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Research Paper

Genetic enhancement of lodging resistance in rice due to the key cell wall polymer lignin, which affects stem characteristics

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Lodging in crops seriously restricts plant growth and grain production. The genetic modification of cell walls to enhance plant mechanical strength has been suggested as a promising approach toward improving lodging resistance. However, because of the complexity of the plant cell wall, the exact effects of its polymers on plant lodging resistance remain elusive. To address this issue, we performed large-scale analyses of a total of 56 rice (*Oryza sativa* L.) varieties that displayed distinct cell wall component and lodging index. Lignin was identified as the key cell wall polymer that positively determines lodging resistance in rice. Correlation analysis between cell wall composition and plant morphological characteristics revealed that lignin enhanced rice lodging resistance by largely increasing the mechanical strength of the basal stem and reducing plant height. Further characterization of four representative rice varieties, ShenNong9903, YanJian218, KongYu131, and ShenNongK33, displaying varied levels of lodging resistance, revealed the multiple candidate genes (*PAL*, *CoMT*, *4CL3*, *CAD2*, *CAD7* and *CCR20*) responsible for increasing lignin level. Hence, our results demonstrate that the high lignin level in the cell wall predominately improves lodging resistance and suggest target genes for the genetic modification of lignin towards breeding rice with high lodging resistance.

Key Words: cell wall, genetic modification, lignin, lodging resistance, rice (*Oryza sativa* L.), stem characteristics.

Introduction

Lodging is a major and integrated agronomic trait in rice, because it causes significant yield loss as well as reduces grain quality and harvesting efficiency (Berry *et al.* 2004). The application of the semi-dwarf trait and improving the mechanical strength of the basal stem have been suggested as effective approaches for increasing lodging resistance in crops (Liu *et al.* 2018, Miller *et al.* 2018, Murai *et al.* 2004). Although plant cell wall composition can greatly affect plant mechanical strength (Tanaka *et al.* 2003, Wang *et al.* 2017, Zhang and Zhou 2011), little is known about the exact functions of three major wall polymers (cellulose, hemicelluloses and lignin) in lodging resistance in rice (Halpin *et al.* 1998, Ma 2009).

Plant cell walls are predominantly composed of cellulose, hemicelluloses, and lignin. Cellulose is made linear chains of β-1,4-linked glucan. Through intra- and inter-chain hydrogen bonding, parallel linear glucan chains are crystallized to form cellulose microfibrils that provide plants with excellent toughness for normal plant growth (Somerville 2006). Hemicelluloses are a heterogeneous class of polysaccharides with various sugar units, and arabinoxylans comprise the majority of hemicelluloses in the mature tissues of rice (Li *et al.* 2015, Scheller and Ulvskov 2010). Lignin is a complex phenolic polymer that is of great significance to plant growth and development as well as environmental adaptability. Three main types of monolignols (p-coumaryl alcohol, H unit; coniferyl alcohol, G unit; and sinapyl alcohol, S unit) are cross-linked by ether-, ester-, and C-C bonds to form a stable and functional lignin complex (Li *et al.* 2014, Ralph *et al.* 2004, Sun *et al.* 2013). More than 90 genes derived from 10 super-families have been estimated to be involved in lignin biosynthesis and polymerization (Raes *et al.* 2003, Xu *et al.* 2009); however, only a few member genes have been functionally identified in rice.

Plants typically contain numerous different cell types with various cell wall components (Li *et al.* 2013), and thus, it is technically difficult to find the crucial factors of plant cell walls for high plant lodging resistance using classic approaches, such as the characterization of one genetic mutant or one gene transgenic plant. Correlation analysis using a large population has been suggested as a powerful approach for finding the relationships among the multiple traits and

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factors (Atias *et al.* 2009, Farrokhi *et al.* 2006). Hence, in the present work on a total of 56 rice varieties, we performed comparative and correlative analyses of cell wall polymers, plant morphological characteristics, and lodging resistance. The lignin level was found to be the key factor of the cell wall that enhances plant lodging resistance. The characterization of four representative varieties revealed several important candidate genes for genetically enhancing lodging resistance in rice.

