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Endoplasmic reticulum-associated N-glycan degradation of cold-upregulated glycoproteins in response to chilling stress in *Arabidopsis*

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Summary

- N-glycosylation has a great impact on glycoprotein structure, conformation, stability, solubility, immunogenicity and enzyme activity. Structural characterization of N-glycoproteome has been challenging but can provide insights into the extent of protein folding and surface topology. We describe a highly sensitive proteomics method for large-scale identification and quantification of glycoproteins in *Arabidopsis* through ¹⁵N-metabolic labeling, selective enrichment of glycopeptides, data-dependent MS/MS analysis and automated database searching.
- In-house databases of *Arabidopsis* glycoproteins and glycopeptides containing Asn-X-Ser/Thr/Cys motifs were constructed by reducing 20% and 90% of the public database size, respectively, to enable a rapid analysis of large datasets for comprehensive identification and quantification of glycoproteins and heterogeneous N-glycans in a complex mixture.
- Proteome-wide analysis identified *c.* 100 stress-related N-glycoproteins, of which the endoplasmic reticulum (ER) resident proteins were examined to be up-regulated. Quantitative measurements provided a molecular signature specific to glycoproteins for determining the degree of plant stress at low temperature.
- Structural N-glycoproteomics following time-course cold treatments revealed the stress-responsive degradation of high-mannose type N-glycans in ER in response to chilling stress, which may aid in elucidating the cellular mechanisms of protein relocation, transport, trafficking, misfolding and degradation under stress conditions.

Keywords

Arabidopsis; chilling stress; endoplasmic reticulum (ER); glycoproteomics; liquid chromatography-tandem mass spectrometry

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Author contributions

Y.-M.S., J.L. and J.-K.Z. planned and designed the research. J.M. and J.S. performed the glycoproteomics experiments. D.W. conducted western blots. J.M., D.W. and J.S. analyzed the data. Y.-M.S., J.L. and J.-K.Z. wrote the manuscript.

Introduction

Degradation of misfolded proteins, through polyubiquitination and degradation in the ubiquitin–proteasome system, is one of the most important plant responses to mitigate the damage caused by accumulation of misfolded proteins under environmental stresses such as cold, heat, drought, salinity, and infections by bacteria and viruses. Proteins fail to fold correctly or function properly as a result of the abnormality of glycan structures or the absence of glycosylation. Glycosylation regulates protein folding, stability, solubility, biogenesis and enzymatic activity, but also prevents proteins from proteolytic degradation. In plants, N-glycosylation of proteins also plays a functional role to regulate endoplasmic reticulum quality control (ERQC), stress tolerance and signal transduction (Ceriotti *et al.*, 1998; von Schaewen *et al.*, 2008; Russell *et al.*, 2009). However, the structural relationship of glycan isoforms with various environmental stresses and developmental biology of plants remains poorly understood. The characterization of N-glycoproteome is of particular importance for understanding the N-glycan distribution, structural topology, cellular location, and molecular adaptation in plant stress responses.

Biosynthesis of plant N-glycans commences with the initial transfer of a dolichol lipid-linked oligosaccharide precursor (i.e., Glc₃Man₉GlcNAc₂) to the asparagine residues localizing at a structural motif Asn-X-Ser/Thr/Cys (to a lesser extent at Cys, X is any amino acid except proline) of nascent proteins in the ER by oligosaccharyltransferase (OST). The terminal glucoses and mannoses are then sequentially trimmed by the ER glucosidases and mannosidases (MNSs) to generate a high-mannose type of N-glycans (Man_{5–9}GlcNAc₂) (Supporting Information Fig. S1) (Rayon *et al.*, 1998; Pattison & Amtmann, 2009). ERQC, through binding of N-glycoproteins to the ER chaperones by calnexin (CNX) and calreticulin (CRT), recognizes unfolded or misfolded protein intermediates to be retained in the ER for another cycle refolding. If this fails, the accumulated misfolding proteins are subsequently targeted and translocated for ER-associated degradation (ERAD). Correctly folded glycoproteins are transported to the Golgi, plasma membrane and other subcellular compartments followed by addition of N-acetylglucosamine, fucose and xylose by N-acetylglucosaminyltransferase (GNT1), fucosyltransferase (α 1,3 FucT) and xylosyltransferase (β 1,2 XylT) to form complex-, hybrid- and paucimannose-type N-glycans in mature proteins (Rayon *et al.*, 1998; Pattison & Amtmann, 2009). As such, N-glycan processing leads to ER-resident glycoproteins having only the high-mannose type N-glycan attachment (e.g. Man_{8–9}GlcNAc₂), whereas the glycoproteins localized in the Golgi apparatus and vacuole have hybrid-, complex-type or paucimannosidic N-glycans on different glycosylation sites. Thus, the presence of distinct glycan modifications on a particular protein of interest could be indicative of the protein at different subcellular locations, whereas variable glycan structures often represent the folding state of glycoproteins in the ER and other cellular environments. Relative abundances of N-glycan isoforms of a protein rely heavily on the protein surface accessibility and the intermolecular interaction with the processing enzymes and glycosyltransferases (Rayon *et al.*, 1998).

In *Arabidopsis*, c. 80% of the gene-encoded proteins are predicted to possess potential N-glycosylation sites based on the Asn-X-Ser/Thr/Cys motif. Previous findings showed that

tunicamycin, as a glycan inhibitor, could induce an unfolded protein response (UPR) of the N-glycan-deficient protein substrates, resulting in growth inhibition of the treated plants (Koizumi *et al.*, 1999). To investigate the physiological significance of different types of N-glycans, several plant mutants of triple knockout MNSs (*mns 123*), N-acetylglucosaminyltransferase (also termed the complex glycan 1, *cgl1*), GDP-D-mannose-4,6-dehydratase fucose (*mur1*) and staurosporin-temperature-sensitive 3-like enzymes (*stt3a/b*), had been utilized to control the biosynthesis of N-glycans in *Arabidopsis*. The *mns123* mutant produced only the high-mannose N-glycan of Man₉GlcNAc₂ on all glycoproteins, and had a phenotype of short root length and small size (Liebminger *et al.*, 2009; Hüttner *et al.*, 2014). Likewise, the *cgl1* mutant accumulated Man₅GlcNAc₂ but no complex glycans on glycoproteins owing to the lack of GnT-I, and displayed a dwarf phenotype and salt-sensitive root growth in rice (Fanata *et al.*, 2013). A study by Kang *et al.* (2008) also reported that the plasma membrane protein, KOR1/RSW2, required N-glycans for its proper function *in vivo*. In the *mur 1* mutant, the plant lacked the GDP-fucose synthesis in glycoproteins but retained a normal phenotype assuming the phenomenon was caused by the galactose replacement of fucose at the side chain of N-linked glycan branches (Bonin *et al.*, 1997; van Hengel & Roberts, 2002). The plant, in the absence of the STT3a gene, displayed a salt-sensitive phenotype, and the double knockout mutant of STT3a/b resulted in gametophytic lethality (Koiwa *et al.*, 2003; Pattison & Amtmann, 2009). As a consequence, N-glycosylation functions in regulating plant growth and development through maintaining the proper structural folding of proteins, and the absence of N-glycan enzymes has been associated with the UPR to salt and osmotic stresses (Koiwa *et al.*, 2003; Kang *et al.*, 2008; von Schaewen *et al.*, 2008; Hüttner *et al.*, 2014).

