Running head: Mixed linkage glucan deficient rice

Corresponding author:
Pamela C. Ronald (pcronald@ucdavis.edu)
Department of Plant Pathology,
225 Robbins Hall
University of California - Davis
One Shields Ave., Davis, CA 95616
Phone: (530) 752-7834
Fax: (530) 752-6088

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Title: Loss of Cellulose Synthase-Like F6 function affects mixed-linkage glucan deposition, cell wall mechanical properties and defense responses in vegetative tissues of rice

Miguel E. Vega-Sánchez⁵, Yves Verhertbruggen⁴, Ulla Christensen⁴, Xuewei Chen⁴, Vaishali Sharma⁴, Patanjali Varanasi⁴, Stephen A. Jobling⁶, Mark Talbot⁶, Rosemary G. White⁶, Michael Joo⁴, Seema Singh⁴, Manfred Auer⁴, Henrik V. Scheller⁴ and Pamela C. Ronald⁴,⁶,⁹,¹₀.

⁴Joint BioEnergy Institute, 5885 Hollis st., Emeryville, CA 94608
⁵Physical Biosciences and ¹Life Sciences Divisions, Lawrence Berkeley National Laboratory, One Cyclotron Road MS 978-4101, Berkeley, CA 94720, USA
⁶CSIRO Food Futures Flagship and ⁷CSIRO Plant Industry, Black Mountain Laboratories, Clunies Ross St., Black Mountain ACT 2601, Australia
⁸Department of Plant Pathology, University of California, Davis, One Shields Ave., Davis, CA 95616
⁹Biomass Science and Conversion Technologies Department, Sandia National Laboratories, Livermore, CA 94551
¹₀Department of Plant Molecular System Biotechnology & Crop Biotech Institute, Kyung Hee University, Yongin 446-701, Korea
¹¹The Genome Center, University of California, Davis, One Shields Ave., Davis, CA 95616
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Present address: Xuewei Chen, Rice Institute, Sichuan Agricultural University at Chengdu 211 Huimin Road, Liucheng, Wenjiang, Chengdu, Sichuan, 611130, China

Corresponding author email: pcronald@ucdavis.edu
Abstract

Mixed-linkage glucan (MLG) is a cell wall polysaccharide containing a backbone of unbranched (1,3)- and (1,4)-linked β-glucosyl residues. Based on its occurrence in plants and chemical characteristics, MLG has primarily been associated with the regulation of cell wall expansion due to its high and transient accumulation in young, expanding tissues. The Cellulose synthase-like F (CslF) subfamily of glycosyltransferases has previously been implicated in mediating the biosynthesis of this polymer. We confirmed that the rice (Oryza sativa) CslF6 gene mediates the biosynthesis of MLG by overexpressing it in Nicotiana benthamiana. Rice cslf6 knockout mutants show a slight decrease in height and stem diameter but otherwise grew normally during vegetative development. However, cslf6 mutants display a drastic decrease in MLG content (97% reduction in coleoptiles and virtually undetectable in other tissues). Immunodetection with an anti-MLG monoclonal antibody revealed that the coleoptiles and leaves retain trace amounts of MLG only in specific cell types such as sclerenchyma fibers. These results correlate with the absence of endogenous MLG synthase activity in mutant seedlings and four week-old sheaths. Mutant cell walls are weaker in mature stems but not seedlings, and more brittle in both stems and seedlings, compared to wild type. Mutants also display lesion mimic phenotypes in leaves, which correlates with enhanced defense-related gene expression and enhanced disease resistance. Taken together, our results underline a weaker role of MLG in cell expansion than previously thought, and highlight a structural role for MLG in non-expanding, mature stem tissues in rice.
Introduction

Plant primary cell walls are typically composed of cellulose, matrix polysaccharides and proteins. Among vascular plants, grasses, such as rice (*Oryza sativa*), have unique primary cell walls with a low content of xyloglucan and pectins, and high levels of feruloylated arabinoxylans and (1,3; 1,4)-β-D-glucan (Carpita, 1996; Smith and Harris, 1999; Vogel, 2008), also known as mixed-linkage glucan (MLG). MLG is a polysaccharide containing a backbone of unbranched (1,3)- and (1,4)-linked β-glucosyl residues. In flowering plants, MLG is only found in cell walls of the taxonomic order Poales, which consists of 16 families including the Poaceae (Smith and Harris, 1999; Trethewey et al., 2005). MLG is also found in a few ascomycete fungi (Honegger and Haisch, 2001; Pettolino et al., 2009) and in some species of bryophytes, pteridophytes and green algae (Popper and Fry, 2003; Fry et al., 2008; Sorensen et al., 2008). The rare occurrence of MLG among angiosperms and a few divergent species suggests that its adoption evolved independently in all these lineages (Burton and Fincher, 2009; Fincher, 2009a).

MLG has primarily been implicated in the regulation of cell wall expansion due to its high and transient accumulation in young, expanding tissues in the coleoptile and seedling (Carpita et al., 2001; Gibeaut et al., 2005; Christensen et al., 2010). However, MLG also accumulates in cells that are not typically associated with growing tissues such as fibers and xylem vessels in the leaf (Trethewey and Harris, 2002; Doblin et al., 2009), which contain thick, lignified, secondary cell walls that provide structural and mechanical support. Another important role of MLG is as a storage compound particularly in barley (*Hordeum vulgare*), oats (*Avena sativa*) and *Brachypodium distachyon* grains where levels can represent as much as 70-80% of the endosperm cell wall (Fincher, 2009b; Guillon et al., 2011). In contrast to other cereals, rice does not accumulate significant amounts of MLG in the grain (Shibuya et al., 1985; Demirbas, 2005).

Two grass-specific subfamilies in the *Cellulose synthase-like* (*Csl*) superfamily of glycosyltransferases have recently been shown to mediate the biosynthesis of MLG: *CslF* and *CslH* (Burton et al., 2006; Doblin et al., 2009). These studies demonstrated the involvement of these gene families in MLG biosynthesis by overexpressing the rice *CslF2* and *CslF4* (Burton et al., 2006) and the barley *CslH1* genes (Doblin et al., 2009) in
Arabidopsis thaliana, a host that does not normally accumulate MLG. These results were later confirmed in grasses by expressing several CslF genes under the control of constitutive and endosperm-specific promoters in barley (Burton et al., 2011). In rice, CslF and CslH are represented as small gene families with eight and three members, respectively (Hazen et al., 2002). Whether the biosynthesis of MLG requires one or more of these proteins acting alone or as part of catalytic complexes still needs to be determined. Nevertheless, recent evidence from barley suggests that distinct CslF members might mediate the biosynthesis of MLG with different physicochemical properties that may affect the polysaccharide’s solubility (Burton et al., 2011).

Despite recent progress in the identification of the genes responsible for MLG biosynthesis in grasses, a detailed understanding of the role of this polysaccharide in any species is still lacking due to the scarcity of characterized mutants. Downregulation of the CslF6 gene by RNAi in wheat grain resulted in decreased MLG levels in the endosperm, but has been reported as an industrial application to modulate MLG levels in the grain (Nemeth et al., 2010). Mutations resulting in the absence of MLG accumulation have recently been mapped to the CslF6 locus in barley (Taketa et al., 2011). However, the characterization of the phenotype was limited to the endosperm and a few tissues in the seedling, and did not discuss or provide evidence for the implications of the absence of MLG in maintaining cell wall integrity (Tonooka et al., 2009; Taketa et al., 2011). Here, we present a detailed analysis of the effects of loss-of-function mutations in the CslF6 gene in rice vegetative tissues. We show that CslF6 is required for MLG accumulation in non-lignified, primary cell walls of rice, and necessary and sufficient for MLG deposition in lignified cells of mature stems but not coleoptiles, developing or expanded leaves. Mutant plants showed a slight growth defect but otherwise grew normally during vegetative development. We also show that mutations in CslF6 affect the mechanical properties of the cell wall in seedlings and stems, and cause upregulation of defenses and enhanced resistance to a bacterial pathogen. Taken together, our results provide unequivocal evidence for the predominant role of CslF6 in MLG biosynthesis in rice and support a cell wall model in which MLG acts as a gel matrix component rather than a cross-linking polysaccharide.
Results

Rice accumulates MLG throughout development. There are very few reports on the content of MLG in most rice tissues (Shibuya et al., 1985; Chen et al., 1999; Demirbas, 2005; Kimpara et al., 2008). The levels of MLG in crude cell wall preparations (alcohol insoluble residue –AIR) of different tissues throughout rice development were determined using a commercial assay specific for MLG. As shown in Figure 1A, MLG accumulates in young and mature organs, mostly in younger, rapidly expanding organs such as the coleoptile, seedlings and immature panicle, which is in agreement with previous reports in maize and barley (Carpita et al., 2001; Gibeaut et al., 2005). Interestingly, the highest MLG content was observed in mature stems after flowering, a tissue containing non-expanding cells with lignified, secondary cell walls. As opposed to many other grasses, we confirmed that rice does not accumulate significant amounts of MLG in grains (Figure 1A).

