Running title: ERAD of glycoproteins

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Studying glycan-dependent ERAD of misfolded glycoproteins in plants

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Abstract

The endoplasmic reticulum (ER) is the site where proteins that are synthesised and destined for the secretory pathway fold into their native conformations. Genetic mutations, oxidative stress, reduced glycosylation and disruption to ER folding and quality control systems are all factors which have been demonstrated to impair this process, leading to the production of misfolded proteins. It is imperative for the survival of cells that these misfolded proteins are recognised by specific signals and directed towards degradation via ER-associated degradation (ERAD), ER-phagy, or related ER-to-lysosome/vacuole transport processes. A pivotal step in this process is the recognition of specific degrons and the distinction between terminally misfolded proteins that necessitate clearance and folding intermediates that have the potential to reach their final conformation. A significant proportion of proteins that traverse the secretory pathway in eukaryotes undergo N-glycosylation. The attached N-glycans not only facilitate the folding and quality control of glycoproteins, but can also be processed to display a glycan degron signal necessary to initiate ERAD of glycoproteins. Here, we describe methods for investigating glycan-related degrons that are key determinants for the ERAD of misfolded glycoproteins in plants.

Key words: N-glycosylation, glycoprotein, quality control, ERAD, protein degradation, *Arabidopsis thaliana*

1. Introduction

Glycosylation is a widespread modification of newly synthesised proteins in the endoplasmic reticulum (ER). Depending on how the oligosaccharide is linked to the amino acid side chain of the protein, there are two main types of glycosylation: N- and O-glycosylation. Both types affect protein folding; this role is however more clearly established for N-glycosylation, whereby the N-glycan, which is attached to an asparagine in a specific sequence motif (Asn-X-Ser/Thr; X can be any amino acid except proline), is often attached co-translationally. Glycosylation can directly contribute to the folding process or facilitate interaction with specific lectins, such as calnexin and calreticulin, which promote folding in association with interaction protein partners. ***(1, 2)***.

A hallmark of N-glycosylation is the *en bloc* transfer of a conserved preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) (**Figure 1A**) from the lipid carrier dolichol pyrophosphate to selected N-glycosylation sites which is catalysed by the oligosaccharyltransferase (OST) complex in all eukaryotes ***(3-5)***. Upon transfer, the attached N-glycans are quickly trimmed by glucosidase I and II which generates a monoglucosylated N-glycan that interacts with calnexin and calreticulin as part of the glycan-dependent ER-quality control ***(6)***. Eventually, however, terminally misfolded glycoproteins are sensed and the N-glycan is trimmed by specific α-mannosidases to expose a glycan degradation signal ***(7)***. Notably, by exposing distinct glucose or mannose residues, the N-glycans determine the fate of glycoproteins in the ER. Glycan recognition and trimming steps are an essential part of the ER-quality control and glycan-dependent ERAD process, which is conserved in yeast, humans and plants. Efficient clearance of misfolded glycoproteins from the ER lumen requires a bipartite signal composed of a specific N-glycan degron and a polypeptide degron.

1.1 The N-glycan degron

For persistently non-native glycoproteins the initiation of ERAD requires the cleavage of mannose residues by specific α-mannosidases in the ER. In the context of mammalian cells, ER-degradation-enhancing α-mannosidase-like (EDEM) 2 cleaves a mannose residue from

the B-branch (**Figure 1B**). This is subsequently followed by mannose trimming on the C-branch, which is catalysed by EDEM1/EDEM3 ***(8, 9)***. In *Saccharomyces cerevisiae*, the mannosidase-like HTM1 enzyme removes the mannose from the C-branch ***(7)***. In the model plant species *Arabidopsis thaliana*, two α-mannosidases (MNS4 and MNS5) are involved in this step ***(10)***. In all these species, the trimming of mannose results in the formation of an exposed α1,6-mannose residue on the C-branch (**Figure 1B**). This glycan moiety is subsequently recognised by the ER-resident mannose-binding lectin YOS9 (yeast), OS9 or XTP3B (humans) or OS9 (plants). The lectin binding directs the misfolded glycoprotein to the membrane-embedded HRD1 E3 ubiquitin ligase complex for retrotranslocation, ubiquitination and proteasomal degradation in the cytosol ***(11, 12)***. The subsequent steps of the ERAD pathway have been extensively documented in yeast, human and the model plant *Arabidopsis thaliana*. In contrast, the recognition step that initiates mannose trimming by the aforementioned enzymes remains less well understood and is hypothesised to involve the recognition of a non-native polypeptide conformation by α-mannosidases and associated proteins like protein disulfide isomerases ***(13, 14)***.