Proteomic methods have been utilized for global characterization of N-glycoproteins, providing valuable information regarding the structure, location and relative abundance of N-glycan isoforms to unravel the secretory pathway related to plant stresses. Such an analysis has been enhanced by the emergence of advanced mass spectrometric technologies with highly improved sensitivity and accuracy, although a full examination of glycoproteome in plants remains a formidable technical challenge owing to the diversity of multiple protein isoforms; the heterogeneity of N-glycan isomeric structures occupying the same site of a protein; the huge variability of glycosylated, nonglycosylated and truncated proteins in dynamic expression; the low intensity of glycopeptide detection; and the complexity of the database search involving the number of protein isoforms and N-glycan structures (Marino *et al.*, 2010; Song *et al.*, 2011). Traditionally, glycoproteins are digested by an endoproteinase and the resulting glycopeptides are captured by a lectin column (e.g. ConA) or hydrophilic interaction liquid chromatography (HILIC). To increase the sensitivity of detection, the negatively charged glycan portion of glycopeptides is often removed by endoglycosidases such as PNGase F, PNGase A or Endo H, to generate completely or partially deglycosylated peptides for mass spectrometric analyses (Song *et al.*, 2013). The technique is feasible to identify N-glycosylation sites of glycopeptides, but misses the structural information of N-glycan isoforms. A full characterization of glycoproteins requires analyses of intact glycopeptides to annotate both peptide sequences and the attached glycans at the side chains. Toward this goal, multistage mass spectrometry (MSⁿ) has been used to identify the complex glycan structure under the low-energy collision-induced

dissociation (CID, MS²) or the higher-energy collision-induced dissociation (HCD), followed by MS³ analysis of high-intensity monoglycosylated peptide precursor ions. The method achieved huge success in the analysis of purified glycoproteins with sufficient starting materials; however, the proteomic application is rather limited owing to the low sensitivity of MS³ acquisition. Alternative approaches using either electron-capture dissociation or electron-transfer dissociation for analyzing intact glycopeptides are promising, but the techniques suffer from a low fragmentation efficiency which requires multiply charged precursor ions of glycopeptides to generate high-density fragments for glycopeptide sequencing. Here we describe a highly sensitive proteomics method for high-throughput structural identification of glycoproteins in *Arabidopsis* through selective enrichment of glycopeptides, data-dependent ultraperformance liquid chromatography (LC)–Orbitrap MS/MS analysis and automated database searching. The method took advantage of the high-sensitivity detection of intact glycopeptides by elucidating the glycan structure with CID and complementary peptide sequencing with HCD in parallel experiments. We also used ¹⁵N-metabolic labeling to quantitatively compare the differential expression of glycoproteins during time courses of plant growth under normal and cold-stressed conditions. The study was aimed at establishing a rapid, straightforward method for large-scale structural analyses of glycoproteins in plants, and discovering the functional role of aberrant N-glycans associated with UPR to cold stress.

Materials and Methods

Plant materials

Arabidopsis thaliana (L.) Heynh. seeds (Columbia-0) were surface-sterilized with 0.5% sodium hypochlorite solution and planted on half-strength Murashige and Skoog (½ MS) medium. After low-temperature induction (i.e. vernalization) for 2 d at 4°C, the seedlings were allowed to grow in a chamber at 22°C for 2 wk and subsequently relocated to the growth chamber at 4°C for another 6, 12 and 18 d. As a comparison, the seedlings were also continually grown at 22°C during the same period of time as control samples. The materials were collected, immediately frozen in liquid nitrogen and stored at –80°C until use.

In vivo ¹⁵N-isotope metabolic labeling of proteins in *Arabidopsis*

Metabolic labeling of proteins in *Arabidopsis* was performed according to the protocol reported previously (Skirycz *et al.*, 2011). The 1/2 MS media were prepared to contain ¹⁴N and ¹⁵N isotopes. In the ¹⁵N-incorporated medium, the heavy labeling reagents of ¹⁵NH₄¹⁵NO₃ and K¹⁵NO₃ (Sigma) were used to replace those containing ¹⁴N-isotope. *Arabidopsis* seedlings were grown at 22°C under long-day conditions (16 : 8 h, light : dark) on ¹⁵N- or ¹⁴N-isotope-containing medium, respectively. After 2 wk, the seedlings grown on the ¹⁴N medium were transferred to a growth chamber at 4°C for 6, 12 and 18 d. As such, ¹⁵N-labeled plants at 22°C were used for control experiments (i.e. forward labeling), or the time-course cold-treated plants at 4°C were also metabolically labeled by ¹⁵N-isotopes in parallel experiments (i.e. reciprocal labeling) (Kline *et al.*, 2010; Qing *et al.*, 2016). The labeled and unlabeled seedlings were harvested separately, and a mixture with the same amount of ¹⁴N- and ¹⁵N-isotope-labeled tissues (w/w, 1:1) at the two different temperatures was utilized for relative quantification of proteins.

Protein extraction

Arabidopsis seedlings were ground under liquid nitrogen using a chilled mortar and pestle, and 3 g of the materials were suspended in a lysis buffer containing 4% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl (pH 7.6) and 100 mM dithiothreitol (DTT). Following ultrasonication for 10 min and boiling for 3 min to reduce disulfide-binding linkage of proteins, the crude protein extract was harvested as supernatants after centrifugation at 2935 g for 20 min. Proteins were precipitated by 10% trichloroacetic acid-aceton at -20°C overnight, and subsequently washed with cold acetone three times to remove lipids and excess trichloroacetic acid.

Protein digestion using filter-aided sample preparation method

The protein pellet was dried and then solubilized in 500 μl of 8 M urea containing 50 mM iodoacetamide to block the free cysteines of proteins, and finally transferred into a spin column filter (10 kDa MWCO; Pall Life Sciences, Ann Arbor, MI, USA). After sequential washes with 8 M urea and buffer exchanges of 25 mM ammonium bicarbonate (NH_4HCO_3), the alkylated proteins were digested by sequencing-grade trypsin (Promega) in 25 mM NH_4HCO_3 at an enzyme: substrate ratio of 1: 200 (w/w) at 37°C in a shaking incubator for 18 h. The tryptic peptides were collected after centrifugation at 13 523 g, and freeze-dried using a refrigerated CentriVap concentrator (Labconco, Kansas, MO, USA).

Glycopeptide enrichment

Glycopeptides were enriched using an HILIC microcolumn that was prepacked with FLASH HILIC resins (20–45 μm , 100 $\text{\AA}$; Bonna-Agela Technologies, Wilmington, DE, USA). The column was preconditioned with 3 ml of 0.1% trifluoroacetic acid (TFA) and equilibrated by 3 ml of 0.1% TFA/80% acetonitrile (ACN). The peptide digest was dissolved in 1 ml of 0.1% TFA/80% ACN, and then loaded onto the column. Nonglycosylated peptides were washed away with 3 ml of 0.1% TFA/80% ACN. The glycopeptides were eluted using 1.5 ml of 0.1% TFA/40% ACN, and subsequently dried using a refrigerated CentriVap concentrator.

