

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

Pravina Soussillane · Cecilia D'Alessio ·
Thomas Paccalet · Anne-Catherine Fitchette ·
Armando J. Parodi · Richard Williamson ·
Carole Plasson · Loïc Faye · Véronique Gomord

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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 $\alpha\beta$ -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.

Keywords Plant · *N*-glycosylation · Glucosidase II · Glucose-trimming

Abbreviations

At	<i>Arabidopsis thaliana</i>
CNX	calnexin
ConA	concanavalin A
CRT	calreticulin
Endo H	endo- β -N-acetylglucosaminidase H
ER	endoplasmic reticulum
GCS	glucosidase
TFA	trifluoroacetic acid

Cecilia D'Alessio and Thomas Paccalet have equal contributions to this work

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P. Soussillane · T. Paccalet · A.-C. Fitchette · C. Plasson ·
L. Faye · V. Gomord (✉)
CNRS, UMR 6037, IFRMP 23, Bâtiment Biologie Extension,
Faculté des Sciences,
Mont-Saint-Aignan, France
e-mail: vgomord@crihan.fr

C. D'Alessio · A. J. Parodi
Instituto Leloir,
Av Patricias Argentinas 435,
Buenos Aires, Argentina

R. Williamson
Plant Cell Biology Group,
Research School of Biological Sciences,
The Australian National University,
G. P. O. Box 475, Canberra, ACT 2601, Australia

Introduction

Protein *N*-glycosylation in eukaryotic cells is initiated in the endoplasmic reticulum (ER) by the *en bloc* transfer of a glycan precursor, $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, from a lipid carrier to specific Asn residues in the nascent polypeptide [1, 2]. After transfer, the *N*-glycan undergoes extensive remodeling that begins with the removal of the outermost (α 1-2)-linked glucose residue by the type II membrane protein glucosidase I. Glucosidase II (GCSII) then removes the middle (α 1-3)-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 (α 1-3)-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 (GCSII α) and beta (GCSII β) subunits. GCSII α 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 GCSII β is to mediate ER localization of the GCSII α /GCSII β complex [12]. Indeed, GCSII β 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 GCSII β . For instance it is probably involved in the folding of recombinant mammalian GCSII α expressed also in mammalian cells, as this last protein is insoluble and forms inactive aggregates when expressed alone [16, 17]. However, once GCSII α is fully folded, GCSII β 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* GCSII β , which is not necessary for retention of GCSII α in the ER, but is required for cleavage of the innermost (α 1-3)-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* GCSII α encoding gene (At5g63840) and the characterization of the protein through complementation of *Schizosaccharomyces pombe* GCSII α 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* GCSII α 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,

Table 1 Primers employed

At5g63840 F1	ATGAGATCTCTTCTCT TTGTACTATCACTCATT
At5g63840 R1	CACCAACCATCAAAGTCTT TTCCAG
At5g63840 F2	CTCATCCAGAGGAGATGCAAAAGAA
At5g63840 R2	TCACAGAAT CTTTACGGTCCAGTCTT
At5g63840 F3	AAGATGGATGCTCCAGAG
At5g63840 R3	CCGAGCTCGCGGCCCGCCAGA ATCTTACGGTCC
B2a	GCATTTTCATTACGCTCATCG

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*, *gls2β::ura4+*) were described in D'Alessio *et al.* [8].

The wild type strains used as positive controls were h⁹⁰, *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 h⁹⁰), 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*, *gls2α::ura4+* and h⁺, *ura4-D18*, *leu1-32*, *ade6-M216*, *gls2α::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 GIAb as already described in Fernandez *et al.* [23]. They yielded 542- and 2306-bp bands for the wild type gene and the *gls2α::ura4+* 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*, *gls2α::ura4+*,

gls2β::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 [¹⁴C]glucose and purification of labelled endo-β-N-acetylglucosaminidase 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 [¹⁴C]Glc₁Man₉GlcNAc, 40 mM

sodium phosphate buffer pH 7.0 and 0.5% Lubrol in a total volume of 100 μ l. 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. Chromatography 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 \times 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 (β 1-2)-xylose or (α 1-3)-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 chemiluminescence (Supersignal West Pico Chemiluminescence 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 \times g for 15 min at 4°C. Proteins were precipitated by the addition of trichloroacetic acid (12.5% v/v). After centrifugation (10,000 \times 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 \times 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% trifluoroacetic 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 II α subunit

