Salt tolerance of *Arabidopsis thaliana* requires maturation of *N*-glycosylated proteins in the Golgi apparatus

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Protein *N*-glycosylation in the endoplasmic reticulum (ER) and in the Golgi apparatus is an essential process in eukaryotic cells. Although the *N*-glycosylation pathway in the ER has been shown to regulate protein quality control, salt tolerance, and cellulose biosynthesis in plants, no biological roles have been linked functionally to *N*-glycan modifications that occur in the Golgi apparatus. Herein, we provide evidence that mutants defective in *N*-glycan maturation, such as *complex glycan 1* (*cgl1*), are more salt-sensitive than wild type. Salt stress caused growth inhibition, aberrant root-tip morphology, and callose accumulation in *cgl1*, which were also observed in an ER oligosaccharyltransferase mutant, *staurosporin and temperature sensitive 3a* (*stt3a*). Unlike *stt3a*, *cgl1* did not cause constitutive activation of the unfolded protein response. Instead, aberrant modification of the plasma membrane glycoprotein KORRIGAN 1/RADIALLY SWOLLEN 2 (KOR1/RSW2) that is necessary for cellulose biosynthesis occurred in *cgl1* and *stt3a*. Genetic analyses identified specific interactions among *rsw2*, *stt3a*, and *cgl1* mutations, indicating that the function of KOR1/RSW2 protein depends on complex *N*-glycans. Furthermore, cellulose deficient *rsw1-1* and *rsw2-1* plants were also salt-sensitive. These results establish that plant protein *N*-glycosylation functions beyond protein folding in the ER and is necessary for sufficient cell-wall formation under salt stress.

complex *N*-glycans | endoplasmic reticulum stress | salt stress

Protein *N*-glycosylation is an essential posttranslational modification for eukaryotic cells (1, 2). It occurs predominantly in the luminal space of the endoplasmic reticulum (ER), which is the entry site into the secretory system. *N*-glycosylation is catalyzed by the oligosaccharyltransferase (OST) complex, a multisubunit enzyme (3, 4) that transfers preassembled core oligosaccharides ($Glc_3Man_9GlcNac_2$) to asparagine residues (Asn-X-Ser/Thr) of the nascent polypeptide chain [supporting information (SI) Fig. S1]. Immediately after covalent coupling to the polypeptide chain, the terminal glucose residues of the core-oligosaccharides are removed by ER glucosidases I and II. Trimmed *N*-glycans are recognized by the lectin chaperones calnexin and calreticulin, which are associated, in animal cells, with the cochaperone ERp57 (5). Interference with any of these processes results in the accumulation of misfolded proteins in the ER lumen and triggers the unfolded-protein response (UPR), a major protein quality-control mechanism of the ER. UPR induces expression of genes that promote proper protein folding in the ER, such as calnexin, calreticulin, binding protein (BiP), and peptide disulfide isomerase (6, 7). In mammals, UPR also inhibits protein synthesis (8) and causes cell-cycle arrest at the G_1 phase (9).

Glycosidases and glycosyltransferases in the Golgi apparatus are responsible for the formation of complex (and hybrid) *N*-glycans. Plant complex *N*-glycans are unique because these contain β1,2-xylose and core α1,3-fucose residues that are not found in human *N*-glycans (10, 11). The high-mannose *N*-glycans on glycoproteins exported from the ER are trimmed by α-mannosidase I in the *cis* Golgi. The first step in the formation of complex *N*-glycans in the Golgi apparatus is catalyzed by β1,2-*N*-acetylglucosaminyltransferase I (GnTI) (12), followed by steps comprising α-mannosidase II (13), GnTII (14), β1,2-xylosyltransferase (15), and α1,3-fucosyltransferase (16). Finally, β1,3-galactosyltransferase (17) and α1,4-fucosyltransferase (18) further modify complex *N*-glycans to produce Lewis a epitopes (19).

In plants, defects in the ER *N*-glycosylation pathway are associated with diverse phenotypes. Null mutations in *Arabidopsis* genes encoding mannose-1-phosphate guanylyltransferase (*CYT1*) (20, 21), ER α-glucosidase I (*KNOPF*/*GCSI*) (22, 23), which function in an early stage of the protein *N*-glycosylation pathway, result in lethality. Plants containing weak alleles of these genes, however, are viable and exhibit deficiency in cellulose biosynthesis, similar to mutants that are compromised for cellulose synthase subunit A1/RADIALLY SWOLLEN 1 (CESA1/RSW1) or endo-β1,4-glucanase KORRIGAN 1/RADIALLY SWOLLEN 2 (KOR1/RSW2) proteins (24, 25). On the other hand, dysfunction of *STT3a*, a catalytic subunit of OST, results in accumulation of underglycosylated proteins and promoter activation of the ER chaperon BiP and confers salt/osmotic stress sensitivity (salt sensitivity). The salt-sensitive response of the mutants is associated with mitotic arrest of the root apical meristem and radial swelling of root tips (26) similar to the phenotype of cellulose deficiency induced by genetic mutations (25) or by treatments with cellulose biosynthesis inhibitors (27). In contrast, no physiological phenotype has been reported for the mutants that are defective in the Golgi-localized *N*-glycan modification pathway, namely, GnTI (*complex glycan 1*; *cgl1*), α-mannosidase II (*hybrid glycan 1; hgl1*), β1,3-galactosyltransferase (*galT1*), and a triple mutant lacking func-

