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FLEXIBLE CULM 1 encoding a cinnamyl-alcohol dehydrogenase controls culm mechanical strength in rice

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Abstract Culm mechanical strength is an important agronomic trait in crop breeding. To understand the molecular mechanisms that control culm mechanical strength, we identified a *flexible culm1* (*fc1*) mutant by screening a rice T-DNA insertion mutant library. This mutant exhibited an abnormal development phenotype, including late heading time, semi-dwarf habit, and flexible culm. In this study, we cloned the *FLEXIBLE CULM1* (*FC1*) gene in rice using a T-DNA tagging approach. *FC1* encodes a cinnamyl-alcohol dehydrogenase and is mainly expressed in the sclerenchyma cells of the secondary cell wall and vascular bundle region. In these types of cells, a deficiency of FC1 in the *fc1* mutant caused a reduction in cell wall thickness, as well as a decrease in lignin. Extracts from the first internodes and panicles of the *fc1* plants exhibited drastically reduced cinnamyl-alcohol dehydrogenase activity. Further histological and biochemical analyses revealed that the *p*-hydroxyphenyl and guaiacyl monomers in *fc1* cell wall were reduced greatly. Our results indicated that *FC1* plays an important role in the biosynthesis of lignin and the control of culm strength in rice.

Keywords *FLEXIBLE CULM 1* · Lignin biosynthesis · Cinnamyl-alcohol dehydrogenase · Rice

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Introduction

Culm mechanical rigidity, which is an important trait for crop yield, has attracted the attention of crop breeders for many years. Lodging may cause a reduction of photosynthesis and results in significant yield losses, especially in crop production (Zuber 1973). Therefore, culm mechanical strength has been considered as a crucial agronomic trait in crop breeding. During the growth and development of crops, those varieties with high stem mechanical strength have an ability to adapt to a wide range of abiotic and biotic environmental conditions, including strong winds, swampy substrates, and pathogen infections (Nicholas 1996). In contrast, those varieties that lodge fall down under those growth conditions due to weak stem strength, resulting in great loss of yield. Even when growing in an appropriate environment, crop varieties with weak stem strength always lodge, especially at the late development stage when plants become heavy with grain.

Early studies showed that the lodging-resistant types of rice had a thicker band of sclerenchyma at the periphery of the stem compared with lodging-susceptible strains (Ramaiah and Mudaliar 1934). Recent studies showed that lodging-resistant rice varieties had more vascular bundles in both the peripheral and the inner section of the outer layers, as compared to lodging-susceptible varieties (Chaturvedi et al. 1995). These findings indicate that the thickness of cell walls in the sclerenchyma and the number of vascular bundles are important factors that affect the stem mechanical strength of rice.

The plant cell wall has a strong fibrillar network that provides mechanical support to cells, tissues, and the entire plant body. The cell wall is a highly organized composite structure that contains cellulose, hemicellulose, lignin, polysaccharides, and proteins. The structure and

composition of plant cell walls are ideally suited to their functions (Li et al. 2003). After cellulose, lignin is the second major biopolymer component of the plant cell wall. Lignin plays an important role in the cell wall by cross-linking with cellulose and hemicellulose, and it is crucial for mechanical support, water transport, and defense against pathogens in vascular plants. During cell wall lignification, lignin polymers embed into cell walls and enhance their rigidity. The resistance of xylem to compressive stresses imposed by water transport and the mass of the plants is important to plant's growth and development. In addition, the insolubility and complexity of the lignin polymer makes it resistant to degradation by most microorganisms (Brill et al. 1999; Chabannes et al. 2001; Jones et al. 2001).

Lignins are composed of three main units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. These components originate from the polymerization of the three monolignols, the *p*-coumaryl, coniferyl, and sinapyl alcohols, respectively (Sibout et al. 2005). The types and amount of lignin units vary across plant species, tissues, cell types, and cell wall layers and are influenced by developmental and environmental factors (Campbell and Sederoff 1996; Donaldson 2001). Many genes have been identified in its biosynthetic pathway. Cinnamyl-alcohol dehydrogenase (CAD; EC 1.1.1.195) is an enzyme in the lignin biosynthetic pathway that catalyzes the final reduction of the hydroxyl-cinnamaldehydes to the corresponding alcohols (Mansell et al. 1974). The relative proportions of these cinnamyl alcohols are important factors that determine the structural and mechanical properties of lignin (Mellerowicz et al. 2001; Sibout et al. 2003). CAD genes in different species display distinct features depending on their phylogenetic origin. Duplication of CAD genes has only been observed in angiosperms, such as tobacco (*Nicotiana tabacum*, Knight et al. 1992), lucerne (*Medicago sativa*, Brill et al. 1999), and rice (Zhang et al. 2006).

Many CAD homologs have been isolated from various plant species, and they have been well studied using traditional mutants and transgenic approaches (Ralph et al. 1997; Halpin et al. 1998; Sibout et al. 2005; Zhang et al. 2006). For example, Halpin et al. (1998) reported that the natural CAD-deficient mutant *brown midrib1* (*bm1*) of maize (*Zea mays*) showed a reddish brown pigment in leaf midribs and stem sclerenchyma; *bm1* had a 20% decrease in lignin content and better digestibility. Using an antisense transgene strategy, down-regulated CAD plants have been produced in many species, including alfalfa (*M. sativa*), poplar (*Populus* spp.), and tobacco. The lignin content of those plants was only modestly affected, and abnormal lignin monomers become incorporated in the lignin, making it more extractable (Halpin et al. 1994; Baucher et al. 1996, 1999).

The completion of *Arabidopsis* and rice whole-genome sequencing and annotation projects (Arabidopsis Genome Initiative 2000; International Rice Genome Sequencing Project 2005) affords opportunities to conduct genome-wide analyses of CAD family members. In *Arabidopsis*, Sibout et al. (2003) reported that there were nine CAD-like proteins distributed among four classes based on their amino acid similarity. Studies on AtCADC and AtCADD revealed that both acted as the primary genes involved in lignin biosynthesis in the floral stem of *Arabidopsis* by supplying both coniferyl and sinapyl alcohols (Sibout et al. 2005). Tobias and Chow (2005) recently reported that there were 12 CAD family members in rice, and four closely related members at the same locus may have been created by a recent gene duplication followed by inversion. *OsCAD2* was cloned using the *gold hull and internode2* (*gh2*) mutant by a map-based cloning approach (Zhang et al. 2006). The mutant *gh2* showed reddish brown pigment in the hulls and internode, suggesting it is an orthologous gene of the *bm1* in rice. *GH2* was confirmed to be a multifunctional enzyme, which had both CAD and sinapyl alcohol dehydrogenase (SAD) activity.