CslF6 is predominantly expressed in young, expanding tissues. A previous gene expression profiling analysis done on 33 rice tissue samples and two rice cultivars revealed that among the CslF subfamily, CslF6 was the most widely and highly expressed family member (Wang et al., 2010). We confirmed these results by extending this analysis using databases of publicly available rice gene expression data that include microarray experiments encompassing 38 rice cultivars and over 1100 hybridizations (rice oligo array database: http://www.ricearray.org/index.shtml) and the rice MPSS database (http://mpss.udel.edu/rice/) (Figure S1). A gene expression analysis done in the same tissues used for MLG accumulation above revealed that, although CslF6 expression did not completely correlate with MLG levels throughout development, it was most highly expressed in young vegetative and reproductive tissues (Figure 1B).

CslF6 mediates the biosynthesis of MLG. To demonstrate that the rice CslF6 gene can mediate the biosynthesis of MLG in planta, we transiently overexpressed CslF6 in leaves of Nicotiana benthamiana, a host that does not accumulate MLG. Leaves expressing CslF6 accumulated remarkably high levels of MLG (about 13% [w/w] of the cell wall preparations), indicating that this gene encodes an MLG synthase (Figure 2A).
Identification of \textit{cslf6} knockout mutants. We had previously identified \textit{Csf6} (MSU ID LOC\_Os08g06380; RAP ID Os08g0160500) as a highly expressed, grass-diverged glycosyltransferase using a phylogenomic approach (Cao et al., 2008). The \textit{Csf6} ORF is 3.9kb, encoding for a protein of 952 amino acid residues with 9 predicted transmembrane domains and central core catalytic domain typical of members of the CESA superfamily (Figure S2B). Through searches on the Rice Functional Genomic Express database (http://signal.salk.edu/cgi-bin/RiceGE), we identified two independent transposon insertion lines within the \textit{Csf6} genomic sequence: one located in the first intron (TT76-19-6-1: \textit{cslf6-1}) and the other on the last exon (TT76-27-6-17: \textit{cslf6-2}) (Figure S2A). RT-PCR analysis indicated that \textit{cslf6-1} and \textit{cslf6-2} are knockout lines since no \textit{Csf6} transcript could be detected in plants homozygous for the insertions (Figure S2C-D).

Morphological phenotypes of \textit{cslf6} mutants. Homozygous insertion lines for \textit{cslf6-1} and \textit{cslf6-2} were selected and grown under controlled conditions in growth chambers. The \textit{cslf6} mutants did not show any gross morphological phenotypes throughout development. Upon closer inspection, however, we observed that \textit{cslf6} plants are moderately reduced in stature compared to wild type plants 3 weeks after germination (33% decrease in height; Figure 3A-B). The decrease in height correlated with a similar percentage of reduction in cell elongation of 3 day-old coleoptiles: elongated cells in longitudinal sections from \textit{cslf6-1} coleoptiles were significantly shorter compared to wild type (Table 1). At maturity, this growth phenotype was manifested by significantly shorter stem internodes and reduced stem internode diameter, compared to controls (Figure 3C-D). The thinner stems in the mutant were primarily due to a reduction in the cross sectional diameter of sclerenchyma fibers and parenchyma cells compared to wild type (Table 1). In contrast, we did not observe changes in diameter or morphology of outer and inner vascular bundles (Table 1). Although both \textit{cslf6} mutants showed seemingly normal inflorescence development, anthesis was compromised which resulted in more than 50% reduced seed set (Figure S3A). A closer analysis revealed that although spikelet size was normal (Figure S3B), \textit{cslf6} mutants had deformed anthers and filaments (Figure S3C), suggesting that the poor seed set in the mutant was due to partial male sterility.
With the exception of coleoptiles and leaves, MLG content and synthase activity are undetectable in cslf6 mutants. In order to assess whether mutations in CslF6 had any effect on MLG accumulation, we measured the polysaccharide content in cell wall samples from wild type and mutants. High performance anion exchange chromatography (HPAEC) analysis of oligosaccharides released by lichenase treatment of MLG, which releases the characteristic tri- and tetrasaccharides G4G3G and G4G4G3G, respectively, revealed that coleoptile samples in cslf6 mutants had a 97% decrease of MLG compared to wild type (Table 2 and Figure S4). This decrease was even more dramatic in seedlings, immature panicles and mature stems where only traces, if any MLG were detectable in the mutants (Table 2 and Figure S4). These results were confirmed by measurement of MLG content using a quantitative, but less sensitive, assay specific for MLG, and by immunodot assays using a monoclonal antibody that specifically recognizes MLG epitope (Meikle et al., 1994) (Figures 4 and S5, respectively). Taken together, these results clearly demonstrate the dramatic decrease in detectable MLG in cslf6 mutants. As CslF6 is only one of 8 CslF genes in rice, we decided to test whether any remaining MLG synthase activity was detectable in the mutants. We measured the endogenous MLG synthase activity in microsomal fractions from 7 day-old seedlings and 4 week-old sheaths of wild type and mutant plants. We could not detect any residual MLG synthase activity above background levels in the mutant samples (Figure 2B-C). We conclude that, with the exception of coleoptiles, the MLG deficiency we observed in the cslf6 mutant samples is due to a lack of MLG synthase activity and that the other CslF family members do not compensate for this loss of enzymatic activity, at least in seedlings and sheaths.

The MLG deficiency in cslf6 mutants occurs in both non-lignified and lignified tissues of the stem but only in non-lignified cells of 3 day-old coleoptiles and leaves. To confirm the results obtained above, immunolabeling using the anti-MLG antibody was carried out on transverse sections of three day-old coleoptiles, expanded leaves and mature stems after grain filling. In the wild type coleoptiles and developing leaf, MLG occurred in every cell type (Figures 5A, S6 and S7). In the cslf6 mutant sections, the
MLG detection was severely reduced: although MLG was still abundant in sclerenchyma cells of the coleoptile and developing leaves, its occurrence was barely visible in the other cell types (Figures 5B, S6 and S7). To investigate whether this pattern of distribution occurred in developed leaf tissue, we tested the occurrence of MLG epitope in fully expanded leaves of wild type and cslf6 mutant. In wild type leaves, the MLG was present in all cell types, including weak labeling in sub-epidermal sclerenchyma fibers, and was most abundant in the bulliform cells and the cell walls of the vascular bundles and phloem (Figure 6A). In the cslf6 mutant, the detection of MLG was low and only observed in a few epidermal and sclerenchyma cells (Figure 6C). Interestingly, the bulliform cells in expanded leaves of cslf6 plants showed an aberrant morphology compared to wild type (Figure 6B and D). The alteration of MLG occurrence in cslf6 was even more significant in mature stems. Indeed, MLG was strongly and weakly detected in the parenchyma and sclerenchyma cells, respectively, of wild type (Figures 5E, S1 and S8) whereas no binding was detected in the mutant (Figures 5F, S1 and S8). Interestingly, the absence of MLG in cslf6 led to opposite patterns of xylan detection in coleoptiles and stems. As shown in Figures 5 and S8, the LM10 (xylan) epitope occurrence was higher in the mutant coleoptiles but lower in stems, compared to wild type. This may reflect differences in cell wall architecture that can either be due to distinct polysaccharide interactions inherent to the two organs or distinct reorganization of the cell wall in response to the absence of MLG in cslf6. Phloroglucinol staining in coleoptiles and developing leaf (Figure S9) and toluidine blue staining of stems (Figure 5K-L) confirmed that sclerenchyma fibers and xylem vessels were lignified in both the wild type and cslf6 mutant. Previous reports using electron microscopy of immunogold labeled sections in grass tissues have shown that MLG largely accumulates in the primary cell wall and very little, if any, in secondary cell walls (Trethewey and Harris, 2002; Trethewey et al., 2005). We confirmed that secondary cell walls of wild type mature stems contain large amount of MLG, while no MLG was present in cslf6 cell walls, by transmission electron microscopy of immunogold-labeled sections with the anti-MLG antibody (Figure S10). Taken together, these results highlight the major contribution of CslF6 to MLG biosynthesis in young and mature tissues, and show that CslF6 function is required for MLG accumulation in secondary cell walls in rice stems.
Mutations in *CslF6* affect the mechanical properties of the cell wall. In order to test whether the MLG deficiency in the *cslf6* mutant affected the mechanical properties of the cell wall, we measured the tensile strength in seedlings and the compression strength in stems, along with their associated strain tolerance parameter. Although we could not detect differences in ultimate strength (force applied per unit of area) between wild type and mutant seedlings (Figure S11A), cell walls of *cslf6* had significantly lower strain (deformation at breaking) than wild type (Figure S11B). In contrast, for stems, both the cell wall strength and strain properties were significantly reduced in the mutant (Figure 7). As the strain is a measure of the brittleness of the material, these results show that MLG contributes to the flexibility properties of the cell wall in both seedlings and mature stems, and to cell wall strengthening in stems but not in seedlings.

