Running title: ER glycosylation

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Analysis of protein glycosylation in the ER

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Abstract

Protein N-glycosylation is an essential posttranslational modification which is initiated in the endoplasmic reticulum (ER). In plants, the N-glycans play a pivotal role for protein folding and quality control. Through the interaction of glycan processing and binding reactions mediated by ER-resident glycosidases and specific carbohydrate binding proteins, the N-glycans contribute to the adoption of a native protein conformation. Properly folded glycoproteins are released from these processes and allowed to continue their transit to the Golgi where further processing and maturation of N-glycans leads to the formation of more complex structures with different functions. Incompletely folded glycoproteins are removed from the ER by a highly conserved degradation process to prevent the accumulation or secretion of misfolded proteins and maintain ER homeostasis. Here, we describe methods to analyze the N-glycosylation status and the glycan-dependent ER-associated degradation process in plants.

Key words: N-glycosylation, glycoprotein, oligosaccharyltransferase, quality control, ERAD

1. Introduction

Glycosylation is a ubiquitous modification of newly synthesized proteins in the endoplasmic reticulum (ER). Dependent on the linkage of the oligosaccharide to the amino acid side chain of the protein there are two major types of glycosylation: N- and O-glycosylation. O-glycosylation is quite diverse and various types of O-glycans have been described for secretory proteins in eukaryotes including mucin-type O-linked N-acetylgalactosamine (GalNAc) or O-mannosylation (1, 2). These O-glycosylation reactions are initiated by the transfer of a single monosaccharide to serine or threonine residues of proteins. While the O-glycan biosynthesis can already be initiated in the ER, the majority of the O-glycan modifications take place in the Golgi. In plants, it has been proposed that the plant-specific serine O-galactosylation of extensins or arabinogalactan proteins is initiated in the ER while further maturation of glycans takes place in the Golgi (3).

In contrast to O-glycosylation, N-glycosylation and early N-glycan processing steps are highly conserved in all eukaryotes (4). A hallmark of N-glycosylation is the *en bloc* transfer of a common preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) from the lipid carrier dolichol pyrophosphate to selected asparagine residues in the sequence Asn-X-Ser/Thr (X ≠ P) within nascent polypeptides (5) (*see* **Note 1**). The transfer of the oligosaccharide takes place at the luminal side of the ER and is catalyzed by the oligosaccharyltransferase (OST) complex. In the ER, the attached N-glycans are crucial for protein folding and for quality control processes. Notably, by exposing defined motifs the N-glycans orchestrate the fate of glycoproteins in the ER (6). In contrast to the conserved role of ER-type oligomannosidic structures, complex N-glycans are generated in the Golgi and serve different protein-specific roles that are still not very well understood in plants (Fig. 1A) (7-9).

1.1 N-glycosylation

The biosynthesis of the lipid-linked oligosaccharide starts on the cytosolic side of the ER membrane by a number of different glycosyltransferases that have been initially characterized in yeast (asparagine linked glycosylation proteins– ALGs) (10). The synthesized $Man_5GlcNAc_2$-PP-Dol is flipped through the ER membrane (Fig. 1B) and further

elongated in the lumen of the ER by three α-mannosyltransferases (ALG3, ALG9 and ALG12) and three α-glucosyltransferases (ALG6, ALG8 and ALG10) (Fig. 1B). Putative orthologs of these enzymes are present in the plant genome and mutants with defects in the assembly of the oligosaccharide precursor have been described (4). For example, knockout of the *Arabidopsis thaliana* ALG10 gene results in an incomplete synthesis of the oligosaccharide precursor leading to reduced glycosylation efficiency of proteins (11). The observed underglycosylation defect affects different glycoproteins and results in a leaf growth phenotype.

The fully assembled oligosaccharide precursor is transferred by OST to asparagine resides within the consensus sequence. In yeast, OST is a heteromeric membrane-bound protein complex (12) consisting of one catalytically active subunit (STT3) and several different non-catalytic subunits that contribute to N-glycosylation by regulation of the substrate specificity, stability or assembly of the complex (12). Humans, harbour two different catalytic isoforms (STT3A and STT3B) that are present in distinct OST complexes and differ in their catalytic activity and acceptor substrate selectivity (13). While STT3A is predominately involved in co-translational glycosylation, STT3B displays a preference for post-translational glycosylation. The transfer of the preassembled oligosaccharide in plants involves a similar OST multi-subunit complex, which is still poorly characterized (14). In *A. thaliana* two proteins (STT3A and STT3B) with homology to yeast and mammalian catalytic subunits have been identified (15). STT3A-deficient plants are viable, but display a protein underglycosylation defect that impairs the biogenesis of different heavily glycosylated proteins, such as the pattern recognition receptor EF-TU RECEPTOR (EFR) or the endo-β1,4-glucanase KORRIGAN1 (16,17,7). By contrast, much less is known about STT3B function and its substrates. Notably, *A. thaliana stt3a stt3b* double knockouts are gametophytic lethal (15) highlighting the importance of the catalytic OST subunit for protein N-glycosylation in plants. Other subunits that have been characterized include DGL1, OST3/6, OST1A/B and DAD1/2 (14,18,19) (Fig. 2A). While DGL1 and OST1A/B appeared essential for plants, the *dad1 dad2* double mutant

is viable but displayed a plant growth and pollen production defect (14). OST3/6-deficient plants do not display any growth phenotype but are compromised in their immunity (19) and OST3/6 is required for the interspecific gametophyte recognition in *A. thaliana* (20).