In this study, we report on the identification of the T-DNA-tagged rice mutant *flexible culm 1* (*fc1*), whose phenotype displays a dramatic reduction in mechanical strength. Isolation and analysis of the T-DNA flanking sequence from the mutant revealed that the interruption caused a deficiency of the *CAD* gene *OsCAD7* (Loc_Os04g52280). Cosegregation analysis revealed the mutant phenotype cosegregated with the insertion of T-DNA. Our findings indicated that FC1 had strong CAD activity and weak SAD activity, which influenced the mechanical strength of rice culms. Identification of the *FC1* gene and its role in cell wall biosynthesis in rice is an important step toward understanding the manipulation of culm strength mechanisms for breeding genetic improved crops.

Material and methods

Plant materials and growth conditions

The transgenic rice plants (*Oryza sativa* L. ssp. *japonica*) used here were developed by Wu et al. (2003). A total of 3,000 T_1 families were planted in the experimental field of Huazhong Agriculture University in Wuhan, China, during the rice-growing season of 2004 with a planting density of 23 plants m^{-2}. The plants were screened mainly for conspicuous morphological changes in the plant mechanical strength characters.

Genotyping of *fc1* mutant plants

PCR genotyping of *fc1*-segregating population was performed by PCR using the following primers: a,

5′-GCGACCATTCTGGCTGTGAT-3′ and b, 5′-AACCTG AAACGGCGGTAGTG-3′ and c, 5′-AATCCAGATCCCC CGAATTA-3′. The PCR was conducted with an initial step of 94°C incubation for 2 min, followed by 30 cycles of 94°C for 1 min, 56°C for 45 s, and 72°C for 1.2 min; with a final extension at 72°C for 10 min.

Measurements of physical properties

The breaking force of *fc1* and wild-type culms ($n = 5$) was measured with a Microforce Tester (model 5848; Instron, High Wycombe, UK). To avoid inaccuracies from sampling, fresh first and second internodes of culms were used for measurement.

Scanning electron microscopy

Samples were prepared as described previously (Mou et al. 2000), with some modifications. Briefly, rice tissues were excised with a razor and immediately placed in the fix solution (70% ethanol, 5% acetic acid, and 3.7% formaldehyde) for 24 h. Samples were dried to the critical point, sputter-coated with gold, and observed with a scanning electron microscope (S570; Hitachi, Tokyo, Japan).

Measurement of cell wall composition

Internodes were collected after the removal of leaves, leaf sheaths, and seeds at harvest stage. The fresh and dry weight (in an oven at 80°C for 3 days) of internodes were measured for calculation their water content. The dried internodes were milled and the powder was sieved through a 0.5 mm screen, washed in phosphate buffer (50 mM, pH 7.2) three times, extracted with 70% ethanol twice at 70°C for 1 h, and vacuum-dried. The dried cell wall materials were assayed for cellulose content with the anthrone reagent (Updegraff 1969) with Whatman 3MM paper as the standard (Whatman, Maidstone, UK). The determination of lignin content was performed on the extract-free samples using the standard Klason procedure (Sibout et al. 2005). The evaluation of lignin structure was assayed using the DFRC protocol (Lu and Ralph 1997).

Plant protein extraction and enzyme assay

Plant total proteins were extracted using the method described by Zhang et al. (2006). Fresh tissues (about 500 mg) at the heading stage were separately milled to a fine powder in liquid nitrogen, then extracted with 1,000 µl extraction buffer (100 mM Tris–HCl, pH 7.5; 2% polyethylene glycol 6000, 5 mM dithiothreitol, 2% polyvinylpolypyrrolidone) for 2.5 h at 4°C. The crude extract was clarified by centrifugation twice (10,000×g for 10 min) at 4°C. The supernatant was decanted and about 20 µg of total proteins were used for the CAD and SAD activity assays. Assays of crude enzyme were carried out in the reverse direction using coniferyl alcohol and sinapyl alcohol as the substrates, and the formation of hydroxy-cinnamaldehydes was monitored spectrophotometrically (DU640; Backman, Fullerton, USA) at 400 nm using the following molar extinction coefficients: coniferaldehyde 2.10×10^{-4} M^{-1} cm^{-1} and sinapaldehyde 1.68×10^{-4} M^{-1} cm^{-1}, as described by Sibout et al. (2003). Assays were carried out at 30°C for 10 min in 100 µl of 100 mM Tris–HCl (pH 8.8), 100 µM NADP, and 250 µM coniferyl alcohol or sinapyl alcohol. The enzyme reactions were initiated by enzyme addition and stopped by holding at 85°C for 10 min. An assay without NADP was used as the control. Protein concentration was determined using the Bradford reagent (Coomassie plus protein assay reagent, Pierce, Rockford, USA). Resulting units are defined as the amount of activity that converts 1 nmol of hydroxycinnamyl alcohol into the corresponding aldehyde per second (1 n Katal) per microgram of crude protein extract.

Histochemical staining

For histochemical localization of the accumulated lignin, phloroglucinol staining was performed according to the method described by Zhang et al. (2006). Fresh hand-cut sections from the second internode of rice plants at the heading stage were incubated for 10 min in phloroglucinol solution (1% in 70% ethanol), the phloroglucinol was removed and treated with 18% HCl for 5 min, then photographed under a light microscope (model DM4000B; Leica, Germany). Mälue staining was performed by first incubating sections in 0.5% $KMnO_4$. After 10 min, sections were washed with water and treated with 10% HCl for 2 min, washed with water again, incubated in concentrated $NH_3 \cdot H_2O$, and photographed immediately under a light microscope as above.

DNA extraction and Southern hybridization

Fresh leaves were harvested from field-grown plants, and genomic DNA was extracted by using the CTAB method (Murray and Thompson 1980). DNA digestion, Southern blotting, and hybridization were conducted following the protocol described by Liu et al. (1997).

Gene expression analysis

To extract total RNA of various tissues from Zhonghua 11 (*O. sativa* ssp. *japonica*), the Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used according the manufacturer's instructions. Gene expression was analyzed three

times by RT-PCR. Before the RT step, total RNA was treated with amplification-grade DNase I (Invitrogen) for 15 min to degrade possibly contaminating residual genomic DNA. The synthesis of the cDNA first strand was carried out with Molony Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega Corp., Madison, WI, USA) according the manufacturer's instructions. An aliquot (1 μl) of the reaction mixture was used for PCR. The PCR was performed in an ABI 9700 thermocycler (Applied Biosystem) with the following cycling profile: 94°C for 3 min; 30 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min; and 72°C for 5 min. Next, 15 μl of the PCR product was separated in a 1.0% agarose gel and stained with ethidium bromide for visualization. The primers for the *FC1* gene were FCRTF (5′-TAGCCAGCTAGCAACC GACAA-3′) and FCRTR (5′-CCGCCGGAGCAGTAGTT CT-3′). The actin primers used as standard in all the RT-PCR assays were ACTF (5′-TGCTATGTACGTCGCCATCC AG-3′) and ACTR (5′-AATGAGTAACCACGCTCCG TCA-3′).

