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N-glycan maturation is crucial for cytokinin-mediated development and cellulose synthesis in *Oryza sativa*

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SUMMARY

To explore the physiological significance of N-glycan maturation in the plant Golgi apparatus, *gnt1*, a mutant with loss of *N*-acetylglucosaminyltransferase I (GnTI) function, was isolated in *Oryza sativa*. *gnt1* exhibited complete inhibition of N-glycan maturation and accumulated high-mannose N-glycans. Phenotypic analyses revealed that *gnt1* shows defective post-seedling development and incomplete cell wall biosynthesis, leading to symptoms such as failure in tiller formation, brittle leaves, reduced cell wall thickness, and decreased cellulose content. The developmental defects of *gnt1* ultimately resulted in early lethality without transition to the reproductive stage. However, callus induced from *gnt1* seeds could be maintained for periods, although it exhibited a low proliferation rate, small size, and hypersensitivity to salt stress. Shoot regeneration and dark-induced leaf senescence assays indicated that the loss of GnTI function results in reduced sensitivity to cytokinin in rice. Reduced expression of A-type *O. sativa* response regulators that are rapidly induced by cytokinins in *gnt1* confirmed that cytokinin signaling is impaired in the mutant. These results strongly support the proposed involvement of N-glycan maturation in transport as well as in the function of membrane proteins that are synthesized via the endomembrane system.

Keywords: *Oryza sativa*, N-acetylglucosaminyltransferase I, N-glycan maturation, development, cytokinin signaling, cellulose synthesis.

INTRODUCTION

The endoplasmic reticulum (ER) and Golgi apparatus in eukaryotic cells are important in the processing of proteins that are destined for the plasma membrane, cell wall, vacuoles, lysosome, or the secretory pathway. In eukaryotic cells, ribosomes bind to the ER once they begin to synthesize proteins that are co-translationally translocated into the lumen of rough endoplasmic reticulum (RER). These proteins subsequently undergo post-translational modifications such as disulfide formation, multisubunit assembly, lipidation, and glycosylation. Among the modifications that take place in the ER, Asn-linked glycosylation (N-glycosylation) is an important co- and post-translational modification affecting the physicochemical properties of proteins in eukaryotic cells (Ruiz-Canada *et al.*, 2009). Various important biological processes including protein folding (Molinari and Helenius, 1999; Sitia and Braakman, 2003; Spiro, 2004), activity (Martina *et al.*, 2000), secretion (Fiedler and Simons, 1995) and intracellular trafficking (Helenius and Aebi, 2001, 2004), and cell–cell communication (Varki, 1993) are reported to be affected by N-glycosylation.

N-glycosylation begins in the ER when the oligosaccharyltransferase (OST) complex transfers an entire glycan ($Glc_3Man_9GlcNAc_2$; Glc = glucose, Man = mannose, GlcNAc = N-acetylglucosamine) *en bloc* from the lipid-linked precursor dolichol–PP–$Glc_3Man_9GlcNAc_2$ to the Asn residue in the sequence Asn–X–Ser/Thr (where X is not proline) of a nascent polypeptide (Hardt *et al.*, 2000). Subsequently, the Glc residues of the 14-sugar oligomannose $Glc_3Man_9GlcNAc_2$ are trimmed by α-glucosidase I (GI) and α-glucosidase II (GII) in the ER (Gomord *et al.*, 2010). Then,

the four α1,2-linked mannose residues are removed by α-mannosidases in the ER and Golgi apparatus, generating the $Man_5GlcNAc_2$ substrate for the formation of mature N-glycans in the Golgi apparatus. Subsequent highly coordinated stepwise N-glycan processing steps in the Golgi apparatus lead to the biosynthesis of high-mannose-, complex-, hybrid-, and paucimannose-type N-glycans (Kornfeld and Kornfeld, 1985; Lerouge *et al.*, 1998). However, N-glycan processing in the Golgi apparatus and the resulting dominant N-glycan structures are quite variable among different taxa and species. The paucimannose-type N-glycans predominantly found in vascular plants are characterized by the presence of β1,2-xylose and α1,3-fucose residues attached to the pentasaccharide core, and by the absence of terminal GlcNAc residues (Bencur *et al.*, 2005). Structural differences in the mature N-glycans between plants and mammals have been a limitation in the use of plant-made pharmaceuticals (PMPs) in human therapy, as epitopes containing paucimannose-type N-glycans are immunogenic in mammals (Bardor *et al.*, 2003).

The addition of β1,2-linked GlcNAc to $Man_5GlcNac_2$ by *N*-acetylglucosaminyltransferase I (GnTI) is a prerequisite for the actions of subsequent N-glycan processing enzymes such as Golgi α-mannosidase II (Altmann and Marz, 1995; Paschinger *et al.*, 2006; Strasser *et al.*, 2006), core α1,3-fucosyltransferases (Fabini *et al.*, 2001; Freeze, 2002), β1,2-xylosyltransferases (Mulder *et al.*, 1995; Strasser *et al.*, 2000; Faveeuw *et al.*, 2003), and *N*-acetylglucosaminyltransferase II (GnTII; Strasser *et al.*, 1999). Thus, eukaryotic cells lacking GnTI activity in the Golgi apparatus are incapable of synthesizing complex and hybrid N-glycans (Metzler *et al.*, 1994). Defects in N-glycan maturation caused by the mutation of several enzymes in the Golgi apparatus lead to type II congenital disorders of glycosylation (CGD) in humans (Freeze, 2002). Severe developmental abnormalities in neural tube formation and vascularization, as well as lethality at embryonic days 9–10, have been observed in mutant *Mus musculus* lacking *GnTI* (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). However, Lec1 Chinese hamster ovary (CHO) and Ric^R14 baby hamster kidney (BHK) cell lines that lack GnTI activity are viable in *in vitro* cultures (Kumar and Stanley, 1989; Opat *et al.*, 1998). Hence, hybrid and complex N-glycans seem not to be essential for the viability of mammalian cell lines. A lack of GnTI activity in invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster* does not cause lethality. However, it results in defective interaction of *C. elegans* with pathogenic bacteria (Zhu *et al.*, 2004) and leads to defective locomotor activity (LMA), brain abnormalities, and a severely reduced lifespan in *D. melanogaster* (Sarkar *et al.*, 2006). In plants, the importance of GnTI activity and N-glycan maturation in the Golgi apparatus is not fully understood due to a lack of identified mutants with obvious phenotypes. The Arabidopsis *cgl1-T* mutant lacking GnTI activity shows salt stress-sensitive responses such as growth inhibition and swollen root tips; however, significant alterations in development were not observed under normal growth conditions (von Schaewen *et al.*, 1993; Kang *et al.*, 2008). Thus, it has been assumed that hybrid and complex glycans are required for stress tolerance but not for plant development. In this study, to expand our knowledge on the physiological role of *GnTI* in plants, a rice mutant containing a T-DNA insertion in the *GnT1* gene was isolated. N-glycan structures of *gnt1* and the wild type (WT) were compared using a variety of biochemical tools. The developmental defects of *gnt1* and the physiological functions of *GnTI*, especially in cytokinin signaling and cell wall biosynthesis, were also examined.