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tional core α1,3-fucosyltransferase and β1,2-xylosyltransferase activities (*fucTa fucTb xylT*) (12, 16, 18). Therefore, it has been believed that the primary function of *N*-linked oligosaccharides in plants is mainly to assist protein folding in the ER that occurs before *N*-glycan modifications in the Golgi apparatus.

We have conducted a systematic salt tolerance analysis of protein *N*-glycosylation pathway mutants, from which it is concluded that maturation of complex *N*-glycans is required for adaptation to salt stress in *Arabidopsis*. Initial studies using *cgl1* mutants indicated that an early defect in *N*-glycan maturation in the Golgi apparatus could confer salt sensitivity comparable to that of *stt3a* mutants. Subsequent analyses of individual Golgi glycosidase and glycosyltransferase mutants determined that several *N*-glycan modification steps contribute to salt tolerance. Furthermore, our biochemical and genetic analyses indicate that plasma membrane RSW2/KOR1 protein is a substrate of STT3a and CGL1 and necessary for the tolerance. These results indicate that maturation of *N*-glycans is necessary for plant stress tolerance and that *N*-glycans are essential not only for protein folding but also for *in vivo* functions of plant glycoproteins.

Results

Golgi-Localized *N*-Acetylglucosaminyltransferase I Is a Determinant of Osmotic Stress Tolerance in *Arabidopsis thaliana*. We have shown that mutations in OST subunit isoform STT3a can confer salt sensitivity in *Arabidopsis* (26). Because OST is upstream of the entire *N*-glycan maturation pathway, and *stt3a* mutations likely cause general decrease in *N*-glycosylations, salt sensitivity in *stt3a* may be caused by either loss of specific *N*-glycans or by general protein-folding defects in the ER. To delineate these effects, we have analyzed salt sensitivity of *Arabidopsis cgl1* mutants, which have wild-type levels of *N*-glycosylations but lack proper *N*-glycan maturations because of the defect in Golgi-localized GnTI (12). Two *cgl1* alleles, *cgl1* C5 (refs. 12 and 28 and J.F., Heidi Kaulfürst-Soboll, Stephan Rips, H.K., and A.v.S., unpublished work) and *cgl1-T* (J.F., Heidi Kaulfürst-Soboll, Stephan Rips, H.K., and A.v.S., unpublished work) lacking complex *N*-glycans (Dataset S1) were used in our analysis. Both mutant alleles exhibited substantial salt sensitivity compared with wild type, albeit both *cgl1* alleles were slightly less salt-sensitive than *stt3a-2* (Fig. 1*A*). Similar to *stt3a-2*, *cgl1* alleles were sensitive to osmotic stress imposed by high concentrations of KCl or mannitol but not to ionic toxicity caused by low concentrations of LiCl (Fig. 1*A* and data not shown). This contrasts with the *sos1-1* mutant that is sensitive to the toxicity of Na^+ and Li^+ (29). Notably, aniline blue staining revealed strong induction of callose accumulation in *cgl1* root tips exposed to salt (Fig. 1*C*). Weaker callose fluorescence was observed in *stt3a-2* but not in wild type grown under the same condition. We recently found that the *stt3a-2 cgl1-T* double mutant, with combined qualitative and qualitative *N*-glycan abnormalities, exhibits mild growth retardation under normal conditions (J.F., Heidi Kaulfürst-Soboll, Stephan Rips, H.K., and A.v.S., unpublished work). Interestingly, root growth of the double mutant was strongly inhibited by NaCl at 50 mM or greater (Fig. 1*B*). The strong additive effect is consistent with the predicted function of both *STT3a* and *CGL1* in the same posttranscriptional-modification process. In the double mutant, *stt3a-2* impairs the OST reaction upstream of *cgl1* but an additional isoform, *STT3b* (26), allows the OST reaction to proceed at a decreased level. Together, these results indicate that not only the attachment of core *N*-glycans to proglycoproteins but also the maturation of *N*-glycans in the Golgi apparatus are necessary to maintain root growth under salt stress.