Materials and Methods

Plant materials

The 56 rice varieties were broadly collected from northeast China (**Supplemental Table 1**) and grown in experimental fields at Shenyang Agricultural University (Shenyang, China). The rice stem tissues were harvested at 30 days after the heading stage. The collected fourth internodes from the top were dried at 55°C in an oven to a constant weight, and ground through 60 mesh (0.3 mm × 0.3 mm), and stored in a dry container until use.

Determination of the lodging index and stem physical properties

The lodging index was determined at 30 days after heading as previously described (Li *et al.* 2017). All of the measurements were conducted using nine independent biological duplicates. The breaking resistance of the fourth internode from the top was detected using a prostrate tester (DIK 7401, Daiki, Osaka, Japan), with the distance between fulcra of the tester at 5 cm. The fresh weight (FW) from the bottom of the fourth internode to the panicle tip was measured. The bending moment (BM) and lodging index (LI) were calculated using the following formulae: BM = length from the fourth internode to the top of panicle × FW; LI = BM/breaking resistance.

Plant cell wall fractionation and determination

The plant cell wall fractionation procedure and assay of the total cellulose and hemicelluloses were conducted as previously reported by Li *et al.* (2013, 2017). The soluble sugar, lipids, and starch of the samples were successively removed from the dry biomass power samples by potassium phosphate buffer (pH 7.0), chloroform-methanol (1:1, v/v), and DMSO-water (9:1, v/v). The remaining pellets were suspended in 4 M KOH containing 1.0 mg/mL of sodium borohydride for 1 h at 25°C, and the combined supernatants were regarded as hemicelluloses. The remaining pellets were regarded as total cellulose. All of the experiments were carried out in biological triplicate.

The determinations of total lignin content, including acid-insoluble (AIL) and acid-soluble lignin (ASL), were performed by a two-step acid hydrolysis method as described previously (Li *et al.* 2014). The sample (0.5 g, W1) was extracted with benzene-ethanol in a Soxhlet extractor for 4 h and then air-dried in a hood. The sample was hydrolyzed with 72% H_2SO_4 (v/v) in a shaker at 30°C for 1.5 h. After hydrolysis, the acid was diluted to 2.88%, then the sample was placed into the autoclave for 1 h at 121°C (15 psi). The autoclaved hydrolysis was vacuum-filtered through the previously weighed filtering crucible. The filtrate was captured as ASL and was measured by UV spectroscopy. The acid-insoluble residue was washed free of acid and dried in an oven at 80°C until it attained a constant weight. The crucible and dry residue were cooled in a desiccator and weighed (W2). Finally, the dried residue was burn into ash in a muffle furnace at 200°C for 30 min and 575°C for 4 h. The crucibles and ash were weighed (W3) after being cooled in a desiccator. The AIL was calculated according to the equation: AIL (%) = (W2 – W3) × 100/W1%.

Scanning electron microscopic observation

The fourth stem internode tissues (0.5 cm sections above the node) at the heading stage were cut into 1–2 mm pieces, then observed and photographed under a scanning electron microscope (SEM; TM1000, Hitachi, Tokyo, Japan). Stem wall thickness (SWT) was measured 8–10 times per image as shown in **Fig. 5A**. Cell wall thickness (CWT) was detected using the third layer cells of small vascular bundles as shown in **Fig. 5A**. Each sample was observed 5–10 times, and the representative image was used in this study.

Expression of genes involved in the lignin biosynthesis

Fresh culm tissues of the fourth internode (0.1 g) were ground to a fine powder in liquid nitrogen. Total RNA was extracted using RNAprep pure Plant Kit (Takara, Japan). The synthesis of the first strand cDNA was carried out with a PrimeScript™ RT reagent Kit (Takara, Japan) according to the manufacturer's instructions. Gene expression was performed three times by quantitative reverse transcription-PCR (qRT-PCR) in a 20 µL reaction system: cDNA template 2.0 µL, 2 × SYBR Green1 Mix 10 µL, primer-F 0.5 µL, primer-R 0.5 µL, MilliQ 7.0 µL with SYBR Green qPCR kit (Cwbio, Beijing, China) on Two Color Real-time PCR Detection System (QuantStudio 3, Thermo Fisher Scientific, Waltham, Massachusetts, USA). *Ubiquitin* gene (AK059011) was used as an internal standard in the qRT-PCR. The gene expression unit was subjective to the percentage of the target gene expression value relative to the internal standard (*Ubiquitin* gene). The gene locus number and the primers used in this study were shown in **Supplemental Table 2**.

