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Increased lodging resistance in long-culm, low-lignin *gh2* rice for improved feed and bioenergy production

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Lignin modification has been a breeding target for the improvements of forage digestibility and energy yields in forage and bioenergy crops, but decreased lignin levels are often accompanied by reduced lodging resistance. The rice mutant *gold hull and internode2* (*gh2*) has been identified to be lignin deficient. *GH2* has been mapped to the short arm of chromosome 2 and encodes cinnamyl-alcohol dehydrogenase (CAD). We developed a long-culm variety, 'Leaf Star', with superior lodging resistance and a *gh* phenotype similar to one of its parents, 'Chugoku 117'. The *gh* loci in Leaf Star and Chugoku 117 were localized to the same region of chromosome 2 as the *gh2* mutant. Leaf Star had culms with low lignin concentrations due to a natural mutation in *OsCAD2* that was not present in Chugoku 117. However, this variety had high culm strength due to its strong, thick culms. Additionally, this variety had a thick layer of cortical fiber tissue with well-developed secondary cell walls. Our results suggest that rice can be improved for forage and bioenergy production by combining superior lodging resistance, which can be obtained by introducing thick and stiff culm traits, with low lignin concentrations, which can be obtained using the *gh2* variety.

When developing new rice varieties for feed and biofuel, the main goals are to enhance biomass production and lodging resistance as well as to improve feed digestibility and lignocellulose saccharification for biofuel production. Cell wall composition affects digestibility and saccharification efficiency. As the lignin concentration in the shoot increases, efficiency decreases^{1,2}. Therefore, lignin modification in feed and bioenergy crops has been the main objective when breeding new plants for improved energy yields¹.

The brown midrib phenotype has been used to identify lignin synthesis mutants in grasses. *Brown midrib* (*Bmr*) mutants have been discovered in maize³, which is a model for studying forage digestibility in livestock. *Bmr* mutants have been used to breed varieties with high forage digestibility^{4,5}, and commercial *Bmr* varieties of sorghum have been bred in many countries for foraging.

In rice, *gold hull and internode 2* (*gh2*) was identified as a lignin-deficient mutant⁶. *gh2* exhibits a reddish-brown pigmentation in the hull and the internode, is located on the short arm of chromosome 2 and codes for cinnamyl-alcohol dehydrogenase (CAD). CAD catalyzes the final step in monolignol biosynthesis, reducing hydroxycinnamaldehydes to hydroxycinnamyl-alcohol monomers, such as p-coumaryl, coniferyl and syringyl alcohols. In the *gh2* mutant, coniferyl alcohol dehydrogenase activity is reduced and sinapyl alcohol dehydrogenase activity is not detectable. *GH2* is a multifunctional enzyme that has both activities. The rice genome contains 12 CAD-like genes⁷. *OsCAD2* was cloned using the *gh2* mutant by map-based cloning⁶. The lignin concentration of the *OsCAD2* null mutant, *gh2*, was reduced in comparison to the wild type (WT). *OsCAD7* is responsible for the *flexible culm 1* (*fc1*) phenotype, which shows reduced mechanical strength⁸. Hirano et al.⁹ suggested that *OsCAD2* is largely responsible for monolignol biosynthesis. Previous studies have not investigated whether *OsCAD2* affects mechanical strength.



Reduced lignin concentrations are often associated with reduced lodging resistance^{8,10–12}. Therefore, reduced lignin concentrations must be accompanied by strong culms for commercial use. In Japan, rice cultivation for animal feed and biofuel has been promoted on abandoned cultivated land. Recently, new high-biomass, long-culm rice varieties have been developed for whole-crop silage (WCS). To fully utilize WCS varieties, it is important to determine which traits are associated with superior lodging resistance and to introduce those traits into new WCS varieties by crossing them with superior lodging-resistant varieties.

We developed a new high-biomass, long-culm WCS variety and named it 'Leaf Star'. Among the improved varieties, Leaf Star has the highest bending moment at the basal culm at breaking because of its large section modulus, which is an indicator of culm thickness and higher bending stress, and thus, culm stiffness¹³. Thus, Leaf Star has superior resistance to lodging because of its combination of culm thickness and culm stiffness. This variety also has the same reddish-brown pigmentation of the hull and the internode as that observed in its parent 'Chugoku 117' and in the *gh* mutants¹³. In spite of its reduced lignin concentration, Leaf Star experiences high bending stress¹³. This is a very important characteristic that contributes to the lodging resistance of rice and other gramineous crops that use rice germplasms with low lignin concentrations.

In this study, we sought to investigate the relationships between the physiological and genetic factors of *gh* and the low lignin concentrations in Leaf Star and its parent Chugoku 117. Specifically, we examined enzyme activity, substrate specificity and the expression of *OsCAD2*. In addition, we examined the effects of a natural CAD mutation on culm strength using Kinmaze, which has been widely cultivated as a semi-dwarf variety in the southern part of Japan and from which many mutant resources have been developed, and a *gh2* null mutant with a Kinmaze background¹⁴. Finally, to investigate which traits are responsible for strong culm strength in Leaf Star, we focused on cell wall components, such as cellulose, hemicellulose and lignin and on the morphological characteristics of the cortical fiber tissue in the culm.

Results

Genetic factors responsible for the low levels of lignin in Leaf Star.

The new long-culm variety Leaf Star showed superior lodging resistance. Leaf Star did not lodge until harvest, while severe lodging occurred with Koshihikari (Fig. 1a, b). Leaf Star had a gold hull and internode phenotype, similar to Chugoku 117 and the Kinmaze-*gh2* mutant (Fig. 1c, d). To determine the effects of *gh* on the lignin concentrations in the leaves and stems of Leaf Star, the lignin concentrations of the fifth internode cell walls of Leaf Star and its parents, Chugoku 117 (*gh* line) and Koshihikari (non-*gh* variety), were compared at 20 days after heading (Fig. 1e). The lignin levels in the Chugoku 117 cell walls were 11% lower than those in Koshihikari, and the lignin levels in Leaf Star were 39% lower than those in Koshihikari.

To identify the factors responsible for the low lignin levels in Leaf Star, the enzyme activity and substrate specificity of CAD in the basal internodes of Leaf Star and its parents were investigated (Fig. 1f, g). CAD activity peaked at 30 days before heading and then declined until 10 days before heading. There were no significant differences in CAD activity among the varieties investigated. Sinapyl alcohol dehydrogenase activity in the non-*gh* variety Koshihikari peaked at 20 days before heading and then declined until 10 days after heading. In the *gh* varieties Leaf Star and Chugoku 117, sinapyl alcohol dehydrogenase activity was markedly lower than in Koshihikari and was not detectable at 10 days before heading.