1.2. The polypeptide degron

The present methods will focus on polypeptides with lesions in a luminal domain (ERAD-L substrates). In the plant kingdom, the aforementioned lesions that are known to target glycoproteins to ERAD comprise the substitution of a single cysteine residue (SUBEX-C57Y; BRI1-5) ***(15, 16)*** and the presence of an additional hydrophobic amino acid in a conserved repeat or β-strand (SUBEX-V64M, BRI1-9) (**Figure 1C**) ***(17, 18)***. In properly folded secretory proteins, cysteines typically from disulfide bonds. Mutation of a single cysteine residue generates and unpaired cysteine which destabilises the protein. In the case of SUBEX-C57Y it has been demonstrated that the formation of the N-glycan degradation signal and the presence of the C57Y mutation are both prerequisites for HRD1 complex mediated ERAD (**Figure 1D**) ***(15)***. In this study, we present protocols designed to identify and characterize glycan-dependent ERAD substrates in plants.

2. Materials

1. *Arabidopsis thaliana* seeds with characterized defects in lipid-linked oligosaccharide assembly can be purchased from the Versailles Arabidopsis Stock Center (e.g., *alg12* - ID: FLAG_310A12; https://publiclines.versailles.inrae.fr/) ***(19)***. Seeds with defects in ERAD are available from the European Arabidopsis Stock Center (*os9* - NASC ID: N529413; *hrd3* - NASC ID: N663635; *mns4 mns5* - double mutant can be obtained by crossing of NASC ID N656907 and NASC ID N162962, https://arabidopsis.info/BasicForm) ***(10)***.
2. *Nicotiana benthamiana* wild type or the glycoengineered ΔXT/FT line that is deficient in the formation of complex N-glycans with core α1,3-fucose ***(20)*** can be requested from the Strasser group.
3. Safe-lock microcentrifuge tubes
4. Mixer mill with steel beads.
5. Container with liquid nitrogen.
6. Refrigerated bench-top centrifuge with a relative centrifugal force of 10.000 or higher and a rotor for 1.5/2 mL microcentrifuge tubes.
7. Thermo block.
8. Orbital shaker.
9. Vertical electrophoresis system.
10. Tank transfer system.
11. Power supply with at least 100V and 400 mA.
12. SDS-PAGE (10 to 12%) gels.
13. Tris/glycine/SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.
14. 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.
15. Pre-stained protein marker, for example Peqlab peqGOLD Protein Marker IV (Avantor).
16. Tris/glycine/methanol transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol.
17. Gel blotting paper.

18. Nitrocellulose blotting membrane, for example Amersham™ Protran® Premium Western Blotting membrane (Cytiva).
19. 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.
20. PBST: 1x PBS + 0.1% (v/v) Tween 20.
21. 1x TBS: Tris buffered saline (25 mM Tris, pH 7.5, 150 mM NaCl, 2 mM KCl).
22. TBST: 1x TBS + 0.1% (v/v) Tween 20.
23. Blocking solution for immunoblots: TBST + 5% (w/v) skimmed milk powder or PBST + 3% (w/v) BSA.
24. Western blotting detection reagent: for example SuperSignal West PICO Plus (Thermo Scientific) or AgriseraECL BRIGHT (Agrisera) chemiluminescent substrates.
25. Mouse monoclonal GFP antibody (Roche: 11814460001). 1:2000 diluted in PBST + 3% (w/v) BSA.
26. Mouse monoclonal actin antibody (Sigma-Aldrich: A0480). 1:3000 diluted in PBST + 1% (w/v) BSA.
27. Mouse monoclonal α-tubulin antibody (Sigma-Aldrich: T6074). 1:5000 diluted in PBST + 1% (w/v) BSA.
28. Mouse monoclonal RFP antibody (Chromotek: 6g6). 1:2000 diluted in PBST + 0.5% (w/v) BSA.
29. Anti-mouse IgG-peroxidase (such as Sigma A9044): 1:10000 diluted in TBST for detection of GFP, actin, tubulin and RFP.
30. Endoglycosidase H (Endo H, 500.000 units/mL, New England Biolabs), Glycobuffer 3 (New England Biolabs) and 10x Glycoprotein Denaturing Buffer (New England Biolabs).
31. Empty microspin chromatography columns.
32. Kifunensine (Santa Cruz Biotechnology): class I α-mannosidase inhibitor, dissolved in ultrapure water.
33. MS medium: 1/2 x Murashige & Skoog (MS) medium (Duchefa) including MES buffer supplemented with 0.8% (w/v) agar and 1 % (w/v) sucrose.

