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N-glycan trimming by glucosidase II is essential for Arabidopsis development

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Abstract

Glucosidase II, one of the early *N*-glycan processing enzymes and a major player in the glycoprotein folding quality control, has been described as a soluble heterodimer composed of α and β subunits. Here we present the first characterization of a plant glucosidase II α subunit at the molecular level. Expression of the Arabidopsis α subunit restored *N*-glycan maturation capacity in *Schizosaccharomyces pombe* α— or αβ—deficient mutants, but with a lower efficiency in the last case. Inactivation of the α subunit in a temperature sensitive Arabidopsis mutant blocked *N*-glycan processing after a first trimming by glucosidase I and strongly affected seedling development.

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Keywords

Plant; *N*-glycosylation; Glucosidase II; Glucose-trimming

Introduction

Protein *N*-glycosylation in eukaryotic cells is initiated in the endoplasmic reticulum (ER) by the *en bloc* transfer of a glycan precursor, $Glc_3Man_9GlcNAc_2$, from a lipid carrier to specific Asn residues in the nascent polypeptide [1,2]. After transfer, the *N*-glycan undergoes extensive remodelling that begins with the removal of the outermost (α1-2)-linked glucose residue by the type II membrane protein glucosidase I. Glucosidase II (GCSII) then removes the middle (α1-3)-linked glucose residue from the glycan thus producing monoglucosylated species that may be recognized by the lectin chaperones calnexin (CNX) and/or calreticulin (CRT). Lectin-glycoprotein interaction enhances folding efficiency and prevents ER exit of folding intermediates and irreparably misfolded glycoproteins. GCSII removes then the innermost (α1-3)-linked glucose residue, which prevents further association with CNX/CRT and allows folded proteins to proceed through the secretory pathway. In contrast, incorrectly folded glycoproteins and folding intermediates are reglucosylated by the ER-localized, glycoprotein conformation sensor UDP-glucose: glycoprotein glucosyltransferase and then engaged in a new folding CNX/CRT cycle. If correctly folded, however, glycoproteins are further deglucosylated by GCSII, but not reglucosylated by the glucosyltransferase. The whole process, based on folding cycles *via* deglucosylation/reglucosylation reactions, shows GCSII to be a major player in glycoprotein folding quality control [3–5].

All GCSII characterized so far are soluble heterodimers composed of two non-covalently linked, but strongly associated subunits, the so called alpha (GCSIIα) and beta (GCSIIβ) subunits. GCSIIα has been described as a soluble protein having a molecular mass of about 110 kDa and displaying sequence homology with glycoside hydrolase family 31 [6–10]. This subunit contains the catalytic domain and carries a conserved WXDMNE motif [11]. It is generally believed that the major function of GCSIIβ is to mediate ER localization of the GCSIIα/GCSIIβ complex [12]. Indeed, GCSIIβ presents a C-terminal ER retention signal (HDEL or VDEL) and a long negatively charged sequence similar to those found in CRT and other luminal ER resident proteins in most species (mammals and *S. cerevisiae*, but not in *S. pombe*)[6,13–16]. Additional data also indicate other functions for GCSIIβ. For instance it is probably involved in the folding of recombinant mammalian GCSIIα expressed also in mammalian cells, as this last protein is insoluble and forms inactive aggregates when expressed alone [16,17]. However, once GCSIIα is fully folded, GCSIIβ is no longer necessary for activity [15]. The only exception to the presence of an ER retention signal known so far occurs in *Saccharomyces cerevisiae* GCSIIβ, which is not necessary for retention of GCSIIα in the ER, but is required for cleavage of the innermost (α1-3)-glucose residue by the last subunit [18].

While GCSII is well characterized in yeasts and many animal species and the biochemical activity of GCSII was described and characterized in mung bean seedlings and soybean suspension-cultured cells more than 15 years ago [19], surprisingly no molecular information on plant GCSII was available when we started this study.

We report here the cloning of an *A. thaliana* GCSIIα encoding gene (At5g63840) and the characterization of the protein through complementation of *Schizosaccharomyces pombe* CGSIIα deficient mutants. We also carried out *N*-glycan biochemical and structural analysis of *A. thaliana* mutants defective in GCSII activity and showed the enzyme to play a key role in *N*-glycan maturation and seedling development.

Materials and methods

Cloning of the *A. thaliana* GCSIIα encoding gene

At5g63840 cDNA, was cloned in two steps by RT-PCR (Access Quick RT-PCR Kit from Promega) from a total RNA extract (RNA extract Kit from Promega) of *A. thaliana* plants using two pairs of primers (At5g63840 F1/R1 and At5g63840 F2/R2) (Table 1) designed according to positions 1–33, 1391–1415, 1250–1274, 2741–2766 of the *A. thaliana* cDNA sequence from Genbank: accession number NM_125779. Two blunt-ended fragments obtained from RT-PCR were cloned separately in pTOPO vector (*Invitrogen*) and the full length cDNA was reconstituted thanks to the Bam HI restriction site in the common part (1250–1415) of these two cDNA fragments and confirmed by sequencing. In order to clone this cDNA in the yeast expression vector pREP3X, the cDNA cloned into pTOPO was digested by Sac I and Xho I before ligation with the pREP3X.

In order to clone the cDNA from At5g63840 into pSGP72 vector, the 3′ end was amplified using primers bearing additional restriction sites. The forward primer At5g63840 F3 matched exactly the cDNA sequence in position 2281–2299, while the reverse primer At5g63840 R3 matched the cDNA sequence in position 2746–2763 and carried two additional restriction sites at 5′ end for Not I and Sac I (Table 1). The 3′ end synthesized was exchanged with the original one from the cDNA already cloned in pTOPO using Sac I and Age I digestions. The construct obtained was transferred to pSGP72 using Not I digestion of both the pTOPO construct and the pSGP72 vector. The final construct carried the full length cDNA from At5g63840 fused at its 3′ end, with the sequence encoding an hemagglutinin-tag (HA-Tag).

Strains and culture media

Escherichia coli Top10 was used for the first steps of cDNAs cloning. *E. coli* JA226 strain was used for cloning in *S. pombe* expression vectors. Bacteria were grown on LB medium supplemented with Kanamycin or Ampicillin, respectively, for constructs in pTOPO or *S. pombe* expression vectors [20].

S. pombe cells were grown in rich medium containing 0.5% yeast extract (Difco), 3% glucose, and 75 $mg.L^{-1}$ adenine. The minimal culture medium was as described in Alfa *et al*. [20], supplemented with adenine (75 $mg.L^{-1}$), uracil (75 $mg.L^{-1}$), or leucine (250 $mg.L^{-1}$). Malt extract medium was used for conjugations [22]. The wild type *S. pombe* strains ADp (h+, *ura4*-D18, *leu1*—32, *ade6*-M216) and Sp61 (h-, *ura4*-D18, *leu1*—32, ade1, *ade6*-M210). SpADIIβ (h-, *ura4*-D18, *leu1*—32, *ade6*-M210, *gls2*β:: *ura4*+) were described in D'Alessio *et al*. [8].

The wild type strains used as positive controls were h^{90}, *ura4-D18, ade6-M216, leu1—32*.

Construction of *gls2α* mutants

Sp61IIα and Sp22IIα mutant constructions were performed as described for the Sp95IIα strain (which is h^{90}), but with strains Sp61 and ADp [23]. The mutants were characterized by Southern blotting analysis as described previously in Fernandez *et al*. [23]. The mutant genotypes were h-, *ura4*-D18, *leu1*—32, ade1, *ade6*-M210, *gls2*α::*ura4*+ and h+, *ura4*-D18, *leu1*—32, *ade6*-M216, *gls2*α::*ura4*+, respectively. Sp61IIα was used for transformation with the putative *A. thaliana gls2α*+ gene. Sp22IIα was used for the *gls2αβ* construction.

