1 Screening of rice mutants with improved saccharification efficiency results in the identification
2 of CONSTITUTIVE PHOTOMORPHOGENIC 1 and GOLD HULL AND INTERNODE 1
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33
34

Abstract

Main conclusion **The screening of rice mutants with improved cellulose to glucose saccharification efficiency (SE) identify reduced xylan and/or ferulic acid, and qualitative change of lignin to impact SE.**

To ensure the availability of sustainable energy, considerable effort is underway to utilize lignocellulosic plant biomass as feedstock for the production of biofuels. However, the high cost of degrading plant cell wall components to fermentable sugars (saccharification) has been problematic. One way to overcome this barrier is to develop plants possessing cell walls that are amenable to saccharification. In this study, we aimed to identify new molecular factors that influence saccharification efficiency (SE) in rice. By screening 22 rice mutants, we identified two lines, *122* and *108*, with improved SE. Reduced xylan and ferulic acid within the cell wall of line *122* were probable reasons of improved SE. Line *108* showed reduced levels of thioglycolic-released lignin; however, the amount of Klason lignin was comparable to the wild-type, indicating that structural changes had occurred in the *108* lignin polymer which resulted in improved SE. Positional cloning revealed that the genes responsible for improved SE in *122* and *108* were rice *CONSTITUTIVE PHOTOMORPHOGENIC 1 (OsCOP1)* and *GOLD HULL AND INTERNODE 1 (GH1)*, respectively, which have not been previously reported to influence SE. The screening of mutants for improved SE is an efficient approach to identify novel genes that affect SE, which is relevant in the development of crops as biofuel sources.

Keywords Cellulose, Ferulic acid, Lignin, Rice mutant, Xylan

Abbreviations

SE	saccharification efficiency
SNP	single nucleotide polymorphism
NGS	next generation sequencing
GA	gibberellic acid
NIL	near isogenic lines
BMB	bending moment at breaking

Introduction

Considerable effort is underway to efficiently convert lignocellulosic biomass to bio-ethanol. Lignocellulosic biomass is primarily derived from plant cell walls; thus, the major cell wall component, cellulose, becomes the predominant feedstock for bio-ethanol production. The advantage of using lignocellulosic biomass compared to corn grain and sugarcane juice which are currently used for commercial bio-ethanol production, is that it is more abundant and does not compete with crops grown for food supply (Yuan et al. 2008). Furthermore, not only the

abundance but also the possibility of so many different sources of lignocellulosic materials would allow countries that cannot produce neither corn nor sugarcane to have their own bioenergy matrix according to their crop of choice. On the other hand, the disadvantage of lignocellulosic biomass is that the complex physical structure and chemical compositions of plant cell wall hamper the efficient conversion of cell wall polysaccharides into fermentable sugars. For example, cellulose must first be degraded to glucose to be used for fermentation, a process termed as saccharification (Weng et al. 2008). This process is difficult and costly due to cellulose crystallinity and also because it is hampered by the complex interactions of cellulose with hemicellulose and lignin in the plant cell wall. One approach to reduce the cost is to develop plants with improved saccharifiication efficiency (SE).

Several genes that impact SE have been identified using reverse genetics, and the most extensive research has been conducted on lignin biosynthesis genes. Lignin is the third most abundant component in plant cell walls and is a phenolic polymer composed primarily of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units (Boerjan et al. 2003). Reducing lignin content by modifying lignin biosynthesis genes often leads to improved SE.

Another important factor in SE are xylans, the major component of rice hemicellulose. Xylans contain a linear backbone of β-(1→4)-linked D-xylosyl residues (Vogel 2008; Scheller and Ulvskov 2010) with the C-2 and/or C-3 positions often modified by acetylation or arabinosylation. In grasses, the O-5 of α-1,3 arabinosyl residues can be further modified with ferulate and coumarate esters (de O Buanafina 2009). It is known that reducing the number of arabinosylated xylan residues leads to improved SE (Chiniquy et al. 2012). This might be attributed to the reduction of arabinose-bound ferulates, since ferulates strengthen the cell wall by intra- and intermolecular cross-linking in both xylan and lignin (Grabber et al. 1998a, 1998b; Lam et al. 2003; Casler and Jung 2006; Anders et al. 2012; Chiniquy et al. 2012; Bartley et al. 2013).

As stated above, genes which influence SE have been identified primarily through reverse genetics. In contrast, a forward genetic approach has been conducted in only a few cases, especially, studies on screening of SE improved plants from a large population is limited (Bartley et al. 2013; Stamatiou et al. 2013; Li et al. 2015; Marriot et al. 2014). Marriot et al. (2014) obtained 2400 M2 plants of *Brachypodium distachyon* using a chemical mutagen and selected 12 lines with improved SE. Although they did not isolate the genes responsible for the improved SE, GT61 glycosyltransferase was identified as a candidate based on the analysis of one mutant. Another large scale screening was conducted by Stamatiou et al. (2013) who used ethyl methanesulfonate to mutagenize Arabidopsis seedlings. From 23000 chemically mutagenized M2 plants, 63 mutants were identified showing increased sugar release compared to the wild-type parental line. Although they did not discriminate whether sugar was released