Based on quantitative trait locus (QTL) analyses using backcross inbred lines (BILs) derived from a cross between Chugoku 117 and Koshihikari and recombinant inbred lines (RILs) derived from a

cross between Leaf Star and Koshihikari, the *gh* locus was found to be located in the same region as *gh2* on chromosome 2 (Supplementary Table S1 and Supplementary Table S2). In the BILs derived from Chugoku 117 and Koshihikari, one QTL mapped to the short arm of chromosome 2 (nearest the simple sequence repeat (SSR) marker RM3703, LOD 18.6, R^2 value 0.40) (Supplementary Table S1). Using backcrossed recombinant homozygous lines (BC₄F₄), the location of *gh* was narrowed down to a 3.83-Mb region between RM3703 and RM3390, which included *OsCAD2* (Fig. 1h). To identify the QTL, we bred a near-isogenic line (NIL) of *gh* with the Koshihikari genetic background. We confirmed that this NIL showed the *gh* phenotype (Fig. 1i). For the RILs derived from Leaf Star and Koshihikari, only one QTL also mapped to the short arm of chromosome 2 (nearest the SNP marker aa02000707, LOD 38.3, R^2 value 0.78) (Supplementary Table S2).

OsCAD2 expression in the fifth internode of Leaf Star and its parents was compared at 30 days before heading up to 10 days after heading (Fig. 2a). In Koshihikari, *OsCAD2* expression peaked at 20 days before heading and then decreased rapidly. In contrast, in Leaf Star and Chugoku 117, no *OsCAD2* expression was detected at 30 days before heading, and peak expression was delayed until 10 days before heading. In addition, the expression levels in Leaf Star and Chugoku 117 were markedly lower than those in Koshihikari during the fifth internode elongation.

To determine the cause of the reduced *OsCAD2* gene expression, the amino acid sequences of *OsCAD2* were compared between Leaf Star and its parents (Supplementary Fig. S1). The amino acid sequence of *OsCAD2* in Chugoku 117 differed from those in Koshihikari and Leaf Star. In Chugoku 117, Tyr85 underwent a point mutation to Cys85 at nucleotide 254 (A → G) in exon 3. Amino acids 342–359 in Chugoku 117 also differed from those in Koshihikari and Leaf Star due to a 59-bp deletion in exon 4 (Fig. 2b). This deletion created a premature stop codon in the Chugoku 117 allele.

The *OsCAD2* gene structure and mutation sites in Leaf Star and Koshihikari are shown in Fig. 2b. There were no sequence differences in the exons of Leaf Star and Koshihikari, but a 506-bp Ty3-gypsy retrotransposon was inserted into the first intron of the Leaf Star allele. Thus, Leaf Star had a natural *OsCAD2* mutation that caused it to be distinct from the Chugoku 117 allele.

Because it has been reported that two gene families contain the primary CAD synthesis genes⁸, the expression of *OsCAD7* in the fifth internode was also compared in Leaf Star and its parents at 20 days before heading. No differences in expression were observed (Supplementary Fig. S2).

Effects of *gh2* on culm stiffness. The *gh2* mutant with the Kinmaze background was more susceptible to lodging compared to the WT for both years. To determine the effects of the *gh2* mutation on culm stiffness, the culm strength, the lignin concentrations of the cell walls and the CAD enzyme activity in the fifth internode in the Kinmaze-*gh2* mutant were compared with those of the Kinmaze-WT at 20 days after heading (Fig. 3).

The lignin concentration in Kinmaze-*gh2* was 23% lower than that in Kinmaze-WT (Fig. 3a). Coniferyl alcohol dehydrogenase activity in Kinmaze-*gh2* was 29% lower than that in Kinmaze-WT. Sinapyl alcohol dehydrogenase activity was high in Kinmaze-WT, but it was not detectable in Kinmaze-*gh2*, indicating that it was involved in lignin synthesis (Fig. 3b).

To investigate the effects of reduced lignin levels on the traits associated with culm strength, the bending moment at breaking (M), section modulus (SM) and bending stress (BS) were measured in Kinmaze-*gh2* and Kinmaze-WT (Fig. 3c). The M value in Kinmaze-*gh2* was 16% lower than that in Kinmaze WT. This difference resulted from a lower BS in Kinmaze-*gh2*.

Based on these results, the *OsCAD2* mutation reduced lignin concentrations and affected culm stiffness.