RESULTS

Oryza sativa GnTI encodes enzymatically and functionally active protein

To examine the *in vivo* activity of rice *GnTI*, a cDNA encoding GnTI was isolated from a rice leaf cDNA library by polymerase chain reaction (PCR) and expressed in the Arabidopsis *cgl1-T* mutant (Frank *et al.*, 2008) under the control of the constitutive CaMV 35S promoter. Formation of complex- and hybrid-type N-glycans was compared by immunoblot analyses using anti-horseradish peroxidase (HRP), anti-fucose, and anti-xylose antibodies. The amount of high-mannose-type N-glycans in the plants was measured by lectin blot analysis using concanavalin A (con A). Results from the immunoblot and lectin blot analyses showed that the expression of rice *GnTI* in *cgl1-T* leads to rescue of defective N-glycan maturation in the *cgl1-T* Golgi apparatus (see Figure S1). The salt stress-sensitive phenotype of *cgl1* was reported to be caused by incomplete N-glycan maturation of glycoproteins (Kang *et al.*, 2008). To examine whether the recovered N-glycan maturation in *cgl1-T* resulted in rescue of the stress-sensitive phenotype, root growth of WT, *cgl1-T*, and rice GnTI-expressing *cgl1-T* (lines C3 and C7) on salt-containing media was analyzed (see Figure S1). The inhibition of *cgl1-T* root growth on salt-containing medium was completely rescued by the expression of rice *GnTI*. These results indicate that not only the GnTI activity in N-glycan maturation but also the salt stress-sensitive phenotype of the mutant can be complemented by the overexpression of rice GnTI, suggesting that rice GnTI is enzymatically and functionally active *in vivo* (see Figure S1). The GlcNAc transferase activity of rice GnTI has been also shown using culture supernatants of *Pichia pastoris* transformed with rice GnTI (Leonard *et al.*, 2004).

gnt1 is a null mutant of rice *GnTI* with a T-DNA insertion

BLAST searches of the TIGR rice genome annotation database with the rice cDNA (AJ457976) sequence as a query revealed that the *GnTI* gene composed of 18 exons is

present as a single copy in the rice genome at the Os02 g58590 locus. To address the physiological roles of GnTI in rice plants, we screened rice mutants associated with Loc_Os02 g58590 among the collection at the Rice Functional Genomic Express Database (http://signal.salk.edu/cgi-bin/RiceGE). A mutant line (SAI3G12) containing a T-DNA insertion in *GnTI* was identified by the search and obtained from the Génoplante rice tagged line collection. Homozygous mutant plants, designated as *gnt1*, were identified by genomic PCR and RT-PCR analyses using gene-specific (P1, P2, P3, and P4) and T-DNA (LB) primers (Figure 1). Heterozygous plants showed an expected Mendelian segregation ratio for hygromycin resistance (see Table S1). We were unable to detect native transcripts of *GnTI* by RT-PCR in homozygous *gnt1* plants, and WT siblings derived from the heterozygous *gnt1* plants were used as WT controls in this study. To examine whether the expression of adjacent genes is affected by the T-DNA inserted in *GnTI*, we measured expression of the genes by quantitative real-time PCR (qRT-PCR) in *gnt1* and WT. However, there was no significant difference in the expressions of the genes encoding cation efflux family protein (Os02 g58580), hypothetical protein (Os02 g58600), and protein kinase (Os02 g58610) between *gnt1* and WT (see Figure S2). Results from DNA gel blot and segregation analyses indicated that *gnt1* contains a single T-DNA insertion (Figure 1). Sequence analysis of the PCR fragments generated with the gene-specific (P1 and P2) and T-DNA (LB and RB) primers revealed that the T-DNA is inserted in *GnTI* and that 44 nucleotides (nt) including 42 and 2 nt of the sixth exon and sixth intron, respectively, are deleted and substituted by the T-DNA (Figure 1). Thus it is likely that the T-DNA insertion in the sixth exon/intron junction leads to aberrant splicing of *GnTI* and the introduction of premature stop codons, which may ultimately result in post-transcriptional mRNA degradation.

N-glycan maturation is abolished in *gnt1*

To address whether the rice *gnt1* mutant produces glycoproteins with high-mannose N-glycans instead of paucimannose or complex N-glycans, we performed immunoblot analyses using anti-HRP, anti-fucose, and anti-xylose antibodies and lectin blot analysis using con A (Figure 2a). The anti-HRP, anti-fucose, and anti-xylose antibodies exhibited reactions only with proteins from WT and not with those of *gnt1*, suggesting that the formation of the paucimannose and complex N-glycans containing core α1,3-fucose and β1,2-xylose residues has been abolished in *gnt1*. By contrast, con A showed a strong reaction with proteins from *gnt1* but a weak reaction with those of WT, indicating that the amount of the high-mannose N-glycans has been increased in *gnt1*. To obtain quantitative and comparative structural information on the N-glycans in *gnt1* and WT, glycoproteins extracted from the embryo-derived calli were subjected to matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The mass

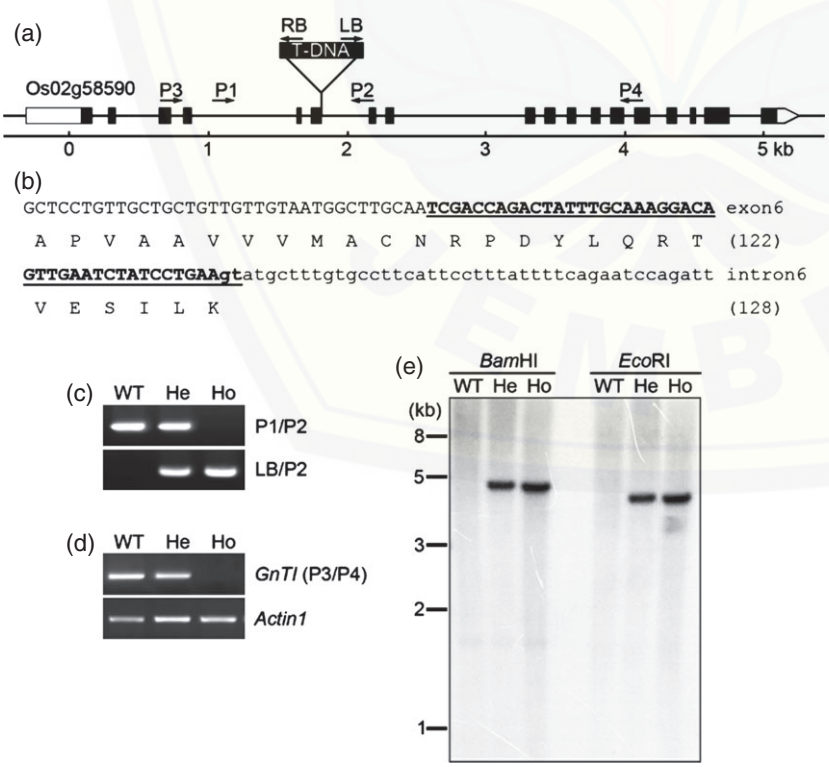

Figure 1. Identification of the *gnt1* T-DNA insertion mutant in rice.
(a) Schematic representation of the genomic structure of rice *GnTI* and the T-DNA insertion site. Black boxes represent exons comprising an open reading frame (ORF), solid lines between the boxes indicate introns, and white boxes represent exons comprising the five- and three-prime untranslated regions (5′ and 3′ UTRs). The location of the T-DNA insertion and orientation of the left and right borders are indicated. Gene-specific (P1, P2, P3, and P4) and T-DNA-specific (LB and RB) primers used in genotyping and RT-PCR are shown with arrows.
(b) The deletion of 44 nucleotides in *gnt1* by the insertion of T-DNA. The nucleotide sequence deleted in *gnt1* is indicated by bold underlining. Uppercase and lowercase indicate exon and intron, respectively. The encoded amino acids are shown as capital letters below the DNA sequence.
(c) Genotyping of heterozygous (He) *gnt1*, homozygous (Ho) *gnt1*, and WT using the indicated primer pairs.
(d) The RT-PCR analysis of He, Ho, and WT. The *Actin1* gene was used as a loading control.
(e) The DNA gel blot analysis of He, Ho, and WT. ^{32}P-labeled *HPT* cDNA was used as a probe.