1 from the cell wall or from starch, they identified genes responsible for improved sugar release
2 from three mutants (*rah1*, *rah22*, and *rah9*). *rah1* and *rah22* contained mutations in genes
3 encoding enzymes related to starch degradation, e.g. DISPROPORTIONATING ENZYME 2
4 (DPE2) and STARCH EXCESS 4 (SEX4), respectively. *rah9*, which is possibly affected in the
5 release of sugars from cell walls, contained a mutation in the gene encoding PINOID (PID), a
6 protein kinase that presumably functions in the localization of the PIN efflux auxin carrier
7 (Christensen et al. 2000). Based on the observation that *rah9* exhibited a pin-shaped
8 inflorescence, the authors measured the release of sugars by other pin-shaped mutants (*pin1* and
9 *arf5*); these also exhibited improved sugar release, suggesting that the impaired auxin transport
10 is associated with improved sugar release. The above studies suggest that screening for
11 SE-improved mutants using forward genetics is an efficient approach to identifying novel
12 factors associated with SE.
13 In the current study, 22 rice mutant lines were screened for improved SE. Two mutants were
14 identified with significantly improved SE, and the mutations were mapped to genes not
15 previously reported to affect SE. Our findings are useful in the context of SE improvement and
16 may also be relevant in efforts to develop grasses as potential biofuels.
17
18 **Materials and methods**
19 **Plant materials**
20 Twenty-two mutants or cultivars and their 5 original cultivars of *Oryza sativa* L. plants kept at
21 Nagoya University were grown in the rice paddy field at the Togo Field Science and Education
22 Center of Nagoya University. Every rice plant was sampled 40 days after heading and used for
23 the analyses since accumulation of secondary cell wall usually completes before 30 days after
24 heading (Hirano et al. 2013).
25
26 **Analysis of SE and cell wall components**
27 Plants were sun-dried for two weeks prior to analysis. After removing the panicles, the
28 above-ground parts of rice were macerated using a juicer. Subsequently, 1 g of the macerated
29 sample was further processed using a Shake Master (Bio Medical Science, Tokyo, Japan) for 1 h
30 at 20.39 *g* in tubes containing 5 mm (15 per tube) and 20 mm (1 per tube) stainless beads. Next,
31 starch was enzymatically hydrolyzed, solubilized, and removed using a Total Starch Assay Kit
32 (K-TSTA, Megazyme International Ireland Ltd., Bray, Ireland) according to the manufacturer's
33 instructions. Finally, the residues were collected, washed twice with purified water and dried at
34 40°C. For the saccharification assays, 50 mg of the destarched samples were dissolved into 997
35 μl of 50 mM acetate buffer (pH 4.8) supplemented with 0.02% (w/v) sodium azide. Next, 3 μl of
36 cellulase (GC220, 15 FPU/g, Genecore Kyowa, Tokyo, Japan) was added to the sample solution

and incubated using Mini Disk Rotor BC-710 (Biocraft, Tokyo, Japan) at maximum speed, 50°C for 24 h. After incubation, the reaction mixture was immediately centrifuged to remove solids. Glucose content in the supernatant was determined by a glucose oxidase method using Glucose CII-Test Wako (Wako Pure Chemical Industries Ltd, Osaka, Japan). For the measurement of cellulose, 0.25 g of destarched sample was hydrolyzed in 3 ml of 72% sulfuric acid at 30°C for 1 h. To completely hydrolyze cellulose, 84 ml of purified water was added to the sample and incubated at 120°C for 1 h. The supernatant (SUP) and residue (RES) fractions were separated by filtration using a 10 μm filter. The amount of glucose in the SUP fraction was measured by the Glucose CII-Test Wako and considered to represent the amount of cellulose. The conversion ratio of cellulose to glucose (cellulose conversion ratio, SE) was calculated as follows: SE (%) = (released glucose / cellulose) × 100.

The amount of xylose in the SUP fraction was measured using the D-xylose assay kit (Megazyme International Ireland Ltd) according to the manufacturer's instructions. To measure the amount of Klason lignin, the RES fraction was washed with water and dried at 105°C overnight; the weight of the RES was considered as a mixture of Klason lignin and ash. Subsequently, RES was incubated at 600 °C for 4 h to vaporize Klason lignin and the weight was considered as ash. The amount of Klason lignin was calculated by subtracting the amount of ash from the amount of Klason lignin and ash. We also applied another method to analyze lignin content, the thioglycolysis method, since lignin content often differs depending on the methodologies used. Thioglycolysis lignin was determined by the method of Suzuki et al. (2009). Ester-linked hydroxycinnamic acids were prepared from destarched samples by alkaline hydrolysis and extracted with ethyl acetate as described by Piston et al. (2010). To measure the monolignol composition, plant samples were ground with the Shake Master as described above and quantified by alkaline nitrobenzene oxidation of lignified cell walls. High-pressure liquid chromatography (HPLC) was performed as described by Pomar et al. (2002).

Proportions of cell wall fractions were measured as follows. The destarched sample was sequentially extracted at 100 °C for 10 min with water, 50 mM EDTA (pH 6.8) (pectin fraction), and 17.5% (w/v) NaOH containing 0.04% $NaBH_4$ (hemicellulose fraction). The residual precipitate was washed with water, ethanol, and diethyl ether, and collected as the cellulose fraction. The sugar content of each fraction was measured by the phenol–sulphuric acid method (Dubois et al. 1956) using glucose as the standard. Hemicellulose fraction was hydrolysed in 2 M TFA at 100 °C for 1 h and sugar composition analysis was carried out by high-performance anion-exchange chromatography (HPAEC) with pulsed amperometry detection (PAD) using a Dionex DX-500 liquid chromatograph fitted with a CarboPac PA-1 column and a pulsed amperometric detector (Dionex, Sunnyvale, CA, USA).

