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# High-level hemicellulosic arabinose predominately affects lignocellulose crystallinity for genetically enhancing both plant lodging resistance and biomass enzymatic digestibility in rice mutants

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## Summary

Rice is a major food crop with enormous biomass residue for biofuels. As plant cell wall recalcitrance basically decides a costly biomass process, genetic modification of plant cell walls has been regarded as a promising solution. However, due to structural complexity and functional diversity of plant cell walls, it becomes essential to identify the key factors of cell wall modifications that could not much alter plant growth, but cause an enhancement in biomass enzymatic digestibility. To address this issue, we performed systems biology analyses of a total of 36 distinct cell wall mutants of rice. As a result, cellulose crystallinity (CrI) was examined to be the key factor that negatively determines either the biomass enzymatic saccharification upon various chemical pretreatments or the plant lodging resistance, an integrated agronomic trait in plant growth and grain production. Notably, hemicellulosic arabinose (Ara) was detected to be the major factor that negatively affects cellulose CrI probably through its interlinking with β-1,4-glucans. In addition, lignin and G monomer also exhibited the positive impact on biomass digestion and lodging resistance. Further characterization of two elite mutants, *Osfc17* and *Osfc30*, showing normal plant growth and high biomass enzymatic digestion *in situ* and *in vitro*, revealed the multiple *GH9B* candidate genes for reducing cellulose CrI and *XAT* genes for increasing hemicellulosic Ara level. Hence, the results have suggested the potential cell wall modifications for enhancing both biomass enzymatic digestibility and plant lodging resistance by synchronically overexpressing *GH9B* and *XAT* genes in rice.

## Introduction

Crop residues are considered to be a major biomass resource for biofuel production (Service, 2007). As one staple food crop worldwide, rice produces about 800 million metric tons of straws annually (Domínguez-Escribá and Porcar, 2009). Principally, biomass conversion into bioethanol involves in three major steps: physical and chemical pretreatments for cell wall disassociation, enzymatic digestion for soluble sugar release and yeast fermentation for ethanol production. However, lignocellulosic recalcitrance is a great hindrance for biomass conversion because plant cell walls have evolved complex structural and chemical mechanisms for resisting the physical and biochemical digestions in nature. Genetic modification of plant cell walls has been proposed as a promising solution to lignocellulosic recalcitrance (Xie and Peng, 2011). As genetic modifications of cell walls are mostly associated with defects in plant growth and development (Abramson *et al.*, 2010; Casler *et al.*, 2002; Li *et al.*, 2008), it becomes critical to find out the key factors of plant cell wall modifications that could not much affect plant growth, but lead to an enhancement in biomass digestibility (Xie and Peng, 2011).

Plant cell walls are primarily composed of cellulose, hemicelluloses, lignin and pectic polysaccharides with minor structural proteins. Cellulose is an unbranched β-1,4-linked glucans and assembled into crystalline microfibrils (Somerville, 2006). The hydrogen bonds formed between β-1,4-glucan chains significantly determine cellulose crystallinity (Bansal *et al.*, 2010; Park *et al.*, 2010). Cellulose crystallinity can be defined as measuring the cellulose crystalline index (CrI) that reflects the relative amount of crystalline cellulose (Park *et al.*, 2010; Zhang *et al.*, 2013). It has been reported that cellulose synthesis is catalyzed by a superfamily of CESA enzymes in plants (Arioli *et al.*, 1998; Pear *et al.*, 1996). In rice, *OsCESA1*, *3* and *8* are involved in primary wall cellulose biosynthesis, whereas *OsCESA4*, *7* and *9* are reported for

secondary cell wall formation (Tanaka *et al.*, 2003; Wang *et al.*, 2010). Recently, *OsGH9B1*, *B3* and *B16* genes have been suggested with a role in cellulose crystallinity modification in rice (Xie *et al.*, 2013). In addition, *COBRA-like 1* gene may have function in cellulose assembly in rice (Liu *et al.*, 2013).

Hemicelluloses are a class of heterogeneous polysaccharides with various monosaccharides, and arabinoxylan is a major hemicellulose in the mature tissues of grass plants (Scheller and Ulvskov, 2010). Several glycosyltransferase (GT) gene families, such as *GT43* and *GT61*, have been reported to involve in both main chain biosynthesis and side chain substitution of hemicelluloses in grasses (Anders *et al.*, 2012; Chiniquy *et al.*, 2013). For instance, a homologue gene *TaXAT2* knock-down mutants strongly decrease α-(1, 3)-linked arabinosyl substitution of xylan in wheat (Anders *et al.*, 2012). *OSIRX9* and *OSIRX14*, homologues of the *Arabidopsis IRX9* and *IRX14* genes, have been identified in building the xylan backbone (Chiniquy *et al.*, 2013). Lignin is a phenolic polymer composed mainly of *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). The three monomers are cross-linked by ether-, ester- and C-C bonds to form a stable and water-proofing lignin complex (Li *et al.*, 2014b; Ralph *et al.*, 2004; Sun *et al.*, 2013). More than 90 genes derived from 10 super-families are 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.

Principally, lignocellulosic recalcitrance is determined by cell wall compositions, wall polymer features and wall network styles. Cellulose CrI has been reported to be the key factor negatively affecting biomass enzymatic digestibility in all plants examined, whereas hemicelluloses can negatively affect lignocellulose CrI for high biomass digestibility in *Miscanthus* (Li *et al.*, 2013; Xu *et al.*, 2012). Notably, arabinose (Ara) substitution degree of xylans is the positive factor on biomass enzymatic saccharification upon various chemical pretreatments in the grass plants examined (Li *et al.*, 2013). By comparison, lignin could play dual roles in biomass enzymatic digestions, due to its structural diversity and distinctive heterogeneity in different plant species (Chen and Dixon, 2007; Grabber, 2005; Jung *et al.*, 1994, 2012; Penning *et al.*, 2014). Despite that the cell wall factors on biomass saccharification have been examined in plants, little is known about the factors associated with both high biomass digestibility and normal plant growth.

In rice, lodging is a major and integrated agronomic trait in plant growth and grain production, because it causes poor grain filling and yield loss and reduces grain quality and mechanical harvesting efficiency (Berry *et al.*, 2004). Rice lodging index (LI) arises from the bending or breaking of the lower culm internodes (Sirajul Islam *et al.*, 2007) and is highly associated with plant height, fresh weight, stem diameter and others (Crook and Ennos, 1994; Sirajul Islam *et al.*, 2007). Although plant cell wall composition and features can greatly affect plant mechanic strength (Tanaka *et al.*, 2003; Zhang and Zhou, 2011), little is known about their impacts on plant LI in rice and other plants (Halpin *et al.*, 1998; Ma, 2009).

Plants constitute numerous different cell types with diverse cell wall components, and thus, it is difficult in technique to identify the key factors of plant cell walls for high biomass enzymatic digestibility and normal plant growth using classic approaches such as selection of one-gene transgenic plant or one genetic mutant. However, systems biology analysis has been considered as a powerful approach for the multiple traits and factors using large population of samples (Atias *et al.*, 2009; Farrokhi *et al.*, 2006; Guo *et al.*, 2014). Hence, in this study, we performed comparative and correlative analyses among cell wall composition and features, biomass enzymatic saccharification and lodging resistance using a total of 36 distinct cell wall mutants of rice, leading to identification of the key factors on cell wall modifications. Characterization of two elite mutants reveals several important candidate genes for genetic enhancing biomass digestibility and lodging resistance in rice.