In order to identify *A. thaliana* GCSII α subunit involved in the early trimming of N-glycans, mammalian GCSII α sequences available were blasted against the *A. thaliana* genome database at NCBI and a gene encoding a putative GCSII α 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://npsa-pbil.ibcp.fr/>) were also identified and one of these two sites (N804) could be actually glycosylated according to NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Furthermore, as observed for yeast and mammalian GCSII α , 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 GCSII α subunit by functional complementation of a *S. pombe* GCSII α deficient mutant

Microsomes prepared from *S. pombe* mutants defective in GCSII α (*gls2 α*) or in both GCSII α and GCSII β (*gls2 $\alpha\beta$*), but transformed with At5g63840 cDNA in the pREP3X vector displayed GCSII activity as they were able to liberate labelled glucose from [*glucose*-¹⁴C]Glc₁Man₉GlcNAc (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 *gls2 α* (GCSII α minus) cells transformed either with an empty vector or with the pREP3X vector containing At5g63840

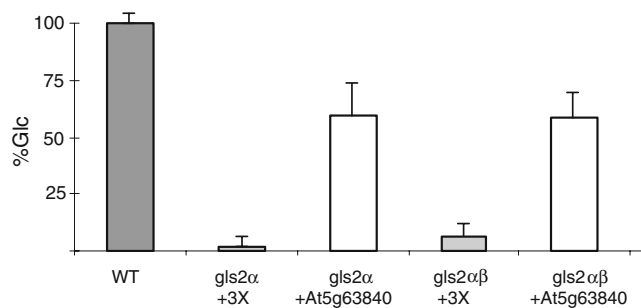


Fig 1 GCSII activity in yeast cells transformed with At5g63840. GCSII activity was measured using [*glucose*-¹⁴C]Glc₁Man₉GlcNAc as substrate in microsomes prepared from wild type (WT) cells, in GCSII α minus (*gls2 α*) mutants transformed with an empty vector (*gls2 α* +3X), in the same cells transformed with At5g63840 cDNA in the pREP3X vector (*gls2 α* +At5g63840), in yeast mutant cells lacking GCSII α and GCSII β (*gls2 $\alpha\beta$*) transformed with an empty vector (*gls2 $\alpha\beta$* +3X) and in the same cells transformed with At5g63840 (*gls2 $\alpha\beta$* +At5g63840)

cDNA were pulse-labelled for 30 min with [¹⁴C]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 (Man₉GlcNAc₂) and traces of Man₈GlcNAc₂ (Fig. 2a). In contrast, the *gls2 α* mutant lacking GCSII activity accumulated the enzyme substrate Glc₂Man₉GlcNAc₂ (Fig. 2b).

The oligosaccharide pattern obtained with the *gls2 α* mutant transformed with At5g63840 cDNA was similar to that obtained from wild type *S. pombe* except for trace amounts of Glc₂Man₉GlcNAc₂ (Fig. 2c). This result clearly illustrates that GCSII activity was restored by the At5g63840 cDNA in the *S. pombe gls2 α* 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 GCSII α subunit cloned so far.

Preliminary characterization of Arabidopsis GCSII α subunit

Kaushal *et al.* [19] showed a 95 kDa protein to be the GCSII α subunit in soybean suspension-cultured cells while GCSII α is a 110 kDa protein in mung bean. The molecular mass of AtGCSII α deduced from its cDNA sequences was 104 kDa, while western blot analysis of protein extracts obtained from *S. pombe gls2 α* mutant expressing HA-tagged AtGCSII α showed a polypeptide of approximately 119 kDa (Fig. 3a). Moreover, the molecular mass of yeast-made AtGCSII α was reduced by about 2 kDa after deglycosylation with Endo H (Fig. 3b). This result shows that when expressed in *S. pombe* AtGCSII α is glycosylated with probably a single *N*-glycan, a data in agreement with previous biochemical characterization of a plant GCSII α subunit [36] and *in silico* prediction from the At5g63840 sequence (see above).