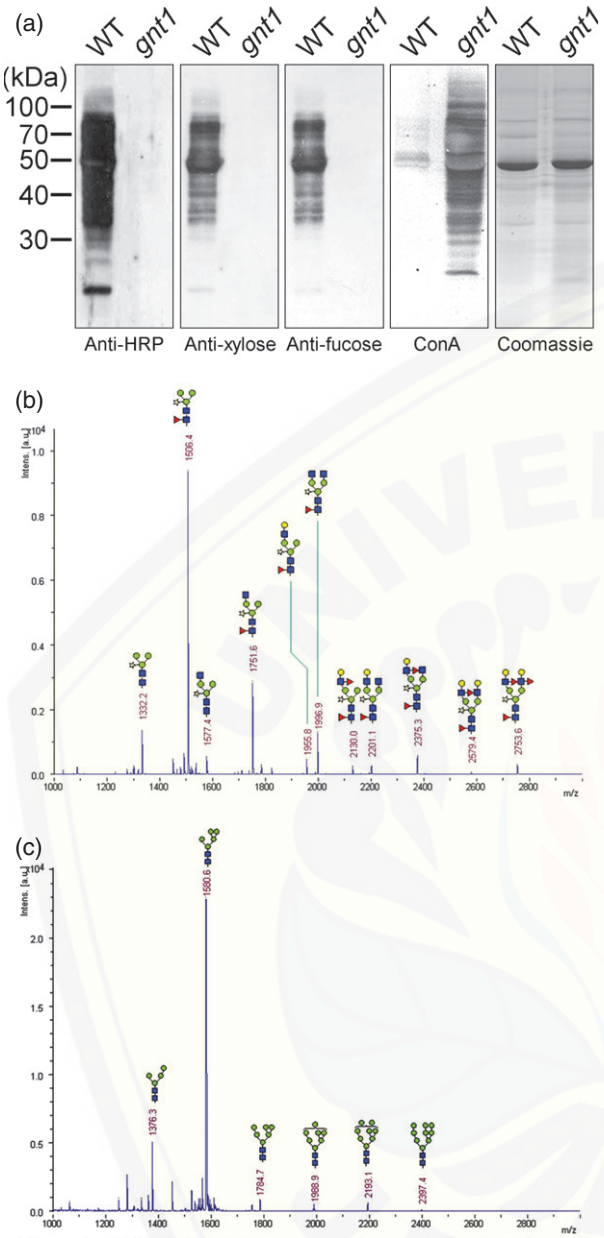

Figure 2. Analyses of the N-glycans produced in *gnt1* and wild type (WT).
(a) Immunoblot and affinoblot analyses of total leaf proteins from *gnt1* and WT. The blots were probed with anti-horseradish peroxidase (HRP), anti-xylose, and anti-fucose antibodies. Affinoblot assays were performed using the concanavalin A (con A)/peroxidase system. Coomassie blue staining was used to show equal loading of the *gnt1* and WT proteins.
(b), (c) Matrix-assisted laser-desorption time-of-flight mass spectra of the PNGase A-released permethylated N-glycans from WT (b) and *gnt1* (c) calli. Diagrams indicate the proposed structures of the peaks that have been denoted with their corresponding *m/z* value. The blue square, red triangle, yellow star, green circle, and yellow circle represent N-acetylglucosamine, fucose, xylose, mannose, and galactose residues, respectively.

spectra of the N-glycans from WT callus revealed that various complex N-glycans containing β1,2-xylose, α1,3-fucose, and Lewis a (Le^a) epitopes were found in WT callus (Figure 2, Table 1). By contrast, N-glycans from *gnt1* callus were composed mostly of high-mannose N-glycans ($Man_{4-9}GlcNAc_2$) with $Man_5GlcNAc_2$ as the major form (76.7%), but paucimannose- and complex-type N-glycans were not found (Figure 2, Table 1). A similar result was also obtained from HPLC analysis of the N-glycans from WT and *gnt1* callus (see Figure S3, Table S2, Methods S1). Taken together, these experiments provide evidence that the lack of GnTI activity in rice also results in complete inhibition of N-glycan maturation in the Golgi apparatus, leading to the accumulation of high-mannose N-glycans, with the $Man_5GlcNAc_2$ form as the major N-glycan.

gnt1 exhibits developmental abnormalities and dies before reproduction

To explore the developmental characteristics of *gnt1* compared with those of WT, plants were grown in soil (Figure 3a–c). Compared with WT, *gnt1* did not show distinctive phenotypes until 8 days after imbibition (DAI). However, severely arrested seedling growth was observed in the mutant at 1 month. Furthermore, the primary tiller, which was observed at 25–30 DAI in WT, failed to appear in *gnt1* during the remainder of the culture time, and the mutant plants ultimately died without transitioning to the reproductive stage. To examine the defective development carefully, *gnt1* and WT were grown hydroponically. Severe growth inhibition, measured by determining fresh weight and root length and number over time, was also observed in *gnt1* from approximately 13 DAI onward (Figure 3d–f). The severe growth arrest in *gnt1* prompted us to examine whether the amount of glycoproteins with N-glycans was changed with plant development at the seedling stage in WT. When the abundance of N-glycans in WT was assessed via immunoblot analyses using anti-HRP, anti-fucose, and anti-xylose antibodies, a discernible increase in the amount of complex N-glycans including β1,2-xylose and α1,3-fucose residues was detected from 13 DAI and the increase became more evident from 16 DAI (Figure 4). On the other hand, lectin blot analysis using con A showed that the amount of the high-mannose N-glycans also increased from 16 DAI (Figure 4). These results provide new insight into the potential effects of glycoproteins on plant development.

gnt1 callus shows a reduced proliferation rate and weak cell–cell interaction

To address the physiological role of GnTI at the cellular level, embryo-derived callus was induced from seeds of *gnt1* and WT (Figure 5a,b). To allow careful examination of the phenotype and proliferation of tissues, suspension cultures were established and maintained by subculturing every 2 weeks. Compared with that of WT callus, markedly reduced proliferation activity was observed in *gnt1* callus (Figure 5d,e). Furthermore, the turbidity of the

Table 1 Matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF MS) analysis of N-glycans from wild type (WT) and *gnt1* calli

Theoretical *m/z*	Observed *m/z*	N-glycan structures	WT	*gnt1*
		Complex-type N-glycans		
1330	1332	$Man_3XylGlcNAc_2$	6.83	ND
1504	1506	$Man_3XylFucGlcNAc_2$	51.63	ND
1575	1577	$GlcNAcMan_3XylGlcNAc_2$	2.79	ND
1750	1752	$GlcNAcMan_3XylFucGlcNAc_2$	15.72	ND
1821	1823	$GlcNAc_2Man_3XylGlcNAc_2$	1.44	ND
1954	1956	$GalGlcNAcMan_3XylFucGlcNAc_2$	2.76	ND
1995	1997	$GlcNAc_2Man_3XylFucGlcNAc_2$	8.07	ND
2128	2130	$GalFucGlcNAcMan_3XylFucGlcNAc_2$	1.59	ND
2199	2201	$GalGlcNAc_2Man_3XylFucGlcNAc_2$	1.81	ND
2373	2375	$GalFucGlcNAc_2Man_3XylFucGlcNAc_2$	3.47	ND
2577	2579	$Gal_2FucGlcNAc_2Man_3XylFucGlcNAc_2$	0.38	ND
2751	2754	$Gal_2Fuc_2GlcNAc_2Man_3XylFucGlcNAc_2$	1.81	ND
		Total	98.3	0
		High mannose-type N-glycans		
1374	1376	$Man_4GlcNAc_2$	ND	17.47
1579	1581	$Man_5GlcNAc_2$	ND	76.72
1783	1785	$Man_6GlcNAc_2$	1.7	2.8
1987	1989	$Man_7GlcNAc_2$	ND	1.4
2191	2193	$Man_8GlcNAc_2$	ND	1.4
2395	2397	$Man_9GlcNAc_2$	ND	0.21
		Total	1.7	100

Values indicate percentage of total peak area.
ND, not detected; Man, mannose; Xyl, xylose; GlcNAc, N-acetylglucosamine; Fuc, fucose; Gal, galactose.
Quantitative analysis of N-glycans from WT and *gnt1* was performed by MALDI-TOF MS using N-glycans derivatized by permethylation prior to analysis (Wada *et al.*, 2007). To attain good ion statistics the spectra presented were generated from several subspectra of 100 laser shots. The integrated peak area for their entire isotopic cluster was measured for relative quantification.

culture medium and the size of the callus clumps in the suspension culture of *gnt1* were higher and smaller, respectively, than those of WT (Figure 5c). Based on this observation, we hypothesized that the lack of GnTI activity may result in reduced proliferation and weak cell–cell interaction, resulting in small-sized callus. To examine salt-stress sensitivity, *gnt1* and WT calli were grown in suspension culture media with NaCl. In the presence of NaCl, reduced proliferation of *gnt1* was more severe (Figure 5d,e). These results implicate N-glycan maturation in cell–cell interaction, proliferation, and salt stress tolerance of rice callus.