## Results

### Selection of large population of rice cell wall mutants

In this study, we initially performed large-scale screening of cell wall mutants using the mutagenesis pools of genome-wide T-DNA insertions and chemical EMS inductions with more than 10 000 individual rice lines (Wu *et al.*, 2003; Xie and Peng, 2011). With several generations of multiplications, a total of 36 rice homozygous mutants were selected as experimental materials in this study (Figure 1). Compared with wild type variety (Nipponbare, NPB), the rice mutants displayed large variations in the three wall polymer levels in the mature straws (Figure 1a). For instance, cellulose levels varied from 14.05% to 31.62%, hemicelluloses levels from 8% to 23.37% and lignin levels from 10.63% to 18.5% on a dry matter basis (Figure 1a).

Biomass enzymatic digestibility (or saccharification) has been defined by calculating either the hexoses yield (% cellulose)

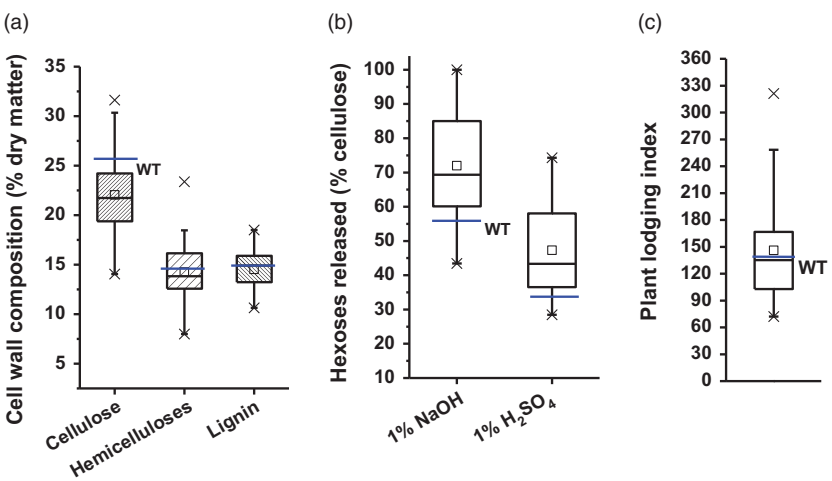


**Figure 1** Variations of total 36 genetic mutants of rice ($n$ = 37). (a) Diverse three major wall polymer levels. (b) Varied hexoses yields released from the mixed-cellulases hydrolysis after 1% NaOH and 1% $H_2SO_4$ pretreatments. (c) Varied plant lodging indexes. Blue line indicated as wild type.

 

released from hydrolysis by a crude cellulase mixture of lignocellulose after pretreatment or total sugars yield (% dry matter) released from both enzymatic hydrolysis and pretreatment (Li *et al.*, 2013; Xu *et al.*, 2012). Due to their diverse cell wall compositions, the 36 rice mutants also exhibited varied biomass enzymatic digestibility upon 1% NaOH and 1% $H_2SO_4$ pretreatments (Figure 1b). In particular, the hexoses yields varied from 43% to 100% (% cellulose) by 1% NaOH pretreatment and from 28% to 74% by 1% $H_2SO_4$ pretreatment.

As plant cell wall greatly affects plant mechanic strength (Ma, 2009; Tanaka *et al.*, 2003; Zhang and Zhou, 2011), we measured plant LI in the rice mutants. As determined in cell wall compositions and biomass digestibility, the mutants also exhibited large variations in plant LI (Figure 1c). Compared with the wild type, more than 50% mutants displayed a higher lodging resistance due to their relative lower LI values. Moreover, we performed a correlation analysis between LI and morphological traits including plant height, fresh weight and breaking force (Table S1). As a result, the plant LI displayed a positive correlation with plant height and fresh weight ($P < 0.01$), but was not significantly correlated with the plant breaking force.

To examine the genetic stability of the homozygous mutants used in this study, we also performed a correlation analysis among the cell wall composition, hexoses yields, breaking forces and LI using a total of 30 mutants samples harvested between 2009 and 2010 seasons (Table S2). Notably, all those parameters measured in two season's samples showed a positive correlation at $P < 0.05$ and 0.01.

## Effects of wall polymer levels on hexoses yields and LI

Pairwise correlation has been extensively applied to investigate biological traits relationships or associations using large populations of samples (Li *et al.*, 2013; Zhang *et al.*, 2013). In this study, a correlation analysis was conducted to find out effects of plant cell wall composition on biomass digestibility and LI in rice mutants (Figure 2). Significantly, cellulose level negatively affected hexoses yields under 1% NaOH or 1% $H_2SO_4$ pretreatment whereas both hemicelluloses and lignin contents displayed a positive impact at $P < 0.05$ and 0.01 ($n = 37$), different from observations in wheat showing hemicelluloses and lignin as negative factors (Wu *et al.*, 2013). By contrast, cellulose level positively affected LI, but hemicelluloses and lignin levels showed the negative impacts at significance levels ($P < 0.01$). Because LI adversely indicates degree of plant lodging resistance, both hemicelluloses and lignin were examined to be the positive factors on plant lodging resistance in rice. Hence, three major wall polymer levels could distinctively affect biomass enzymatic digestibility and plant lodging resistance in rice.