Probable characteristics of *A. thaliana* GCSII β subunit

As mentioned above, depending on the organism, different roles for GCSII β subunits have been proposed. In most cells GCSII β is apparently required for ER retention and/or proper folding of GCSII α 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*

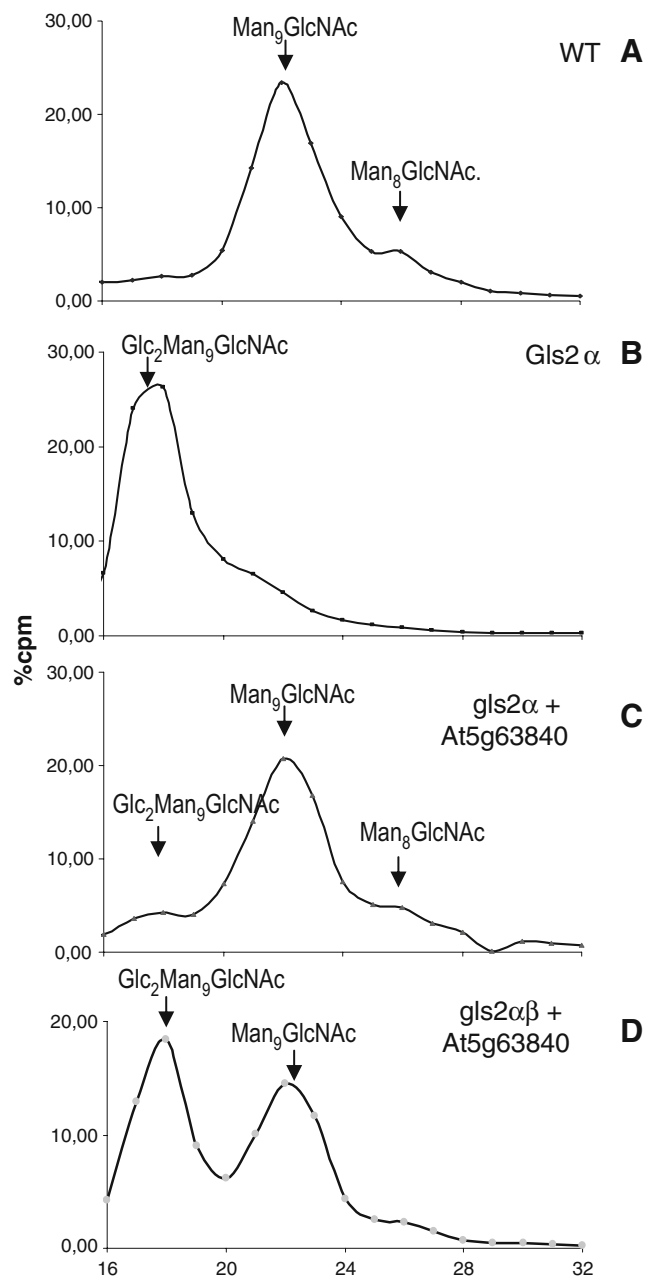


Fig 2 N-glycan processing in yeast cells transformed with At5g6380. Wild type (A), *gls2α* (B), *gls2α* transformed with At5g63840 cDNA in the pREP3X vector (C) or *gls2αβ* cells transformed with the same cDNA (D) were incubated with [¹⁴C]glucose for 30 min and N-glycans released from whole cell glycoproteins with Endo H were run on paper chromatography