Analyses of agronomic traits

Plants were sun-dried for two weeks prior to measurement of grain yield and above-ground biomass. Grain yield was calculated as the total grain weight per plant. Above-ground biomass was calculated by weighing plant material (excluding panicles and roots).

Analyses of lodging resistance

Lodging resistance parameters were measured from rice plants 40 days after heading. Culm bending resistance was determined by measuring the cLr value as described by Grafius and Brown (1954). Bending moment at breaking (BMB) was measured at a distance of 4 cm between two supporting points, and the physical parameters were calculated as described by Ookawa and Ishihara (1992).

Microscopic observation of rice internodes

The second internodes of rice were selected, cut into pieces, fixed in 5% (w/v) agar dissolved in water, and sectioned with a microtome (Microslicer DTK-1000, Ted Pella, Inc., CA, USA) at a thickness of 80 to 100 μm. Sections were incubated for 5 min in phloroglucinol solution (2% in ethanol:water [95:5]); after the phloroglucinol was removed, sections were treated with 18% HCl for 5 min and then inspected by microscopy (BX51, Olympus, Tokyo, Japan). For Mäule staining, sections were treated with 1% $KMnO_4$ for 5 min, rinsed with water, treated with 25% HCl for 20 s, rinsed with water again, mounted in 28% $NH_3 \bullet H_2O$, and examined immediately by microscopy.

Positional cloning

Positional cloning was conducted by the MutMap method (Abe et al. 2012). The principle of MutMap is identical to conventional positional cloning, but includes whole genome sequencing of F2 plants and the original parental line. Genomic DNA of F2 individuals showing the *122* mutant phenotype were combined and next-generation sequencing (NGS) was conducted. If all the F2 individuals contained a SNP at a certain genome position, then the SNP frequency (SNP index) was considered to be 1.0. If all the F2 individuals contained nucleotides identical to the WT, the SNP index was 0. After sequencing, the *122* F2 population and T65 were compared, and regions with a SNP index = 1.0 were determined.

Complementation tests

The *OsCOP1* genomic and *GH1* coding regions were amplified by PCR and cloned into the *Asc*I site of pCAMBIA and *Sma*I site of pUBi/pCAMBIA, respectively. Sequences of primers used in this study are listed in Supplementary Table S1. Constructs were introduced into

Agrobacterium tumefaciens strain EHA105 and used to transform rice callus of mutants *122* and *108* according to Ozawa (2012). Transformants were selected by screening for hygromycin resistance. Regenerated plants were grown to maturity in a greenhouse, and SEs of T0 plants were measured 40 days after heading.

Results

Screening for rice mutants with improved SE

To search for rice mutants with improved SE, we selected 22 mutants with potential secondary cell wall defects from over 2000 mutant lines maintained at the Nagoya University Togo Field (Fig. 1). These mutants were chosen based on morphologies such as culm brittleness, flexible culms, golden colored grain, and early senescence, which are phenotypes often associated with altered secondary cell walls (Li et al. 2003; Zhang et al. 2006; Li et al. 2009; Zhou et al. 2009; Aohara et al. 2009; Hirano et al. 2010; Xiong et al. 2010; Dai et al. 2011; Kotake et al. 2011; Li et al. 2011; Prashant et al. 2011; Wu et al. 2012). Details of each mutant and their heading date are listed in Supplementary Table S2 and in Supplementary Figure S1, respectively. To evaluate whether the alteration of cell walls impacts SE, we additionally analyzed three mutants and one cultivar that were previously characterized as cell wall mutants. These were *brittle culm 1* (*bc1*) and *bc3* showing reduced cellulose content (Li et al. 2003; Hirano et al. 2010; Xiong et al. 2010), *fukei71*, which ectopically accumulates cell wall polysaccharide-linked ferulate and *p*-coumarate (Mase et al. 2005), and Leaf Star, a cultivar with reduced lignin content due to a mutation in *CAD2*, which encodes a lignin biosynthesis gene (Ookawa et al. 2014). Two gibberellin (GA) dwarf mutants with attenuated GA signaling (*gid1-8* and *slr1d-4*) were also included because GA is known to positively regulate cellulose synthesis (Ueguchi-Tanaka et al. 2007; Asano et al. 2009; Petti et al. 2015; Huang et al. 2015). To mention, mutants showing similar phenotypes (such as brittle culm and gibberellin mutants) are in many cases not caused by a unique factor and the loss-of-function of genes belonging to unrelated families might lead to the same phenotype.

The SE of mutants *bc1* and *bc3* was not significantly different from the parental cultivar, Kinmaze (Fig. 2). The SE of *fukei71* showed a significant decrease (32.1%) compared to Fujiminori (40.3%), and the SE of Leaf Star increased (32.9%) compared to its presumed ancestor, Chugoku117 (29.0%). Although their cellulose contents were reduced to original cultivars, the SE of the two GA mutants, *gid1-8* and *slr1d-4,* was comparable to original cultivars, T65 and Nipponbare, respectively (Fig. 2). Thus, although GA is known to promote cellulose synthesis, it does not necessarily affect SE.

