Characterizing the Link between Glycosylation State and Enzymatic Activity of the Endo-β1,4-glucanase KORRIGAN1 from Arabidopsis thaliana*

Received for publication, April 6, 2013, and in revised form, June 12, 2013; Published, JBC Papers in Press, June 19, 2013, DOI 10.1074/jbc.M113.475558

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Background: N-Glycosylation affects the biosynthesis of the endo-β1,4-glucanase KORRIGAN1 which is crucial for cell wall synthesis in plants.
Results: Underglycosylated KORRIGAN1 displays reduced enzymatic activity. This is not observed upon changes to the N-glycan composition.
Conclusion: Modified N-glycan processing exerts no direct effect on KORRIGAN1 function in planta.
Significance: The relevance of several N-glycosylation sites for KORRIGAN1 activity is revealed.

Defects in N-glycosylation and N-glycan processing frequently cause alterations in plant cell wall architecture, including changes in the structure of cellulose, which is the most abundant plant polysaccharide. KORRIGAN1 (KOR1) is a glycoprotein enzyme with an essential function during cellulose biosynthesis in Arabidopsis thaliana. KOR1 is a membrane-anchored endo-β1,4-glucanase and contains eight potential N-glycosylation sites in its extracellular domain. Here, we expressed A. thaliana KOR1 as a soluble, enzymatically active protein in insect cells and analyzed its N-glycosylation state. Structural analysis revealed that all eight potential N-glycosylation sites are utilized. Individual elimination of evolutionarily conserved N-glycosylation sites did not abolish proper KOR1 folding, but mutations of Asn-216, Asn-324, Asn-345, and Asn-567 resulted in considerably lower enzymatic activity. In contrast, production of wild-type KOR1 in the presence of the class I α-mannosidase inhibitor kifunensine, which abolished the conversion of KOR1 N-glycans into complex structures, did not affect the activity of the enzyme. To address N-glycosylation site occupancy and N-glycan composition of KOR1 under more natural conditions, we expressed a chimeric KOR1-Fc-GFP fusion protein in leaves of Nicotiana benthamiana. Although Asn-108 and Asn-133 carried oligomannosidic N-linked oligosaccharides, the six other glycosylation sites were modified with complex N-glycans. Interestingly, the partially functional KOR1 G429R mutant encoded by the A. thaliana rsw2-1 allele displayed only oligomannosidic structures when expressed in N. benthamiana, indicating its retention in the endoplasmic reticulum. In summary, our data indicate that utilization of several N-glycosylation sites is important for KOR1 activity, whereas the structure of the attached N-glycans is not critical.

Glycosylation of proteins is a central co- and post-translational modification of secretory and membrane-bound proteins in all eukaryotes. N-Glycans covalently attached to asparagine residues have a vast number of diverse functions including support of protein folding, quality control processes, protein targeting, and modulation of protein-protein interactions (1). In plants, several N-glycan biosynthesis and processing mutants have been identified with defects in synthesis of cellulose (2–5), which is a major component of the plant cell wall. Consequently, it has been proposed that one or several proteins involved in cellulose biosynthesis are glycosylated, and abolished N-glycosylation or changes of their N-glycan structures could result in altered cellulose content or composition (6). The Arabidopsis thaliana cellulose synthase proteins that form the functional multisubunit cellulose synthase complex contain several potential N-glycosylation sites. However, due to the proposed membrane protein topology, most of these N-glycosylation sites do not face the luminal or extracellular space (7) and are therefore not accessible for the oligosaccharyltransferase that initiates protein glycosylation by transfer of the preassembled oligosaccharide precursor in the endoplasmic reticulum (ER). This hypothesis is also supported by experimental data (3). Among other A. thaliana proteins implicated in cellulose biosynthesis, the membrane-bound endo-β1,4-glucanase KORRIGAN1 (KOR1) is a possible candidate glycoprotein that could be directly affected by changes in N-glycosylation. The precise molecular function of KOR1 and its orthologs from other species during synthesis of cellulose is still unclear (8–10). A. thaliana mutants partially deficient in KOR1 exhibit reduced cellulose content in roots and minor changes in other cell wall polysaccharides as well as cell elongation defects (11–16). Close-to-null mutations of KOR1 display also defects in 1,4-glucanase KORRIGAN1 which is crucial for cell wall synthesis in plants.