Maturation of Complex *N*-Glycans in the Golgi Apparatus Is Essential for Root Salt Tolerance. Reduced protein *N*-glycosylation caused by the *stt3a* mutation constitutively activates UPR, an ER stress response (26). The *cgl1* mutant defect is confined to *N*-glycan modifications in the Golgi apparatus (ref. 12 and J.F., Heidi Kaulfürst-Soboll, Stephan Rips, H.K., and A.v.S., unpublished work) and predicted to leave the protein folding machinery in the ER intact. Thus, expression of an UPR-marker gene, *BiP-GUS* (30), was evaluated in the two mutants. $Col_{BiP\text{-}GUS}$ *stt3a-2* constitutively exhibited strong GUS expression even without stress (Fig. S2), whereas only basal-level GUS expression was observed in $Col_{BiP\text{-}GUS}$ wild type and $Col_{BiP\text{-}GUS}$ *cgl1* C5 in the

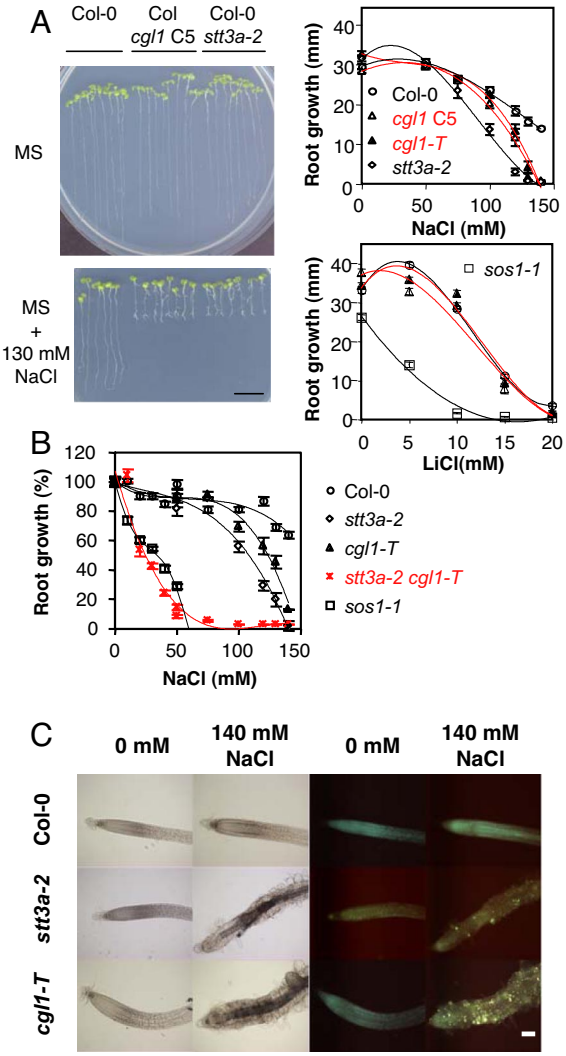

Fig. 1. Mutated alleles of β1,2-*N*-acetylglucosaminyltransferase I (*CGL1*) confer salt sensitivity to *Arabidopsis*. (*A*) (*Left*) Wild-type (Col-0), *cgl1* C5, and *stt3a-2* seedlings that were grown on MS-agar medium for 5 days and then transferred to MS-agar medium without (*Upper*) or with (*Lower*) 130 mM NaCl and grown for an additional 5 days. (Scale bar, 10 mm.) (*Right*) Root growth of *cgl1* seedlings under various concentrations of NaCl and LiCl ($n = 15 \approx 25$). Bars represent standard errors. (*B*) The root growth response of *stt3a-2 cgl1-T* double-mutant plants to NaCl was compared with those of wild type, *stt3a-2*, *cgl1-T*, and *sos1-1* (29) single mutants. Because of temperature sensitivity of the double mutant, the assay was conducted at 18°C, and 7-day-old seedlings were transferred to the test media. Root growth was measured 7 days after transfer. Data are presented as percentage relative to the growth without salts. Bars represent standard errors. (*C*) *N*-glycosylation mutants accumulate callose under salt stress. Five-day-old Col-0 wild-type, *stt3a-2*, *cgl1-T* plants were salt stressed with 140 mM NaCl for 4 days and stained with 50 μg/ml discolorized aniline blue. Yellow-green fluorescent patches of callose deposition were visualized by using a fluorescent microscope equipped with a DAPI filter set. (Scale bar, 0.1 mm.)

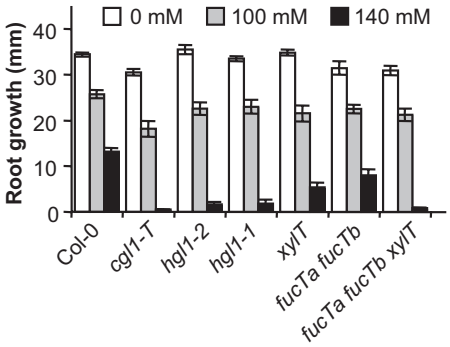

Fig. 2. Golgi *N*-glycan modification mutants are salt-sensitive. Root growth responses of various complex *N*-glycan mutants to salt stress were analyzed as described in Fig. 1. Bars represent standard errors (n = 15–20).