In situ hybridization

The RNA in situ hybridization and immunological detection were performed according to De Block and Debrouwer (1993). The cDNA clone EI107N06 was used as a template to generate RNA probes. Transverse sections (10 μm thick) were probed with digoxigenin-labeled antisense probes (DIG Northern Starter Kit, Roche, Indianapolis, IN, USA). The slides were observed with a microscope (model DM4000 B, Leica) and photographed using a 3CCD color video camera (model DFC480, Leica).

Vector construction and rice transformation

An 8.8 kb genomic DNA fragment containing the entire *FC1* coding region, the 3,253 bp upstream sequence, and the 1,646 bp downstream sequence was inserted into the binary vector pCAMBIA2301 to generate the transformation plasmid pCFC1 for the complementation test. An empty pCAMBIA2301 vector was used as a control. The entire *FC1* coding region was amplified using primers FC1OEF (5′-GAT**CCCGGG**GCCGCCACTGGCTACT-TAAA-3′ with a *Sma*I site in bold letters) and FC1OER (5′-AAA**CCCGGG**TGGCTCGTTCTTGGATAGGA-3′ with a *Sam*I site in bold letters) for overexpression analysis. PCR products were digested by *Sma*I and inserted into the binary vector PU1301 (contains a maize ubiquitin promoter) in the correct direction (Chu et al. 2006). The two binary plasmids were introduced into *A. tumefaciens* EHA105 by electroporation, and the rice transformation was according to the method of Hiei et al. (1994).

Results

Morphological characterization of the *fc1* mutant

To understand the mechanisms that regulate stem mechanical strength and the biosynthesis of plant cell walls, we identified a *flexible culm* (*fc1*) mutant (Fig. 1a) by screening about 3,000 T_1 transformant families from our T-DNA insertional mutant library (Wu et al. 2003). Compared with the wild-type variety, Zhonghua 11, *fc1* plants exhibited a reduction in mechanical strength and a slight delay in growth that resulted in one week delay in heading date. At the heading stage, the length of mutant plants was decreased to about 80% of that in wild-type plants, and some of the third nodes bent downward compared to those of the wild-type (Fig. 1b). We also investigated the changes of some agronomic traits of *fc1*. The panicle length and tiller numbers of *fc1* were similar to those of the wild-type. The fertility and 1.000-grain weight of *fc1* plants were decreased by 9.4 and 28.8%, respectively, which resulted in a reduction in yield per plant to about 64% of the wild-type. This yield reduction in *fc1* may be caused by its retarded growth habit and related small plant size. The details of the agronomic traits of *fc1* compared to the wild-type are shown in Table 1.

We quantitatively compared *fc1* mutant and wild-type plants with regard to the forces required to break the segments of internodes. The forces required to break the first and second internodes of *fc1* mutant were decreased to 34% and 28% of those required for the wild-type, respectively (Fig. 1c). The dramatic decrease in the breaking forces of the *fc1* mutant suggests that it has altered mechanical tissues in the cell wall structure.

The *fc1* mutant plant has altered mechanical tissues

To reveal whether the reduction of mechanical strength of culms was caused by alterations in cell wall structure, composition, or fiber length, we examined transverse sections of culms by scanning electron microscopy. The mechanical support of plants arises mainly from several mechanical cell layers, especially those around the peripheral vascular tissues and under the epidermal layer in culms. At the heading stage, the sclerenchyma cell walls of culms (Fig. 2a) were dramatically thickened and nearly completely filled in wild-type rice. However, no significant thickening of the sclerenchyma cell walls was observed in the *fc1* plants (Fig. 2b). In addition, the structure of peripheral vascular tissues in *fc1* was much looser than those in the wild-type, and the shape of vascular tissues in *fc1* also differed from that of the wild-type (Fig. 2b). These results indicated that the reduction in the mechanical strength of *fc1* plants likely resulted from a defect in the