Statistical analysis

All of the statistical analyses were performed using SPSS. Significance was measured at the levels of $P < 0.05$ and $P < 0.01$. Correlation coefficients were calculated by performing Pearson correlation analysis.

Results

Diverse cell wall composition and varied lodging index in the selected rice samples

In this study, we collected a wide range of 56 rice varieties from northeast China (**Supplemental Table 1**) that showed different genotypes or ecological types. The selected rice varieties displayed large variations in the three wall polymer levels in the mature straws (**Fig. 1A**). For instance, cellulose levels ranged from 23.4% to 40.9%, hemicelluloses from 10.3% to 24.3%, and lignin from 8.5% to 21.7% on a dry matter basis (**Fig. 1A**).

The lodging in rice arises from the bending or breaking of the basal culm internodes (Sirajul Islam *et al.* 2007). As the plant cell wall greatly affects plant mechanical strength (Ma 2009, Tanaka *et al.* 2003, Zhang and Zhou 2011), we measured the LI in the rice varieties. The 56 rice varieties exhibited largely varied LI ranging from 74.9% to 247.3% (**Fig. 1B**). This finding is consistent with their diverse cell wall composition as described above.

To examine the genetic stability of the 56 rice varieties used in this study, we also performed a correlation analysis of the plant LI between the 2016 and 2017 seasons. Notably, the LI measured in varieties from the 2016 and 2017 showed a significantly positive correlation at $P < 0.01$ (**Supplemental Fig. 1**).

Effects of cell wall polymer levels and morphological characteristics on LI

Correlation analysis has been extensively applied to investigate biological trait relationships or associations using large populations of samples (Li *et al.* 2013). In the current work, a correlation analysis was conducted to determine the effects of plant cell wall composition on LI in 56 rice varieties (**Fig. 2**). The LI showed a significant correlation with lignin level ($P < 0.01$) but it did not show a clear correlation

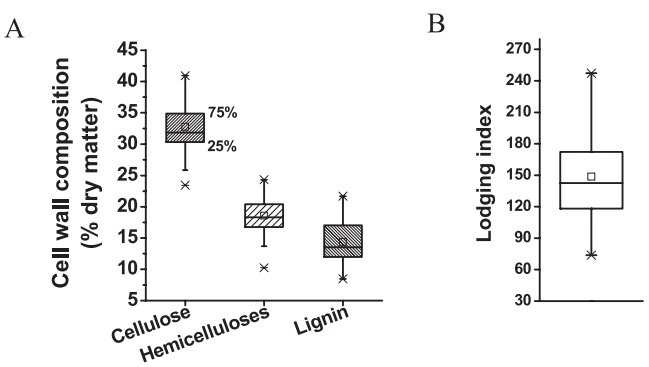

Fig. 1. Variation of cell wall composition and lodging index in rice varieties. (A) Cell wall composition (n = 56); (B) Lodging index (n = 56). The line and square within the box represent the median and mean values of all of the data; the bottom and top edges of the box indicate the 25th and 75th percentiles of all of the data, respectively; and the bottom and top bars represent the maximum and minimum values of all of the data, respectively.

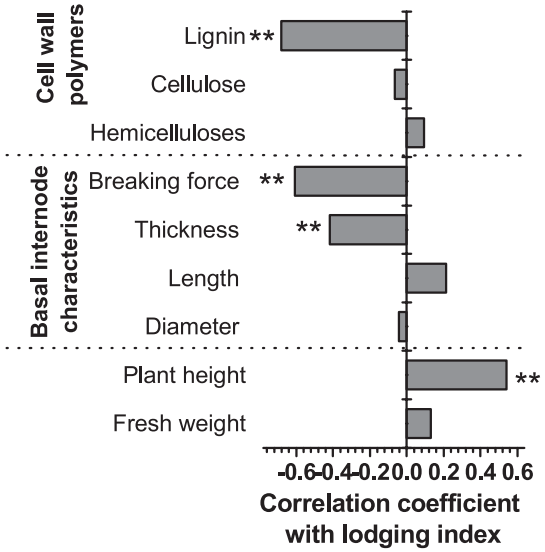

Fig. 2. Effects of cell wall polymer levels and plant morphological traits on lodging index (n = 56). ** indicate a significant correlation at $P < 0.01$.

with cellulose and hemicelluloses (**Fig. 2**), which suggests that lignin is the crucial cell wall polymer that positively affects LI in rice.