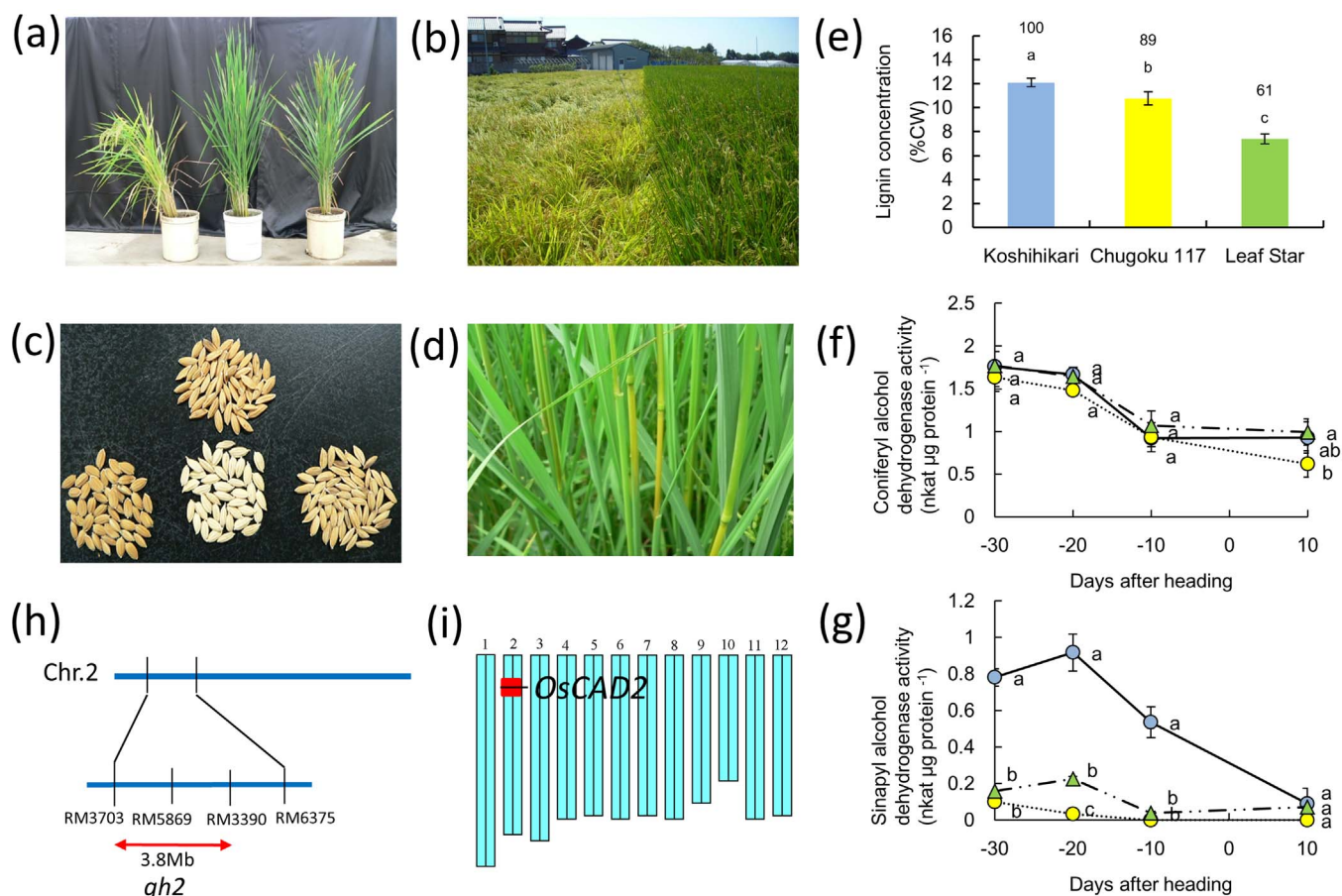


Figure 1 | The natural *gh2* mutation in Chugoku 117 and Leaf Star. (a) Plant appearance. Koshihikari (left); Leaf Star (center); Chugoku 117 (right). (b) Lodging during grain ripening in a paddy field immediately after a strong typhoon hit. Koshihikari (left); Leaf Star (right). (c) Gold hull phenotype. Kinmaze-*gh2* mutant (left); Koshihikari (lower center); Chugoku 117 (upper center); Leaf Star (right). (d) Gold internode phenotype in Leaf Star. (e) Lignin concentrations of fifth internodes 20 days after heading. Blue bar: Koshihikari; yellow bar: Chugoku 117; green bar: Leaf Star. Each column represents the mean \pm s.d. (three replicates). The means followed by different letters are significantly different (5% confidence level) according to Tukey's test. The values in parentheses are the percentages relative to Koshihikari. (f, g) Coniferyl alcohol dehydrogenase activity (f) and sinapyl alcohol dehydrogenase activity (g) in fifth internodes from 30 days before heading to 10 days after heading. Blue circles: Koshihikari; yellow circles: Chugoku 117; green triangles: Leaf Star. The means followed by different letters are significantly different (5% confidence level) according to Tukey's test. (h) Location of *gh2* estimated by RILs and introgression lines between Chugoku 117 and Koshihikari. The location of *gh2* was predicted to be between markers RM3703 and RM6375 on chromosome 2. The putative *gh2* region was narrowed down to the 3.8 Mb region between RM3703 and RM3390 by mapping. (i) Graphical genotype of NIL-*gh2* on rice chromosomes. The red bar indicates the chromosome segment of *gh2* including *OsCAD2*. Photographs (a)–(d) were taken by Taiichiro Ookawa.

Factors responsible for the stiff culm of Leaf Star. In our previous study¹³, we have found that the M value of Leaf Star is higher than that of its parents because it has a larger SM and a higher BS. In this study, the same differences in M, SM and BS were observed among these varieties (Supplementary Fig. S3). Thus, Leaf Star, unlike Kinmaze-*gh2*, has a stiff culm despite its low lignin concentration.

To investigate whether the cell wall densities in Leaf Star are responsible for its stiff culm, the densities of cellulose and hemicellulose in the fifth internode were measured in Leaf Star and its parents at 20 days after heading (Fig. 4a). The densities of both cellulose and hemicellulose in Leaf Star were higher than those in Chugoku 117.

To analyze the morphological characteristics of the culm tissues in the stiff culm of Leaf Star, cross-sections of the basal internodes were stained with crystal violet to visualize the lignified cell walls. Violet staining was observed in the cortical fiber tissues and in the small vascular bundles (Fig. 5).

Next, cross-sections of the basal internodes were treated with phloroglucinol or with calcofluor to detect lignin and glucans. Red phloroglucinol staining was observed in the cortical fiber tissues and

small vascular bundles of Leaf Star, confirming that it had a well-developed secondary cell wall (Fig. 6). The blue-white calcofluor staining was stronger in the thick cell walls of the parenchyma cells in Leaf Star than in those of both parents (Fig. 6). This was due to the high densities of cellulose and hemicellulose in the basal culm of Leaf Star.

The cortical fiber tissue in the fifth internode was approximately 40% thicker in Leaf Star than in Chugoku 117 (Fig. 4b and Fig. 5). The ratio of cortical fiber tissue thickness to culm wall thickness was higher in Leaf Star than in Chugoku 117. Koshihikari had a higher percentage of cortical tissue than Chugoku 117. The secondary walls of the cortical fiber cells of Leaf Star and Koshihikari were well-developed, whereas many hollow cortical fiber cells were observed in Chugoku 117 (Fig. 7). Thus, Leaf Star inherited extremely thick cortical fiber tissues with well-developed secondary walls from Koshihikari.

Discussion

Reduction of lignin concentration and mechanical strength in an *OsCAD2* mutant. *OsCAD2* is the major *CAD* gene responsible for