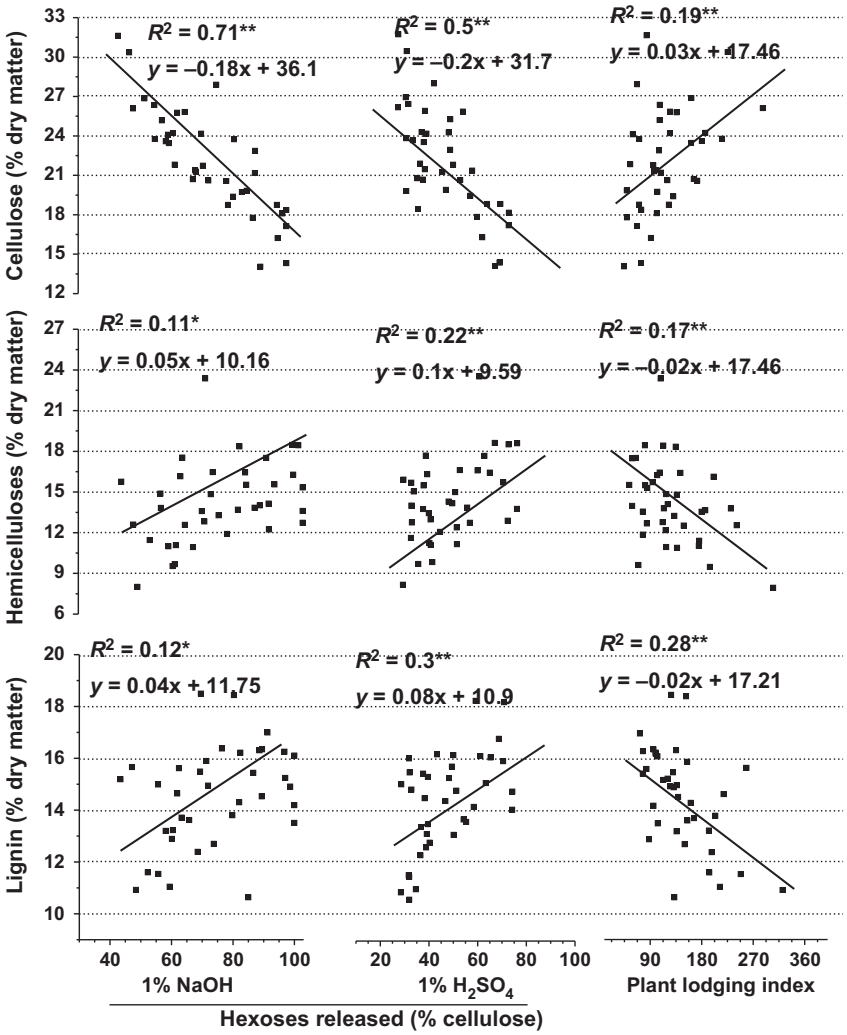


**Figure 2** Effects of the wall polymer levels on hexoses yields and lodging indexes in rice mutants and wild type ($n = 37$). * and ** indicated significant correlations at $P < 0.05$ and 0.01, respectively.

## Effects of wall polymer features on biomass saccharification and lodging resistance

It has been characterized that three major wall polymer features, rather than wall polymer levels, could predominately affect biomass enzymatic digestibility in the grass plants examined (Li *et al.*, 2014a; Wu *et al.*, 2013). However, little is yet known about the wall polymer feature impacts on plant agronomic traits. In this work, three major wall polymer features were detected including cellulose CrI, monosaccharide composition of hemicelluloses and three monomer constitution of lignin in a total of 36 rice mutants and wild type (Table S3). As expected, the cellulose CrI showed a negative impact on hexoses yields under 1% NaOH and 1% $H_2SO_4$ pretreatments at $P < 0.01$ (Figure 3a), consistent with the findings in other plants (Li *et al.*, 2014a; Wu *et al.*, 2013; Zhang *et al.*, 2013). However, the cellulose CrI exhibited a positive impact on the LI at $P < 0.05$ (Figure 3a). As the LI was a negative parameter on lodging resistance, to our knowledge, this was the first time to report that the cellulose CrI was the negative factor on both biomass digestibility and lodging resistance in plants.

With respect to the hemicelluloses feature, Xyl and Ara were determined to be two major monosaccharides covering 80% and 12% of total (Table S3), similar to other grass plants (Li *et al.*, 2014a; Wu *et al.*, 2013; Zhang *et al.*, 2013). However, only Ara level of hemicelluloses, other than Xyl and Xyl/Ara, showed a significant correlation either positively with hexoses yields upon 1% NaOH and 1% $H_2SO_4$ pretreatments or negatively with LI at $P < 0.01$ (Figure 3b). Hence, the Ara level was the positive factors on both biomass digestibility and lodging resistance in rice, in contrast to the cellulose CrI being the negative factors. In addition, despite that the Xyl/Ara showed a negative impact on hexoses yields, similar to the findings in other grasses examined (Li *et al.*, 2013, 2014a; Wu *et al.*, 2013), it did not exhibit correlation with LI at significant level in rice mutants. In terms of three monomers of lignin in rice mutants, the G monomer exhibited a significant impact on both hexoses yields from 1% NaOH or 1% $H_2SO_4$ pretreatment and LI in rice mutants at $P < 0.05$ (Figure 3c). As a comparison, the H and S monomers showed a positive correlation with the hexoses yield from 1% $H_2SO_4$ pretreatment, other than from 1% NaOH pretreatment. Therefore, the Ara and G monomer levels could predominately determine hemicelluloses and lignin's positive impacts on biomass enzymatic digestibility and lodging resistance in rice mutants, respectively.

## Association among three wall polymers

Using the 36 rice mutants, we further performed a correlative analysis among the three major wall polymers (Figure 4). Both cellulose level and CrI were negatively correlated with hemicelluloses levels at $P < 0.01$, but did not show any significant correlation with lignin levels (Figure 4a,b), indicating that cellulose should mainly have an interaction with hemicelluloses other than lignin in rice mutants as reported in *Miscanthus* (Xu *et al.*, 2012). Notably, the cellulose CrI exhibited the significant correlation with hemicellulosic Ara, but remained no correlation with G monomer and other two monomers (S and H) of lignin (Figures 4c and S1a). Despite that cellulose CrI is basically affected by cellulose level (Figure S1b), the results revealed that the Ara level, other than G monomer, could negatively affect cellulose CrI via its interaction with β-1,4-glucans for positively enhancing lignocellulosic enzymatic digestibility and lodging resistance in rice mutants.

Furthermore, the hemicelluloses and Ara levels, respectively, exhibited a positive correlation with lignin and G monomer levels at $P < 0.01$, indicating that both polymers should have an association (Figure 4d). In addition, the H monomer was also positively correlated with Ara at $P < 0.05$ (Figure S1c), but it remains lower coefficient values than that of G monomer. Therefore, the data suggested that the hemicellulosic Ara may mainly interlink with G monomer, other than H and S monomers of lignin in rice mutants.

## Characterization of two standard rice mutants

Among the 36 rice mutants, we characterized two standard rice mutants termed as *Osfc17* and *Osfc30* (Figure 5). In general, the two mutants, like wild type (NPB), exhibited normal agronomic traits in the field (Figure 5a,b). However, the *Osfc17* mutant could even show the higher dry spike (total grain yield) by 17% than that of the wild type at $P < 0.05$, whereas the *Osfc30* mutant had much lower LI by 27% at $P < 0.01$ (Table S4). Notably, both mutants showed much higher hexoses yields (% cellulose) or total sugar yields (% dry matter) up to 1.3-fold to twofold than that of the wild type after pretreatments with $H_2SO_4$ and NaOH at three concentrations (Figure 5c and Table S5). By comparison, the *Osfc30* mutant could even exhibit higher hexoses and total sugars yields up to onefold than that of the *Osfc17* mutant. Hence, both mutants might be directly applied as desirable bioenergy crops for biofuel purpose.

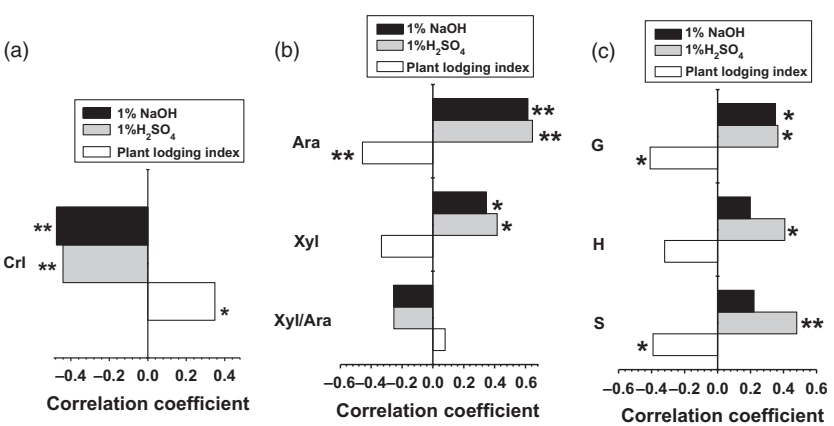


**Figure 3** Effects of the wall polymer features on hexoses yields and lodging indexes in rice mutants and wild type ($n = 37$). (a) Cellulose crystallinity (CrI). (b) Hemicellulosic Ara and Xyl. (c) Three monomers (G, S, H) of lignin. * and ** indicated significant correlations at $P < 0.05$ and 0.01, respectively.