LC-MS/MS analysis of intact glycopeptides

Samples were reconstituted in 10 μl of 0.1% formic acid (FA) and analyzed by online nanoAcquity ultraperformance LC (Waters, Milford, MA, USA) coupled with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Nanospray was controlled by a PicoView Nanospray Source (PV550; New Objective, Woburn, MA, USA) at a spray voltage of 1.9 kV. The peptides were trapped by a 2G-V/MT Trap symmetry C18 column (5 μm particles, 180 μm ID $\times$ 20 mm length) at the flow rate of 5 $\mu\text{l min}^{-1}$ for 3 min, and separated on a BEH130 C18 analytical column (1.7 μm particles, 100 μm ID $\times$ 250 mm length) at 250 nl min^{-1} . Peptides were eluted using mobile phases consisting of solvent A (0.1% FA) and solvent B (ACN/0.1% FA) through a linear gradient from 10% to 30%, and then 85% of solvent B at a duration of 60 or 120 min. Data-dependent MS/MS acquisition was performed following a full MS survey scan by Orbitrap at a resolution of 60 000 over the m/z range of 350–1800, and MS/MS measurements of the top 20 most intense precursor ions. The target values of automatic gain controls (AGC) were

set up to 200 000 for Orbitrap MS and 10 000 for ion-trap MS/MS detection. The fragmentations of the selected multiply charged ions were achieved using helium gas and argon at a normalized collision energy of 35% for CID and HCD, respectively. Dynamic exclusion was enabled for 60 s.

Identification of glycoproteins and N-glycan structure by database search

An in-house N-glycoprotein database was constructed by refining amino acid sequences of the *Arabidopsis* proteins or peptides containing the Asn-X-Ser/Thr/Cyr motif (X, not proline) from The *Arabidopsis* Information Resource (TAIR10; downloaded on 6 January 2014; 35 386 sequences) or the National Center for Biotechnology Information (NCBI) nonredundant database (downloaded on 6 January 2014; 226 526 sequences) to exclude non N-glycosylated proteins and redundant peptides, where the generated glycopeptide sequences containing two missed trypsin cleavage sites away from the N-glycosylation motif in each putative glycoprotein were collected (Fig. S2). The MS raw data from the LC-MS/MS analyses of the biological duplicate and three technical repeats were separately converted to MGF files using the Proteome Discoverer 1.4 software (Thermo Fisher Scientific Inc.) and then searched against the in-house databases using Mascot Daemon 2.4 (Matrix Science, London, UK). The search parameter for tryptic digestion was restricted to a maximum of two missed cleavages of proteins. Cysteine carbamidomethylation was designated as a fixed modification. Deamidation of Asn or Gln, oxidation of Met, and a glycan library incorporating 161 common plant N-glycans (Fig. S3) were considered as variable modifications. Mass tolerances were set up to 20 ppm for the Orbitrap-MS ions, and 0.8 Da for ion-trap MS/MS fragment ions. Peptide assignments were filtered by an ion score cutoff of 20, and the significance threshold was adjusted to 0.001 to achieve a false discovery rate (FDR) of < 1%. The retrieved glycopeptide sequences and N-glycan structures were validated by manual inspection of the CID and HCD fragments, as well as the accurate masses that calculated using the predicted sequences. Gene ontology (GO) annotation and functional categorization were conducted using the website tool at <https://www.arabidopsis.org/tools/bulk/go/index.jsp>.

Protein quantitation and validation by Western blots

Mascot Distiller software (v.2.5.1.0, Matrix Sciences, London, UK) was utilized for the accurate quantification of proteins by ¹⁵N-metabolic labeling. The MS data were centroided at a peak half-width of 0.02 and 200 points Da⁻¹. The maximum ion charge state was set to 6, and the Sum method was used as the scan group aggregation at the precursor mass range of 700–16 000 Da. MS/MS processing of the ion-trap data was centroided at a peak half-width of 0.2 and 20 points Da⁻¹, and regridded with the same value. The MS peak picking was accomplished with 500 iterations, and filtered through a correlation threshold of 0.6, minimum signal-to-noise ratio of 2, and *m/z* range from 50 to 100 000. MS peak profile was determined at a minimum width of 0.002 Da and maximum peak width of 1 Da.

The identified peptides from the MS/MS search were then imported into Mascot Distiller to generate a quantification report of light: heavy (L: H) ratios. The output of L: H ratios was thus limited to the ¹⁵N-labeled peptides that were matched with the confident sequence identification and the identical charge state. Precursor ion protocol was used for peptide

quantification, and the ratios were calculated using the peak areas of extracted ion chromatograms (XICs) based on the trapezium integration method. To improve the accuracy of the results, the impurity correction of ^{15}N -labeled reagents (98% K^{15}NO_3 and 99% $^{15}\text{NH}_4^{15}\text{NO}_3$) was incorporated into the quantification method. The protein ratios were manually examined on the abundances of ^{14}N - and ^{15}N -containing peptides based on the valid sequence identification by Mascot search and calculation by the integrated XIC peak area or the intensity. Reliable quantification of individual proteins was achieved by the mean $\pm$ SD value of triplicate experiments on either the glycopeptides or minimum two nonglycopeptides free of methionine (/oxidized methionine).

The relative ratio of protein abundances was validated by western blots. The *Arabidopsis* proteins were extracted by a buffer containing 4% SDS, 100 mM DTT, and 100 mM Tris-HCl (pH 7.6), and then centrifuged for 20 min at 4°C. Protein concentrations were determined by Bradford assay (Bio-Rad); equal amounts of samples from the control and the cold-treated materials were separated in parallel by 7% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto a polyvinylidene fluoride membrane on a transfer apparatus at 250 mA for 1.5 h. The membrane was subsequently blocked with 5% nonfat milk and incubated with primary antibodies against maize-CRTs (Pagny *et al.*, 2000), protein disulfide isomerase-like protein (PDI; Rose Biotechnology, MA, USA), and actin (CWBIO, Beijing, China) as described previously (Hong *et al.*, 2008).

The in-house databases of the modified glycoproteins and glycopeptides are publicly accessible at the website of the Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences (<http://www.psc.ac.cn/facility.asp#4>).

Results

High-throughput analysis of N-glycosylated proteins in *Arabidopsis*

N-glycans attached to asparagine residues of a protein are usually localized at the sequence motif of Asn-X-Ser/Thr/Cys. However, the presence of such a motif is often not sufficient to generate N-glycosylation at a given site in nature, which also heavily depends on the flanking structural surface topology and the close interaction with the processing enzymes and glycosyltransferases. Examination of the whole protein sequences in TAIR10 and NCBI databases indicated that *c.* 80% of them possess potential N-glycosylation sites. To annotate the putative glycoproteins, we established a proteomic approach for automated analysis of naturally existing N-glycosylated proteome in *Arabidopsis*. Fig. 1 shows the overall workflow of the experimental procedure used for glycoprotein identification and quantification. Proteins were cleaved by trypsin and the resulting glycopeptides were purified using a HILIC column for unbiased extraction of N-glycosylated species, followed by LC-MS/MS analysis. Owing to a huge data size acquired by highly frequent MS/MS scans of a sample on the Orbitrap Fusion (e.g. 3.7 GB size per 2 h run, 65 000 spectra), the routine database searching was always time-consuming. To rapidly identify heterogeneous N-glycan isoforms, we constructed two in-house databases of N-glycoproteins and N-glycopeptides of *Arabidopsis* consisting of the N-glycosylation motif. The first refinement of glycoprotein sequences reduced *c.* 20% of the database size of proteins in TAIR10 and NCBI (Fig. S4a). The second assortment of glycopeptides allowed two missed cleavage sites

of trypsin away from the N-glycosylation motif, which dramatically reduced 90.7% and 90.4% of the public database size over the total number of peptides in TAIR10 and NCBI databases, respectively (Figs S2, S4b). In addition, a plant N-glycan library collected from previous reports was also incorporated as variable modifications, which covered 161 known glycan isoforms (Fig. S3; Table S1). Automated database search of LC-MS/MS datasets combined with high-mass accuracy Orbitrap mass spectrometry thus provided a rapid method for large-scale identification of glycoproteins in a complex mixture. The retrieved N-glycan structure was verified manually by CID spectra of the precursor ions based on sequential neutral losses of monosaccharides, and the characteristic oxonium ions of m/z 204.1 [HexNAc⁺], m/z 366.2 [HexHexNAc⁺] and m/z 528.2 [HexHexHexNAc⁺] at the low mass range (Fig. 2a), whereas the peptide sequence was validated by a series of C-terminal y_n fragments and N-terminal b_n fragments derived from the HCD spectra of the corresponding precursor ions (Fig. 2b).

