Oligomerization of the reversibly glycosylated polypeptide: Its role during rice plant development and in the regulation of self-glycosylation

ARTICLE *in* PROTOPLASMA · FEBRUARY 2012

Impact Factor: 2.65 · DOI: 10.1007/s00709-012-0382-x · Source: PubMed

3 AUTHORS, INCLUDING:

Veronica De Pino

National Scientific and Technical Research …

3 PUBLICATIONS **12** CITATIONS

SEE PROFILE

Silvia Moreno

National Scientific and Technical Research …

29 PUBLICATIONS **513** CITATIONS

SEE PROFILE

Protoplasma
DOI 10.1007/s00709-012-0382-x

ORIGINAL ARTICLE

Oligomerization of the reversibly glycosylated polypeptide: its role during rice plant development and in the regulation of self-glycosylation

Verónica De Pino · Cristina Marino Busjle · Silvia Moreno

Received: 5 December 2011 / Accepted: 23 January 2012

Abstract A multigenic family of self-glycosylating proteins named reversibly glycosylated polypeptides, designated as RGPs, have been usually associated with carbohydrate metabolism, although they are an enigma both at the functional, as well as at the structural level. In this work, we used biochemical approaches to demonstrate that complex formation is linked to rice plant development, in which class 1 *Oryza sativa* RGP (OsRGP) would be involved in an early stage of growing plants, while class 2 OsRGP would be associated with a late stage linked to an active polysaccharide synthesis that occurs during the elongation of plant. Here, a further investigation of the complex formation of the *Solanum tuberosum* RGP (StRGP) was performed. Results showed that disulfide bonds are at least partially responsible for maintaining the oligomeric protein structure, so that the nonreduced StRGP protein showed an apparent higher molecular weight and a lower radioglycosylation of the monomer with respect to its reduced form. Hydrophobic cluster analysis and secondary structure prediction revealed that class 2 RGPs no longer maintained the Rossman fold described for class 1 RGP. A 3D structure of the StRGP protein resolved by homology modeling supports the possibility of intercatenary disulfide bridges formed by exposed cysteines residues C79, C303 and C251 and they are most probably involved in complex formation occurring into the cell cytoplasm.

Keywords Complex formation · Plant polysaccharide · Self-glycosylation · Reversibly glycosylated polypeptide

Abbreviations

C	Cysteine
RGP	Reversibly glycosylated polypeptide
Os	*Oriza sativa* RGP
StRGP	*Solanum tuberosum* RGP
AtRGP	Arabidopsis thaliana
TaRGP	Triticum aestivum
UDP	Uridine diphosphate
UDP-Glc	Uridine dipjosphate glucose

Introduction

Plant cell walls, which consist mainly of polysaccharides and glycoproteins, constitute most of the world's biomass and the bulk of the earth renewable source of energy. Nowadays, researcher's community of the plant cell walls is making steady progress in defining biochemical functions of the glycosyltransferases, not only that polymerizes the carbon backbone but also the ones that decorate the main chain. However, the biochemical details of this large and coordinated process of plant cell wall synthesis are still unknown.

During long time it was thought that autocatalytic glycosyltransferases of plants are implicated in the first steps of plant polysaccharide biosynthesis (Moreno et al. 1986,

Handling Editor: Klaus Harter

V. De Pino
Facultad de Farmacia y Bioquímica, Cátedra de Farmacognosia, INQUIMEFA–Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET),
Junín 954,
Ciudad Autónoma de Buenos Aires (1113), Argentina

C. Marino Busjle · S. Moreno (✉)
Instituto de Investigaciones Bioquímicas Buenos Aires–CONICET,
Fundación Instituto Leloir,
Patricias Argentinas 435,
Ciudad Autónoma de Buenos Aires (1405), Argentina
e-mail: smoreno@leloir.org.ar

Published online: 25 February 2012

1987). In this sense, looking for an enzyme capable of self-glycosylation, reversibly glycosylated polypeptide (RGP) was discovered in the early 1990s (Dhugga et al. 1991, 1997; Delgado et al. 1998; Bocca et al. 1999). Even now the precise function of the RGP protein remains an enigma. Genomic and proteomic data indicate that RGP protein is involved in a variety of metabolic and developmental pathways in plants (Chen et al. 2006; Drakakaki et al. 2006; Masuko et al. 2006; Dai et al. 2007; Zavaliev et al. 2010). Moreover, this enzyme is associated with several defense response phenomena (Selth et al. 2006), impairment of virus spreading (Zavaliev et al. 2010), response to abiotic stresses (Jiang et al. 2007; Hukkanen et al. 2008; Tran and Plaxton 2008; Ge et al. 2009), in processes involving reactive oxygen species (Kim et al. 2008), and in random gravitational fields (Barjaktarovic et al. 2009). All the aforementioned processes have in common an active synthesis of cell wall components such as cellulose and hemicellulose and/or the mobilization of sugars in order to respond to diverse stimulus.

Different strategies have been addressed in order to understand the role of RGP in plants, including phylogenetic analysis, which revealed that dicot and monocot plants have different RGP subfamilies, class 1 RGP that are well separated from the class 2 (Wald et al. 2003). It was proposed that the two classes of genes might be involved in the biosynthesis of different plant polysaccharides (Langeveld et al. 2002; Wald et al. 2003). Earlier biochemical studies revealed that glucosylation of the *Solanum tuberosum* RGP (StRGP) is reversible after the incubation with micromolar concentration of radioactive uridine diphosphate (UDP-Glc), since radioactive UDP-Glc was obtained in a chase-out experiment (Testasecca et al. 2004). This report showed that one residue of glucose, xylose, and galactose are linked to the protein in a β-configuration, and all of them are linked to the protein. In sweet corn and rice, it was found that the sugar moiety was bound to a single arginine residue (Singh et al. 1995; Konishi et al. 2010b). In addition, a UDP-arabinopyranose mutase activity was report to be associated with the rice RGP protein (Konishi et al. 2007), and site-directed mutagenesis studies revealed that a DXD motif is required for the catalytic activity of the arabinose mutase activity of class 1 RGP, while for class 2 RGP, neither arabinose mutase activity (Konishi et al. 2010a) nor other self-glycosylating activity has been described (Langeveld et al. 2002), suggesting that some other element may be required for self-glycosylation activity.