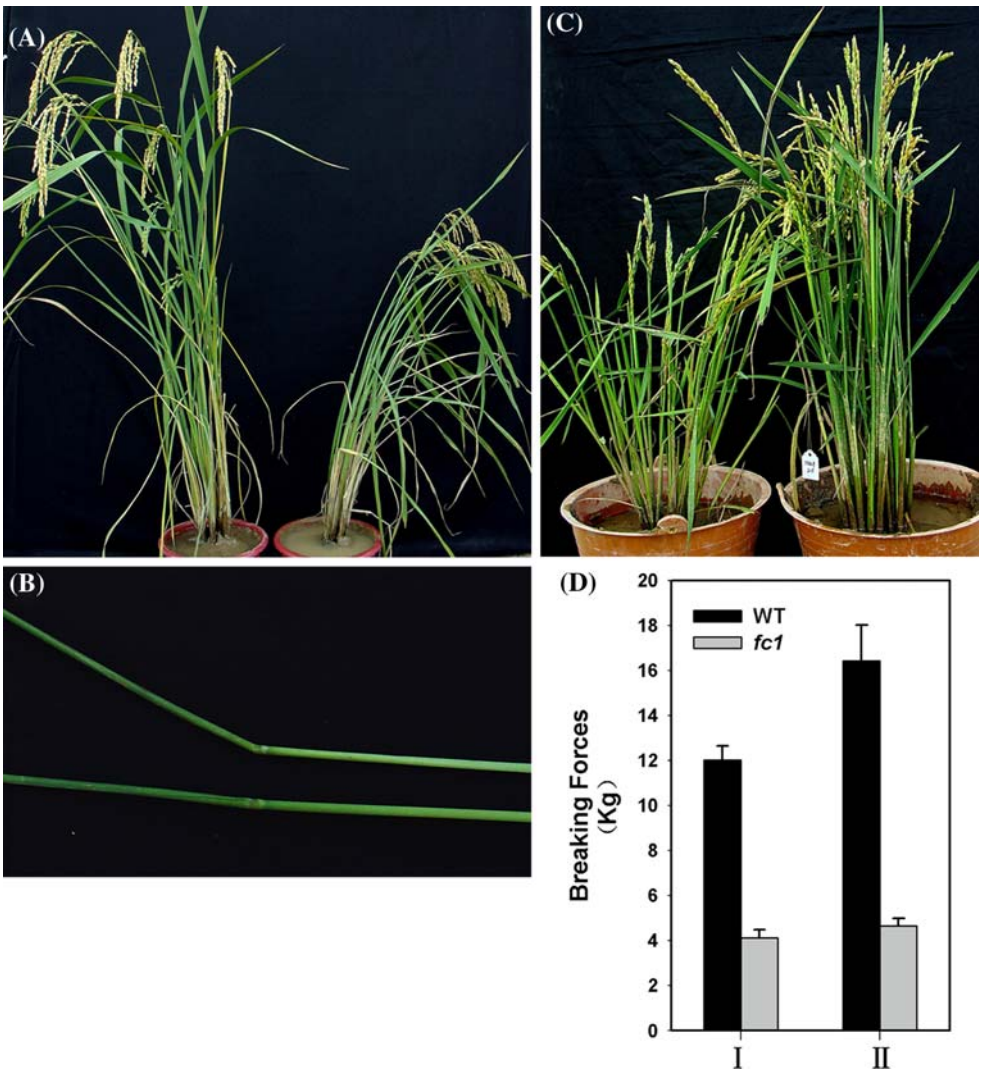

Fig. 1 Phenotypes and physical properties of wild-type, *fc1* mutant plants and genetic complementary plants. **a** The phenotypes of wild-type (left) and *fc1* mutant (right) at the heading stage. **b** The internodes of the wild-type (below) and *fc1* mutant (upper) at the heading stage. **c** *fc1* mutant transformed by empty pCAMBIA2301 vector (left) and PCFC1 (right). **d** The force required to break first (I) and second (II) internodes of wild-type and *fc1*

Table 1 Comparison of agronomic traits of *fc1* and its wild-type under field conditions (Wuhan, China, 2006)

Plants	Plant height (cm)	Panicle length (cm)	Tillers/plant	Fertility (%)	1,000-grain weight (g)	Yield/plant (g)
Wild-type	109.0 ± 3.9	22.3 ± 0.8	11.0 ± 1.6	83.0 ± 5.4	21.9 ± 1.1	26.1 ± 4.9
fc1	73.8 ± 3.3	20.3 ± 0.7	12.0 ± 3.4	73.6 ± 5.2	15.0 ± 0.7	16.7 ± 4.4

The data correspond to the mean ± standard error from 18 individuals

cell wall mechanical tissues, such as sclerenchyma cells and peripheral vascular tissues.

The *fc1* mutant plant has altered cell wall structure

As the main composition of cell wall, cellulose and lignin link with other cell wall components to provide mechanical strength to plants. Therefore, we compared the cellulose and lignin contents in culms from mutant and wild-type plants. The amount of cellulose in *fc1* culms was reduced to about 86% of the wild-type, and the amount of Klason lignin in *fc1* culms was reduced to about 82% of the wild-type. In addition, the water content of the fresh culms of *fc1* was increased by about 12% compared with that of wild-type. The lignin composition of the wild-type and *fc1* culms was also detected by the derivatization followed by reductive cleavage (DFRC) method. The results showed that rice lignin consists of the three basal monomers, the H, G, and S monomers (Table 2). In wild-type rice, the G monomer was the major component and the G:S ratio was 9.57 in the internodes. In the *fc1* plants, the H monomer was reduced greatly (by ~36.84%); the G and S monomers

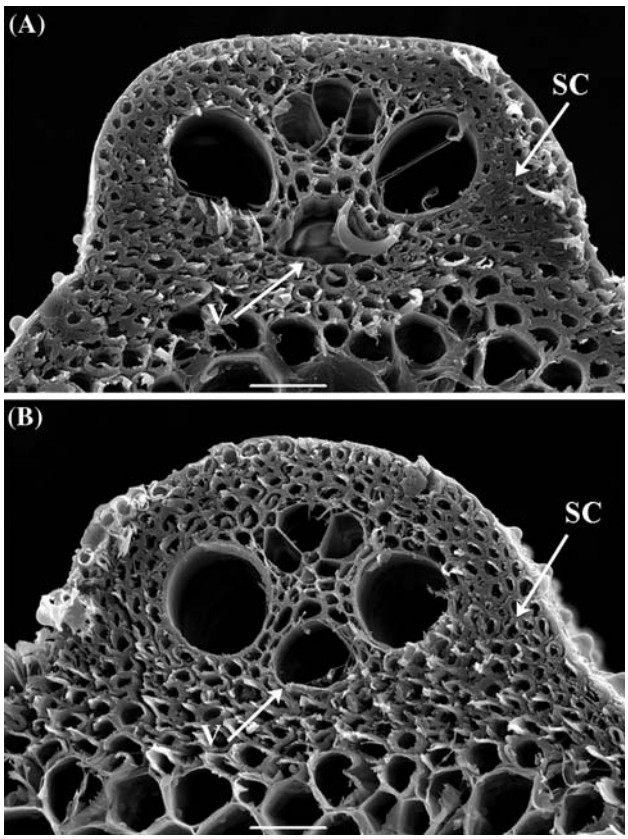

Fig. 2 Scanning electron micrographs showing the differences between sclerenchyma cells (SC) and vascular bundles (V) in wild-type and *fc1* plants. **a** Cross-section of a wild-type culm. **b** Cross-section of an *fc1* mutant culm. Bars = 20 μm

were reduced slightly (by ~19.9 and 16.69%, respectively), resulting in a G:S ratio of 9.20. The reduction of all three monomers was also observed in *gh2* plants (Zhang et al. 2006), but the reductions of H, G, and S monomers in the same tissues were of a comparable degree. The uneven reduction of three monomers in *fc1* mutant indicated that the *FC1* gene is necessary for normal synthesis of H, G, and S monomer precursors in the internodes.

To further examine whether the alterations of cellulose and lignin were localized in particular cells, we examined histochemical transverse sections of the second culm internodes of wild-type and mutant plants stained with phloroglucinol reagent and Mäule solutions. Wiesner stain is known to react with cinnamaldehyde residues in lignin, and the color intensity consistent with the total lignin content. There were obvious color differences in mechanical tissues between wild-type (Fig. 3a, c) and mutant (Fig. 3b, d) plants, especially in the sclerenchyma cells below the epidermis. The cortex and vascular bundle regions were stained dark red in wild-type, but were light red in *fc1* mutant (Fig. 3). These results indicated a decrease in lignin quantity in mutant plants. In addition, compared with the thickened walls of sclerenchyma cells of the wild-type (Fig. 3e, f), those of mutant plants were thin (Fig. 3). This finding was consistent with the scanning electron microscopy observations (Fig. 2) and indicated that the *fc1* mutant was deficient mainly in the secondary cell walls.