Construction of *gls2αβ* double mutant

Strains SpADIIβ and Sp22IIα were conjugated at 28°C and diploids were selected in minimal medium supplemented with leucine. Diploids spontaneously sporulated after 7 days in this medium. Tetrads were treated with β-glucuronidase (Sigma) for 5 min at room temperature

and vigorously shaken in the presence of acid-washed glass beads. Resulting spores were diluted in water and spread in minimal medium supplemented with adenine and leucine. Haploid colonies and their mating type were identified by colony PCR with primers MT1, MP and MM as described in D'Alessio *et al*. [24]. Genomic DNA was prepared from 10 mL of culture and the genotypes of spores were determined by PCR with primers specific for each disrupted gene. For *gls2α+*, the primers used were GIISb and GIIAb as already described in Fernandez *et al*. [23]. They yielded 542- and 2306-bp bands for the wild type gene and the *gls2α*::*ura*4+ insertion, respectively. For gene *gls2β+*, the primers used were B1s as described in D'Alessio *et al*. [8] and B2a (Table 1). They yielded 672- and 2436-bp bands for the wild type gene and the *gls2β*::*ura4+* insertion, respectively. Germinated spores were grown in rich medium without adenine for determination of the adenine genotype. The resultant ADIIαβ double mutant genotype was h-, *ura4*-D18, *leu1*—32, *ade6*-M216, *gls2*α::*ura4*+, *gls2*β::*ura4* +. This mutant was used for transformation with the putative *A. thaliana* GCSIIα encoding gene.

S. pombe transformation

S. pombe mutants *gls2α* and *gls2αβ* were, respectively, lacking the α and both α and β GCSII subunits, respectively. Transformation was performed by electroporation. Both *gls2α* and *gls2αβ* mutants were transformed by constructions in pREP3X and in pSGP72 (see The Forsburg Lab *S. pombe* pages, http://www.pombe.net). In pREP3X the full length cDNA from At5g63840 was cloned unmodified. In pSGP72 the cDNA from At5g63840 was cloned without the stop codon and fused at the 3′terminus with the HA sequence tag. In both cases the signal peptide from the plant was unchanged. Transformants were isolated in selective medium (minimal medium + adenine) and transformation by Atg63840 was checked by PCR.

Preparation of microsomes

Cells from the exponential phase (approximately 1 g in 250 mL of appropriate medium) were harvested (3,000 *g*, 5 min), resuspended in water, centrifuged (3,000×*g*, 5 min), and resuspended in 5 mL of Solution A (0.25 M sucrose, 20 mM imidazole, pH 7.5, 1 mM EDTA) containing protease inhibitors (1 mM tosylphenylalanyl chloromethyl ketone, 1 mM phenylmethylsulfonyl fluoride, 1 μM E-64, 1 μM pepstatin, 10 μM leupeptin). Cells were then broken with 10 pulses of 1 min each of vortexing with glass beads and the suspension centrifuged at 5,000×*g* for 7 min. The supernatant was saved and the pellet resuspended in solution A plus protease inhibitors and treated with the beads as above. The supernatants were pooled and centrifuged at 45 000×*g* rpm for 60 min. The pellet was then resuspended in 40 mM sodium phosphate buffer pH 7.0, 1 mM EDTA plus the same protease inhibitors as mentioned above.

Characterization of *S. pombe* N-glycans

Short term (30 min) *in vivo* labelling of *S. pombe* cells with [^{14}C]glucose and purification of labelled endo-β-*N*-acetyl-glucosaminidase H (Endo H)-sensitive oligosaccharides were performed as described previously in Fernandez *et al.* [25] for *S. cerevisiae* cells, but with the addition of 50 μL of 50 mM Kifunensine and no 1-deoxynojirimycin. Whatman 1 papers were used for chromatographies. Solvent employed was 1-propanol//water (5:2:4).

Cell-free GCSII assay

Microsomes (100 μg protein) were incubated for 30 min with 1,400 cpm of [*glucose*-^{14}C] $Glc_1Man_9GlcNAc$, 40 mM sodium phosphate buffer pH 7.0 and 0.5% Lubrol in a total volume of 100 μl. The reaction was stopped by adding 1 volume of methanol and heating at 60°C for 5 min. The reaction mix was centrifuged and the supernatant loaded on a Whatman 1 paper.

Chromatograhy was run using 2-propanol/acetic acid/water (29:4:9) as solvent. This method was described before [8].

Western-blot N-glycan analysis

A. thaliana seeds from wild type Colombia and *rsw3* mutant were grown for 2 days at the permissive temperature (21°C) and then at 30°C (non-permissive temperature) for 5 days. Seedlings were then collected and homogenized in an Eppendorf tube containing hot denaturing buffer (20 mM Tris—HCl pH 6.8, 0.3% β-mercaptoethanol, 5% (*v*/*v*) glycerol and 1% (*w*/*v*) SDS). The homogenate was then boiled for 5 min and centrifuged for 10 min at 12,000×*g*. Proteins were separated by SDS-PAGE in 15% polyacrylamide gels according to Laemmli [26]. Analytical gels were silver stained according to Blum *et al.* [27]. After transfer from the gel onto a nitrocellulose membrane, glycoproteins were immunodetected using purified rabbit antibodies specific for (β1-2)-xylose or (α1-3)-fucose residues constitutive of plant complex *N*-glycans [28] and the ECL (Amersham) amplification procedure. Affinodetection of glycoproteins on blot was carried out as previously described by Faye and Chrispeels [29] and using ECL (Amersham) for detection. Immunodetection of the HA-tagged proteins was performed as described in Abe *et al*. [30] with high affinity monoclonal antibodies (3F10, Roche). Immunoreactive bands were visualized by staining with horseradish-conjugated goat anti rat IgG (Sigma) and chemiluminiscence (Supersignal West Pico Chemiluminiscence substrate, Pierce).

Purification of N-linked glycans isolated from wild-type and rsw3 *A. thaliana* seedlings

A crude protein extract was obtained from 7-days-old *A. thaliana* seedlings after homogenization of 60 mg of freeze dried seedlings in 10 ml of 50 mM HEPES buffer pH 7.5, 2 mM sodium metabisulfite and 0.1% SDS. Insoluble material was eliminated by centrifugation at 4,400×*g* for 15 min at 4°C. Proteins were precipitated by the addition of trichloroacetic acid (12.5% *v*/*v*). After centrifugation (10,000×*g*, 15 min), the pellet was washed twice in 80% acetone and air-dried. The protein pellet was then solubilized by heating for 4 min in 1 mL of 50 mM sodium acetate buffer pH 5.5, 0.1% SDS. The volume was completed to 5 mL with 50 mM sodium acetate buffer pH 5.5 before adding Endo H (0.1 U). The solution was incubated for 18 h at 37°C. Proteins were precipitated by the addition of 4 volumes of ethanol at -20°C. After centrifugation (10,000×*g*, 15 min), the supernatant was lyophilized. Released *N*-glycans were purified by chromatography on 200-mg SepPack C18 column (Varian) followed by a Carbograph column (LudgerClean E10 Cartridge). Bound *N*-glycans were eluted in 50% acetonitrile, 0.1% trifluoracetic acid (TFA). The oligosaccharides were lyophilized before mass spectrometry analysis.

N-glycan mannosidase degradation

Two hundred milliunits of Jack bean α-mannosidase (Sigma) were desalted by ultrafiltration (Microcon YM-10, Millipore) and incubated overnight with the glycan mixture. The digest was then directly analyzed using MALDI-TOF MS [31,32].