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

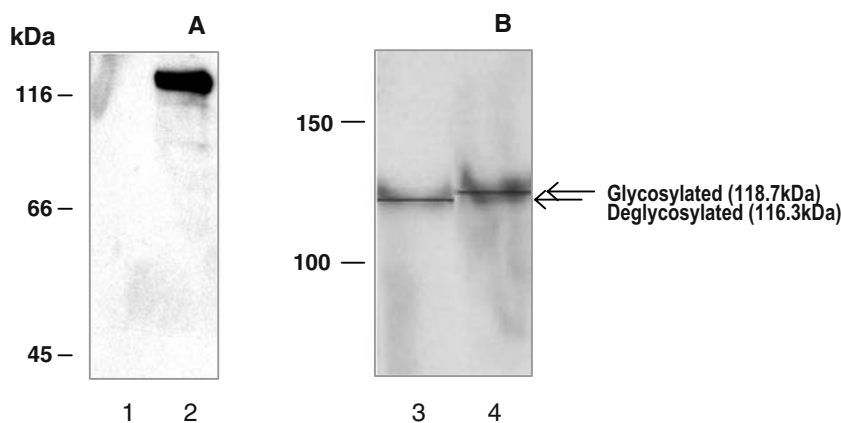
subunit to display similar roles as those of homologous proteins in most eukaryotic cells: as shown in Fig. 1, AtGCSIIα expressed in *gls2αβ* (both GCSIIα and GCSIIβ minus *S. pombe* mutant cells) was able to release the glucose unit from [¹⁴C]Glc₁Man₉GlcNAc. Moreover, *in vivo* processing of the N-glycan in yeast double mutant cells expressing AtGCSIIα resulted in the formation of almost similar amounts of Glc₂Man₉GlcNAc₂ and Man₉GlcNAc₂, thus confirming the ability of the plant catalytic subunit to remove both the middle and innermost glucose residues. Lack of complete N-glycan deglycosylation 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 cycloheximide, 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).

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.



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 (β 1-2)-xylose- (Fig. 6b) or (α 1-3)-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).

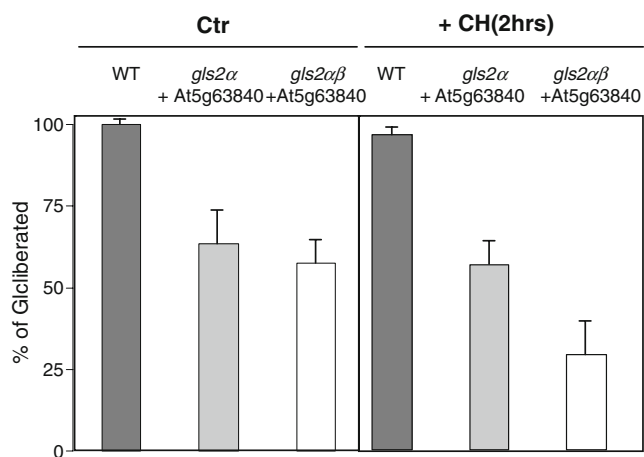


Fig 4 GCSII activity in yeast cells transformed with At5g63840 preincubated with cycloheximide. GCSII activity was assayed using [14 C]Glc₁Man₉GlcNAc as substrate in microsomes prepared from wild type (WT), from *gls2α* mutants transformed with At5g63840 cDNA in the pREP3X vector (*gls2α*+At5g63840) or from *gls2αβ* mutants transformed with the same cDNA (*gls2αβ*+At5g63840). Ctr and CH(2 h) indicate control and a previous 2 h incubation of cells with cycloheximide, respectively

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 Man₅GlcNAc to Man₉GlcNAc, 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