The lignin composition of the wild-type and *fc1* plants was also estimated by Mälue staining. The Mäule reagent stains G residues yellow and S residues red. The fresh hand-cut internode sections of wild-type plants were stained reddish yellow in the cortex region and pure yellow in the vascular bundle region, indicating that cortex region contained abundant G monomers and a small amount of S monomer, while the vascular bundle region mainly contained G monomers (Fig. 3g). In the corresponding tissues of the *fc1* plants, the cortex region and vascular bundle region were stained pure light yellow (Fig. 3h), suggesting that G monomer was the major monomer in *fc1*. The staining degree is decreased in the *fc1* internodes compared with that of the wild-type internodes (Fig. 3h), indicating the G and S monomers decreased greatly in *fc1* plants. This result fit well with the previous measurement using the DFRC method.

FC1 encodes a CAD protein

The copy number of integrated T-DNA in *fc1* was detected by DNA blot hybridization with the GAL4/VP16 fragment as the probe; it was found to be a single copy insertion event (data not shown). We amplified the flanking region of the inserted T-DNA in *fc1* by thermal asymmetric interlaced PCR (TAIL-PCR; Liu et al. 1995). Sequence analysis

Table 2 Klason and DFRC analysis of the culms of wild-type and *fc1* mutant

Plants	Lignin content[a]	Lignin monomer composition			
	(% CWR)	Total H monomers (μmol g^{-1} Klason lignin)	Total G monomers	Total S monomers	G:S
Wild-type	17.29 ± 0.29	40.88 ± 4.81	128.47 ± 6.02	13.42 ± 0.12	9.57
fc1	14.98 ± 0.64	25.82 ± 3.53	102.86 ± 8.39	11.18 ± 1.17	9.20

The data correspond to the mean ± standard error from triplicate analyses

[a] Klason lignin content (percentage of weight of the extract-free dried cell wall from the internode)

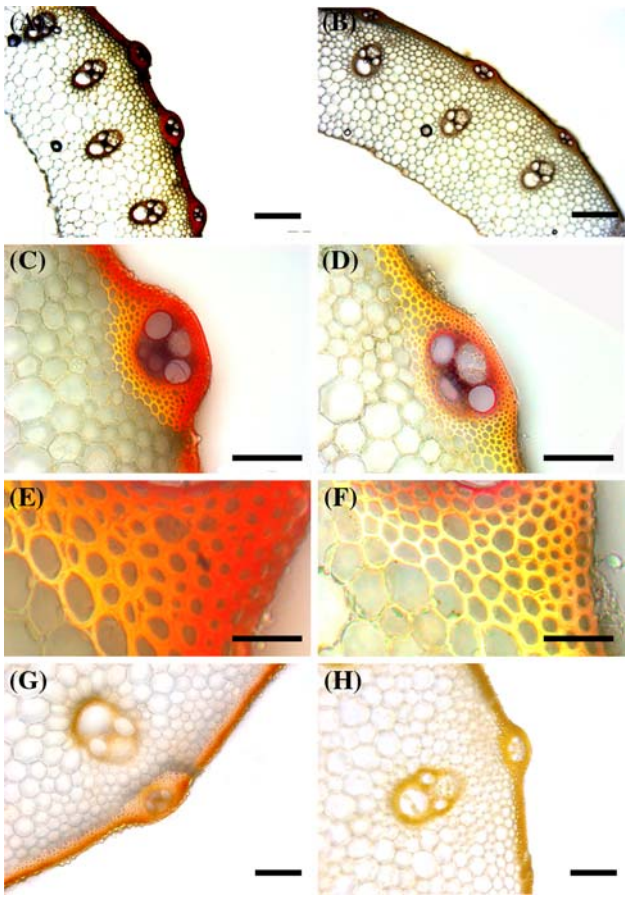

Fig. 3 Histochemical staining of wild-type and *fc1* plants with phloroglucinol and Mälue reagent. Phloroglucinol staining of the transverse internode sections of wild-type (**a**) and *fc1* (**b**) plants (bars = 400 μm), and magnified sections (**c**, **d**) showing the decreased level of lignin in the walls of sclerenchyma cells and vascular bundles in the mutant culm (bars = 50 μm). (**e**, **f**) Magnified sections of (**c**) and (**d**) showing the reduced thickness of the sclerenchyma cell walls of *fc1* (bars = 20 μm). Mälue staining of transverse internode sections of wild-type (G) and *fc1* (H; bars = 100 μm)

of that region revealed that T-DNA was inserted into a gene located on chromosome 4 in the BAC clone OSJNBa0070C17. Its full-length cDNA was identified as AK102452 in the KOME (Knowledge-Based Oryza Molecular biological Encyclopedia) rice full-length cDNA database (http://cdna01.dna.affrc.go.jp/cDNA). The transcript of the tagged gene was 1,329 bp long, including a 1,140 bp coding sequence. The locus name of this gene had been registered as LOC_Os04g52280 in The Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml). The primary structure of the gene comprised five exons and four introns (Fig. 4a). The T-DNA insertion was located at 1,576 bp downstream from the ATG start codon, in the first intron of the gene (Fig. 4a). The predicted protein encoded by the tagged gene contains 379 amino acid residues with a molecular mass of 39.6 kD. BLAST analysis indicated that the protein belonged to the CAD gene family and was named as OsCAD7 by Tobias (Tobias and Chow 2005). We renamed the gene *FC1* (*Flexible Culm1*) according to its mutant phenotype.

The cosegregation assay of T-DNA tag with the mutant phenotype was performed by PCR analysis of T_1 and T_2 families. We isolated genomic DNA from 20 seedlings of T_1 or T_2 families to determine their homo- or heterozygous genotype by PCR reaction using the primers a, b, and c. As shown in Fig. 4b, c, the DNA fragments of 1.0 and 0.8 kb from the primer combinations of a + b and a + c, respectively, should be amplified if the *fc1* locus was heterozygous, and single PCR fragment amplified only by a + c combination should be produced from a homozygous seedling because the amplification fragment expected from a + b combination is too large to be amplified. As we expected, 10 and six transgenic plants were found to be heterozygous and homozygous, respectively, and four transgenic plants were wild-type. The six homozygous *fc1* mutants showed a semi-dwarf and flexible culm phenotype, whereas the heterozygous and wild-type plants grew normally (Fig. 4c). We concluded that the T-DNA insertion cosegregated with the mutant phenotype, and this flexible culm phenotype segregated as a single recessive mutation.

To further confirm that the gene correspond to the *fc1* mutant phenotype, we performed a genetic complementation experiment. Construct pCFC1 containing the entire open reading frame, 3,253 bp upstream sequences, and the 1,646 bp downstream region and an empty pCAMBIA2301 vector were introduced into the *fc1* mutant by *Agrobacterium tumefaciens*—mediated transformation. The flexible culm phenotype of *fc1* was rescued in 28 of 42 independent transgenic plants with pCFC1 (Fig. 1c); all eight plants regenerated with empty vector failed to rescue the *fc1* mutant. The next progeny family from the rescued plants also showed the cosegregation wild phenotype with *FC1* gene. These results confirm that the *fc1* phenotype is caused by the loss of function of the rice *FC1* gene.