gnt1 displays cytokinin-insensitive phenotypes

It is a great challenge to analyze the underlying molecular mechanisms of the multiple phenotypes caused by deficiency of N-glycan maturation in *gnt1*. Since *gnt1* showed severe developmental defects, including a failure in the formation of tiller buds and early lethality, we asked whether hormone signaling was affected by the deficiency of N-glycan maturation in *gnt1*. Cytokinins are a class of plant hormones that play essential and crucial roles in regulating the proliferation and differentiation of plant cells (Sakakibara, 2006; Argueso *et al.*, 2009; Werner and Schmulling, 2009). To examine whether cytokinin signaling was affected by the deficiency of N-glycan maturation in *gnt1*, we performed shoot regeneration assays on media containing kinetin. Whereas efficient differentiation and shoot regeneration were observed for WT calli, late formation of only a few differentiated green spots resulted in the absence of shoot regeneration in *gnt1* calli, suggesting that *gnt1* exhibits a cytokinin-insensitive phenotype (Figure 6a). Arabidopsis cytokinin receptor histidine kinases (AHKs) transmit the cytokinin signal that regulates leaf longevity, cell division, chloroplast biogenesis, and senescence (Hwang and Sheen, 2001; Kim *et al.*, 2006). We investigated the possible involvement of N-glycan maturation in cytokinin signaling by assaying dark-induced senescence in detached leaves. When the fourth leaves of 3-week-old WT and *gnt1* plants were kept in the dark for 6 days, chlorophyll degradation (a characteristic feature of senescence) was increased in *gnt1* leaves compared with those of WT in the absence or presence of cytokinins (Figure 6b,c). These data suggest that N-glycan maturation may play a crucial role in the function of cytokinin signaling components.

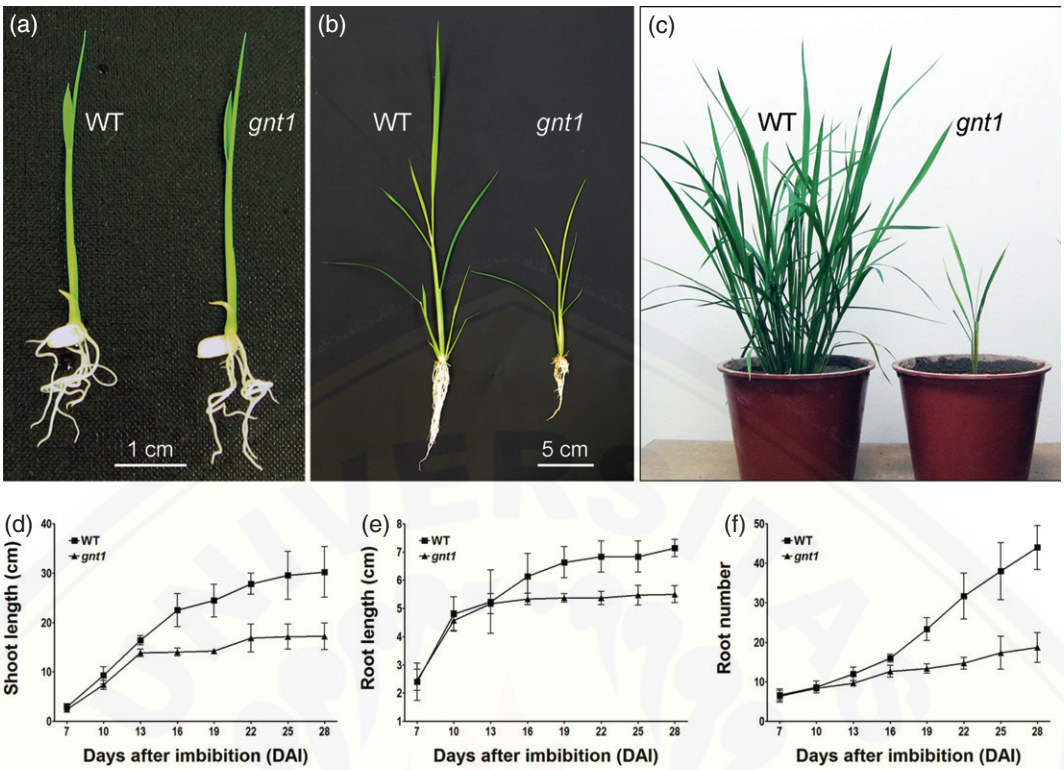

Figure 3. Developmental analysis of *gnt1*.
(a) Growth phenotypes of *gnt1* and wild type (WT) seedlings at 8 days after imbibition.
(b) One-month-old *gnt1* and WT.
(c) Three-month-old *gnt1* and WT.
(d)–(f) Measurement of shoot length (d), root length (e), and root number (f) of hydroponically grown *gnt1* and WT. Data represent the mean ± SE from 10 independent plants.

Cell wall thickness and cellulose content are reduced in *gnt1*

During the experiments, we found that the leaves of *gnt1* exhibited a brittle phenotype when treated with mechanical forces such as tension or pulverization. To examine the brittleness of *gnt1* more carefully, the fourth leaves of 3-week-old *gnt1* and WT were subjected to bend tests and tension assays. When the leaves of *gnt1* and WT were subjected to bending stress, most leaves from both *gnt1* and WT did not show snapping, suggesting that the brittle phenotype of *gnt1* does not result from a loss in flexibility, which would cause the tissues to snap instead of bend (Figure 7A). However, the breaking forces measured using a texture analyzer showed that the average maximum force required to break the fourth leaves of 3-week-old *gnt1* was about 26% lower than that required for WT, indicating that the brittle phenotype of *gnt1* results from loss of tensile strength (Figure 7B). To determine if the brittleness of *gnt1* arises from morphological changes in the cells, sections prepared from the fourth leaves of 3-week-old *gnt1* were compared with those of WT using light and transmission electron microscopy (LM and TEM). Although most cells in *gnt1* had thinner cell walls than their WT, the difference was prominent in the cell walls of sclerenchyma underlying the epidermis and surrounding the vascular bundles (Figure 7Cb). A TEM analysis revealed that the cell wall thickness of the sclerenchyma cells in *gnt1* was significantly (about 50%) reduced compared with that of WT (Figure 7C,D). Similar results were obtained when the calli of *gnt1* and WT were analyzed by TEM, although the difference in cell wall thickness was much more severe (about two-fold more) than that observed in the leaf tissues (Figure 7E,F). Furthermore, TEM analysis also showed that the thicknesses of both primary and secondary cell walls were affected by loss of GnTI function (Figure 7Ea–d). These results indicate that loss of GnTI function resulted in decreased cell wall synthesis activity, which might be responsible for the brittle phenotype and reduced tensile strength in *gnt1*.

The brittleness and reduced cell wall thickness of *gnt1* prompted us to investigate the cell wall composition in *gnt1* and WT. When the amounts of cellulose and lignin in cell walls from *gnt1* and WT were compared using the

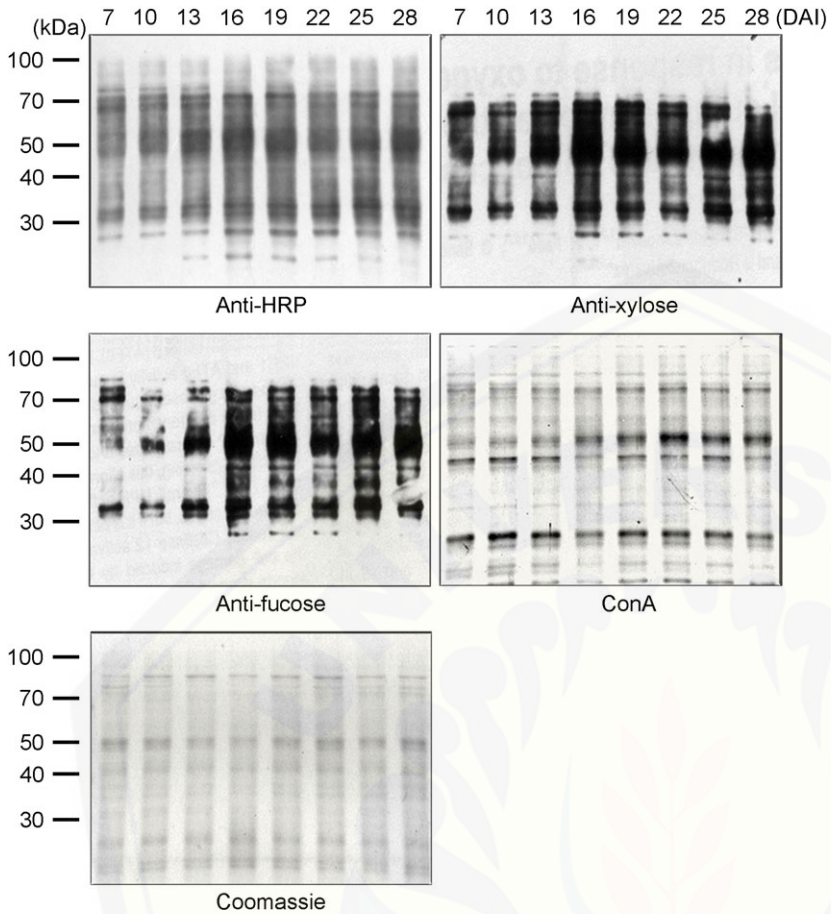

Figure 4. Analyses of the N-glycans in wild type (WT) during seedling development.
Total proteins extracted from roots at the indicated stages of seedling development (days after imbibition, DAI) were subjected to immunoblot and affinoblot analyses. The blots were probed with anti-horseradish peroxidase (HRP), anti-xylose, and anti-fucose antibodies. Affinoblot assays were performed using the concanavalin A (con A)/peroxidase system. Coomassie blue staining was used to show equal loading of the proteins.