LI is a complex and integrated agronomic trait that is directly associated with plant height, fresh weight, stem mechanical strength, and others (Crook and Ennos 1994, Sirajul Islam *et al.* 2007). We thus performed a correlation analysis between LI and the morphological traits of plant height, fresh weight, and basal internode characteristics. Notably the LI displayed a significant correlation with the breaking force of the basal internode, stem wall thickness, and plant height ($P < 0.01$), but it was not correlated with the plant fresh weight or the diameter and length of basal internode (**Fig. 2**). In addition, a positive correlation was observed between the breaking force and the stem wall thickness, indicating that the wall thickness of the stem was an important contributor to the mechanical strength of the stem (**Supplemental Fig. 2**).

Correlations of cell wall polymers with lodging-related morphological traits

Due to the observed significant correlations of LI with lignin level and plant morphological traits (**Fig. 2**), we further examined the relationships of cell wall polymers and lodging-related morphological characteristics. Correlative analyses of three major wall polymers with the breaking force, stem wall thickness, and plant height were conducted using the 56 rice varieties (**Fig. 3**). The lignin level positively correlated with both the breaking force and stem wall thickness and negatively correlated with plant height (**Fig. 3**). In contrast, cellulose and the hemicelluloses did not show any significant correlation with either the breaking force or stem wall thickness (**Fig. 3**), indicating that the stem breaking force was mainly decided by lignin level

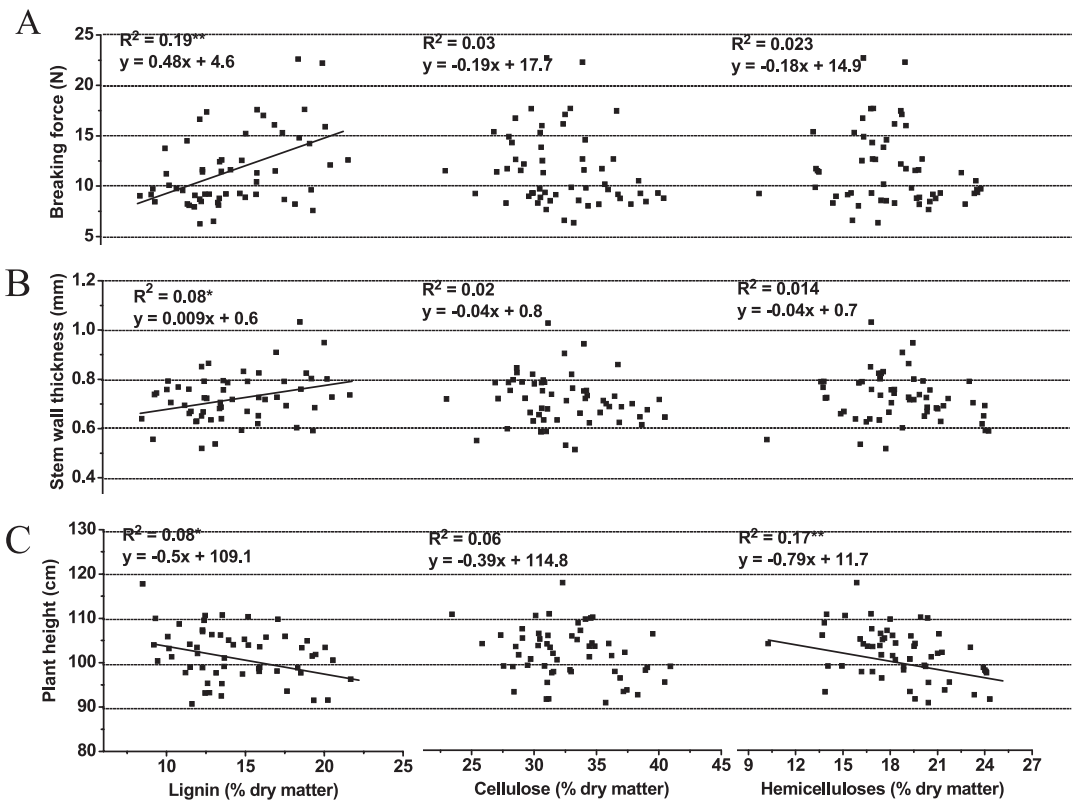

Fig. 3. Effects of the cell wall polymer levels on lodging-related morphological characteristics in rice (n = 56). * and ** indicate a significant correlation at $P < 0.05$ and 0.01, respectively.

rather than cellulose or hemicelluloses in rice as reported in wheat (Ma 2009). These results further confirm that the lignin was the predominant cell wall factor that enhances lodging resistance in rice.