FC1 expression pattern

We attempted to examine the expression pattern of *FC1* in various rice organs with RNA gel-blot analysis, but it could not be detected due to its low expression level. Reverse transcription (RT)-PCR revealed that *FC1* was expressed a bit more strongly at the seedling stage than at the heading stage, and expression levels were similar in leaf, stem, and root (Fig. 5a). At the heading stage, a strong *FC1* expression level was detected in the first internodes, panicles, and roots (Fig. 5b). These expression patterns are closely related to the performance of *FC1* in rice during growth

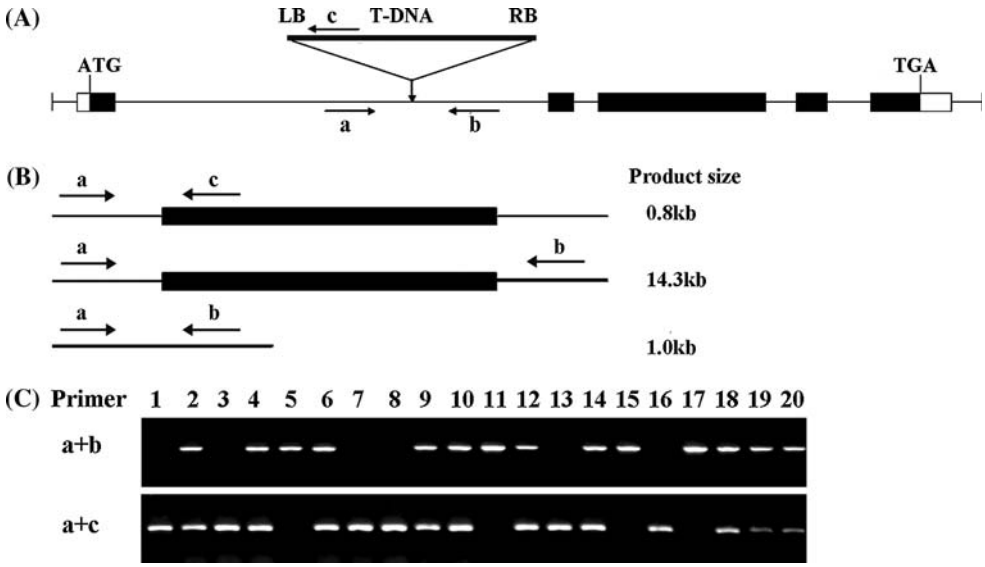

Fig. 4 Molecular analysis of the *fc1* mutant. **a** Structure of the *FC1* gene and position of T-DNA insertion. Black boxes indicate exons encoding the protein, and open boxes indicate the 5′ and 3′ UTR regions. Arrows identify primers for genotyping T-DNA insertion. **b** Schematic diagrams of the genotyping: a, forward primer generated from the first intron region of *FC1* gene; b, reverse primer generated from the first intron region of the *FC1* gene; c, reverse primer from the T-DNA region. Combination of a + c primers produces a 0.8 kb PCR fragment; the T_1 plants that allow this amplification could only be homozygous because amplifying the 14.3 kb fragment by the combination of a + b primers is too large. Wild-type plant, however, allows the production of a single 1.0 kb PCR fragment by the a + b primer combination because there is no T-DNA insertion in the genome, while heterozygous T_1 plants allow both 0.8 kb and 1.0 kb PCR amplifications by a + c and a + b combinations, respectively. **c** Confirmation of cosegregation of T-DNA insertion with flexible phenotype in 20 T_1 plants. Lanes 1, 3, 7, 8, 13, and 16 are homozygous; lanes 2, 4, 6, 9, 10, 12, 14, 18, 19, and 20 are heterozygous; and lanes 5, 11, 15, and 17 are wild-types

and development. The null of *FC1* in stem and flower caused reduced mechanical strength and fertility of *fc1*.

The precise expression patterns of *FC1* were explored further by RNA in situ hybridization. *FC1* was expressed mainly in the highly lignified cells, including the vascular bundles and the sclerenchyma cells below the epidermis in stem (Fig. 5c–f). The expression pattern of *FC1* in these tissues explained the deficiency in lignin biosynthesis observed with scanning electron microscopy and histochemical staining.

CAD activities assay in total proteins

To explore the catalytic ability of native FC1 in rice, we assayed CAD and SAD activities of the total proteins in various organs from wild-type and *fc1* plants at the heading stage. Strong CAD activity and weak SAD activity were detected in all tissues of the wild-type and *fc1* plants (Fig. 6). The CAD activity was stronger in the first internodes, leaf (blade and midrib), and leaf sheath. The CAD and SAD activities were dramatically reduced in the first internodes of *fc1* compared with wild-type plants. In contrast, in leaves, second internodes, and third internodes, in which *FC1* gene had a relatively lower expression level, the CAD and SAD activities were slightly reduced. The reductions were consistent with the expression pattern of *FC1* described above (Fig. 5b). These results indicated that FC1 protein had strong CAD activity and weak SAD activity. In some tissues, such as the leaf, leaf sheath, the second and third internodes, in which the *FC1* gene were only slightly expressed, the total proteins of the *fc1* mutant still exhibited nearly the same or slightly lower CAD activity compared to wild-type plants. The reduction of CAD activity depended greatly on tissue type in *fc1* plants. It was concluded that additional CAD isozymes with CAD activity were also unevenly distributed in various tissues. Based on the *FC1* expression pattern, its CAD activity sites, and *fc1* mutant phenotype, we conclude that FC1 plays an important role in lignin biosynthesis in the culm of rice, especially in the first internodes, and *FC1* deficiency in the first internodes is the main cause of the lodging trait in *fc1* plants.

