioenergy and bio-based products [1]. Three major steps are necessary for conversion of lignocellulose to ethanol: i) physical and chemical pretreatments to enhance cell wall destruction, ii) enzymatic digestion to release soluble sugars, and iii) microbial fermentation to produce ethanol [37]. The first two of the three steps are mainly affected by lignocellulose recalcitrance of the secondary cell wall. Therefore, it is great significance for providing a strategy for manipulating plant biomass production to understand the mechanism underlying SCWs biosynthesis. So far, many brittle culm mutants have been isolated, and there are idea materials for studying SCWs biosynthesis [4, 29–31, 34–36]. In this study, we obtained a novel culm easily fragile3 (cef3) mutant, which alters wall component (Table 1) and decreases the thickness of sclerenchyma cell walls (Fig. 2). These implying that CEF3 may be involved in SCWs biosynthesis. In rice, OsCESA4, OsCESA7 and OsCESA9 comprise the CSCs necessary for SCWs biosynthesis [38]. All of these mutants show the brittle culm phenotype due to decreases in cellulose content [39–41]. We detected OsCESA4, OsCESA7 and OsCESA9 gene expression were downregulated in cef3 plants (Fig. 8A), indicating that CEF3 is involved in SCWs biosynthesis may through regulating these gene expression. CEF3 encodes a homologous protein of Arabidopsis STOMATAL CYTOKINESIS DEFECTIVE2 (SCD2), which together with SCD1 constitute the SCD complex that cooperates with members of the exocyst complex to mediate post-Golgi trafficking to the PM [22]. The subcellular localization results showed CEF3 is a Golgi-localized protein (Fig. 6B), and quantification of FM4-64 internalization assay revealed that CEF3 participated in membrane trafficking (Fig. 9), similar with its homolog of AtSCD2. We also found that the abundance of OsCESA9 was lower at the PM in cef3 plants (Fig. 9). Therefore, as a critical protein involved in membrane trafficking, CEF3 may also contribute to the intracellular distribution of CSCs for regulating SCWs biosynthesis. BC3, a rice DRP OsDRP2B was also function in membrane trafficking pathways, its mutation also affects the distribution of proteins essential for cellulose biosynthesis in SCWs [13]. Not like bc3 mutant, cef3 exhibits brittle culm phenotype at after heading stage (Fig. 1) and no brittle culm phenotype at seedling stage (Additional file 1: Figure S1). Therefore, the biochemical and genetic relationships between BC3 and CEF3 in regulating secondary cell wall component trafficking in rice need further investigate. In
In addition to the culm easily broken phenotype, cef3 plant exhibits pleiotropic defects in plant height and expression. The hemi-cellulose is produced in the Golgi apparatus, and then transported to the walls through secretory pathways [10]. Therefore, CEF3 not only affects the distribution of cellulose synthase at the PM, but also may affects the transport of hemicellulose from Golgi apparatus to the wall, and these hypotheses require further experimental verification.

Fig. 8 Expression analysis of cell wall biosynthesis-related genes. A Relative expression levels of BC genes. B Relative expression levels of xylan biosynthesis genes. C Relative expression levels of lignin biosynthesis genes. OsActin2 was used as the internal control and data from three independent biological replicates were analyzed.

the cef3 plants, in addition to decreased cellulose content, other cell wall components were altered (Fig. 2H). The content of xylose, a major sugar of hemi-cellulose, was substantially decreased by approximately 18% (Table 1). We also detected some of xylan biosynthesis genes, such OsIRX10, OsCSLF6, OsGT61-1, OsIRX8L and OsIRX14 gene expression were downregulated in cef3 plants (Fig. 8B), indicating that CEF3 is involved in hemi-cellulose biosynthesis may through regulating these gene expression.
panicle morphology (Fig. 1). We detected CEF3 is ubiquitously expressed in all organs (Fig. 6A). This expression pattern also fits with the pleiotropy phenotypes (dwarfism, culm brittleness, small panicle etc.) of cef3. BC12, encodes a dual-targeting kinesin-4 protein, its mutation display dwarfism resulting from a significant reduction in cell number and brittleness due to an alteration in cellulose microfibril orientation and wall composition [33]. We detected decreased BC12 expression level in cef3 plants, suggesting CEF3 may regulate plant height development by regulating BC12 gene expression.

As one of the most important staple food crops, rice produces significant quantities of agronomic biomass residue every year. In recent years, rice straw has been highlighted as an important material for biofuel production, but the high cellulose content and crystallinity determine lignocellulose recalcitrance, leading to costly biomass processing [31, 32]. Biomass enzymatic saccharification efficiency is a key parameter for determining lignocellulosic straw digestibility. In addition, we detected higher saccharification efficiency of the lignocellulosic material derived from the cef3 mutants (Fig. 5 and Additional file 5: Figure S5), suggesting that the CEF3 mutation can enhance biomass enzymatic saccharification. Therefore, we can used CRISPR/Cas9 system to edit the CEF3 homologs in energy crops, such as poplar and switchgrass to improve biomass conversion efficiency.