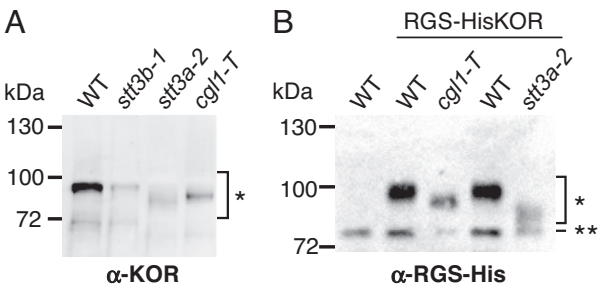

Fig. 3. Altered SDS/PAGE mobility of KOR1/RSW2 proteins in *N*-glycosylation mutants. (*A*) Anti-KOR1 immunoblot. Twenty-five micrograms of buffer-insoluble proteins isolated from wild-type and mutant leaves were separated in an 8% SDS gel, electroblotted to the nitrocellulose filter, and probed with anti-KOR1 antibodies. (*B*) Anti-RGS-His immunoblot. Buffer-insoluble proteins were isolated from plants with or without the introgressed RGS-His-KOR1 gene and analyzed. The bracket marked by a single asterisk indicates KOR1/RSW2 proteins specifically recognized by the antibodies, and the double asterisks mark the position of a nonspecific band.

absence of stress. Upon exposure to salt stress, $Col_{BiP\text{-}GUS}$ *stt3a-2* and $Col_{BiP\text{-}GUS}$ *cgl1* C5 plants exhibited root-growth arrest and swelling of root tips similar to that of the parental mutants. The level of GUS staining in *cgl1* C5 root tips was minimal regardless of the salt treatment, suggesting that UPR was not the primary cause of the salt sensitivity in *cgl1*.

To further verify that salt sensitivity of *cgl1* is caused by a lack of *N*-glycan maturation in the Golgi apparatus, we tested salt tolerance of mutants with defects in other Golgi glycosidases and glycosyltransferases (Fig. 2), i.e., α-mannosidase II (*hgl1-1*, *hgl1-2*) (13), β1,2-xylosyltransferase (*xylT*), and two α1,3-fucosyltransferase isoforms (*fucTa*, *fucTb*) (16) (Fig. 2). Both *hgl1* mutant alleles and the *fucTa fucTb xylT* triple mutant were as salt sensitive as the *cgl1* alleles. In contrast, *xylT* single and *fucTa fucTb* double mutants showed a much weaker to no phenotype. These results show that maturation of complex *N*-glycans is important for salt tolerance and that β1,2-xylose and α1,3-fucose of complex *N*-glycans might have an overlapping function in salt tolerance.

KOR1/RSW2 Protein Is a Target of *N*-Glycosylation and *N*-Glycan Modification. The above results indicate that the salt sensitivity in *Arabidopsis N*-glycan mutants is caused by deficiencies in mature protein-*N*-glycans rather than by the activation of UPR. To identify glycoprotein determinants involved in salt tolerance, we searched for glycoproteins that met the following criteria: (*i*) loss of function mutations cause growth arrest and root morphological changes similar to *stt3a* and *cgl1* and (*ii*) the activity of the protein is affected by its *N*-glycosylation status. A set of mutants, *radially swollen* (*rsw*) *1* to *3* met the first criterion. Temperature-sensitive *rsw* mutants exhibit cellulose deficiency, root radial swelling, and growth arrest at restrictive temperatures. *RSW* genes encode cellulose synthase catalytic subunit (*RSW1*) (25), membrane-bound endo-β1,4-D-glucanase (*KOR1/RSW2*) (24, 31, 32), and ER α-glucosidase II (*RSW3*) (33). Interestingly, RSW3 catalyzes ER *N*-glycan processing at a step between *STT3a* and *CGL1*. Of these, only KOR1/RSW2, which was suggested to function in relieving tensions from cellulose microfibrils (34, 35), met the second criterion. KOR1/RSW2 belongs to the family of class II membrane proteins and contains a luminal catalytic domain with eight potential *N*-glycosylation sites (32). *N*-glycosylation was previously shown to be essential for *in vitro* catalytic activity of a recombinant *Brassica napus* KOR1/RSW2 protein homolog (36). The *in vivo N*-glycosylation state of KOR1/RSW2 in selected *N*-glycosylation mutants was assessed by immunoblot analyses. The analyses were performed by using KOR1 antibodies (32) for detection of endogenous KOR1/RSW2 (Fig. 3*A*) and RGS-His antibodies for detection of $RGS(His)_4$-tagged KOR1/RSW2 proteins (37) (Fig. 3*B*) with similar results. In *cgl1-T* extracts, KOR1/RSW2 proteins migrated faster than in those of the wild type, in accordance with lack of *N*-glycan maturation. In *stt3a-2* plants, KOR1/RSW2 proteins migrated even faster and as a faint smear, indicating a general reduction and heterogeneity of the *N*-glycan attachment pattern. In contrast, the mobility of KOR1/RSW2 in salt-tolerant *stt3b-1* (26) was similar to that in wild type (Fig. 3*A*). These results show that KOR1/RSW2 protein undergoes core *N*-glycosylation in the ER and is subject to *N*-glycan modifications in the Golgi apparatus, i.e., constitutes a substrate of STT3a and CGL1.