We have previously described that native StRGP from potato tuber is associated with membranes as an oligomeric protein (Moreno et al. 1986; Bocca et al. 1999) and incubation at high ionic strength produced a high level of RGP protein self-glycosylation, while low ionic strength led to a low level of protein glycosylation favoring the formation of high molecular weight RGP-containing forms (De Pino et al. 2007). Here, we further analyze the nature of the OsRGP oligomerization during development through biochemical approaches. In addition, several information regarding protein–protein interactions of class 1 and class 2 of the RGP protein was inferring using hydrophobic cluster analysis, secondary structure prediction, and homology modeling of the StRGP.

Materials and methods

Plant materials

Rice seeds (*Oriza sativa* cv. El Paso 144) were provided by La Arrocera Argentina, Entre Rios, Argentina. For in vitro plant system, seeds were dehulled and surface-sterilized in 30 ml of NaClO and put in 70 ml of sterilized distilled water with 100 μl of Triton X100 for 30 min in agitation. Then they were rinsed several times in distilled water and grown aseptically in flasks containing Murashige and Skoog (1962) medium supplemented with 2% sucrose, 0.8% agar. They were kept at 22–24°C under a 16 h photoperiod (40–60 $\mu Em^{-2}\ s^{-1}$) with cool white fluorescent tubes (OSRAM, USA). At 2, 5, 8, and 10 days after germination, plants were collected, immediately frozen in liquid nitrogen, and stored at -80°C. Protein was extracted from plants at different stages. Plant tissues were ground in liquid nitrogen in buffer A (100 mM Tris–HCl, pH 7.4, 20 mM 2-mercaptoethanol) with 0.1 mM PMSF (Sigma, St. Louis, MO, USA). Rice proteins were obtained as described (Lavintman et al. 1974). The 100,000×*g* supernatant was used as a source of soluble enzyme.

Potato tuber enzyme was obtained as described (Testasecca et al. 2004). The supernatant of 100,000×g was precipitated by adding 50% (w/v) ammonium sulfate. The protein was dissolved in 10 mL of 50 mM Tris–HCl, pH 7.4, 10 mM 2-mercaptoethanol. This soluble potato preparation was applied to a Mono Q HR 10/10 column (GE Healthcare, Buckinghamshire, UK) and eluted with a KCl gradient (0–0.5 M), and RGP activity was obtained eluting with a maximum active peak eluting at 0.2 M KCl. In experiments in the presence or absence of reducing agent, the Mono Q fraction F1 was divided into four aliquots of 500 μl, preincubated in the presence of reducing and assayed for RGP activity after glucosylation with UDP [^{14}C]Glc. Proteins were submitted to SDS-PAGE and autoradiography as described below.

Size exclusion chromatography on Superose 12

Aliquot of the RGP protein (200 μl) was applied to a Superose 12 column HR 10/30 (GE Healthcare). The column was equilibrated with 50 mM Tris–HCl, pH 7.4, 10 mM 2-mercaptoethanol and eluted at a flow rate of 0.5 ml min^{-1}.

Fractions of 500 µl were taken and RGP activity was determined in 250 µl of each fraction. The column was calibrated with molecular mass standard proteins trypsinogen (29 kDa), carbonic anhydrase (48 kDa), and lactate dehydrogenase (176 kDa). For RGP glycosylation assays, proteins were incubated with UDP-[^{14}C]Glc (0.2 nmol, 100,000 cpm) for 2 h. The radioactive sugar incorporated into the protein was measured into a 10% trichloroacetic acid pellet as described (Wald et al. 2003). Liquid scintillation counting was carried out with Ultima Gold mixture (Perkin-Elmer Life Sciences, Wellesley, MA, USA) in a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA, USA). UDP-[^{14}C]Glc (11,100 GBq mol^{-1}) was obtained according to Thomas et al. 1968.

SDS-PAGE and immunoblots

SDS-PAGE analysis was performed on 10% (w/v) acrylamide gels according to Laemmli (1970). An aliquot of 50 µg of total protein was precipitated with 10% trichloroacetic acid, centrifuged, and resuspended in 20 µl 1× Laemmli buffer and heated for 1 min with or without 2-mercaptoethanol. For Western blot analysis, proteins were transferred to polyvinylidene fluoride membranes (PVDF-Hybond-P, GE Healthcare) using a mini Trans-Blot cell (Bio Rad, Hercules, California, USA). Membranes were blocked with 3% (w/v) nonfat milk in PBS. The blots were incubated with anti-potato tuber RGP antibody (1:1,000 dilution) for 90 min as described (Bocca et al. 1999). This antibody recognizes StRGP and OsRGP proteins. Immunoreactive bands were visualized by the ECL system (GE Healthcare). Alternatively, precipitated proteins were separated by SDS-PAGE and subjected to autoradiography using a phosphoimager (Molecular Dynamics STORM 840, GE Healthcare) as reported by (Testasecca et al. 2004). Signals were quantified by phosphoimaging.

Homology modeling of RGP

The RGP from *S. tuberosum* formerly named UPTG (E2 clone: AJ310910; Wald et al. 2003) was chosen for homology modeling. Protein residues from 1 to 337 was modeled using UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferase N-terminal domain as template (pdb code 1xhb from residues 103 to 422; Fritz et al. 2004). The template structure (1XHB) belongs to the nucleotide-diphospho-sugar transferases superfamily, polypeptide *N*-acetylgalactosaminyltransferase 1, N-terminal domain family, according to Scop classification (Andreeva et al. 2008). Other well-ranked templates were 2ffuA, 3ckjA, 3e25A, 2d7iA, according to BioInfoBank and 3bcv, 1qg8, 2z86, 3f1y, 3ckj, 2d7i, according to same HHpred superfamily. Although both servers recognized the match between residues 95 and 346 of 1hxb approximately and residues 1 and 270 of RGP sequence, we extended the model till the end of the N-terminal 1hxb domain (residue 422 1xhb and 337 StRGP) since secondary structure prediction methods predicted four helices between residues 270 and 337 of StRGP, comparable to the ones present in 1hxb between residues 346 and 422. The model validation supported the veracity of that hypothesis. Several fold recognition methods were tested through the BioInfoBank (Ginalski et al. 2003) and HHpred metaservers (Soding et al. 2005). Once the template was selected, clustal alignment followed by manual adjustment was done. The model was built using MODELLER 9v8 (Eswar et al. 2007). The cycle of realignment, modeling, and structure evaluation were repeated until no further improvements on the structure were observed. Evaluation of the model (excluding loops) was carried out with Verify 3D (Luthy et al. 1992) and Procheck (Laskowski et al. 1993) and by superposition with its own template structure using the combinatorial extension method (Shindyalov and Bourne 1998). All graphics were done with Chimera (Pettersen et al. 2004).