**Conclusions**

In this study, we isolated a novel culm easily broken mutant cef3, which exhibits altered cell wall composition and reduced secondary wall thickness. Map-based cloning revealed that CEF3 encodes a homologous protein of Arabidopsis STOMATAL CYTOKINESIS DEFECTIVE2 (SCD2). Expression pattern analysis indicated that CEF3 is ubiquitously expressed in many organs at different developmental stages. Subcellular localization revealed that CEF3 is a Golgi-localized protein. The FM4-64 uptake assay revealed CEF3 is involved in endocytosis. Furthermore, mutation of CEF3 not only affected cellulose synthesis-related genes expression, but also altered the abundance of cellulose synthase catalytic subunit 9 (OsCESA9) in the PM and in the endomembrane systems. The saccharification assays revealed that CEF3 mutation can improve biomass enzymatic saccharification. Hence, this study has provided a powerful strategy for genetic modification of plant cell walls in bioenergy crops.
Materials and methods
Plant materials and growth conditions
The culm easily fragile (cef3) mutant was isolated from a japonica cultivar, xiushu63 (XS63) by heavy ion beam treatment. An F2 mapping population was generated from the cross between cef3 and a polymorphic indica cultivar, 93–11. All plants used in this research were grown in the experimental fields at Hefei Institute of Physical Science, Chinese Academy of Sciences (Hefei, China) and Sanya (Hainan province, China) during the natural growing season.

Measurement of extension force and microscopy
Extension force of the 2nd internodes and flag leaves of XS63, cef3, WYJ7 and cef3-c1 plants were determined according to [29]. The maximum force required to break apart the internodes and leaves were considered as the extension strength of these plants at heading stage.

For transmission electron microscopy, the 2nd internodes of WT and cef3 plants were cut with a razor and immediately post-fixed in 70% ethanol (V/V), 5% acetic acid (V/V), and 3.7% formaldehyde (V/V) mixture 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). The thickness of sclerenchyma cell wall was measured by Image J software.

Cell wall composition analysis and saccharification assays
The second internodes of XS63, cef3, WYJ7 and cef3-c1 plants at mature stage were used to prepare alcohol-insoluble residues (AIRs) of the cell walls. De-starched AIRs were produced as previously described [35]. The samples were hydrolyzed in 67% v/v H2SO4 for 1 h at room temperature, and then in 2 M H2SO4 at 121 °C for 1 h (h). The released sugars in the supernatant were measured by reading the A540 on an ELISA reader (Tecan) as described previously [43].

Expression analysis
total RNA was extracted from various rice tissues using TRIzol reagent (Invitrogen), as described previously [4]. 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 3 times, the OsActin2 gene was used as an internal control. The primers were used in expression analysis are listed in Additional file 8.

Subcellular localization of CEF3
To observe the subcellular localization of CEF3, a green fluorescent protein (GFP) fused to the C-terminus of CEF3 and inserted into the pCAMBIA1300 between the KpnI and BamHI sites to create the p35S::CEF3-GFP vector, which was introduced into A. tumefaciens GV3101 and transformed into 1-month-old N. benthamiana leaves together with a vector harboring the cis-Golgi marker Man49-mCherry. The fluorescent signals were observed with a confocal laser scanning microscope (Leica TCS SP5) after 3 days.

Binary vectors construction and rice transformation
We used CRISPR/Cas9 system for creating cef3 mutants. The CRISPR/Cas9 binary vectors were constructed as previously described [44]. The Cas9 plant expression vector (pYLCRISPR/Cas9Pubi-H) and sgRNA expression vector (pYLgRNA) were provided by Prof. Yao-Guang Liu (South China Agricultural University). We selected the target (TCAAACCCGACGACCATTTG) in the 2nd exon of CEF3 (Fig. 4a) as candidate target sequences according to the design principles of the target sequences in the CRISPR/Cas9 system. Then, they were ligated into
two sgRNA expression cassettes of a Cas9 binary vector, driven by OsL3 promoter. The construct was introduced into a *japonica* cultivar, wuyunjing7 (WY17) by the *Agrobacterium*-mediated transformation procedure as described previously [7].