***stt3a* and *cgl1* Genetically Interact with *rsw2*.** To determine the biological significance of aberrant KOR1/RSW2 *N*-glycan modification in *stt3a-2* and *cgl1-T*, genetic interactions among *stt3a-2*, *cgl1-T*, and *rsw2-1* were analyzed. *rsw2-1* is a temperature-sensitive allele of *KOR1/RSW2* caused by a missense mutation (24). Combining *rsw2-1* with *stt3a-2* or *cgl1-T* had pronounced effects on plant growth even at the permissive temperature (Fig. 4*A*). Root growth of *rsw2-1 cgl1-T* double-mutant seedlings was strongly inhibited and also associated with increased root branching (Fig. 4*A* and Fig. S3). On the other hand, shoot morphology was relatively normal, albeit shoots exhibited slower growth. In contrast, *rsw2-1 stt3a-2* double-mutant seedlings had a severe dwarf shoot phenotype and produced very short roots (Fig. 4*A* and Fig. S3). Similar symptoms were observed in *rsw2-1* mutants grown at restrictive temperatures (24) or in combination with another temperature-sensitive cellulose deficient mutant, *rsw1-1* (Fig. 4*A*), indicating that *stt3a-2* and *cgl1-T* function as genetic enhancers of *rsw2-1*. Aniline blue staining analysis detected deposition of callose in growth-arrested primary roots of *rsw2-1 cgl1-T* and to lesser extent in *rsw1-1 rsw2-1* and *rsw2-1 stt3a-2* double mutants but not in roots of wild type or the single mutants (Fig. S4). This indicated that *stt3a-2* and *cgl1-T* mutations induced a cell-wall biosynthesis defect, most likely a cellulose biosynthesis defect, in the *rsw2-1* background.

We hypothesized that, if *stt3a-2* and *cgl1-T* indirectly regulated plant cell wall formation in the *rsw2-1* background via *N*-glycans attached to KOR1/RSW2 proteins, the genetic interaction would be specific to *rsw2* alleles and not to other cellulose-deficient mutants, and also, *stt3a* and *cgl1* (with wild-type *RSW2*) would not exhibit strong cellulose deficiency by themselves. Therefore, cellulose content in cell walls (alcohol-insoluble residues) of wild type and *stt3a-2* and *cgl1-T* mutants was determined. Crystalline cellulose contents of wild-type, *stt3a-2*, and *cgl1-T* cell walls were 545.42 ± 12.04, 458.41 ± 43.24, and 509.03 ± 13.07 (nmol

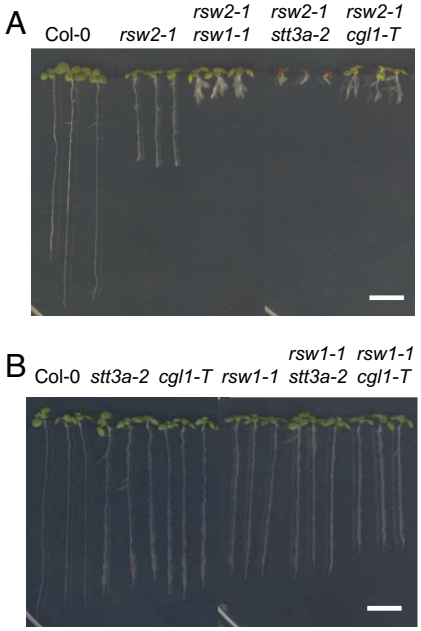

Fig. 4. Genetic interaction among *stt3a-2*, *cgl1-T*, and *rsw* mutations. (*A*) Strong genetic interaction is obvious among *stt3a-2*, *cgl1-T*, and *rsw2-1*. (*B*) Only weak genetic interactions can be observed among *stt3a-2*, *cgl1-T*, and *rsw1-1*. (Scale bars, 5 mm.) Plants were grown on 1× MS salts and 3% sucrose for 11 days at 18°C and photographed.

