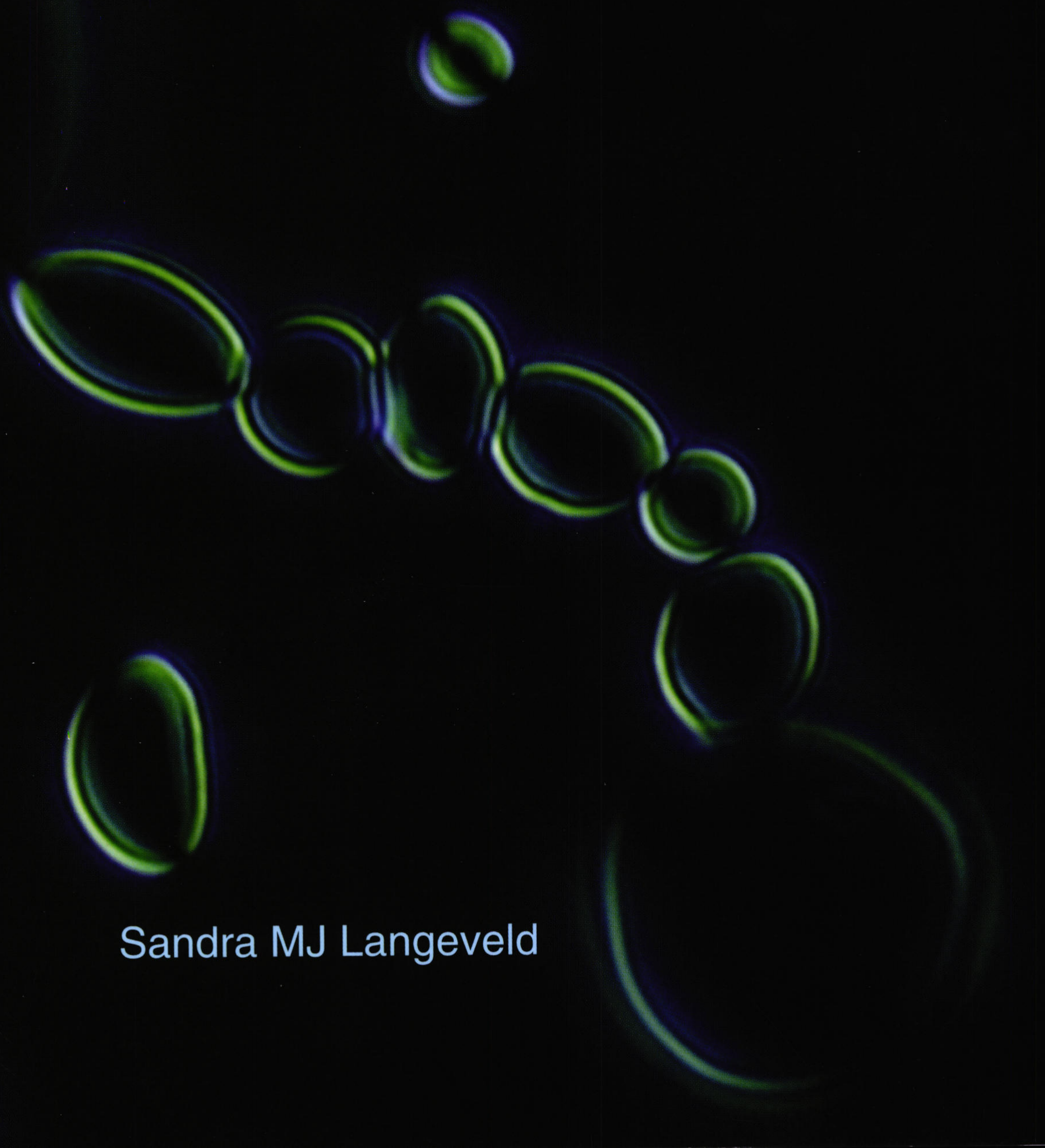
Role of reversibly glycosylated polypeptides in starch granule biosynthesis



Sandra MJ Langeveld

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Sandra Mariska Jeanette Langeveld

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Picture of potato starch granules, taken by Marco Vennik

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"Role of reversibly glycosylated polypeptides in starch granule biosynthesis"
by Sandra MJ Langeveld

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De erkenning dat het universum een mysterie is,

is de weg van alle ware wetenschap

Albert Einstein

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Chapter 1

General Introduction

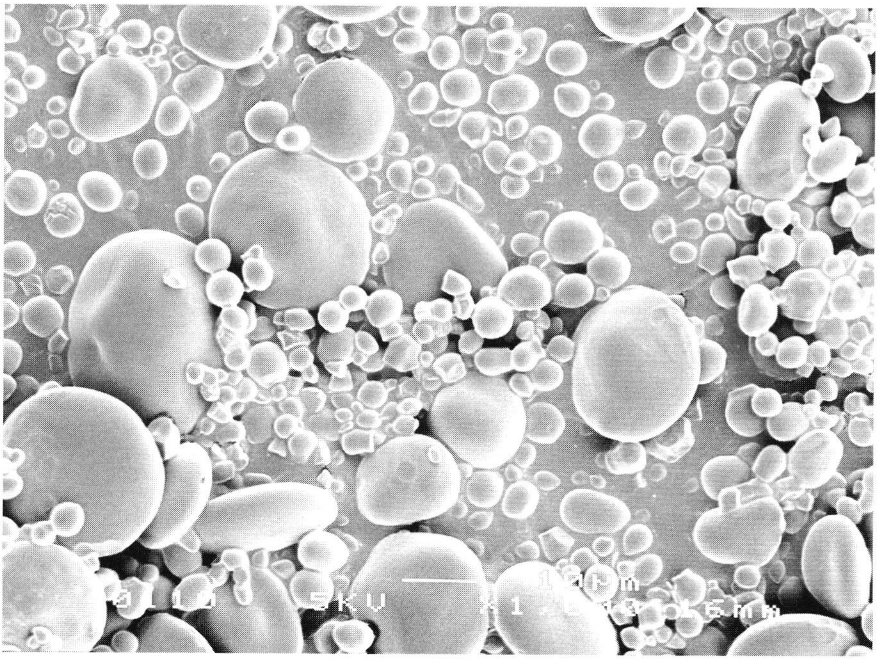

Figure 1. Scanning Electron Microscope image of small and large starch granules isolated from mature wheat endosperm.