aerial parts of 3-week-old seedlings, *gnt1* was found to exhibit a 38% decrease in cellulose and a 69% increase in lignin compared with WT (Figure 7G,H). By contrast, it has been reported that the expression of an Arabidopsis transcription factor, SHINE (SHN), in rice led to a 34% increase in cellulose and a 45% reduction in lignin content (Ambavaram *et al.*, 2011). These results suggest that a mechanism regulating the coordinated activation of cellulose and repression of lignin biosynthesis pathways, or *vice versa*, might exist in plants. To verify the reduced cellulose content in *gnt1*, the neutral monosaccharide composition of the cell walls was determined using the aerial parts of 3-week-old seedlings. *gnt1* showed a 37% decrease in glucose compared with WT, whereas the amounts of arabinose, galactose, and xylose were similar to those of WT (Table 2). Since cellulose is composed of repeated β1,4-glycosidic bonds of glucose units, this result is consistent with the finding of an altered cellulose content in *gnt1*. Taken together, these results indicate that GnTI also plays an important role in cellulose biosynthesis in the plant.

Cytokinin signaling is impaired in *gnt1*

In Arabidopsis, AHKs function as cytokinin receptors and histidine phosphotransfer proteins (AHPs) transmit the signal to nuclear response regulators (ARRs), which can activate or repress transcription of downstream genes (Inoue *et al.*, 2001; Ueguchi *et al.*, 2001a,b; Yamada *et al.*, 2001). A recent report provided evidence that an Arabidopsis histidine protein kinase CKI1 is mainly localized to the plasma membrane and expression of the CKI1-GFP was completely abolished by treatment of an N-glycosylation inhibitor, tunicamycin in Arabidopsis protoplasts (Hwang and Sheen, 2001). Five histidine kinase (HK) genes have been identified in the rice genome suggesting that the cytokinin signal is transduced by a phosphotransfer mechanism as in Arabidopsis (Ito and Kurata, 2006). The reduced cytokinin sensitivity in *gnt1* led us to examine if *Oryza sativa* cytokinin receptor histidine kinases (OHKs) are containing potential N-glycosylation sites to be the substrates of GnTI. Amino acid sequence analysis using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc) indicated that five OHKs have potential N-glycosylation sites in their sequences. We hypothesized that if the functions of OHKs were impaired by deficient N-glycan maturation in *gnt1*, expressions of A-type *OsRR*s that are rapidly induced by cytokinins should be decreased in the mutant. Quantitative RT-PCR results showed that treatment with kinetin, 6-benzylaminopurine (BAP), or *trans*-zeatin riboside (ZR)

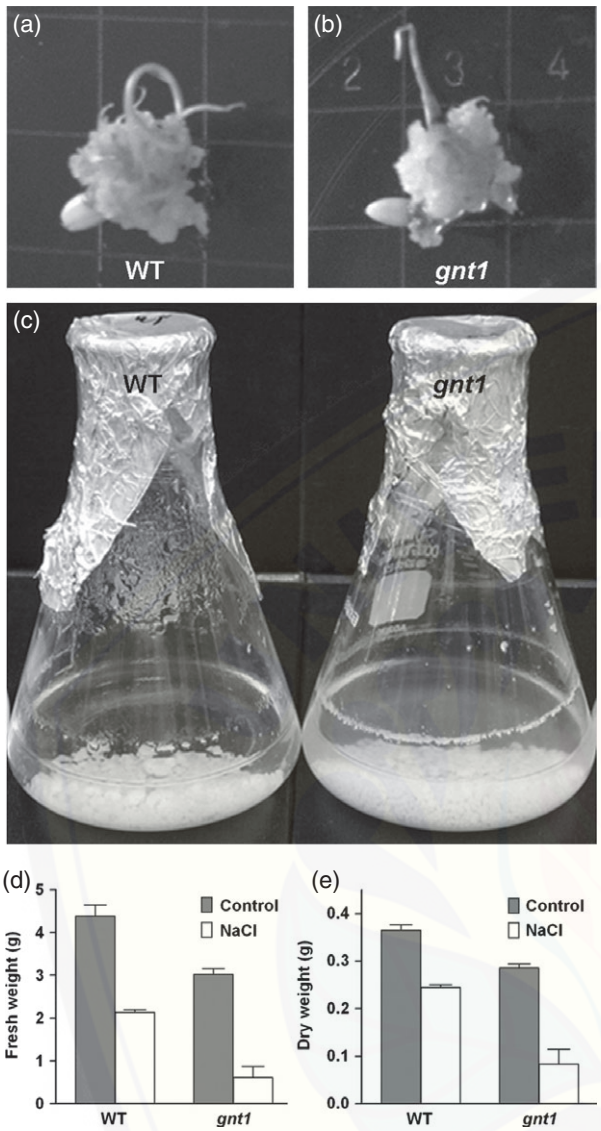

Figure 5. Proliferation rates and suspension culture characteristics of *gnt1* and wild type (WT) callus tissues.
(a), (b) One-month-old embryonic calli induced from WT (a) and *gnt1* (b) seeds.
(c) WT (left) and *gnt1* (right) calli grown in 2N6 liquid medium.
(d), (e) Proliferation of the *gnt1* and WT calli assessed by fresh weight (d) and dry weight (e). Calli were grown for 15 days in 2N6 liquid medium in the absence (grey bar) or presence (white bar) of 100 mM NaCl. Data represent the mean ± SE from three independent experiments.