N-glycan structural analysis by MALDI-TOF

MALDI-TOF mass spectra of purified *N*-glycans were obtained on a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems, USA) equipped with a 337-nm nitrogen laser. Mass spectra were performed in the reflector, delayed extraction mode using 2, 5-dihydroxybenzoic acid (Sigma-Aldrich) as matrix. The matrix, freshly dissolved at 5 $mg.mL^{-1}$ in a 70:30% acetonitrile/0.1% TFA was mixed with the oligosaccharides, solubilized in water with 0.1% TFA, in a ratio 1:1 (*v*/*v*). The spectra were recorded in a positive mode, using an acceleration voltage of 20,000 V with a delay time of 100 ns. They were smoothed once and externally calibrated using commercially available mixtures of peptides and proteins (Applied

Biosystems). In this study, the spectra have been calibrated using des-Arg1-Bradykinin (904.4681 Da), Angiotensin I (1296.6853), Glu1-Fibrinopeptide B (1570.6774 Da), ACTH clip 18–39 (2465.1989) and bovine insulin (5730.6087). Laser shots were accumulated for each spectrum in order to obtain an acceptable signal to noise ratio.

Results and discussion

Cloning of the Arabidopsis GCS IIα subunit

In order to identify *A. thaliana* GCSIIα subunit involved in the early trimming of *N*-glycans, mammalian GCSIIα sequences available were blasted against the *A. thaliana* genome database at NCBI and a gene encoding a putative GCSIIα subunit (At5g63840) was identified. This gene shows a high degree of similarity (>60%) with alpha subunits cloned from yeasts and mammals, [6,16–18,33–35]. At5g63840 encodes a protein containing a 16- or 20 amino acid long, cleavable N-terminal signal peptide according to Psort (http://www.psort.nibb.ac.jp) or to SignalP (http://www.cbs.dtu.dk/services/SignalP/), respectively. Two potential *N*-glycosylation sites in position N689 (NVT) and N804 (NSS) according to Proscan (http://npsapbil.ibcp.fr/) were also identified and one of these two sites (N804) could be actually glycosylated according to NetN-Glyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Furthermore, as observed for yeast and mammalian GCSIIα, none of the ER retention or retrieval signals identified so far is present in the amino acid sequence derived from At5g63840. In contrast, the protein sequence carries a conserved WXDMNE motif, which is the typical consensus sequence for the active site of glycosidases in CAZy family-31 [11].

The corresponding cDNA was cloned in two PCR-steps and subcloned in pTOPO and *S. pombe* expression vectors as described under Material and methods.

Demonstration that At5g63840 encodes a catalytically active GCSIIα subunit by functional complementation of a *S. pombe* GCSIIα deficient mutant

Microsomes prepared from *S. pombe* mutants defective in GCSIIα (*gls2α*) or in both GCSIIα and GCSIIβ (*gls2αβ*), but transformed with At5g63840 cDNA in the pREP3X vector displayed GCSII activity as they were able to liberate labelled glucose from [*glucose*-^{14}C] $Glc_1Man_9GlcNAc$ (Fig. 1). This result strongly suggested that At5g63840 encodes a protein bearing the *A. thaliana* GCSII catalytic activity. To further confirm this result we studied *N*-glycan maturation *in vivo* in *S. pombe* cells. Wild type and *gls2α* (GCSIIα minus) cells transformed either with an empty vector or with the pREP3X vector containing At5g63840 cDNA were pulse-labelled for 30 min with [^{14}C]glucose in the presence of both Kifunensine and dithiothreitol. The former was added to minimize *N*-glycan ER mannosidase degradation and the latter to prevent ER exit of glycoproteins and thus further *N*-glycan enlargement at the Golgi. Dithiothreitol impedes disulfide bond formation and thus proper folding of most glycoproteins. Proteins from incubated cells were proteolytically degraded with Protease Type XIV (Pronase from Sigma) and *N*-glycans released from glycopeptides by Endo H treatment.

Consistent with the expected effect of Kifunensine, under described conditions wild type *S. pombe* mainly accumulated the product of GCSI and GCSII (Man_9- $GlcNAc_2$) and traces of $Man_8GlcNAc_2$ (Fig. 2a). In contrast, the *gls2α* mutant lacking GCSII activity accumulated the enzyme substrate $Glc_2Man_9GlcNAc_2$ (Fig. 2b).

The oligosaccharide pattern obtained with the *gls2α* mutant transformed with At5g63840 cDNA was similar to that obtained from wild type *S. pombe* except for trace amounts of $Glc_2Man_9GlcNAc_2$ (Fig. 2c). This result clearly illustrates that GCSII activity was restored by the At5g63840 cDNA in the *S. pombe gls2α* mutant at an almost wild type level. Altogether

these results obtained both *in vitro* and *in vivo* clearly indicate that At5g63840 encodes the first plant GCSIIα subunit cloned so far.

Preliminary characterization of Arabidopsis GCSIIα subunit

Kaushal *et al.* [19] showed a 95 KDa protein to be the GCSIIα subunit in soybean suspension-cultured cells while GCSIIα is a 110 kDa protein in mung bean. The molecular mass of AtGCSIIα deduced from its cDNA sequences was 104 kDa, while western blot analysis of protein extracts obtained from *S. pombe gls2α* mutant expressing HA-tagged AtGCSIIα showed a polypeptide of approximately 119 kDa (Fig. 3a). Moreover, the molecular mass of yeast-made AtGCSIIα was reduced by about 2 kDa after deglycosylation with Endo H (Fig. 3b). This result shows that when expressed in *S. pombe* AtGCSIIα is glycosylated with probably a single *N*-glycan, a data in agreement with previous biochemical characterization of a plant GCSIIα subunit [36] and *in silico* prediction from the At5g63840 sequence (see above).

Probable characteristics of *A. thaliana* GCSIIβ subunit

As mentioned above, depending on the organism, different roles for GCSIIβ subunits have been proposed. In most cells GCSIIβ is apparently required for ER retention and/or proper folding of GCSIIα whereas in *S. cerevisiae* it is not required for those roles but to allow cleavage of the innermost glucose unit. Although we have not yet identified *A. thaliana* GCSIIβ encoding gene (a Blast search showed the At55g56360 gene to be the best candidate; a comparison of this gene with those encoding mouse, *S. pombe* and *S. cerevisiae* GCIIβ subunits is provided in supplemental Fig. S1) or fully characterized the role of the protein, preliminary results strongly suggest *A. thaliana* GCSIIβ subunit to display similar roles as those of homologous proteins in most eukaryotic cells: as shown in Fig. 1, AtGSIIα expressed in *gls2αβ* (both GCSIIα and GCSIIβ minus *S. pombe* mutant cells) was able to release the glucose unit from [*glucose*-^{14}C]$Glc_1Man_9GlcNAc$. Moreover, *in vivo* processing of the *N*-glycan in yeast double mutant cells expressing AtGCSIIα resulted in the formation of almost similar amounts of $Glc_2Man_9GlcNAc_2$ and $Man_9GlcNAc_2$, thus confirming the ability of the plant catalytic subunit to remove both the middle and innermost glucose residues. Lack of complete *N*-glycan deglucosylation might reflect a shorter ER permanence of the plant catalytic subunit when expressed in the absence of GCSIIβ subunits. Accordingly, cell free enzymatic assays performed with yeast microsomes prepared from cells preincubated for two h with cyclo heximide, a protein synthesis inhibitor, showed that the plant catalytic subunit synthesized in *S. pombe* cells expressing its endogenous GCSIIβ subunit (*gls2α* mutant) had an active ER localization that was longer than when synthesized in yeast cells expressing no GCSIIβ proteins (*gls2αβ* mutant) (Fig. 4). These results put together strongly suggest that the role of AtGCSIIβ is to determine the ER localization of the catalytic subunit rather than to allow removal of the innermost glucose unit or to allow proper folding of AtGCSIIα.