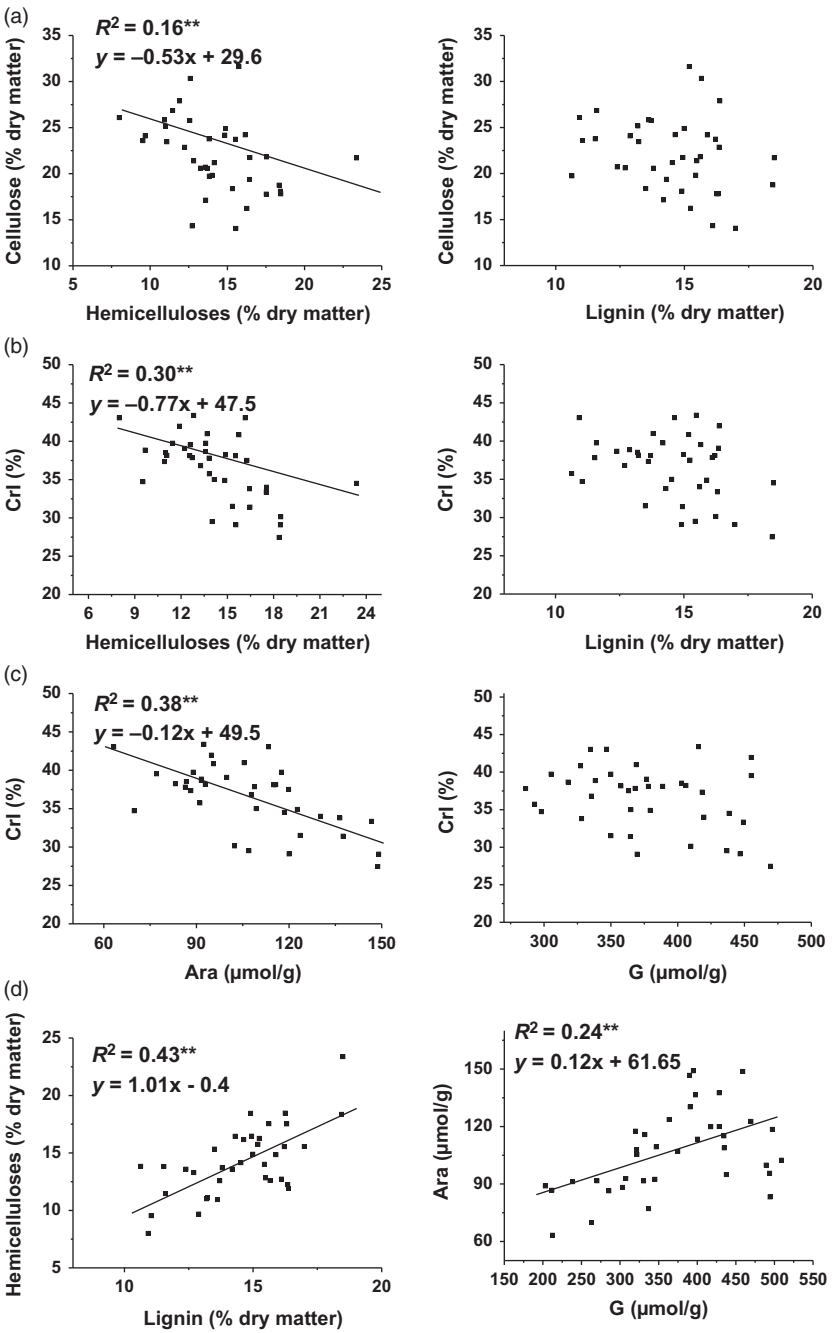


**Figure 4** Correlations among the wall polymer levels and features in rice mutants and wild type ($n$ = 37). (a) Correlations of cellulose with hemicelluloses and lignin. (b) Correlations of CrI with hemicelluloses and lignin. (c) Correlations of CrI with hemicellulosic Ara and G monomer. (d) Correlations between hemicelluloses and lignin. ** indicated significant correlations at $P < 0.01$.

Furthermore, the two mutants were determined with distinct alternations in cell wall compositions and features (Table 1). Compared with the wild type, the *Osfc17* mutant exhibited increased hemicelluloses and lignin levels by 57% and 23% at $P < 0.01$, whereas the *Osfc30* mutant showed a major decrease in cellulose level by 29%. In terms of the wall polymer features, both mutants exhibited decreased cellulose CrI by 10% and 21% than that of the wild type (Table 1), confirming that the cellulose CrI is the negative factor on biomass enzymatic digestibility. In addition, the *Osfc30* mutant showed much lower cellulose CrI value than that of the *Osfc17* mutant, consistent with their difference in biomass enzymatic digestibility (Table S5). Meanwhile, compared with the wild type, the *Osfc17* and *Osfc30* mutants exhibited increased Ara levels by 42% and 23%, but did not show much different G monomer levels (Table 1), which support the previous findings about the negative impact of Ara (other than G monomer) on cellulose CrI for high biomass digestibility (Figure 4c). As the *Osfc17* mutant showed much less decreased cellulose level (−19%, relative to the wild type) than that of the *Osfc30* mutant (−29%), its much more increased Ara level (42%, relative to 23% in *Osfc30*) may mainly attribute for the reduced cellulose CrI (Table 1). Hence, the reduced cellulose level and increased Ara level in the *Osfc30* mutant should both attribute for the much reduced cellulose CrI. Taken all together, it demonstrated that the cell wall modifications could not only maintain plant normal growth and grain yield, but also enhance plant lodging resistance and biomass enzymatic digestibility in rice mutants.

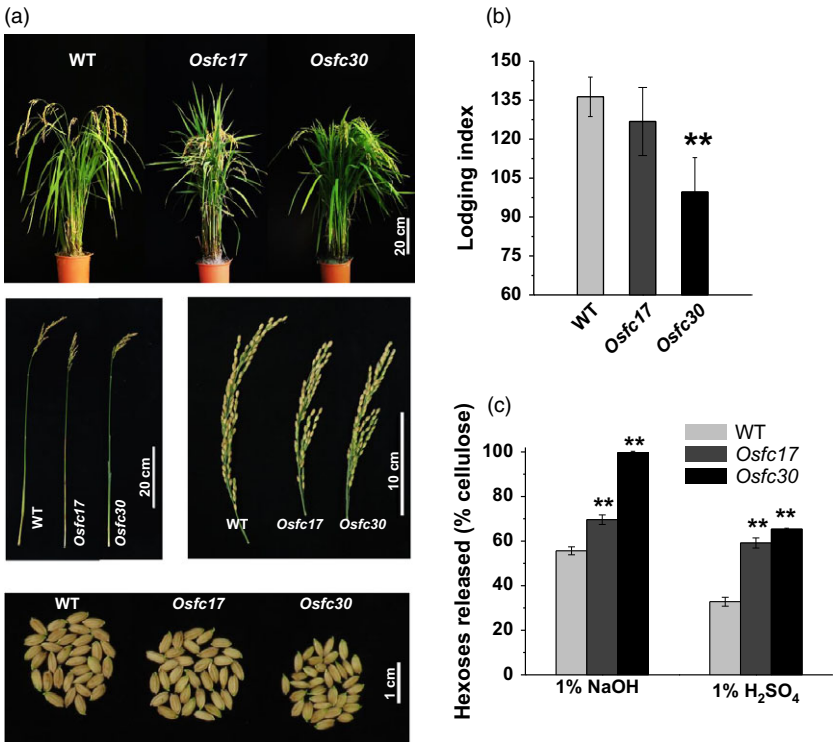


**Figure 5** Comparisons between two elite mutants (*Osfc17*, *Osfc30*) and wild type (NPB). (a) Plant growth and grain phenotypes. (b) Plant lodging index. (c) Hexoses yields released from enzymatic hydrolysis after 1% NaOH and 1% $H_2SO_4$ pretreatments. ** indicated significant difference between the mutants and wild type by *t*-test at $P < 0.01$.