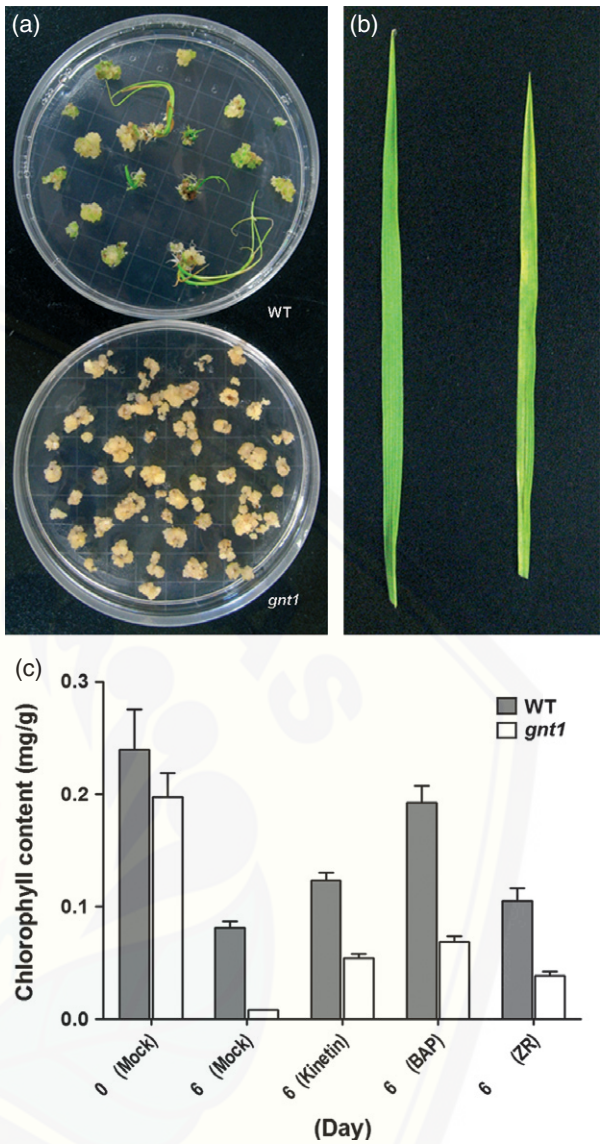

Figure 6. Shoot regeneration and dark-induced leaf senescence assays of *gnt1* and wild type (WT).
(a) Two-month-old WT and *gnt1* calli were placed on a regeneration medium containing NAA (1 μg ml^{-1}) and kinetin (5 μg ml^{-1}) and grown for 1 month.
(b), (c) Fourth leaves of 3-week-old WT and *gnt1* plants were kept in the dark for 6 days in the absence or presence of cytokinins and (c) chlorophyll was extracted and measured as described previously (Chi *et al.*, 2008). Fourth leaves of 3-week-old WT and *gnt1* were mock-treated with 3 mM 2-(*N*-morpholine)-ethanesulfonic acid (pH 5.7) or treated with 0.2 μM kinetin, 0.2 μM 6-benzylaminopurine (BAP), or 0.2 μM *trans*-zeatin riboside (ZR) for 6 days. Data represent the mean ± SE from five independent experiments.

effectively induced the expressions of A-type OsRR genes such as *OsRR2*, *OsRR4*, and *OsRR6* in WT, whereas cytokinin-induced expressions were significantly lower or absent in *gnt1* (Figure 8a). A previous report showed that cell wall biosynthesis of suspension-cultured *Phaseolus vulgaris* cells is stimulated by increased cytokinin (Robertson *et al.*, 1999). *OsCesA4*, *OsCesA7*, *OsCesA9*, and *OsCSLD4* genes were reported to be involved in cell wall biosynthesis of rice (Tanaka *et al.*, 2003; Li *et al.*, 2009). Therefore, the expression of cellulose synthase (*CesA*) and cellulose synthase-like (*CSLD*) genes in WT and *gnt1* plants was analyzed. Quantitative RT-PCR analysis showed that the expression levels of *OsCesA3*, *OsCesA4*, *OsCesA7*, *OsCesA9*, and *OsCSLD4* genes were significantly lower in *gnt1* compared with those in WT in the absence or presence of cytokinins (Figures 8b and 4S). Consistent

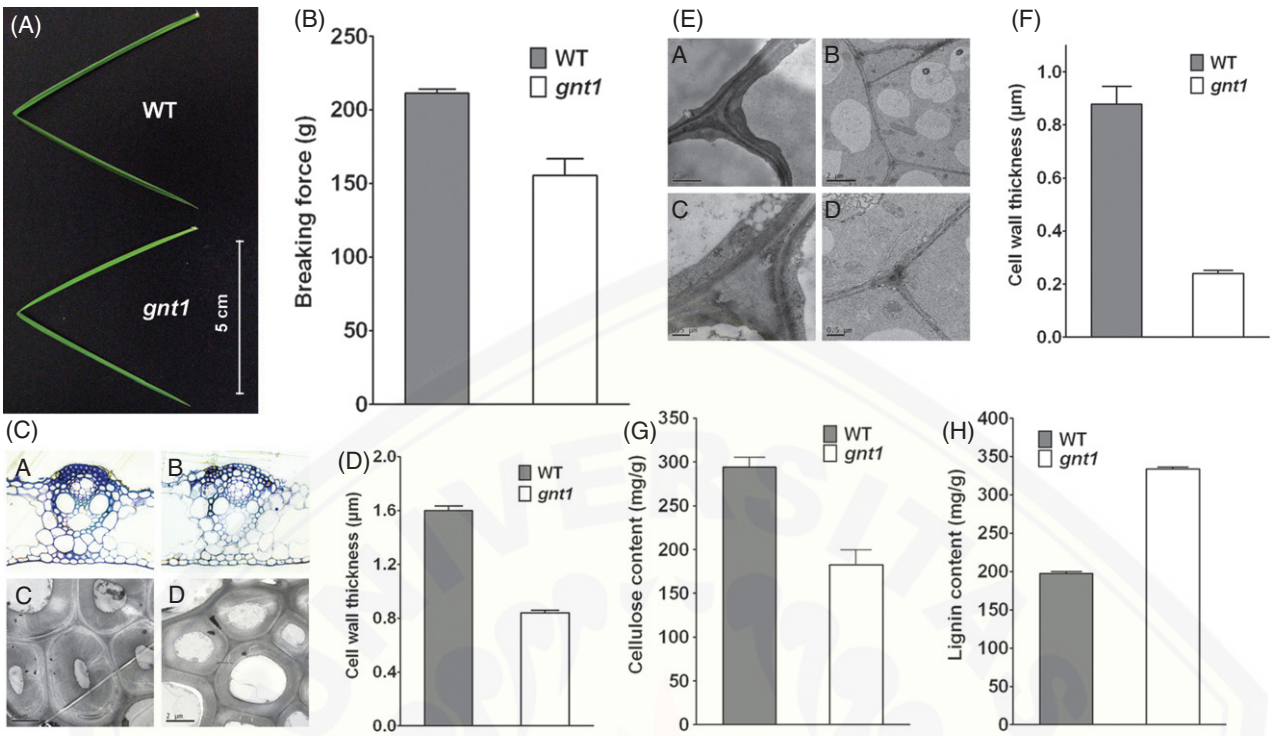

Figure 7. Analyses of mechanical properties and cell walls of *gnt1* and wild type (WT).
(A) Bending analysis of the leaves of *gnt1* and WT.
(B) Breaking force of the leaves of *gnt1* and WT. Data represent the mean ± SE from ten independent experiments.
(C) Cross-sections of the culms from 3-week-old WT (a, c) and *gnt1* (b, d) seedlings. Transmission electron micrographs of the sclerenchyma cells of WT (c) and *gnt1* (d) seedlings.
(D) Cell wall thickness of the sclerenchyma cells of WT and *gnt1* seedlings. Mean ± SE values were calculated from 50 cells per line.
(E) Transmission electron micrographs of the cell walls of WT (a, c) and *gnt1* (b, d) calli with different magnification. Scale bars: 2 μm (Cc,d and Ea,b) and 0.5 μm (Ec,d).
(F) Cell wall thickness of the callus cells. Mean ± SE values were calculated from 50 cells per line.
(G), (H) Cellulose (G) and lignin (H) contents of *gnt1* and WT. Data represent the mean ± SE from six and three independent experiments for cellulose and lignin, respectively.

Table 2 Comparison of cell wall sugar contents of wild type (WT) and *gnt1* seedlings

Cell wall component	WT[a]	*gnt1*[a]	Difference	*P*-value[b]
Arabinose	21.62 ± 1.68	24.88 ± 2.77	3.26	0.361
Galactose	8.67 ± 1.77	13.16 ± 2.44	4.49	0.193
Glucose	234.80 ± 9.85	148.82 ± 10.3	−85.98	0.0009
Xylose	16.10 ± 1.51	12.92 ± 1.53	−3.18	0.191

[a]Cell wall components of WT and *gnt1* seedling shoots are given as means ± SE of four independent assays and calculated as milligrams per gram total cell wall residue.
[b]Determined using Student's two-sample *t*-test.

cytokinin-induced expression was observed for *OsCesA3* and *OsCesA9* but was not evident for *OsCesA4*, *OsCesA7*, and *OsCSLD4* in WT (Figures 8b and 4S).

DISCUSSION

Our findings provide new evidence for the hypothesis of a physiological function in plant development for N-glycan maturation in the Golgi apparatus. A Mendelian segregation ratio of 3:1 (WT/mutant) indicates that the *gnt1* phenotype is caused by a single recessive nuclear mutation. Because additional *gnt1* mutant allele is not available, we attempted to obtain genetically complemented transformants in *gnt1* mutants using GnTI genes driven by the rice thioredoxin *m* or maize ubiquitin 1 promoters. However, we were unsuccessful in obtaining regenerated transformants, possibly because the calli were not competent to produce somatic embryos for regeneration under our experimental conditions, or reduced cell division and differentiation in *gnt1* make the calli less competent or not competent for transformation.