**Western blot**
The extraction of total membrane protein and separation of the plasma membrane (PM) and endomembrane fractions were performed as previously described [41]. The proteins in the PEG and DEX fractions were separately collected and concentrated at 100,000 g for 1 h. The pellets were dissolved in suspension buffer (2 mM Tris, pH 6.5, 1 mM DTT and 0.25 M sucrose). Ten micrograms of protein was run on an SDS–PAGE gel and probed with corresponding monoclonal antibodies and the secondary antibody HRP-conjugated anti-mouse IgG (Sigma). The reactions were determined by the ECL Plus Western Blotting Detection System kit (GE Healthcare) and the chemiluminescent signal intensity was detected with a Tanon-5200 Chemiluminescent Imaging System (Tanon Science and Technology). The antibodies of Anti-Flag, Anti-HRP90, Anti-PIP1s and Anti-BiP2 were purchased from Agrisera (http://www.agrisera.com).

**Supplementary Information**
The online version contains supplementary material available at https://doi.org/10.1186/s13068-022-02205-y.

**Additional file 1**: Figure S1. Phenotype of wild-type (WT), cef3 and cesa9 plants at seedling stage. The seedling of cesa9 is easily broken, while seedlings of WT and cef3 are normal after hand flexing

**Additional file 2**: Figure S2. CEF3 amino acid sequence alignment of WT and cef3 mutant

**Additional file 3**: Figure S3. Complementary assay. The phenotype (A) and the plant height (B) show the rescued properties in the complemented plants. Scale bars: 10 cm

**Additional file 4**: Figure S4. Deduced CEF3 amino acid sequence alignments for the three homozygous mutants generated by CRISPR–Cas9 system and WT.

**Additional file 5**: Figure S5. Saccharification analysis of the wall residues: Additional files 1: S1, Additional files 2: S2, Additional files 3: S3, Additional files 4: S4, Additional files 5: S5, Additional files 6: S6, Additional files 7: S7, Additional files 8: Table S1, and Supplementary data—primers were used in this study).

**Declarations**

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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**Author contributions**
YY, BL, and YW together designed the experiments. YY wrote the manuscript. HJ and YR performed most of the experiments. JG and HY performed the vectors construction. XZ, WL and LT preformed the field experiment. YZ and QW performed the subcellular localization of OsCEF3. BL performed the expression pattern analysis. All authors have discussed the results and contributed to the drafting of the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
All data supporting the conclusions of this article are provided within the article and its Additional files 1: Figure S1, Additional files 2: S2, Additional files 3: S3, Additional files 4: S4, Additional files 5: S5, Additional files 6: S6, Additional files 7: S7, Additional files 8: Table S1, and Supplementary data—primers were used in this study.

**References**
1. Keegstra K. Plant cell walls. Plant Physiol. 2010;154(2):483–6.
2. Burton RA, Fincher GB. Plant cell wall engineering: applications in biofuel production and improved human health. Curr Opin Biotech. 2014;26:79–84.
3. Huang X, Huang S, Han B, Li J. The integrated genomics of crop domestication and breeding. Cell. 2022. https://doi.org/10.1016/j.cell.2022.04.036.
4. Ye Y, Liu B, Zhao M, Wu K, Cheng W, Chen X, Liu Q, Liu Z, Fu X, Wu Y. CEF1/ OsMYB103L is involved in GA-mediated regulation of secondary wall biosynthesis in rice. Plant Mol Biol. 2015;89(4–5):385–401.
5. Sathitsuksanoh N, Xu B, Zhao B, Zhang YH. Overcoming biomass recalcitrance by combining genetically modified switchgrass and cellulose-solvent-based lignocellulose pretreatment. PLoS ONE. 2013;8(9):e73523.

6. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foist TD. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science. 2007;315(5813):804–7.

7. Ye Y, Wu K, Chen J, Liu Q, Wu Y, Liu B, Fu X. OsSND2, a NAC family transcription factor, is involved in secondary cell wall biosynthesis through regulating MYB expression in rice. Rice (N Y). 2018;11(1):36.

8. Zhang B, Gao Y, Zhang L, Zhou Y. The plant cell wall: biosynthesis, construction, and functions. J Integr Plant Biol. 2021;63(1):251–72.

9. McFarlane HE, Doring A, Persson S. The cell biology of cellulose synthesis. Annu Rev Plant Biol. 2014;65:69–94.

10. Kim S-J, Brandizzi F. The plant secretory pathway for the trafficking of cell wall polysaccharides and glycoproteins. Glycobiology. 2016;26(9):940–9.

11. Wilkop T, Paptallith S, Ren G, Davis DJ, Bao W, Duan D, Peralta AG, Domozycz DS, Hahn MG, Drakakaki G. A hybrid approach enabling large-scale genomic analysis of post-golgi vesicles reveals a transport route for polysaccharides. Plant Cell. 2019;31(3):627–44.

12. Kim S-J, Brandizzi F. The plant secretory pathway: an essential factory for building the plant cell wall. Plant Cell Physiol. 2014;55(4):687–93.