34. MS liquid medium: 1/2 x MS medium including MES buffer supplemented with 1 % (w/v) sucrose.
35. Ponceau S (Sigma-Aldrich) in 5 % (v/v) acetic acid.
36. Fusion XS (Vilber) chemiluminescence imaging system.
37. Flatbed scanner.
38. Cycloheximide (Sigma-Aldrich).
39. 6 well plates.
40. Infiltration buffer: 50 mM MES, pH 5.6, 2 mM Na_3PO_4 $12H_2O$, 5 mg/mL D-glucose, 0.1 mM acetosyringone (Sigma-Aldrich).
41. OD_{600} photometer and cuvettes.

3. Methods

3.1 Monitoring of N-glycan dependent protein degradation in Arabidopsis seedlings

1. Respective *A. thaliana* seeds were germinated on 1/2 x MS agar plates supplemented with 1 % (w/v) sucrose. Plates were incubated for 8-12 days (*see* **Note 1**) in a dedicated growth chamber at 21°C under controlled light (16 h light/8 h dark) conditions.
2. 8-to-12-day-old *A. thaliana* seedlings were detached from the plates and submerged in 6 well plates filled with MS liquid medium supplemented with 20 µM kifunensine or as a control in liquid MS medium without kifunensine.
3. Incubate at 21°C under controlled light (16 h light/8 h dark) conditions with gentle shaking on an orbital shaker for a duration of 24 hours.
4. Harvest 10–100 mg of plant material (e.g., seedlings) from the mutants and *A. thaliana* wild type, then transfer the plant material to 2 mL safe-lock microcentrifuge tubes containing two 5 mm diameter steel beads per tube.
5. Submerge the 2 mL tubes containing the plant material in a container of liquid nitrogen. Remove the tubes after 3 minutes, place them in a mixer mill and run the mixer mill for 2 minutes at an amplitude of 50–60. Add 4 µL of PBST extraction buffer for every mg of plant

material. Mix briefly and transfer the mixture to a 1.5 mL tube. Incubate on ice for 15 minutes, inverting the tube every 3 minutes.