*cslf6* mutants have a significant reduction in matrix polysaccharide glucose content. We tested whether mutations in *CslF6* cause any changes in cell wall monosaccharide composition. HPAEC analysis of trifluoroacetic acid-treated samples, which hydrolyzes matrix polysaccharides into monosaccharides (2 M TFA at 121°C for 1 h), revealed that *cslf6* mutants have a severe decrease in glucose content (Table 3), which is consistent with the MLG deficiency. These changes were accompanied by significant increases in galacturonic acid and moderate decrease in cellulose content in mutant seedlings, and by increases in rhamnose, galactose, galacturonic acid and lignin content in stems of *cslf6* mutants (Tables 3 and 4).

Mutations in *CslF6* cause lesion mimic formation, upregulated defenses and enhanced disease resistance. After flowering, we observed the appearance of spontaneous, discrete, necrotic lesions in flag and old leaves of the *cslf6* mutant plants (Figure 8F), suggesting the development of a lesion mimic phenotype. Lesion mimic mutations are characterized by spontaneous lesion formation in plants in the absence of an external stimulus, such as a pathogen (Lorrain et al., 2003). Because many lesion mimic mutations are characterized by enhanced disease resistance and upregulation of defenses (Lorrain et al., 2003), we tested the response of *cslf6* mutants to bacterial
inoculation in leaves. The \textit{cslf6} plants had both decreased lesion size and reduced bacterial growth compared to wild type (Figures 8A-B and S12), following inoculation with a virulent isolate of \textit{Xanthomonas oryzae pv. oryzae}, the causal agent of bacterial blight in rice. To confirm whether the lesion mimic appearance correlates with upregulated defense responses in the \textit{cslf6} mutants, we tested the expression of three pathogenesis related (\textit{PR}) genes in intact leaves. \textit{PR} genes are generally induced after pathogen infection or other stresses, and are widely used as markers for cell death and defense responses such as systemic acquired resistance (van Loon et al., 2006). Pathogen and salicylic acid-responsive genes \textit{PR1a} and \textit{PR1b} (Mitsuhara et al., 2008) as well as the cell death-related marker gene \textit{PR10} (Kim et al., 2011) were all significantly upregulated in non-inoculated leaves of \textit{cslf6} mutants (Figure 8C-E). Interestingly, the same \textit{PR} marker genes were also upregulated in non-inoculated, lesion mimic-free fully expanded leaves of younger plants prior to flowering in \textit{cslf6} mutant plants compared to wild type (Figure S13). These results indicate that loss of MLG activates defense responses and cell death, which correlates with enhanced disease resistance in \textit{cslf6} plants.

\textbf{Discussion}

Previous work has demonstrated that members of the \textit{CslF} and \textit{CslH} subfamilies of glycosyltranferases mediate the biosynthesis of MLG, both in heterologous and native hosts (Burton et al., 2006; Doblin et al., 2009; Burton et al., 2011). \textit{CslF6} is the most widely and highly expressed family member in barley (Burton et al., 2008), \textit{Brachypodium} (Christensen et al., 2010), and rice (Wang et al., 2010). Through functional analysis of \textit{cslf6} loss-of-function mutants, we have shown here that, with the exception of sclerenchyma fibers in coleoptiles, developing leaf and fully expanded leaves, MLG was undetectable in any other tissues or developmental stages examined, including mature stems. These results demonstrate that the \textit{CslF6} gene has a predominant and non-redundant role in the biosynthesis and accumulation of MLG in non-lignified, primary cell walls of young, expanding tissues in rice, as well as being necessary and sufficient for MLG deposition in both non-lignified and lignified tissues in stems. The presence of large amounts of MLG in rice mature stems (after grain filling) is noteworthy, as most other studies in grasses have heavily focused on MLG analysis in coleoptiles,
grains or leaves (Carpita et al., 2001; Gibeaut et al., 2005; Doblin et al., 2009; Christensen et al., 2010; Guillon et al., 2011). Importantly, using electron microscopy, we were able to show that secondary cell walls of sclerenchyma fibers and xylem vessels of rice stems are heavily labeled with anti-MLG epitopes. In a previous study looking at the distribution of MLG in various species representative of the order Poales by electron microscopy, Trethewey et al (2005) reported that grasses have what they termed Type I MLG distribution, with heavy primary cell wall labeling and light secondary cell wall labeling. However, in their study, only leaf samples were labeled for the grass species they selected. It remains to be determined whether other grass species also contain elevated content of MLG in mature stems. However, a preliminary survey revealed very low MLG content in mature stem tissues of switchgrass, *Brachypodium* and maize compared to rice (Vega-Sanchez, *unpublished results*), which suggests that the high levels of MLG in mature stems may be unique to rice among the grasses. It is known that *Equisetum arvense*, a non flowering vascular plant remotely related to the grasses, accumulates a significant amount of MLG in stem cell walls (Sorensen et al., 2008), while species in the Restionaceae and Xyridaceae families in the Poales primarily accumulate MLG in secondary cell walls rather than primary cell walls (Trethewey et al., 2005).

The finding that the other *CslF* family members in rice do not seem to compensate for the loss of *CslF6* function was predicted based on our earlier analysis of large gene families, which addressed putative functional redundancy in gene families by focusing the functional analysis on the most highly expressed family members (Jung et al., 2008). Most of the *CslF* genes are clearly expressed, albeit at much lower levels and in fewer tissue types than *CslF6*, as shown here in Figure S1 and in (Wang et al., 2010). Whether these genes encode functional proteins needs to be determined, but at least rice *CslF2* and *CslF4* can mediate the biosynthesis of MLG when overexpressed in Arabidopsis, albeit in very small amounts (Burton et al., 2006). It is possible that these genes are involved in the deposition of MLG at stages of development and/or very specific tissue types not represented in our study. In any case, our data strongly suggest that a different gene, other than *CslF6*, is required for deposition of MLG in the sclerenchyma of coleoptiles and leaves. *In situ* PCR experiments have recently shown that barley *CslH1* expression is
restricted to sclerenchyma fiber and xylem cells in leaves (Doblin et al., 2009). Thus, rice
CslH1 is a strong candidate for this function in coleoptiles, developing and fully
expanded leaves as, unlike the other CslF genes, CslH1 and CslF6 are strongly expressed
in coleoptile samples, and CslH1 is highly expressed in leaves (Figure S1).
Our results are in agreement with a recent report describing loss-of-function mutations in
the barley CslF6 (HvCslF6) gene, which are also known as ‘betaglucanless’ (bgl)
mutations (Taketa et al., 2011). Barley bgl mutants lack detectable MLG in the
endosperm and seedling tissues, including coleoptiles, and in mature leaves (Tonooka et
al., 2009; Taketa et al., 2011). The lack of detectable MLG in barley coleoptiles suggests
that HvCslF6 may be required for MLG deposition in sclerenchyma fibers in this tissue,
in contrast to rice. However, the bgl seedling tissues including the coleoptiles, were also
considerably older than the rice coleoptiles analyzed here (10 days vs 3 days) and it is
possible that if there was any MLG in the bgl coleoptiles it may have been degraded since
it is known that the MLG content of coleoptiles declines after elongation (Gibeaut et al.,
2005). Analysis of younger bgl tissues and immunolabelling studies are needed to
confirm this. Nevertheless, it is clear that the CslF6 gene has a unique and non-redundant
role in MLG biosynthesis in both rice and barley, and likely in other grasses as well, such
as wheat (Nemeth et al., 2010).
In this study, we used several independent methods to confirm the deficiency of MLG in
the cslf6 mutants. One explanation for the trace levels of MLG in cslf6 mutants could be
enhanced degradation by the activity of the (1,3; 1,4)-β-D-glucan endohydrolases, which
are believed to hydrolyze MLG in growing tissues (Inouhe and Nevins, 1991). Although
we cannot completely rule out this possibility, we hypothesize that the lack of MLG in
the mutants is primarily due to a loss of synthase activity, as clearly shown in our
endogenous MLG synthase activity assays in seedlings and sheaths (Figure 2B-C).
Moreover, the presence of high amounts of MLG in wild type mature stem samples, a
tissue no longer expanding, coupled to the low expression levels of CslF6 in these tissues
(Figure 1B), suggests that MLG is deposited in stems prior to maturity and is not
hydrolyzed by (1,3; 1,4)-β-D-glucan endohydrolases. The fact that CslF6 is expressed in
young stems (tillers in Figure 1B) and MLG synthase activity is detectable at this stage in
wild type plants (Figure 2C), but not in mature stems (Christensen and Vega-Sanchez, unpublished results), is in agreement with this hypothesis.