Measurements of a tryptic digest of glycoproteins from wild-type *Arabidopsis* reliably identified 2000 glycopeptides resulting from a 2 h gradient LC-MS/MS run. Parallel analyses were conducted on three sets of *Arabidopsis* seedlings grown at two different temperature conditions (22 and 4°C) for periods of 6, 12 and 18 d (Fig. S5). We identified 912 glycoproteins by Mascot database search at the FDR < 1%, c. 90% of which were shared in each biological sample. A total of 100 glycoproteins were found to be related to abiotic and biotic stresses based on the GO functional annotation (Fig. 3a). Some proteins have been reported to be involved in cold stress responses. The GO cellular categorization revealed a wide range of glycoprotein locations, especially in ER, plasma membrane and cell walls (Fig. 3b). The identified N-glycans of high-mannose, hybrid, paucimannosidic and complex-type are summarized in Fig. 3(c). Compared with those variations of N-glycans (labeled with a dashed pink line in Fig. 3c) isolated from the plants grown under the normal condition as a control, the relative distribution of high-mannose N-glycans (labeled with a yellow line in Fig. 3c) dramatically increased with the time period of cold treatments.

N-glycan structure and functional annotation of glycoproteins

Glycoproteome analysis revealed that many glycoproteins are encoded by genes involved in plant defense responses to abiotic stresses, including temperature and salts (Table S2). These included CRT1b, PDIL1-1, thioglucoside glucohydrolases, UDP-glucose-glycoprotein glucosyltransferase (UGGT), germin-like proteins, beta-glucosidase 35 and STT3a/b. The N-glycosylation sites of these proteins were all occupied by exclusively oligomannosidic glycoforms of Man₈₋₉GlcNAc₂, which support the cellular location as ER-resident proteins. By contrast, the presence of only complex N-glycans, instead of mannose-type, in the receptor-like protein kinases (e.g. RLK, AT5G16590) implies the continuous N-glycan processing of the glycoproteins to ensure its proper folding and subsequent transfer from ER to Golgi apparatus.

Glycosylphosphatidylinositol (GPI)-anchor functions in the attachment of proteins to the surface of the membrane. LC-MS/MS analysis reliably identified two distinct glycopeptides of ICSIHSSNLTSSSCPVINVDEFESTVDTAK and NKPCTND-TGSATVPGTEPQFANYPQILAK with the different types of N-glycoforms

Man₆₋₈GlcNAc₂ and (GlcNAc)₀₋₂Man₃(Xyl)(Fuc)(GlcNAc)₂, corresponding to the tryptic fragments of two uncharacterized GPI-anchored membrane proteins with the accession numbers of gi|30387511 and gi|18397452, respectively (data not shown). Considering that the GPI-anchored protein precursor normally contains an N-terminal signal peptide and a C-terminal tail of GPI attachment signal sequence (CAAX), the encoding large protein (gi|30387511, 433 aa) from the specific gene AT1G61900 possessed exclusively high-mannose N-glycans of Man₆₋₈GlcNAc₂, suggesting that the precursor protein might lose GPI signal at the C-terminus, causing the extensive accumulation mainly in ER (Rivier *et al.*, 2010). In comparison, the existence of complex-type (GlcNAc)₀₋₂Man₃(Xyl)(Fuc)(GlcNAc)₂ in the small GPI membrane glycoprotein (gi|18397452, 200 aa), derived from the gene AT3G06035, indicated that the glycoprotein sequence has an N-terminal signal peptide that is part of residues 1–106, as seen in the NCBI database (gi|24417290), which was likely cleaved, resulting in a mature protein transported to the cell surface membrane. As such, our results suggest possible subcellular location-specific GPI-anchored membrane proteins in *Arabidopsis*.

Two distinct glycosylation sites of GDSL esterase/lipase isoenzymes (AT1G54000, AT1G54010 and AT3G14210), a known cold stress-responsive protein, were identified by MS/MS analyses as the complex N-glycans of (GlcNAc)₀₋₁Man₃(Xyl)(Fuc)(GlcNAc)₂ at varying sequence regions of Asn105 and Asn146 and the high mannose-type of Man₃₋₉GlcNAc₂ at the highly conserved residues of Asn288 and Asn290, respectively (Fig. S6). Structure modeling of GDSL esterase/lipase (AT1G54000; gi|332194912) using the Phyre2 server indicated that the two glycosylation sites of Asn146 and Asn288 are all localized at the structural loop regions (Kelley *et al.*, 2015), and Asn288 is positioned nearby the cluster of four α -helices (Fig. S7). The observation of trimmed high-mannose N-glycans suggests that the Asn288-glycosylation might function in regulating the protein conformation, which is sensitive to cold stress. As reported previously (Zielinska *et al.*, 2010; Song *et al.*, 2013), the identification of heterogeneous N-glycan isoforms by mass spectrometry thus offers a method to determine the surface topology and different subcellular locations of the same protein or different enzyme isoforms.

ER-associated N-glycan degradation in the early stage of cold treatments

Among the identified 912 glycoproteins, *c.* 100 proteins are stress-related and 3.5% of these (i.e. 32 glycoproteins) are localized in the ER (Table S3). The N-glycan analyses showed that most ER glycoproteins of *Arabidopsis* grown at 22°C have merely the predominate high-mannose N-glycans of Man₈₋₉GlcNAc₂. To examine the possible effect on the glycan structure and glycoprotein misfolding caused by cold stress, we conducted a thorough inspection on N-glycan modifications of the individual ER-resident proteins (Fig. S8), except for CNXs, which are known as nonglycosylated type I integral membrane proteins (Miernyk, 1999).

As shown in Fig. 4(a), a predominate glycopeptide at *m/z* 1538.0936 (2+) was identified as a Man₈GlcNAc₂ attached to the residue Asn59 at peptide 55–67 of CRT1 (AT1G56340) of plants grown under normal conditions, whereas this unique glycopeptide was detected to have additional trimming by MNSs into heterogeneous Man₅₋₈GlcNAc₂ during plant growth

at the cold temperature of 4°C between 6 and 18 d (Fig. 4a). Similar phenomena occurred in the other ER stress-related glycoproteins, such as OST1B and UGGT; the N-glycans of these glycoproteins retained the stable glycan iso-forms of Man₈₋₉GlcNAc₂ under the normal growth condition, but degraded under the cold-stressed environment (Figs S9a, b). It is worth noting that PDIL1-1, a protein folding assistant, originally possessed a series of high-mannose N-glycans of Man₅₋₈GlcNAc₂ at 22°C, and the intensities of these trimmed N-glycans were all enhanced by the low temperature (Fig. S9c). We thus concluded that these cold-regulated ER glycoproteins all have the N-glycan degradation resulting from chilling stress.