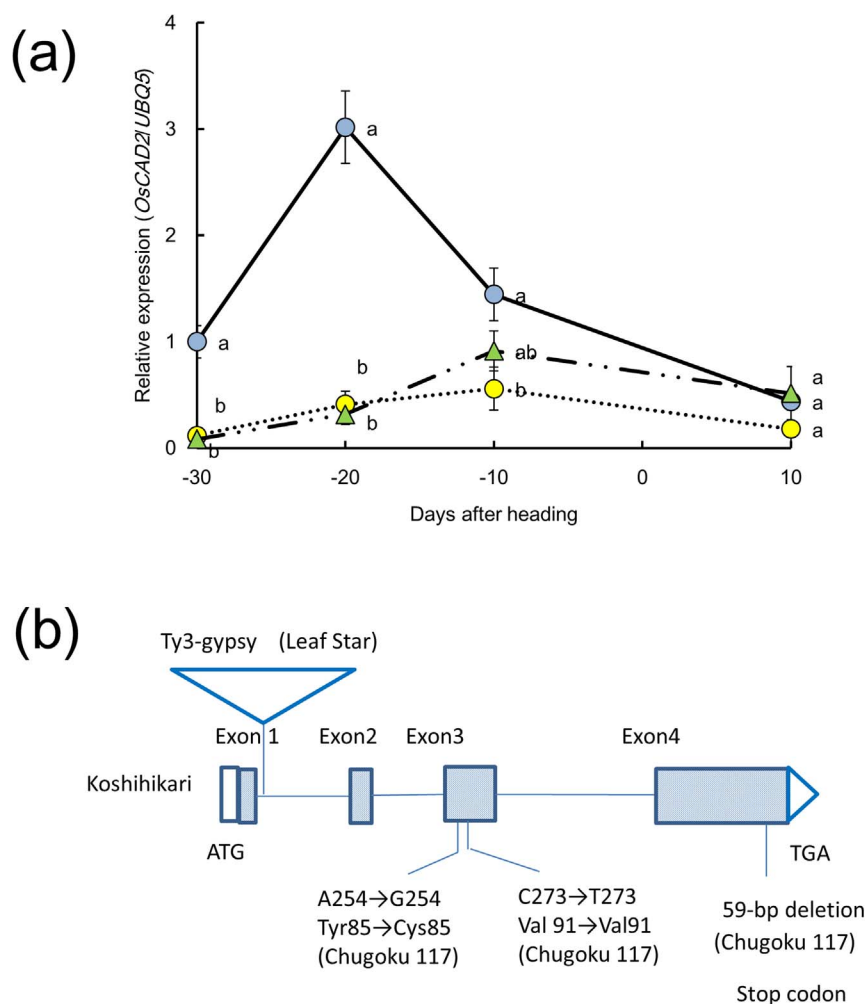


Figure 2 | Properties of *OsCAD2* in the *gh2* varieties Leaf Star and Chugoku 117. (a) Expression of *OsCAD2* relative to *UBQ5* in the fifth internode from 30 days before heading to 10 days after heading. Blue circles: Koshihikari; yellow circles: Chugoku 117; green triangles: Leaf Star. The means followed by different letters are significantly different (5% confidence level) according to Tukey's test. (b) *OsCAD2* gene structure and mutation sites in Leaf Star. There were no differences in the exon sequences of Leaf Star and Koshihikari, but a 506-bp region of a retrotransposon is inserted in the first intron in Leaf Star.

lignin biosynthesis in rice culms⁹. A deficiency in *OsCAD2* causes the *bm* phenotype, which is observed in grains, such as maize and sorghum, and the *gh* phenotype¹⁵. However, it is not known whether *OsCAD2* affects the mechanical strength of rice culms. The lignin concentration in the *OsCAD2*-null mutant Kinmaze-*gh2* was 23% lower than that in the WT (Fig. 3a), and Kinmaze-*gh2* had low coniferyl alcohol dehydrogenase activity and no detectable sinapyl alcohol dehydrogenase activity (Fig. 3b). The loss of sinapyl alcohol dehydrogenase activity has also been observed in other *gh2* mutants⁶.

Regarding the characteristics associated with culm strength, the mechanical strength of the Kinmaze-*gh2* culms was 42% lower than that of the WT culms, and severe lodging occurred in this mutant as a result of a weak BS (Fig. 3c). This is the first study demonstrating that *OsCAD2* affects the mechanical strength of the basal internode involved in lodging. A T-DNA-tagged *OsCAD7* mutant, *fc1*, also shows 13.4% less lignin and a 34% reduction in mechanical strength at the uppermost internode⁸. Because weak culms cause lodging problems, these mutants cannot be used in rice breeding for the production of livestock feed or biofuel.

A natural mutation in *OsCAD2* reduces lignin concentrations in some rice varieties. Leaf Star and Chugoku 117 exhibited natural *gh2* mutations in chromosome 2 (Fig. 1h, Supplementary Table S1 and

Supplementary Table S2). For Leaf Star, the lignin concentrations of the culms were significantly reduced compared with those of the two parents, Koshihikari and Chugoku 117 (Fig. 1e). The two varieties with the *gh2* mutations had different amounts of lignin. The sinapyl alcohol dehydrogenase activities of both of the *gh2* varieties were significantly lower than those in Koshihikari (Fig. 1g). *OsCAD2* expression was repressed in the developing internodes of Leaf Star and Chugoku 117. No differences in *OsCAD7* expression were observed in Leaf Star and its parents (Supplementary Table S2). The reason for the smaller reduction in lignin levels in Chugoku 117 compared to that in Leaf Star is unclear. In some studies, no differences in the lignin concentrations of the *gh2* mutants and WT have been found. Because the lignin biosynthetic pathway is very complex, the activities of other lignin biosynthetic enzymes may be higher in Chugoku 117; this possibility needs further investigation.

Leaf Star and Chugoku 117 have different natural *OsCAD2* mutations. There were large differences in the nucleotide and amino acid sequences of *OsCAD2* in both varieties (Fig. 2b and Supplementary Fig. S1). The mutations in the Chugoku 117 allele included a Tyr85-to-Cys85 point mutation in exon 3 and a 59-bp deletion in exon 4. Furthermore, the deletion in exon 4 created a premature stop codon in the Chugoku 117 allele. It has been reported that the premature stop codon not only produces a truncated and inactive protein product but also destabilizes the mRNA and leads to its degradation¹⁶.