Results

In order to further analyze the complex formation of RGP, we study the OsRGP protein profile during development in an in vitro rice plant system, in which germination occurs after 1–2 day, coleoptiles and nodal roots emerge from seed after day 2, and shoots including several leaves are observed after day 4 (Fig. 1a). Soluble protein extracts of different developmental stages were analyzed by SDS-PAGE followed by Western blot (Fig. 1b). A prominent 40 kDa protein corresponding to the monomeric size of OsRGP was observed in ungerminated seeds (day 0), germinated seed (day 2) and growing plants (days 4, 8 and 10). However, upon day 4 of culture, the OsRGP protein profile was complex appearing a 116 kDa protein, which was the main form after 10 days of culture. The identity of the monomer was confirmed by sequencing the 40 kDa band (data not shown). Densitometric analysis showed that the high molecular weight protein increases its amount along development while the OsRGP monomer decreases its expression (Fig. 1c).

To investigate the molecular weight of the OsRGP in ungerminated seeds and in plantlets after 8 days of culture, separation of RGP by native size exclusion chromatography on Superose 12 followed by measurement of radioactive glucose incorporation to the protein as previously described,

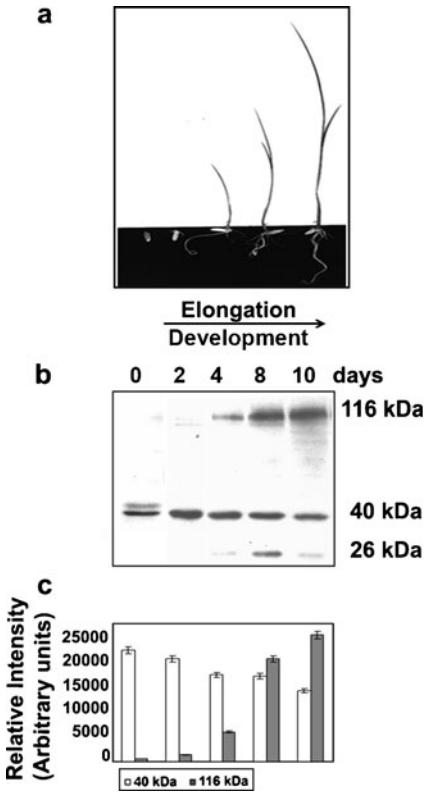


Fig. 1 OsRGP protein profile during rice development. **a** In vitro rice plant system from ungerminated seeds to platelets after 10 days of culture. **b** Western blot analysis of protein extracts of rice plants from 0 to 10 days of culture. **c** Quantification of protein bands by Scion image program

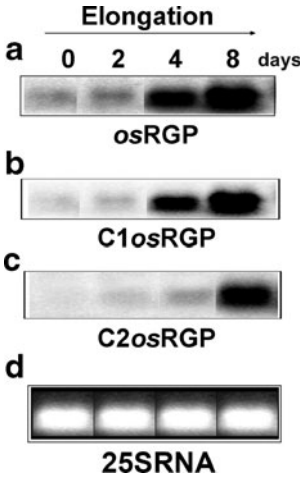


Fig. 3 OsRGP mRNA expression by northern blot analysis during rice development. Total RNA was extracted from ungerminated seeds (0 days), germinated seed (2 days), and growing plants after 4 and 8 days and revealed with specific probes: **a** RGP universal probe, **b** class 1 RGP and **c** class 2 RGP and **d** loading control gel stained with ethidium bromide

was performed (Fig. 2; De Pino et al. 2007). As expected the main active peak present in the ungerminated seeds had a molecular weight analogous to the RGP monomer (40 kDa, Fig. 2, full squares), minor peaks probably corresponded to a self-association of the protein. By contrast, the foremost active peak in the plantlets after 8 day of culture had a high molecular weight of approximately 176 kDa (Fig. 2, full circles).

To study whether OsRGPs can form homo or heterodimers during development, northern blot analysis was performed using a universal probe of the RGP and two highly specific probes directed against class 1 or class 2 RGPs. A remarkable increase in OsRGP mRNA abundance is observed at day 4 when the universal or the class 1-specific OsRGP probes were used (Fig. 3a and b). In contrast, an increase in the class 2 OsRGP expression was not observed until day 8.

Sequence analysis revealed that both classes of RGPs have several cysteines along their sequences: class 1 RGPs (StRGP1 12, StRGP2 10, OsRGP1 9, AtRGP1 9, and AtRGP4 8) and class 2 RGPs (OsRGP2 7, AtRGP5 8, TaRGP2 7). Therefore, we focused on the reduction of disulfide bonds. SDS PAGE separation of a radioglycosylated sample was performed in the presence or absence of 2-mercapthoethanol followed by western

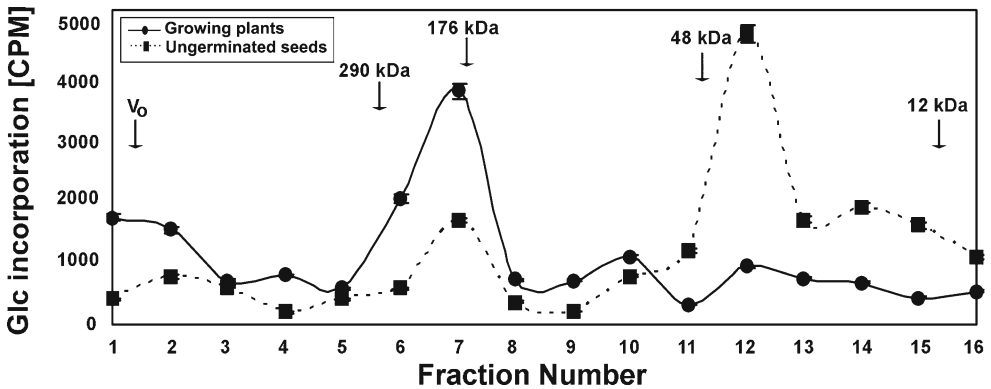


Fig. 2 OsRGP molecular weight analysis by size exclusion chromatography. Protein extracts of rice ungerminated seeds or growing plants after 8 days of culture were chromatographed on Superose 12. RGP activity was measured by the incorporation of glucose after incubation with UDP-[^{14}C]Glc for 2 h as described in material and methods. Vo corresponds to the dead volume and arrows to molecular weight of protein markers