* This work was supported by Austria Science Fund Grants P23906-B20 and P20817.
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2 The abbreviations used are: ER, endoplasmic reticulum; LC-ESI-MS, liquid chromatography-electrospray ionization mass spectrometry; ΔXTFT, N. benthamiana glycosylation mutant deficient in β1,2-xylosyltransferase and core α1,3-fucosyltransferase; KOR1, A. thaliana KORRIGAN1; rKOR1, recombinant KOR1; Endo H, endoglycosidase H; PNGase F, peptide N-glycosidase F; KIF, kifunensine.
cytokinesis and severe morphological abnormalities (17). KOR1 is a type II membrane protein with eight predicted N-glycosylation sites in its extracellular domain (11), and most of these N-glycosylation sites are conserved in different KOR1 orthologs from other species (18, 19). The catalytic domain of Brassica napus Cel16, which shares 94% sequence identity with A. thaliana KOR1 was found to be heavily glycosylated when expressed in Pichia pastoris (20). Moreover, deglycosylation with endoglycosidases almost completely abolished the endoglu- conce activity of Cel16, indicating that N-glycosylation might play a crucial role for KOR1 enzymatic activity. Similarly, the enzymatic activity of recombinant PttCel9A, a KOR1 homolo- from Populus tremula x tremuloides, was reduced by 80% when subjected to deglycosylation (18). Altered mobility upon SDS-PAGE separation and reduced accumulation of endoge- nous KOR1 in different A. thaliana glycosylation mutants fur- nally, it has been proposed that KOR1 requires complex N-glycans for its in vivo function in plants (21, 22). These findings are also corroborated by genetic evidence showing drastically enhanced root growth phenotypes of the weak KOR1 mutants were generated using the following primers: N216Q-F, CTTTCTCAAGACTTTTCCAAAGTACTGCTGATTTCC and N216Q-R, GGAATCAGCAGTACTTTGGAAAGTCTTGA- AAGG; N324Q-F, TCTAGCAACTTCTATCATGCAATG- ATGTATTGG and N324Q-R, CCAATACATCTTGAAGC- ATAGAAGCTTGATAGA; N345Q-F, TATATTGCTAGGG- ACAAGTAACTGTATCTCATT and N345Q-R, ATTTGAT- AGCTGTATCGCTTCGCTAGCTAAATA; N408Q-F, GACT- ATGCTGTATGTTGGAAGGTCTTTTAG and N408Q-R, CTAAGGACCTTTCCACAAACACAGCCCACTAG; N425Q- F, GCCATTTCATCAAAATTTCAAGAAACAATGAGGG- TTTAA and N425Q-R, TTAAACCCTCATTGTTTGA- AATTTGTGAAATAGGC; and N567Q-F, TGTCCGATAG- AACCTACAAACTGAAACCAGCT and N567Q-R, GAGG- TCGGTCAGTGTAATTGAGTTCATACGGCA. The single mutant N324Q was used as template to generate the double mutant N324Q/N345Q, and this double mutant was subsequently used as template to obtain the triple mutant N216Q/N324Q/ N345Q. To confirm the introduced mutations, all constructs were subjected to DNA sequencing using the following primers: for N216Q and N216Q/N324Q/N345Q primer, KOR1–3F (TTTTGGTGGAATGGTTTTGATGATG); for N345Q, N345Q, and N324Q/ N345Q primer, KOR1–14F (TGGACTATAAAAGGCCGTGA- CTAAGGACCTTTCCACAAACACAGCCCACTAG; N425Q- F, GCCATTTCATCAAAATTTCAAGAAACAATGAGGG- TTTAA and N425Q-R, TTAAACCCTCATTGTTTGA- AATTTGTGAAATAGGC; and N567Q-F, TGTCCGATAG- AACCTACAAACTGAAACCAGCT and N567Q-R, GAGG- TCGGTCAGTGTAATTGAGTTCATACGGCA. The single mutant N324Q was used as template to generate the double mutant N324Q/N345Q, and this double mutant was subsequently used as template to obtain the triple mutant N216Q/N324Q/ N345Q. To confirm the introduced mutations, all constructs were subjected to DNA sequencing using the following primers: for N216Q and N216Q/N324Q/N345Q primer, KOR1–3F (TTTTGGTGGAATGGTTTTGATGATG); for N345Q, N345Q, and N324Q/ N345Q primer, KOR1–14F (TTTTGGTGGAATGGTTTTGATGATG); for N345Q, N324Q, and N567Q, primer KOR1–12F (TTTTGGTGGAATGGTTTTGATGATG); for N345Q, N324Q, and N567Q, primer KOR1–14F (TTTTGGTGGAATGGTTTTGATGATG); for N345Q, N324Q, and N567Q, primer KOR1–12F (TTTTGGTGGAATGGTTTTGATGATG). Plant Materials—A. thaliana ecotype Col-0 was used as a wild-type control. The rsw2-1 seeds (14) were obtained from the European Arabidopsis Stock Centre, and the fut11 fut12 double knock-out line was described previously (27). All plants were grown under long day conditions at 22 °C. Nicotiana benthamiana plants were grown under long day conditions at 24 °C. Protein extraction and immunoblotting was carried out as described previously (23). Transient Expression of KOR1 in N. benthamiana—The KOR1 coding region was amplified using the following primers: KOR1–4F, TATATTGCTGAAAGTACGACCATCATTG- GAGTGGTTCTGTTGGGAGGTCCTTAG and N567Q-R, GAGG- TCGGTCAGTGTAATTGAGTTCATACGGCA; and for the PCR fragment was subcloned using a Zero Blunt® TOPO PCR cloning kit (Invitrogen). Subse- quently, KOR1 was excised and ligated into the Xbal/BamHI digested binary expression vector p29-Fc-GFP. p29 is derived from p27 (28) and contains an enhanced CaMV 35S promoter derived from vector pVKH18En6 (29) and the Fc–GFP domains for purification and subcellular localization (30). Mutated ver-
sions of KOR1 were generated using site-directed mutagenesis as described above. Transient expression in N. benthamiana wild-type and ΔXTFT lines (31) was done by infiltration of leaves with agrobacteria containing the binary expression vectors as described previously (30). Infiltrations were carried out with agrobacteria diluted to an A_{600} of 0.2, and infiltrated leaf material was harvested 48–72 h post infiltration. KOR1 was purified from 800 mg of infiltrated leaves by incubation with rProtein A-Sepharose (GE Healthcare) as described previously (30). To generate KOR1\_SW2-1\_Fc-GFP, the respective KOR1 DNA fragment was amplified as described above from cDNA derived from A. thaliana rsw2-1 plants. The presence of the mutation that leads to the G429R change (14, 15) was confirmed by DNA sequencing.