6. Centrifuge twice for 15 minutes at 9600 x g at 4 °C, transferring the supernatant to a new tube each time. Mix the samples with 3x Laemmli sample buffer and heat to 95 °C for 5 minutes. Load the SDS-PAGE gel with 20 µL (*see* **Note 2**) and run the protein extract for 1.5 hours at 100 V. In order to monitor the accumulation of the ERAD substrate, immunoblotting should be performed with appropriate antibodies (e.g., GFP antibody for ERAD substrate fused to GFP).
7. For a quantitative approach, the same membrane should be probed with an antibody directed against an unrelated control protein (e.g., actin or tubulin) and the membrane should be stained with Ponceau S solution to visualize the total protein in each lane (*see* **Note 3**). Detection of the signals on the membrane is done using a chemiluminescence imaging system and Ponceau S stained membranes are scanned using a flatbed scanner.
8. An increased signal of the ERAD substrate protein in the protein extracts of seedlings that were incubated with kifunensine indicates the involvement of a glycan-dependent degradation pathway that depends on trimming of mannose residues by class I α-mannosidases (*see* **Note 4**) (**Figure 2A and 2B**).
9. To substantiate this finding, the misfolded glycoprotein can be stably expressed in different genetic mutants (e.g., *mns4 mns5, alg12 and os9*), which have distinct defects in glycan-dependent ERAD ***(15)***. In lines with blocked glycan-dependent ERAD there is no increase of the misfolded glycoprotein in the presence of kifunensine (**Figure 2A and 2B**).
10. A fast approach to assess the impact of kifunensine on an expressed protein is the transient expression of the glycoprotein by agroinfiltration of *N. benthamiana* leaves. An overnight culture of Agrobacteria carrying an expression vector for the misfolded glycoprotein is harvested by low-speed centrifugation. The Agrobacteria are resuspended in infiltration buffer (final OD_{600} = 0.1 to 0.3) and infiltrated with a needle-free syringe into the abaxial side of 5-week-old leaves. After 24 hours, 20 µM kifunensine in infiltration buffer without acetosyringone is infiltrated into the same leaf area and after another 24 hours the

infiltrated leaves are detached form the plants. Homogenization, protein extraction and immunoblotting are carried out as described for *A. thaliana* (**Figure 2C**).

3.2. CHX treatment of seedlings

1. *A. thaliana* seedlings are grown as described in 3.1.
2. Prepare a cycloheximide (CHX) working solution (final concentration 100 µg/mL) by dilution of a 100 mg/mL stock in liquid MS medium.
3. 7-to-9-day-old seedlings are removed from the plate and transferred to 6 well plates filled with 6 mL/per well liquid MS medium supplemented with CHX.
4. Incubate plates on an orbital shaker with gentle shaking (60 rpm).
5. Harvest 200 mg of seedlings at different time points (e.g., 0, 1, 2 and 4 hours), dry seedlings briefly on a paper towel and transfer the seedlings into 2 mL safe-lock microcentrifuge tubes with steel beads. Freeze in liquid nitrogen, extract and analyze the proteins as described in 3.1. Analyze the abundance of an unrelated protein (e.g., actin or tubulin) at all time points (**Figure 2C**) and normalize the protein signal of the misfolded protein to the unrelated protein to obtain quantitative data.
6. Compare the degradation of the misfolded glycoprotein in different genetic backgrounds or in the presence of kifunensine or proteasome inhibitors.

3.3 Characterization of ER-type oligomannosidic N-glycans

It is a well-established fact that ER-retained proteins typically bear oligomannosidic N-glycans. This phenomenon occurs because these proteins do not come into contact with the Golgi-localised machinery responsible for processing of complex N-glycans ***(6)***. Oligomannosidic N-glycans are susceptible to digestion by endoglycosidase H (Endo H), an enzyme that cleaves the oligosaccharide at the chitobiose core. Consequently, digestion of oligomannosidic N-glycans by Endo H will result in a reduction in molecular weight, which can be monitored by SDS-PAGE and immunoblotting (**Figure 3A**). By contrast, proteins that traffic through the Golgi apparatus usually carry complex N-glycans which are resistant to Endo H digestion and do not

display a similar change in molecular weight. N-glycans processed in the Golgi apparatus in mammals can be cleaved off by PNGase F digestion. However, this enzyme is blocked by the core α1,3-fucose commonly found on plant complex N-glycans. ***(21, 22)*** (*see* **Note 5**).

1. Incubate 22.5 µL of the protein extract (1-20 µg of protein) with 2.5 µL 10x Glycoprotein denaturing buffer for 10 minutes at 95°C, transfer to ice and cool for 5 minutes (*see* **Note 6**).
2. Mix 22.5 µL of the denatured glycoproteins with 3 µL 10x Glycobuffer 3 (NEB), 3 µL ultrapure water and 1.5 µL Endo H (*see* **Note 7**). For the control reaction replace Endo H with ultrapure water.
3. Incubate for 120 minutes at 37°C and stop the reaction by heating to 95°C for 5 minutes.
4. Load samples on the SDS-PAGE gel and perform immunoblotting, for example, with a GFP antibody to detect the ERAD substrate protein fused to GFP.
5. To confirm the presence of the oligomannosidic N-glycans and the exposed α1,6-mannose residue, the misfolded glycoprotein can be purified from total soluble proteins by affinity chromatography using (e.g., a GFP-nanobody coupled to agarose beads is commonly used to purify GFP-tagged proteins ***(15)***). The purified glycoprotein can be proteolytically digested and glycopeptides subjected to LC-ESI-MS analysis to obtain the site-specific glycan composition (**Figure 3B**) ***(15, 23)*** (*see* **Note 8**).