Among the other mutants, only *122* and *108* showed significant increases in SE (Fig. 2). *122* showed a 33.7% increase in SE compared to T65, and *108* showed a 37.0% increase relative to

1 Nipponbare.
2
3 **Phenotypic characterization of *122* and *108***
4 Based on the screening described above, two mutants with improved SE, *122* and *108*, were
5 analyzed in more detail. *122* was initially selected based on the presence of a mild brittle culm
6 phenotype (Suppl. Table S2); it also exhibits dark green leaves, a thin apical internode, and a
7 delayed heading of 21 days as compared to the original cultivar, T65 (Fig. 3a-c). The only
8 obvious phenotype for mutant *108* was the accumulation of gold pigment in the panicle and
9 internodes (Fig. 3d, e).
10
11 **Positional cloning of *122* and *108***
12 Positional cloning was conducted to determine the genes responsible for improved SE in *122*
13 and *108*. F1 generations of both mutants were created by crossing with their original cultivars,
14 T65 and Nipponbare, respectively. The F1 plants of *122* and *108* did not show obvious mutant
15 phenotypes, revealing that they are recessive mutants. For positional cloning, we applied a
16 recently developed method designated as MutMap (Abe et al. 2012). In brief, DNA of F2
17 individuals showing mutant phenotypes were bulked, whole genome sequencing was conducted,
18 and searches were undertaken to identify nucleotide differences with respect to the original
19 cultivars. If all the read sequence differed to its original cultivar, SNP index was calculated as
20 1.0, whereas if all were identical to its original cultivar, SNP index was calculated as 0. Total
21 reads of bulked *122* and *108* F2 were 459,237,486 (46.383 Gb) and 466,336,552 (47.100 Gb),
22 respectively, which both account to approximately 12 coverage of the rice genome.
23 After Mutmap analysis of *122*, a peak (a region with SNP index = 1.0) was detected at
24 chromosome 2 around the 30.0-33.5 Mb region (Suppl. Fig. S2a). Within this region, there was
25 a nonsynonymous mutation in the *OsCOP1* gene, which encodes a RING E3 ubiquitin ligase
26 that is involved in photomorphogenesis, flowering, and phase change (Tsuge et al. 2001; Tanaka
27 et al. 2011). Consistent with these functions, *122* showed a delay in flowering (Fig. 3c). The
28 mutation (C to T, resulting in a proline to leucine change) mapped to the first exon of *OsCOP1*,
29 which encodes a RING finger domain (Suppl. Fig. S2b).
30 A distinct region with a SNP index = 1.0 was not observed for *108*. However, the genome
31 sequence of the bulked F2 plants contained a 28-kb deletion in chromosome 3 at the 34 Mb
32 region (Suppl. Fig. S3). *GOLD HULL AND INTERNODE 1 (GH1)* was mapped within the
33 deleted region. *GH1* encodes a chalcone isomerase involved in flavonoid biosynthesis, and a
34 *gh1* mutant was previously identified with a golden grain phenotype similar to *108* (Hong et al.
35 2012). Thus, these results suggest that a mutation in *GH1* resulted in the *108* phenotype.
36 Complementation tests were conducted to investigate whether *OsCOP1* and *GH1* were the

causal genes of the *122* and *108* phenotypes, respectively. The introduction of *OsCOP1* and *GH1* into the *122* and *108* genetic backgrounds, respectively, restored SE to levels similar to their original cultivars (Suppl. Fig. 4a, b) confirming that *OsCOP1* and *GH1* are the causal genes of SE improvement in *122* and *108*, respectively.

_122_ shows reduced xylan and ferulic acid (FA) content

Since *122* is a late-flowering mutant (Fig. 3c), we hypothesized that a prolonged vegetative phase leads to improved SE. To investigate this possibility, we compared SE in cultivar Koshihikari and three near isogenic lines (NILs) with different flowering times. The NILs (Kanto HD1, HD2 and IL5) each contains a unique substituted genome segment from *indica* cultivar Kasalath, but the other genome regions are nearly identical to *japonica* cultivar Koshihikari (Takeuchi 2011). Consistent with the previous report (Takeuchi 2011), Kanto HD1 flowered 5 days earlier, and HD2 and IL5 flowered 4 and 8 days later than Koshihikari, respectively (Suppl. Fig. S5). The SE of the three NILs did not differ significantly from the SE of Koshihikari (Suppl. Fig. S5), indicating that there is no relationship between flowering time and SE at least in these four cultivars.

Mutant *122* has a thinner uppermost internode compared to T65 (Fig. 3b); thus, we next speculated that the ratio of leaf to culm biomass should differ between *122* and T65, and this might be the reason why *122* has improved SE relative to T65. When the biomass of leaf blades, sheath, and the uppermost to 4th internodes of *122* and T65 were compared, the ratio of the uppermost internode of *122* was low compared to T65 (6 and 8% of the total plant biomass, respectively) (Fig. 4a). However, when the SE of each plant component was compared for *122* and T65, *122* showed improvement in all the organs analyzed except for an insignificant increase in the leaf sheath (Fig. 4b). These results indicate that the morphological differences between *122* and T65 do not explain why *122* shows improved SE.