**LC-ESI-MS Analysis of Tryptic Glycopeptides**—To analyze the N-glycans of purified recombinant KOR1, proteins were separated by SDS-PAGE (10%) under reducing conditions and detected with Coomassie Brilliant Blue staining. The corresponding band was excised from the gel, destained, carboxymethylated, in-gel digested with trypsin/chymotrypsin, and analyzed by LC-ESI-MS as described recently (32, 33).

**Enzymatic Deglycosylation and Immunoblot Analysis**—Purified KOR1 or total protein extracts were subjected to enzymatic deglycosylation as described in detail recently (34). Deglycosylated proteins were then either analyzed by LC-ESI-MS or were subjected to SDS-PAGE (10%) followed by immunoblot analysis with anti-GFP-HRP (Milenyi Biotech), anti-human IgG (Promega), or anti-KOR1 antibody (raised in rabbits against the synthetic peptide CGEDEATGKIDKNT; Genscript). The detection was performed with Super Signal West Pico chemiluminescent substrate (Pierce).

**KOR1 Activity Assays**—Solutions (10 μl) of purified recombinant KOR1 (rKOR1) produced in insect cells (0.35–4 μg) in 50 mM MES (pH 6.0), 250 mM NaCl, and 30 mM CaCl_2 were incubated for 60 min at 30 °C with 90 μl of 0.1% (carboxymethyl)-cellulose 4M (Megazyme) resuspended in the same buffer. The 50 mM MES (pH 6.0), 250 mM NaCl, and 30 mM CaCl_2 were incubated for 30 to 240 min at 30 °C prior to activity tests. Statistical analyses were performed using Student’s t test, with p < 0.05 considered significant.

**RESULTS**

**KOR1 Expressed in Insect Cells Is Heavily Glycosylated**—The extracellular domain of A. thaliana KOR1 contains eight potential N-glycosylation sites (Asn-X-Ser/Thr, X can be any amino acid except proline). To analyze the role of N-glycosylation for KOR1 enzymatic activity we expressed an A. thaliana KOR1 variant lacking the N-terminal 90 amino acids (which include the cytoplasmic tail and the single transmembrane domain) in S. frugiperda Sf21 cells using a baculovirus expression system. Immunoblot analysis of cell extracts and culture supernatants revealed that recombinant wild-type KOR1 (rKOR1 WT) was successfully expressed in Sf21 cells and secreted into the culture medium. The secreted His_{6}-tagged rKOR1 protein was purified by means of nickel-chelate affinity chromatography and subjected to enzymatic deglycosylation with Endo H, which cleaves oligomannosidic N-glycans, and PNGase F, which cleaves oligomannosidic and complex N-glycans lacking core α1,3-fucose (35), followed by immunoblotting with anti-KOR1 antibody. Both deglycosylation reactions led to distinct mobility shifts of rKOR1 WT, indicating that it is decorated with oligomannosidic and processed complex or paucimannosidic N-glycans (Fig. 1). To gain a more detailed insight into the glycan structures present on each N-glycosylation site of rKOR1 WT, glycopeptides derived from proteolytic digestion were analyzed by LC-ESI-MS. Mass spectrometry revealed that all eight N-glycosylation sites are occupied with N-glycans. Except for the peptide with glycosylation site Asn-567, which harbored mainly oligomannosidic N-glycans (in particular Man_{5}GlcNAc_{2}), the predominant structures present on the other seven N-glycosylation sites were Golgi-processed paucimannosidic N-glycans (Man_{4}GlcNAc_{2} and Man_{4}FucGlcNAc_{2}). Only minor peaks with masses that match to oligomannosidic N-glycans were found (Fig. 2).