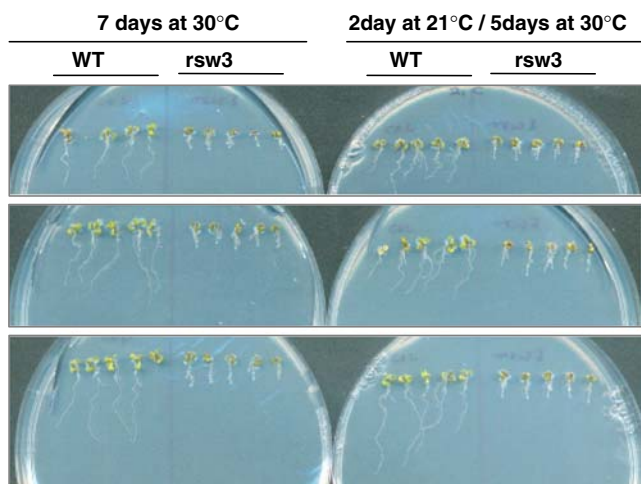
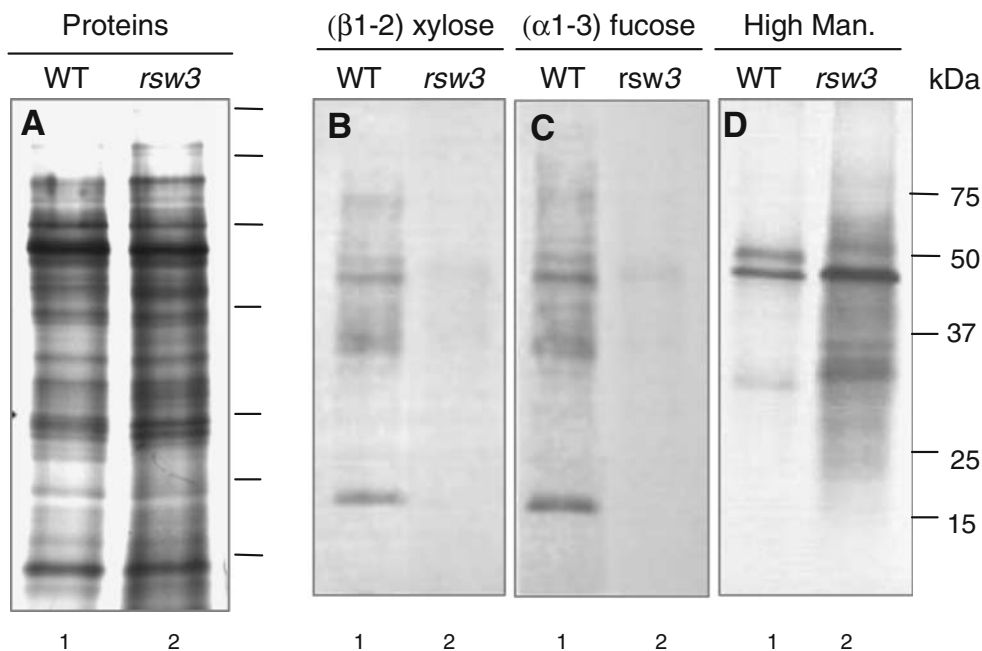


Fig 5 Germinating study of *rsw3* mutant grown at restrictive temperature. Wild type and *rsw3* mutant seeds have been grown in petri-dish in the same conditions. *Left side* seedlings have germinated and were kept at restrictive temperature (7 days at 30°C). *Right side* seedlings have germinated at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

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).

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)



Conclusion

Using heterologous expression in the yeast *S. pombe*, we have shown that the protein encoded by At5g63840 is an authentic GCSII α subunit bearing GCSII catalytic activity. Inactivation of GCSII α 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 GCSII α 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 deglycosylation 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 deglycosylation pathway involving a *cis/medial* Golgi endomannosidase, that releases a Glc_{1,2}Man di- or tri-saccharide from the Glc_{1,2}Man₉GlcNAc₂ [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 GCSII α -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