Starch

Starch is the main source of carbohydrates in the human diet and therefore plays an important part in human nutrition. In addition, many industrial processes use starch as a raw material. Starch types of different plant species show distinctive properties, which make them useful for different industrial applications. Recently, the use of starch as a biodegradable polymer is becoming increasingly attractive because of environmental concerns.

Starch consists of two different glucans, amylose and amylopectin, which differ in their degree of branching. Amylose mainly consists of linear α-1,4-glucan polymers, whereas amylopectin is a more highly branched glucan, linked by α-1,6 glucosidic bonds. Although considerable progress has been made in the identification of the enzymes and the biosynthetic steps involved in the formation of these glucan polymers, the way in which starch synthesis and granule formation is initiated is still an enigma.

The endosperm of mature wheat and barley seeds contains two major classes of starch granules: large, early formed, elliptical A-type granules and small, later formed, spherical B-type granules (Figure 1). The quality of starch from wheat and barley is greatly influenced by the ratio of large and small granules. For example, during the brewing process a large proportion of the small B-type granules from barley is not degraded and blocks the filters, which results in increased run-off times (Tillett and Bryce, 1993). Therefore, for the brewing industry a large number of small granules is unfavourable. On the other hand, small granules are suitable as a paper coating and for cosmetic products such as face powders (Ellis *et al.*, 1998, and references therein). Therefore, production of cultivars containing starch enriched in A-type granule fractions will improve the quality of raw material for the brewer, whereas enrichment in B-type granule fractions is favourable for the paper industry and the cosmetic industry.

Starch granules are synthesised in amyloplasts

Starch granules are synthesised and stored in specialised organelles, the amyloplasts. Amyloplasts belong to the family of plastids, which are plant specific organelles with various functions, surrounded by an envelope consisting of a double membrane. Plastids are derived from proplastids; small, colourless, undifferentiated plastids that occur in meristematic cells. Depending on the function of the cell, proplastids develop into specialised plastids with functions such as photosynthesis (chloroplasts), synthesis and storage of starch (amyloplasts) or pigments (chromoplasts; Figure 2). The various kinds of plastids can transform from one type into the other (Bonora *et al.*, 2000).

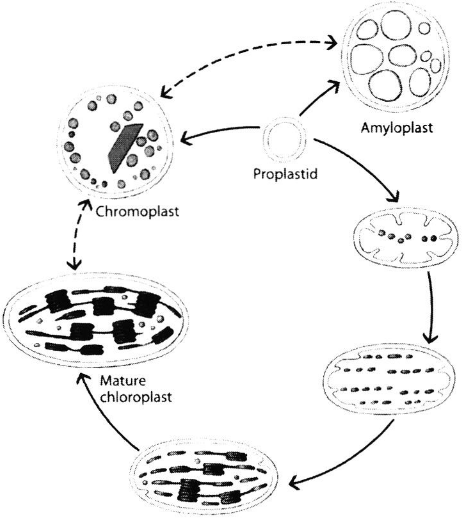

Figure 2. Plastid developmental cycle. Adapted from Raven *et al.* (1999)

In 1958, Badenhuizen suggested that the small B-type granules in wheat endosperm arose in mitochondria (Badenhuizen, 1958). However, Buttrose concluded after investigation of B-granule formation in barley (1960) and wheat (1963) that the small granules were formed in vesicles budded off from outgrowths of A-type granule-containing amyloplasts. Hughes (1976) endorsed this view, but Duffus (1979) as well as Czaja (1982) could not confirm budding-off of B-type granule-containing vesicles. However, Czaja (1982) studied granule formation by use of light microscopy only. Since organelle protrusions are hardly visible by light microscopy, conclusive evidence cannot be obtained by use of this method. Parker (1985) showed by using electron microscopy (EM) the presence of narrow protrusions between B-type granules and the parent amyloplast, but also failed to find evidence for budding-off of B-type amyloplast parts.

From sucrose to starch

Sucrose metabolism

Sucrose, the primary product of photosynthesis, is also the form in which carbon is transported from source to sink tissues (ap Rees, 1993). In sink tissues, the available carbon is partitioned into cell wall synthesis, starch synthesis and other carbon-demanding routes. The network of sucrose metabolism in sink tissue is depicted in Figure 3. Sucrose can be cleaved by two enzymes, sucrose synthase (SuSy) and invertase (Huber and Akazawa, 1986). SuSy catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose (Avigad, 1982), whereas invertase irreversibly hydrolyzes sucrose into glucose and fructose. Studies with plant mutants indicated that SuSy is mainly responsible for the cleavage of imported sucrose used for starch synthesis. For example, in maize endosperm the loss of 90% of SuSy activity in the *sh1* mutant resulted in starch deficiency (Chourey, 1981). In SuSy-antisense potato plants, starch content decreased by 66% (Zrenner *et al.*, 1995), whereas over-expression of a yeast invertase in the cytosol of potato tubers led to a 10-15% reduction in starch content (Sonnewald *et al.*, 1997). Data on sucrose synthase activities and amounts of substrate and metabolites indicated that sucrose synthase cleavage was the preferred pathway of sucrose catabolism in developing wheat endosperm (Riffkin *et al.*, 1995). In maize, two sucrose synthase isozymes have been identified. One of these, encoded by *Shrunken1*, was shown to be critical for cell wall integrity whereas the other, encoded by *Sucrose synthase1*, was found to generate precursors for starch synthesis (Chourey *et al.*, 1998).

UDP-glucose is a precursor for both cell wall synthesis and starch synthesis and, therefore, represents a branchpoint in sugar metabolism. For starch synthesis, UDP-glucose is converted into glucose-1-phosphate (G1P) by UDP-glucose pyrophosphorylase (UGPase). This enzyme was found to be linked to QTLs for potato tuber yield and starch content (Schäfer-Pregl *et al.*, 1998). However, using an antisense approach Zrenner *et al.* (1993) showed that only 4% of the wildtype UGPase activity is sufficient for growth and development of potato tubers.

Fructose and glucose are converted to hexose phosphates by fructokinase (FK) and hexokinase (HK), respectively. Subsequently, phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM) form G1P. Studies on isolated wheat amyloplasts strongly suggested that these events take place in the cytoplasm (Entwistle and ap Rees, 1988).