1.2. N-glycan processing in the ER

Specific processing of N-glycans in the ER generates distinct oligosaccharide structures that expose signals for quality control processes or degradation. Upon transfer of the preassembled oligosaccharide to the polypeptide chain, glucosidase I (GCSI) and II (GCSII) remove the two outermost glucose residues. The resulting monoglucosylated N-glycan can interact with the lectins calnexin (CNX) and calreticulin (CRT) to promote folding. In the current models, release from the CNX/CRT-interaction requires the trimming of the remaining glucose by GCSII. Re-addition of the single glucose by the folding sensor UDP-glucose:glycoprotein glucosyltransferase (UGGT) facilitates prolonged interaction with CNX/CRT (21). Several rounds of glucose trimming, re-glucosylation and interaction with CNX/CRT are possible until proteins attain their final conformation and are released from this glycan-dependent quality control process.

The N-glycans from properly folded proteins or from folding intermediates may be subjected to further processing in the ER which is catalyzed by the α-mannosidase MNS3. Analysis of the substrate specificity revealed that MNS3 displays the typical ER-α-mannosidase I activity that has been described in mammals and yeast. In contrast to yeast, however, MNS3 is mainly located in small punctate structures resembling Golgi bodies (22). This specific subcellular localization is a means to spatially separate MNS3 from ER-localized mannose trimming steps that generate the glycan signal required for flagging terminally misfolded glycoproteins for ER-associated degradation (ERAD). Further processing takes place in the Golgi and is initiated by Golgi α-mannosidase I (MNS1/MNS2)-mediated trimming of mannose residues resulting in the formation of $Man_5GlcNAc_2$.

1.3. N-glycan-dependent quality control and ER-associated degradation

To maintain protein homeostasis in the ER all eukaryotic cells have a conserved machinery that selects aberrant or incompletely assembled proteins for degradation. In mammals and yeast, this ERAD pathway involves recognition of the misfolded protein, retro-translocation from the ER to the cytoplasm, ubiquitination of the protein and its subsequent disposal by the 26S proteasome (23). While the latter steps have not been characterized in detail in plants, the selection and delivery reactions that take place in the lumen of the ER appear highly conserved. If folding attempts are unsuccessful, the α-mannosidases MNS4 and MNS5 cleave a terminal mannose residue form the C-branch of the oligomannosidic N-glycan (24). The exposed α1,6-linked mannose residue is recognized by the lectin-like protein OS9 and delivered to the SEL1L-HRD1 ERAD complex resulting in the degradation of the aberrant glycoprotein (25) (Fig. 1B).

Here, we provide protocols to address different aspects of protein N-glycosylation, N-glycan-processing and glycan-dependent ERAD that take place in the lumen of the ER.

2. Materials

1. *A. thaliana* seeds with characterized underglycosylation defects can be purchased from the European Arabidopsis Stock Center (*alg10-1*: NASC ID: N874475) (15,11). Seeds with defects in glycan-dependent ERAD are available from NASC (*os9-1*: N529413; *mns4 mns5*: double mutant can be obtained by crossing of N656907 and N162962) (26,24). The *bri1-9* seeds were kindly provided by Frans E. Tax. The *ost3/6-1* seeds can be requested from the Strasser group (19).
2. Mixer mill with steel beads.
3. Container with liquid nitrogen.
4. Refrigerated bench-top centrifuge with a relative centrifugal force of 9.400 or higher and a rotor for 1.5/2 mL microcentrifuge tubes.
5. Thermo block.

6. Orbital shaker.

7. Vertical electrophoresis system.

8. Tank transfer system.

9. Power supply with at least 100V and 400 mA.

10. SDS-PAGE (10 to 12%) gels.

11. Tris/glycine/SDS-PAGE running buffer: (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.

12. 3x Laemmli sample buffer: 187.5 mM Tris pH 6.8, 30% (w/v) glycerol, 6% (w/v) SDS, 15% (v/v) β-mercaptoethanol, 0.15 % (w/v) bromophenol blue.

13. Prestained protein standard.

14. Tris/glycine/methanol transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol.

15. Blotting paper.

16. Nitrocellulose blotting membrane.

17. 1x PBS: phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.

18. PBST: 1x PBS + 0.1% (v/v) Tween 20.

19. 1x TBS: Tris buffered saline (25 mM Tris, pH 7.5, 150 mM NaCl, 2 mM KCl).

20. TBST: 1x TBS + 0.1% (v/v) Tween 20.

21. Blocking solution for immunoblots: TBST + 5% (w/v) skimmed milk powder or PBST + 3% (w/v) BSA.

22. Western blotting detection reagent: as an example we use SuperSignal West PICO Chemiluminescent Substrate or ECL Prime Western Blotting Detection Reagent.

23. Sensitive films for detection: for example we use Amersham Hyperfilm ECL (GE Healthcare).

24. Film developer and fixer solutions.

25. Rat monoclonal HA antibody (Roche 11867423001): 1:2000 diluted in TBST + 1% (w/v) BSA.

26. Polyclonal TGG1 antibody (Agrisera: AS20 4416): 1:1000 diluted in TBST.

27. Polyclonal BRI1 antibody (Agrisera: AS12 1859): 1:5000 diluted in TBST + 5% (w/v) skimmed milk powder.

28. Rat monoclonal RFP antibody (ChromoTek: 5F8). 1:1000 diluted in PBST + 1% (w/v) BSA.

29. Mouse monoclonal GFP antibody (Roche: 11814460001). 1:2000 diluted in PBST + 3% (w/v) BSA.

30. Mouse monoclonal α-tubulin antibody (Sigma-Aldrich: T6074). 1:5000 diluted in PBST + 1% (w/v) BSA.

31. Concanavalin A-peroxidase (ConA-HRP, Sigma-Aldrich: L6397): ConA solution: 0.5 µg/mL Con A-HRP in PBST containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$.