**Table 1** Three major wall polymer levels and features in the two mutants and wild type

| | Wall polymer level (% dry matter) | | | Wall polymer feature | | |
|---|---|---|---|---|---|---|
| Sample | Cellulose | Hemicelluloses | Lignin | CrI[†] (%) | Ara (μmol/g) | G (μmol/g) |
| WT | 26.34 ± 1.64 | 14.9 ± 1.46 | 14.99 ± 0.13 | 38.23 | 83.17 ± 1.7 | 494.42 ± 4.64 |
| *Osfc17* | 21.29 ± 0.61* | 23.37 ± 0.63** | 18.5 ± 0.14** | 34.52 | 118.50 ± 3.18** | 497.27 ± 14.01 |
| | (−19%)[‡] | (57%) | (23%) | (−10%) | (42%) | (1%) |
| *Osfc30* | 18.75 ± 0.36** | 18.47 ± 0.47** | 16.25 ± 0.1* | 30.13 | 102.37 ± 1.87** | 509.15 ± 11.32 |
| | (−29%) | (24%) | (8%) | (−21%) | (23%) | (3%) |

[†]CrI method was detected at ±0.05–0.15 at $P < 0.01$ using five representative samples.

* and ** indicated significant difference between the mutants and wild type by *t*–test at $P < 0.05$ and 0.01, respectively.

[‡]Percentage of the increased and decreased level between the mutants and wild type.

## Observations of cell tissues *in situ* and biomass residues *in vitro*

To confirm the biomass enzymatic digestibility of the two standard mutants (*Osfc17*, *Osfc30*), we observed their stem cell tissues *in situ* and biomass residues *in vitro* under scanning electron microscopy. Without any pretreatment and enzymatic digestion, the young stems of two mutants and wild type did not show any visible difference *in situ* (Figure 6). However, both mutants exhibited much more destructed and lost cells than that of the wild type after 1% NaOH or 1% $H_2SO_4$ pretreatment and sequential enzymatic hydrolysis. Hence, the effective destruction *in situ* of stem tissues in the mutants could attribute for high biomass enzymatic digestibility *in vitro*. Furthermore, the two mutants were observed with much rougher surfaces of biomass residues *in vitro* compared with the wild type under 1% NaOH or 1% $H_2SO_4$ pretreatments and sequential enzymatic hydrolysis (Figure S2), consistent with the findings in other grass plants (Li *et al.*, 2013, 2014a; Xu *et al.*, 2012). Therefore, both observations *in situ* and *in vitro* confirmed a high biomass enzymatic digestibility in the two mutants.

## Detection of the major gene candidates for cell wall modifications

It has been reported that more than one thousand genes should be associated with plant cell wall formation and modification (Ralph *et al.*, 2004; Scheller and Ulvskov, 2010; Somerville, 2006; Xie *et al.*, 2013). In this study, we detected the transcription alterations of the major genes that lead to reducing cellulose CrI and increasing hemicellulosic Ara levels in the *Osfc17* and *Osfc30* mutants (Figure 7). Relative to the wild type, the *Osfc17* and *Osfc30* mutants exhibited much higher transcription levels of *GH9B1*, *3*, *16* genes by onefold to sevenfold, with lower *CESAs* gene expressions by 0.5- to twofold (Figure 7a). As CESA enzymes are involved in cellulose biosynthesis and GH9Bs have enzymatic activity for decreasing cellulose CrI (Xie *et al.*, 2013),

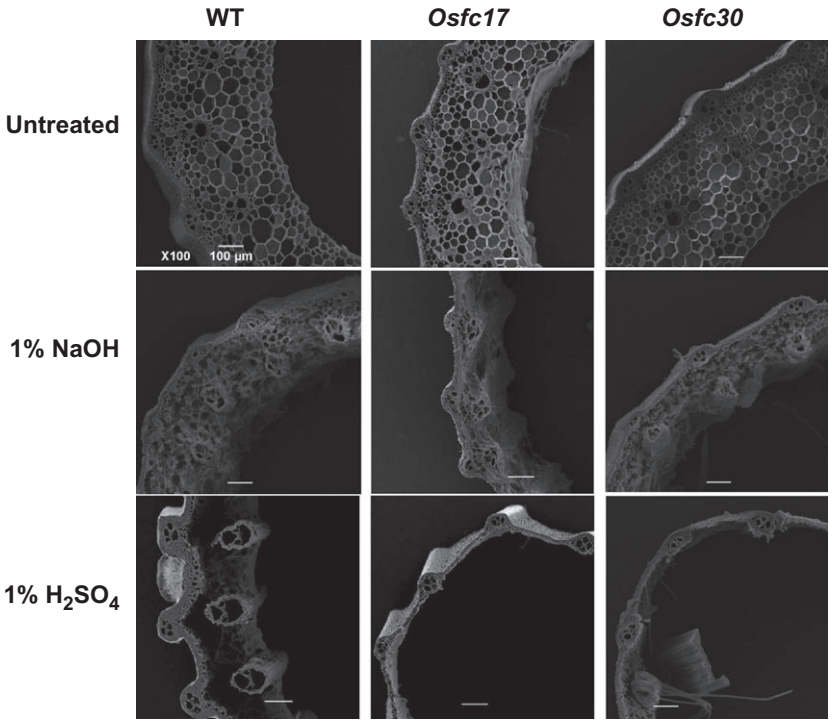


**Figure 6** Observations *in situ* of stem tissues in the two mutants (*Osfc17*, *Osfc30*) and wild type (NPB) after pretreated with 1% NaOH and 1% $H_2SO_4$ and sequentially digested with mixed-cellulase enzymes using scanning electron microscopy.

the data were consistent with the reduced cellulose level and CrI in the mutants (Table 1). With regard to the increased hemicellulose and Ara levels, the two mutants were detected with much higher transcription levels of *OsXAT2*, *3* and *OsIRX9*, *14* genes up to 0.4- to 2.1-fold compared with the wild type (Figure 7b). However, the *OsXAT2*, *3* transcription levels were higher than that of *OsIRX9*, *14*. As the OSIRX9 and OSIRX14 are involved in backbone chain synthesis of hemicelluloses (Chiniquy *et al.*, 2013), and OSXAT2 and OSXAT3 are specific for the Ara side chain elongation (Anders *et al.*, 2012), the increased *OsXAT2* and *OsXAT3* expression levels were in support of the findings about much higher Ara levels in the two mutants. Hence, three *GH9B* and two *OsXAT* genes could be considered for genetic enhancing biomass enzymatic digestibility and plant lodging resistance in rice.