4. Notes

1. The seedlings can also be younger or slightly older and similar protocols are available for detached leaves from plants grown on soil ***(11)***. Seedlings older than 10 days typically have longer roots that can be recalcitrant to fully submerging in the 6 well plates filled with MS liquid medium.
2. Loading the same volume here usually produces highly comparable total protein amounts. Therefore, it is normally not necessary to measure the protein concentration prior to SDS-PAGE.

3. Bands can be quantified and the signal intensity can be normalized by the signal intensity of the detected control protein or Ponceau S. Be aware that the linear range for quantification of bands is typically quite narrow. Densitometric analysis can be performed using a specific software provided by the used imaging system like the Fusion EvolutionCapt software (Vilber) or the Fiji open-source image processing package based on Image J.
4. Quantitative RT-PCR (RT-qPCR) may be performed to rule out that kifunensine has any effect on the transcription of the ERAD substrate.
5. Expression of the glycoprotein in plants that are deficient in the formation of the core α1,3-fucose are available ***(20, 22)*** and can be used to examine the presence of complex N-glycans with PNGase F digestion.
6. Denaturation of the glycoproteins is necessary to facilitate the access of the endoglycosidase to all N-glycans.
7. Oligomannosidic N-glycans have been identified on secretory proteins that do not undergo trafficking through the Golgi, for instance as a consequence of direct ER-to-vacuole protein trafficking. Furthermore, 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 secreted or plasma membrane-anchored proteins. Consequently, further experimentation is required, such as confocal microscopy of fluorescent fusion proteins, to confirm the ER localization.
8. To distinguish between different isobaric mannosidic N-glycans, *in vitro* digestion with specific α1,2-, α1,3- or α1,6-mannosidases might be required prior to MS analysis. Alternatively, released N-glycans can be subjected to porous graphitic carbon (PGC)-lquid chromatography (LC) in conjunction with MS analysis ***(24)***.

Acknowledgements

We would like to thank Yun-Ji Shin (BOKU University) for help with immunoblots. We thank Daniel Maresch (BOKU Core Facility Mass Spectrometry) for help with conducting MS

experiments. This work was funded by the Austrian Science Fund (FWF) P35621 (Grant-DOI 10.55776/P35621).

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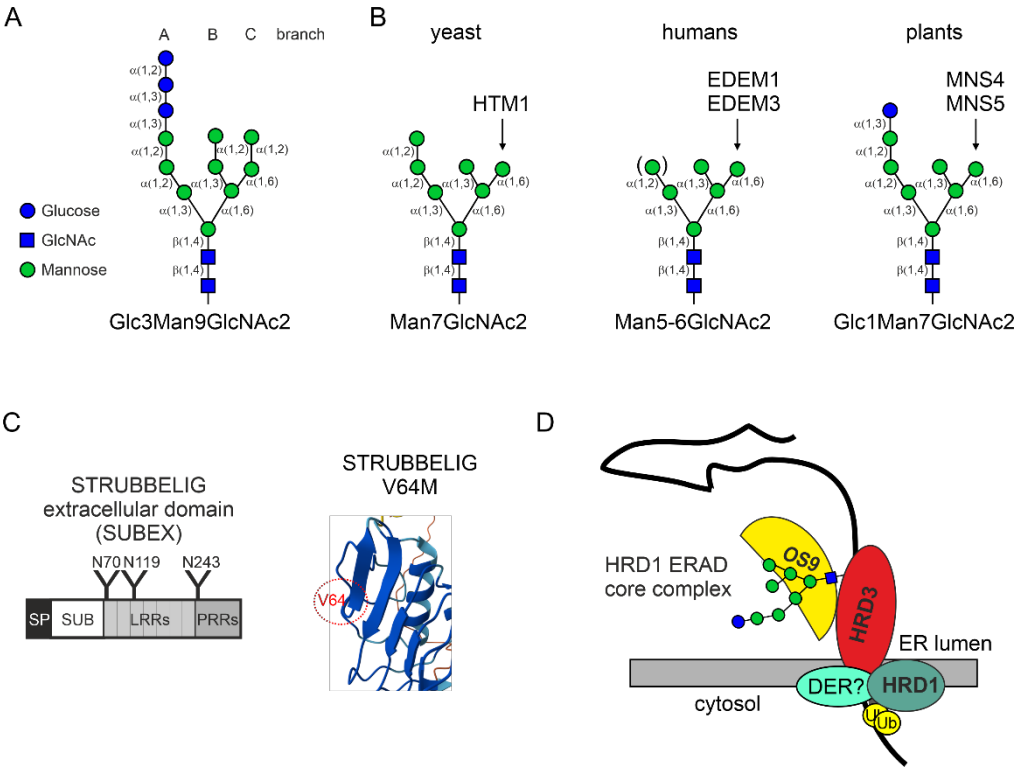