The extent of the severity of phenotypes linked to altered N-glycan structures has been reported in plants. The Arabidopsis mutants *knf-14/gcs1*, *cyt1-1*, and *lew3* with loss-of-function of ER-localized N-glycosylation enzymes α-glucosidase I, mannose-1-phosphate guanylyltransferase, and α1,2-mannosyltransferase (ALG11), respectively, show pleiotropic effects including strongly reduced cellulose

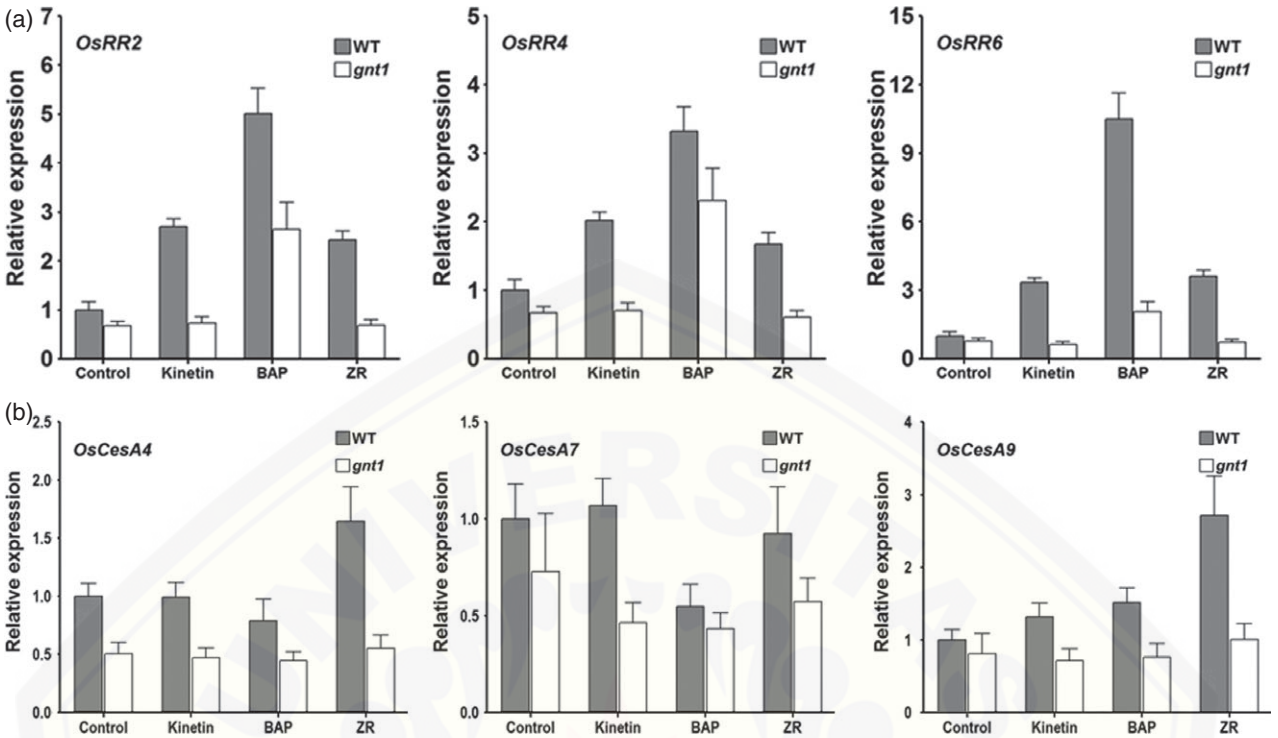

Figure 8. Analyses of the expressions of cytokinin-responsive and cell wall synthesizing genes in *gnt1* and wild type (WT).
(a) Expression of cytokinin-responsive genes and (b) cell wall synthesizing genes in *gnt1* and WT. Three-week-old WT and *gnt1* were treated with 5 μM kinetin, 5 μM 6-benzylaminopurine (BAP), or 10 μM *trans*-zeatin riboside (ZR) for 24 h and the expression levels of A-type *OsRR* (a) and *OsCesA* (b) genes were analyzed by quantitative real-time PCR. Data represent the mean ± SE from three independent experiments.

contents (Boisson *et al.*, 2001; Lukowitz *et al.*, 2001; Gillmor *et al.*, 2002; Zhang *et al.*, 2009). However, the phenotypes and cellulose contents of *cgl1*, and *mns3-1* mutants with defects in Golgi-localized N-glycosylation enzymes are not significantly changed compared with those of WT (Kang *et al.*, 2008; Liebminger *et al.*, 2009). In this study, the *gnt1* mutant exhibited severe developmental defects and significantly reduced expressions of A-type *OsRR*s that are important for cytokinin-mediated cellular responses and development, and of *OsCesA*s and *OsCSLD4*, which are important for cellulose synthesis.

Only a small amount (1.7%) of the mannose-type N-glycan ($Man_6GlcNAc_2$) was observed in WT rice callus under our experimental conditions. Low levels of mannose-type N-glycans have also been reported in the leaves and seeds of rice (Leonard *et al.*, 2004). However, 26.1% of the total N-glycans of WT Arabidopsis leaves was shown to be mannose-type N-glycans, with $Man_5GlcNAc_2$ as the major (10.8%) component and the other 73.9% of N-glycans composed of hybrid, paucimannose, and complex types (Strasser *et al.*, 2004, 2006). These findings may provide insight into the evolutionary differences between monocot and dicot plants in N-glycan processing in the Golgi apparatus.

The fact that the developmental abnormities in *gnt1* appeared at the same time as the amount of complex and high-mannose N-glycans increased in WT indicates that glycoproteins with N-glycans are crucially important in the early development of rice. It is possible that deficiency of N-glycan maturation in *gnt1* disrupts the translocation and function of newly synthesized membrane proteins involved in cell differentiation and development. Unlike in Arabidopsis *cgl1*, our results indicate that GnTI activity is developmentally required at the late seedling stage, especially for secondary tiller formation, in rice plants. It can be speculated that the tillering process, through the initiation and outgrowth of axillary buds, is accompanied by hormonal regulation of cell differentiation. Auxin has been proposed to inhibit the outgrowth of tiller buds in rice by downregulating cytokinin biosynthesis in nodes (Liu *et al.*, 2011). By contrast, cytokinins have been proposed to exhibit stimulatory effects on lateral bud growth, and externally applied cytokinin stimulates tiller bud growth in wheat (Langer *et al.*, 1973). *In vitro* results using *gnt1* callus suggest that GnTI activity is not only required for organ development during differentiation but is also important for the proliferation of callus. In addition, when the *gnt1* callus was grown on a regeneration medium it did not lead to

regeneration (Figure 6). The cytokinin-insensitive phenotypes and lack of induction of A-type *OsRR*s by cytokinin treatment indicate that the developmental abnormalities are markedly associated with impaired cytokinin signaling in *gnt1*. Potential N-glycosylation sites with the motif N–X–S/T are also found in the Arabidopsis cytokinin receptor AHKs, and experimental studies have shown that AHKs contain N-glycosylation sites (Hwang and Sheen, 2001; Caesar *et al.*, 2011); however, the role and relevance of N-glycosylation in their functions remain to be fully unraveled. Taken together, these results suggest that GnTI activity may be involved in cell differentiation and organ development.