32. Anti-rabbit IgG-peroxidase (such as Sigma A0545): 1:5000 diluted in TBST + 5% (w/v) skimmed milk powder for BRI1 detection and 1:50000 diluted in TBST for TGG1 detection.

33. Anti-mouse IgG-peroxidase (such as Sigma A9044): 1:10000 diluted in TBST for detection of GFP and α-tubulin.

34. Anti-rat IgG-peroxidase (Jackson): 1:10000 diluted in PBST + 0.5% (w/v) BSA for detection of RFP and HA-tagged proteins.

35. Endoglycosidase H (Endo H, we use 500.000 units/mL, New England Biolabs) + GlycoBuffer 3 (New England Biolabs) + 10x Glycoprotein Denaturing Buffer (New England Biolabs).

36. Empty micro spin chromatography columns.

37. Nonidet P-40 (10% solution).

38. Kifunensine (Santa Cruz Biotechnology): class I α-mannosidase inhibitor, dissolved in ultrapure water.

39. RIPA buffer: 150 mM NaCl, 1.0 % (v/v) Nonidet P-40, 0.5 % (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris, pH 8.0.

40. RFP-Trap®_A beads (ChromoTek) for purification of RFP-tagged proteins.

41. Protein A Sepharose (such as rProtein A Sepharose Fast Flow from GE Healthcare).

42. Coomassie Brilliant Blue staining solution: 0.25% (w/v) Coomassie Brilliant Blue R-250, 10% (v/v) acetic acid, 50% (v/v) methanol.

43. Infiltration buffer: 0.5% (w/v) D-glucose, 50 mM 2-(N-morpholino)ethanesulphonic acid (MES), 2 mM Na_3PO_4, 0.1 mM acetosyringone.

44. *Agrobacterium tumefaciens* strain UIA143 pMP90 (27).

45. LB Broth (Lennox): 10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract. Sterilize by autoclaving, supplement with 50 mg/L kanamycin 25 mg/L gentamicin.

46. MS medium: 0.5x Murashige & Skoog (MS) medium including MES buffer supplemented with 0.8% (w/v) agar and 1% (w/v) sucrose.

3. Methods

3.1 Monitoring of protein underglycosylation

N-glycosylation efficiency can be monitored by comparison of differences in mobility upon SDS-PAGE and immunoblotting of glycoproteins (*see* **Note 2**). Reduced N-glycosylation efficiency causes underglycosylation of glycoproteins which typically results in a faster migrating protein due to the reduced molecular weight (*see* **Note 3**). As a result a single faster migrating band representing a partially or non-glycosylated variant can be detectable (Fig. 2B) or several bands might appear representing a mixture of differentially glycosylated proteins (fully-glycosylated, partially glycosylated and unglycosylated variants) (*see* **Note 4**). Protein underglycosylation has been described in several *A. thaliana* mutants which can be used as controls (e.g. mutants with defects in one of the OST subunits such as *stt3a-2* (15) or *ost3/6-1* (19) or mutants with defects in the assembly of the oligosaccharide precursor like *alg10* (11) (*see* **Note 5**). *A. thaliana* beta-thioglucoside glucohydrolase 1 (TGG1) is a heavily glycosylated protein that is expressed in high amounts in leaves (28) and has been used in several studies to show a reduction in protein glycosylation efficiency (15, 11). In addition, protein underglycosylation can also be detected by an overall reduced signal with the lectin

ConA that recognizes terminal α-linked mannose residues from oligomannosidic N-glycans (*see* **Notes 6** and **7**).

1. Harvest 10-100 mg of plant material (e.g. rosette leaves) from mutants and Columbia (Col-0) wild-type and transfer to 2 mL Eppendorf Safe-lock microcentrifuge tubes containing 2 steel beads (5 mm diameter) per tube.
2. Submerge 2 mL tubes with plant material in container with liquid nitrogen.
3. Mount 2 mL tubes in mixer mill and run mixing for 2 min at 50-60 amplitude.
4. Add 4 µL extraction buffer such as PBST per mg of plant material, vortex shortly, transfer mixture into a 1.5 mL tube.
5. Incubate on ice for 15 min, invert tube every 3 min.
6. Centrifuge 2 times 15 min, 9.600x g at 4°C, transfer supernatant each time to a new tube.
7. Mix samples with 3x Laemmli sample buffer and heat to 95°C for 5 min.
8. Load SDS-PAGE gel with 20 µL and run protein separation for 1.5 h at 100V.
9. Soak nitrocellulose membrane, sponges and blotting paper in transfer buffer.
10. Perform the gel-membrane assembly according to the user manual.
11. Blot for 1 h at 100V, disassemble gel-membrane sandwich and carefully rinse the membrane with ultrapure water.
12. Incubate in blocking solution for 1 h at room temperature.
13. Rinse briefly with PBST and incubate membrane on a shaker for 1.5 h at room temperature in the antibody solution (in our example use a TGG1 antibody to detect changes in N-glycosylation and an antibody against a non-glycosylated protein like α-tubulin as a control).
14. After incubation, wash 4 times 5 min with TBST and add secondary antibody solution to the membrane, incubate 1.5 h at room temperature on a shaker. Omit this step for ConA detection, because the used ConA protein is covalently linked to horseradish peroxidase.
15. Wash 4 times 5 min in TBST, perform detection using the chemiluminescent substrate and develop the film.