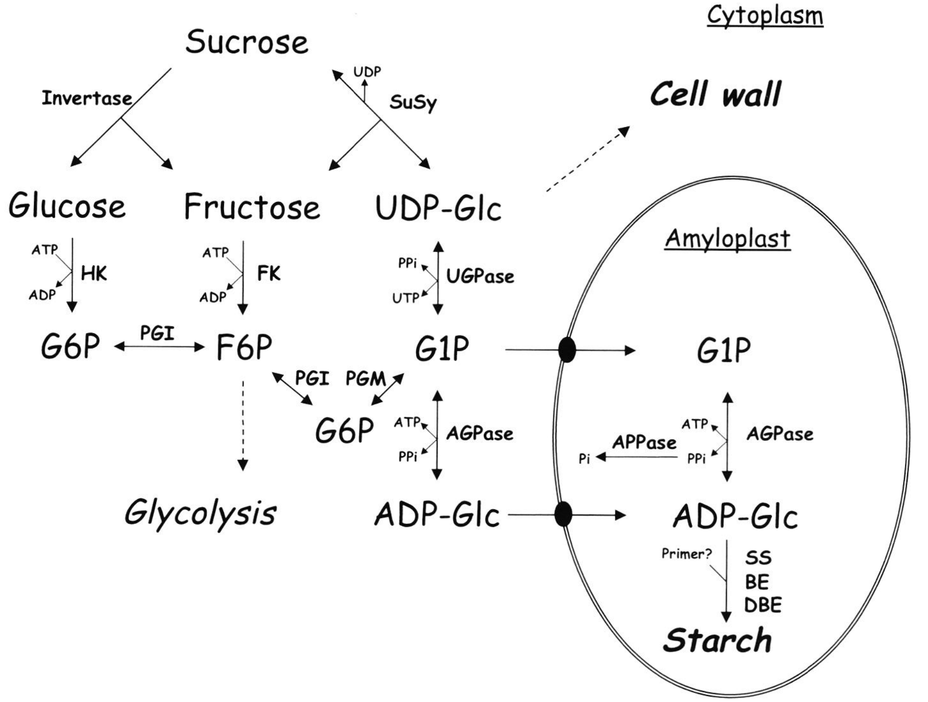

Figure 3. Schematic representation of carbohydrate metabolism in wheat endosperm. Details are given in the text.

Starch biosynthesis

In starch-synthesising tissues the product of UGPase, G1P, is converted into ADP-glucose by ADP-glucose pyrophosphorylase (AGPase). Subsequently, ADP-glucose is used as a substrate for starch synthesis by starch synthases (SS)(Figure 3). *In vitro* SS are able to utilise both amylose and amylopectin as substrates. However, it was shown by Nelson and Rines (1962) that the *waxy* mutant of maize, which lacks the amylose component, also lacks a granule-bound starch synthase (GBSS) activity. Visser *et al.* (1991) and Salehuzzaman *et al.* (1993) demonstrated that by using an antisense GBSSI gene from potato and cassava respectively, GBSS activity can be reduced, leading to complete absence of amylose in potato starch.

The form in which glucose enters the amyloplast has been a matter of debate. For many years it was generally accepted that AGPase was localised in amyloplasts (Entwistle and ap Rees, 1988), and that triose and hexose phosphates were transported over the amyloplast envelope membranes. Keeling *et al.* (1988) suggested that hexose phosphates, and not triose phosphates, were imported into the amyloplast. In wheat endosperm, G1P appeared to be the exclusive substrate for starch synthesis (ap Rees and Entwistle, 1989; Tetlow *et al.*, 1998), whereas in pea endosperm, only G6P was taken up by amyloplasts (Hill and Smith, 1991). Two reports on potato presented evidence that G1P is imported (Kosegarten and Mengel, 1994; Naeem *et al.*, 1997), whereas two other reports demonstrated that G6P is the preferred transported metabolite in potato tuber (Schott *et al.*, 1995; Wischmann *et al.*, 1999). AGPase activity was reported to be associated with the amyloplast fraction obtained from developing maize endosperm (Echeverria *et al.*, 1988). This observation was supported by immunocytochemical data for potato tuber (Kim *et al.*, 1989) and maize endosperm (Brangeon *et al.*, 1997; Miller and Chourey, 1995). However, the amyloplastic localisation of AGPase was questioned when cDNAs of the AGPase subunits from maize endosperm did not exhibit plastid transit-peptide signals (Giroux and Hannah, 1994). Denyer *et al.* (1996) showed that maize endosperm cells contain two isoforms of AGPase. More than 95% of total AGPase activity was found to be extra-amyloplastic. Similar results were described for barley endosperm by Thorbjörnsen *et al.* (1996). With immunolocalisation studies, Chen *et al.* (1998) showed that cytosolic localisation of AGPase is not restricted to cereals, but is also found in tomato. The fact that isolated amyloplasts from sycamore cells (Pozueta-Romero *et al.*, 1991) and wheat endosperm (Tetlow *et al.*, 1994) can take up ADP-glucose supported the idea that AGPase is cytosolic. Shannon *et al.* (1998) provided evidence that in maize endosperm an adenylate translocator present in the amyloplast envelope membranes facilitates the transfer of ADP-glucose into the amyloplast stroma.

Initiation of starch synthesis

Considerable effort has been put in the study of enzymes involved in starch elongation and branching. However, the way in which the initial primers for glucan chain formation are produced is still unclear. Enzymes capable of elongating glucans seem to display activity *in vitro* independent of added primer. Pollock and Preiss (1980) reported that a starch synthase found in maize endosperm is capable of unprimed synthesis if the reaction medium contains citrate. Also, starch phosphorylase II has been reported to be capable of synthesising α-1,4-glucans in the absence of a primer (Sivak *et al.*, 1981a; Sivak *et al.*, 1981b). Whether these unprimed reactions occur *in vivo* is unknown. Since it is hard to eliminate all glucan from the enzyme preparations, it is questionable whether these activities represent *de novo* synthesis of starch (Sivak and Preiss, 1998). Holmes and Preiss (1979) showed that the glucose incorporated into minute amounts of glucan primer by glycogen synthase from *E. coli*, was not covalently bound to the enzyme.