Characterization of four representative rice samples with different plant lodging resistance

Among the 56 rice varieties, we characterized four representative rice varieties ShenNong9903 (SN9903), YanJian (YJ218), KongYu (KY131), and ShenNongK33 (SNK33) that displayed different LI. SN9903 and YJ218 exhibited two-fold lower LI (100.3 and 103.9, respectively) compared to those of KY131 and SNK33 (213.8 and 231.8, respectively) (**Fig. 4A**, **4B**). The four varieties were determined with distinct alternations in cell wall composition and stem breaking force (**Fig. 4C**, **4D**). Compared with the KY131 and SNK33 samples, SN9903 and YJ218 showed a significant increase in lignin level, but did not show much difference in cellulose and hemicellulose levels (**Fig. 4C**, **Supplemental Table 3**). With regard to the plant morphological characteristics, SN9903 and YJ218 showed a significantly higher breaking force of the basal stem internode and lower plant height compared to the KY131 and SNK33, but the other examined traits were not found to have changed much in these varieties (**Fig. 4D**, **4E**, **Supplemental Table 3**). Taken all together, the results demonstrate that lignin is the key cell wall polymer that enhances the stem breaking force, which thereby increases the lodging resistance.

Observations of cell wall in the representative rice samples

To explore how lignin enhances the mechanical strength of the basal stem, we observed the cell wall structure of the basal internode under the SEM (**Fig. 5A**). The stem wall thickness was much higher in the high lodging resistance varieties, SN9903 and YJ218, compared to those of KY131 and SNK33 (**Fig. 5A**, **5B**). In addition, the four varieties were observed to have different cell wall thicknesses. The cell wall thickness in SN9903 and YJ218 were significantly higher than that in KY131 and SNK33 (**Fig. 5A**, **5C**). The increased cell wall thickness should largely enhance stem stiffness, and thereby improve the stem breaking force for high lodging resistance.

Detection of the major gene candidates for lignin modification

It has been reported that more than 10 gene families might be involved in the lignin biosynthesis in monocot and dicot plants (Raes *et al.* 2003, Xu *et al.* 2009). In this study, we compared the transcript abundance of the major lignin biosynthesis-associated genes in the above four representative rice varieties by qRT-PCR (**Fig. 6**). The expression levels of *PAL*, *CoMT*, and *4CL3*, which are involved in the phenylpropanoid pathway for lignin biosynthesis (Aohara