Overexpression of *FC1* does not affect the cell wall composition

We also studied the cell wall composition in the internode of *FC1*-overexpressing transgenic plants. A total of 14 independent transgenic plants were generated for the *FC1* overexpression. Among them, seven plants showed

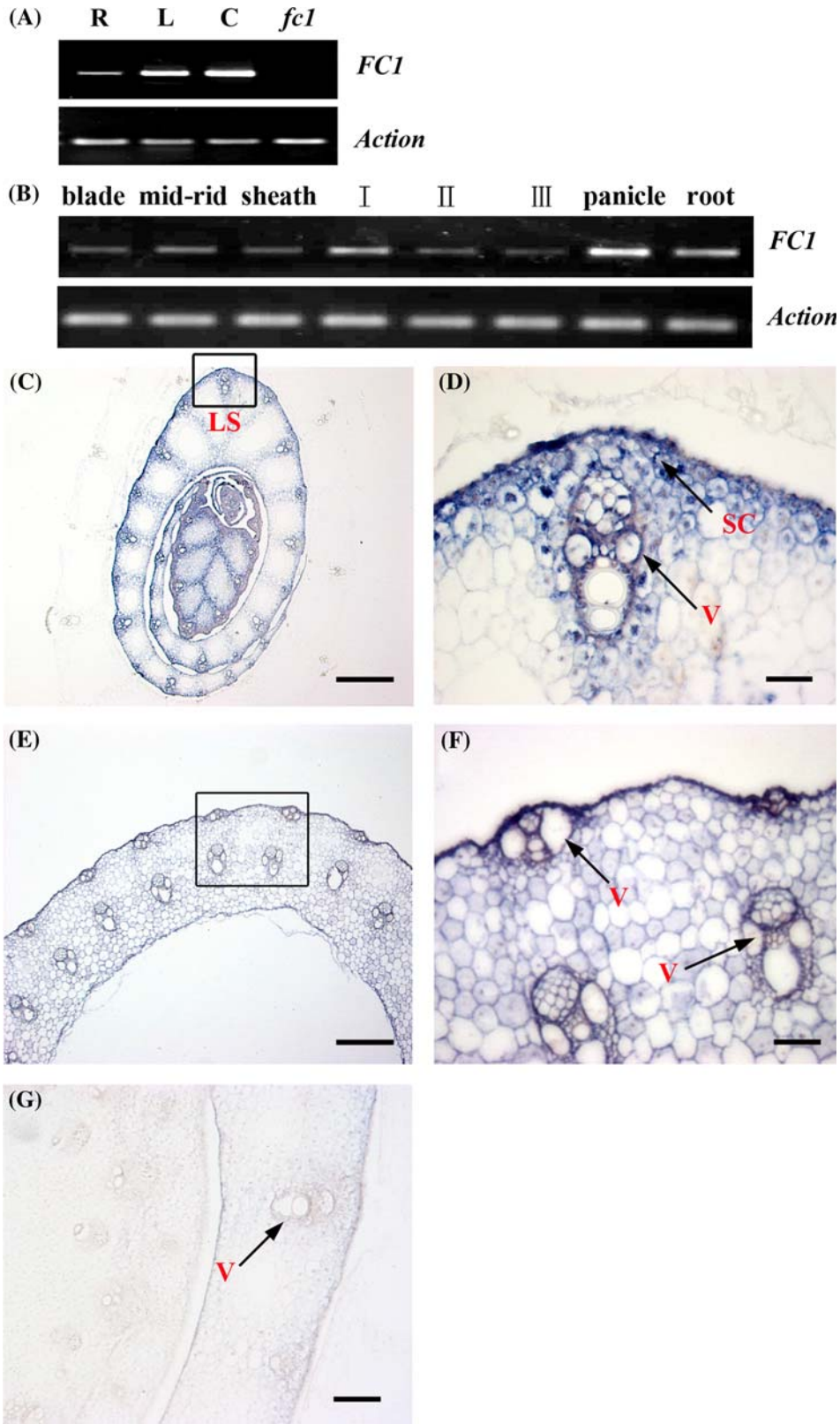

Fig. 5 *FC1* expression analysis and RNA in situ hybridization in transverse sections of wild-type rice plants. **a** RT-PCR analysis of *FC1* expression in wild-type and *fc1* mutant. Total RNA samples were isolated from roots (R), leaves (L), culms (C) of wild-type plants, and from leaves of *fc1* mutant at the seedling stage. Amplification of *actin* cDNA was used to ensure that approximately equal amounts of cDNA were loaded. **b** RT-PCR analysis of *FC1* expression in different tissues of the wild-type at the heading stage. Amplification of *actin* cDNA was used to ensure that approximate equal amounts of cDNA were loaded. The tissue include leaf blades, midribs, sheaths, first internodes (I), second internodes (II), third internodes (III), panicle, and roots. The leaves eliminated of midrib (blades), midribs, and sheaths were all derived from flag leaves. **c** Leaf and leaf sheath (bars = 500 μm). **d** A magnified section from (C) (bars = 50 μm). **e** Stem (bars = 500 μm). **f** A magnified section from (E) (bars = 100 μm). **g** Background control, in situ hybridization of young leaf sheath with a sense probe (bars = 100 μm). Sc, sclerenchyma cells; V, vascular bundles

overexpression of *FC1*, as determined by RNA gel-blot analysis (Fig. S1). The transverse sections of the culms of the *FC1*-overexpressing plants were histochemically stained with phloroglucinol reagent, but we did not find obvious difference compared to the wild-type phenotype (Fig. S1). We inferred that the overexpression of *FC1* did

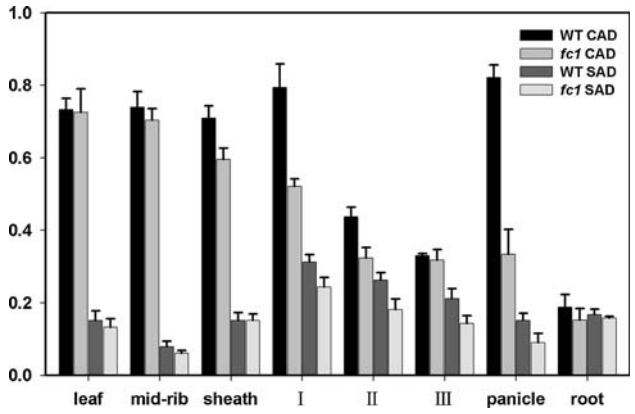

Fig. 6 CAD activity and SAD activity of the total proteins from different tissues of the wild-type and *fc1* plants at the heading stage. The standard error bars were obtained from triplicate independent measurements. The tissues include leaf blades, midribs, sheaths, first internodes (I), second internodes (II), third internodes (III), panicle, and roots. The leave blades, midribs, and sheaths were all derived from flag leaves

not alter the cell wall composition. This may be because normal FC1 activity is high enough so that it does not represent a rate limitation. There are many enzymes involved in the biosynthesis of monolignols, and one or more of these steps may limit flux through the monolignol pathway.

Discussion

In this study, we identified a flexible culm rice mutant caused by T-DNA insertion into a CAD-encoding gene. We studied the phenotype of the *fc1* mutant, *FC1* gene cloning, and its expression pattern, especially in the mechanical tissues. Based on knockout of the *FC1* gene, we deduced that the reduction of CAD activities in all the examined tissues altered the biosynthesis of lignin and cellulose, resulting in a reduction of secondary cell wall thickness and decreased mechanical strength of rice plants.