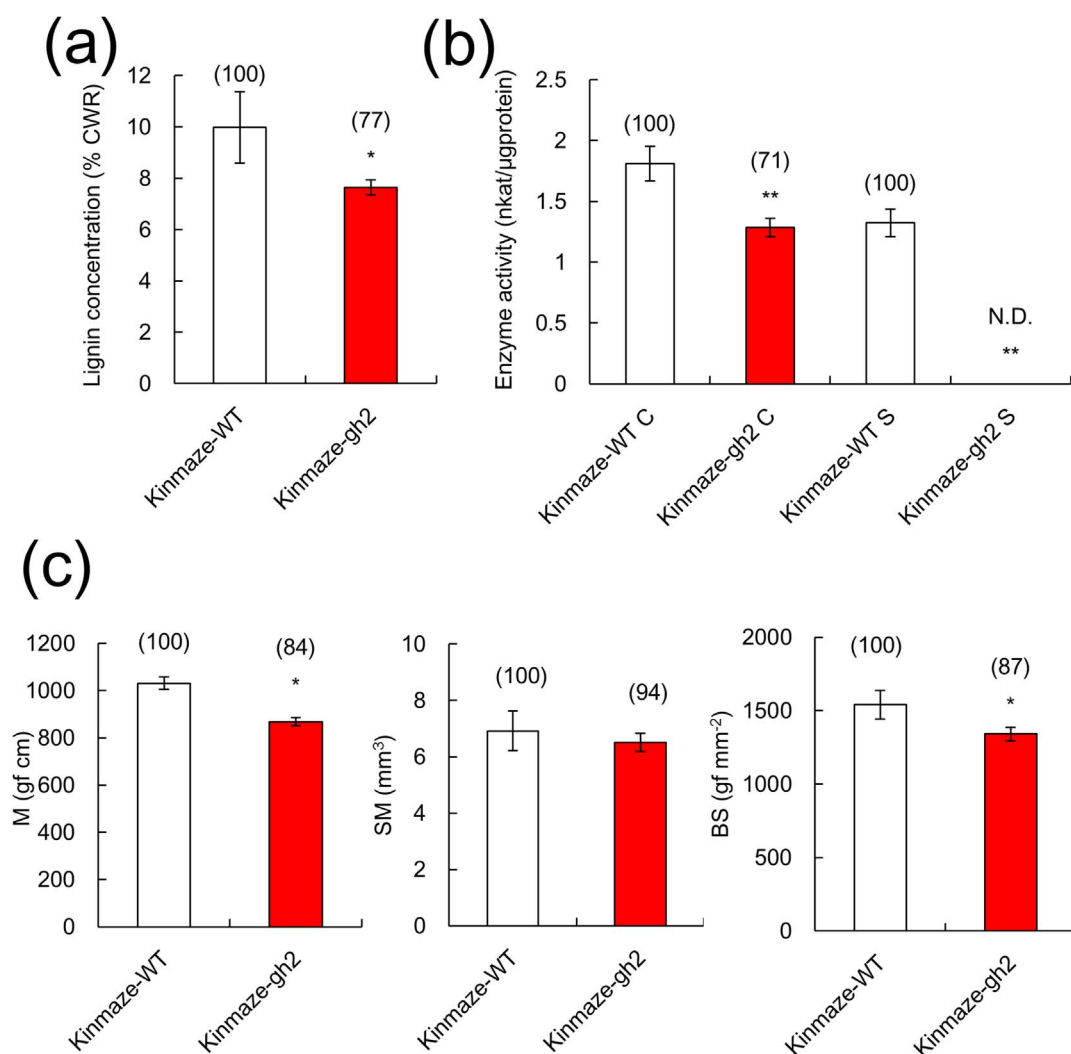


Figure 3 | Lignin properties and culm strength in a *gh2* mutant. (a) Lignin concentration in the fifth internode 20 days after heading. (b) Coniferyl alcohol dehydrogenase activity (C) and sinapyl alcohol dehydrogenase activity (S) in the fifth internode 20 days before heading. (c) M, SM and MS of the fifth internode 20 days after heading. Open bars: Kinmaze-WT; red bars: Kinmaze-*gh2*. Each column represents the mean \pm s.d. (three replicates). The means followed by different letters are significantly different (5% confidence level) according to Tukey's test. The values in parentheses are the percentages relative to Kinmaze-WT.

This mutation may be responsible for the weak expression of *OscAD2* and the low activity of CAD2 in Chugoku 117.

Although there were no differences in the exon sequences of Leaf Star and Koshihikari, the Ty3-gypsy retrotransposon was inserted into the first intron of the Leaf Star allele (Fig. 2b). It has been reported that the insertion of a transposon into intron 1 of maize *CAD2* in the maize strain *bm1* is responsible for the nonsense or the frame shift mutations that create the truncated CAD2 protein¹⁷. Additionally, in *bm1*, *cad2* mRNA expression was reduced and the lignin concentration was low¹⁸.

This natural mutation accidentally occurred in the Koshihikari allele during the individual or the line selection in the breeding of Leaf Star, which was derived from a cross between Chugoku 117 and Koshihikari. During these selections, a retrotransposon may have inserted in the first intron region of the Koshihikari allele. This new *OscAD2* allele in Leaf Star can be utilized in future breeding projects to further reduce lignin concentrations for feed and bioenergy production.

Key factors associated with strong culms in Leaf Star. Leaf Star experienced high bending stress, and thus, possessed a high

mechanical strength despite its low lignin concentration conferred by its natural *OscAD2* mutation (Fig. 1e). The densities of cellulose and hemicellulose were higher in Leaf Star than in Chugoku 117 (Fig. 4a). The densities of cell wall components are correlated with culm stiffness^{13,19,20}. Cellulose and hemicellulose usually accumulate in the secondary cell wall. In the basal culms of Leaf Star, they accumulated in the secondary cell walls of the cortical fiber cells (Fig. 4b and Fig. 5). In rice plants that highly express cell wall biosynthetic genes, reduced lignin concentrations have been reported, which lead to compensatory increases in cellulose and hemicellulose²¹. Transgenic aspen trees with the suppression of the lignin biosynthetic gene *Pt4CL1* exhibit a reduction in lignin, which is compensated for by an increase in cellulose²². This compensatory regulation may be one of the causal factors for the high accumulation of cellulose and hemicellulose in Leaf Star. Further investigations are needed to clarify the regulatory mechanisms underlying the accumulation of cell wall materials in mechanical tissues.

Leaf Star has been utilized as a commercial rice variety for whole-crop silage and used as a model variety for studying the efficiency of saccharification to produce biofuel from rice straw in Japan^{13,23}. Rice varieties with high cellulose and hemicellulose levels and low lignin