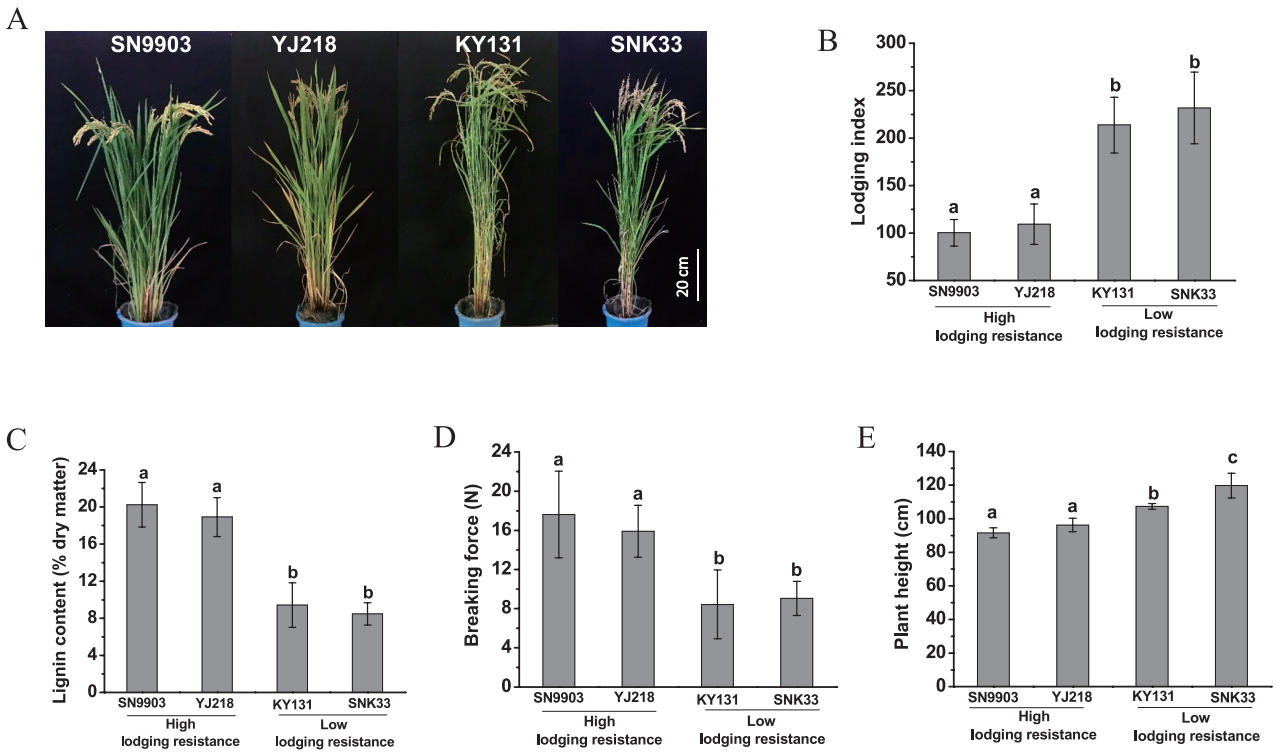

Fig. 4. Comparisons among four representative rice varieties. (A) Plant growth; (B) Plant lodging index; (C) Lignin level; (D) Breaking force of the basal internode; (E) Plant height. One-way ANOVA and Tukey's HSD *post-hoc* test were performed, and significant differences ($P < 0.05$) are represented with a, b and c. The results represent the mean ± standard deviation.