3.2. Co-IP and subcellular localization of OST subunits

Agroinfiltration of *Nicotiana benthamiana* leaves is a fast procedure for protein expression and subsequent co-localization studies by confocal laser scanning microscopy or for monitoring of complex formation by co-immunoprecipitation (co-IP). These protocols have, for example, been used to characterize unknown subunits of the *A. thaliana* OST complex (19) (Fig. 2A and 2C).

1. Grow *A. tumefaciens* in LB liquid culture supplemented with the appropriate antibiotics for 16 h at 29°C.
2. Dilute the overnight culture containing the construct for expression of the glycan-modifying enzyme with infiltration buffer to an OD600 (optical density at 600 nm) of 0.1.
3. For co-localization of GFP and mRFP fusion proteins mix two or more agrobacteria suspensions and co-infiltrate the whole mixture into fully expanded leaves of 5-6-week-old *N. benthamiana* plants using a 1 mL syringe without a needle. The analysis of subcellular localization by confocal microscopy has been described in detail previously (22,29).
4. Briefly, sections of infiltrated leaves are analyzed 1-3 days post-infiltration (dpi) on a Leica TCS SP5 confocal microscope equipped with x63 and x100 oil immersion objectives using appropriate spectral selections. Post-acquisition image processing was performed in IMAGEJ and ADOBE PHOTOSHOP CS (Fig. 2 D-G).
5. For co-IP, harvest 500 mg of leaves and extract the proteins with RIPA buffer following the grinding steps as described in 3.1.
6. Per sample equilibrate 50 µL RFP-Trap®_A bead slurry in 500 µL of RIPA buffer.
7. Centrifuge at 2.500x g for 2 min, discard the supernatant and repeat wash twice.
8. Add 2 mL of the clear protein extract to equilibrated RFP-Trap®_A beads and incubate for 1 h at 4°C.

9. The RFP-Trap®_A beads with the bound proteins are washed 4 times with RIPA buffer and proteins are eluted by incubation at 95°C for 10 min in 50 µL 1x Laemmli buffer.
10. To collect the bound proteins, perform centrifugation at 2.500x g for 2 min using micro spin columns.
11. The protein extracts and the eluate are subjected to SDS-PAGE and immunoblotting with anti-HA and anti-mRFP antibodies as described above for monitoring of protein underglycosylation (*see* **Note 8**) (Fig. 2C).

3.3 Characterization of ER-type oligomannosidic N-glycans

ER-resident proteins typically carry oligomannosidic N-glycans because these proteins are not in contact with the Golgi-located machinery for complex N-glycan processing (Fig. 1B). Such oligomannosidic N-glycans are sensitive to digestion by endoglycosidase H (Endo H) which cleaves between the chitobiose core to release the majority of the oligosaccharide (Fig. 3A). As a consequence, Endo H digestion of oligomannosidic N-glycans will result in a reduced molecular weight that can be monitored by SDS-PAGE and immunoblotting. Proteins with complex N-glycans are resistant to Endo H digestion and do not cause a similar change in the molecular weight (**Fig. 3A**). Endo H treatment has been used to show that a misfolded variant of the brassinosteroid receptor BRI1 accumulates in the ER in the *bri1-9* mutant (30). By contrast, correctly folded BRI1 is secreted and carries primarily Golgi-processed complex N-glycans (*see* **Note 9**).

1. Incubate 22.5 µL protein extract with 2.5 µL 10x Glycoprotein Denaturing Buffer for 10 min at 50°C, transfer to ice and cool for 5 min.
2. Mix 22.5 µL of the denatured glycoproteins with 3 µL 10x GlycoBuffer 3, 3 µL ultrapure water and 1.5 µL Endo H.
3. For the control reaction replace the 1.5 µL Endo H with ultrapure water.
4. Incubate for 90 min at 37°C and stop the reaction by heating to 50°C for 5 min.

5. Subject samples to SDS-PAGE and perform immunoblotting, for example with anti-BRI1 antibody (Fig. 3A) to distinguish between ER-retained and secreted BRI1 variants.
6. To confirm the presence of oligomannosidic N-glycans, glycoproteins can be purified and subjected to mass spectrometry.

The attachment of the HDEL or KDEL ER-retrieval peptide sequence to the C-terminal end leads to the steady-state accumulation of proteins in the ER. An example for such a protein is a chimeric protein consisting of an N-terminal signal peptide, the Fc domain region from human IgG, GFP and the HDEL peptide (SP-Fc-GFP-HDEL). This protein can be expressed transiently in *N. benthamiana* leaves as described in 3.2 and ER accumulation can be monitored by confocal microscopy. Efficient one-step purification of this protein is achieved by affinity chromatography using binding of the Fc domain region to bacterial Protein A (*see* **Note 10**).