In addition, as total 10 gene families are involved in the lignin biosynthesis in monocot and dicot plants (Raes *et al.*, 2003; Xu *et al.*, 2009), we also detected transcription levels of the 19 representative genes based on microarray data and coexpression analysis in rice (Guo *et al.*, 2014). Compared with the wild type, the *Osfc17* and *Osfc30* mutants showed much higher expression levels in the 14 genes, but remained lower transcription levels in four genes (Figure S3).

## Discussion

### Large-scale screening of rice mutants for wall factor identification

Selection of genetic mutants and transgenic plants with cell wall modifications has been considered for enhancing biomass enzy-

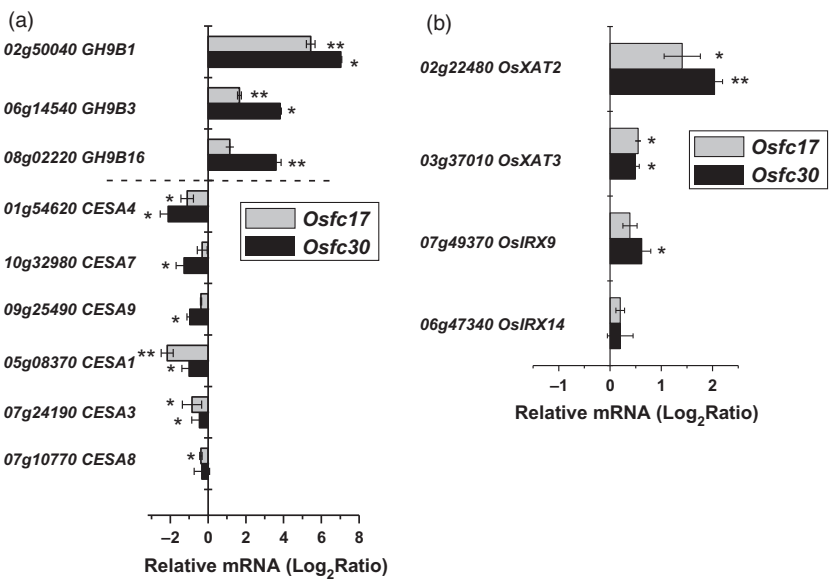


**Figure 7** Gene expression ratios of the two mutants (*Osfc17*, *Osfc30*) relative to the wild type (NPB) by qRT-PCR analysis. (a) Genes involved in cellulose biosynthesis (*CESAs*) and cellulose crystallinity modifications (*GH9Bs*). (b) Genes involved in Xylan backbone synthesis (*OsIRXs*) and branched Ara elongation (*OsXATs*). * and ** indicated significant different transcription levels between the two mutants and wild type by *t*-test at $P < 0.05$ and 0.01, respectively.

matic digestibility (Pauly and Keegstra, 2010; Xie and Peng, 2011). Due to the complicated structures and diverse functions of plant cell walls, however, the selected mutants and transgenic plants have exhibited the defects to some degrees in plant growth and development (Abramson *et al.*, 2010; Li *et al.*, 2008). Hence, it becomes essential to find out the key factors of plant cell wall modifications for normal plant growth and high biomass enzymatic saccharification using systems biology approach. Principally, the systems biological approach is powerful for analysis of the multiple traits and factors, but it requires large population of samples (Atias *et al.*, 2009; Farrokhi *et al.*, 2006; Guo *et al.*, 2014). As rice is a cultivar, large-scale screening of rice cell wall mutants is optimal for collecting large populations of samples. In this study, a total of 36 cell wall mutants have exhibited diverse biomass enzymatic digestibility and varied agronomic traits, in particular on the plant LI that is a major and integrated agronomic trait in plant growth and grain production. Notably, the mutant samples harvested from two field seasons exhibit a significantly positive correlation in various agronomic traits and biological parameters measured, indicating that the rice mutants are homozygous and genetic stable for any experiments in this work. Using those mutants, therefore, we could perform a correlative and comparative analysis, leading to finding out the key factors on cell wall modifications in rice. Notably, we have identified the several rice mutants (for instance, *Osfc30*) that exhibit both lodging resistance and biomass enzymatic digestibility higher than that of the wild type, suggesting that the mutants could be directly used as bioenergy crops.

## Hypothetical model of the key factors that direct for cell wall modifications

Based on the systems biology analysis of the 36 cell wall mutants, to our knowledge, this study at the first time has reported the distinct effects of three major wall polymer features on both biomass enzymatic digestibility and plant lodging resistance. Hence, a hypothetical model could be proposed to elucidate the key factors on cell wall modifications (Figure 8). First, the cellulose crystallinity (CrI) is the key factor that negatively determines biomass enzymatic hydrolysis and plant lodging resistance (Figure 3a). Second, the hemicellulosic Ara is the major factor negatively affecting cellulose CrI probably through its interlinking with β-1,4-glucans via hydrogen bonds (Figure 4b,c). Third, the lignin and G monomer may positively affect biomass digestibility and lodging resistance by interaction with hemicellulosic Ara (Figures 3b,c and 4d). In addition, the G monomer and lignin may also have a direct and minor impact on biomass digestion and lodging resistance, but the mechanism remains unclear.

Despite the cellulose CrI has been demonstrated to be the key negative factor on biomass enzymatic saccharification in all grass plants examined (Abramson *et al.*, 2010; Li *et al.*, 2014a; Wu *et al.*, 2013; Zhang *et al.*, 2013), it remains under test in terms of its negative impact on lodging resistance in all other crops, because the LI is the integrated parameter associated with the multiple agronomic traits that are distinctive in different crops (Ma, 2009; Sirajul Islam *et al.*, 2007). As hemicellulosic Ara is the major branched sugar in grass plants, other branched monosaccharides in hemicelluloses may also negatively affect cellulose CrI for enhancing biomass digestibility and lodging resistance. In addition, lignin is important for lodging resistance in plants (Ma, 2009), but to our surprise, it has also exhibited significantly positive impact on biomass enzymatic hydrolysis in rice mutants.

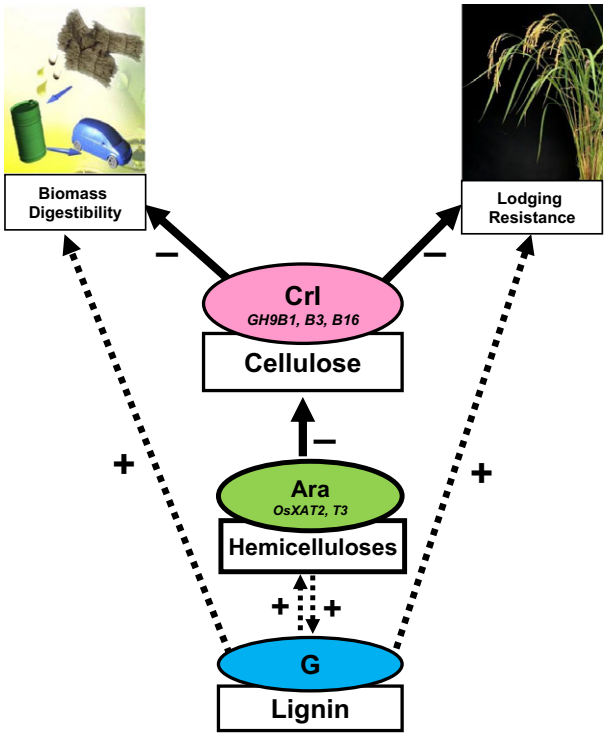


**Figure 8** Hypothetical model on the key factors of cell wall modifications for genetic enhancing both biomass enzymatic digestibility and plant lodging resistance in rice. Cellulose crystallinity (CrI) is the key factor negatively determining biomass enzymatic saccharification and plant lodging resistance, and GH9Bs enzymes have activities for reducing cellulose crystallinity; hemicellulosic Ara is the main factor negatively affecting cellulose CrI through its direct interaction with β-1,4-glucans, and OSXATs enzymes have activities for Ara elongations; G monomer and lignin may directly affect biomass digestibility and lodging resistance and/or have an interaction with hemicelluloses and Ara as an indirect impact.