The role of the primary cell wall in the control of cell expansion has been largely documented, and various models exist that describe the arrangement of cellulose microfibrils among a matrix or network of cross-linking hemicellulosic polysaccharides (Cosgrove, 2001, 2005). In grass vegetative tissues, MLG has been implicated in the regulation of cell expansion (Carpita, 1996; Gibeaut et al., 2005; Christensen et al., 2010) and two complementary models of its association with cellulose microfibrils have been suggested: one where MLG primarily coats the cellulose microfibrils while arabinoxylans serve as the cross-linking units (Carpita et al., 2001), and another in which MLG primarily forms a gel-like matrix around the microfibrils with occasional formation of junction zones with cellulose and other polysaccharides (Fincher, 2009a, b). Thompson has argued that polysaccharides forming these cross-links or “tethers” between microfibrils will share the main load-bearing functions of the cell wall with cellulose (Thompson, 2005), and this suggests that plants with deficiencies in these polysaccharides should show similar severe growth and developmental phenotypes as primary cell wall cellulose synthase (cesa) mutants (Arioli et al., 1998; Persson et al., 2007). The dramatic reduction of MLG (>99% in most tissues), accompanied by an absence of major growth and developmental defects in cslf6 mutants suggests that MLG does not act as a major load-bearing component of the primary cell wall in rice and thus may not participate as a cross-linking polysaccharide. These observations are supported by the tensile strength results that suggest that MLG acts more like a gel matrix that provides flexibility meanwhile strengthening the cell wall in growing tissues such as seedlings, in agreement with a model proposed by Fincher (Fincher, 2009a, b). We found that mutant seedlings had a moderate decrease in cellulose content (Table 4), which can also explain the reduced resistance to deformation in cslf6 plants. In addition, our data suggests that MLG plays a role in cell elongation, but less predominantly so than would have been expected for a polysaccharide strongly associated with growing tissues. The reduction in cell elongation in coleoptiles was moderate (28-30%) and is in agreement with the 33% height difference in fully developed plants. One possibility for MLG’s transient accumulation in growing tissues is that it serves as an energy source, especially
in conditions of sugar depletion, as shown in wheat leaves (Roulin and Feller, 2001; Fincher, 2009a). In contrast, we found that in non-growing tissues, such as mature stems, the distribution of MLG in vascular bundles and sclerenchyma fibers is in agreement with a role in cell wall reinforcement, which was supported by the compression test results that revealed a weaker and more brittle cell wall in the cslf6 mutant. It is interesting to note that the lack of MLG in stems is accompanied by increases in lignin, rhamnose, galactose and galacturonic acid content (Table 3). These likely reflect cell wall changes that compensate for the lack of MLG. Mutants with mild developmental phenotypes and lacking detectable levels of xyloglucan (Cavalier et al., 2008) and stem glucomannan (Goubet et al., 2009) have been recently characterized in Arabidopsis. These and our results with rice cslf6 mutants suggest that the plant can adapt and compensate, to a certain extent, for losses in major hemicellulosic polysaccharides in the cell wall. Taken together, our results add to a growing number of observations suggesting that current models of cross-linking of cellulose microfibrils as a fundamental principle to ensure cell wall integrity need revision (Scheller and Ulvskov, 2010).

The discovery of lesion formation and upregulated defenses leading to enhanced disease resistance in the cslf6 mutant adds to the growing number of cell wall mutations that have broad effects on cellular homeostasis beyond the well-characterized mechanical stress support functions typically associated with plant cell walls (Pilling and Hofte, 2003; Cantu et al., 2008; Seifert and Blaukopf, 2010). For example, mutations in the Arabidopsis MUR3 gene, which is involved in xyloglucan biosynthesis, also cause lesion formation, upregulated defense gene expression and enhanced resistance to a downy mildew pathogen (Tedman-Jones et al., 2008). It has been widely documented that plants can sense and monitor disturbances or changes in cell wall integrity and activate signaling pathways in response to these wall feedback stimuli (Pilling and Hofte, 2003; Bart et al., 2010; Seifert and Blaukopf, 2010). Our data suggests that the absence of MLG in the cell wall triggers a signaling cascade that activates stress and defense-related gene expression, and eventually, a cell death program. This is supported by the finding that PR marker genes are upregulated in younger leaves even in the absence of lesion mimics, which suggest that cslf6 plants are primed for the onset of cell death at later stages of development. For this reason, we believe that the enhanced resistance phenotype we
observed in *cslf6* plants is a consequence of such a program. The actual mechanism that
activates these defenses in *cslf6* remains to be elucidated. It is possible that MLG
oligosaccharides can function as signaling molecules to repress defense responses in wild
type plants, analogous to bacterial cyclic β-(1,2)-glucans that have been shown to
suppress the plant’s defense response (Rigano et al., 2007).

In summary, we have shown that *CslF6* is required for MLG accumulation in non-
lignified, primary cell walls of rice, and necessary and sufficient for MLG deposition in
lignified, secondary cell walls of mature stems but not coleoptiles, developing and fully
expanded leaves. Our results demonstrate the importance of MLG in maintaining the
mechanical properties of the cell wall, as well as highlight how the plant can adapt and
rearrange its cell wall environment to compensate for the lack of this polysaccharide. The
absence of gross morphological phenotypes observed in plants with low or no content of
xyloglucan (Cavalier et al., 2008), glucomannan (Goubet et al., 2009) or, as shown here,
MLG, underscores the necessity to redefine current models of cell wall architecture.

Materials and methods

Plant material and growth conditions

Rice was grown in growth chambers with the following conditions: 12 h daylight, 470
µmol.m⁻².s⁻¹ illumination intensity, 80% relative humidity, 26°C for 1 h at the beginning
and end of the cycle, and 28°C for the remaining 10h; 12h dark, 80% relative humidity,
26°C. Transposon insertion lines in the Nipponbare cultivar background were obtained
from the CSIRO collection in Australia and homozygous lines for each independent
insertion were identified by PCR. The generation of the transposon insertion lines is
described in (Upadhyaya et al., 2006).

Preparation of Alcohol Insoluble Residue (AIR) and mixed-linkage glucan

oligosaccharide fingerprinting. Samples were collected from various rice organs or
infiltrated *N. benthamiana* leaves, frozen in liquid nitrogen and freeze-dried overnight
using a lyophilizer. AIR preparation and destarching was done according to methods
described in (Harholt et al., 2006). For the detection of MLG oligosaccharides, AIR
(5mg) was sequentially extracted as follows: 0.05 M CDTA (pH 6.5) for 24 h at room
temperature; 0.05 M Na₂CO₃ containing 0.01 M NaBH₄ for 24 h; and then with 4 M KOH containing 0.01 M NaBH₄ for 24 h at room temperature (4 M KOH fraction). The pH of the 4 M KOH fraction was adjusted to pH 5 with glacial acetic acid, dialyzed against deionised water and then lyophilized. The KOH fraction was digested with 0.5 U lichenase (from Bacillus subtilis, Megazyme, Ireland) in 0.02 M sodium phosphate buffer (pH 6.5) at 50°C with continuous mixing for 24 h. The reaction was stopped by boiling at 100°C for 2 min, cooled to ambient temperature, and centrifuged prior to HPAEC analysis. The released oligosaccharides after enzymatic digestion were separated by HPAEC on a Dionex DX600 system (Sunnyvale, CA) equipped with a pulsed amperometric detector (PAD). A CarboPac PA200 column (3x250 mm) eluted with a gradient of 0-250 mM sodium acetate in 100 mM NaOH over 40 min at 0.4 ml/min was used. G₄G₄G₃G, G₄G₃G (Megazyme) and cellodextrins (Sigma-Aldrich) were run as standards.

Quantitative Reverse-Transcriptase PCR
Total RNA was extracted using the RNeasy plant mini kit (Qiagen, Valencia, CA) following manufacturer’s instructions. RNA preparations were treated with DNase I (Sigma-Aldrich) to remove traces of DNA contamination. One μg of RNA was used for reverse transcription with the Transcriptor high fidelity cDNA synthesis kit (Roche) and oligo dT primers. After synthesis, the cDNA reaction was diluted three times in ultrapure water, and 1 μl was used for PCR using the Fast SYBR Green master mix (Applied Biosystems, Carlsbad, CA) and gene-specific primers (Table S1) in a StepONE plus Q-PCR machine (Applied Biosystems).

Plasmids and constructs
Full-length cDNA clones for CslF6 (AK065259.1) and CslA1 (OsJNEa07E18) were identified and ordered from the Knowledge-based Oryza Molecular biological Encyclopedia (KOME: http://cdna01.dna.affrc.go.jp/cDNA/) and the Arizona Genomics Institute (http://www2.genome.arizona.edu/) collections, respectively. The cDNA for CslA6 was amplified by PCR using gene-specific primers (Table S1) from first strand cDNA made from pooled rice samples. Entry clones for cloning using the Gateway
technology (Invitrogen) were made by amplifying the coding sequences for each gene using Gateway-compatible primers (Table S1), as follows: PCR reactions were gel-purified using the MinElute Gel extraction kit (Qiagen) and used for BP recombination reactions into pDONR-Zeocein (Invitrogen) for CslF6 and CslA1, and pENTR-D-TOPO cloning (Invitrogen) for CslA6, following manufacturer’s instructions. Entry clones were finally used for Gateway recombination into the destination vector pEarleyGate 201 (Earley et al., 2006) using LR clonase enzyme mix (Invitrogen).