Lavintman and Cardini (1973) proposed the concept of a distinct enzyme for the initiation of starch synthesis. This idea was based on studies with mammalian cells, which synthesise glycogen by means of a process similar to starch synthesis in plants. In glycogen synthesis, a protein primer named glycogenin has been identified which possesses both a glucosyltransferase activity and a glucose acceptor site (Lomako *et al.*, 1988). UDP-glucose:protein transglucosylase (UPTG) was identified using a membrane-containing fraction of potato tuber, which suggested *de novo* synthesis of protein-bound α-1,4-glucan (Lavintman and Cardini, 1973). Subsequent studies indicated that synthesis of α-glucan covalently linked to protein involved at least two steps. During the first reaction, which is catalysed by UPTG, glucosyl moieties are transferred to specific sites on an endogenous protein acceptor (Tandecarz and Cardini, 1978). In the second step, the resulting 38 kD glucosylated protein subsequently serves as a primer for enzymatic glucan chain elongation (Moreno *et al.*, 1987; Rothschild and Tandecarz, 1994). Ardila and Tandecarz (1992) showed that purified UPTG undergoes self-glucosylation in a Mn^{2+}-dependent reaction using UDP-glucose as a substrate and is, therefore, both the enzyme and the 38 kD endogenous protein acceptor. Polyclonal antibodies raised against this 38 kD protein were able to neutralise potato tuber and maize endosperm UPTG activity (Bocca *et al.*, 1997). Singh *et al.* (1995) isolated a similar autocatalytic self-glucosylating protein from maize and coined it "amylogenin" by analogy to glycogenin. They showed that the glucosylated amino acid was arginine.

Although some reports claim that beside UDP-glucose, also ADP-glucose is used as a substrate, this observation was cultivar-dependent (Cura *et al.*, 1994).

Since UDP-glucose is synthesised in the cytoplasm and is not known to enter the amyloplast, the question arises where this putative primer of starch synthesis is localised.

Reversibly Glycosylated Polypeptides

A protein designated Reversibly Glycosylated Polypeptide (RGP) with characteristics similar to putative primers of starch synthesis was found to be localised to trans-Golgi dictyosomal cisternae (Dhugga *et al.*, 1997). This 41 kD protein can be glucosylated by UDP-glucose, UDP-xylose or UDP-galactose in a ratio similar to the composition of xyloglucan. Therefore, it was hypothesised that RGP is involved in cell wall synthesis rather than in starch biosynthesis. In *Arabidopsis*, RGP1 was found to be present in the soluble as well as the microsomal cell fractions, suggesting a function of RGP1 in the transport of UDP-sugars from the cytoplasm towards membranes (Delgado *et al.*, 1998)

Molecular cloning of the UDP-glucose:protein transglucosylase from potato, and sequence analysis of amylogenin from maize both showed a high homology with RGP1 (Bocca *et al.*, 1999). This implied that both UPTG and amylogenin are in fact RGPs. Alignment of RGP1 sequences with cellulose synthase and other β-glycosyltransferases failed to reveal a consensus UDP-glucose-binding region (Delgado *et al.*, 1998; Dhugga *et al.*, 1997). However, RGP1 contains a region similar to domain A of cellulose synthase and other β-glycosyltransferases (Saxena and Brown Jr, 1999). Since domain B, characteristic of processive β-glycosyltransferases is lacking in RGP1, these authors suggested RGP1 to represent a non-processive β-glycosyltransferase.

Outline of this thesis

As part of the EUREKA "Wheat for Industrial Needs (WIN)" programme, this project aimed at studying possibilities for improvement of the starch quality in wheat. Since the ratio of large A-type and small B-type starch granules influences the quality of wheat starch, factors regulating this ratio were investigated.

Little is known about the formation of B-type granules. Therefore, first wheat amyloplasts and the B-type granules therein were studied using transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). This research is described in Chapter 2. It is shown that B-type granules are present in protrusions emanating from the A-type granule-containing amyloplast and that amyloplasts are connected by these protrusions.

The other chapters describe the search for a protein involved in the priming of starch synthesis. An increased amount or activity of putative primer during endosperm development may shift the ratio of large A-type and small B-type starch granules. Initially, this putative primer was cloned and designated "amylogenin". However, the function of this protein in starch synthesis was questioned and it was renamed Reversibly Glycosylated Polypeptide (RGP). Chapter 3 describes the cloning and classification of two RGPs and their localisation in wheat endosperm.

In Chapter 4, the subcellular localisation of RGP1 and RGP2 is described. The activity of both RGPs is analysed in Chapter 5. Glucosylation assays revealed that RGP1-containing tobacco extracts as well as in RGP2-containing tobacco extracts incorporated UDP-glucose. Furthermore, it was shown that RGP1 and RGP2 were present as high molecular weight complexes, the composition of which was studied. Yeast two-hybrid screening and purification of the RGP1 complex by an antibody affinity column did not reveal the presence of other proteins besides RGPs in this complex.

The function of RGPs was studied using a transgenic approach. Because in contrast to wheat, potato is easy to transform, transgenic potato plants overexpressing *Rgp1* or *Rgp2* were generated. The analysis of these plants is described in Chapter 6 and the possible function of RGPs is discussed.

Chapter 2

B-type granule containing protrusions and interconnections between amyloplasts in developing wheat endosperm revealed by transmission electron microscopy and GFP expression

Sandra MJ Langeveld, Ringo van Wijk, Nico Stuurman, Jan W Kijne and Sylvia de Pater

Modified from: Langeveld *et al.* (2000) J Exp Bot 51: 1357-1361

Abstract

Starch granules in mature wheat endosperm show a bimodal size distribution. The formation of small starch granules in wheat endosperm cells was studied by transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) after expression and targeting of fluorescent protein into amyloplasts. Both techniques demonstrated the presence of protrusions emanating from A-type granules-containing amyloplasts and the presence of B-type starch granules in these evaginations. Moreover, CLSM recordings demonstrated the interconnection of the amyloplasts by these protrusions, suggesting a possible role of these protrusions in interplastid communication.

Introduction

Endosperm of mature wheat and barley contains two classes of starch granules. Formation of the large lenticular A-type granules is initiated about 4 to 5 days post anthesis (dpa). About 4 days later, the final number of A-type granules is achieved (Briarty *et al.*, 1979). Their size reaches up to 45 µm (Briarty *et al.*, 1979), depending on cultivar (Dengate and Meredith, 1984) and season (Baruch *et al.*, 1979). B-type starch granules are reported to be first initiated between 12 and 16 dpa by Parker (1985) and between 16 and 22 dpa by Briarty *et al.* (1979). At grain maturity B-type granules vary in size between 1 and 10 µm and are spherical in shape. The final number of B-type granules per grain is affected by environmental conditions during grain growth such as temperature (Cochrane *et al.*, 1996; Tester *et al.*, 1991).