7. Harvest 1 g of leaves 2 dpi and extract proteins in RIPA buffer as described in 3.2.
8. Add 50 µL protein A sepharose that has been washed several times with 1x PBS.
9. Incubate for 1.5 h at 4°C using an orbital shaker, spin down briefly and discard the supernatant.
10. Wash the sepharose 3 times with 250 µL 1x PBS using micro spin columns.
11. Elute the Fc domain-containing protein from the column by adding 50 µL 1x Laemmli buffer.
12. Elute as described for the RFP-Trap®_A purification and separate by SDS-PAGE.
13. Stain the gel with Coomassie Brilliant Blue and excise the glycoprotein band from the gel. Perform S-alkylation, digestion with trypsin (*see* **Note 11**) and analyze the resulting glycopeptides by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) (**Fig. 3B**). The detailed procedure for this MS-based glycopeptide analysis has been described recently (31).

3.4. Monitoring of glycan-dependent ERAD

Glycan-dependent ERAD is a major pathway for disposal of misfolded glycoproteins from the ER. To monitor whether a protein is subjected to glycan-dependent ERAD the class I α-mannosidase inhibitor kifunensine can be used. Kifunensine blocks the trimming of terminal α-linked mannose residues which is mediated by class I α-mannosidases (32). The exposure of an α1,6-linked mannose residue (Fig. 1B) to the ERAD machinery (including proteins like OS9, SEL1L and HRD1) is a highly conserved hallmark of glycan-dependent ERAD in different species (33-35,24). Kifunensine blocks the formation of the exposed α1,6-linked mannose residue and thus blocks the degradation of ERAD substrates (Fig. 4A) (*see* **Note 12**).

1. Detach leaves from 4-week-old *A. thaliana* plants, cut them into small pieces (alternatively whole seedlings can be used in the same way) and submerge in 0.5x MS medium supplemented with 20 µM kifunensine.
2. Incubate at 22°C with gentle shaking under long day conditions (16 h light/8 h dark) for 24 h.
3. Harvest seedlings, remove excess liquid and extract protein as described under 3.1. To monitor the accumulation of the ERAD substrate, perform immunoblotting with appropriate antibodies (Fig. 4B).
4. An increased signal in the presence of the inhibitor indicates the involvement of a glycan-dependent degradation pathway.
5. To substantiate the finding the fate of the misfolded glycoprotein can be analyzed in different mutants with distinct defects in glycan-dependent ERAD (26, 24).

4. Notes

1. Apart from Asn-X-Ser/Thr there is the rare possibility for the use of non-canonical glycosylation sites like Asn-X-Cys (36, 37). The sequon is necessary but not sufficient for N-glycosylation. N-glycosylation efficiency is dependent on many factors involving the amino acid sequence close to the N-glycosylation site, the secondary structure, the positioning of

the site within the protein (13) and organism-specific difference in the composition and function of the OST subunits (12).

2. A shift in mobility can also be caused by other posttranslational modifications including proteolytic processing. Digestion of protein extracts with endoglycosidases that remove oligomannosidic or complex N-glycans is a way to support the observation of underglycosylation. However, complex N-glycans carrying core α1,3-fucose cannot be efficiently removed from whole proteins using endoglycosidases which can complicate the interpretation of the results. Additional blots with antibodies against complex/paucimannosidic N-glycans and lectins that bind to mannose residues can be used to further support a protein underglycosylation defect (see also the procedure for detection of glycoproteins that are recognized by ConA). Apart from the described immunoblot-based protocols, purification of the protein with subsequent MS analysis and quantification of glycosylated versus non-glycosylated peptide should be used to confirm the underglycosylation defect (31).

3. In some cases a clear protein band cannot be detected anymore in a protein underglycosylation mutant (e.g. KORRIGAN1 in *stt3a-2*) (7). Most likely, the absence of distinct N-glycans interferes with proper folding leading to degradation or aggregation (exposure of hydrophobic sequence stretches that are otherwise shielded by N-glycans) of these proteins.

4. These differences depend on the number of N-glycans and other protein intrinsic features that are involved in N-glycosylation. For example, some of the non-catalytic OST subunits have accessory function and can slow down protein folding to keep the polypeptide in a glycosylation-competent state (13).

5. Additional approaches can be used that suggest a possible reduction in glycosylation efficiency. These approaches involve treatment of seedlings with tunicamycin. Tunicamycin is an inhibitor that blocks N-glycosylation by interfering with one of the early steps of the lipid-linked oligosaccharide biosynthesis pathway. Plants with defects in N-glycosylation are typically more sensitive to tunicamycin (38).

6. An altered signal with ConA could also result from changes in complex N-glycan formation (e.g. increased signal in the *cgl1* mutant that lacks complex N-glycans). It is therefore recommended to combine the ConA lectin blot with immunoblots using antibodies against complex N-glycans (39).

7. In an alternative approach protein underglycosylation can be detected in *A. thaliana* by restoration of complex N-glycan formation in the *cgl1* mutant. This approach has been described in detail recently (39).

8. While co-IP can be helpful to identify the components of a protein complex, it does not provide spatio-temporal information on direct interactions of the investigated proteins. Therefore, alternative approaches like FRET-FLIM should be applied for monitoring of real-time protein-protein interactions in cells (40).

9. Oligomannosidic N-glycans can also be found on secreted proteins that are not trafficking through the Golgi (e.g. due to direct ER-to-vacuole protein trafficking). In addition, some N-glycans are not accessible for Golgi-mediated N-glycan processing (e.g. steric hindrance of N-glycan processing) resulting in the presence of oligomannosidic N-glycans on some secreted or plasma-membrane anchored proteins (see also Endo H digestion of BRI1 in Col-0 in Fig. 3A). It is therefore necessary to perform additional experiments (e.g. confocal microscopy of fluorescent fusion proteins) to confirm ER localization.