A. thaliana plants affected in AtGCSIIα encoding gene (At5g63840) show strong glycosylation defects

Burn *et al*. (2002) reported a Ds-insertional mutant in At5g63840 and found that plants did not survive if both copies of the gene carry the mutation. Inheritance of the tagged allele in heterozygotes was further reduced by severe effects on male and, to a lesser extent, female gametogenesis. Examination of two T-DNA insertional mutants (N_503451 and N_624837) again showed that plants mutated in both copies did not survive. The *rsw3* mutant of Arabidopsis shows a one nucleotide change in the At5g63840 sequence that results in the substitution of Ser599 by a Phe residue in AtGCSIIα. The phenotype is severe, but strongly temperature-sensitive allowing ready propagation of the mutant at its permissive temperature and analysis of the mutant phenotype in plants grown at or transferred to the restrictive temperature [37].

In order to get further information on the effect of this gene mutation in the early trimming of plant N-glycans, we characterized N-glycan structures in *rsw3* seedlings that had germinated from seeds collected from plants grown at the permissive temperature (21°C). Seeds were germinated either for 2 d at 21°C followed by 5 d at 30°C or for 7 d entirely at 30°C. Roots emerge on about day 4 and mutant roots were very stunted and swollen with slightly longer roots if the first 2 days were spent at 21°C (Fig. 5).

As illustrated in Fig. 6a, protein patterns obtained after SDS-PAGE and silver staining of the gel were quantitatively and qualitatively similar in both cases. The seedling protein extracts were further analysed for glycosylation patterns by either immunodetection of glycoproteins containing plant complex *N*-glycans using purified antibodies specific for (β1-2)-xylose- (Fig. 6b) or (α1-3)-fucose-containing (Fig. 6c) glycoepitopes [28] or by lectin affinity of glycoproteins containing high mannose-type *N*-glycans using the lectin Concanavalin A (ConA) as probe [29], (Fig. 6d). Several glycoproteins with complex *N*-glycans reacting with anti-xylose and anti-fucose antibodies, and at least three glycoproteins with high-mannose *N*-glycans reacting with ConA were detected in blots from wild type (WT) Arabidopsis seedlings. In contrast, glycoproteins reacting with antibodies specific for complex *N*-glycans were almost completely absent from *rsw3* seedling extracts, while glycoproteins reacting with ConA were strongly increased. Moreover, Lewis A *N*-glycan-containing glycoproteins immunodetected in wild type seedling extracts using an antibody specific for this glycan structure were not detected in glycoproteins from *rsw3* seedlings (data not shown). These results indicate that in the *rsw3* mutant seedlings grown for 5 days under restrictive conditions maturation of *N*-glycans is blocked at an early stage, this block leading to the accumulation of glycoproteins with high-mannose type *N*-glycans and to a lack of complex type ones. Trace amounts of glycoproteins with complex *N*-glycans detected in *rsw3* seedling extracts were either synthesized during the first 2 days of growth at the permissive temperature, or may reflect residual enzyme activity in the protein product of the *rsw3* allele of At5g63840. The latter seems likely since development, albeit highly abnormal, can continue through to flowering even at 30°C (Burn *et al*. 2002).

To further characterize the structure of the *N*-glycans reacting with ConA in the *rsw3* mutant *N*-glycans were released from glycoproteins after an Endo H treatment of seedling protein extracts and analysed by MALDI-TOF mass spectrometry. Analyses of WT *A. thaliana N*-glycans revealed the presence of ions at *m/z*=1,054, 1,216, 1,378, 1,540 and 1,702, assigned to the sodium adducts of high mannose type *N*-glycans from $Man_5GlcNAc$ to Man_9-GlcNAc, previously described in Arabidopsis (Fig. 7). Interestingly, two oligosaccharide structures at *m/z*=1,864 and 2,026 were exclusively detected in the *rsw3* mutant. These molecular ions corresponded to structures containing one GlcNAc and 10 and 11 hexose residues, respectively. Consistent with GCSII inactivation at non-permissive temperature, these structures could be attributed to the presence of one or two terminal glucose residues on the oligosaccharide (Fig. 7A, structures F to K). The presence of *N*-glycans displaying only one glucose unit might reflect a leaky characteristic of the *rsw* mutation.

Enzymatic degradation using Jack bean α-mannosidase coupled with MALDI-TOF MS analysis was conducted as described previously, [32] to further identify the structures of *N*-glycans specific for *rsw3* (Fig. 7). Ions at *m/z*=1,054, 1,216, 1,378, 1,540 and 1,702 were detected exclusively in the *rsw3* mutant after digestion with this glycosidase. Observed products corresponded to oligosaccharide structures containing one GlcNAc and five to eight hexoses, including one to two terminal glucose residues (the mannosidase employed is an exoglycosidase). These structures are presented Fig. 7B, structures d to j).

Conclusion

Using heterologous expression in the yeast *S. pombe*, we have shown that the protein encoded by At5g63840 is an authentic GCSIIα subunit bearing GCSII catalytic activity. Inactivation of GCSIIα in *A. thaliana* blocks *N*-glycan maturation at an early stage, this block resulting in the accumulation of *N*-glycans with one or two terminal glucose residues and in the absence of complex *N*-glycans. Consequently, At5g63840 encodes a GCSIIα subunit playing a key role in *N*-glycan maturation that cannot be balanced by other *N*-glycan trimming enzymes.

Indeed, in some mammalian tissues, prevention of *N*-glycan trimming by ER glucosidases using either specific inhibitors or mutant cell lines never completely hinders deglucosylation of the oligosaccharide precursor and formation of complex *N*-glycans. The GCSII-independent trimming of the terminal glucosyl residues is due to the presence of an alternative deglucosylation pathway involving a *cis/medial* Golgi endomannosidase, that releases a $Glc_{1,2}Man$ di- or tri-saccharide from the $Glc_{1,2}Man_9GlcNAc_2$ [38]. Our results clearly demonstrate that complex *N*-glycans containing (α1-3)-fucose and (β1-2)-xylose residues are neither present in plant cells after treatment with glucosidase inhibitors [39,], nor in GCSI [40] or GCSIIα-deficient Arabidopsis mutants (the present paper). Together these observations indicate that plants lack an alternative pathway to *N*-glycan trimming by ER glucosidases. This result agrees with the reported absence of the endomannosidase from plant tissues [41].

Removal of terminal glucose residues from *N*-glycans by GCSI and GCSII plays a central role in the ER quality control of newly synthesized *N*-glycoproteins [42–44]. *N*-glycan trimming by ER-resident glucosidases is not essential for suspension-cultured plant cell viability [39, and Gomord *et al*, unpublished results] the same as for yeast and cultured mammalian cells, thus implying that not only *N*-glycan processing, but also the CNX/CRT cycle is not essential for these cells. This situation strongly contrasts with lethal phenotypes obtained after inactivation or knocking out GCSI and/or GCSII in multicellular organisms including the model plant Arabidopsis. Indeed, it was previously shown in *A. thaliana* that glucose trimming by GCSI is required for seed development [40] and cellulose biosynthesis [45]. The present study, together with results previously published by Burn *et al*. [37] illustrate that GCSII activity is also essential for normal plant and seed development in *A. thaliana*. These results suggest that glucose trimming from the oligosaccharide precursor is strictly required for proper folding through CNX/CRT cycles of some glycoproteins essential for plant growth and cell differentiation, but not for single cell viability. Defects in plant growth and cell differentiation in Arabidopsis *rsw3* mutants do not seem to be a consequence of the blockage of complex *N*-glycan biosynthesis as other complex *N*-glycan deficient *A. thaliana* mutants affected in glycosyltranferase activities show no obvious phenotypes except for the *N*-glycan patterns [46,47].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

At, Arabidopsis thaliana; CNX, calnexin; ConA, concanavalin A; CRT, calreticulin; Endo H, endo-β-N-acetylglucosaminidase H; ER, endoplasmic reticulum; GCS, glucosidase; TFA, trifluoracetic acid.