The quality of starch extracted from wheat and barley is greatly influenced by the ratio of A- versus B-type granules. During the brewing process, a significant proportion of the B-type granules from barley is not gelatinised in the mash. Subsequently, this undegraded residue blocks the filter-beds in lauter tuns, which results in increased run-off times (Tillett and Bryce, 1993). Therefore, a large number of small granules is unfavourable in the brewing industry. On the other hand, small granules are suitable as a paper coating and also find application in cosmetic products such as face powders (Ellis *et al.*, 1998, and references herein). Thus, the production of cultivars containing starch enriched in the A-type granule fractions will improve the quality of the raw material for the brewer, whereas enrichment in the B-type granule fractions is favourable for the paper and cosmetic industry.

In the history of starch research there has been some disagreement about the origin of B-type granules. In 1958, Badenhuizen suggested they arose in mitochondria (Badenhuizen, 1958). Buttrose investigating B-granule formation in barley (1960) and wheat (1963), concluded that the small granules were formed in vesicles budded off from outgrowths of the A-type granule-containing amyloplasts. Hughes (1976) endorsed this view, but Duffus (1979) could not confirm the budding off of B-type granule-containing vesicles. Czaja (1982) studied granule formation by light microscopy only and concluded that evidence for the formation of vesicles in which B-type granules developed was absent. However, using this method exclusive evidence cannot be obtained, since protrusions are hardly, if at all, visible at the light microscopy level. By using electron microscopy (EM), the presence of narrow protrusions between B-type granules and the parent amyloplast has been shown, but evidence for the budding off of B-type amyloplasts was lacking (Parker, 1985).

Since these EM observations were obtained, new technologies have been developed. Green Fluorescent Protein (GFP) of the jellyfish *Aequorea victoria* has been discovered as a powerful reporter enabling visualisation of dynamic processes in living cells or organisms (for a review, see Gerdes and Kaether, 1996). Modified versions of this reporter protein, such as Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP), are available. These fluorescent proteins can be targeted to specific subcellular organelles such as mitochondria (Köhler *et al.*, 1997b; Rizzuto *et al.*, 1995) and plastids (Köhler *et al.*, 1997a) by including a specific targeting sequence at the amino terminus. Thus, monitoring these proteins by confocal laser scanning microscopy (CLSM) and generation of 3D images are possible.

In order to gain more knowledge about B-type granule formation in fixed and in living endosperm cells, the presence of these small granules and the structure of amyloplasts were studied by EM as well as by CLSM. Here, the presence of B-type granules in protrusions of A-type granule-containing amyloplasts is demonstrated, conclusively confirming some of the earlier observations (Buttrose, 1960; 1963; Parker, 1985). Moreover, the interconnection of amyloplasts by these protrusions is shown using CLSM.

Materials and methods

Plant material and growth conditions

Wheat grains, *Triticum aestivum* L., cv. Minaret, were germinated in pots with a diameter of 6 cm (containing potting compost, perlite and peat in a 1:1:1 by vol. ratio) in a climate chamber at 20°C/ 80% humidity with a 16h photoperiod. After one week the seedlings were transferred to pots with a diameter of 15 cm in a phytotron with day/night temperatures of 15/12°C, 80% humidity and a 16h photoperiod. Light intensity at ear level was 24 klx. Ears were tagged when the first stamen appeared and harvested at the desired age, indicated as Days Post-Anthesis (dpa).

Transmission electron microscopy

Transverse sections, about 1.5 mm thick, were cut across the centre of each grain, fixed at room temperature in 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1M Na-cacodylate, pH 7.2, for 16 h and post-fixed at room temperature for 2 h in 1% (w/v) osmium tetroxide in 0.1M Na-cacodylate, pH 7.2. Samples were dehydrated in a graded ethanol series and infiltrated and embedded in Epon. Serial sections (100 nm) were cut on an ultramicrotome, collected on Formvar-coated grids, stained with uranyl acetate and examined in a Jeol Transmission Electron Microscope 1010 (TEM).

Confocal laser scanning microscopy

A plasmid containing the *gusA* reporter gene under control of the rice actin promoter (McElroy *et al.*, 1990) and the rice *RbcS* terminator (Pactin-GUS) was obtained from Sören Knudsen (McElroy group) of the Carlsberg Research Laboratory, Copenhagen. This construct was derived from plasmid pDM803, which is a pSP72 vector (Promega) containing two expression cassettes. The cassette consisting of the *Bar* selection marker gene under control of the maize ubiquitin promoter and *nos* terminator was deleted, resulting in the Pactin-GUS plasmid. Pactin-GFP and Pactin-YFP were constructed by replacing the *gusA* coding region from Pactin-GUS by GFP(S65T) (Chiu *et al.*, 1996) and YFP coding sequences (Clontech), respectively. *NcoI-NotI* fragments (the *NotI* sites made blunt using the Klenow fragment of DNA polymerase I) from pGFP(S65T) and pEYFP were cloned into the *NcoI* and *SmaI* sites of Pactin-GUS. A DNA fragment encoding the transit peptide (TP) from wheat granule bound starch synthase (Ainsworth *et al*, 1993) was constructed by PCR using genomic DNA from wheat as a template. The primers SP74 5'-CGCG**CCATGG**CGGCTCTG-3' and SP75 5'-GG**CCATGG**TGGCGCGCA-CCACCATAGAGAGGCACC-3' were used to remove an internal *NcoI* site and to introduce *NcoI* sites at both ends of the TP fragment. The PCR fragment was digested with *NcoI*, sequenced, isolated and introduced into the *NcoI* site of the Pactin-GFP and Pactin-YFP plasmids, resulting in Pactin-TP-GFP and Pactin-TP-YFP. The orientation was checked by restriction analysis.

Wheat grains (8-13 dpa) were cut in half longitudinally, perpendicular to the crease and transferred to Petri dishes containing Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with 0.8% (w/v) agar, the cut side facing up. Low melting agarose (1%, w/v) containing MS medium was used to stick the grains to the solid medium. Grains were transiently transformed with one of the constructs described above, using the Biorad Biolistic Particle Delivery System-1000/He. The Petri dishes were transferred to a phytotron with a 16 h photoperiod (2 klx) and a temperature of 21°C. Fluorescent cells were examined one day after bombardment in a Leica TCS/SP Confocal Laser Scanning Microscope using an excitation wavelength of 488 nm. Some of the recordings were restored by deconvolution with the Huygens System 2 program (Scientific Volume Imaging, The Netherlands) using an experimentally determined point spread function.