10. Purification with an immobilized antibody against GFP could also be used instead of binding to Protein A. While such protocols work equally well with our fusion proteins, there are more suppliers for Protein A-related reagents than for reagents that can be used for purification of GFP-tagged proteins. Consequently, Protein A-based purification is typically less expensive than purifications based on GFP-binding proteins. Alternatively, an antibody specific for an (ER-resident) glycoprotein can be used for purification and subsequent N-glycan analysis. Due to the low expression levels such protocols typically require extensive optimization.

11. The choice of the protease is dependent on the amino acid sequence. Separate or sequential digestion with alternative enzymes, such as Glu-C, Asp-N or chymotrypsin is sometimes necessary to improve the detection of glycopeptides (31).

12. Although the components of the glycan-dependent ERAD pathway are already well known, the steps that lead to the recognition of aberrant glycoproteins as well as the downstream steps that lead to their disposal are less clear. In mammals and yeast, ERAD involves translocation into the cytoplasm, ubiquitination and proteasomal degradation. In plants, the involvement and role of the proteasome in degradation of glycoprotein ERAD substrates is not so well established (41,42). While distinct misfolded glycoproteins are subjected to proteasome-dependent degradation in plants (43), aberrant glycoproteins with powerful ER export signals are subjected to alternative pathways including vacuolar degradation (44,45).