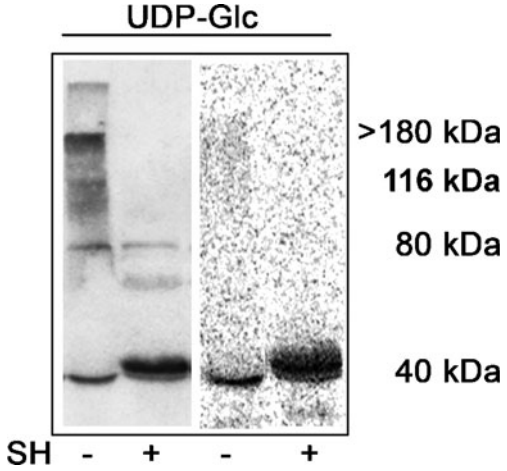


Fig. 4 Effect of reducing agent on the size and activity of StRGP protein. StRGP protein was preincubated during 30 min in the presence of 2-mercaptoethanol (*lanes 2–4*) or absence (*lanes 1–3*), then samples were incubated with UDP [^{14}C]Glc during 30 min and subjected to SDS PAGE analysis. The gel was revealed by western blot (*left panel*) and autoradiography (*right panel*)

blot analysis (Fig. 4 lane 1 and 2) and autoradiography (Fig. 4, lanes 3 and 4). As expected, a prominent 40 kDa monomer appeared in the presence of reducing agent (Fig. 4, lane 2), suggesting an impairment in the formation of disulfide bonds taking place between different monomers. Accordingly, in non-reducing conditions, high-molecular weight bands were clearly observed (Fig. 4, lane 1). Similarly, a detailed analysis of the autoradiography revealed radioactive high-molecular weight bands in the absence of the reducing agent (Fig. 4, lane 3), and the radioglycosylation of the RGP monomer was significantly lower under reducing conditions (Fig. 4, lane 3 vs. lane 4).

To obtain additional information regarding protein–protein interactions, we performed *in silico* characterization of three class 1 RGPs (OsRGP1, AtRGP1, TaRGP1) and three class 2 RGPs of different species (OsRGP2, AtRGP5 and TaRGP2) using hydrophobic cluster analysis (HCA) (Gaboriaud et al. 1987) HCA predicts several hydrophobic domains in class 2 RGP, which are absent in class 1 RGPs (Fig. 5a). In addition, an alignment of all members of class 1 or class 2 RGP performed by the Predict Protein algorithm (www.predictprotein.org) showed that class 2 RGPs display a