Results

In order to visualise putative protrusions emanating from A-type granule-containing amyloplasts, serial sections of wheat grains ranging from 8-13 dpa were examined by transmission electron microscopy (TEM). Except for the aleurone layer, protrusions were observed throughout the endosperm from 8 dpa onwards, although in older cells the protrusions were less prominent. B-type granule formation was first visible at 11 dpa in plants grown under the conditions described in the Materials and methods section, in 2 - 3 cell layers from the sub-aleurone cell layer. Figures 1A and B show two serial sections of a string of B-type granules (Figure 1A), which at first sight did not seem to be connected to the amyloplast with the A-type starch granule and protrusion (Figure 1B). However, mounting the two sections together (Figure 1C)

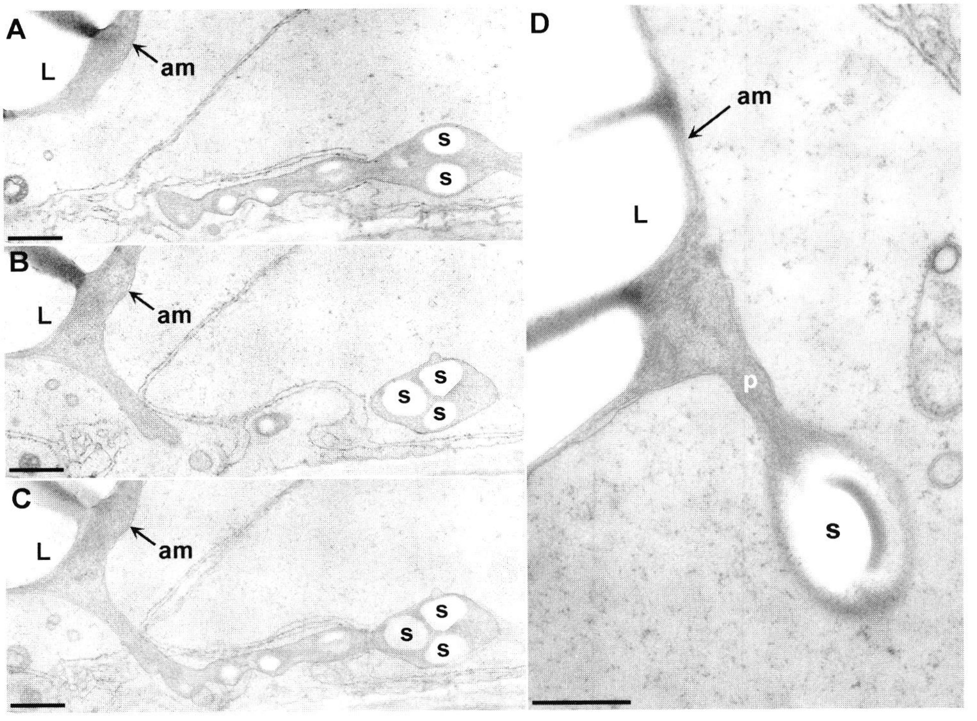

Figure 1. TEM images of wheat endosperm. (A,B) sequential sections of an amyloplast (am) with A-type (L) and B-type (s) starch granules in 11 dpa endosperm. (C) Overlay of (A) and (B) strongly suggesting that the string of B-type granules is connected to the A-type granule-containing amyloplast. (D) B-type starch granule in a protrusion (p) emanating from the A-type granule-containing amyloplast in 13 dpa endosperm. Bars represent 1 µm.

suggested that the B-type granules are present in the protrusion emanating from the parent amyloplast. Figure 1D shows a B-type granule in a protrusion directly visible to be connected to an A-type granule-containing amyloplast (13 dpa).

In order to visualise these protrusions and their dynamics in living cells, GFP and YFP constructs were introduced into wheat endosperm of 9-13 dpa by particle bombardment. Bombardments with seeds older than 13 dpa were not successful. Most frequent transformations were obtained in cells 1-3 cell layers from the sub-aleurone layer. The constructs used contained a transit peptide from granule-bound starch synthase, which enables the delivery of the fluorescent protein into the stroma of amyloplasts and other plastids. CLSM observations indeed showed targeting of GFP and YFP to the amyloplast stroma (Figures 2A and B), visualising starch granules as black areas within the amyloplast. In all cells examined amyloplast