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Figures

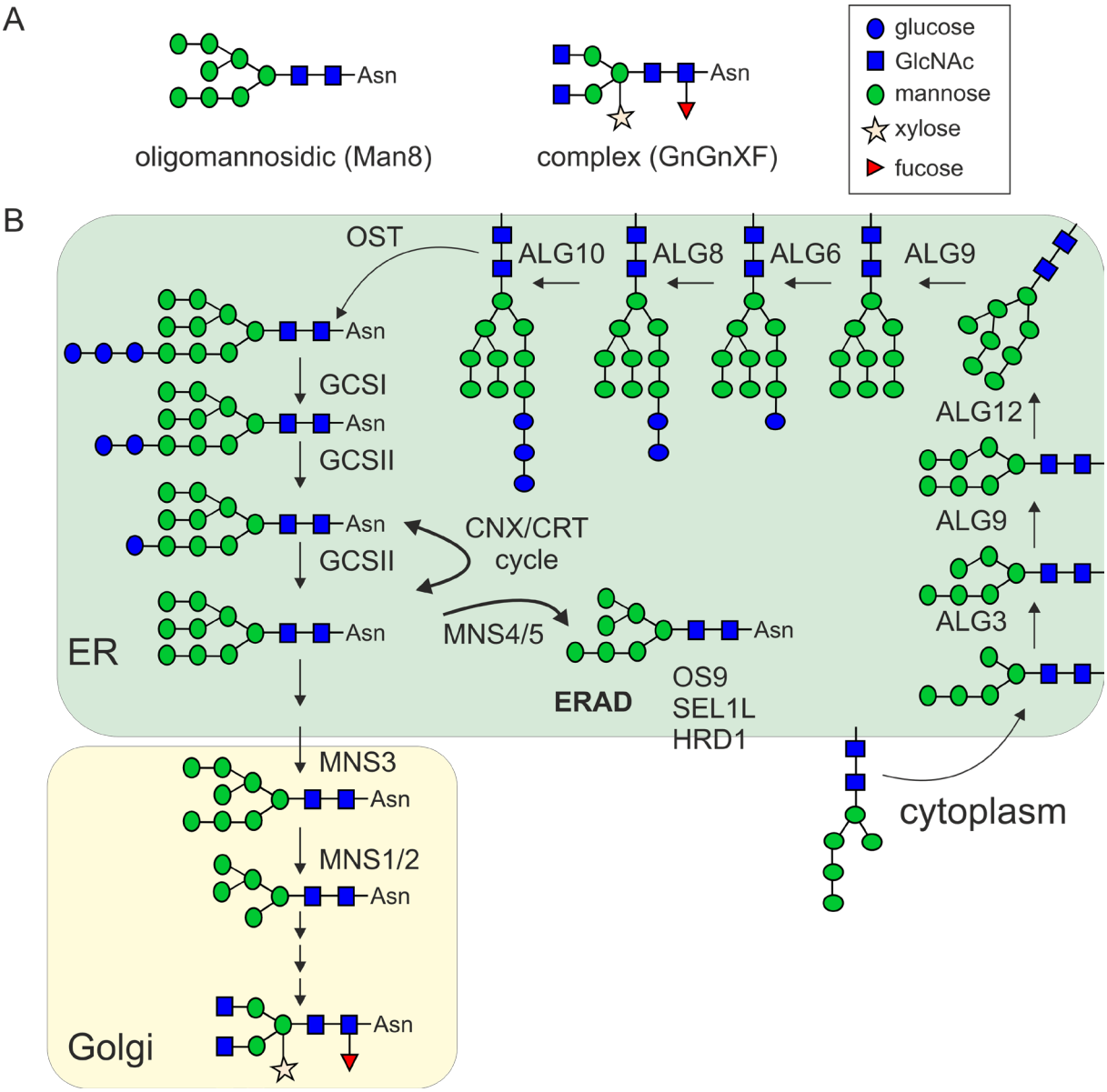

Fig. 1. (**A**) Schematic representation of two major N-glycan structures in plants: ER-type oligomannosidic and Golgi-processed complex N-glycans. (**B**) N-glycosylation and N-glycan processing in the ER of plants. The dolichol-linked $Man_5GlcNAc_2$ intermediate is translocated from the cytosol into the lumen of the ER (the preceding cytosolic biosynthesis steps are not shown). In the lumen of the ER, mannose residues are sequentially added by the α-mannosyltransferases ALG3, ALG9 and ALG12. Further elongation of the oligosaccharide is carried out by the α-glucosyltransferases ALG6, ALG8 and ALG10. The fully assembled $Glc_3Man_9GlcNAc_2$ oligosaccharide is transferred to selected asparagine residues by the oligosaccharyltransferase (OST) complex. Upon transfer, terminal glucose residues are trimmed by α-glucosidase I (GCSI) and II (GCSII). A terminal glucose residue is required for the interaction with calnexin (CNX) and calreticulin (CRT) that support folding of the glycoproteins. Properly folded proteins may be further processed in the ER by the α-mannosidase MNS3 (22) and are allowed to exit to the Golgi which is the site for complex N-glycan formation. In the ER, terminally misfolded proteins are subjected to processing by the α-mannosidases MNS4 and MNS5 which results in the degradation by the OS9-SEL1L-HRD1 complex (24). The symbols for representation of the glycan structures follow the style of the Consortium for Functional Glycomics (www.functionalglycomics.org).