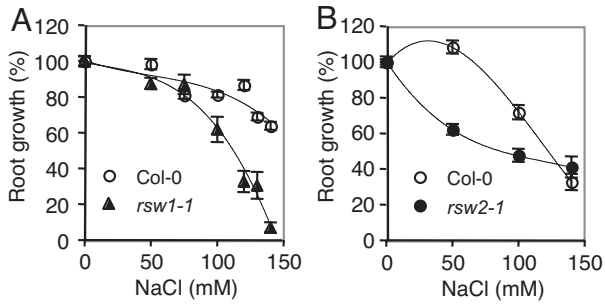

Fig. 5. Cellulose-deficient *rsw1-1* and *rs2-1* are salt-sensitive. (*A*) Salt sensitivity of *rsw1-1* plants. Root growth assays using *rsw1-1* were conducted as described in Fig. 1 at 18°C. Root growth was measured 7 days after transfer. (*B*) Salt sensitivity of *rsw2-1* plants. The seedlings were treated similarly to *A* except that media containing 1/4× MS salts, 0.5% sucrose, and 1.5% agar were used as basal medium. Data are presented as percentage relative to the growth without salts. Bars indicate standard errors.

glucose/mg dry weight). *stt3a-2* and *cgl1-T* showed slightly lower cellulose content than that of wild type (84% and 93% of wild type); however, the observed differences were not highly significant ($P = 0.052$ and 0.044, respectively, by Student's t test).

The specificity of the genetic interaction was confirmed by testing a possible enhancer function of *stt3a-2* and *cgl1-T* in the *rsw1-1* background (Fig. 4*B*). *rsw1-1* encodes a temperature-sensitive variant of cellulose synthase catalytic subunit A1 with a missense mutation (25). Importantly, RSW1 protein seems not to be *N*-glycosylated (22). In contrast to the case of *rsw2-1*, combining *rsw1-1* with *stt3a-2* or *cgl1-T* resulted in only slight growth retardations (Fig. 4*B*). This establishes that enhancer activity of *stt3a-2* and *cgl1-T* is more specific for *rsw2-1* compared with *rsw1-1*. The lack of substantial cellulose deficiencies in cell walls of *stt3a-2* and *cgl1-T* single mutants and the specificity of genetic interaction suggest that the nature of interactions between *rsw2-1* and *stt3a-2* or *cgl1-T* is not a mere additive effect of individual cellulose deficiencies.

Cellulose-Deficient *rsw1* and *rsw2* Are Salt-Sensitive. The results that salt-sensitive *N*-glycan mutants genetically interact with cellulose biosynthesis mutant *rsw2-1* led to the question of whether impaired cellulose synthesis itself could cause hypersensitivity to salt stress. Indeed, at the permissive temperature, root growth of *rsw1-1* single-mutant plants was salt-sensitive (Fig. 5*A*). Similar to *cgl1*, salt-induced growth arrest of *rsw1-1* was associated with radial swelling and only minor activation of *BiP-GUS* expression (data not shown). On the other hand, it was not possible to analyze the salt response of *rsw2-1* mutant seedlings under the same conditions, because *rsw2-1* grew slowly on the control media. This was perhaps because of the slight dehydration stress imposed by the cellophane membrane used in the assay. Growth of *rsw2-1* seedlings improved when the basal medium was adjusted to 1/4× Murashige and Skoog (MS) salts, 0.5% sucrose. Under this condition, *rsw2-1* was more salt-sensitive than wild type at 50–100 mM NaCl (Fig. 5*B*). A higher concentration of NaCl strongly affected the root growth of both wild-type and *rsw2-1* mutant plants, probably because of a higher Na^+ toxicity or insufficient K^+ nutrient that occurred in medium with lower concentrations of K^+ and Ca^{2+}. Together, these results indicate that reduced cellulose biosynthesis causes salt sensitivity.

Discussion

Arabidopsis cgl1 mutants lacking complex *N*-glycans were discovered more than a decade ago, however, associations with biological functions have not been established (12, 13, 16, 18). Here, we provide evidence that complex *N*-glycans are essential for root growth under salt stress in the model plant *Arabidopsis*. Two *cgl1* mutant alleles showed greater sensitivity to high concentrations of various solutes including NaCl, KCl, and mannitol but did not show hypersensitivity to ionic toxicity of osmotically-inert concentrations of LiCl. In addition, mutations in other complex *N*-glycan biosynthesis genes also conferred salt sensitivity to the host plants. The similarity of salt-sensitive phenotype of *cgl1* to that of *stt3a* implies that these genes function in the same pathway. The fact that a mutation in Golgi apparatus-resident CGL1 did not activate the *BiP* promoter confirmed that salt sensitivity was not caused by general protein-folding defects in the ER. Instead, *stt3a-2* and *cgl1-T* exhibited callose deposition in root tips exposed to salt, suggesting that salt stress induces cell wall-biosynthesis defects in *stt3a-2* and *cgl1-T*. This may be a direct cause for the salt sensitivity of *stt3a* and *cgl1-T*, because cellulose-deficient *rsw1-1* and *rsw2-1* plants are also salt-sensitive, and *STT3a* and *CGL1* both affect the maturation of KOR1/RSW2 protein.