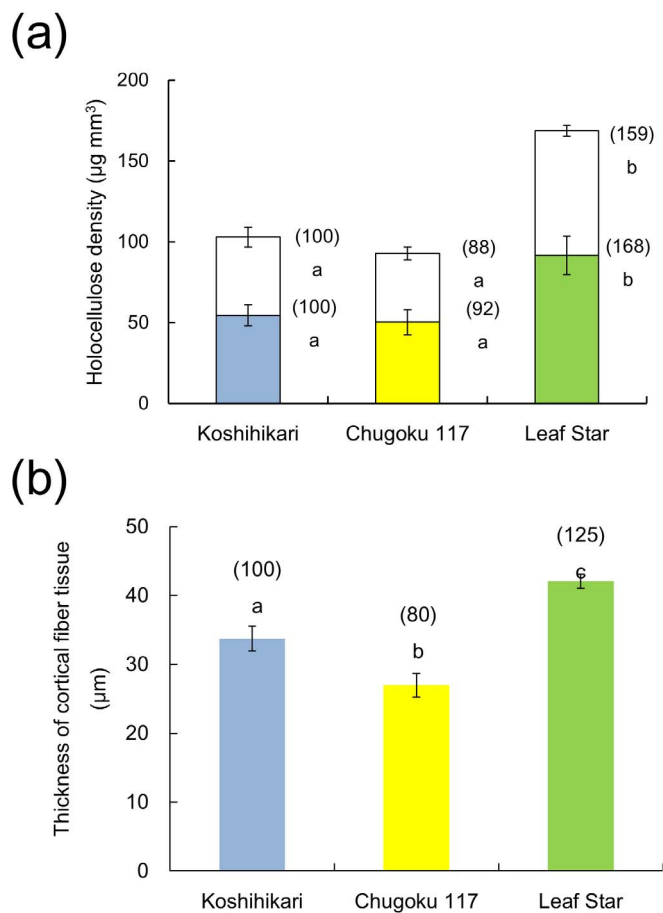


Figure 4 | Accumulation of cell wall components and thickness of cortical fiber tissue in culms. (a) The densities of cellulose (shaded) and hemicellulose (unshaded). (b) Thickness of cortical fiber tissue. Blue bars: Koshihikari; yellow bars: Chugoku 117; green bars: Leaf Star. Each column represents the mean \pm s.d. (three replicates). The means followed by different letters are significantly different (5% confidence level) according to Tukey's test. The values in parentheses are the percentages relative to Koshihikari.

concentrations, such as Leaf Star, may improve lodging resistance and the efficient utilization of lignocellulose for feed and biofuel production.

Furthermore, calcofluor staining showed that Leaf Star has well-developed parenchymal cells with thick cell walls (Fig. 6). These structural properties may be responsible for the stiff culm of Leaf Star. It has been reported that cortical fiber tissues are very important to bending stress²⁴. Leaf Star had well-developed and thick cortical fiber tissues, which were inherited from the japonica variety Koshihikari, compared with the tropical japonica line Chugoku 117 (Fig. 7). In our previous study¹⁹, the bending stresses in the typical japonica fine-culm varieties in Japan were shown to be higher than those in the typical tropical japonica and indica thick-culm varieties. Further investigation is needed to determine the genetic basis for the structural traits associated with cortical fiber tissues.

Physiological factors associated with differentiation and development of the cortical fiber tissues in rice internodes are unknown. The duration of periclinal division in developing internodes has been found to be associated with cortical fiber tissue development²⁵. It has been reported that in *brittle culm* (*bc*) mutants, the accumulation of cellulose, hemicellulose and lignin and the thickness of the secondary walls in the sclerenchyma tissues are reduced; additionally, *BC* genes have been found to regulate the development of secondary cell walls^{26,27}. Further studies are needed to investigate the physio-

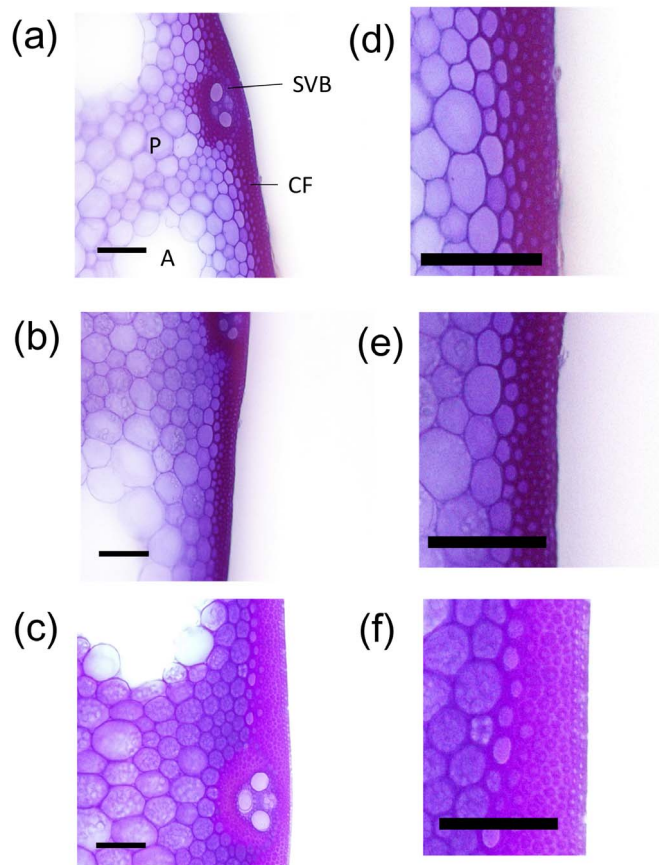


Figure 5 | Morphological characteristics of transverse sections of cortical fiber tissues in culms. (a, d) Koshihikari, (b, e) Chugoku 117, (c, f) Leaf Star. Scale bar: 100 μm . Transverse sections of fifth internodes 20 days after heading were stained with crystal violet. CF: cortical fiber tissue; SVB: small vascular bundle; P: parenchyma; A: aerenchyma.

logical and genetic aspects of morphogenesis, such as cell division and cell elongation, that are associated with the formation of the thick cortex fiber tissues and with the thickening of the secondary walls in the cortical fiber cells.

This study demonstrated that superior lodging resistance could be introduced into lignin-deficient varieties by incorporating rice genes that confer thick, stiff culms, such as the natural *OsCAD2* mutation found in Leaf Star. To successfully develop long-culm and lignin-deficient varieties with superior lodging resistance and high biomass using marker-assisted selection, it is important to determine the causes of the thick and stiff culms and to identify the QTLs associated with the strong culms. We have identified some QTLs responsible for strong culms and culm thickness, such as *SCM2*²⁸ and *SCM3*. Further genetic studies are needed to investigate the molecular regulation of culm thickness and stiffness. These studies will provide new strategies for improving lodging resistance in rice, wheat and other grassy crops during the next green revolution.

Methods

Cultivation. The rice (*Oryza sativa* L.) cultivar Leaf Star and its parents, Koshihikari and Chugoku 117, were cultivated in 2010 and 2011. The *gh2* mutant with the Kinmaze background (*Kinmaze-gh2*) was generated by the ethyl-methanesulfonate (EMS) treatment of immature embryos. *Kinmaze-gh2* and *Kinmaze-WT* were also cultivated during both years. Field experiments using these varieties and the *gh2* mutant were conducted in 2010 and 2011. The growth conditions were nearly the same for both years. Here, we describe the 2010 cultivation method.