**Infiltration of Agrobacterium tumefaciens into Nicotiana benthamiana**

Infiltration of leaves of 4 week-old *N. benthamiana* plants was done using *A. tumefaciens* strain C58 (OD=1), following the method described in (Jensen et al., 2008). AIR preparations and MLG content measurements were done on leaves five days post-infiltration, as described below. The CslA1 and CslA6 constructs were infiltrated as negative controls to show that only CslF6 mediates the biosynthesis of MLG in this heterologous host (CslA1, CslA6 and CslF6 all belong to the same GT2 family of glycosyltranferases).

**Mixed-linkage glucan content determination**

For MLG content measurement, the mixed-linkage beta-glucan assay from Megazyme was used, adapting the manufacturer’s instructions for small-scale (5 mg AIR) preparations in microtubes by dividing all reaction volumes by 10. In this assay, cell walls are digested with lichenase, a (1,3; 1,4)-β-D-glucan endohydrolase, which releases specific oligosaccharides that are then digested to glucose by β-glucosidase treatment. The glucose is then quantified colorimetrically by a reaction involving glucose oxidase and peroxidase.

**Determination of (1,3; 1,4)-β-D-glucan synthase activity**

Microsomes were extracted as described in (Christensen and Scheller, 2012) with slight modifications using approximately 0.5 g (fresh weight) of tissue. A reaction mixture consisting of 100 µg microsome protein, 0.2 mM UDP-Glc (Sigma-Aldrich), 0.56 kBq UDP-14C-Glc (ARC, USA), 20 mM MgCl2, 200 mM sucrose, 50 mM Tris-HCl pH 8.5 in
a total volume of 50 µl was incubated at 25°C with agitation for 1h. The product was subsequently precipitated and detected according to (Christensen and Scheller, 2012).

Preparation of plant material for microscopy

Three day-old coleoptiles, fully expanded leaves and mature second stem internode pieces were fixed for 24h at 4°C in 4% paraformaldehyde in PEM (50 mM piperazine-N,N'-bis[2-ethanesulphonic acid], 5 mM EGTA, 5 mM MgSO4; pH 6.9) as described in (Verhertbruggen et al., 2009). The coleoptile pieces were cut from the basal region of the organ and were 4.0 mm and 6.0 mm long for longitudinal and transverse sections, respectively. Stem sections (1 cm long) were 2 cm distal from the second node with the first internode being the youngest internode (from the top). Coleoptiles were embedded in 7% agarose and sectioned (60 µm) using a Leica VT1000S vibratome as described in (Manabe et al., 2011). For cell length and diameter measurement, the coleoptile sections were analyzed under light microscopy. Cell measurements were done using Metamorph software calibrated with a 1 mm micrometer certified by National Physical Laboratory. Fully expanded leaves and mature second stem internode were embedded in LR white resin as described in (Yin et al., 2011). Stems and leaves were washed once in distilled water for 10 min, dehydrated at room temperature (RT) by incubation in a gradient of aqueous alcohol (10, 20 and 30% (v/v) ethanol (EtOH) (10 min for each solution), 50, 70, 90, 100% (v/v) EtOH (30 min for each solution) and overnight in 100% (v/v) EtOH)) and infiltrated with LR white resin by incubation in a gradient of LR White/ethanol solution (10, 20, 30, 50, 70 and 90% (v/v) resin (60 min for each solution, RT) and three times 100% (v/v) resin (overnight, 8 h, overnight at 4°C)). After polymerisation at 65°C for 5 days, LR white embedded fully expanded leaves and stem internodes were sectioned (2 and 0.5 µm thick, respectively) with an ultramicrotome and disposed on 10 well-slides coated with Vectabond.

Immunofluorescence microscopy

For each genotype, over 50 transverse sections from twelve coleoptiles, 12 transverse sections from two stems and 12 sections from 2 leaves were immunolabeled and analyzed as described in Verhertbruggen et al. (2009). Rice material was incubated for 30 min at
RT in a 5% milk powder protein/phosphate buffer saline (PBS) solution, extensively washed with PBS and incubated for 90 min at room temperature with primary monoclonal antibodies [10-fold dilution rat anti-xylan LM10 (McCartney et al., 2005) or 1000-fold mouse anti-MLG (Meikle et al., 1994)]. The anti-xylan LM10 was first used to demonstrate that polysaccharides other than MLG could be detected in cslf6 plants. After extensive washes in PBS, the sections were incubated for 90 min at RT in the darkness with anti-rat or anti-mouse monoclonal antibodies coupled with FITC. Secondary monoclonal antibodies were diluted 100-fold in 5% milk powder protein/PBS solution. After incubation with secondary antibody, the samples were washed in PBS, incubated at RT for 1 min in the darkness with 0.1 M Calcofluor, washed again in PBS, and mounted on slides with Citifluor AF1. Immunofluorescence detected in stems and coleoptiles was captured with a Q-imaging camera mounted on a Leica DM4000B epifluorescence microscope. Fully expanded leaves were visualized with a Zeiss Axioimager M1 compound microscope; FITC antibody label was detected using blue excitation and a GFP narrow-band emission filter, and calcofluor staining was observed in the same section with UV excitation and a long pass emission filter. Sections incubated without primary antibody were used as negative control and are shown in Figures S7 and S8, labeling of wild type or with LM10 are positive controls. Immunolabelling was done at least in triplicate. Wild type and mutant micrographs were captured and processed in Adobe Photoshop CS4 with the exact same values.

**Bacterial inoculation**

Inoculation of rice leaves with *Xanthomonas oryzae* pv. *oryzae* isolate PXO99, lesion size measurement and bacterial titer determination were carried out as described in (Bart et al., 2010).

**Tensile strength assay**

Ultimate stress and strain were measured using an in-house tensile testing instrument, which consists of a stepper motor and a sensitive load cell. The stepper motor was calibrated every day, prior to measurements, with an in line screw gauge. The force sensor, as suggested by the manufacturer, was calibrated with known quantities of water.
from 0 to 1500 gram force (0, 250, 500, 750, 1000 and 1500 gf were used as calibration points; 1 N =101.97 gf). Further, rectangular strips of Whatman glass microfiber filters were subjected to tensile tests and the resulting stress and strain values had standard deviations of less than 5%. Six day-old seedlings were used. The diameter of the seedling near its base and its total length were measured. The seedlings were then glued on to the sample holders using hot glue (Stanley DualMelt). Only 5 mm of the culm remained unglued between the sample holders. The sample holders were then screwed on to the apparatus and the tensile strength measurements were taken at room temperature. The sample holders consisted of a support system for the unglued portion of the culm to prevent it from damage during the holder installation. The support was removed from the holder just before starting the analysis. The stepper motor (IMC17-S04-A, RMS motion) was used to maintain a constant speed rate of 50 μm/s. The force sensor (ADW 15, LCM systems) was used to measure the force applied by the motor on the seedling. The instrument was digitally controlled and data was also collected digitally. The constant speed rate was used to calculate the elongation of the culm as a product of strain rate and time elapsed. Strain was then calculated as a ratio of elongation to the length of the suspended culm (5 mm). Stress was calculated as a ratio of the force and cross-sectional area of the culm. The ultimate stress and the corresponding strain at the breaking point were established from a stress vs strain plot (Figure S14). The ultimate stress represents the maximum load a material can take before failure and the strain corresponding to ultimate stress represents a measure of the brittle/ductile nature of the material. Materials with low strain are characterized as brittle. For the stem strength assay, mature (after grain filling) floral bolt stems from 4 month-old plants were subjected to compression tests. Compression strength was measured on the same instrument but the motor was driven in the opposite direction to tensile testing. The compression yield strength, based on the first deformation of the stem, and the corresponding strain were recorded from the stress-strain graph. Representative stress vs strain plots are shown in figure S14.

Monosaccharide composition analysis

Cell wall monosaccharide composition following TFA hydrolysis (2 M TFA at 121°C for 1 h) was determined from AIR samples of 7 day-old seedlings and mature second
internode stems using HPAEC according to methods described in (Obro et al., 2004). For
the separation of xylose and mannose peaks, samples were run on a CarboPac SA10
column (Dionex, Sunnyvale, CA) with 1 mM KOH eluent over 11 min at a flow rate of 1
ml/min, using a Dionex ICS3000 unit equipped with a pulse amperometric detector.

**Acetyl bromide lignin determination**
Lignin content was estimated from AIR samples using the method from (Fukushima and
Hatfield, 2001). The extinction coefficient used for rice samples was 17.75.