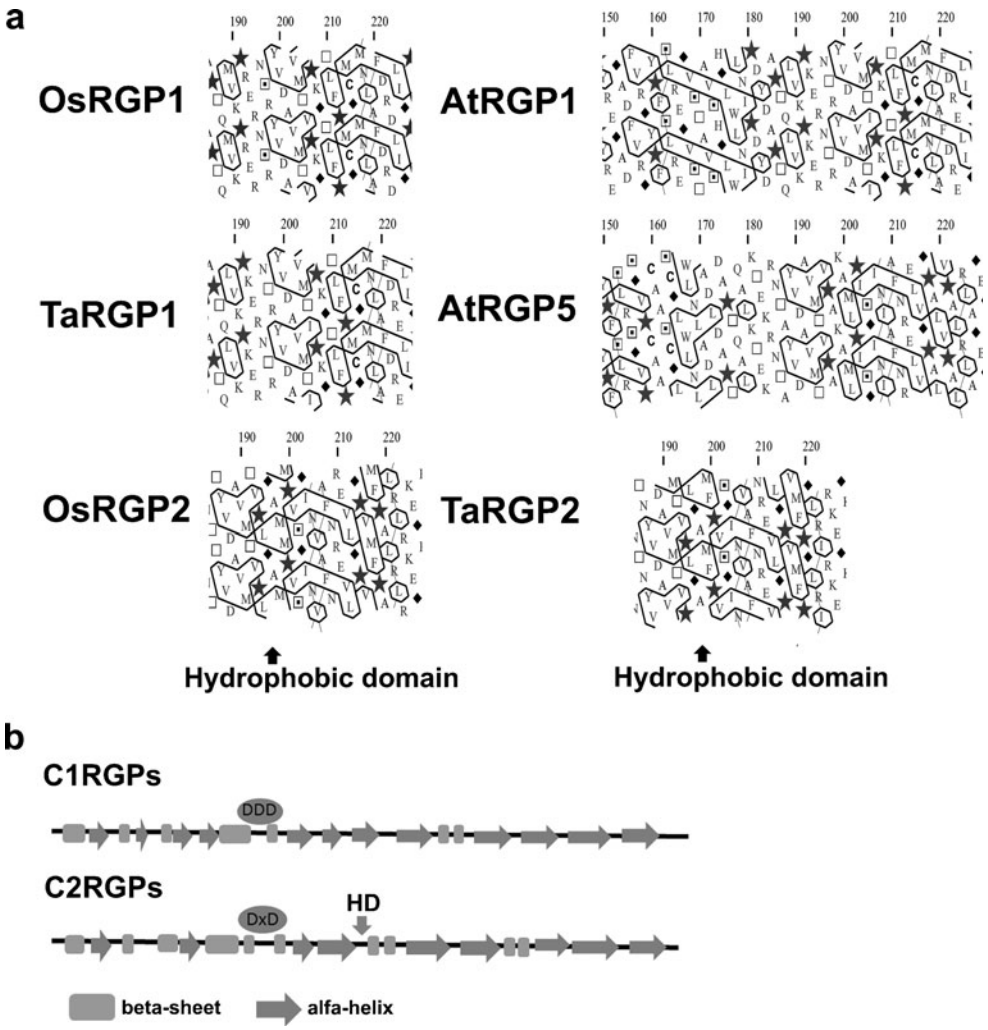
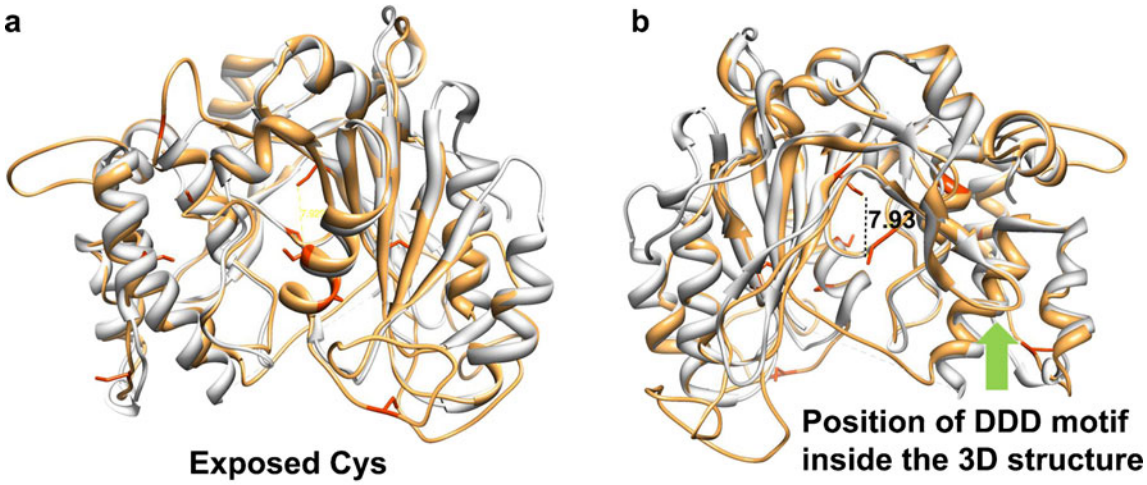
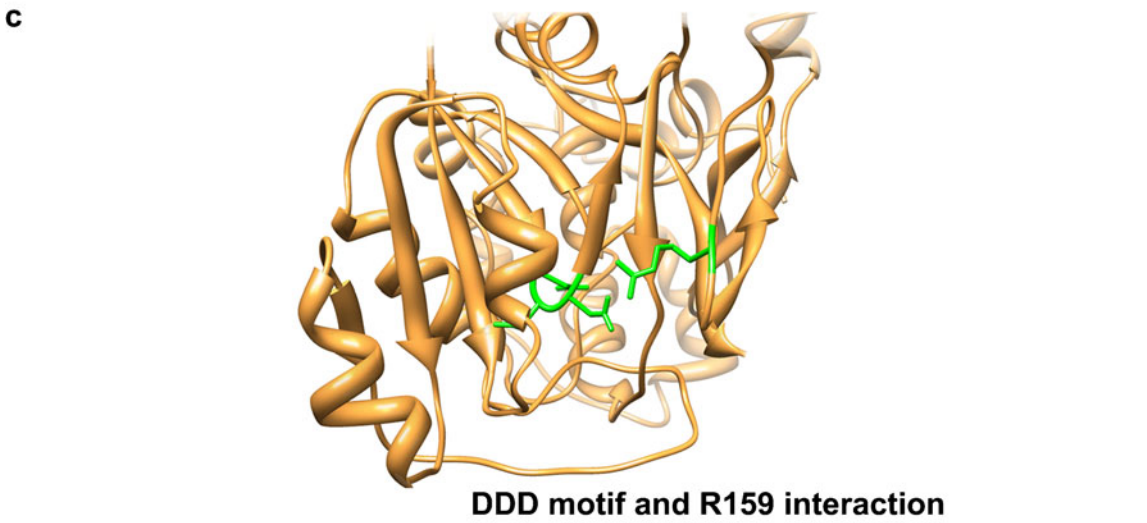


Fig. 5 *In silico* characterization of RGP protein secondary structure. HCA analysis of three proteins belong to class 1 and class 2 RGPs (**a**). Arrows indicated the hydrophobic domain present in class 2 RGP absent in class 1 RGP. Secondary structure elements of both classes of RGP after alignment of all members of class 1 or class 2 performed by the Predict Protein algorithm (**b**). HD represents the position of the hydrophobic domain predicted for class 2 RGP






```
1XhB   9/104    LEGCKTKVYPDNLPTTSVVIVFHNEAWSTLLRTVHSVINRSPRHMIEEIVLVDDASERDF
StRGP  1/2      MAGSSVTPTPLLKDELDIVIPTIRNLD-FLEMWRPFF-------QPYHLIIVQDGDPSKI

1XhB   69/164   LKRPLESYVKKLKVPVHVIRME------------QRSGLIRARLKGAAVSRGQVITFLDA
StRGP  53/54    INVPEGFD-------YELYNRNDINRILGPKASCISFKDSACRCFGYMVSKKKYIYTIDD

1XhB   117/212  HCECTAGWLEPLLARIKHDRRTVVCPIIDVISD--------------------DTFEYM
StRGP  106/107  DCFVAKDPSGKDINALEQHIKNLLCPSTPHFFNTLYDPYREGADFVRGYPFSMREGAATA

1XhB   156/251  AGSDMTYGGFNWKLNFRWYPVPQREMDRRKGDRTLPVRT-PTMAGGLFSIDRDYFQEI--
StRGP  166/167  VSHGLWLNIPDYDAPTQLVKPRERNTRYVDAVMTIPKGTLFPMCGMNLAFDRELIGPAMY

1XhB   213/308  -GTYDAGMDIWGGENLEISFRIWQC----GGTLEIVTCSHVGHV
StRGP  226/227  FGLMGDGQPIGRYDDMWAGWCIKVICDHLGLGVKTGLPYIWHSK
```

Fig. 6 3D prediction of the class 1 StRGP protein structure by homology modeling. **a** and **b** Ribbon representation of 1xhb (*grey*) and class 1 StRGP (*orange*) superimposed. **a** StRGP model showing the position of Cys residues represented as sticks coloured red and the distance between Cys 113 and Cys 209 represented as dashed line. **b** Ribbon model of StRGP presenting the position of the DDD motif (*green arrow*) and the distance represented as green sticks, between two adjacent Cys residues (130 and 209) and R159. **c** Zoom of the DDD motif and R159 interaction

different order in their secondary structure elements, no longer adopting the typical Rossman fold found in class 1 RGPs (consisting of three repeats of beta sheets-alpha helices) involved in the binding of nucleotide sugars (Fig. 5b).