By contrast, identical MS profiles of glycopeptides were observed on the nonregulated proteins isolated from plants grown at the two different temperatures (22 and 4°C). As shown in Fig. 4(b), the spectra of a glycopeptide of embryo defective 3012 (EMB3012) exhibited a pair of nascent peaks after expanding m/z scale, corresponding to an N-glycan of Man₈GlcNAc₂ attached to the peptide 285–304 at m/z 1240.5605 (3+) and the other component having a mass increase of 17 Da at m/z 1246.2371 (3+). Accurate mass measurements of the triply charged ions showed a difference of 17.0299 ± 0.0001 Da (Fig. 4c), matching one molecule of ammonia (calculated mass: 17.0265 Da). Based on a set of similar C-terminal y fragment ions resulting from the peptide bond cleavages in the HCD condition, MS/MS analysis of the larger peptide ion at m/z 1246.24 (3+) identified the same peptide sequence as the peptide of m/z 1240.56 (3+). The 17 Da increment at the larger peptide was localized at the terminal mannose of Man₈GlcNAc₂ by the preferential fragmentation of low-energy CID at relatively weak glycosidic bonds, which resulted in the initial neutral loss of 179 Da at m/z 1187.02 ($[M + 3H - 59.55]^{3+}$), that is, the 17 Da adduct of mannose from the precursor ion (Fig. 4d). Similar results were also obtained for a pair of tryptic glycopeptides of the transmembrane CLPTM1 family protein (Fig. S10). Accurate mass analysis on the EMB3012 glycopeptide pairs in a protein mixture of unlabeled and ¹⁵N-labeled *Arabidopsis* seedlings determined that the mass difference of 17.0280 Da (i.e. ¹⁴NH₃ adduct) between the ¹⁴N-coded glycopeptides had shifted to 18.0210 Da between the ¹⁵N-coded glycopeptide counterparts, corresponding to one molecule of ¹⁵N-labeled ammonia (theoretical mass 18.0236 Da) (Fig. S11). The reproducible results in biological triplets indicated that the modification of 17 Da on the terminal mannose of N-glycans was caused by *in vivo* ammonolysis of plants. Structural glycoproteomics therefore provided evidence for N-glycan ammonolysis of nonregulated glycoproteins in *Arabidopsis*, which might protect the N-glycan from degradation under chilling stress.

Chilling stress induces ER glycoprotein up-regulation

To further investigate the effect on protein expression by cold-induced ER stress, we used a quantitative proteomics strategy by means of the forward and reciprocal ¹⁵N-isotope metabolic labeling approaches for accurate measurements of protein abundances at the plant growth stages of 6, 12 and 18 d. Time-course qualification of glycoproteins was performed by LC-MS/MS analyses at the levels of both glycopeptides and nonglycopeptides. The protein abundances of *Arabidopsis* seedlings were compared between two plant growth environments of 22°C (heavy ¹⁵N-isotope labeled proteins) and 4°C (light ¹⁴N-isotope labeled proteins) at the same period of time, and vice versa in the reciprocal ones (Fig. 5a).

Initial analysis confirmed the similar L: H ratio of relative intensities of ^{14}N - and ^{15}N -labeled glycopeptides to that of the integrated XIC peak areas (data not shown). Using the N-linked glycopeptides of $\text{Man}_8\text{-}_9\text{GlcNAc}_2$ as references, the abundance of ER-resident glycoproteins after cold treatments for 18 d was determined to be twofold higher than that from plants grown at the normal condition. The quantitative results from LC-MS/MS analyses on the L: H ratios (more than twofold) of ^{14}N - and ^{15}N -isotope-labeled glycopeptide pairs are summarized in Table 1 (Table S4), in which the ER stress-responsive glycoproteins all showed a significant stepwise up-regulation during the time periods of cold treatments.

A similar comparison was performed on the nonglycosylated peptides using LC-MS/MS analyses, and the L: H ratios of the proteins containing an ER retention [K/R/H]DEL motif were selectively examined as shown in Table 2 (Table S5). The relative abundances of nonglycosylated peptides in the stress-responsive proteins showed up-regulated L: H ratios in the forward ^{15}N -isotope metabolic labeling and further verified by the reciprocal experiments, which were consistent with the quantitative results of glycopeptides combined from the multiple N-glycan distributions. As expected, the normal- and chilling stress-grown plants showed no differences in the overall expression levels of actin, rubisco activase, ribulose biphosphate carboxylase and other housekeeping proteins as measured by the L: H ratios at *c.* 1. By contrast, an increase of four- to sixfold in the protein abundances was determined in the amounts of PDIL1-1 (4.43), PDIL1-2 (5.39) and PDIL1-4 (4.52). In terms of the chaperone-like activity of protein disulfide isomerases (Cai *et al.*, 1994), the finding suggests that a large amount of PDI isoforms are required to help the refolding of unfolded or misfolded proteins caused by chilling stress.

To ensure the reliability of the quantitative proteomics method, we also used western blotting to verify the expression level of stress-responsive proteins when reliable antibodies were available. A direct comparison of densitometry analyses on the protein bands of plant materials at 0, 6, 12 and 18 d determined the relative abundance change, which was equivalent to that of up-regulated proteins detected by LC-MS/MS quantification (Fig. 5b). The small amount of low-molecular-weight protein bands in Fig. 5(b) was also visualized by western blots to have an increasing abundance with the time of cold treatments of 12 and 18 d for CRT and PDI, but not nonglycosylated CNX and actin, suggesting the existence of degraded products of the glycoproteins. Thus, the data show that LC-MS/MS analysis of glycoproteins using ^{15}N -metabolic labeling provides a reliable and accurate method for comparing the difference in abundance of stress-related glycoproteins between plants grown under normal and stressed conditions.

Discussion

Structural N-glycoproteomics targets proteins in response to chilling stress

Endoplasmic reticulum stress is one of the important responses to mitigate the plant damage caused by adverse environmental conditions, and is activated by unfolded proteins that accumulate in the ER (Howell, 2013). The rapid determination of stress-responsive protein targets and the quantitative assessment on the extent of stress-caused protein misfolding represent a significant bottleneck currently in plant stress proteomics research. Recent

advances in mass spectrometry allowed measurements of protein folding, unfolding intermediate and conformational change using hydrogen-deuterium exchange of protein backbone amides and LC-MS/MS (Engen & Wales, 2015); the technology has proved successful but possesses drawbacks such as difficult sample handling, hydrogen/deuterium scrambling, a back-exchange issue and complicated data interpretation from the nonspecific protease cleavage of proteins in the acidic condition (e.g. pepsin) (Hoofnagle *et al.*, 2003). The utility of this method is therefore restricted to purified proteins or isolated protein complexes. As an alternative strategy, we report here a structural glycoproteomics method for large-scale analysis of glycoproteins to provide insights into the folding status of chilling stress-responsive proteins in *Arabidopsis*.