13. Xiong G, Li R, Qian Q, Song X, Liu X, Yu Y, Zeng D, Wan J, Li J, Zhou Y. The rice dnamin-related protein DRP2B mediates membrane trafficking, and thereby plays a critical role in secondary cell wall cellulose biosynthesis. Plant J. 2010;64(1):56–70.

14. Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof YD, Schumacher K, Gonneau M, Hofte H, Vernhettes S. Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. Plant Cell. 2009;21(4):1141–54.

15. Zhang Y, Nikolovski N, Soneul M, Vellisollo S, McFarlane HE, Dupree R, Kesten C, Schneider R, Dremerle C, Lathe R, et al. Golgi-localized STELLO proteins regulate the assembly and trafficking of cellulose synthase complexes in Arabidopsis. Nat Commun. 2016;7:11656.

16. Viotti C, Bubek J, Stierhof YD, Krebs M, Langhans M, van den Berg W, van Dongen W, Richter S, Geldner N, Takano J, et al. Endocytic and secretory traffic in arabidopsis merge in the trans-golgi network/early endosome, an independent and highly dynamic organelle. Plant Cell. 2010;22(4):1344–57.

17. Drakakaki G, van de Ven P, Pan SQ, Miao YS, Wang JQ, Keinath NF, Weatherly B, Jiang LW, Schumacher K, Hicks G, et al. Analysis of the SYP61 compartment reveal its role in exocytic trafficking in arabidopsis. Plant Physiol. 2011;155(3):1115–25.

18. Dahhan DA, Reynolds GD, Cardenas JJ, Eeckhout D, Johnson A, Yperman TD. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science. 2007;315(5813):804–7.

19. Gonneau M, Hofte H, Vernhettes S. Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. Plant Cell. 2009;21(4):1141–54.

20. Li J, Jiang J, Qian Q, Xu Y, Zhang C, Xiao J, Du C, Luo W, Zou G, Chen M, et al. Mutation of rice BC12/GDD1, which encodes a kinesin-like protein that binds to a GA biosynthesis gene promoter, leads to dwarfism with increased biomass content. Plant Physiol. 2011;155(3):626–40.

21. Zhang M, Zhang B, Qian Q, Yu Y, Li R, Zhang J, Liu X, Zeng D, Li J, Zhou Y. Brittle Culm 12, a dual-targeting kinesin-4 protein, controls cell-cycle progression and wall properties in rice. Plant J. 2010;63(3):312–28.

22. Zhou Y, Li S, Qian Q, Zeng D, Zhang M, Guo L, Liu X, Zhang B, Deng L, Liu X, et al. BC10, a DUF266-containing and Golgi-located type II membrane protein, is required for cell-wall biosynthesis in rice (Oryza sativa L.). Plant J. 2009;57(3):446–62.

23. Li YH, Qian O, Zhou YH, Yan MX, Sun L, Zhang M, Fu ZM, Wang YH, Han B, Pang XM, et al. BRITLE CULM1, which encodes a COBRA-like protein, affects the mechanical properties of rice plants. Plant Cell. 2003;15(9):2010–21.

24. Wang Y, Fan C, Hu H, Li Y, Sun D, Wang Y, Peng L. Genetic modification of plant cell walls to enhance biomass yield and biofuel production in bioenergy crops. Biotechnol Adv. 2016;34(5):997–1017.

25. Tanaka K, Murata K, Yamazaki M, Onosato K, Miyao A, Hirochika H. Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. Plant Physiol. 2003;133(1):73–83.

26. Wang D, Qin Y, Fan Y, Jiao L, Peng L, Zhao J, Li X. A Missense mutation in the zinc finger domain of oscesa7 deleteriously affects cellulose biosynthesis and plant growth in rice. PLoS ONE. 2011;6(4): e1053993.

27. Wang D, Yuan S, Yin L, Zhao J, Guo B, Lan J, Li X. A missense mutation in the transmembrane domain of CESAA9 affects cell wall biosynthesis and plant growth in rice. Plant Sci. 2012;196:117–24.

28. Zhang B, Deng L, Qian Q, Xiong G, Zeng D, Li R, Guo L, Li J, Zhou Y. A missense mutation in the transmembrane domain of CESAA4 affects protein abundance in the plasma membrane and results in abnormal cell wall biosynthesis in rice. Plant Mol Biol. 2009;71(4–5):509–24.

29. Updegraff DM. Semimicro determination of cellulose in biological materials. Anal Biochem. 1969;32(3):420–4.

30. Gao Y, He C, Zhang D, Liu X, Xu Z, Tian Y, Liu XH, Zang S, Pauly M, Zhou Y, et al. Two trichome birefringence-like proteins mediate xylan acetylation, which is essential for leaf bight resistance in rice. Plant Physiol. 2017;173(1):470–81.

31. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y, et al. A Roselt CRSIPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant. 2015;8(8):1274–84.