**KOR1 Single N-Glycosylation Site Mutants Are Functionally Expressed in Insect Cells**—To examine the impact of individual N-glycans on rKOR1 expression, stability, and activity, selected glycosylation sites were eliminated by site-directed mutagenesis where asparagine was changed to glutamine. N-Glycosylation sites Asn-216, Asn-324, and Asn-345 were chosen due to their proximity to the proposed catalytic cleft of KOR1 (18). In addition, glycosylation sites Asn-408, Asn-425, and Asn-567 were mutated because they are located in the catalytic domain of KOR1 and mostly also present in KOR1 orthologs from other species. The first two sites (Asn-108 and Asn-133) that are close to the transmembrane domain of native A. thaliana KOR1 were not mutated because they are not evolutionarily conserved (18–20). To further investigate the role of glycosylation for KOR1 expression and stability, we generated a double
mutant (N324Q/N345Q). All of the single N-glycosylation mutants were efficiently expressed and could be purified from the culture medium. However, the level of secretion was lower for rKOR1 N567Q than for the other single mutants (Fig. 3A), although the rKOR1 content of the respective cell extracts was similar (data not shown). This suggests that absence of the N-glycan attached to Asn-567 interferes with proper folding of the enzyme in the endoplasmic reticulum of the insect cells in a subtle manner. In contrast, the amount of secreted rKOR1 N324Q/N345Q was strongly reduced as compared with wild-type and rKOR1 N345Q (Fig. 3B and C), indicating that folding of the double mutant was heavily impaired. LC-ESI-MS analysis of purified rKOR1 forms confirmed that all single-mutant proteins lacked only the N-glycan usually attached to the mutated asparagine residue (data not shown).

**Removal of Individual N-Glycosylation Sites Affects KOR1 Activity**—We chose to assay enzymatic activity of rKOR1 WT and the mutant variants using carboxymethylcellulose ((carboxymethyl)cellulose 4M) as substrate and a reducing sugar assay to monitor the released glucose. In previous studies, (carboxymethyl)cellulose 4M was proven to be the best substrate, when KOR1 orthologs were analyzed with different known cellulose derivatives (18, 20). rKOR1 WT and the mutated variants hydrolyzed (carboxymethyl)cellulose 4M, albeit to a varying extent. rKOR1 N408Q and rKOR1 N425Q displayed similar hydrolysis rates as rKOR1 WT, indicating that these two N-glycosylation sites are not critical for maximal enzymatic activity. Mutations at glycosylation sites Asn-216, Asn-324, and Asn-345 reduced KOR1 enzymatic activity by 15–30%. Removal of the oligomannosidic N-glycan on glycosylation site Asn-567 resulted in the most pronounced difference with 50% of wild-type activity (Table 1).

To ensure that the effect on KOR1 activity is not a matter of protein instability due to the loss of individual N-glycans, rKOR1 WT and mutated forms were incubated at 30 °C over a time range from 30 to 240 min prior to activity assays. rKOR1 WT and all single mutants except for N425Q retained at least 90% of their enzymatic activity after preincubation for 120 min (data not shown). In contrast, the activity of rKOR1 N425Q was found to be reduced to 69% (120 min) or 44% (240 min). The sensitivity of the activity assay was tested using varying amounts of purified proteins. At least 350 ng of recombinant protein per assay were needed to obtain reliable results. The hydrolysis rate of (carboxymethyl)-
KORRIGAN1 N-Glycans

![Image](https://example.com/image.png)

**FIGURE 3.** Comparison of rKOR1 WT and N-glycosylation mutant expression in insect cells. A, the indicated recombinant KOR1 variants were expressed in insect cells using a baculovirus expression system and purified from cell supernatants by means of affinity chromatography. Equal volumes were separated by SDS-PAGE and analyzed by immunoblotting. B, comparison of expression levels in cell extracts (cells) and cell supernatants (medium) of rKOR1 N345Q and the double mutant rKOR1 N324Q/N345Q. Extracts of uninfected cells were included as negative control, and supernatant expressing rKOR1 WT was included as positive control. C, comparison of rKOR1 WT and rKOR1 N324Q/N345Q expression levels in cell supernatants. 10 μl of cell supernatant from cells expressing rKOR1 WT and rKOR1 N345Q/N345Q were separated by SDS-PAGE and analyzed by immunoblotting with anti-KOR1 antibodies. Kif, kifunensine.

**TABLE 1**

Enzymatic activity of rKOR1 WT and mutated rKOR1 forms using (carboxymethyl)cellulose 4M as substrate

| rKOR1 protein | Enzyme activity (nmol min⁻¹ mg⁻¹) |
|---------------|---------------------------------|
| rKOR1 WT      | 374 ± 18                        |
| rN216Q        | 244 ± 25*                      |
| rN324Q        | 307 ± 8*                       |
| rN345Q        | 371 ± 25*                      |
| rN408Q        | 379 ± 18                       |
| rN425Q        | 394 ± 34                       |
| rN567Q        | 200 ± 15*                      |
| rKOR1 KIF     | 402 ± 40                       |

* p < 0.05 as compared to rKOR1 WT.