Specific genetic interactions between *rsw2-1* and *cgl1-T* or *stt3a-2* indicate that not only the attachment of core *N*-glycans but also maturation of complex *N*-glycans are necessary for full functionality of KOR1/RSW2 protein. The importance of *N*-glycosylation for the function of KOR1/RSW2 protein was first proposed because deglycosylation of a recombinant *Brassica* KOR1/RSW2 homolog expressed in *Pichia pastoris* yeast cells resulted in the loss of its catalytic activity (36). However, because *P. pastoris* produces only high-mannose *N*-glycans, this demonstrates that KOR1/RSW2 protein requires some level of *N*-glycosylation for its catalytic activity. *In planta*, the exact number, modification types, and mode of function of *N*-glycans attached to KOR1/RSW2 have not been determined. Nevertheless, our results show that KOR1/RSW2 is indeed *N*-glycosylated *in planta* and that both *stt3a-2* and *cgl1-T* mutations alter the *N*-glycosylation state of KOR1/RSW2 proteins. Furthermore, these *N*-glycosylation defects specifically enhanced the *rsw2-1* phenotype. A more severe growth defect of *rsw2-1 stt3a-2* than that of *rsw2-1 cgl1-T* is consistent with the severity of salt-sensitive

phenotypes exhibited by *stt3a-2* and *cgl1-T* single mutants. In the *stt3a-2* mutant, which is impaired in the oligosaccharide-transfer reaction, some KOR1/RSW2 proteins may fail to fold and are degraded in the ER, whereas other KOR1/RSW2 proteins are produced with a smaller number of correctly processed *N*-glycans. Some mature KOR1/RSW2 proteins in *stt3a-2* may entirely lack *N*-glycans at essential positions. This may cause greater loss of KOR1/RSW2 activity in *stt3a* than in *cgl1-T*, in which the KOR1/RSW2 defect is confined in maturation of complex *N*-glycans attached to KOR1/RSW2. In support of this hypothesis, it has been shown that recombinant KOR1/RSW2 with unorthodox *N*-glycan maturation in *P. pastris* cells was active, but when deglycosylated, recombinant KOR1/RSW2 lost catalytic activity (36).

Interestingly, Lerouxel *et al.* (38) reported that the *dgl1* mutation, affecting another *Arabidopsis* OST subunit, caused underglycosylation of several proteins but not of KOR1/RSW2. The *dgl1* mutant showed a dwarf phenotype like *kor1* but had normal cellulose contents. This further confirms that even a severe *N*-glycosylation defect (and perhaps UPR) does not cause cellulose deficiency when it does not affect *N*-glycosylation of KOR1/RSW2. It is not clear why mild underglycosylation induced by *stt3a-2* but not severe underglycosylation induced by *dgl1-1* affects KOR1/RSW2. Because electrophoretic mobility of KOR1/RSW2 did not change in the *stt3b-1* mutant extracts (Fig. 3), one possibility is that KOR1/RSW2 is preferentially glycosylated by OST complexes containing STT3a rather than STT3b. Because *dgl1-1* is a leaky allele (38), residual activity of STT3a-containing OST complexes in the *dgl1-1* mutant may result in sufficient *N*-glycosylation of KOR1/RSW2. Similar to this observation, *N*-glycosylation of myrosinase is less in *stt3a* than in *stt3b* (ref. 26 and data not shown). On the other hand, *N*-glycosylation of ER protein disulfide isomerase is less in *dgl1* than in *stt3a* (38). Whether these apparent target specificities are determined by differences in subunit composition of the OST complex or not has yet to be determined.

Biosynthesis of *N*-glycans globally affects the status of membrane proteins and secreted proteins. Considering that *N*-glycosylation constitutes a major posttranslational modification, and a substantial proportion of *N*-glycans are modified to complex *N*-glycans (16), deficiency in complex glycan formation should affect more than just KOR1/RSW2 glycoproteins. Indeed, among the genes that regulate root growth, salt tolerance, and cellulose biosynthesis, the amino acid sequences of COBRA and SOS5 proteins contain multiple *N*-glycosylation sites (39, 40). Although our results demonstrate that root growth and stress tolerance are determined by functional interactions between the *N*-glycosylation pathway and KOR1/RSW2, they do not exclude the possibility that salt sensitivity of *N*-glycosylation mutants is caused by simultaneous failure of several glycoproteins including KOR1/RSW2. Indeed, the short and swollen root phenotype of *cobra-1* appears only when the mutant is grown at elevated sucrose concentration (39), where osmotic signals may play a role in triggering the observed phenotype. The functions of *N*-glycans on these proteins are currently unknown.