Due to the failure in the crystallization of RGP proteins of different sources, we decided to perform a 3D prediction of the class 1 RGP protein structure by homology modeling (Fig. 6). The class 1 StRGP (formerly named UPTG; E2 clone: AJ310910) was chosen since it was reported to be a more active isoform (Wald et al. 2003). The template used was the enzyme *N*-acetylgalactosaminyltransferase (PDB code: 1XHB_A), which is also a UDP-glycosyltransferase that transfers GalNAc from the sugar donor UDP-GalNAc in the presence of divalent cations, it has only one nucleotide binding site DXD sugar, and is a type II membrane protein (Fritz et al. 2004). Even though this protein is longer than RGP and has a C-terminal lectin domain, it has a highly unstructured area that would serve to link several acceptors, and its secondary structure elements are well aligned with the RGP. These features supported the choice of the *N*-acetylgalactosaminyltransferase as a suitable template for the homology modeling process. When superimposed (Fig. 6a) the structure is quite similar, showing a RMSD of 1.2 Å. Evaluation of the model with Verify 3D (http://nihserver.mbi.ucla.edu/Verify_3D; (Luthy et al. 1992) produced all positive scores. In addition, despite the low sequence identity between the model and the template (12.1 %) the accuracy of the modeling was confirmed using the Procheck algorithm (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK), which showed that 99% residues are in allowed regions of the Ramachandran plot.

The observation of the 3D structure model (Fig. 6b) shows that three cysteine residues (C79, C303 and C251) are in external highly flexible loops, suggesting that none of them would be involved in the formation of intrachain disulfide bonds. The distance between C113 and C209 is 7.9 Å, which is not optimal to form disulfide bridges (5 Å approx.). We can also see a conserved cysteine in all RGPs, close to the DDD motif (residues 100, 101 and 102; Fig. 6c) which is also preserved in the template, interacting with the nucleotide sugar. The DDD motif is placed in a structurally conserved region in the two proteins, template and model, as well as in some other glycosyltransferases. The arginine residue R159, capable to be glycosylated seems to be mobile enough, showing no obstruction to allow an interaction with the DDD motif (see figure loop).

Discussion

Throughout this work we showed that OsRGP oligomers occur at a specific time in development. RGP complexes are the main form in plantlets, while in ungerminated seeds little or no oligomerization is observed. Therefore, the appearance of complex formation in rice is associated with an active polysaccharide synthesis that occurs during the elongation of the plant. In addition, a differential mRNA expression of class 1 and class 2 RGP was observed. A remarkable increment in the class 1 OsRGP mRNA is observed at day 4 (Fig. 3a and b), a time in which appeared high molecular weight complexes (Fig. 1). Nevertheless, an increment in the class 2 RGP expression was observed at day 8, suggesting that complexes between class 1 and class 2 OsRGP, might be under regulation of developmental cues.

The formation of RGP complexes during development in rice could indicate a specific physiological role in this process. Our findings suggest that both classes of OsRGP are associated with vegetative development when there is an active polysaccharide synthesis. In this scenario, class 1 OsRGP would be involved in an early stage of plant growth, while class 2 RGP would be associated with an active elongation process. Previously, RGPs were observed in association with the Golgi apparatus as high molecular weight complexes (De Pino et al. 2007) and Konishi et al (2010a) reported self-association of RGPs. However our data showed for the first time that the formation of RGP

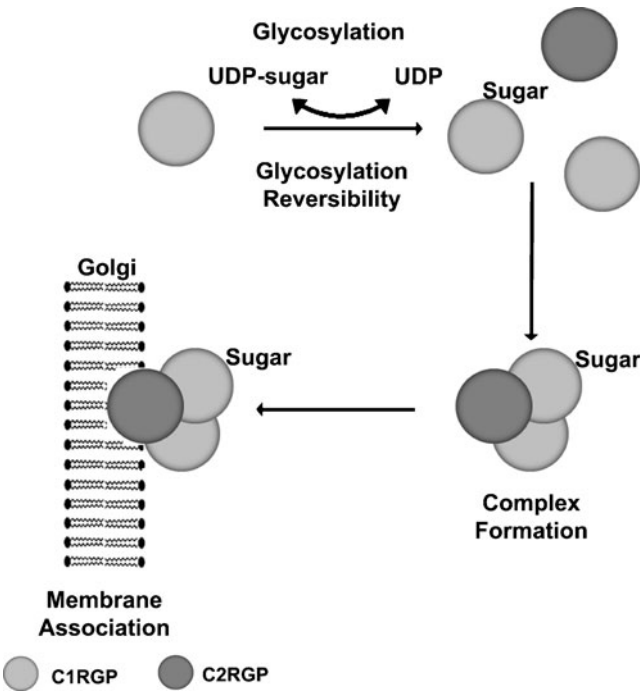


Fig. 7 Schematic representation of RGP Oligomerization. Expansion of the earlier reported schema showing the correlation of RG complex occurrence with the expression of class 1 RGP (*C1RGP*) and class 2 RGP (*C2RGP*). The class 1 RGP would be glycosylated in the cytoplasm and its glycosylation would be able to recruit other RGPs: class 1 RGPs as well class 2 RGPs. Once formed the RGP oligomer, the class 2 RGP would link the complex to the Golgi membranes

complexes occurred during development of rice plantlets by combining different forms of the enzyme.