**FIGURE 4.** rKOR1 WT and single N-glycosylation mutants display reduced enzymatic activity upon deglycosylation with Endo H and PNGase F. A, rKOR1 variants were expressed in insect cells, purified, and subjected to enzymatic deglycosylation with Endo H and PNGase F under non-denaturing conditions followed by immunoblotting with anti-KOR1 antibodies. B, digested and non-digested samples were subjected to activity assays with (carboxymethyl)cellulose 4M as substrate. Data are expressed as mean ± S.D. of three independent experiments and presented as percentage of the specific activity of untreated rKOR1 WT (control). It was not possible to perform these assays with the rKOR1 N567Q mutant due to insufficient amounts of purified enzyme.

Enzymatic Deglycosylation Results in Reduced KOR1 Activity—Previously, it was shown that enzymatic deglycosylation of the heterologously produced B. napus and P. tremula x tremuloides KOR1 orthologs caused an almost complete loss of enzymatic activity (18, 20). To test the impact of deglycosylation on the activity of the A. thaliana enzyme, rKOR1 WT and mutant forms were treated with PNGase F and Endo H under non-denaturing conditions. The extent of deglycosylation was monitored by immunoblotting with anti-KOR1 antibodies. For all analyzed rKOR1 mutants, a reduction in molecular mass was observed (Fig. 4A). In accordance with data for rKOR1 WT, the shift in mobility was more pronounced after treatment with PNGase F. The slight shift of rKOR1 N567Q in the Endo H-digested sample results very likely from removal of minor amounts of oligomannosidic N-glycans present on the other seven N-glycosylation sites.

Upon deglycosylation with PNGase F, rKOR1 displayed ~50% residual enzymatic activity (Fig. 4B), whereas Endo H treatment of rKOR1 resulted in a loss of 20–30% compared...
KORRIGAN1 N-Glycans

FIGURE 5. Recombinant KOR1 with predominantly oligomannosidic N-glycans is stably expressed in insect cells and fully active. rKOR1 WT was expressed in insect cells in the absence or presence of kifunensine (KIF), purified, and subjected to PNGase F or Endo H treatment. A, immunoblot analysis with anti-KOR1 antibodies after enzymatic deglycosylation under denaturing conditions. B, LC-ESI-MS analysis of rKOR1 WT expressed in the presence of kifunensine (rKOR1 KIF). The glycosylation pattern of the peptide containing Asn-425 is depicted. The N-glycosylation site is shown in bold and underlined. MM, Man$_5$GlcNAc$_2$; Man$_6$, Man$_7$GlcNAc$_2$; Man$_7$, Man$_8$GlcNAc$_2$; Man$_8$, Man$_9$GlcNAc$_2$; Man$_9$, Man$_9$GlcNAc$_2$. C, activity assays were performed using (carboxymethyl)cellulose 4M as substrate after enzymatic deglycosylation under non-denaturing conditions. Data are expressed as mean ± S.D. of three independent experiments and presented as percentage of the specific activity of untreated rKOR1 WT (control).

FIGURE 6. KOR1-Fc-GFP is N-glycosylated. KOR1-Fc-GFP was transiently expressed in N. benthamiana wild-type or ΔXTFT plants, purified, and digested with Endo H or PNGase F and then subjected to immunoblot analysis with anti-human IgG or anti-KOR1 antibodies. Deglycosylation experiments with purified KOR1-Fc-GFP were performed under denaturing conditions.

with the initial enzymatic activity (Fig. 4B). Interestingly, the specific activity of the single mutants N216Q, N324Q, N345Q, N408Q, and N425Q after deglycosylation with PNGase F was at least as high as that of deglycosylated rKOR1 WT. The activities of glycosylation mutants treated with Endo H were comparable with that of Endo H-digested rKOR1 WT, being reduced by 20–30% as compared with the undigested forms.