Structural characterization of the N-glycosylated proteome through analysis of intact glycopeptides by LC-MS/MS has traditionally been difficult as a result of the large protein database and heterogeneous N-glycan structures. The unprecedented LC-MS/MS data acquisition leads to a huge data set from a complicated biological sample. To reduce the complexity of sample analysis and the time-consuming process of database search, we thus established a proteomic method based on selective HILIC enrichment of glycopeptides, and database refinements of glycoproteins or glycopeptides, as well as an N-glycan library of *Arabidopsis*. Automated LC-MS/MS identification and accurate quantification of glycopeptides and the attached N-glycans were achieved through two highly sensitive data-dependent acquisitions by CID and HCD on the Orbitrap Fusion, and *in vivo* ¹⁵N-metabolic labeling of plant materials. The stress-related protein targets were discriminated from the nonstressed proteins according to the abnormal N-glycan structure together with a significant abundance change of glycoproteins between the plants grown under two different temperature conditions. Using this method, we have identified 912 glycoproteins consisting of 2000 glycopeptides in a 2 h LC-MS/MS run, and resolved the N-glycan isoform structures of cellular location-specific proteins which enabled an in-depth understanding of structural folding and stability of ER chilling stress-responsive glycoproteins.

Chilling stress leads to ER N-glycan degradation and protein up-regulation

As described earlier, mass spectrometry-based glycoproteomic analysis provided a direct comparison on N-glycan components of the same peptide of individual proteins. The results showed that the N-glycan degradation occurred in the ER stress-regulated proteins of plants under cold treatment, but not in the nonregulated glycoproteins (Figs 4b, S10). Further quantitative analysis of MNSs (MNS1, MNS2 and MNS3) revealed the up-regulation of the proteins by increasing abundances over twofold in the chilling stress condition (Fig. S12), and this may be responsible for trimming of N-linked high-mannose of the interacting protein substrates. Protein mislocalization resulting from either the retention of partial MNSs in the ER by trafficking slowdown at low temperature or the escape of ER stress-related proteins to the Golgi could cause the N-glycan cleavage of the proteins by alpha-MNSs. The stable N-glycan forms of nonregulated glycoproteins could be contributing to the possible protection of reversible N-glycan ammonolysis from the N-glycan degradation, in which the modified terminal mannose might not be recognized by MNS for its cleavage (Fig. S13). Mannose trimming of ER stress-regulated proteins from Man₈₋₉GlcNAc₂ to Man₆₋₇GlcNAc₂ appeared as an ERAD signal for misfolded proteins (Määttä *et al.*,

2010), and the continued removal of mannose residues to Man₄₋₅GlcNAc₂ might result in protein transport and exit from the ER (Fig. S14). Our results thus suggest that ER N-glycan trimming is a consequence of plant responses to environmental stress, which may be caused by mislocalization of stress-responsive proteins. The proper regulation of protein ERQC via UPR is probably an important mechanism for plant adaptation to chilling stress.

Quantitative analyses of glycoproteins at the levels of both glycopeptides and nonglycosylated peptides showed a correlation consistent with the protein abundance changes vs the time of plant cold treatments (Tables 1, 2, S4, S5). The relative abundances of ER stress-responsive proteins were all up-regulated following a stepwise increase after the first 12 d cold treatment and then a dramatic escalation at 18 d. Among the ER glycoproteins examined, the stress-related proteins of PDIs and CRTs were of particular interest, as quantitative differences were detected ranging from four- to sixfold at the top of the list (Fig. 5a; Table 2). The heat shock protein 70 family proteins (BIP1, BIP2, HSP70) had a stable increase in the L : H ratio of one- to threefold after the initial cold treatments of 6 and 12 d followed by a dramatic increase (to fivefold) after 18 d of treatment. Considering the tight interaction of CNX, CRT and UGGT with misfolded glycoproteins, the ER chaperone-binding proteins (BIP1, BIP2, HSP70) and PDI isoforms assist in the formation of intramolecular disulfide bonds and protein refolding to prevent the proteins from misfolding and aggregation (Sung *et al.*, 2001). Our quantification results are highly consistent with previous findings on the up-regulation of PDI and HSPs in *Arabidopsis* and *Abelmoschus moschatus* grown at low temperature (Bae *et al.*, 2003; Li *et al.*, 2012). Collective evidence thus suggests that the fold change of ER stress-regulated proteins can be considered a molecular signature for evaluating the degree of plant stress.

Enhancement of plant tolerance to chilling stress

Endoplasmic reticulum stress activates the UPR signaling network and causes cell dysfunction and death (Kim *et al.*, 2008). Investigation of ER stress-induced protein misfolding and aberrant N-glycans could help us to understand the structure and function of stress-responsive glycoproteins, and to develop strategies to improve plant resistance to the ER stress-caused disorders. Based on the glycoproteomics results and the previous reports on the direct involvement of MNSs in targeting misfolding glycoproteins for ERAD to relieve ER stress (Tokunaga *et al.*, 2000; Hosokawa *et al.*, 2001; Termine *et al.*, 2009), we can speculate that there is a general plant chilling stress response pathway, as shown in Fig. 6. ER stress initially triggers the N-glycan trimming of Man₈₋₉GlcNAc₂ of stress-related glycoproteins to yield smaller Man₅₋₇GlcNAc₂ isoforms at the protein sidechains, resulting in misfolded proteins and subsequent escape from the ER lumen. With a certain amount of misfolded proteins degraded by ubiquitin–proteasome system, ER chaperones and PDI enzymes are up-regulated to maintain the proper function of ERQC and the refolding cycle of misfolded proteins during cold acclimation. With extensive cold treatment, ER stress continues to produce ERQC dysfunction and accumulation of misfolded proteins, leading to subsequent ERAD degradation of the proteins and consequently cell death and damage of plants (Kim *et al.*, 2008).

To reduce ER stress and the downstream UPR, the stress-responsive ER-resident glycoproteins were up-regulated to cope with the cold-induced disorder in ER. With the knowledge gained from this study, it may be possible to increase plant tolerance to chilling stress by enhancing ER retrieving signals. Partial escape of ER stress-related proteins resulted in dysfunction of ERQC and the refolding cycle of misfolded proteins during cold acclimation; the overexpression of the ERD2 transmembrane protein, a KDEL receptor, may be helpful to retrieve the escaped ER-resident proteins bearing the C-terminal ER retention sequence motif of [K/R/H]DEL (Table S5) from the Golgi apparatus to the ER. The protein retention could assist in the CNX/CRT chaperone function of the ERQC system. It may also be useful to develop reliable chemical chaperones to complement the refolding role of CRT, HSP70 and PDI isoenzymes to rescue the folding-defective proteins that could eventually alleviate ER-associated chilling stress for plant survival (Molinari, 2007).