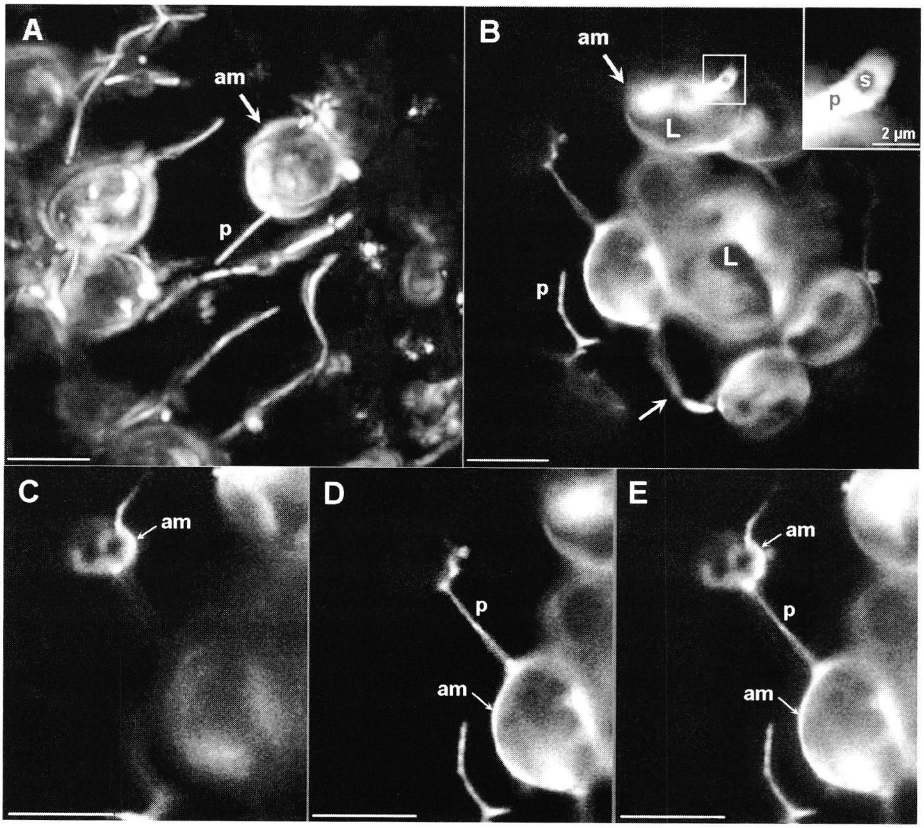

Figure 2. CLSM images of wheat endosperm bombarded with either the Pactin-TP-GFP or the Pactin-TP-YFP construct. (A) GFP labelled amyloplasts (am) with protrusions (p) in 13 dpa endosperm. The resolution of this image was computationally enhanced by deconvolution. (B) Optical section of YFP labelled amyloplasts (am) with A-type (L) and B-type (s) starch granules in 11 dpa endosperm. The inlay shows a protrusion (p) in which a B-type starch granule is present. (C-D) Two optical sections (4 µm apart) of YFP labelled amyloplasts (am) and a protrusion (p) in 11 dpa endosperm. (E) Overlay of C and D showing the interconnection of the amyloplasts by the protrusion. Bars represent 10 µm.

protrusions were clearly observed, and in some of these protuberances small B-type granules were visible (Figure 2B, inlay). Moreover, amyloplasts seemed to be connected to each other (Figure 2B, arrow). Figure 2C and D show two optical sections (4 µm apart) from the same cell as shown in Figure 2B. A mount of these sections (Figure 2E) demonstrated the interconnection of two amyloplasts by a protrusion. Study of the optical sections in between (each 0.20 µm apart) confirmed the physical connection between the amyloplasts (not shown). When a time-lapse recording with intervals of 7 seconds was performed, movement of the amyloplast protrusions was visible (results shown at wwwimp.leidenuniv.nl/tno.html).

The protrusions varied in length from 2 µm to 30 µm and ranged in width between 0.5 µm and 1.5 µm, depending on the presence of B-type granules when observed by CLSM. This corresponds with the TEM observations, where the width of the protrusions ranged from 0.35 -1.4 µm. The maximum length of the protuberances inferred from serial sections obtained by TEM was 16 µm. Considering the resistance of starch-containing material to sectioning and examination by TEM, the latter is probably an underestimation.

Taken together, results obtained by both TEM and CLSM techniques showed the presence of B-type granules in protrusions emanating from A-type granule-containing amyloplasts. Moreover, these observations showed the interconnection of amyloplasts by these protrusions.

Discussion

Amyloplast structure and B-type granule formation in wheat endosperm were studied by TEM and CLSM. Both techniques showed protrusions in which B-type granules were present, confirming some of the earlier results (Buttrose, 1960; 1963; Parker, 1985). B-type granules were first detected in seed of 11 dpa. Buttrose (1963) and Parker (1985) observed B-type granule initiation about 14 dpa. This difference can be explained considering the wheat cultivar and growth conditions used. Although grains of different ages were analysed, the variation in development within one age was huge. This is likely to be due to the development of the endosperm, the place of the grain in the ear, the sequential order of the ear on the plant and the labelling procedure.

CLSM enabled the visualisation of amyloplasts in living cells and showed the interconnection of amyloplasts by these protrusions. These observations are consistent with the results from Köhler *et al.* (1997a) who showed connections between chloroplasts, starting as protrusions emanating from the chloroplasts. These connections are 0.35-0.85 µm wide with a maximum length of 15 µm. They demonstrated the exchange of molecules through these protrusions, suggesting the presence of a communication system facilitating the co-ordination of plastid activities.

In all endosperm cells examined by CLSM, protrusions were present. Because only the outer cell layers (1-3 layers from the sub-aleurone layer) were transiently transformed, it is possible that the interplastid connections are characteristic for young endosperm cells. When older cell layers were examined by TEM, protrusions were less abundant, augmenting the possibility that protrusions are a developmental phenomenon.

Up to now, it has not been possible to visualise a starch granule larger than 20 μm using bombardment labelling and there has been no success in obtaining GFP or YFP expression in grains older than 13 dpa, both possibly due to the increasing amount of starch, impeding delivery of the construct to the nucleus. In order to get an overall view of amyloplast development in older grains, transgenic plants expressing the TP-YFP construct would be useful tools.

It can be concluded that new microscopic techniques such as CLSM in combination with the use of fluorescent protein labelling permit the visualisation of protrusions in three dimensions. It has been demonstrated that amyloplasts are interconnected by these protrusions. More research is required to elucidate the exact function of the connections between these plastids.

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Chapter 3

Two classes of reversibly glycosylated polypeptide cDNA clones and localisation of their gene products in wheat

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Abstract

Reversibly glycosylated polypeptides (RGPs) have been implicated in polysaccharide biosynthesis. In plants these proteins may function, for example, in cell wall synthesis or in synthesis of starch. We have isolated wheat and rice *Rgp* cDNA clones in order to study the function of these RGPs in monocots. Sequence comparisons showed the existence of two classes of RGP proteins, designated RGP1 and RGP2, which may correlate to different functions in polysaccharide biosynthesis. Northern blot analysis, *in situ* hybridisation, Western blot analysis and immunolocalisation experiments showed the presence of RGP2 mRNA and protein in storage tissue, whereas RGP1 mRNA and protein were detected in all tissues examined. Interestingly, levels of RGP1 and RGP2 protein were high in pericarp at different developmental stages, including those stages in which the tissue starts to degenerate. At this stage, these findings do not hint at a specific role for RGPs, and leave the possibility of a more general role as, for example, in transport of nucleotide sugars.