References

1. Kornfeld R, Kornfeld S. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem 1985;54:631–664. [PubMed: 3896128]doi:10.1146/annurev.bi.54.070185.003215
2. Moremen KW, Trimble RB, Herscovics A. Glycosidases of the asparagine-linked oligosaccharide processing pathway. Glycobiology 1994;4:113–125. [PubMed: 8054711]doi:10.1093/glycob/4.2.113
3. Ellgaard L, Helenius A. Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol 2003;4:181–191. [PubMed: 12612637]doi:10.1038/nrm1052
4. Helenius A, Aebi M. Roles of N-linked glycans in the endoplasmic reticulum. Annu. Rev. Biochem 2004;73:1019–1049. [PubMed: 15189166]doi:10.1146/annurev.biochem.73.011303.073752
5. Moremen KW, Molinari M. N-linked glycan recognition and processing: the molecular basis of endoplasmic reticulum quality control. Curr. Opin. Struct. Biol 2006;16:592–599. [PubMed: 16938451]doi:10.1016/j.sbi.2006.08.005
6. Trombetta ES, Simons JF, Helenius A. Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit. J. Biol. Chem 1996;271:27509–27516. [PubMed: 8910335]doi:10.1074/jbc.271.44.27509
7. Hentges A, Bause E. Affinity purification and characterization of glucosidase II from pig liver. Biol. Chem 1997;378:1031–1038. [PubMed: 9348113]
8. D'Alessio C, Fernandez F, Trombetta ES, Parodi AJ. Genetic evidence for the heterodimeric structure of glucosidase II. The effect of disrupting the subunit-encoding genes on glycoprotein folding. J. Biol. Chem 1999;274:25899–25905. [PubMed: 10464333]doi:10.1074/jbc.274.36.25899
9. Henrissat B, Davies G. Structural and sequence-based classification of glycoside hydrolases. Curr. Opin. Struct. Biol 1997;7:637–644. [PubMed: 9345621]doi:10.1016/S0959-440X(97)80072-3
10. Henrissat B, Romeu A. Families, superfamilies and subfamilies of glycosyl hydrolases. Biochem. J 1995;311:350–351. [PubMed: 7575477]
11. Henrissat B, Bairoch A. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J 1993;293:781–788. [PubMed: 8352747]
12. Lucocq JM, Brada D, Roth J. Immunolocalization of the oligosaccharide trimming enzyme glucosidase II. J. Cell Biol 1986;102:2137–2146. [PubMed: 3519622]doi:10.1083/jcb.102.6.2137
13. Arendt CW, Ostergaard HL. Identification of the CD45-associated 116-kDa and 80-kDa proteins as the alpha- and beta-subunits of alpha-glucosidase II. J. Biol. Chem 1997;272:13117–13125. [PubMed: 9148925]doi:10.1074/jbc.272.20.13117
14. Flura T, Brada D, Ziak M, Roth J. Expression of a cDNA encoding the glucose trimming enzyme glucosidase II in CHO cells and molecular characterization of the enzyme deficiency in a mutant mouse lymphoma cell line. Glycobiology 1997;7:617–624. [PubMed: 9254044]doi:10.1093/glycob/7.5.617
15. Trombetta SE, Fleming KG, Helenius A. Quaternary and domain structure of glycoprotein processing glucosidase II. Biochem 2001;40:10717–10722. [PubMed: 11524018]doi:10.1021/bi010629u
16. Treml K, Meimaroglou D, Hentges A, Bause E. The alpha- and beta-subunits are required for expression of catalytic activity in the hetero-dimeric glucosidase II complex from human liver. Glycobiology 2000;10:493–502. [PubMed: 10764838]doi:10.1093/glycob/10.5.493
17. Pelletier MF, Marcil A, Sevigny G, Jakob CA, Tessier DC, Chevet E, Menard R, Bergeron JJM, Thomas DY. The heterodimeric structure of glucosidase II is required for its activity, solubility, and localization *in vivo*. Glycobiology 2000;10:815–827. [PubMed: 10929008]doi:10.1093/glycob/10.8.815
18. Wilkinson BM, Purswani J, Stirling CJ. Yeast GTB1 Encodes a subunit of glucosidase II required for glycoprotein processing in the endoplasmic reticulum. J. Biol. Chem 2006;281:6325–6333. [PubMed: 16373354]doi:10.1074/jbc.M510455200

19. Kaushal GP, Pastuszak I, Hatanaka K, Elbein AD. Purification to homogeneity and properties of glucosidase II from mung bean seedlings and suspension-cultured soybean cells. J. Biol. Chem 1990;265:16271–16279. [PubMed: 2168886]
20. Forsburg SL. Comparison of *Schizosaccharomyces pombe* expression systems. Nucleic Acids Res 1993;21:2955–2956. [PubMed: 8332516]doi:10.1093/nar/21.12.2955
21. Alfa, C.; Fantes, P.; Hyams, J.; McLeod, M.; Wabrik, E. Experiments with fission yeast: A laboratory manual. Cold Spring Harbor Laboratory; New York: 1993. p. 133-136.ISBN 0 87969 424 6
22. Moreno S, Klar A, Nurse P. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol 1991;194:795–823. [PubMed: 2005825]doi:10.1016/0076-6879(91)94059-L
23. Fernandez F, D'Alessio C, Fanchiotti S, Parodi AJ. A misfolded protein conformation is not a sufficient condition for *in vivo* glucosylation by the UDP-Glc:glycoprotein glucosyltransferase. EMBO J 1998;17:5877–5886. [PubMed: 9774332]doi:10.1093/emboj/17.20.5877
24. D'Alessio C, Trombetta ES, Parodi AJ. Nucleoside diphosphatase and glycosyltransferase activities can localize to different subcellular compartments in *Schizosaccharomyces pombe*. J. Biol. Chem 2003;278:22379–22387. [PubMed: 12686557]doi:10.1074/jbc.M300892200
25. Fernandez FS, Trombetta ES, Hellman U, Parodi AJ. Purification to homogeneity of UDP-glucose:glycoprotein glucosyltransferase from *Schizosaccharomyces pombe* and apparent absence of the enzyme from *Saccharomyces cerevisiae*. J. Biol. Chem 1994;269:30701–30706. [PubMed: 7982990]
26. Laemmli U. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 1970;227:680–685. [PubMed: 5432063]doi:10.1038/227680a0
27. Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 1987;8:93–99.doi:10.1002/elps.1150080203
28. Faye L, Gomord V, Fitchette-Laine A-C, Chrispeels MJ. Affinity purification of antibodies specific for Asn-linked glycans containing [alpha]1 -> 3 fucose or [beta]1 -> 2 xylose. Anal. Biochem 1993;209:104–108. [PubMed: 7682035]doi:10.1006/abio.1993.1088
29. Faye L, Chrispeels MJ. Characterization of N-linked oligosaccharides by affinoblotting with concanavalin A-peroxidase and treatment of the blots with glycosidases. Anal. Biochem 1985;149:218–224. [PubMed: 4073477]doi:10.1016/0003-2697(85)90498-1
30. Abe H, Shimma Y, Jigami Y. *In vitro* oligosaccharide synthesis using intact yeast cells that display glycosyltransferases at the cell surface through cell wall-anchored protein Pir. Glycobiology 2003;13:87–95. [PubMed: 12626410]doi:10.1093/glycob/cwg014
31. Harvey DJ. Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates. Mass Spectrom. Rev 1999;18:349–450. [PubMed: 10639030]doi:10.1002/(SICI)1098-2787(1999)18:6<349::AID-MAS1>3.0.CO;2-H
32. Bakker H, Bardor M, Molthoff JO, Gomord V, Elbers I, Stevens LH, Jordi W, Lommen A, Faye L, Lerouge P, Bosch D. Galactose-extended glycans of antibodies produced by transgenic plants. Proc. Natl. Acad. Sci. U S A 2001;98:2899–2904. [PubMed: 11226338]doi:10.1073/pnas.031419998
33. Freeze HH, Lammertz M, Iranfar N, Fuller D, Panneerselvam K, Loomis WF. Consequences of disrupting the gene that encodes α-glucosidase II in the N-linked oligosaccharide biosynthesis pathway of *Dictyostelium discoideum*. Dev. Genet 1997;21:177–186. [PubMed: 9397534]
34. Geysens S, Pakula T, Uusitalo J, Dewerte I, Penttila M, Contreras R. Cloning and characterization of the glucosidase II alpha subunit gene of *Trichoderma reesei*: a frameshift mutation results in the aberrant glycosylation profile of the hypercellulolytic strain *Rut-C30*. Appl. Environ. Microbiol 2005;71:2910–2924. [PubMed: 15932985]doi:10.1128/AEM.71.6.2910-2924.2005
35. Taylor MA, Ross HA, McRae D, Stewart D, Roberts I, Duncan G, Wright F, Millam S, Davies HV. A potato alpha-glucosidase gene encodes a glycoprotein-processing alpha-glucosidase II-like activity. Demonstration of enzyme activity and effects of down-regulation in transgenic plants. Plant J 2000;24:305–316. [PubMed: 11069704]doi:10.1046/j.1365-313x.2000.00873.x
36. Kaushal GP, Zeng Y, Elbein AD. Biosynthesis of glucosidase II in suspension-cultured soybean cells. J. Biol. Chem 1993;268:14536–14542. [PubMed: 8314807]
37. Burn JE, Hurley UA, Birch RJ, Arioli T, Cork A, Williamson RE. The cellulose-deficient Arabidopsis mutant *rsw3* is defective in a gene encoding a putative glucosidase II, an enzyme processing *N*-