Concluding remarks

We have established a highly sensitive proteomics approach for large-scale structural analysis of N-linked glycoproteins and utilized it to determine the chilling stress-related proteins in *Arabidopsis*. Our results suggest that the ER-associated N-glycan degradation serves as a signature in response to chilling stress, which could aid in elucidating cellular mechanisms of protein relocation, transport, trafficking, misfolding and degradation under stress conditions. The stable ¹⁵N-isotope metabolic labeling and LC-MS/MS analysis further provide a quantitative strategy to accurately measure the differential protein expression, and thus the effects of chilling stress on individual glycoproteins. Our observations suggest that the molecular signature of N-glycan degradation and the stoichiometry of stress-regulated proteins could be considered as criteria for evaluating the physiological effect of plants under stress conditions. The findings increase our understanding of the underlying mechanisms of ER-associated N-glycan signaling, low temperature-responsive protein regulation, and the structure–function relationship of chilling stress-related glycoproteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Experimental workflow of glycopeptide identification and protein quantification in *Arabidopsis*. *Arabidopsis* seedlings of Col-0 were treated with cold at 4 and 22°C, followed by protein extraction, enzymatic digestion, affinity purification of glycopeptides, and Orbitrap LC-MS/MS analysis. Data were analyzed by Mascot, searching against in-house databases of glycoproteins, glycopeptides and a plant glycan library. Quantification of proteins and glycopeptides was achieved by the relative intensity or the extracted ion chromatogram (XIC) peak area of ^{14}N -labeled peptides and ^{15}N -labeled counterparts. HILIC, hydrophilic interaction liquid chromatography.

Fig. 2. Identification of the intact calreticulin-1 glycopeptide at m/z 2016.06 (3+) using LC-MS/MS. The N-glycan structure was defined by the sequential losses of monosaccharides and the characteristic oxonium ions of m/z 366.2 (HexHexNAc⁺) and m/z 528.2 (HexHexHexNAc⁺) in the low-energy collision-induced dissociation (CID) mass spectrum (a). M_{ASCOT} database search identified the peptide sequence matching a series of C-terminal y_n fragments (doubly and triply charged ions) in the higher-energy collision-induced dissociation (HCD) mass spectrum (b).

Fig. 3.

Structural function, cellular localization and relative distribution of N-glycans of *Arabidopsis* N-glycoproteome obtained by LC-MS/MS. (a) Pie chart shows the functional categorization of 912 glycoproteins by gene ontology (GO) annotations in which 100 glycoproteins are stress-related. (b) Cellular localization indicated 32 stress-related glycoproteins in endoplasmic reticulum (ER). (c) The N-glycan distribution of glycoproteins determined that the cold-treated *Arabidopsis* (at 4°C) overall yielded more high-mannose N-glycans than those of plants grown at the normal growth condition (at 22°C). To help view the relative distribution change of N-glycans, the dashed pink line and the solid yellow line show the tendency of increasing percentage of high-mannose N-glycans at the time periods of 6, 12 and 18 d for the control and cold-treated plants, respectively. FDR, false discovery rate.

Fig. 4.

Discriminating the N-glycan profiles of endoplasmic reticulum (ER)-resident glycoproteins in plants under normal and cold-treated conditions for 18 d. (a) The mass spectra show the distinguishable high-mannose type N-glycans of the stress-responsive calreticulin-1 under two different conditions of plant growth: $\text{Man}_8\text{GlcNAc}_2$ (upper panel, 22°C) vs $\text{Man}_{5-8}\text{GlcNAc}_2$ (lower panel, 4°C). (b) Similar N-glycan profiles of $\text{Man}_8\text{GlcNAc}_2$ in the spectra were observed at the two glycosylation sites of Asn289 and Asn1743 of EMB3012 in plants grown under both conditions. The expanded m/z range of 1230–1260 from (b) showed the exact mass difference of 17.0299 ± 0.0001 Da between two peptide ions (c) at m/z 1240.56 (3+) and m/z 1246.24 (3+), which is close to that calculated from ammonium. (d) MS/MS spectra of the peptide precursor ion at m/z 1246.24 identified the same peptide sequence as the peptide of m/z 1240.56 at the higher-energy collision-induced dissociation (HCD, upper panel), and the additional modification of 17 Da was localized at the terminal mannose by the low-energy collision-induced dissociation (CID, lower panel) according to the initial neutral loss of 179 Da at m/z 1187.02 ($[\text{M} + 3\text{H} - 59.55]^{3+}$), that is, the 17 Da adduct of mannose from the precursor ion.

Fig. 5.

Validation of up-regulated endoplasmic reticulum (ER)-resident glycoproteins in *Arabidopsis* under chilling stress. (a) Accurate protein quantification was performed by the forward and reciprocal ^{15}N -metabolic labeling and LC-MS/MS analyses as shown of two representative proteins of calreticulin-1 (CRT1) and the protein disulfide isomerase-like 1-1 (PDI). In forward experiments ('FORWARD'), the ^{14}N -isotope was labeled on plants grown at 4°C (peaks in blue) whereas the ^{15}N -isotope was labeled on plants grown at 22°C (peaks in pink), and in the reciprocal experiments ('RECIPROCAL') the reverse was the case. (b) Total protein extracts from the cold-treated *Arabidopsis* at 4°C and the plants grown at 22°C for 0, 6, 12 and 18 d were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blots using antibodies specific to actin, calnexin (CNX), CRT and PDI.

Fig. 6.

Diagram of the stress-related glycoproteins in the endoplasmic reticulum (ER) and the effects of chilling stress in *Arabidopsis*. The N-glycan degradation of up-regulated ER glycoproteins caused by extensive chilling stress leads to partial misfolding of the proteins, ER-associated degradation (ERAD) and escape from the ER lumen, which will eventually cause cell death of plants. Thus, a possible strategy to use ER retention of stress-regulated proteins through the retrieval transport from the Golgi apparatus back to the ER by overexpressed ER retention receptors selectively binding the molecules containing a C-terminal motif [K/R/H]DEL or to treat with chemical chaperones is suggested for enhancing plant resistance to the chilling stress. ERQC, endoplasmic reticulum quality control; CRT, calreticulin; CNX, calnexin.

Table 1

Quantitative measurements of the endoplasmic reticulum (ER)-resident glycoproteins using ^{15}N -metabolic labeling glycopeptides