Lastly, we suspected that the secondary cell walls of mutant *122* might have an altered structure or/and composition with respect to cultivar T65, and this might explain the differences in SE. The amount of cellulose, Klason lignin, and thioglycolic-released lignin in mutant *122* was comparable to T65 (Fig. 5a, c-d); however, when all the cell wall polysaccharides within the cell wall were degraded into simple sugars and analyzed, the xylose content of *122* was 78% of that detected in T65 (Fig. 5b). To investigate the cause of reduced xylose in *122*, proportion of cell wall fraction against total cell wall polysaccharides was analyzed. The ratio of hemicellulose was reduced in *122*, whereas other fractions (hot-water extractable, pectin, and cellulose fractions) did not significantly differ to T65 (Fig. 5e). When sugar composition of hemicellulose were analyzed and calculated as the amount against total cell wall polysaccharides, xylose was significantly reduced in *122* together with decrease in fucose and

1 increase in glucose (Fig. 5f, see also Suppl. Fig. S6). These suggest that the polysaccharides
2 which contain xylose such as xylans may be reduced in the cell wall of *122*.
3 To analyze the cell wall composition of *122* in more detail, we measured the ferulic acid (FA)
4 and *p*-coumaric acid (*p*CA) content in the hemicellulose fraction of *122*. In monocots, FA is
5 attached to the xylan backbone via arabinose, and cell-wall localized FA is known to negatively
6 influence SE (Grabber et al. 1998a, 1998b; Lam et al. 2003; Casler and Jung 2006; Bartley et al.
7 2013). The FA content within the hemicellulosic fraction of *122* was significantly reduced
8 compared to T65 (Fig. 5g), whereas the *p*CA content was not different in the two rice lines.
9 When the monolignol composition of the lignin polymer in mutant *122* was analyzed, FA
10 showed a significant decrease relative to T65, whereas other monolignol constituents were
11 similar to T65 (Fig. 5h). Thus, mutant *122* contains reduced FA in comparison to the original
12 cultivar, T65. Taken collectively, reduction of xylan and/or FA seems to be the reason of
13 improved SE in *122*, which is consistent with the prior reports that reduction in xylan and FA
14 improves SE in rice (Grabber et al. 1998a; Brown et al. 2011; Petersen et al. 2012; Chen et al.
15 2013; Kuang et al. 2016; Pawar et al. 2016).
16
17 ***108* shows reduced lignin content**
18 Next, the cause of improved SE in *108* was investigated. *108* shows a golden hull phenotype
19 which is similar to a lignin deficient mutant *gh2* possessing mutation in a gene encoding lignin
20 biosynthesis enzyme CINNAMYL ALCOHOL DEHYDROGENASE 2 (CAD2) (Zhang et al.
21 2006). Thus we suspected that some cell wall alteration also occurred in *108* which led to
22 improved SE. The amounts of cellulose, xylose, and Klason lignin within the cell wall, and FA
23 and *p*CA content in the *108* hemicellulose fraction were comparable to those of Nipponbare (Fig.
24 6a-c, e). In contrast, the amount of thioglycolic-released lignin in *108* was only 53% of the level
25 in Nipponbare (Fig. 6d). Analysis of monolignol composition revealed that S lignin [syringic
26 acid (SA) and syringaldehyde (S)] was significantly reduced in *108* compared to Nipponbare
27 (Fig. 7f). Consequently, the ratios of H lignin [*p*-hydroxybenzaldehyde (pHA)], G lignin
28 [vanillic acid (VA) and vanillin (V)], and FA increased. When transverse sections of the 2nd
29 internodes were stained with phloroglucinol, *108* exhibited an orange color compared to the
30 pink hue detected in Nipponbare (Fig. 7a). Phloroglucinol interacts with the aldehyde
31 end-groups of hydroxycinnamaldehydes and thus the different coloration of phloroglucinol
32 staining might result from a change in aldehyde composition of lignin. Nipponbare showed a
33 red coloration when stained with Mäule; however, the red staining changed to a more yellowish
34 pigment in *108* (Fig. 7b). This is consistent with the reduction in S lignin in *108*, since Mäule
35 stains S lignin red and G lignin yellow. To summarize, mutation in *GH1* results in qualitative
36 change in lignin which seems to be the reason of improved SE in *108* (see Discussion for

details).

Biomass and lodging resistance of *122* and *108*

Lastly, we measured biomass parameters of *122* and *108* to evaluate whether they were affected by the loss of function of the targeted genes. Although grain yield of *122* and *108* were dramatically reduced compared to their original cultivars (Suppl. Fig. S7a), the total dry weight biomass of both mutant lines (excluding roots and panicles) was comparable to their original cultivars (Suppl. Fig. S7b). Lodging resistance, which helps plants withstand wind and rain and support the weight of grain, was also analyzed. We evaluated two types of lodging resistance, namely the cLr value and the bending moment at breaking (BMB). The cLr value measures the ability of plants to resist culm bending, whereas BMB evaluates resistance to culm breaking (Ookawa and Ishihara 1992, 1993; Watanabe 1997; Kashiwagi et al. 2007). *122* and *108* both showed an increase in cLr relative to the original cultivars, T65 and Nipponbare, respectively (Suppl. Fig. S8a, c). The BMB value was significantly lower in *122* than T65 in all internodes analyzed (Suppl. Fig. S8b). Mutant *108* showed a significant increase in BMB at the 2nd internode with other internodes similar to Nipponbare (Suppl. Fig. S8d).

