EDITORIAL EXPRESSION OF CONCERN.

PNAS is publishing an Editorial Expression of Concern regarding the following three articles:

(i) PHYSIOLOGY. “NCX-1000, a NO-releasing derivative of ursodeoxycholic acid, selectively delivers NO to the liver and protects against development of portal hypertension,” by Stefano Fiorucci, Elisabetta Antonelli, Olivia Morelli, Andrea Mencarelli, Alessandro Casini, Tommaso Mello, Barbara Palazetti, Dominique Tallet, Piero Del Soldato, and Antonio Morelli, which appeared in issue 15, July 17, 2001, of Proc Natl Acad Sci USA (98:8897–8902; first published July 10, 2001; 10.1073/pnas.151136298);

(ii) PHARMACOLOGY. “NCX-1015, a nitric-oxide derivative of prednisolone, enhances regulatory T cells in the lamina propria and protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice,” by Stefano Fiorucci, Elisabetta Antonelli, Eleonora Distritti, Piero Del Soldato, Roderick J. Flower, Mark J. Paul Clark, Antonio Morelli, Mauro Perretti, and Louis J. Ignarro, which appeared in issue 24, November 26, 2002, of Proc Natl Acad Sci USA (99:15770–15775; first published November 11, 2002; 10.1073/pnas.232583599); and


The editors wish to note that a reader has raised questions about the apparent duplication in the use of certain figures in the foregoing articles. We have been informed by the University of Perugia, Italy, of an ongoing review conducted by an inquiry committee at the university. We are awaiting the findings of the committee to determine the appropriate next steps.

Randy Schekman, Editor-in-Chief

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PLANT BIOLOGY. For the article “Salt tolerance of Arabidopsis thaliana requires maturation of N-glycosylated proteins in the Golgi apparatus,” by Jae Sook Kang, Julia Frank, Chang Ho Kang, Hiroyuki Kajiura, Meenu Vikram, Akhiho Ueda, Sewon Kim, Jeong Dong Bahk, Barbara Triplett, Kazuhiyo Fujiyama, Sang Yeol Lee, Antje von Schaewen, and Hisashi Koiwa, which appeared in issue 15, April 15, 2008, of Proc Natl Acad Sci USA (105:5933–5938; first published April 11, 2008; 10.1073/pnas.0800237105), the authors note that the affiliation for Kazuhiyo Fujiyama should have appeared as Osaka University. The corrected author line, the affiliation line, and a related footnote appear below.

Jae Sook Kang*, Julia Frank†, Chang Ho Kang‡, Hiroyuki Kajiura§, Meenu Vikram¶, Akhiho Ueda†, Sewon Kim*, Jeong Dong Bahk*, Barbara Triplett§, Kazuhiyo Fujiyama‡, Sang Yeol Lee¶, Antje von Schaewen†, and Hisashi Koiwa§

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NEUROSCIENCE. For the article “Organization of the core structure of the postsynaptic density,” by Xiaobing Chen, Christine Winters, Rita Azzam, Xiang Li, James A. Galbraith, Richard D. Leapman, and Thomas S. Reese, which appeared in issue 11, March 18, 2008, of Proc Natl Acad Sci USA (105:4453–4458; first published March 7, 2008; 10.1073/pnas.0800897105), the authors note that on page 4455, right column, in “AMPA-Type Structures,” paragraph 2, line 10, the phrase “from replica labeling (20 ± 4 nm) (28)” should instead read: “from replica labeling (20 ± 4 nm) (48).” The authors also wish to add the following reference citation:


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

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Edited by Maarten J. Chrispeels, University of California at San Diego, La Jolla, CA, and approved February 26, 2008 (received for review January 9, 2008)

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 (cgf1), are more salt-sensitive than wild type. Salt stress caused growth inhibition, aberrant root-tip morphology, and callose accumulation in cgf1, which were also observed in an ER oligosaccharyltransferase mutant, stausporin and temperature sensitive 3a (stt3a). Unlike stt3a, cgf1 did not cause constitutive activation of the unfolded protein response. Instead, aberrant modification of the plasma membrane glycoprotein KORRIGAN 1/RADIIALLY SWOLLEN 2 (KOR1/RSW2) that is necessary for cellulose biosynthesis occurred in cgf1 and stt3a. Genetic analyses identified specific interactions among rsww, stt3a, and cgf1 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.