**Cellulose content determination**
For the determination of cellulose content, AIR samples were first treated with TFA (2 M
TFA at 121°C for 1 h) to remove matrix polysaccharides and then treated with 72% w/w
sulphuric acid at 120°C as described in (Bart et al., 2010). Cellulose was estimated as
glucose equivalents from the sulphuric acid hydrolysates using HPAEC.

**Supplemental material**

**Table S1.** List of primers used in this study.

**Figure S1.** Digital expression analysis of CsIF genes in rice.

**Figure S2.** CsIF6 gene structure and identification of knockout lines.

**Figure S3.** cslf6 mutant flower phenotypes.

**Figure S4.** HPAEC analysis of oligosaccharides released from MLG by digestion with
lichenase.

**Figure S5.** Immuno dot assays showing MLG content in different tissues.

**Figure S6.** Indirect immunodetection of transverse sections through the coleoptile and
developing leaf.

**Figure S7.** Immunolabeling of coleoptiles sections.

**Figure S8.** Immunolabeling of stem internode sections.

**Figure S9.** Phloroglucinol-HCl staining of 3 day-old coleoptile and developing leaf
sections of wild type (NPB) and cslf6-1 mutant.

**Figure S10.** Transmission electron micrographs of mature stem sections immunogold
labeled for the anti-MLG antibody.
**Figure S11.** Mechanical properties of cell walls of *cslf6* and WT seedlings.

**Figure S12.** Lesion length on rice plants inoculated with *X. oryzae pv. oryzae* isolate PXO99.

**Figure S13.** Upregulated defenses in *cslf6* mutant plants prior to flowering stage.

**Figure S14.** In-house tensile strength testing instrument and representative stress vs strain plots.

**Supplemental Materials and Methods**

**Acknowledgements**

We would like to thank Dr. Narayana Upadhyaya, CSIRO Plant Industry, Australia, for providing seeds of *cslf6* insertion mutants; Jeemeng Lao and Jacob Katsnelson for technical assistance; Sherry Chan for plant growth and care; Lina Prak for assistance with LR white embedding and microtome sectioning of rice stems; and Drs. Brad Holmes and Berit Ebert for help with HPAEC protocols and equipment setup.

**References**


Bart RS, Chern M, Vega-Sanchez ME, Canlas P, Ronald PC (2010) Rice Snl6, a cinnamoyl-CoA reductase-like gene family member, is required for NH1-mediated immunity to *Xanthomonas oryzae pv. oryzae*. PLoS Genet **6**


Christensen U, Scheller HV (2012) Regulation of (1,3;1,4)-β-D-glucan synthesis in developing endosperm of barley lys mutants. Journal of Cereal Science 55: 69-76


Fincher GB (2009a) Exploring the evolution of (1,3;1,4)-beta-D-glucons in plant cell walls: comparative genomics can help! Curr Opin Plant Biol 12: 140-147

Fincher GB (2009b) Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. Plant Physiol 149: 27-37

Fry SC, Nesselrode BHWA, Miller JG, Mewburn BR (2008) Mixed-linkage (1,3; 1,4)-beta-D-glucan is a major hemicellulose of Equisetum (horsetail) cell walls. New Phytologist 179: 104-115


Honegger R, Haisch A (2001) Immunocytochemical location of the (1,3) (1,4)-beta-glucan lichenin in the lichen-forming ascomycete Cetraria islandica (Icelandic moss). New Phytologist 150: 739-746


beta-glucan-specific monoclonal antibody and its use in the quantitation and
immunocytochemical location of (1-->3,1-->4)-beta-glucans. Plant J 5: 1-9
Mitsuhara I, Iwai T, Seo S, Yanagawa Y, Kawahigasi H, Hirose S, Ohkawa Y,
Ohashi Y (2008) Characteristic expression of twelve rice PR1 family genes in
response to pathogen infection, wounding, and defense-related signal compounds
(121/180). Mol Genet Genomics 279: 415-427
Nemeth C, Freeman J, Jones HD, Sparks C, Pellny TK, Wilkinson MD, Dunwell J,
Andersson AA, Aman P, Guillou F, Saulnier L, Mitchell RA, Shewry PR
(2010) Down-regulation of the CSLF6 gene results in decreased (1,3;1,4)-beta-D-
glucan in endosperm of wheat. Plant Physiol 152: 1209-1218
tuberosum tubers contains complex arabinogalactan structures. Phytochemistry
65: 1429-1438
Persson S, Paredez A, Carroll A, Palsdottir H, Doblin M, PoinINDEXER P, Khitrov N,
Auer M, Somerville CR (2007) Genetic evidence for three unique components in
primary cell-wall cellulose synthase complexes in Arabidopsis. Proc Natl Acad
Sci USA 104: 15566-15571
(2009) Hyphal cell walls from the plant pathogen Rhynchosporium secalis contain
(1,3; 1,6)-beta-D glucans, galacto- and rhamnomannans, (1,3; 1,4) beta-D-glucans
and chitin. FEBS Journal 276: 4122-4133
Popper ZA, Fry SC (2003) Primary cell wall composition of bryophytes and
Rigano LA, Payette C, Brouillard G, Marano MR, Abramowicz L, Torres PS, Yun
M, Castagnaro AP, Oirdi ME, Dufour V, Malamud F, Dow JM, Bouarab K,
Vojnov AA (2007) Bacterial cyclic beta-(1,2)-glucan acts in systemic suppression
of plant immune responses. Plant Cell 19: 2077-2089
Roulin S, Feller U (2001) Reversible accumulation of (1-->3,1-->4)-beta-glucan
Seifert GJ, Blaukopf C (2010) Irritable walls: the plant extracellular matrix and
cell wall preparations from rice bran, germ, and endosperm. Cereal Chemistry 62:
252-258
Smith BG, Harris PJ (1999) The polysaccharide composition of Poales cell walls:
Poaceae cell walls are not unique. Biochemical Systematics and Ecology 27: 33-
53
Sorensen I, Pettolino FA, Wilson SM, Doblin MS, Johansen B, Bacic A, Willats WG
(2008) Mixed-linkage (1-->3),(1-->4)-beta-D-glucan is not unique to the Poales
and is an abundant component of Equisetum arvense cell walls. Plant J 54: 510-
521
Taketa S, Yuo T, Tonooka T, Tsumuraya Y, Inagaki Y, Haruyama N, Larroque O,
Jobling SA (2011) Functional characterization of barley betaglucanless mutants
demonstrates a unique role for CslF6 in (1,3;1,4)-\{beta\}-D-glucan biosynthesis. J
Exp Bot
Tedman-Jones JD, Lei R, Jay F, Fabro G, Li X, Reiter WD, Brearley C, Jones JD
(2008) Characterization of Arabidopsis mur3 mutations that result in constitutive
activation of defence in petioles, but not leaves. Plant J 56: 691-703
2285
beta-D-glucanless grain on barley (Hordeum vulgare L.) chromosome 7H.
Breeding Science 59: 47-54
Trethewey JA, Campbell LM, Harris PJ (2005) (1->3),(1->4)-\{beta\}-d-Glucans in the
cell walls of the Poales (sensu lato): an immunogold labeling study using a
monoclonal antibody. Am J Bot 92: 1660-1674
Trethewey JAK, Harris PJ (2002) Location of (1-->3)- and (1-->3), (1-->4)-beta-D-
glucans in vegetative cell walls of barley (Hordeum vulgare) using immunogold
Upadhyaya NM, Zhu QH, Zhou XR, Eamens AL, Hoque MS, Ramm K,
Dissociation (Ds) constructs, mapped Ds launch pads and a transiently-expressed
transposase system suitable for localized insertional mutagenesis in rice. Theor
Appl Genet 112: 1326-1341
van Loon LC, Rep M, Pieterse CM (2006) Significance of inducible defense-related
proteins in infected plants. Annu Rev Phytopathol 44: 135-162
Verhertbruggen Y, Marcus SE, Haeger A, Verhoef R, Schols HA, McCleary BV,
polysaccharides and their processing in plant cell walls. Plant J 59: 413-425
profiling and integrative analysis of the CESA/CSL superfamily in rice. BMC
Plant Biol 10: 282
Figure legends

Figure 1. Mixed-linkage glucan (MLG) content and CslF6 expression in rice. A. MLG accumulation throughout development and in different tissues of rice cultivar Nipponbare. B. Quantitative RT-PCR showing CslF6 expression in rice tissues throughout development. col: coleoptile (3 day-old); sdl: seedling (7 day-old); till: tiller (30 day-old); yl: young leaf (30 day-old); ol: old leaf (30 day-old); ip4: immature panicle (4 cm); ip6: immature panicle (6 cm); elp: elongated panicle (11 cm); emp: emerging panicle; ms: mature stem (80 day-old); grn: mature seed (grain). AIR: alcohol insoluble residue.