glycans during ER quality control. Plant J 2002;32:949–960. [PubMed: 12492837]doi:10.1046/j.1365-313X.2002.01483.x

38. Fujimoto K, Kornfeld R. alpha-Glucosidase II-deficient cells use endo alpha-mannosidase as a bypass route for N-linked oligosaccharide processing. J. Biol. Chem 1991;266:3571–3578. [PubMed: 1825311]
39. Lerouge P, Cabanes-Macheteau M, Rayon C, Fitchette-Laine A-C, Gomord V, Faye L. N-glycoprotein biosynthesis in plants: recent developments and future trends. Plant Mol. Biol 1998;38:31–48. [PubMed: 9738959]doi:10.1023/A:1006012005654
40. Boisson M, Gomord V, Audran C, Berger N, Dubreucq B, Granier F, Lerouge P, Faye L, Caboche M, Lepiniec L. Arabidopsis glucosidase I mutants reveal a critical role of N-glycan trimming in seed development. EMBO J 2001;20:1010–1019. [PubMed: 11230125]doi:10.1093/emboj/20.5.1010
41. Dairaku K, Spiro RG. Phylogenetic survey of endomannosidase indicates late evolutionary appearance of this *N*-linked oligosaccharide processing enzyme. Glycobiology 1997;7:579–586. [PubMed: 9184840]doi:10.1093/glycob/7.4.579
42. Hammond C, Braakman I, Helenius A. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc. Natl. Acad. Sci. U S A 1994;91:913–917. [PubMed: 8302866]doi:10.1073/pnas.91.3.913
43. Zhang JX, Braakman I, Matlack KE, Helenius A. Quality control in the secretory pathway: the role of calreticulin, calnexin and BiP in the retention of glycoproteins with C-terminal truncations. Mol. Biol. Cell 1997;8:1943–1954. [PubMed: 9348535]
44. Fanchiotti S, Fernandez F, D'Alessio C, Parodi AJ. The UDP-Glc:glycoprotein glucosyltransferase is essential for *Schizosaccharomyces pombe* viability under conditions of extreme endoplasmic eeticulum stress. J. Cell Biol 1998;143:625–635. [PubMed: 9813085]doi:10.1083/jcb.143.3.625
45. Gillmor S, Poindexter P, Lorieau J, Palcic MM, Somerville C. α-Glucosidase I is required for cellulose biosynthesis and morphogenesis in *Arabidopsis*. J. Cell Biol 2002;156:1003–1013. [PubMed: 11901167]doi:10.1083/jcb.200111093
46. von Schaewen A, Sturm A, O'Neill J, Chrispeels MJ. Isolation of a mutant Arabidopsis plant that lacks N-acetyl glucosaminyl transferase I and is unable to synthesize Golgi-modified complex N-linked glycans. Plant Physiol 1992;102:1109–1118. [PubMed: 8278542]doi:10.1104/pp.102.4.1109
47. Strasser R, Altmann F, Mach L, Glössl J, Steinkellner H. Generation of Arabidopsis thaliana plants with complex N-glycans lacking beta1,2-linked xylose and core alpha1,3-linked fucose. FEBS Lett 2004;561:132–136. [PubMed: 15013764]doi:10.1016/S0014-5793(04)00150-4

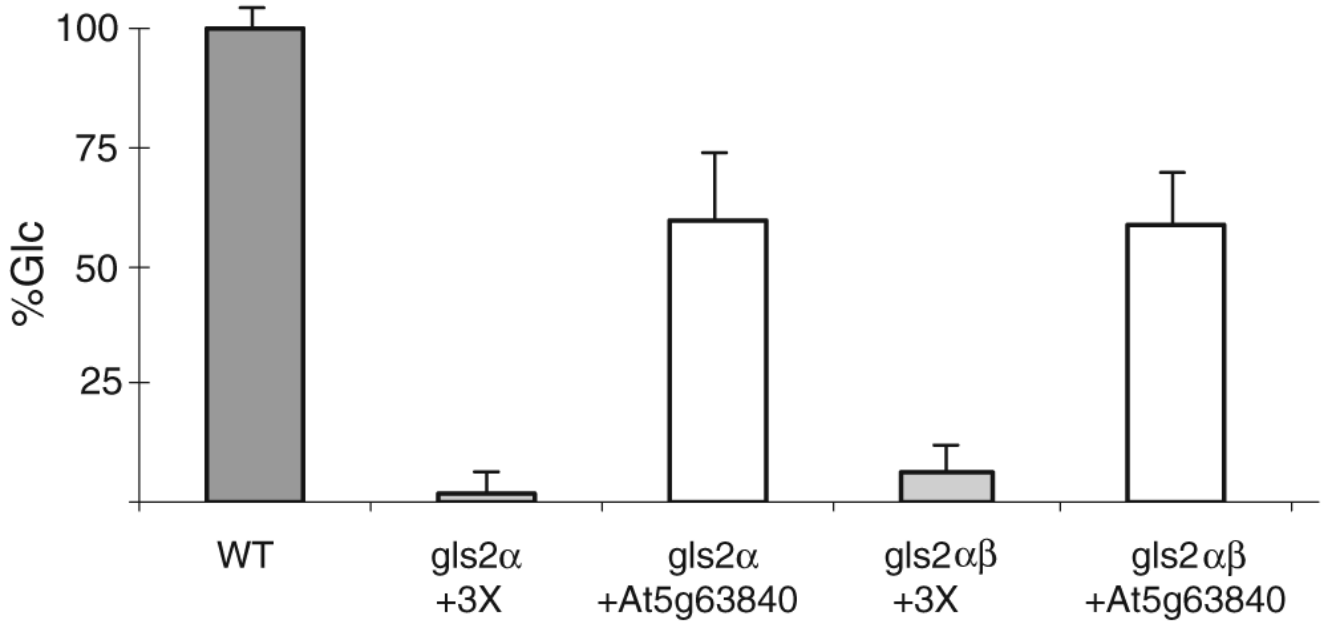

Fig 1.