Discussion

If we could identify plants with altered SE from phenotypes that can be evaluated in the field, screening from a large plant population would be possible. To test this possibility, from over 2000 mutant lines, we first selected 22 mutants which were evaluated in the field based on phenotypes that are often associated with altered cell wall. Among them, only four showed significant difference in SE as compared to their original cultivars. *fukei71*, which over-accumulates cell wall polysaccharide-linked ferulate, showed reduced SE, whereas, Leaf Star and *108* with altered lignin composition showed improved SE (Fig. 2, Mase et al. 2005; Ookawa et al. 2014). With the exception of *bc1* and *bc3*, the cell walls of the brittle culm mutants used in this study have not been characterized; however, brittle culm mutants generally contain cell wall modifications (Li et al. 2003; Hirano et al. 2010; Xiong et al. 2010; Zhang et al. 2010; Li et al. 2011; Dai et al. 2011; Wu et al. 2012). Although we analyzed seven brittle culm mutants, only mutant *122* showed a significant increase in SE due to reduced xylan and FA (Figs. 2, 5). Thus it seems plausible that some types of cell wall alterations affect SE, whereas some do not, which is difficult to simply predict from the visual phenotype. For the flexible culm rice, none of the 11 mutants analyzed showed significant change in their SE (Fig. 2). It is noteworthy that even the KN1 mutant which cannot withstand to support their own body (Fig. 1) did not show any significant alteration in SE. This again highlights the difficulty in identifying plants with improved SE merely based on phenotypes that can be evaluated in the

1 field.
2 In the present study, the analysis of two new mutants, *108* and *122*, revealed a role for *GH1*
3 and *OsCOP1* in SE, respectively. The causal gene in *108* was allelic to *gh1*, which has a
4 mutation in a flavonoid biosynthesis gene, chalcone isomerase (*OsCHI*) (Hong et al. 2012).
5 Despite that *gh1* has a loss of function mutation in *OsCHI*, it accumulates increased total
6 flavonoids (Hong et al. 2012). Although the reason that *OsCHI1* deficiency results in improved
7 SE is unknown, since flavonoid and lignin synthesis share the same phenylpropanoid pathway
8 in the early stages of biosynthesis, altered flavonoid biosynthesis in *108* might have impacted
9 lignin biosynthesis, leading to improved SE in *108* (see below for further discussion).
10 Based on the phenotypes exhibited by mutant *122*, multiple hypotheses were developed and
11 tested to investigate the basis for improved SE. Prolonged vegetative stages (late heading) and
12 differences in organ ratios seemed unlikely reasons; however, reduced xylan and FA levels
13 seemed plausible due to the following facts. Arabidopsis with reduced xylan often shows
14 improved SE (Brown et al. 2011; Petersen et al. 2012; Kuang et al. 2016; Pawar et al. 2016),
15 and in rice, inactivation of OsIRX10, an enzyme possibly responsible for xylan biosynthesis,
16 also results in SE improvement (Chen et al. 2013). These are in accordance with the idea that
17 reduced xylan enhances the ability of cellulase to access cellulose. Concerning the relationship
18 between FA and SE, the FA located within xylan forms a dimer with the FA of another xylan
19 molecule and also cross-couple with monolignols of lignin, which makes the cell wall more
20 rigid and negatively affects saccharification (Grabber et al. 1998a, 1998b; Lam et al. 2003;
21 Casler and Jung 2006). A rice mutant *xylosyl arabinosyl substitution of xylan 1* (*xax1*) which is
22 deficient in feruloyl and coumaroyl esters exhibits improved SE, probably due to a lower
23 amount of FA within the xylan (Chiniquy et al. 2012), which is consistent with the above idea.
24 In our study, *fukei71*, which accumulates cell wall polysaccharide-linked ferulate (Mase et al.
25 2005), showed reduced SE, further supporting that FA levels affect SE in rice as previously
26 reported (Bartley et al. 2013).
27 On the other hand, the mechanistic basis of improved SE in mutant *108* may be more complex.
28 In this study, we measured the amount of lignin using two different methods. Although
29 thioglycolic-released lignin was dramatically reduced in *108*, the amount of Klason lignin was
30 similar to Nipponbare. Although we cannot define the reason for the different results obtained
31 by the two measurements, it may derive from the different measuring principles of the two
32 assays. The Klason lignin measurement is a gravimetric assay that directly quantifies the weight
33 of lignin. One disadvantage of this method is that some phenolic compounds can be included,
34 which may lead to an inaccurate measurement. On the other hand, the thioglycolic acid method
35 is based on the formation of thioethers of benzyl alcohol groups within the lignin polymer,
36 which results in solubilization of lignin under alkaline conditions (Moreira-Vilar et al. 2014).

Although the β-O-4 linkage, the main linkage that connects propenyl chains of monolignols, can be solubilized by thioglycolic acid (Hatfield and Fukushima 2005), there are other linkages within the lignin polymer that do not react with thioglycolic acid. In fact, G lignin units, which were increased in *108*, form a condensed structure within the lignin polymer that does not react with thioglycolic acid. Thus, the different amount of solubilized thioglycolic acid-released lignin in *108* and Nipponbare may derive from the compositional differences between the two plants. Moreover, although histochemical analyses are not precise methods to define qualitative differences, phloroglucinol and Mäule staining also suggests that lignin structural changes have occurred in *108*. Of interest, the flavone tricin (5,7-dihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-4*H*-chromen-4-one) was recently found to be a constituent of the lignin polymer in various monocotyledonous species, including wheat and maize (del Río et al. 2012; Zeng et al. 2013; Rencoret et al. 2013; Lan et al. 2015, 2016; Eloy et al. 2017).