Figure 2. CslF6 mediates biosynthesis of mixed linkage glucan. A. Accumulation of MLG in Nicotiana benthamiana leaves following agroinfiltration of constructs overexpressing CslF6. p19 (viral silencing suppressor), CslA1 and CslA6 are negative controls. B and C: endogenous mixed linkage glucan synthase activity assay using purified microsomal fractions from 7 day-old rice seedlings and 4 week-old sheaths, respectively, from wild type and cslf6 mutants. NPB: Nipponbare (wild type); WT: segregant of cslf6-2 without the insertion. n = 3 biological replicates; error bars: sem; AIR: alcohol insoluble residue.

Figure 3. cslf6 mutant anatomical phenotypes. A. 3 week old plants. B. Height of 3 week old plants. n = 10, error bars: sem. C. Second internode length of mature plants after flowering. n = 8, error bars: sem. D. Stem diameter of mature plants after flowering. n = 8, error bars: sem. WT: segregating sibling of cslf6-2 without the insertion. Asterisks indicate statistically significant differences at p<0.05 (*) and p<0.01 (**) using Student’s t test.

Figure 4. Mixed-linkage glucan accumulation in cslf6 mutant. MLG content in 7-day seedlings (A), immature panicles (B), mature stems after flowering (C), and coleoptiles (D). NPB: Nipponbare (wild type); WT: segregant of cslf6-2 without the insertion. AIR: Alcohol insoluble residue.
Figure 5. Immunolabeling and staining of coleoptile and stem sections. Wild type (A) and cslf6 (B) coleoptile sections labeled with anti-MLG monoclonal antibody. Inset: low exposure micrograph of vascular bundles is shown for clear depiction of cell types in the bundles. Wild type (C) and cslf6 (D) coleoptile sections labeled with LM10 xylan antibody. Wild type (E) and cslf6 (F) stem internode sections labeled with anti-MLG antibody. Wild type (G) and cslf6 (H) stem sections labeled with LM10 antibody. Close-up of wild type (I) and cslf6 (J) stem sections of the sclerenchyma labeled with anti-MLG antibody. Toluidine blue staining of wild type (K) and cslf6 mutant (L) stems showing the presence of secondary cell wall thickenings (light blue). ep: epidermis, p: parenchyma, sc: sclerenchyma, vb: vascular bundle, usc: upper sclerenchyma, lsc: lower sclerenchyma, ivb: inner vascular bundle, ovb: outer vascular bundle. Scale bar: A – D: 50 μm; E – H: 70 μm; I, J: 15 μm; K, L: 65 μm.

Figure 6. Immunolabeling and staining of fully expanded leaves. Wild type (A) and cslf6 (C) leaves labeled with anti-MLG monoclonal antibody. Arrowheads in C show weak MLG labeling in sclerenchyma fibers, vascular bundle sheaths and lower epidermis. B and D show the anatomy wild type and cslf6 mutant, respectively, using calcofluor staining. Scale bar: 50 μm; bc: bulliform cells, bs: bundle sheath, ep: epidermis, m: mesophyll, f: sclerenchyma fibers, vb: vascular bundle.

Figure 7. Mechanical properties of cell walls from wild type and cslf6 mutant mature stems. A. Stress expressed as compression yield strength (strength at first deformation or bending force). B. Strain (deformation tolerance) as a measure of material brittleness. N=12, ± SD. * Significant differences at P<0.05 and P<0.001 (**).

Figure 8. Enhanced disease resistance and upregulated defenses in cslf6 mutant. A. Lesion length of rice leaves inoculated with a bacterial suspension of Xanthomonas oryzae pv. oryzae isolate PXO99. Measurements taken 14 days post inoculation. B. Bacterial titer in PXO99 inoculated leaves, 14 days post inoculation. CFU: colony forming units. NPB: Nipponbare (wild type); WT: segregant of cslf6-2 without the insertion. Quantitative RT-PCR showing the expression of pathogenesis-related genes.
*PR1a* (Os07g03710) (C), *PR10* (Os12g36850) (D), and *PR1b* (Os01g28450) (E) in flag and old leaves of wild type and mutant plants, prior to inoculation. Values are presented as fold change expression from the wild type. **F.** Spontaneous lesion formation on flag leaves of *cslf6* mutants. 1: NPB (WT); 2: *cslf6*-1; 3: WT; 4: *cslf6*-2.
Table 1. Average cell and inner vascular bundle\(^1\) dimensions in rice stems and coleoptiles

<table>
<thead>
<tr>
<th></th>
<th>NPB(^2) (µm)</th>
<th>cslf6-(t)(^2) (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coleoptile longitudinal sections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell length</td>
<td>39 ±17.0</td>
<td>29 ±8.0 **</td>
</tr>
<tr>
<td><strong>Mature stem cross sections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular bundle tangential diameter</td>
<td>143.2 ±17.1</td>
<td>145 ±17.3</td>
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<tr>
<td>Vascular bundle radial diameter</td>
<td>104.8 ±11.6</td>
<td>88.3 ±6.1</td>
</tr>
<tr>
<td>Sub-epidermal sclerenchyma cells</td>
<td>5.6 ±1.6</td>
<td>4.3 ±1.7 **</td>
</tr>
<tr>
<td>Parenchyma cells (first layer)</td>
<td>15.6 ±14.1</td>
<td>14.9 ±7.9</td>
</tr>
<tr>
<td>Parenchyma cells (excluding first layer)</td>
<td>37.4 ±13.3</td>
<td>29.0 ±12.2 **</td>
</tr>
</tbody>
</table>

\(^1\)See figure 5 L
\(^2\)Measurement in micrometers ± standard deviation
\(^*\)Statistically significant at p < 0.001 (Student \(t\) test)
\(n=400\) and 866 cells for cslf6 and NPB coleoptiles, respectively; \(n=20\) (vascular bundles);
\(n=120\) cells for parenchyma and sclerenchyma.
NPB: Nipponbare wild type
Table 2. Oligosaccharide composition from mixed-linkage glucan hydrolyzed by lichenase and extracted from different tissues as determined by HPAEC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>G4G3G$^1$</th>
<th>G4G4G3G$^1$</th>
<th>Relative amount (%)</th>
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<tr>
<td>Coleoptile</td>
<td></td>
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<tr>
<td>NPB</td>
<td>44.0±0.4</td>
<td>26.0±0.1</td>
<td>100</td>
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<tr>
<td>cs/lf6-1</td>
<td>1.5±0.1</td>
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<tr>
<td>cs/lf6-2</td>
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<tr>
<td>Seedling</td>
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<tr>
<td>NPB</td>
<td>51.6±0.3</td>
<td>32.5±0.1</td>
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<tr>
<td>cs/lf6-1</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>WT</td>
<td>60.9±0.1</td>
<td>36.8±0.1</td>
<td>100</td>
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<tr>
<td>cs/lf6-2</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>Immature panicle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPB</td>
<td>35.1±0.1</td>
<td>24.0±0.1</td>
<td>100</td>
</tr>
<tr>
<td>cs/lf6-1</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>WT</td>
<td>20.8±1.6</td>
<td>12.0±1.0</td>
<td>100</td>
</tr>
<tr>
<td>cs/lf6-2</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>Mature stem</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>NPB</td>
<td>63.7±3.8</td>
<td>35.2±0.1</td>
<td>100</td>
</tr>
<tr>
<td>cs/lf6-1</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>WT</td>
<td>59.0±0.6</td>
<td>35.0±1.0</td>
<td>100</td>
</tr>
<tr>
<td>cs/lf6-2</td>
<td>trace</td>
<td>ND</td>
<td>trace</td>
</tr>
</tbody>
</table>

Relative amount calculated by adding total tri and tetra-saccharide amounts

$^1$μg/mg of alcohol insoluble residue
ND: not detected
NPB: Nipponbare wild type
WT: wild type segregant without the insertion
Table 3. Cell wall monosaccharide composition\(^1\) (μg/mg AIR)