Thus, we assume that the increased G and other two monomers (H, S) may not well form the interlinking networks via various chemical bonds, leading to an easy removal by chemical pretreatments. In other case, the specific selection of cell wall mutants for improved biomass digestibility and agronomic traits may result in the deposition of lignin with altered cell wall ultrastructure or anatomical distributions in the rice tissues. Therefore, the modified lignin may be required for structural integrity of cell walls, but apparently not enough to impede cell wall polysaccharides digestibility after acid or base pretreatment. A similar result was also reported in *Lucene* (*Medicago sativa* L.) with improved forage quality (Jung *et al.*, 1994). Obviously, the detailed mechanism of lignin and monomers in the cell wall digestibility deserves further investigation.

## Potential genetic modification of cell walls for enhancing both biomass digestibility and plant lodging resistance

Genetic modification of plant cell walls has been considered as a promising solution for reducing biomass recalcitrance and maintaining plant normal growth (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 plant cell walls towards enhancing both biomass enzymatic

 

digestibility and plant lodging resistance in rice (Figure 8). With respect to the cellulose crystallinity that is the key and negative factor, genetic reducing cellulose CrI becomes critical by synchronically overexpressing both three *GH9B* and two *OsXAT* candidate genes (Figure 7a,b), which have been demonstrated in the two elite mutants (*Osfc17* and *Osfc30*) (Figure 5). It has been assumed that GH9B enzymes may have activities to produce the noncrystalline cellulose in the surface of microfibers (Xie *et al.*, 2013), and thus, more hemicellulosic Ara catalyzed by XAT may be required to fill in the noncrystalline cellulose regions to maintain cell wall strength and integrity, which supports that the Ara may have interaction with noncrystalline cellulose via hydrogen bonds (Li *et al.*, 2013). Furthermore, our preliminary data have showed that over-expressing single *OsGH9B* gene could result in plant growth defects in the transgenic rice lines (data not shown). Hence, overproduction of both GH9B and XAT enzymes should be the potential approach for largely enhancing both biomass enzymatic digestibility and lodging resistance in transgenic rice. In addition, as described above, the enhanced G monomer and lignin may also aid to maintain cell wall strength and integrity in the transgenic plants or mutants.

However, there may be an alternative approach by genetic manipulation of single genes that regulate multiple genes (such as *GH9B*, *XAT* and others), as observed in *Osfc17* and *Osfc30* mutants. Our preliminary data have showed that *Osfc17* may encode the dynamic-related protein and *Osfc30* may be the upstream transcription regulator (data not shown). Hence, characterization of *Osfc17* and *Osfc30* mutants could offer other desire single genes for genetic modification of plant cell walls in rice and beyond.

### A rapid evaluation of biomass digestibility using young stem tissues

Fourier transform infrared attenuated total reflectance spectroscopy has been used to predict cell wall composition and biomass digestibility in various plants such as *Switchgrass*, *Bluestem* grass, prairie biomasses, hardwood and corn stover (Sills and Gossett, 2012). A near infrared spectroscopic approach was also applied to evaluate biomass enzymatic digestibility in *Miscanthus* (Huang *et al.*, 2012). However, this machine-based method requires the equation formula based on chemical analysis of large populations of biomass samples. In this study, we could pre-evaluate biomass enzymatic saccharification by observing *in situ* the enzymatic digestion of young stem tissues at heading stage of rice (Figure 6). To confirm the desire mutants, we have further performed time-course observations of the stem tissue digestions between wild type and mutants. Therefore, this method can be used to quickly identify the genetic mutants and transgenic plants with high biomass enzymatic digestibility.

## Conclusions

Using systems biology analysis of a total of 36 cell wall mutants, cellulose crystallinity (CrI) has been examined to be the key factor negatively determining biomass enzymatic saccharification and plant lodging resistance, whereas hemicellulosic Ara is the major factor that negatively affects cellulose CrI. Characterization of two elite mutants (*Osfc17* and *Osfc30*) further suggests the potential cell wall modifications for enhancing both biomass enzymatic digestibility and plant lodging resistance by synchronically expressing *GH9B* and two *OsXAT* candidate genes in rice.

## Experimental procedures

### Plant materials

Selections of rice T-DNA insertion and EMS induction mutagenesis pools were performed in 2008 and 2009 as described by Xie *et al.* (2013) and Wu *et al.* (2013). The samples of a total of 36 homozygous rice mutants were collected from the Huazhong Agricultural University experimental fields in 2009 and 2010. The collected mature straws were dried at 60 °C to the constant weight, grounded into powders through 40 mesh (0.425 mm × 0.425 mm) and stored in the dry container until use.

### Measurement of LI in rice

Lodging index was detected using rice stem tissue in 30 days after heading. Length of the fourth stem internode was measured, and its breaking force was detected using a Prostrate Tester (DIK 7401; Daiki, Osaka, Japan), with the distance between fulcra of the tester at 5 cm. Fresh weight (*W*) of the upper portion of the plant was measured including panicle, four internodes and leaves. Bending moment (BM) was calculated using the following formula: BM = length of the fourth internode × W, and thus, LI was calculated as follows: LI = BM/breaking force × 100%. Measurements of lodging indexes of all samples were duplicated with six independent biological experiments.

### Dry spike measurement

The spike weights of rice mutants were calibrated after these samples were dried in the oven at 60 °C to a constant weight.

### Plant cell wall fractionation

The plant cell wall fractionation procedure was applied to extract cellulose and hemicelluloses, as described by Peng *et al.* (2000) with minor modification by Wu *et al.* (2013). The soluble sugar, lipids and starch of the samples were successively removed by potassium phosphate buffer (pH 7.0), chloroform-methanol (1 : 1, v/v) and DMSO-water (9 : 1, v/v). The remaining pellets as total crude cell wells were suspended in 0.5% (w/v) ammonium oxalate and heated for 1 h in a boiling water bath, and the supernatants were combined as total pectin. The remaining pellets were suspended in 4 M KOH containing 1.0 mg/mL sodium borohydride for 1 h at 25 °C, and the combined supernatants were neutralized, dialysed and lyophilized as KOH-extractable hemicelluloses. The remaining pellets were sequentially extracted with TFA as non-KOH-extractable hemicelluloses. The pellets were further extracted with acetic–nitric acids–water (8 : 1 : 2) for 1 h at 100 °C, and the remaining materials were regarded as crystalline cellulose. All experiments were carried out in biological triplicate.