At least 11 *OsCesA* genes have been identified in rice and substantial expressions of *OsCesA3–9* in rice seedlings are estimated according to the microarray analysis results in the gAtlas data source (http://signal.salk.edu/RiceGE/RiceGE_gAtlas_Source.html). Analyses of the amino acid sequences of 11 OsCesA proteins (OsCesA1–11) indicate that they contain CesA multidomains including six to eight membrane-spanning regions (absent in OsCesA10) and putative N-glycosylation sites. It is interesting that one of the conserved potential N-glycosylation sites of OsCesA proteins is located near the QxxRW motif, which is essential for the binding of UDP-Glc (Saxena *et al.*, 1995; Tanaka *et al.*, 2003). On the other hand, quantitative RT-PCR analysis revealed that *OsCesA3* and *OsCesA9* exhibit cytokinin-induced expression patterns and that expression of *OsCesA3*, *OsCesA4*, *OsCesA9*, and *OsCSLD4* is significantly reduced in *gnt1* (Figures 8b and S4). However, it is difficult to conclude whether the reduced cell wall thickness in *gnt1* is caused mainly by impaired N-glycosylation of OsCesA proteins and/or reduced transcription of *OsCesA* and *OsCSLD* genes. Further study of GnTI in association with functional regulation of membrane proteins required for cellulose synthesis, differentiation, and development may give us insights into the pleiotropic phenotypes of *gnt1*.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Surface-sterilized rice (*O. sativa* L cv. Nipponbare) seeds were germinated on half-strength Murashige and Skoog (MS) agar medium (Duchefa, http://www.duchefa-biochemie.nl/) with 1% sucrose. The seedlings were grown for 2 weeks in a growth chamber with supplemental lighting (8 h dark/16 h light; 200 μmol m^{-2} sec^{-1}) and a day/night temperature regime of 30/25°C, and then were transferred to a greenhouse (sunlight). Embryo-derived rice callus was induced and maintained as described previously (Kim *et al.*, 2011). *Arabidopsis thaliana* Columbia (Col-0), *cgl1-T*, and complemented *cgl1-T* seeds were sterilized and grown for 5 days on MS medium containing salt mixture (Duchefa), pH 5.8 supplemented with 3% sucrose and 0.25% gellan gum in a growth chamber with supplemental lighting (8 h dark/16 h light; 120 μmol m^{-2} sec^{-1}) at 22°C and 70% humidity.

Genotyping and analysis of flanking sequences

Mutant lines with a T-DNA insertion in *GnTI* were obtained from the T-DNA mutant collections generated at Génoplante (http://www.genoplante.com/). Genomic DNA was extracted from rice leaf and callus tissues using phenol–chloroform. PCR reactions with a combination of specific primers P2 + LB and P1 + P2 were used to verify the insertion site and homozygosity of the T-DNA. The flanking regions of the T-DNA insertion were rescued by PCR using the specific primers P2 + LB and P1 + RB, cloned into the pGEM-T vector (Promega, http://www.promega.com/), and analyzed by sequencing. Primers used in this study are presented in Table S3.

Reverse transcription-PCR and qRT-PCR analyses

Total RNA was extracted from various tissues of rice with the NucleoSpin RNA Plant Kit (Macherey-Nagel, http://www.mn-net.com/) following the manufacturer's instructions. For each tissue, 1 μg of the purified RNA was used for first-strand cDNA synthesis using the RevertAid™ kit (Fermentas, http://www.thermoscientificbio.com/fermentas/) or ReverTraAce-α kit (Toyobo, http://www.toyobo-global.com/) and a poly$(T)_{18}$ primer according to the manufacturer's instructions. One microliter of the first-strand cDNA was used as the template for subsequent PCR. The forward and reverse primers used were P3 and P4, respectively; *Actin1* primers were used as a control for RNA content. The thermal profile used was: one cycle, 94°C for 2 min (denaturation); 28 cycles, 94°C for 15 sec (denaturation), 60°C for 30 sec (annealing), 70°C for 1 min (extension); and one cycle, 70°C for 5 min. Quantitative real-time PCR was performed using the CFX96® Real-Time PCR Detection System (Bio-Rad, http://www.bio-rad.com/) and SsoFast® EvaGreen® Supermix (Bio-Rad) real-time PCR mixture. *OsAct1* was amplified and used as an internal positive control. Primers used in this study are presented in Table S3.

Plasmid construction and Arabidopsis transformation

For complementation analyses, rice *GnTI* cDNA was amplified by RT-PCR from WT using the GnTI-F and GnTI-R primers. The resulting cDNA was cloned into the *Sac*I and *Xba*I sites of a modified pCAMBIA 1380 binary vector (Hajdukiewicz *et al.*, 1994) containing a CaMV 35S promoter. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101, and the resulting strain was used to transform Arabidopsis Col-0 plants by the floral dip method (Clough and Bent, 1998).

DNA gel blot analysis

Ten micrograms of genomic DNA, isolated from rice calli of WT, heterozygous *gnt1*, or homozygous *gnt1*, was digested with *Bam*HI and *Eco*RI. The fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to a nylon membrane (Hypobond-N^+, GE Healthcare, http://www.gelifesciences.com) by capillary transfer using the salt transfer protocol according to the manufacturer's instructions. The DNA was immobilized on the membrane by UV cross-linking at 1200 μJ cm^{-2} and washed in 2 × SSC for 5 min. Then HPT-F and HPT-R were used to generate a T-DNA-specific probe by PCR using the genomic DNA from *gnt1* as a template. The membrane was hybridized with ^{32}P-labeled T-DNA-specific probe and washed under low-stringency conditions. The blot was visualized by autoradiography via exposure to X-ray film in a cassette with an intensifying screen for 24–72 h at –70°C.

Immunoblotting and affinoblot analyses

Plant protein extraction for immunoblot and affinoblot analyses was conducted using 3-week-old *gnt1* and WT seedlings as

described previously (Strasser *et al.*, 2004). Ten micrograms of total protein extracts was resolved in 10% SDS-PAGE gels and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham). For immunoblotting to detect N-glycans with plant-specific sugar residues, the membranes were probed with antibodies against α1,3-fucose, β1,2-xylose (Agrisera), or HRP (Sigma, http://www.sigmaaldrich.com/). Concanavalin A was used for affinoblotting to detect high-mannose N-glycans.

Measurement of cellulose, lignin, and sugars

The aerial parts of 1-month-old rice seedlings were ground to a fine powder in liquid nitrogen using a mortar and pestle. Extraction of cell wall materials was conducted as described previously (Li *et al.*, 2003). The lignin and cellulose contents were determined as described previously (Updegraff, 1969; Kirk and Obst, 1988) and the cell wall sugars were analyzed by high-performance ion chromatography (ICS-2500, Dionex).

N-glycan purification and analysis

Protein for N-glycan purification was extracted from callus as described previously (Bakker *et al.*, 2006). The protein was digested with trypsin and bound N-glycans were released by PNGase A (Roche, http://www.roche.com/). After digestion, the sample was passed through a C18 Sep-Pak cartridge and the carbohydrate fraction was eluted with 5% acetic acid and dried by lyophilization. The carbohydrate fraction was dissolved in dimethylsulfoxide and then permethylated as described previously (Anumula and Taylor, 1992).

Matrix-assisted laser-desorption time-of-flight mass spectrometry

We performed MALDI-TOF MS in the reflector positive ion mode using α-dihydroxybenzoic acid (DHBA; 20 mg ml^{-1} solution in 50% methanol:water) as a matrix. The spectrum was obtained by using a Microflex LRF (Bruker, http://www.bruker.com/).

Specimen preparation and TEM

Sections of 2 mm^2 fresh leaf and callus samples were fixed in a mixture of 2% glutaraldehyde in 100 mM phosphate buffer, pH 7.2. After washing in the same buffer, the samples were post-fixed in 2% OsO_4 in 100 mM phosphate buffer, pH 7.2. The samples were washed with the same buffer, and the specimens were dehydrated in an ethanol series and embedded in Epon. Semi-thin sections were cut with an ultramicrotome (Ultracut S, Leica, http://www.leica.com/) and stained with toluidine blue and basic fuchsin. Observation was performed under a light microscope. Ultrathin sections were obtained with an ultramicrotome and collected on copper grids. The specimens were stained with uranyl acetate and lead citrate and observed via TEM, with a Tecnai G^2 Spirit (FEI, http://www.fei.com/) operated at 120 kV.

Breaking force measurement

The breaking forces for the fourth true leaves of 3-week-old seedlings were measured with a digital texture analyzer according to the manufacturer's instructions (TA.XTExpress, Stable Micro Systems, http://www.stablemicrosystems.com/).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Complementation of Arabidopsis *cgl* by the expression of rice *GnTI*.

Figure S2. Analyses of the expressions of *GnTI* and adjacent genes in *gnt1* and wild type.

Figure S3. The HPLC profiles of the fluorescently labeled N-glycans in *gnt1* and wild type.

Figure S4. Analyses of the expressions of cell wall synthesizing genes in *gnt1* and wild type.

Table S1. Segregation analysis of heterozygous *gnt1* derived progenies on medium containing hygromycin.

Table S2. The HPLC analysis of N-glycans from wild type and *gnt1* calli.

Table S3. Primers used in this study.

Methods S1. The HPLC profiling of N-glycan.

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