<table>
<thead>
<tr>
<th>Seedling</th>
<th>Fuc</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glc</th>
<th>Xyl</th>
<th>Man</th>
<th>GalA</th>
<th>GlcA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPB</td>
<td>2.4±0.3</td>
<td>1.2±0.0</td>
<td>25±2.8</td>
<td>12.4±1.7</td>
<td>34.6±4.4</td>
<td>67.4±5.0</td>
<td>5.7±0.8</td>
<td>8.4±0.8</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td><em>cslf6-1</em></td>
<td>3.1±0.4</td>
<td>1.5±0.1*</td>
<td>30.2±2.7</td>
<td>15.3±1.1*</td>
<td>7.6±0.7**</td>
<td>69.2±5.9</td>
<td>2.3±0.7*</td>
<td>10.0±0.6*</td>
<td>2.9±0.4*</td>
</tr>
<tr>
<td>WT</td>
<td>2.6±0.3</td>
<td>1.2±0.2</td>
<td>24.6±1.4*</td>
<td>12.5±0.9</td>
<td>30.6±1.6</td>
<td>71.6±2.9</td>
<td>3.1±0.7</td>
<td>7.6±0.3</td>
<td>2.2±0.4</td>
</tr>
<tr>
<td><em>cslf6-2</em></td>
<td>3.1±0.5</td>
<td>1.2±0.2</td>
<td>28.5±1.7</td>
<td>13.7±0.2</td>
<td>7.2±0.2**</td>
<td>73.1±6.5</td>
<td>2.7±0.7</td>
<td>9.9±1.3*</td>
<td>2.8±0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stem</th>
<th>Fuc</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glc</th>
<th>Xyl</th>
<th>Man</th>
<th>GalA</th>
<th>GlcA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPB</td>
<td>0.2±0.0</td>
<td>1.3±0.1</td>
<td>28.6±0.8</td>
<td>10.8±0.6</td>
<td>106.0±9.3</td>
<td>151.6±5.9</td>
<td>0.7±0.02</td>
<td>7.9±0.2</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td><em>cslf6-1</em></td>
<td>0.3±0.0</td>
<td>1.6±0.0*</td>
<td>33.5±0.4**</td>
<td>12.8±0.6*</td>
<td>49.8±2.2**</td>
<td>179.2±4.1*</td>
<td>0.7±0.03</td>
<td>9.2±0.3*</td>
<td>2.2±0.1*</td>
</tr>
<tr>
<td>WT</td>
<td>0.2±0.0</td>
<td>1.3±0.1</td>
<td>31.2±0.7</td>
<td>9.4±0.5</td>
<td>78.5±6.2</td>
<td>161.9±10.1</td>
<td>0.8±0.2</td>
<td>8.4±0.5</td>
<td>1.7±0.4</td>
</tr>
<tr>
<td><em>cslf6-2</em></td>
<td>0.4±0.0</td>
<td>1.7±0.0*</td>
<td>31.7±0.4</td>
<td>11.5±0.1</td>
<td>28.3±0.8**</td>
<td>157.8±1.5</td>
<td>1.0±0.1</td>
<td>10.9±0.2**</td>
<td>2.3±0.4</td>
</tr>
</tbody>
</table>

Fuc: fucose; Rha: rhamnose; Ara: arabinose; Gal: galactose; Glc: glucose; Xyl: xylose; Man: Mannose; GalA: galacturonic acid; GlcA: glucuronic acid.

\(^1\)HPAEC analysis after TFA hydrolysis

Values are average of 3 replicates ± standard deviation

NPB:  Nipponbare wild type

WT: sibling of *cslf6-2* without the insertion

(*) statistically significant at p<0.05  and p<0.001 (**)
Table 4. Cellulose and lignin content (µg/mg ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Cellulose</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Seedling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPB</td>
<td>202.7±6.6</td>
<td>ND</td>
</tr>
<tr>
<td>cslf6-1</td>
<td>171.0±16.3*</td>
<td>ND</td>
</tr>
<tr>
<td>WT</td>
<td>192.6±32.3</td>
<td>ND</td>
</tr>
<tr>
<td>cslf6-2</td>
<td>167.5±9.1</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPB</td>
<td>468.3±13.4</td>
<td>131.5±3.7</td>
</tr>
<tr>
<td>cslf6-1</td>
<td>461.1±22.0</td>
<td>161.1±15.7*</td>
</tr>
<tr>
<td>WT</td>
<td>455.5±11.6</td>
<td>114.4±6.2</td>
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<tr>
<td>cslf6-2</td>
<td>373.7±16.3*</td>
<td>142.4±9.5*</td>
</tr>
</tbody>
</table>

1Presented as glucose equivalents of crystalline cellulose
2Acetyl bromide lignin
ND: not determined
NPB:  Nipponbare wild type
WT:  sibling of cslf6-2 without the insertion
(*) statistically significant at p<0.05  and p<0.001 (**)
Figure 1. Mixed-linkage glucan (MLG) content and CsIF6 expression in rice. 

A. MLG accumulation throughout development and in different tissues of rice cultivar Nipponbare. 

B. Quantitative RT-PCR showing CsIF6 expression in rice tissues throughout development. col: coleoptile (3 day-old); sdl: seedling (7 day-old); till: tiller (30 day-old); yl: young leaf (30 day old); ol: old leaf (30 day-old); ip4: immature panicle (4cm); ip6: immature panicle (6cm); elp: elongated panicle (11cm); emp: emerging panicle; ms: mature stem (80 day-old); grn: mature seed (grain). AIR: alcohol insoluble residue.
Figure 2. CslF6 mediates biosynthesis of mixed linkage glucan. A. Accumulation of MLG in Nicotiana benthamiana leaves following agroinfiltration of constructs overexpressing CslF6. p19 (viral silencing suppressor), Cs/A1 and Cs/A6 are negative controls. B and C: endogenous mixed linkage glucan synthase activity assay using purified microsomal fractions from 7 day-old rice seedlings and 4 week-old sheaths, respectively, from wild type and cslf6 mutants. NPB: Nipponbare (wild type); WT: segregant of cslf6-2 without the insertion. n = 3 biological replicates; error bars: SEM; A/f: alcohol insoluble residue.
**Figure 3.** *cslf6* mutant anatomical phenotypes. **A.** 3-week old plants. **B.** Height of 3 week old plants. \( n = 10 \), error bars: sem. **C.** Second internode length of mature plants after flowering. \( n = 8 \), error bars: sem. **D.** Stem diameter of mature plants after flowering. \( n = 8 \), error bars: sem. WT: segregating sibling of *cslf6-2* without the insertion. Asterisks indicate statistically significant differences at \( p<0.05 \) (*) and \( p<0.01 \) (**) using Student’s *t* test.
Figure 4. Mixed-linkage glucan accumulation in cslf6 mutant. MLG content in 7-day seedlings (A), immature panicles (B), mature stems after flowering (C), and coleoptiles (D). NPB: Nipponbare (wild type); WT: segregant of cslf6-2 without the insertion. AIR: Alcohol insoluble residue.
Figure 5. Immunolabeling and staining of coleoptile and stem sections. Wild type (A) and cslf6 (B) coleoptile sections labeled with anti-MLG monoclonal antibody. Inset: low exposure micrograph of vascular bundles is shown for clear depiction of cell types in the bundles. Wild type (C) and cslf6 (D) coleoptile sections labeled with LM10 antibody against xylan. Wild type (E) and cslf6 (F) stem internode sections labeled with anti-MLG antibody. Wild type (G) and cslf6 (H) stem sections labeled with LM10 antibody. Close-up of wild type (I) and cslf6 (J) stem sections of the sclerenchyma labeled with anti-MLG antibody. Toluidine blue staining of wild type (K) and cslf6 mutant (L) stems showing the presence of secondary cell wall thickenings (light blue). ep: epidermis, p: parenchyma, sc: sclerenchyma, vb: vascular bundle, usc: upper sclerenchyma, lsc: lower sclerenchyma, ivb: inner vascular bundle, ovb: outer vascular bundle. Scale bar: A – D: 50 µm; E – H: 70 µm; I, J: 15 µm; K, L: 65 µm.
**Figure 6.** Immunolabeling and staining of fully expanded leaves. Wild type (A) and csI6 (C) leaves labeled with anti-MLG monoclonal antibody. Arrowheads in C show weak MLG labeling in sclerenchyma fibers, vascular bundle sheaths and lower epidermis. B and D show matching calcofluor labeling pictures of wild type and csI6 mutant, respectively. Scale = 50 µm; bc = bulliform cells, bs = bundle sheath, ep = epidermis, m = mesophyll, f = sclerenchyma fibers, vb = vascular bundle.
Figure 7. Mechanical properties of cell walls from wild type and *cslf6* mutant mature stems. A. Stress expressed as compression yield strength (strength at first deformation or bending force). B. Strain (deformation tolerance) as a measure of material brittleness. N=12, ± SD. * Significant differences at P<0.05 and P<0.001 (**).
Figure 8. Enhanced disease resistance and upregulated defenses in csfl6 mutant. A. Lesion length of rice leaves inoculated with a bacterial suspension of Xanthomonas oryzae pv. oryzae isolate PXO99. Measurements taken 14 days post inoculation. B. Bacterial titer in PXO99 inoculated leaves, 14 days post inoculation. CFU: colony forming units. NPB: Nipponbare (wild type); WT: segregant of csfl6-2 without the insertion. Quantitative RT-PCR showing the expression of pathogenesis-related genes PR1a (Os07g03710) (C), PR10 (Os12g36850) (D), and PR1b (Os01g28450) (E) in flag and old leaves of wild type and mutant plants, prior to inoculation. Values are presented as fold change expression from the wild type. F. Spontaneous lesion formation on flag leaves of csfl6 mutants. 1: NPB (WT); 2: csfl6-1; 3: WT; 4: csfl6-2.