To analyze the significance of the observed N-glycosylation profile for rKOR1 activity, we expressed rKOR1 WT in insect cells in the presence of kifunensine (rKOR1 KIF), a class I α-mannosidase inhibitor that prevents processing of oligomannosidic glycans in the endoplasmic reticulum. As a consequence of kifunensine treatment, all eight N-glycans on rKOR1 should contain Man$_5$–9GlcNAc$_2$ oligosaccharides instead of the paucimannosidic structures detected on the wild-type enzyme. Immunoblot analysis and subsequent affinity purification showed that rKOR1 KIF was present in the culture supernatant in amounts comparable with untreated rKOR1 WT (Fig. 3A). The reduced mobility on SDS-PAGE indicated that rKOR1 KIF indeed contains largely unprocessed oligomannosidic N-glycans (Fig. 5A). LC-ESI-MS analysis of glycopeptides from rKOR1 KIF confirmed the presence of predominantly oligomannosidic N-glycans (Man$_n$GlcNAc$_2$ to Man$_n$GlcNAc$_n$) (Fig. 5B and data not shown). Notably, the enzymatic activity of rKOR1 KIF was indistinguishable from rKOR1 WT (Table 1), indicating that processing of N-glycans from oligomannosidic to paucimannosidic structures is not required for KOR1 activity. Interestingly, the activity of rKOR1 KIF was more sensitive to Endo H than that of the wild-type enzyme. As expected, this was not observed upon treatment with PNGase F (Fig. 5C). Collectively, these data suggest that N-glycosylation is important for KOR1 activity, but a distinct N-glycan structure is not required for its function.

KOR1 Expressed in N. benthamiana Carries Complex and Oligomannosidic N-Glycans—Next, we attempted to analyze the glycosylation pattern of KOR1 expressed in plants. To this end, we transiently expressed the full-length KOR1 protein, including its cytoplasmic region and single transmembrane domain in N. benthamiana leaf epidermal cells. To facilitate purification and subcellular localization, the Fc domain of the human IgG1 heavy chain and GFP were fused to the C-terminal end of KOR1. To distinguish between complex and oligomannosidic N-glycans, KOR1-Fc-GFP was expressed in N. benthamiana wild-type and ΔXTFT lines (31) prior to treatment with Endo H and PNGase F and analysis by immunoblotting with anti-KOR1 antibodies. The complex N-glycans produced by the ΔXTFT line are almost completely devoid of core α1,3-fucosic residues and thus sensitive to digestion by PNGase F. KOR1-Fc-GFP produced in these plants was sensitive to Endo H as well as PNGase F. However, the digested polypeptides displayed different migration behavior indicative of the simultaneous presence of oligomannosidic and complex N-glycans (Fig. 6). On immunoblots, intense 130-kDa bands as well as faint bands with a molecular mass of ~160 kDa were detected. Immunoblot analysis with anti-GFP antibody revealed that the upper band represents the full-length KOR1-Fc-GFP protein (data not shown), whereas the lower band corresponds to KOR1-Fc lacking the fluorescent protein tag. The glycan structures of KOR1 from the more abundant lower band were analyzed by mass spectrometry. In accordance with data from the insect cell-derived rKOR1, all eight N-glycosylation sites of the extracellular domain were occupied with glycans. N-Glycosylation sites Asn-108 and Asn-133 carried mainly oligomannosidic N-glycans, whereas all other sites showed processed complex N-glycans with GlcNAc$_2$, Man$_n$, GlcNAc$_2$ (GnGnXF) as predominant glycoform (Fig. 7).
To determine whether individual N-glycans contribute to expression and subcellular targeting of KOR1 in plants, we introduced all N-glycosylation site mutations described above also into KOR1-Fc-GFP. Furthermore, a triple mutant (N216Q/N324Q/N345Q) was generated, which lacks all N-glycosylation sites in the potential catalytic cleft of KOR1. All detectable glycopeptides derived from the mutant variants displayed a similar glycosylation profile as observed for wild-type KOR1 (Fig. 8, A and B). These findings demonstrate that the N-glycans of the wild-type enzyme and the single, double, and triple mutants tested are all properly processed in the plant Golgi. However, enzymatic analysis of wild-type and mutant KOR1-Fc-GFP could not be performed due to insufficient amounts of purified protein.

**KORRIGAN1 N-Glycans**

![Figure 7](image-url)

**FIGURE 7.** KOR1-Fc-GFP is glycosylated with oligomannosidic and complex N-glycans when transiently expressed in N. benthamiana wild-type plants. LC-ESI-MS analyses of all eight KOR1 glycopeptides. N-glycosylation sites are shown in **bold** and **underlined**. The **asterisks** denote the presence of ammonium adducts. GnGnXF, GlcNAcXyIFucMan3GlcNAc2; GnMXF, GlcNAcXylFucMan3GlcNAc2; Man5, Man5GlcNAc2; Man6, Man6GlcNAc2; Man7, Man7GlcNAc2; Man8, Man8GlcNAc2; Man9, Man9GlcNAc2; N708, Asn-108; N133, Asn-133; N216, Asn-216; N324, Asn-324; N345, Asn-345; N408, Asn-408; N425, Asn-425; N567, Asn-567.