If *stt3a* and *cgl1* mutations impair the function of KOR1/RSW2 and possibly also of various other cell wall-regulating proteins, why do *stt3a* and *cgl1* mutants show growth inhibition and cell cycle arrest (26) only under salt stress? First, cellulose contents of unstressed *stt3a* and *cgl1* were similar to wild type under normal growth conditions, and second, callose deposition, a marker of cell-wall defects, was observed only when roots of the mutants were exposed to salt stress. Therefore, it is likely that the cell wall defect is triggered by salt stress rather than that the mutants have weakened cell walls that are sensitive to salt stress. Perhaps in wild type, *N*-glycoproteins important for salt tolerance, for example KOR1/RSW2, exist in excess so that plants can tolerate salt-induced attenuation of *N*-glycoprotein functions. Therefore, *N*-glycosylation defects or salt stress alone does not decrease net KOR1/RSW2 activity below a certain threshold level unless they are combined. Salt stress could negatively regulate KOR1/RSW2 via multiple mechanisms. Salt stress may impair KOR1/RSW2 function by affecting catalytic activity and stability and/or by interfering with trafficking of KOR1/RSW2-containing vesicles. In support of the second possibility, the same genetic screen that identified *stt3a-1* also identified a trans-Golgi apparatus-specific syntaxin mutant (*osm1*), suggesting that salt stress also affects trans-Golgi function (41). Notably, a recent study has shown that KOR1/RSW2 proteins undergo cycling between the plasma membrane and intracellular compartments (42). Clearly, further studies are necessary for delineating individual mechanisms that may affect KOR1/RSW2 activity at the root tip.

In conclusion, we have determined that, after all, *N*-glycans of plant glycoproteins have biological significance beyond the facilitation of protein folding in the ER. Our study also indicates that phenotypes of many *N*-glycosylation and *N*-glycan maturation mutants are conditional. Similar conditional and tissue-specific phenotypes have been reported in *N*-glycan maturation mutants of invertebrates (43–45). Thus, careful genetic and biochemical analyses are required to determine the role of *N*-glycans in a given process. With available *Arabidopsis N*-glycan maturation mutants, such efforts are expected to be fruitful particularly in the area of abiotic and biotic stress responses, because the lack of complex *N*-glycans should result in substantial changes in cell-surface protein profiles and thus affect both intercellular and interorganismal communications.

Materials and Methods

Plant Materials. Salk T-DNA insertion lines *cgl1-T* (Salk_073650), *hgl1–1* (Salk_052443), *hgl1-2* (Salk_141821), *fucTa* (Salk_087481), *fucTb* (SALK_063355), *rsw2-1* (CS6555), *stt3a-2* (Salk_058814), and *xylT* (Salk_042226) were provided by the Arabidopsis Biological Resource Center. *rsw1-1* was a gift of H. Höfte (Institut National de la Recherche Agronomique, Versailles, France). A *BiP-GUS* reporter line (30) was provided by N. Koizumi (Osaka Prefecture University, Osaka). RGSHis-KOR1 transgenic plant was provided by S. R. Turner (University of Manchester, Manchester, U.K.) (37). Mutant combinations were prepared by genetic crosses. Double-mutant individuals were identified by PCR. Point mutations in temperature-sensitive *rsw1-1* and *rsw2-1* lines were identified by dCAPS analyses.

Growth Assay for Osmotic Stress Tolerance. Root-growth assays were performed as described (26, 41). Briefly, *Arabidopsis* seeds were sown onto cellophane membrane placed on MS agar medium (46) [1× MS salts, 30 g/liter sucrose, and 16 g/liter agar (pH 5.7)], stratified for 2 days, and then incubated at 25°C for 1 week. The membranes with seedlings were transferred to basal MS medium supplemented with the indicated concentrations of test compounds, and root growth was scored 5 days later. Earlier experiments used 130 mM NaCl as a standard salt stress condition, which was later adjusted to 140 mM NaCl for more consistent response. For temperature-sensitive mutants, growth assays were carried out at 18°C. In low temperature, plants were grown for 7 days before the transfer and root growth was scored 7 days after the transfer. Plants were photographed with a Nikon Coolpix7200 camera at the end of the assay period, and root lengths were determined by using Image J software (National Institutes of Health, Bethesda).

Callose Staining. For detection of callose, seedlings were immersed in 0.005% discolorized aniline blue in 0.1 M K_2HPO_4 (47) for 5 min and documented by using the Olympus BX51 microscope equipped with a DAPI filter set (Chroma).

Immunoblot Analyses. Anti-cgly immunoblot analyses were conducted essentially as described (12). The detailed procedure for immunodetection of KOR1/RSW2 protein is described in *SI Materials and Methods*.

Cellulose-Content Analysis. The crystalline cellulose analysis was conducted as described with minor modifications (48). The detailed procedure is described in *SI Materials and Methods*.

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