GCSII activity in yeast cells transformed with At5g63840. GCSII activity was measured using [*glucose*-^{14}C]$Glc_1Man_9GlcNAc$ as substrate in microsomes prepared from wild type (WT) cells, in GCSIIα minus (*gls2α*) mutants transformed with an empty vector (*gls2α*+3X), in the same cells transformed with At5g63840 cDNA in the pREP3X vector (*gls2α*+At5g63840), in yeast mutant cells lacking GCSIIα and GCSIIβ (*gls2αβ)* transformed with an empty vector (*gls2αβ*+3X) and in the same cells transformed with At5g63840 (*gls2αβ*+At5g63840)

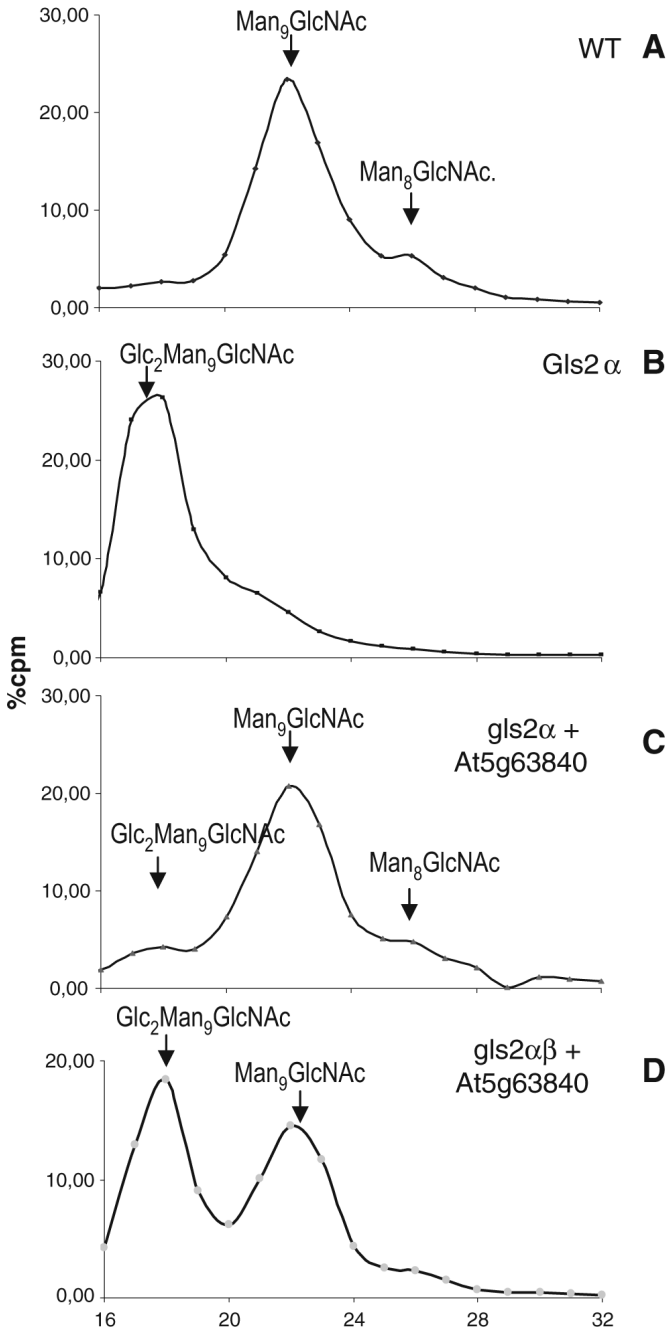

Fig 2.

N-glycan processing in yeast cells transformed with At5g6380. Wild type (*A*), *gls2α* (*B*), *gls2α* transformed with At5g63840 cDNA in the pREP3X vector (*C*) or *gls2αβ* cells transformed with the same cDNA (*D*) were incubated with [^{14}C]glucose for 30 min and *N*-glycans released from whole cell glycoproteins with Endo H were run on paper chromatography

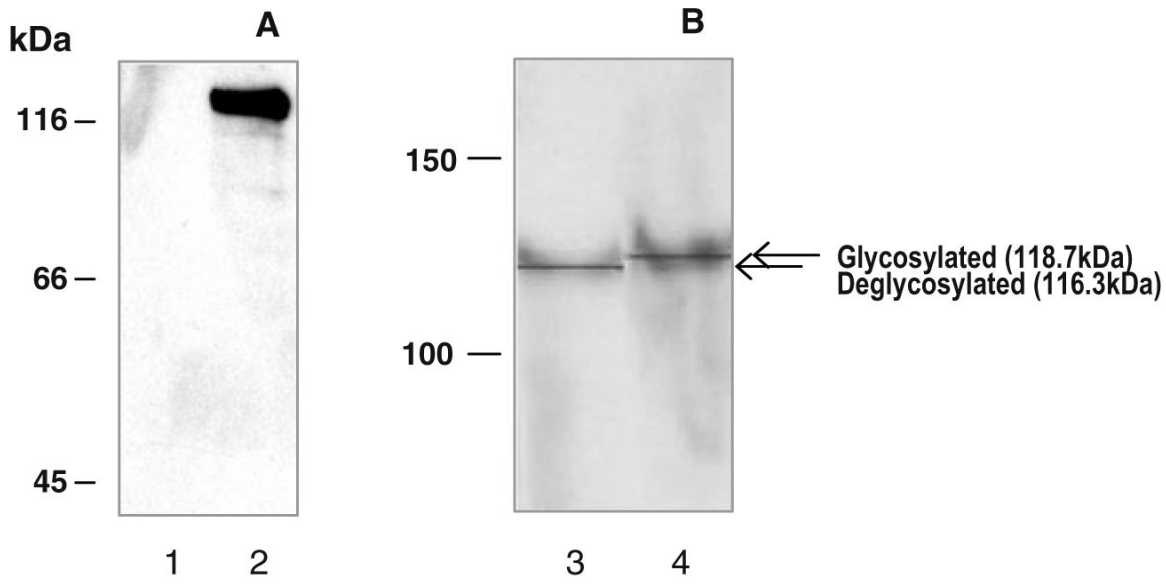

Fig 3.

Expression of A. thaliana GCSIIa encoding gene in S. pombe. Extracts prepared from wild type *S. pombe* cells (lane *1*) or the same transformed with HA-tagged At5g63840 (lanes *2–4*) were run in SDS-PAGE and developed with anti HA serum. Sample in lane *3* had been previously treated with Endo H.

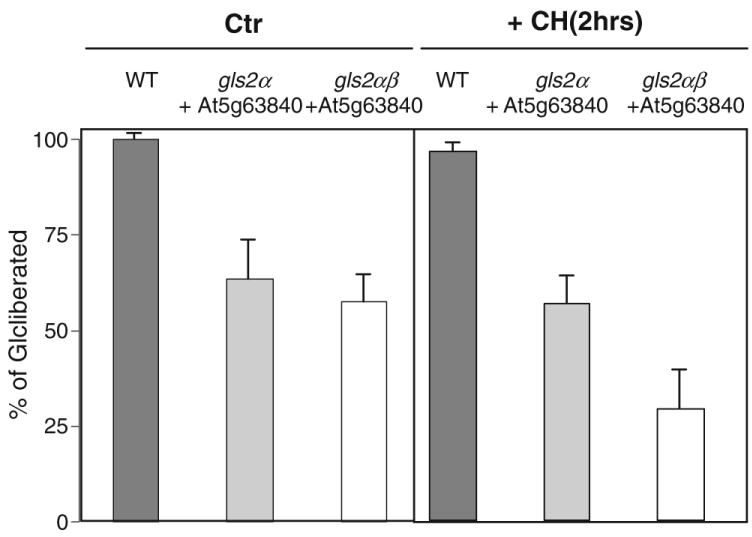

Fig 4.