The evidence regarding complex formation of RGPs is a challenging hypothesis, and makes us wonder about the nature of the interaction among monomers. It is well known that adjacent cysteines can form disulfide bonds in the tertiary structure as well as in the quaternary structure of the protein. In general the reduction of intracatenary disulfide bonds affects the tertiary structure of the protein with a direct impairment of enzymatic activity, so the preservation of these bonds is linked to the conservation of the structure. Our data showed that oligomers would be stabilized by disulfide bonds, since the presence of reducing agent raises activity and decreases the apparent size of the RGP bands (Fig. 4). By contrast, in nonreducing conditions there was an increase in size and a decrease in the incorporation of UDP [14 C]-Glc, suggesting that disulfide bonds are, at least in part, responsible for maintaining the oligomeric structure. Moreover, when we study the probability of intracatenary disulfide bonds formation by DISULFIND software (Ceroni et al. 2006), we observe that the probability is zero for all RGPs studied, both in class 1 as in class 2. In addition, the homology modeling of the StRGP (Fig. 6) supports the formation of intercatenary disulfide bridges by exposed cysteines in both RGP classes, which would be responsible for maintaining the quaternary structure of the oligomeric RGP.

Several hydrophobic domains present in class 2 RGP predicted by HCA analysis and the Predict Protein algorithm, are absent in class 1 RGPs (Fig. 5). In addition, class 2 RGPs have a different order in their secondary structure elements (Fig. 5b), precluding the formation of the Rossman fold which is clearly observed in the class 1 RGP. Moreover, class 2 RGP has a DDN motif in spite of the DXD present in class 1 RGP. To this regard, Konishi et al. (2010b) had no success in the activation of the class 2 RGP after substitution of an asparagine residue from the DDN motif by an aspartic acid using site directed mutagenesis. These results suggested that the context of the DXD motif is also important for the RGP activity.

All these findings are consistent with previous results where lower molecular weight forms have a greater self-glycosylating capability (De Pino et al. 2007) and led to expand the reaction mechanism previously proposed for RGP (De Pino et al. 2007, see Fig. 7). We think that both kinds of RGPs are free in the cytoplasm, with class 1 being capable of glycosylation and once this form gets glycosylation, it could be able to form complexes with other class 1 RGPs and the incorporation of class 2 RGP to the complex would be responsible for linking the overall complex to Golgi membranes (Fig. 7). The hydrophobic regions present in class 2 RGP may be responsible for its association with the class 1 RGP and/or with the Golgi membranes.

Konishi et al. (2007) reported that RGP and mutase are same protein and that function of RGP is mutase. Results regarding the lack of activity associated with class 2 RGPs (Langeveld et al. 2002; Konishi et al. 2010b; Rautengarten et al. 2011) and evidence shown above open the idea for a possible role of class 2 RGPs as a scaffold protein. However, many questions regarding the exact function in different metabolic pathways of the RGPs still remain unanswered. Our work sheds some light on the assembly of multiprotein complexes of RGP during development. So far, all findings point to the notion that RGP oligomers linked two key pathways inside plant cells: the metabolism of nucleotide sugars occurring in the cytoplasm, and the synthesis of cell wall polysaccharide through their interaction with the membranes of the Golgi apparatus.

Acknowledgments We would like to thank the National Council for Scientific and Technological Research (CONICET). S. Moreno is the career investigator of the CONICET. V De Pino (Fellowship, CONICET, Argentina, 2005–2010). We also thank Susana Raffo and Marta L. Bravo for excellent technical assistance.

Conflicts of Interest None

References

Andreeva A, Howorth D, Chandonia JM, Brenner SE, Hubbard TJ, Chothia C, Murzin AG (2008) Data growth and its impact on the SCOP database: new developments. Nucleic Acids Res 36:D419–D425

Barjaktarovic Z, Schutz W, Madlung J, Fladerer C, Nordheim A, Hampp R (2009) Changes in the effective gravitational field strength affect the state of phosphorylation of stress-related proteins in callus cultures of *Arabidopsis thaliana*. J Exp Bot 60:779–789

Bocca SN, Kissen R, Rojas-Beltran JA, Noel F, Gebhardt C, Moreno S, du Jardin P, Tandecarz JS (1999) Molecular cloning and characterization of the enzyme UDP-glucose: protein transglucosylase from potatodaggerdaggerThis paper is specially dedicated to the memory of Dr Juana S. Tandecarz, deceased on December 10, 1996. Plant Physiol Biochem 37:809–819

Ceroni A, Passerini A, Vullo A, Frasconi P (2006) DISULFIND: a disulfide bonding state and cysteine connectivity prediction server. Nucleic Acids Res 34:W177–W181

Chen Y, Chen T, Shen S, Zheng M, Guo Y, Lin J, Baluska F, Samaj J (2006) Differential display proteomic analysis of *Picea meyeri* pollen germination and pollen-tube growth after inhibition of actin polymerization by latrunculin B. Plant J 47:174–195

Dai S, Chen T, Chong K, Xue Y, Liu S, Wang T (2007) Proteomics identification of differentially expressed proteins associated with pollen germination and tube growth reveals characteristics of germinated *Oryza sativa* pollen. Mol Cell Proteomics 6:207–230

De Pino V, Boran M, Norambuena L, Gonzalez M, Reyes F, Orellana A, Moreno S (2007) Complex formation regulates the glycosylation of the reversibly glycosylated polypeptide. Planta 226:335–345

Delgado IJ, Wang Z, de Rocher A, Keegstra K, Raikhel NV (1998) Cloning and characterization of AtRGP1. A reversibly autoglycosylated arabidopsis protein implicated in cell wall biosynthesis. Plant Physiol 116:1339–1350

Dhugga KS, Ulvskov P, Gallagher SR, Ray PM (1991) Plant polypeptides reversibly glycosylated by UDP-glucose. Possible components of Golgi beta-glucan synthase in pea cells. J Biol Chem 266:21977–21984

Dhugga KS, Tiwari SC, Ray PM (1997) A reversibly glycosylated polypeptide (RGP1) possibly involved in plant cell wall synthesis: purification, gene cloning, and trans-Golgi localization. Proc Natl Acad Sci U S A 94:7679–7684

Drakakaki G, Zabotina O, Delgado I, Robert S, Keegstra K, Raikhel N (2006) Arabidopsis reversibly glycosylated polypeptides 1 and 2 are essential for pollen development. Plant Physiol 142:1480–1492

Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, Pieper U, Sali A (2007) Comparative protein structure modeling using MODELLER. Curr Protoc Protein Sci Chap. 2: Unit 2 9

Fritz TA, Hurley JH, Trinh LB, Shiloach J, Tabak LA (2004) The beginnings of mucin biosynthesis: the crystal structure of UDP-GalNAc:polypeptide alpha-*N*-acetylgalactosaminyltransferase-T1. Proc Natl Acad Sci U S A 101:15307–15312

Gaboriaud C, Bissery V, Benchetrit T, Mornon JP (1987) Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. FEBS Lett 224:149–155

Ge C, Ding Y, Wang Z, Wan D, Wang Y, Shang Q, Luo S (2009) Responses of wheat seedlings to cadmium, mercury and trichlorobenzene stresses. J Environ Sci (China) 21:806–813

Ginalski K, Elofsson A, Fischer D, Rychlewski L (2003) 3D-jury: a simple approach to improve protein structure predictions. Bioinformatics 19:1015–1018

Hukkanen A, Kokko H, Buchala A, Hayrinen J, Karenlampi S (2008) Benzothiadiazole affects the leaf proteome in arctic bramble (*Rubus arcticus*). Mol Plant Pathol 9:799–808

Jiang Y, Yang B, Harris NS, Deyholos MK (2007) Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. J Exp Bot 58:3591–3607

Kim SG, Kim ST, Kang SY, Wang Y, Kim W, Kang KY (2008) Proteomic analysis of reactive oxygen species (ROS)-related proteins in rice roots. Plant Cell Rep 27:363–375

Konishi T, Takeda T, Miyazaki Y, Ohnishi-Kameyama M, Hayashi T, O'Neill MA, Ishii T (2007) A plant mutase that interconverts UDP-arabinofuranose and UDP-arabinopyranose. Glycobiology 17:345–354

Konishi T, Miyazaki Y, Yamakawa S, Iwai H, Satoh S, Ishii T (2010a) Purification and biochemical characterization of recombinant rice UDP-arabinopyranose mutase generated in insect cells. Biosci Biotechnol Biochem 74:191–194

Konishi T, Ohnishi-Kameyama M, Funane K, Miyazaki Y, Ishii T (2010b) An arginyl residue in rice UDP-arabinopyranose mutase is required for catalytic activity and autoglycosylation. Carbohydr Res 345:787–791

Laemmli UK (1970) Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227:680–685

Langeveld SM, Vennik M, Kottenhagen M, Van Wijk R, Buijk A, Kijne JW, de Pater S (2002) Glucosylation activity and complex formation of two classes of reversibly glycosylated polypeptides. Plant Physiol 129:278–289

Laskowski RA, MacArthur MW, Moss DS, Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Crystallogr 26:8

Lavintman N, Tandecarz J, Carceller M, Mendiara S, Cardini CE (1974) Role of uridine diphosphate glucose in the biosynthesis of starch. Mechanism of formation and enlargement of a glucoproteic acceptor. Eur J Biochem 50:145–155

Luthy R, Bowie JU, Eisenberg D (1992) Assessment of protein models with three-dimensional profiles. Nature 356:83–85

Masuko H, Endo M, Saito H, Hakozaki H, Park JI, Kawagishi-Kobayashi M, Takada Y, Okabe T, Kamada M, Takahashi H, Higashitani A, Watanabe M (2006) Anther-specific genes, which expressed through microsporogenesis, are temporally and spatially regulated in model legume, Lotus japonicus. Genes Genet Syst 81:57–62

Moreno S, Cardini CE, Tandecarz JS (1986) Alpha-glucan synthesis on a protein primer, uridine diphosphoglucose: protein transglucosylase I. Separation from starch synthetase and phosphorylase and a study of its properties. Eur J Biochem 157:539–545

Moreno S, Cardini CE, Tandecarz JS (1987) Alpha-glucan synthesis on a protein primer. A reconstituted system for the formation of protein-bound alpha-glucan. Eur J Biochem 162:609–614

Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassay with tobacco tissues cultures. Physiol Plant 15:6

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 25:1605–1612

Rautengarten C, Ebert B, Herter T, Petzold CJ, Ishii T, Mukhopadhyay A, Usadel B, Scheller HV (2011) The interconversion of UDP-arabinopyranose and UDP-arabinofuranose is indispensable for plant development in arabidopsis. Plant Cell 23:1373–1390

Selth LA, Dogra SC, Rasheed MS, Randles JW, Rezaian MA (2006) Identification and characterization of a host reversibly glycosylated peptide that interacts with the tomato leaf curl virus V1 protein. Plant Mol Biol 61:297–310

Shindyalov IN, Bourne PE (1998) Protein structure alignment by incremental combinatorial extension (CE) of the optimal path. Protein Eng 11:739–747

Singh DG, Lomako J, Lomako WM, Whelan WJ, Meyer HE, Serwe M, Metzger JW (1995) beta-glucosylarginine: a new glucose–protein bond in a self-glucosylating protein from sweet corn. FEBS Lett 376:61–64

Soding J, Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res 33:W244–W248

Testasecca P, Wald FA, Cozzarin ME, Moreno S (2004) Regulation of self-glycosylation of reversibly glycosylated polypeptides from *Solanum tuberosum*. Physiol Plant 121:27–34

Thomas JA, Schlender KK, Larner J (1968) A rapid filter paper assay for UDPglucose–glycogen glucosyltransferase, including an improved biosynthesis of UDP-14 C-glucose. Anal Biochem 25:486–499

Tran HT, Plaxton WC (2008) Proteomic analysis of alterations in the secretome of Arabidopsis thaliana suspension cells subjected to nutritional phosphate deficiency. Proteomics 8:4317–4326

Wald FA, Kissen R, du Jardin P, Moreno S (2003) Characterization of UDP-glucose:protein transglucosylase genes from potato. Plant Mol Biol 52:705–714

Zavaliev R, Sagi G, Gera A, Epel BL (2010) The constitutive expression of Arabidopsis plasmodesmal-associated class 1 reversibly glycosylated polypeptide impairs plant development and virus spread. J Exp Bot 61:131–142