Figure 1. (**A**) Schematic representation of the initially transferred oligosaccharide with the three branches. (**B**) Processed N-glycans exposing the α1,6-mannose residue on the C-branch. This N-glycans have been detected on glycoproteins subjected to ERAD in different species. The α-mannosidases (HTM1, EDEM1, EDEM2, MNS4, MNS5) responsible for the generation of the glycan degradation signal are indicated. (**C**) Illustration of the domain structure of the extracellular domain from Arabidopsis STRUBBELIG (UniProt: Q8RWZ1). The N-glycosylation sites are indicated. SP, signal peptide; SUB-domain; LRR, leucine-rich repeat; PRR, proline-rich repeat. A snapshot from the predicted STRUBBELIG structure is given to illustrate the location of the V64 amino acid that is mutated in SUBEX-V64M (Image was taken from the AlphaFold Protein Structure Database - AF-Q8RWZ1-F1-v4) ***(25)***. (**D**). Schematic illustration of the plant HRD1 core complex consisting of the lectin OS9, the adaptor protein HRD3, the E3 ubiquitin ligase HRD1 and membrane-embedded DER proteins that are likely involved in retrotranslocation. The misfolded protein with the attached glycan is shown in black.

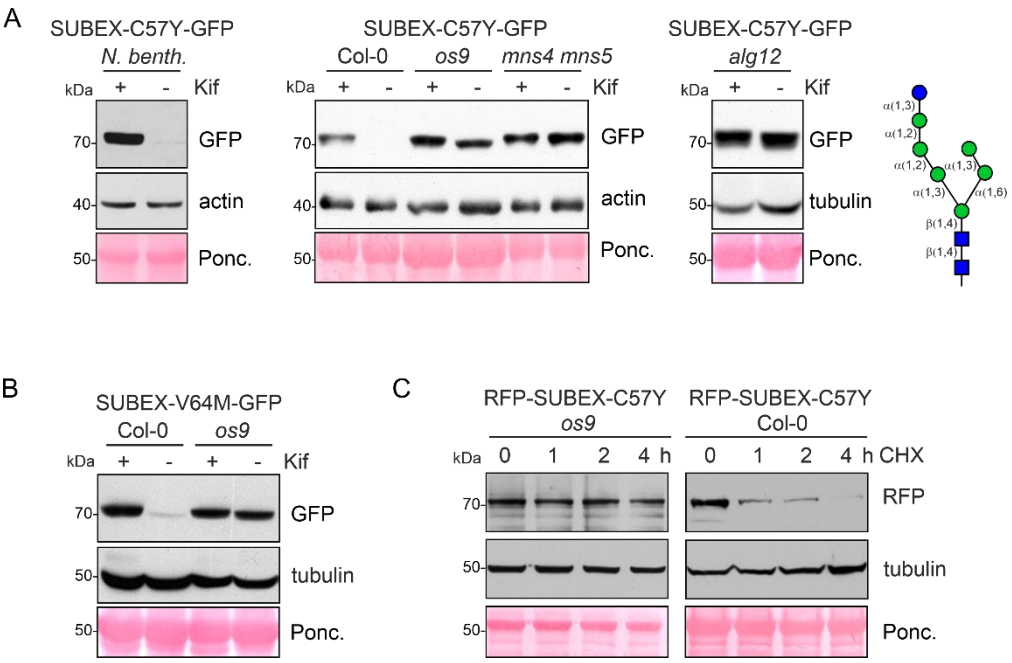