Endogenous A. thaliana KOR1 Is Glycosylated with Oligomannosidic and Complex N-Glycans—Expression of KOR1-Fc-GFP in N. benthamiana showed the presence of oligomannosidic and complex N-glycans. To investigate the glycosylation of endogenous A. thaliana KOR1, proteins extracted from A. thaliana Col-0 seedlings as well as from the fut11 fut12 mutant (27), which generates complex N-glycans lacking core α1,3-fucose were incubated in the presence or absence of Endo H and PNGase F and then subjected to immunoblotting with anti-KOR1 antibodies. In both genetic backgrounds, small shifts in mobility were observed after Endo H treatment indicating the presence of oligomannosidic N-glycans. In the fut11 fut12 line, PNGase F digestion resulted in a mobility shift that was more pronounced than in wild-type plants (Fig. 9), indicating that most of the N-glycosylation sites of endogenous KOR1 are decorated with complex N-glycans carrying core α1,3-fucose residues. In summary, these data are fully in accordance with the N-glycan structures detected on KOR1-Fc-GFP transiently expressed in N. benthamiana.

KOR1 G429R Contains only Oligomannosidic N-Glycans—The phenotype of the rsw2-1 mutant is caused by a point mutation in the KOR1 gene leading to an amino acid change from glycine to arginine (KOR1 G429R) (14). The described additive growth phenotype of the cgl1 rsw2-1 double mutant suggests that the N-glycans of rsw2-1 KOR1 are usually processed to complex N-glycans in the Golgi (21). The amounts of endogenous KOR1 in rsw2-1 seedlings are quite low, precluding direct analysis of its N-glycosylation status (Fig. 10 A). Consequently, to investigate the nature of the N-glycans on KOR1 G429R, we expressed a KOR1 G429R-Fc-GFP variant in N. benthamiana, purified the protein, and analyzed its glycopeptides by LC-ESI-MS. Although the obtained amounts of purified KOR1 G429R-Fc-GFP were considerably lower than for KOR1-Fc-GFP, we could identify four of the eight glycopeptides. The identified glycopeptides displayed a predominant peak corresponding to the mass of Man8GlcNAc2, with additional peaks corresponding to Man6GlcNAc2 and Glc1Man8GlcNAc2 (Fig. 10B and data not shown). The presence of oligomannosidic structures and glucose-containing oligosaccharides instead of complex N-glycans indicates that KOR1 G429R-Fc-GFP is retained by quality control processes in the ER and hence does not reach the Golgi. Collectively, these data strongly suggest that the severe phenotype of cgl1 rsw2-1 is not caused by an N-glycan processing defect that directly affects KOR1 glycosylation.
DISCUSSION

N-Glycosylation is a major co- and post-translational modification in plants and the majority of all proteins destined for different compartments of the endomembrane pathway are glycosylated. The N-glycan biosynthesis and processing pathways of *A. thaliana* are already quite well understood (36, 37), and it has been shown that N-glycosylation is crucial for plant development (3, 23, 38, 39) and plays an important role in tolerance and response to biotic and abiotic stress (21, 22, 40–46).

However, the analysis of the N-glycoproteome and the N-glycosylation state of individual glycoproteins from *A. thaliana* and other plant species is still in its infancy (34, 47–49). In this study, we investigated the N-glycans of *A. thaliana* KOR1 and their role for protein stability and enzymatic activity.
KORRIGAN1 N-Glycans

Overall, our data for recombinant insect cell-derived A. thaliana KOR1 are consistent with enzyme activity assays reported in previous studies for KOR1 orthologs (18, 20) and confirm that N-glycosylation of KOR1 is important for its activity. In contrast to the previous findings, where 80% and more of the enzymatic activity was lost upon deglycosylation we could still detect a considerable amount of residual enzymatic activity in our study. In addition, our data suggest that different N-glycans contribute to optimal KOR1 enzymatic activity and proper folding of KOR1 when recombinantly expressed in insect cells. Individual mutation of Asn-216, Asn-324, Asn-345, and Asn-567 into glutamine resulted in considerably lower enzymatic activity. In the case of Asn-216, Asn-324, and Asn-345, this effect could be due to the proximity of these residues to the putative active site of KOR1. However, this does not apply to Asn-567. Because removal of the latter N-glycosylation site also resulted in reduced secretion of the recombinant enzyme, it appears likely that absence of the N-glycan attached to Asn-567 interferes with proper folding of KOR1 rather than exerting a direct effect on its enzymatic activity.

Notably, the effects of the different N-glycosylation site mutations on KOR1-Fc expression in plants is less pronounced. For example, the double mutant lacking N-glycans on two conserved KOR1 N-glycosylation sites (Asn-324, Asn-345) could not be efficiently expressed in insect cells, whereas the full-length KOR1-Fc-GFP variant with the same mutations was produced in comparable amounts as wild-type KOR1 in N. benthamiana. The presence of complex N-glycans demonstrates further that this mutant variant is processed in the plant Golgi, suggesting normal transport through the endomembrane system. In future studies, it remains to be shown whether the different N-glycan mutants can complement kor1 mutants and whether N-glycosylation of KOR1 is required for its function in plants.