The early steps of tricin biosynthesis require two enzymes, Chalcone Synthase (CHS) and CHI, which together convert *p*-coumaroyl CoA and three molecules of malonyl CoA into the flavanone naringenin, a common precursor of various flavonoids including tricin (Shih et al., 2008; Dixon and Pasinetti 2010). As expected, a maize mutant defective in CHALCONE SYNTHASE C2 (C2-ldf) produces reduced amounts of tricin-related flavonoids and also displays lower incorporation of tricin into lignin polymers. Moreover, Klason lignin content in leaves was reduced and the lignin polymer was enriched in β-β and β-5 units due to the lower incorporation of tricin, which together possibly explains the reduced SE found in this mutant (Eloy et al. 2017).

Interestingly, down-regulation or even loss-of-function of *CHS* in other species do not necessarily phenocopy the *C2-idf* mutant in maize. For instance, in flax, down-regulation of *CHS* results in reduced acetyl bromide-measured lignin (Zuk et al. 2016), whereas no changes in lignin content were observed in the *chs* null mutant in *Arabidopsis thaliana* (Li et al., 2010). Whether these inconsistent results derive from the different species used or differences in the lignin measurement methodology applied, or other unknown reasons is yet to be determined. Although the amount of tricin was not studied for *108*, the fact that *108* is defective in CHI raises the possibility that altered flavonoid biosynthesis in *108* might have affected not only the amount of S, G, and H lignins, but also the amount of tricin incorporated into lignin polymers, which might also contribute to the better SE found for this mutant. Recently, the artificial introduction of ester linkages into the lignin polymer backbone was shown to improve SE (Wilkerson et al. 2014). A detailed structural analysis of lignin in mutant *108* should provide a more in-depth understanding of the effect of lignin structure on SE.

Regarding lodging resistance, *108* and *122* both showed improved bending-type lodging

resistance. For the breaking-type lodging resistance (BMB), *122* showed inferior values compared to its original cultivar in all the internodes analyzed; whereas, *108* showed similar or improved values depending on the internode. BMB is determined by the morphology and quality of the culm, which is influenced by the amount and/or quality of cellulose and lignin (Ookawa and Ishihara 1992). In fact, a *cad2* mutant, *gh2*, shows reduced BMB possibly due to reduced lignin (Ookawa et al. 2014). *122*, which is a mild brittle culm mutant, showed reduced BMB, consistent with various brittle culm mutants requiring reduced breaking force due to alteration in their cell wall structures (Li et al. 2003; Zhou et al. 2009; Zhang et al. 2010; Wu et al. 2012). For *108*, although it remains unknown how lignin structure potentially affects BMB, the fact that a mutation in *GH1* in *108* results in improved SE without negatively affecting BMB indicates that targeting *GH1* may be an ideal strategy for developing cereals for bio-ethanol production.

In order to utilize genes related to SE in plant breeding programs, it is important to improve SE without compromising the yield of plant biomass. Unfortunately, alterations in cell walls often cause detrimental effects on plant growth and result in reduced biomass (Zhong et al. 2008; McCarthy et al. 2009; Hirano et al. 2013). Consistent with these observations, the grain yield of *122* and *108* were drastically reduced. However, the above-ground biomass of *122* and *108* (after excluding panicles) was not significantly different from the original cultivars. This is a desirable trait if *OsCOP1* and *GH1* are to be deployed for bio-ethanol production and grain is not utilized.

Curiously, screening of SE improved mutants has not been widely conducted. Our results and the previous work of Stamatiou et al. (2013) and Marriot et al. (2014) show that SE screening is an efficient strategy to identifying novel factors associated with SE. Such studies will deepen our understanding on the underlying mechanisms of SE, which will become critical when developing bioenergy crops.

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Figure legends

Fig. 1 Phenotype of rice mutants screened for improved saccharification efficiency (SE). Original cultivars (T65, Kinmaze, Nipponbare, Fujiminori, and Chugoku117) are presented in the uppermost panels. The cultivars used to generate the mutants are as follows: T65, mutant lines *TN1* to *TN8*, *122*, and *gid1-8*; Kinmaze, mutants *KN1* to *KN5*, *bc1*, and *bc3*; Nipponbare, mutants *NN1*, *108*, and *slr1d-4*; Fujiminori, *fukei71*; and Chugoku117, Leaf Star. Plants were photographed 40 days after heading. Bar = 20 cm

Fig. 2 Saccharification efficiency (SE) of rice mutants. The SE of original cultivars and mutant lines are shown in blue and green bars, respectively. Three or more individual plants were evaluated for SE. Error bars indicate the standard error. Asterisks indicate statistically significant differences with respect to original cultivars ($P < 0.01$; Dunnett 1955)

Fig. 3 Phenotypes of *122* and *108*. **a**-**c** Phenotypes of *122* were compared with its original cultivar T65. *122* shows darker green leaves (**a**), a thinner uppermost internode (**b**), and delayed heading (**c**). **d**, **e** *108* shows golden colored grain (**d**) and culm (**e**) compared to its original

cultivar Nipponbare. Bar = 0.5 cm in (**a**) and 1 cm for other panels. Asterisks in **c** indicate statistically significant differences with respect to original cultivars ($P < 0.01$; two-tailed Student's *t*-test)