Figure 2. (**A**) Immunoblot analysis of the ERAD substrate SUBEX-C57Y-GFP. Leaves of infiltrated *N. benthamiana* or Arabidopsis seedlings from Col-0 wild type, *os9*, *mns4 mns5* or *alg12* ERAD mutants stably expressing SUBEX-C57Y-GFP were incubated with/without kifunensine (Kif). Protein extracts were subjected to SDS-PAGE and immunoblotting. Detection of endogenous actin or α-tubulin serves as a control. The proposed N-glycan structure present on misfolded glycoproteins in the *alg12* mutant is shown by the illustration. The protein is not subjected to ERAD because it lacks the free α1,6-mannose. (**B**). Immunoblot analysis of the ERAD substrate SUBEX-V64M-GFP stably expressed in Col-0 or *os9*. (**C**) Cycloheximide (CHX)-chase assay. Seedlings were incubated for the indicated time points in CHX and subsequently subjected to immunoblotting.

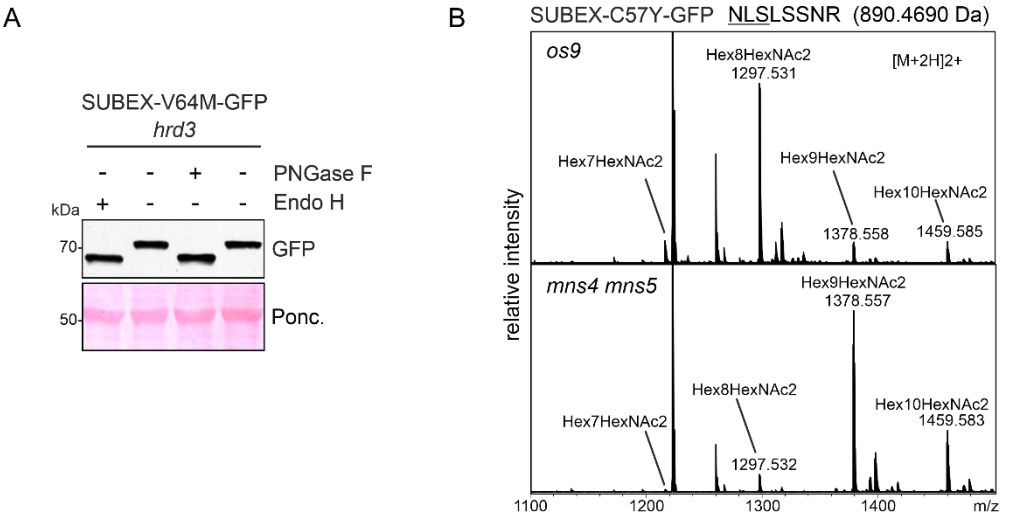

Figure 3. (**A**) Endoglycosidase (Endo H or PNGase F) digestion of protein extracts from the Arabidopsis *hrd3* ERAD mutant stably expressing SUBEX-V64M-GFP. The shift in mobility following Endo H digestion indicates the presence of oligomannosidic N-glycans, which are characteristic of ER-retained proteins. (**B**) LC-ESI-MS analysis of the SUBEX-C57Y glycopeptide carrying the N-glycosylation site Asn119. SUBEX-C57Y-GFP was purified from the indicated Arabidopsis mutants. The N-glycan from the *mns4 mns5* mutant carries an additional hexose residue which is consistent with the mannose trimming activity that leads to the formation of the exposed α1,6-mannose residue on the C-branch.