Seeds were sown in nursery boxes on April 27, 2010. Seedlings at the fourth-leaf stage were transplanted into a paddy field at the University Farm in Tokyo containing alluvial soil of the Tama River at a rate of three plants per hill on May 19, 2010. The planting density was 22.2 hills m^{-2} , with a spacing of 15 cm \times 30 cm. Manure was

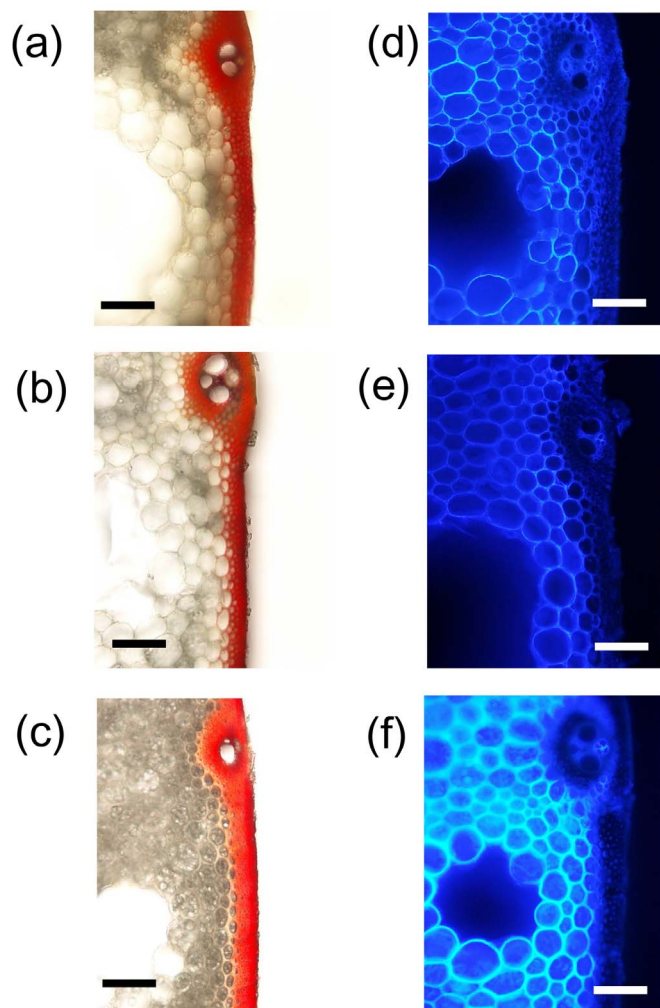


Figure 6 | Accumulation of cell wall components in basal culms of Leaf Star and both parents. Transverse sections of fifth internodes 20 days after heading were stained with phloroglucinol (left) or calcofluor (right). Phloroglucinol staining (red color) was observed in cortical fiber tissues and in small vascular bundles. Calcofluor staining was stronger in the parenchyma cells of Leaf Star than in the parenchyma cells of both parents. (a,d) Koshihikari, (b,e) Chugoku 117, (c,f) Leaf Star. Scale bar: 100 μm .

applied at a rate of approximately 20 t ha^{-1} before puddling, and compound fertilizer containing 14% each of N, P_2O_5 and K_2O was applied as basal dressing at a rate of 50 kg each per hectare. The top dressing of N and K_2O was applied at a rate of 3.0 kg each per hectare at 20 days before heading and at a rate of 20 kg each per hectare on the day of heading. The field was kept submerged throughout the course of the experiments. In 2010, the headings (50%) occurred on August 5 for Koshihikari, August 13 for Chugoku 117, September 2 for Leaf Star and August 31 for Kinmaze-WT and Kinmaze-*gh2*. In 2011, the headings occurred on August 3 for Koshihikari, August 10 for Chugoku 117, August 30 for Leaf Star and August 18 for Kinmaze-WT and Kinmaze-*gh2*.

The experiments were designed with three randomly arranged replicates (36 m^2 per replicate) each year.

Construction of mapping populations and QTL analyses. The two mapping populations used for the QTL analysis of the gold hull trait were bred as follows: for the first population, Koshihikari was crossed with Chugoku 117 to produce F_1 plants. The F_1 plants were backcrossed with Koshihikari. The BC_1F_1 plants were self-pollinated six times to construct BILs using the single-seed descent method. We constructed 93 lines that were backcrossed with Koshihikari. To map the desired QTLs, we selected one BC_1F_1 plant that was heterozygous for the genomic region containing the putative QTL of interest but was homozygous for the other QTLs. Self-pollinated progeny (BC_4F_2 plants) from the BC_1F_1 plants were raised, and BC_4F_2 plants were selected that, when compared to the Koshihikari and Chugoku 117 reference genotypes, appeared to have undergone recombination in the vicinity of the putative QTL of interest. The self-pollinated progenies (BC_4F_4 lines) were used to determine the genotype at the QTL. For the second population, Koshihikari was

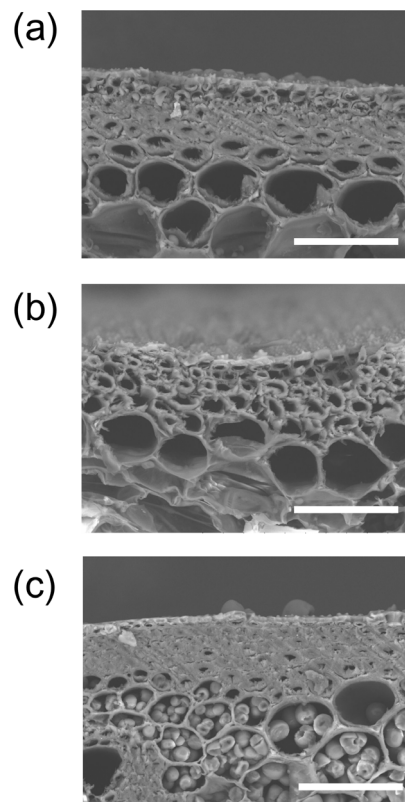


Figure 7 | Development of cortical fiber cells in culms. (a) Koshihikari, (b) Chugoku 117, (c) Leaf Star. Scale bar: 50 μm . Transverse sections of fifth internodes 20 days after heading were observed using a scanning electron microscope.

crossed with Leaf Star, and the F_1 plants were self-pollinated five times to construct RILs using the single-seed descent method. A total of 140 RILs were used for the QTL analysis.

Linkage maps of 102 SSR markers were constructed for the BILs derived from the Chugoku 117 and Koshihikari cross. The orders of and the genetic distances between the marker loci were calculated using the MAPMAKER/Exp 3.0 software²⁹. For the RILs derived from the Leaf Star and Koshihikari cross, 140 single-nucleotide polymorphism (SNP) markers were used. QTL analyses were performed using the QTL Cartographer 2.5 software (<http://statgen.ncsu.edu/qlcart/WQTLCart.htm>)³⁰.