Fig. 4 *122* shows improved saccharification efficiency (SE) in most of the organs. (**a**) Ratios showing the organ composition of mutant *122* and cultivar T65. (**b**) SE of each organ. Three or more individuals were used in the analyses. Asterisks indicate statistically significant differences relative to T65 (*$P < 0.05$, **$P < 0.01$; two-tailed Student's *t*-test)

Fig. 5 Cell wall composition of mutant *122* and its original cultivar T65. The content of cellulose (**a**), xylose (**b**), Klason lignin (**c**), thioglycolysis-released lignin (**d**), proportions of cell wall polysaccharide fractions (**e**), sugar composition of the hemicellulose fraction (**f**), *p*-coumaric (*p*CA) and ferulic acid (FA) of the esterified phenolic fraction (**g**), and monolignol composition of lignin (**h**) are shown. Asterisks indicate statistically significant differences relative to T65 (*$P < 0.05$, **$P < 0.01$; two-tailed Student's *t*-test). Abbreviation: n.s, indicates no significant difference, Fuc: L-Fucose, Rha: L-Rhamnose, Ara: L-Arabinose, Gal: Galactose, Glc: Glucose, Xyl: Xylose, GalA: Galacturonic acid, GluA: Glucuronic acid, *p*HA: *p*-hydroxybenzaldehyde, VA: vanillic acid, V: vanillin, SA: syringic acid, S: syringaldehyde, *p*CA: *p*-coumaric acid, and FA: ferulic acid

Fig. 6 Composition of cell wall components of *108* and its original cultivar Nipponbare. Cellulose (**a**), xylose (**b**), Klason lignin (**c**), thioglycolysis released lignin (**d**), *p*CA and FA of the esterified phenolic fraction (**e**), and monolignol composition of lignin (**f**) are shown. Asterisks indicate statistically significant differences relative to Nipponbare (*$P < 0.05$, **$P < 0.01$; two-tailed Student's *t*-test). n.s, indicates not significant, *p*HA: *p*-hydroxybenzaldehyde, VA: vanillic acid, V: vanillin, SA: syringic acid, S: syringaldehyde, *p*CA: *p*-coumaric acid, and FA: ferulic acid

Fig. 7 Histochemical analysis of lignin in cross-sections of rice culms. The second internodes from the top of Nipponbare and *108* were transversely sectioned to 80-100 µm and stained with Phloroglucinol (**a**) or Mäule (**b**). Bar = 100 µm

Fig. S1 Heading date of mutants used in this study in comparison to their original cultivars. Days to head of original cultivars and mutant lines are shown in blue and green bars, respectively. Three or more individual plants were used. Error bars indicate the standard error.

Fig. S2 MutMap analysis of *122*. **a** The SNP index of chromosome 2 as calculated from *122* and its parental cultivar T65 is represented by blue dots. Note that the SNP index = 1.0 is observed with high frequency at the 30 Mb region (green circle) and *OsCOP1* (green arrow) is located at 32.5 Mb. **b** Functional structure of OsCOP1 and the location of the *122* mutation. *122* contains a proline to leucine mutation in the RING-finger domain. Multiple sequence alignment of OsCOP1, AtCOP1, SlCOP1, and GmCOP1 refer to COP1 of rice, *Arabidopsis thaliana*, *Solanum lycopersicum*, and *Glycine max*, respectively. Note that the proline residue is conserved among angiosperms

Fig. S3 Visualization of the read alignment of bulked *108* F2 DNA using Integrative Genomics Viewer (Thorvaldsdóttir et al. 2012). Sequences were aligned against the Nipponbare reference genome sequence, which was obtained from the TIGR database (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all.dir/). The region around the deletion (chromosome 3, location: 34383316-34411532) is presented. Note that individual sequences are aligned in the regions flanking the deletion (small rectangles). *GH1* maps within the deleted region

Fig. S4 Complementation of the *122* and *108* mutant phenotypes with *OsCOP1* and *GH1*, respectively. The introduction of *OsCOP1* and *GH1* restored the SE of *122* (**a**) and *108* (**b**) to the level exhibited by the original cultivars, respectively. n.s., not significantly different from original cultivar (**, $P < 0.01$; two-tailed Student's *t*-test)

Fig. S5 Saccharification efficiency (SE) of Koshihikari and three near isogenic lines (NILs), Kanto HD1, Kanto HD2 and Kanto IL5. The days to heading of each NIL with respect to Koshihikari (0 day) is shown above the graph

Fig. S6 Proportions of each cell wall fraction within the cell wall polysaccharides of *122* and its original cultivar T65. Sugar compositions of hemicellulose fraction (blue) against cell wall polysaccharides are also presented.

Fig. S7 Grain yield and biomass of *122*, *108* and their original cultivars (T65 and Nipponbare, respectively). Rice was sampled 40 days after heading, sun-dried for 2 weeks, and total grain yield (**a**) and biomass (excluding panicles and roots) (**b**) were measured (n=5). Asterisks indicate statistically significant differences with respect to the original cultivars (n.s., not significantly different from original cultivar. (**$P < 0.01$; two-tailed Student's *t*-test)

1 **Fig. S8** Lodging resistance of *122* and *108* with their original cultivars (T65 and Nipponbare,
2 respectively). Bending-type resistance was evaluated by the cLr value (**a**, **c**) and breaking-type
3 resistance was evaluated by the BMB value (**b**, **d**). 10 individuals were analyzed. Asterisks
4 indicate statistically significant differences relative to the original lines (*$P < 0.05$, **$P < 0.01$;
5 two-tailed Student's *t*-test)