### Colorimetric assay of hexoses and pentoses

UV–VIS Spectrometer (V-1100D; Shanghai MAPADA Instruments Co., Ltd. Shanghai, China) was applied for total hexoses and pentoses assays. Hexoses were detected by anthrone/$H_2SO_4$ method, and pentoses were detected by orcinol/HCl method (Dische, 1962). Regarding the high pentoses level can affect the absorbance reading at 620 nm for hexoses content by the anthrone/$H_2SO_4$ method, the deduction from pentoses reading at 660 nm was carried out for final hexoses calculation. A series of xylose concentrations were analysed for plotting the standard curve referred for the deduction, which was verified by

GC-MS analysis. Cellulose level was measured by the anthrone/$H_2SO_4$ method, and hemicelluloses level was calculated according to total hexoses and pentoses detected. Total sugar yields of biomass samples released from pretreatment and enzymatic hydrolysis were subject to the sum total of hexoses and pentoses. All experiments were carried out in biological triplicate.

### Detection of cellulose crystallinity

X-ray diffraction method described by Zhang *et al.* (2013) was applied for detection of cellulose crystallinity index (CrI) using Rigaku-D/MAX instrument (Uitima III, Tokyo, Japan). The well-mixed powders of biomass samples were detected under plateau conditions. Ni-filtered Cu Kα radiation (λ = 0.154056 nm) generated at voltage of 40 kV and current of 18 mA and scanned at speed of 0.0197°/s from 10° to 45°. The crystallinity index (CrI) was calculated using the intensity of the 200 peak ($I_{200}$, θ = 22.5°) and the intensity at the minimum between the 200 and 110 peaks ($I_{am}$, θ = 18.5°) as the follow: $CrI = 100 \times (I_{200} - I_{am})/I_{200}$. $I_{200}$ represents both crystalline and amorphous materials while $I_{am}$ represents amorphous material. Standard error of the CrI method was detected at ±0.05–0.15 using five representative samples in triplicate.

### Hemicelluloses monosaccharide determination by GC-MS

The sample preparations and GC-MS analysis were conducted as previously described by Li *et al.* (2013).

### Total lignin assay

Total lignin determinations were performed by two-step acid hydrolysis method according to Laboratory Analytical Procedure of the National Renewable Energy Laboratory. The acid-insoluble lignin (AIL) was calculated gravimetrically as acid-insoluble residues after subtraction for ash, and the acid-soluble lignin (ASL) was detected by UV spectroscopy.

#### *Acid-insoluble lignin assay*

0.5 g sample as $W_1$ was extracted with benzene-ethanol (2 : 1, v/v) in a Soxhlet for 4 h, and air-dried in hood overnight. The sample was hydrolyzed with 10 mL 72% $H_2SO_4$ (v/v) in shaker at 30 °C for 1.5 h. After hydrolysis, the acid was diluted to 2.88% and placed in 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 in a filtering flask for ASL. The lignin was washed free of acid with hot distilled water and the crucible, and the acid-insoluble residue was dried in an oven at 80 °C. The weight of the crucible and dry residue was recorded to the nearest 0.1 mg ($W_2$). 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 to the nearest 0.1 mg as $W_3$. AIL was calculated according to the equation: $AIL\,(\%) = (W_2 - W_3) \times 100/W_1\%$.

#### *Acid-soluble lignin assay*

The hydrolysis liquor obtained previously was transferred into 250-mL volumetric flask and brought up to 250 mL with 2.88% sulphuric acid. The absorbance was read at 205 nm on a UV–Vis spectroscopy (Du800; Beckman Coulter Inc. Brea, California, USA), and 2.88% sulphuric acid was used as blank. The calculation of the ASL was based on the equation: $ASL\,(\%) = (A \times D \times V/1000 \times K \times W_1) \times 100\%$. $A$ (absorption value), $D$ (Dilution ratio of the sample), $K$ (absorptivity constant) = 110 L/g/cm. Total lignin (%) = ASL% + AIL%. All experiments were carried out in biological triplicate.

### Lignin monomer detection by HPLC

Lignin monomers were determined by HPLC as previously described by Xu *et al.* (2012) and Wu *et al.* (2013).

### Determination of biomass digestibility

Chemical pretreatments and the following residues enzymatic hydrolysis were performed as previously described by Huang *et al.* (2012).

### Scanning electron microscopic (SEM) observation

We prepared a transverse section of the 2nd stem internode tissues at heading stages. Stem transverse sections were pretreated with 1% NaOH or 1% $H_2SO_4$ as described above; the stem transverse sections were washed with distilled water until pH 7.0 and hydrolyzed with the mixed-cellulase for 2 h at 50 °C. The mixed-cellulases enzyme containing β-glucanase (≥$6 \times 10^4$ U), cellulase (≥600 U) and xylanase (≥$1.0 \times 10^5$ U) was purchased from Imperial Jade Bio-technology Co., Ltd. (Ningxia, China). After enzymatic hydrolysis, the sample surfaces were sputter-coated with gold and observed the tissue degradation intensity under scanning electron microscope (SEM JSM-6390/LV; Hitachi, Tokyo, Japan). The well-mixed biomass residues from pretreatment and sequential enzymatic hydrolysis were observed as previously described by Wu *et al.* (2013). Each sample was observed for 5–10 times, and the representative image was used in this study.

### qRT-PCR analysis

Total RNA was isolated using RNAprep pure Plant Kit (DP432; TIANGEN BIOTECH, Beijing, China), and 5 μg total RNA was reverse transcribed with an oligo(dT)18 primer in a 50 μL reaction using an M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The qRT-PCR was performed 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 (ZOMANBIO, Beijing, China) on Two Color Real-time PCR Detection System (MyiQ2; Bio-Rad, Hercules, California, USA) using the following program: 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, 25 s at 72 °C. *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). All quantitative PCR experiments were performed in biological triplicate. All the gene-specific primers used were listed in Table S6.

### Statistical calculation of correlation coefficients

Correlation coefficients were calculated by performing Spearman rank correlation analysis for all pairs of measured traits across the whole population. This analysis used average values calculated from all original determinations for a given traits pair.

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## Supporting information

Additional Supporting information may be found in the online version of this article:

**Figure S1** Correlative analysis among the wall polymer features in rice mutants and wild type ($n$ = 37).
**Figure S2** Observations *in vitro* of biomass residues in the two mutants (*Osfc17*, *Osfc30*) and wild type (NPB) after pretreated with 1% NaOH and 1% $H_2SO_4$ and sequentially digested with mixed-cellulase enzymes using scanning electron microscopy.
**Figure S3** Transcription ratios of monolignol synthase genes of the two mutants (*Osfc17*, *Osfc30*) relative to the wild type (NPB) by qRT-PCR analysis.
**Table S1** Correlation coefficients among lodging-related traits in rice mutants and wild type ($n$ = 37).
**Table S2** Correlation coefficients of wall polymer levels, hexoses yield and agronomic traits between 2009 and 2010 season in rice mutants ($n$ = 30).
**Table S3** Cellulose CrI, hemicellulosic monosaccharides and three monolignols.
**Table S4** Agronomic traits in the two mutants and wild type.
**Table S5** Hexoses yields (% cellulose) and total sugar yields (% dry matter) released from enzymatic hydrolysis after various chemical pretreatments in the two mutants and wild type.
**Table S6** Primers used for qRT-PCR analysis.