Enzyme assay. The CAD enzyme activity was assayed according to Zhang et al.⁶. A total of 120 mg of culm tissues was milled to a fine powder in liquid N_2 and was extracted with 800 μl of extraction buffer (100 mM Tris-HCl pH 7.5, 2% polyvinylpyrrolidone, 5 mM dithiothreitol, 2% polyethylene glycol 6000) at 4°C. The supernatants were decanted, and 10 μg of total soluble protein was used for the enzyme assays. The enzyme activities were measured using coniferyl alcohol and sinapyl alcohol as substrates. The production of hydroxycinnamaldehydes was monitored spectrophotometrically using a PowerWave HT microplate spectrophotometer (BioTek, Winooski, USA) set at 400 nm.

RNA isolation and expression analysis. Total RNA from the various organs was prepared by the phenol-SDS method and treated with DNase I. First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Quantitative real-time PCR was performed using the EXPRESS SYBR GreenER qPCR SuperMix, universal (Invitrogen, Carlsbad, USA), using a StepOnePlus instrument (Applied Biosystems, Foster City, USA) with the following cycling profile: 95°C for 2 min, 40 cycles at 95°C for 15 s, and 60°C for 60 s. The primer sets used for the real-time PCR are listed in Supplementary Table S3. Housekeeping genes were used as internal standards^{8,31}.

Sequence analysis of *OsCAD2*. The *OsCAD2* DNA sequences were determined for both strands using ABI PRISM dye-labeled terminators and the automated ABI 3100 sequence analyzer (Applied Biosystems). The primer sets used for sequencing are listed in Supplementary Table S4.

Measurements of culm strength. The physical parameters of the main culms, which were closely associated with lodging resistance, were used for the precise phenotyping. Eight main culms were sampled from each plot to determine the average leaf number, and the bending load at breaking was measured at a distance of



4 cm between two supporting points according to Ookawa et al.¹³ using the Tensilon RTG-1210 universal testing machine (A&D, Tokyo, Japan). The Tensilon is a useful tool for precisely evaluating lodging resistance traits in rice. The physical parameters were calculated as follows:

$$M = \text{section modulus} \times \text{bending stress}$$

where M is the bending moment of the basal internode at breaking (gf · cm); and

$$\text{section modulus (SM)} = \pi/32 \times (a_1^3 b_1 - a_2^3 b_2) / a_1$$

where a_1 is the outer diameter of the minor axis in an oval cross-section, b_1 is the outer diameter of the major axis in an oval cross-section, a_2 is the inner diameter of the minor axis in an oval cross-section, and b_2 is the inner diameter of the major axis in an oval cross-section.

BS is a mechanical parameter for lodging resistance that is influenced by the chemical composition of the culm, such as its cellulose and lignin concentrations.

Determinations of cell wall compositions. After the bending tests, the hand-cut internodes were collected, dried in an oven at 80°C until their weights were constant and powdered with a ball mill (TissueLysers, Qiagen, Hilden, Germany). The powdered samples were extracted with 80% ethanol three times at 80°C for 1 h and washed with hexane to remove any residual oil. The residues were hydrolyzed with α -amylase (Roche, Mannheim, Germany) and amyloglucosidase (Roche) at 37°C for 16 h to remove storage starch and were then analyzed for their holocellulose, hemicellulose and lignin concentrations.

Holocellulose concentrations were measured according to the sodium chlorite method of Wise et al.³². A total of 150 mg of each residual sample was delignified with three additions of sodium chlorite and glacial acetic acid at 80°C. The residues were washed on glass filters, dried in an 80°C oven and weighed. Holocellulose concentrations were calculated by subtracting the weight of the delignified material from the weight of the starting material.

Hemicellulose was measured according to Schädel et al.³³. For each residual sample, 50 mg was dissolved in a neutral detergent solution (18 mM sodium tetraborate decahydrate, 66 mM ethylenediaminetetraacetic acid, 10.4 mM sodium dodecyl sulfate, 32 mM dibasic sodium phosphate and 1% (v/v) triethylene glycol), boiled for 1 h, washed with acetone and dried in an oven at 80°C. This 'total cell wall fraction' was dissolved in an acidic detergent solution (1 M H₂SO₄, 55 mM hexadecyl trimethylammonium bromide) and boiled for 1 h to hydrolyze the hemicellulose. After centrifugation, the residual 'cellulose and lignin fraction' was dried and weighed, and the hemicellulose concentration was calculated by subtracting the 'cellulose and lignin fraction' from the 'total cell wall fraction'. The cellulose concentration was calculated by subtracting the hemicellulose concentration from the holocellulose concentration.

The lignin concentration was directly measured according to the high-throughput determination of thioglycolic acid lignin (TGAL) method of Suzuki et al.³⁴. A total of 20 mg of each residual sample was transferred to a microcentrifuge tube, and 1 ml of 3 M HCl and 0.1 ml of thioglycolic acid were added. The samples were heated at 80°C for 3 h. After centrifugation, the pellets were washed with distilled water and resuspended in 1 ml of 1 M NaOH. After centrifugation, 1 ml of supernatant was acidified with 200 μ l of concentrated HCl. After chilling at 4°C for 4 h, the samples were centrifuged, and the pellets were dissolved in 1 ml of 1 M NaOH. These TGAL solutions were diluted 50-fold with 1 M NaOH, 200 μ l of each diluted TGAL solution was transferred to a 96-well glass microplate and absorbances were read at 280 nm using the PowerWave HT microplate reader (BioTek).

Observations of transverse sections of basal internodes. The fifth internodes were sampled at 20 days after heading and fixed in FAA. To observe the lignified cell walls, the tissue samples were stained with 0.05% crystal violet³⁵. Transverse sections of the internodes were visualized using the BX-53 fluorescence digital microscope (Olympus, Tokyo, Japan), and the thickness of the cortical fiber tissues was measured using the CellSens digital image processing software (Olympus). For the histochemical staining of the lignin, the sections were stained with a phloroglucinol solution. For the calcofluor staining, the sections were treated with 0.05% calcofluor white (Becton, Dickinson and Company, Maryland, USA) and were observed by UV excitation. The cortical fiber cell development was observed using a TM3000 scanning electron microscope (Hitachi High Technologies, Tokyo, Japan).

Statistical analysis. The statistical comparisons of multiple datasets were conducted using Dunnett's multiple comparison test and Tukey's significant difference test.

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Author contributions

T.O., T.E., T.T., T.Y., T.U., H.K. and M.V. performed the phenotyping and genotyping of gold hull trait using RILs and BILs for QTL analysis. T.O., T.Y. and M.M. performed to the sequencing of *OsCAD2*. K.I., C.S., S.N. and R.F. performed the morphological observations using microscope. K.I., T.O., S.A. and M.K. analyzed the enzyme activity and the gene expression of CAD. K.T. and T.M. contributed to the practice of field experiments. T.O., K.I., M.M., S.T. and T.H. wrote the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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