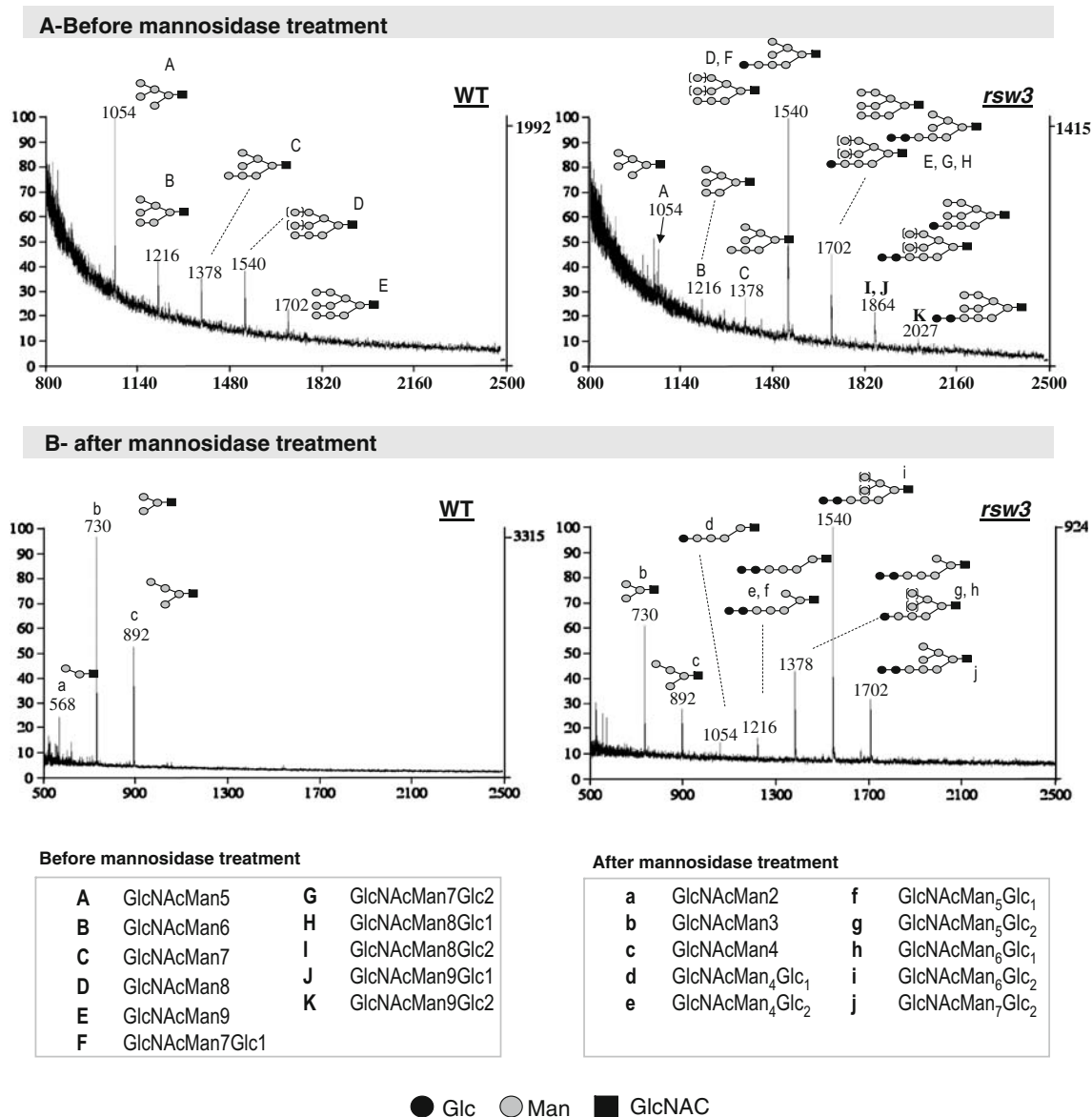


Fig 7 MALDI-TOF analysis of high mannose type N-glycans synthesized in wild type and *rsw3* mutant Arabidopsis seedlings. N-glycans released from whole cell glycoproteins prepared from wild type (WT) and *rsw3* mutant Arabidopsis seedlings by Endo H

treatment were analyzed by MALDI-TOF mass spectrometry before (a) and after (b) α -mannosidase degradation. Letters refer to N-glycan structures

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 glycosyltransferase activities show no obvious phenotypes except for the N-glycan patterns [46, 47].

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