FC1 plays an important role in plant mechanical tissue development

The results from scanning electron microscopy and histochemical analyses indicated that the mechanical tissues of *fc1* plants were altered greatly. The deficiency of FC1 in *fc1* mutant at the early stage and the heading stage caused a slight delay in growth and a notable reduction in mechanical strength. These phenomena were not found in the other rice CAD-deficient mutants. For example, the deficiency of *GH2* caused golden hull and internodes, and the development of *gh2* was nearly normal (Zhang et al. 2006). There are significant differences in phenotypes of *fc1* and *gh1* and the expression patterns of *FC1* and *GH1*. *GH2* could be detected only at the heading stage. The expression pattern of *GH2* is consistent with the phenotype of *gh2* (Zhang et al. 2006). In contrast, the expression level of *FC1* gene is higher at the seedling stage than at the heading stage (data not shown). The expression pattern of *FC1* indicated it may play other roles in plant development, such as controlling plant height and determining flower fertility, because *fc1* plants showed retarded growth at the vegetative stage and decreased fertility in panicle development. The great phenotypic differences between *fc1* and *gh2* mutants may due to the different expression patterns of *FC1* and *GH2*.

The previous phylogenetic analysis of CAD family members indicated that they could be divided into two subfamilies. SubA members are evolutionarily conserved CAD genes in gymnosperms and angiosperms and comprise only *GH2* in rice. *GH2* was regarded as the only primary enzyme that catalyzes the last step of lignin precursor biosynthesis (Zhang et al. 2006). SubB is an angiosperm-specific subfamily that is regarded as the duplication of SubA, and the members were likely to gain new functions over evolutionary time (Zhang et al. 2006). According to the histochemical and molecular analyses of *FC1* in this study, our findings indicate clearly that *FC1* also plays important roles in plant development, especially in lignin biosynthesis in rice. It seems likely that some of the SubB members in rice retained lignin biosynthesis function and exhibit the ability to enhance cell wall thickness.

The *fc1* mutant has a delay in growth, combined with reduced size and mechanical strength. Similar growth characteristics have been described in the *cad-c cad-d* double mutant of *Arabidopsis* (Sibout et al. 2005). Xylem elements in the *cad-c cad-d* double mutant were distorted and collapsed, however, and these traits were not found in *fc1* plants. This may be due to the different structure of vascular tissues in rice and *Arabidopsis*. Whether the alteration in the conducting vessels could impact water and nutrient movements and thus affect the growth habit of the *fc1* mutant requires further study. Another possible explanation for the delay in growth and shorter stems in *fc1* could be the deficiency of lignin biosynthesis. In transgenic plants inhibited in the expression of one lignin biosynthetic gene (by gene knockout or RNAi technology), a wide range of abnormal phenotypes was observed, but few clear relationships could be established between the extent of lignin decrease or modification and the observable phenotypes (Boerjan et al. 2003). The lignin content of the hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) repression in *Arabidopsis* was decreased greatly, and flavonoid accumulated in HCT^{-} plants, which was responsible for the inhibition of auxin transport and the

reduction of plant growth (Besseau et al. 2007). Flavonoid accumulation and auxin transport in the *fc1* mutan should be further studied.

Altered cell wall composition of *fc1* leads to reduced mechanical strength of the culm

Our results clearly demonstrated that mutations in *FC1* affected the biosynthesis of secondary cell walls and resulted in alterations in the contents of cellulose and lignin. The great differences between the *fc1* mutant and the wild-type were localized mainly in the supportive and conductive tissues, such as sclerenchyma cells and cortical vascular bundles. During biosynthesis of the plant secondary cell wall, lignin imparts mechanical strength and water resistance. Thus, a deficiency of lignin may cause stem weakness as well as affecting water and nutrient transport.

Similar growth characteristics have been observed in other mutants with reduced mechanical strength. For example, the rice *brittle culm1* (*bc1*), that has 43% lower stem strength than wild-type, has reduced cellulose content of 70% and increased lignin content of 30% (Li et al. 2003). *Arabidopsis irx4* mutant, which has reduced mechanical strength of 65%, has reduced lignin content of 50% (Jones et al. 2001). The altered cellulose and lignin content in these mutants suggests that proper mechanical strength requires a precise balance between the cellulose and lignin content in plants. In addition, the relatively high water content in *fc1* indicates a loose mechanical structure, which is also one of the causes of the decreased mechanical strength.

Defects in the secondary cell wall are characterized by a collapse of xylem vessels that are unable to withstand the negative pressure generated during water transport through the xylem. This phenotype, described as irregular xylem (*irx*), has been used to identify *Arabidopsis* mutants defective in the biosynthesis of both cellulose (Turner and Somerville 1997) and lignin (Jones et al. 2001). Some *irx* mutants were also found by using expression profiling and reverse genetics in *Arabidopsis* (Brown et al. 2005), and many of them have reduced cellulose content (Taylor et al. 2004). Similarly, in tobacco (*N. tabacum*) defects in lignin biosynthesis (Joël Piquemal et al. 1998) and in phenylpropanoid biosynthesis (Ranocha et al. 2002) are also characterized by irregular or distorted xylem vessels. However, the *irx* phenotypes were not found in the rice mutants with deficient secondary cell walls, such as *bc1* and *fc1*. This may be because rice has different xylem structure and water transport system with *Arabidopsis* and tobacco.

In addition, the *fc1* mutant does not have an obvious change in the color of culms, as was found in other CAD-deficient mutants. The reddish coloration of CAD-deficient plants has been attributed to the incorporation of cinnamaldehydes in polymer. Especially dark red coloration occurred after staining with phloroglucinol reagent, and the color intensity was stronger in the *gh2* and *bm1* mutants than in their respective wild-types (Halpin et al. 1998; Zhang et al. 2006). However, an opposite phloroglucinol staining pattern existed between *fc1* and its wild-type. These differences between *fc1* and other CAD-deficient mutants suggest that the deficiency of FC1 activated its competitive enzymes using cinnamaldehyde as a substrate in the lignin biosynthesis pathway, which caused a decreased cinnamaldehyde content of *fc1*.

Lodging is one of the major problems in crop production and has been studied for many years. Lodging at the early stage may cause significant yield lost; at the late stage, lodging may reduce the quality of grain and will affect the usefulness of cereal strews as animal forage. As a gene with important effects on the mechanical strength, *FC1* would make a significant contribution to understanding the mechanism of lodging and the genetic improvement of rice. Because the *fc1* mutant has a significantly reduced lignin content, this could benefit the use of rice residue as material in the paper industry and as a biofuel.

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