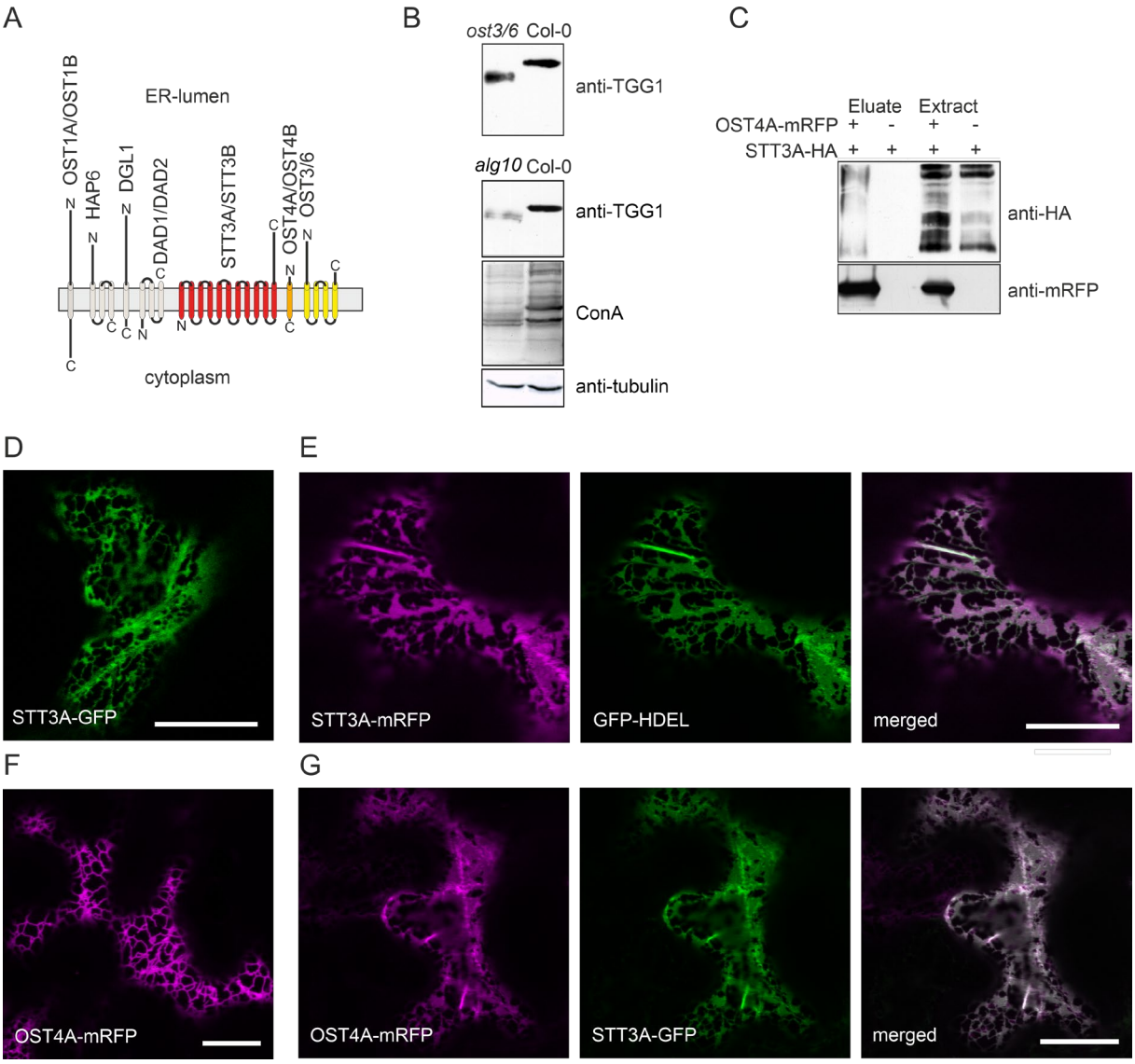

Fig. 2. (**A**) Schematic representation of the OST subunits and their predicted topology (4). Subunits which likely interact (based on co-IP experiments) (19) are depicted in red, orange and yellow. Other subunits are shown in grey. (**B**) Protein extracts from *A. thaliana* leaves were analyzed by immunoblotting with anti-TGG1 or anti-tubulin (non-glycosylated control) antibody. The shift in mobility indicates underglycosylation of TGG1 in the *ost3/6* and *alg10* mutants. The ConA lectin blot shows an overall weaker signal in the *alg10* mutant and some bands display an altered migration which is consistent with the underglycosylation defect. (**C**) OST4A-mRFP interacts with STT3A-HA. Proteins were transiently co-expressed in *N. benthamiana* leaves and extracts were analyzed 2 dpi. OST4A-mRFP was purified by RFP-Trap®_A beads and co-isolated STT3A-HA was monitored by immunoblotting with anti-HA antibodies. Note: due to the presence of numerous transmembrane helices STT3A-HA displays anomalous gel mobility. (**D-G**) Analysis of fluorescent proteins was done by confocal microscopy. Bars = 20 µm. The indicated constructs were transiently expressed in *N. benthamiana* leaves and analyzed 2 dpi.

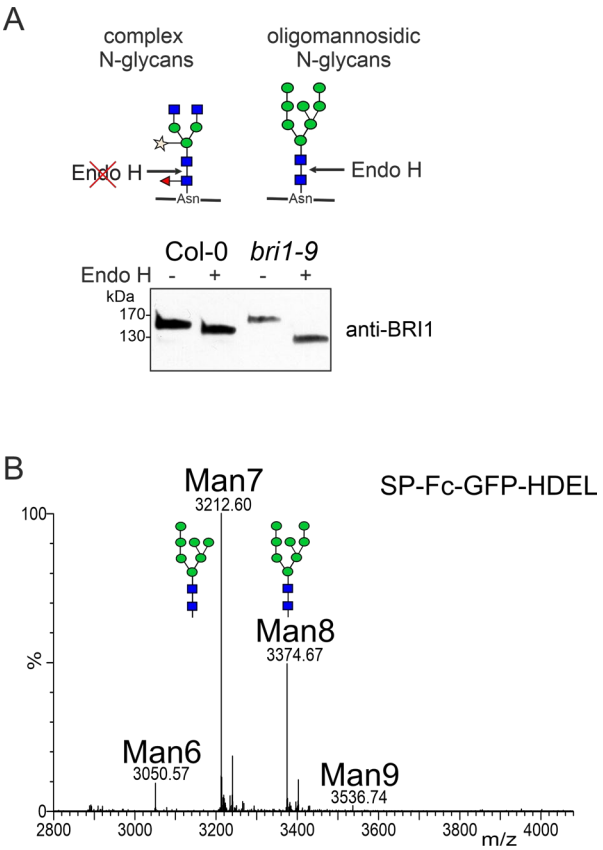

Fig. 3. (**A**) Endo H digestion and detection of BRI1 glycosylation in protein extracts from Col-0 wild-type and the *bri1-9* mutant. BRI1 in Col-0 is only partially deglycosylated (few oligomannosidic N-glycans are present) as visible by the minor shift in mobility (band > 130 kDa). Endo H treatment of BRI1 in the mutant results in complete deglycosylation (band < 130 kDa). The cartoon illustrates the cleavage specificity of Endo H. For description of sugar symbols see Fig. 1. (**B**) Transiently expressed SP-Fc-GFP-HDEL was purified and the glycopeptide (peptide sequence: TKPREEQYNSTYR – N-glycosylation site is underlined) from the Fc domain was subjected to LC-ESI-MS analysis. Peak labels were made according to the ProGlycAn system (www.proglycan.com).

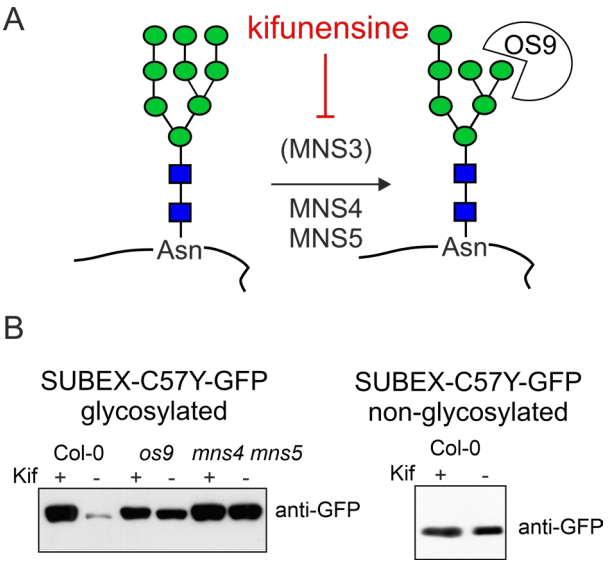

Fig. 4. (**A**) Illustration of the processing by MNS4/MNS5 which generates the N-glycan that serves as degradation signal for misfolded glycoproteins. The class I α-mannosidase inhibitor kifunensine (Kif) blocks the mannose trimming and leads to accumulation of misfolded glycoproteins (ERAD substrates) in the ER. Likewise, OS9 or MNS4/MNS5 deficiency blocks the degradation. MNS3 may act on the $Man_9GlcNAc_2$ N-glycan but is not necessary for glycan-mediated ERAD (24). (**B**) The ERAD substrate SUBEX-C57Y-GFP consisting of a misfolded variant of the glycosylated extracellular domain from STRUBBELIG fused to GFP is degraded in Col-0 wild-type and accumulates in the *os9* or *mns4 mns5* mutants as well as in the presence of Kif (42). A non-glycosylated variant of the misfolded protein is not degraded by the same pathway highlighting the glycan-dependency of the protein disposal pathway.