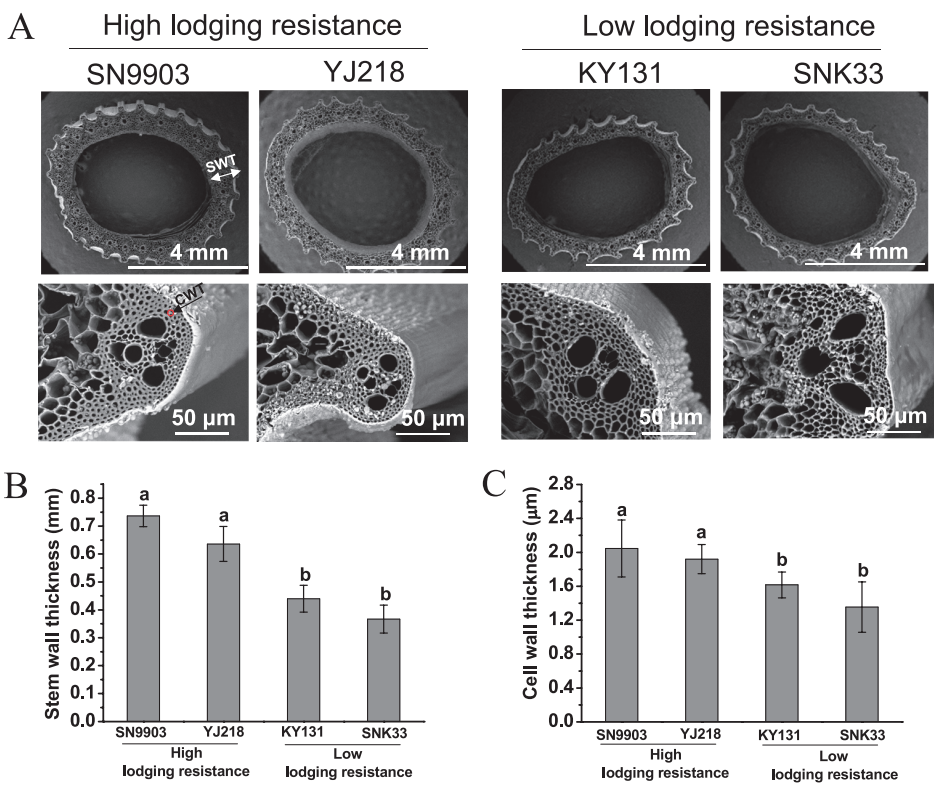

Fig. 5. Observations of the cell wall in four representative rice varieties. (A) Scanning electronic microscope (SEM) images of the basal stem internode at the heading stage of rice. SWT: stem wall thickness. CWT: cell wall thickness; (B) Quantification of stem wall thickness based on SEM images; (C) Quantification of cell wall thickness based on SEM images. One-way ANOVA and Tukey's HSD *post-hoc* test were performed, and significant differences ($P < 0.05$) are represented with a and b. The results represent the mean ± standard deviation.

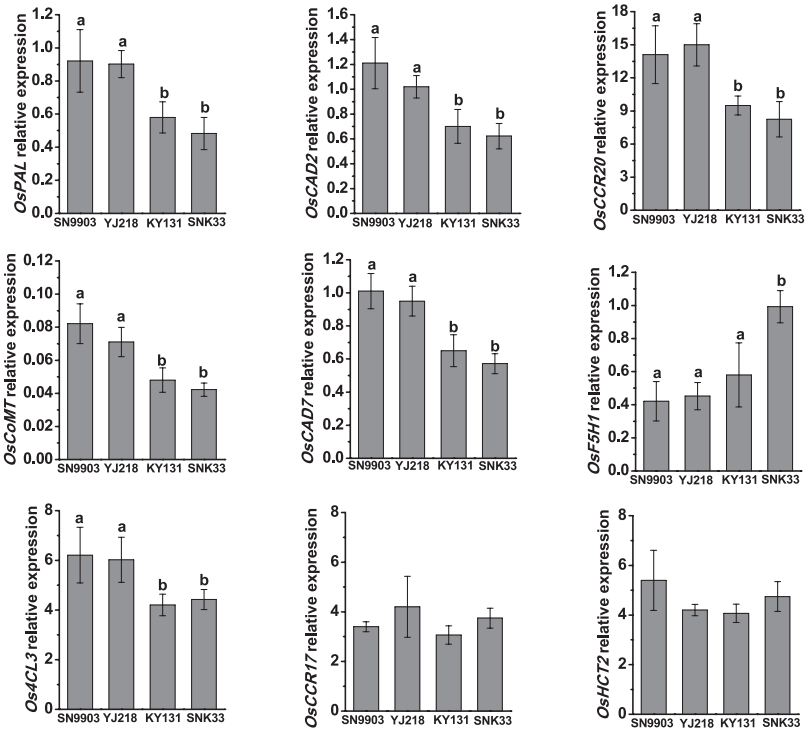

Fig. 6. The expression profile of lignin biosynthetic genes analyzed by quantitative reverse transcription-PCR (qRT-PCR). One-way ANOVA and Tukey's HSD *post-hoc* test for qRT-PCR data were performed, and significant differences ($P < 0.05$) are represented with a and b. The results represent the mean ± standard deviation.

et al. 2009, Gui *et al.*, 2011, Okuno *et al.* 2014), were significantly higher in SN9903 and YJ218 compared to KY131 and SNK33 (**Fig. 6**). Furthermore, the *CAD2*, *CAD7*, *CCR17*, *CCR20*, *F5H1* and *HCT2* genes, which encode enzymes catalyzing the specific steps for lignin biosynthesis (Borah and Khurana 2018, Hirano *et al.* 2012, Li *et al.* 2009, Park *et al.* 2017, Takeda *et al.* 2017, Zhang *et al.* 2006), were also examined (**Fig. 6**). Relative to KY131 and SNK33, the SN9903 and YJ218 exhibited significantly higher transcriptional levels of *CAD2*, *CAD7* and *CCR20* (**Fig. 6**). The *CCR17*, *F5H1*, and *HCT2* genes did not show much change between the higher and lower lodging resistance varieties. Since rice culm is mainly composed of secondary cell wall, we detected the expression of genes involved in biosynthesis of secondary wall cellulose and hemicelluloses. SN9903 and YJ218 did not exhibit significant change compared with KY131 and SNK33 in the expression of *CESA4*, *CESA7* and *CESA9* (**Supplemental Fig. 3**), which are involved in secondary wall cellulose biosynthesis (Li *et al.* 2017). With regard to hemicelluloses, they also did not show significant alterations in the expression of genes (*XAT2*, *XAT3*, *IRX9* and *IRX14*) involved in arabinoxylan biosynthesis (Anders *et al.* 2012, Chiniquy *et al.* 2013) (Supplemental **Fig. 3**). Taken together, these results support the findings of much higher lignin levels being in the two high lodging resistance plants. Hence, the overexpression of these genes would be able to facilitate the breeding of high lodging resistance rice.