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-xylene 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), GnTIII (14), β1,2-xyllosyltransferase (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 mannnose-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/RADIIALLY SWOLLEN 1 (CESA1/RSW1) or endo-β1,4-glucanase KORRIGAN 1/RADIIALLY 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; cgf1), α-mannosidase II (hybrid glycan 1; hgl1), β1,3-galactosyltransferase (galTI), and a triple mutant lacking function.


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tional core α1,3-fucosyltransferase and β1,2-xyllosyltransferase activities (fuTa fuTB 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-Acetylgalactosaminyltransferase 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 maturation because of the defect in Golgi-localized GnTI (12). Two cgl1 alleles, cgl1 C5 (refs. 12 and 28 and J.F., Heidi Kaulfurst-Soboll, Stephan Rips, H.K., and A.v.S., unpublished work) and cgl1-T (J.F., Heidi Kaulfurst-Soboll, Stephan Rips, H.K., and A.v.S., unpublished work) lacking complex N-glycans 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. 1A). 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. 1A 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. 1C). 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 quantitative N-glycan abnormalities, exhibits mild growth retardation under normal conditions (J.F., Heidi Kaulfurst-Soboll, Stephan Rips, H.K., and A.v.S., unpublished work). Interestingly, root growth of the double mutant was significantly inhibited by NaCl at 50 mM or greater (Fig. 1B). 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 glycoproteins 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 Kaulfurst-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. ColBiP-GUS stt3a-2 constitutively exhibited strong GUS expression even without stress (Fig. S2), whereas only basal-level GUS expression was observed in ColBiP-GUS wild type and ColBiP-GUS cgl1 C5 in the

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.0800237105)
absence of stress. Upon exposure to salt stress, Col_{BIP-GUS} stt3a-2 and Col_{BIP-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 glycosidoses and glycosyltransferases (Fig. 2, i.e., α-mannosidase II (hgl1-1, hgl1-2) (13), β1,2-xylosyltransferase (xyfT), and two α1,3-fucosyltransferase isoforms (fucTa, fucTb) (16) (Fig. 2). Both hgl1 mutant alleles and the fucTa fucTb xyfT triple mutant were as salt sensitive as the cgl1 alleles. In contrast, xyfT 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-xylene 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) T 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 (RWS1) (25), membrane-bound endo-β1,4-d-glucanase (KOR1/RSW2) (24, 31, 32), and ER α-glucosidase II (RWS3) (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 vivo 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. 3A) and RGS-His antibodies for detection of RGS(His)_5-tagged KOR1/RSW2 proteins (37) (Fig. 3B) 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 stt5b-1 (26) was similar to that in wild type (Fig. 3C). 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 cgl1-1, rsw2-1 genetic interactions among stt3a-2, cgl1-1, 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 cgl1-1 had pronounced effects on plant growth even at the permissive temperature (Fig. 4A). Root growth of rsw2-1 cgl1-1 double-mutant seedlings was strongly inhibited and also associated with increased root branching (Fig. 4A 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. 4A 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. 4A), indicating that stt3a-2 and cgl1-1 function as genetic enhancers of rsw2-1. Aniline blue staining analysis detected deposition of callose in growth-arrested primary roots of rsw2-1 cgl1-1 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 cgl1-1 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 cgl1-1-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
NaCl strongly affected the root growth of both wild-type and rsw2 mutant at 50–100 mM NaCl (Fig. 5). Under this condition, adjusted to 1/4 strength medium, root growth was improved when the basal medium was covered more than a decade ago, however, associations with biological functions have not been established (12, 13, 16, 18). High concentrations of 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 CG11 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

**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. 5A). 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. 5B). 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 Ca2+. 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 CG11 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 Bractica 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 recombiant KOR1/RSW2 with unorthodox N-glycan maturation in P. pastoris cells was active, but when deglycosylated, recombiant 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 myosinase 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), rsw1-2 (C56555), stt3a-2 (Salk.058814), and xyIT (Salk.042226) were provided by the Arabidopsis Biological Resource Center. rswl-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). RSHis-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 rswl-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 membranes 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% discolored aniline blue in 0.1 M K2HPO4 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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