GCSII activity in yeast cells transformed with At5g63840 preincubated with cycloheximide. GCSII activity was assayed using [*glucose*-^{14}C]$Glc_1Man_9GlcNAc$ as substrate in microsomes prepared from wild type (WT), from *gls2α* mutants transformed with At5g63840 cDNA in the pREP3X vector (*gls2α*+At5g63840) or from *gls2αβ* mutants transformed with the same cDNA (*gls2αβ*+Atg63840). Ctr and CH(2 h) indicate control and a previous 2 h incubation of cells with cycloheximide, respectively

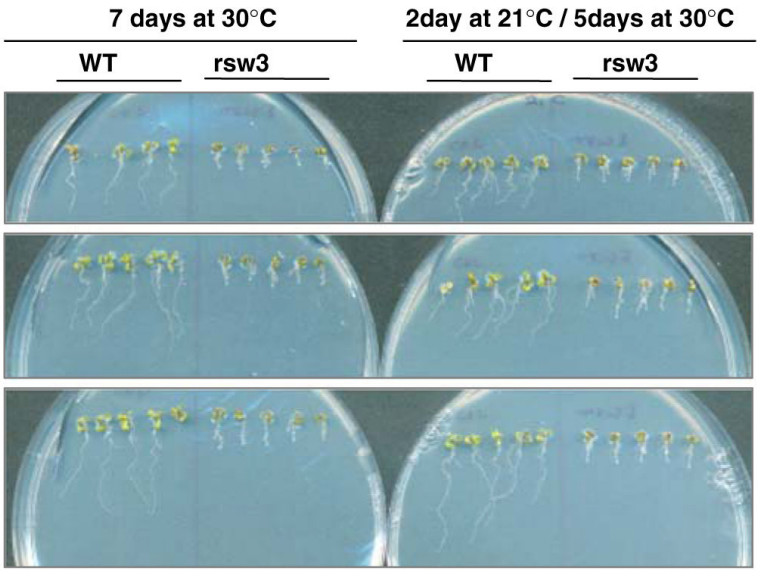

Fig 5.

Germinating study of rsw3 mutant grown at restricitive temperature. Wild type and rsw3 mutant seeds have been grown in petri-dish in the same conditions. *Left side* seedlings have germinated and were kept at restrictive temperature (7 days at 30°C). *Right side* seedlings have germinated at permissive temperature (2 days at 21°C), they were then transferred to restrictive temperature (31°C) for 5 days

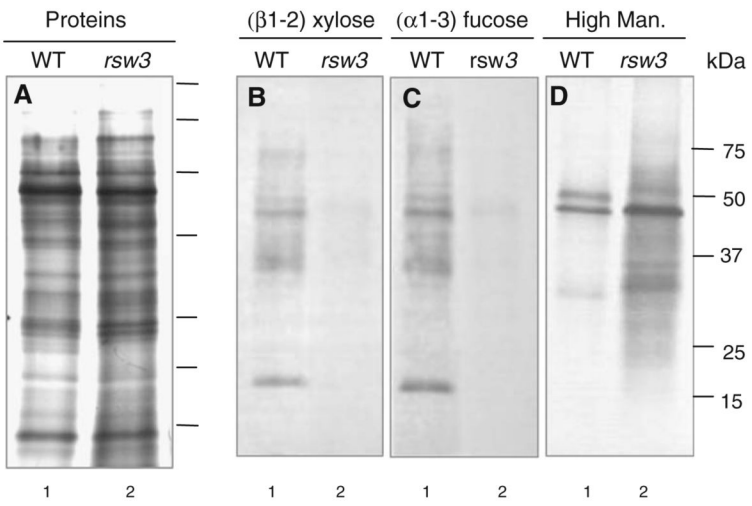

Fig 6.

Characterization of glycoproteins accumulated in Arabidopsis wild type and rsw3 mutant seedlings. Proteins obtained from Arabidopsis wild type (WT, lanes *1*) as well as from *rsw3* mutant seedlings (*rsw3*, lanes *2*) grown for 2 days at 21°C and for 5 days at 30°C were run on SDS-PAGE and either silver stained (**a**) or immunoblotted using antibodies specific for (β1-2)-xylose (**b**) or (α1-3)-fucose (**c**) residues constitutive of complex N-glycans, or submitted to ConA blotting analysis for high mannose N-glycan detection (**d**)

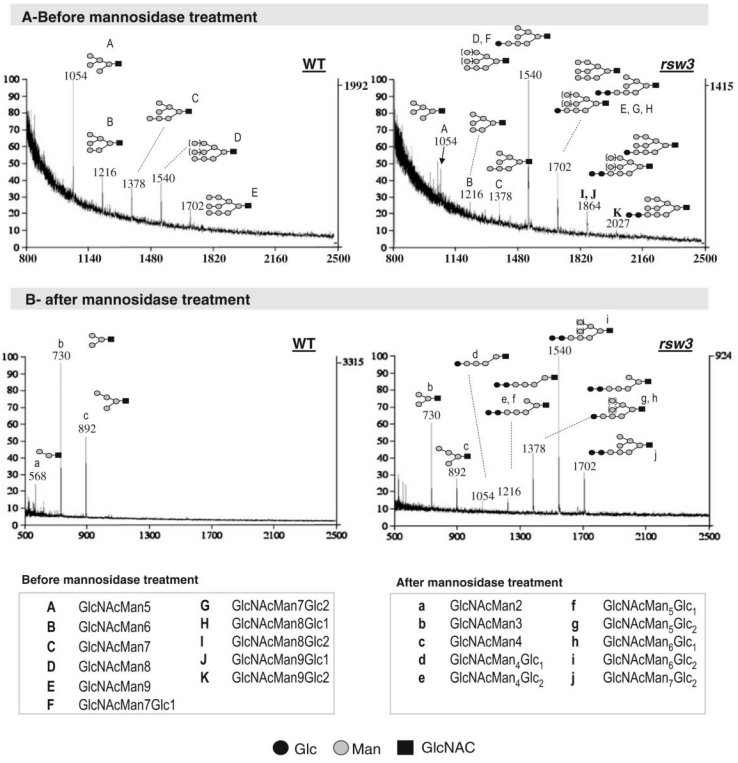

Fig 7.

MALDI-TOF analysis of high mannose type N-glycans synthesized in wild type and rsw3 mutant Arabidopsis seedlings. N-glycans released from whole cell glycoproteins prepared from wild type (WT) and rsw3 mutant Arabidopsis seedlings by Endo H treatment were analyzed by MALDI-TOF mass spectrometry before (**a**) and after (**b**) α-mannosidase degradation. *Letters* refer to N-glycan structures

Table 1

Primers employed

At5g63840 F1	ATGAGATCTCTTCTCT TTGTACTATCACTCATT
At5g63840 R1	CACCAACCATCAAAGTCTT TTCCAG
At5g63840 F2	CTCATCCAGAGGAGATGCAAAAGAA
At5g63840 R2	TCACAGAAT CTTTACGGTCCAGTCTT
At5g63840 F3	AAGATGGATGCTCCAGAG
At5g63840 R3	CCGAGCTCGCGGCCGCCCAGA ATCTTTACGGTCC
B2a	GCATTTCATTACGCTCATCG