Discussion

LI is an integrated agronomic trait in plant growth and development, which is affected by multiple plant morphology characteristics. Plants consist of different cell types with extremely diverse cell wall composition. Therefore, identifying a specific cell wall polymer that dominantly affects plant LI using one gene mutant or small-scale samples is difficult. One practical approach is to analyze large populations using systems biology to correlate the plant LI with wall polymers. Principally, the systems biological approach is powerful for analysis of the multiple traits and factors, but it requires a large sample population (Atias *et al.* 2009, Farrokhi *et al.* 2006). In this study, a total of 56 rice varieties were widely collected from northeast China. They exhibited diverse cell wall composition and varied agronomic traits. The varieties grown in two field seasons exhibited a significant positive correlation in LI, indicating that the rice varieties are genetic stable for the experiments performed in this study. Using those samples, therefore, we could perform a correlative and comparative analysis, leading to finding out the key factor of the cell wall that significantly influences LI in rice.

Based on the systems biology analysis of the 56 rice varieties, we revealed the correlations among cell wall polymers, plant morphological characteristics, and LI. A hypothetical model could be proposed to elucidate how the lignin level positively influences plant LI in rice (**Fig. 7**).

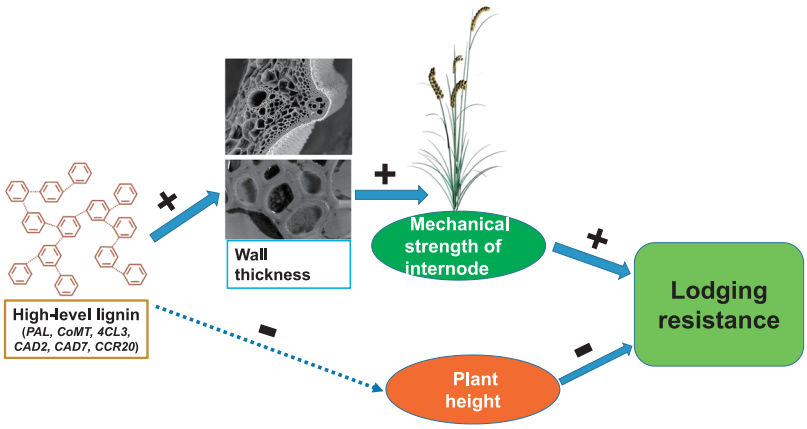

Fig. 7. Hypothetical model of lignin modifications to genetically enhance lodging resistance in rice.

Overexpression of *PAL*, *CoMT*, *4CL3*, *CAD2*, *CAD7* and *CCR20* genes increases the lignin content in the cell wall of stem tissues, which could significantly enhance the stem wall thickness and cell wall thickness. The enhancements of the stem wall thickness and cell wall thickness further positively affect the stem mechanical strength that greatly enhances lodging resistance in rice. In addition, the lignin might also affect lodging resistance in rice by impacting the plant height.

Genetic modification of plant cell walls has been considered as a promising approach for improving plant agricultural traits (Xie and Peng 2011). Based on the proposed hypothetical model, we have further identified the major genes that could be applied for genetic modifications of lignin towards enhancing lodging resistance in rice (**Fig. 7**). Genetically increasing lignin level becomes critical for enhancing lodging resistance by simultaneously overexpressing the *PAL*, *CoMT*, and *4CL3* genes (**Fig. 6**), which have been demonstrated in the two high lodging resistance varieties (SN9903 and YJ218) (**Fig. 4**). Furthermore, our data shows that the genes (*CAD2*, *CAD7*, and *CCR20*) catalyzing the specific steps for biosynthesis of the lignin monomers (G, S, H) also were altered among the four representative varieties. Hence, these results indicate that the G, S, and H monomers of lignin may differently contribute to the cell wall strength and plant lodging resistance, but the mechanism remains unclear.

Acknowledgements

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