Protein (gene no.)	Peptide sequence	N-Glycan modification	m/z (L : H) (charge state)	Forward L : H (6 d)	Reciprocal L : H (6 d)	Forward L : H (12 d)	Reciprocal L : H (12 d)	Forward L : H (18 d)	Reciprocal L : H (18 d)
PDIL1-1 (AT1G21750)	K.EFVLTL ³⁹ LDHTN ³⁹ <u>FT</u> DTINK.H	Man ₈ GlcNAc ₂	1237.5332 (3+); 1245.5095 (3+)	1.13 ± 0.03	0.85 ± 0.02	2.31 ± 0.21	0.42 ± 0.03	4.12 ± 0.3	0.24 ± 0.02
PDIL1-4 (AT5G60640)	K.IGPGVY ²¹ N ²¹ <u>LT</u> LTLDAAEK.V	Man ₇ GlcNAc ₂	1623.7006 (20+); 1633.6709 (2+)	1.11 ± 0.04	0.89 ± 0.02	1.65 ± 0.04	0.61 ± 0.04	2.52 ± 0.12	0.41 ± 0.02
CRT1b (AT1G56340)	K.HTAGN ⁵ <u>9</u> WGDANDK.G	Man ₈ GlcNAc ₂	1538.0887 (2+); 1548.5575 (2+)	1.11 ± 0.05	0.86 ± 0.03	2.27 ± 0.03	0.44 ± 0.005	3.98 ± 0.30	0.25 ± 0.02
UGGT (AT1G71220)	K.LN ³⁷ <u>3</u> ESIKDEILSNQR.M	Man ₈ GlcNAc ₂	1121.1561 (3+); 1128.8000 (3+)	1.10 ± 0.08	0.87 ± 0.03	2.40 ± 0.03	0.43 ± 0.02	4.54 ± 0.44	0.22 ± 0.01
HSP70 (AT4G16660)	R.N ⁵¹⁸ <u>18</u> LSAPIK.A	Man ₈ GlcNAc ₂	1223.0177 (2+); 1228.5014 (2+)	1.42 ± 0.09	0.71 ± 0.07	6.63 ± 0.45	0.16 ± 0.01	10.33 ± 0.24	0.10 ± 0.02
STT3a (AT5G19690)	R.TVIVDN ⁶⁰⁴ <u>NT</u> WNNTHIATVGTAMSSPEK.A	Man ₈ GlcNAc ₂	1501.6515 (3+); 1513.6159 (3+)	1.21 ± 0.06	0.81 ± 0.04	2.13 ± 0.12	0.46 ± 0.01	3.54 ± 0.18	0.28 ± 0.01
	R.TVIVDN ⁶⁰⁴ <u>NT</u> WNNTHIATVGTAMSSPEK.A	Man ₉ GlcNAc ₂	1555.6691 (3+); 1567.6336 (3+)	1.17 ± 0.11	0.85 ± 0.06	1.66 ± 0.06	0.59 ± 0.03	3.49 ± 0.04	0.28 ± 0.01
STT3b (AT1G34130)	R.TVIVDN ⁵⁸¹ <u>NT</u> WNNTHIATVGR.A	Man ₈ GlcNAc ₂	1276.5587 (3+); 1286.8614 (3+)	1.05 ± 0.02	0.92 ± 0.03	1.90 ± 0.09	0.52 ± 0.02	3.38 ± 0.26	0.29 ± 0.02
	R.TVIVDN ⁵⁸¹ <u>NT</u> WNNTHIATVGR.A	Man ₉ GlcNAc ₂	1330.5763 (3+); 1340.8789 (3+)	1.06 ± 0.04	0.91 ± 0.005	1.40 ± 0.07	0.68 ± 0.04	2.86 ± 0.15	0.35 ± 0.02
AQ1 (AT4G38220)	R.N ³¹⁷ <u>17</u> MSFEFK.Q	Man ₈ GlcNAc ₂	1302.9987 (2+);	1.11 ± 0.05	0.89 ± 0.05	1.50 ± 0.02	0.66 ± 0.008	2.14 ± 0.05	0.44 ± 0.04

Protein (gene no.)	Peptide sequence	N-Glycan modification	<i>m/z</i> (L : H) (charge state)	Forward L : H (6 d)	Reciprocal L : H (6 d)	Forward L : H (12 d)	Reciprocal L : H (12 d)	Forward L : H (18 d)	Reciprocal L : H (18 d)
	R <u>N</u> ³¹ <u>N</u> ⁷ MSFEFKQ	Man ₉ GlcNAc ₂	1308.4824 (2+)	1.06 ± 0.04	0.96 ± 0.009	1.55 ± 0.05	0.62 ± 0.01	2.88 ± 0.16	0.37 ± 0.03
			1384.0251 (2+); 1389.5088 (2+)						
<i>P</i> -value				< 0.01		0.01		< 0.01	

LC-MS/MS analysis was performed in biological duplicate samples using 'forward' and 'reciprocal' isotope labeling approaches, and mean ± SD light : heavy (L : H) ratios were calculated based on manual inspection of the isotope peak intensity of the ¹⁴N-labeled (L) and ¹⁵N-labeled (H) peptide ion pairs. Student's *t*-test was used to compare data from two groups. In forward experiments, the ¹⁴N-isotope was labeled on plants grown at 4°C whereas the ¹⁵N-isotope was labeled on plants grown at 22°C, and in the reciprocal experiments the reverse was the case. A *P*-value < 0.05 was considered to be statistically significant. The underlined residues are shown the glycosylation sites localizing at the structural motif Asn-X-Ser/Thr.

Table 2

Quantification of the *Arabidopsis* proteins containing a C-terminal endoplasmic reticulum (ER) retention motif by LC-MS/MS analyses of nonglycopeptides

Protein (gene no.)	ER motif (H/K/RDEL)	Forward L : H (6 d)	Reciprocal L : H (6 d)	Forward L : H (12 d)	Reciprocal L : H (12 d)	Forward L : H (18 d)	Reciprocal L : H (18 d)
BIP1 (AT5G28540)	HDEL	1.22 ± 0.04	0.86 ± 0.04	3.32 ± 0.17	0.35 ± 0.02	5.60 ± 0.28	0.25 ± 0.01
BIP2 (AT5G42020)	HDEL	1.22 ± 0.04	0.86 ± 0.04	3.32 ± 0.17	0.35 ± 0.02	4.25 ± 0.05	0.25 ± 0.01
CRT1a (AT1G56340)	HDEL	1.46 ± 0.04	1.09 ± 0.06	2.50 ± 0.11	0.52 ± 0.03	5.02 ± 0.26	0.32 ± 0.02
CRT1b (AT1G09210)	HDEL	1.27 ± 0.09	0.96 ± 0.01	2.86 ± 0.01	0.52 ± 0.02	4.69 ± 0.22	0.38 ± 0.03
PDIL1-1 (AT1G21750)	KDEL	1.23 ± 0.03	0.89 ± 0.02	2.55 ± 0.14	0.44 ± 0.03	4.43 ± 0.25	0.21 ± 0.01
PDIL1-2 (AT1G77510)	KDEL	1.52 ± 0.08	0.99 ± 0.07	2.24 ± 0.05	0.60 ± 0.03	5.39 ± 0.13	0.22 ± 0.03
PDIL1-4 (AT5G60640)	KDEL	1.32 ± 0.20	0.83 ± 0.13	2.67 ± 0.11	0.35 ± 0.02	4.52 ± 0.17	0.16 ± 0.01
BGLU22 (AT1G66280)	KDEL	0.97 ± 0.02	1.60 ± 0.16	1.36 ± 0.08	0.89 ± 0.04	1.40 ± 0.04	0.80 ± 0.01
PYK10 (AT3G09260)	KDEL	1.14 ± 0.04	1.74 ± 0.02	1.64 ± 0.03	1.55 ± 0.04	1.37 ± 0.05	1.17 ± 0.11
AQ1 (AT4G38220)	RDEL	0.86 ± 0.03	1.631 ± 0.25	1.61 ± 0.11	0.77 ± 0.11	3.57 ± 0.15	0.33 ± 0.01
BGLU21 (AT1G66270)	RDEL	0.97 ± 0.02	1.60 ± 0.16	1.36 ± 0.08	0.89 ± 0.04	1.40 ± 0.04	0.80 ± 0.01
<i>P</i> -value		< 0.01		0.04		0.03	

The list includes only the ER-resident proteins containing a C-terminal ER-retrieving H/K/RDEL motif. The light : heavy (L : H) values were determined by LC-MS/MS analyses of biological duplicate samples using 'forward' and 'reciprocal' isotope labeling approaches at mean ± SD, and validated manually on a minimum of two peptides for each protein. Student's *t*-test was used to compare data sets from two groups, in which the ¹⁴N-isotope was labeled on plants grown at 4°C and the ¹⁵N-isotope was labeled on plants grown at 22°C in forward experiments, and the reverse was true in reciprocal experiments. A *P*-value < 0.05 was considered to be statistically significant.