We attempted to purify native KOR1 from A. thaliana seedlings using affinity purification with different antibodies against KOR1. However, despite numerous efforts, it was not possible to enrich sufficient amounts of endogenous KOR1 to perform glycosylation site analysis by mass spectrometry. Consequently, we transiently expressed a KOR1 fusion protein in leaves of N. benthamiana for analysis of its N-glycan composition when produced in planta. In accordance with data from endoglycosidase treatment and immunoblotting of endogenous KOR1 from A. thaliana seedlings, we detected oligomannosidic as well as processed complex N-glycans. Notably, we observed minor differences between N-glycans present on recombinant KOR1 produced in insect cells and KOR1 expressed in N. benthamiana leaf epidermal cells. These differences are found on the first two and the last N-glycosylation sites and are very likely caused by the presence/absence of different protein domains. In insect cells, we expressed a soluble KOR1 variant lacking the N-terminal cytoplasmic region and the single transmembrane domain to obtain a secreted form of the protein that can be readily purified from cell supernatants and used for in vitro activity assays. These constructs were designed to allow a direct comparison with previous studies relying on recombinant enzymes produced in P. pastoris (18, 20). In plants, we expressed full-length KOR1 fused to GFP and the Fc domain of human IgG. Although we cannot rule out that the protein tags for purification and localization influence protein folding and/or accessibility of individual glycosylation sites, it is more likely that the major effect comes from the membrane anchoring of KOR1. The first two N-glycan sites of KOR1 are very close to the transmembrane domain and consequently might not be as accessible for processing as sites that are more exposed in the catalytic domain. This proximity to the membrane could explain the presence of partially or unprocessed oligomannosidic N-glycans on Asn-108 and Asn-133. In contrast, these two sites are probably accessible (and thus modified with paucimannosidic N-glycans) in the variant produced in insect cells, which lacks the 90 most N-terminal amino acid residues.

Furthermore, we cannot exclude that KOR1 folding and ER-mediated quality control are different in insect cells and in plants. The presence/absence of certain molecular chaperones, lectins, or folding catalysts could contribute to the observed differences in N-glycan maturation as well as the different fate of the double N-glycosylation site mutant. Plants contain, for example, two calnexin and three calreticulin proteins, and calreticulin 3 seems specific for plants and has been found to play an important role in ER quality control processes of heavily glycosylated leucine-rich repeat receptor kinases (43, 44, 50).

The finding that the A. thaliana mutants cgl1 and mns3, which both harbor alterations in complex N-glycan formation, enhance the growth phenotype of the temperature-sensitive rsw2-1 allele even at the permissive temperature (21, 23) led to the hypothesis that processing of KOR1 N-glycans in the Golgi could play a direct role for KOR1 function (21, 24). Block of N-glycan maturation in the cgl1 mutant causes the accumulation of Man5GlcNAc2 structures because all subsequent N-glycan processing reactions leading to the formation of hybrid and complex N-glycans depend on GnTI activity, which is impaired in this mutant (51–53). The mns3 mutant accumulates mainly Man5GlcNAc2 and displays considerably reduced amounts of GlnGndF, the most common complex N-glycan in A. thaliana leaves and seedlings (23). Our results for the recombinant KOR1 variant carrying only oligomannosidic N-glycans indicates that N-glycan processing in the Golgi is not important for KOR1 enzymatic activity. As a consequence, it is possible that the complex N-glycan processing defects in cgl1 and mns3 do not directly affect the KOR1 protein variant present in rsw2-1 but have additional as yet unidentified glycoprotein targets that could be important for KOR1 function(s) in planta.

The presence of oligomannosidic N-glycans on KOR1-G429R-Fc-GFP (which is indicative of ER retention) further corroborates this finding. The observed additive phenotype of cgl1 rsw2-1 and mns3 rsw2-1 could be caused by a combination of the KOR1 defect in rsw2-1 and the N-glycan processing defect involving one or several other glycoproteins that could (but not necessarily have to) act in the same cellular pathway (cellulose synthesis). Alternatively, the less severe phenotype of rsw2-1 compared with other kor1 mutants (14, 17) indicates that a small amount of KOR1 G429R escapes from the ER quality control process and is targeted through the Golgi to its final destination, where it is partially or fully functional. In this scenario, altered N-glycan processing in mns3 and cgl1 could still directly affect the subcellular localization or function of KOR1 G429R.
Further studies such as the combination of rsw-2-I with other N-glycosylation and N-glycan processing mutants (27, 54, 55) and the characterization of additional glycoproteins are necessary to finally understand the role of complex N-glycans in cellulose biosynthesis.

Acknowledgments—We are grateful to Barbara Svoboda for help with KOR1 production in insect cells. We also thank Ulrike Vavara, Saída Reddy Bhavanam, Jakub Jez, Pia Gättinger, and Martina Dicker (Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna, Austria) for assistance in cloning, protein purification, and glycopeptide preparation. We also thank Johannes Stadtmann and Daniel Maresch (Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria) for MS analysis.

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