Introduction

Reversibly glycosylated polypeptides (RGPs) are probably involved in polysaccharide metabolism. Polysaccharides are the main components of the plant cell wall. For example, in dicots 20% of the primary cell wall consists of the polysaccharide xyloglucan, whereas in monocots this hemicellulose makes up 2% of the primary cell wall (Darvill *et al.*, 1980). Xyloglucan consists of a β-1,4-glucan backbone with xylosyl and xylosyl-galactosyl-fucosyl side-chains (Hayashi, 1989). The transferases responsible for the addition of these side-chains are localised to the Golgi apparatus (Brummell *et al.*, 1990; Driouich *et al.*, 1993; Staehelin and Moore, 1995). RGP1 has been implicated in xyloglucan biosynthesis in pea (Dhugga *et al.*, 1997). This protein, a 40 kD doublet which could be glycosylated with radiolabelled UDP-glucose in the presence of Mn^{2+} or Mg^{2+} is associated with Golgi membranes as shown by density gradient centrifugation (Dhugga *et al.*, 1991). Immunolocalisation experiments showed RGP1 to be present in trans-Golgi dictyosomal cisternae (Dhugga *et al.*, 1997). An *Arabidopsis* homologue was found to be mostly soluble, but also to be associated with membranes (Delgado *et al.*, 1998). Glycosylation of the pea protein with UDP-glucose, UDP-xylose and UDP-galactose in a ratio similar to that of the typical sugar composition of xyloglucan (UDP-Glc:-Xyl:-Gal=10:7:3) indicated its involvement in xyloglucan synthesis.

Besides being components of the cell wall, polysaccharides are also major energy reserves. In plants, glucose is mainly stored as starch, an α-1,4-linked glucose polymer with α-1,6 branches. Although considerable progress has been made in the identification of the enzymes and the biosynthetic steps leading to the formation of the glucan polymers, the way in which starch synthesis and granule formation are initiated is still an enigma. In mammalian systems, a 38 kD protein has been identified as the primer for the biosynthesis of the soluble α-1,4-glucan polymer glycogen and was named glycogenin (Lomako *et al.*, 1988). This self-glucosylating protein utilises UDP-glucose as the glucose donor to elongate an α-1,4-glucan chain covalently linked to the polypeptide through a single tyrosine residue in a Mn^{2+}-dependent reaction (Alonso *et al.*, 1995). Likewise, starch biosynthesis is thought to be initiated on a protein primer. The enzyme catalysing the glucose-protein linkage was previously termed UDP-glucose:protein transglucosylase (UPTG) (Lavintman and Cardini, 1973). *In vitro* studies showed self-glucosylation of this protein in a Mn^{2+}-dependent reaction, resulting in the hypothesis that glucosylated UPTG might represent the primer for enzymatic glucan chain elongation (Moreno *et al.*, 1987). In a self-glucosylating protein from sweet corn, glucose was found to be bound to a single arginine residue via a novel glucose-protein bond (Singh *et al.*, 1995). Sequence analysis of this protein revealed a high homology to RGP1. Molecular cloning of the UDP-glucose protein transglucosylase from potato also revealed a high homology (86-93% identity at amino acid level) with RGP1 from several species (Bocca *et al.*, 1999).

Up to now, the relation between the different self-glucosylating proteins described is unclear. Since RGP1 may function in cell wall polysaccharide biosynthesis and a related RGP2 has been found in rice (Dhugga *et al.*, 1997), we adopted the working hypothesis that RGP1-related (RGP2) proteins function in the initiation of starch biosynthesis. Therefore, RGP-homologous cDNAs were isolated from wheat and rice endosperm. Our results show that two classes of RGP homologues can be distinguished, as judged from their amino acid sequences. In a first attempt to find indications about their role in polysaccharide synthesis, we studied their expression patterns and the localisation of the gene products of both classes.

Materials and methods

Plant material and growth conditions

Wheat plants (*Triticum aestivum* cultivar Minaret) were grown in a phytotron with a 16h photoperiod and temperatures of 15°C (day) and 12°C (night). Ears were tagged when the first stamen appeared, and harvested at the desired age. In these experiments the age of the developing wheat grains is indicated in days post anthesis (dpa).

SP76	GCGCCTCGAGCTGACTTTGTCCGTGGTTACC
SP79	GGCCGAGCTCATCACAGCATCCACATAC
SP80	CCGGCCATGGAGTAACATATGTCTTTGGAGGTTCAC
SP90	GCCACAGGCCGTGAG
SP91	GATCGGTACCATATGGCAGGGACGGTGAC

Table 1. Nucleotide sequences of primers used for PCR reactions. *Nde*I restriction sites are underlined.

cDNA cloning

Rgp probes were obtained by RT-PCR using endosperm RNA isolated 10 days post anthesis (dpa) from wheat (*Triticum aestivum* cv. Minaret). After denaturing 10 µg total RNA for 10 min at 60°C, cDNA was made in a final volume of 10 µl containing 1 mM dNTPs, 2 mM T_{20}, 200 units M-MLV reverse transcriptase (Promega) in PCR buffer for 1 h at 37°C. *Rgp*-homologous sequences were amplified after addition of 15 µl of 1.6 mM SP76 and 1.6 mM SP90 for *Rgp1*, or 1.6 mM SP76 and 1.6 mM SP79 primers for *Rgp2* (Table 1) and 2.5 units *Taq* DNA polymerase in PCR buffer during 35 cycli of 1 min at 94°C, 2 min at 60°C and 1 min at 72°C. The resulting fragments were labelled in a second PCR reaction containing 10 µM dCTP (0.5 mCi [^{32}P]dCTP) in 200 µl, and used for screening a cDNA library in λ uni-ZAP XR (Stratagene) made on polyA$^+$RNA from 10-dpa wheat endosperm. Lambda phages (50000 p.f.u./150 mm plate) were grown on XL1-Blue MRF' for 8h at 37°C. Plaques were lifted on Hybond-N filters (Amersham), DNA was denatured in 0.5 M NaOH, 1.5 M NaCl, and the filters were neutralised in 0.5 M Tris/HCl (pH 7.5), 1.5 M NaCl, and washed in 2XSSPE (20x SSPE: 3.6 M NaCl, 0.2 M NaH_2PO_4 (pH 6.5), 20 mM EDTA). Subsequently, the filters were exposed to UV light for 2 min, prehybridised, hybridised and washed as described previously (Memelink *et al*, 1994). The resulting positive plaques were purified by a second and third screening.

To obtain full-length rice *Rgp1* and *Rgp2* cDNA clones, cDNA libraries from etiolated shoot (Meijer *et al.*, 1997) and 7-days old somatic embryo (Postma-Haarsma *et al.*, 1999) were screened with rice ESTs S5091 and C2546, respectively.

Sequence analysis

After *in vivo* excision, the cDNA inserts were sequenced from the 5' and 3' ends with the Pharmacia T7 sequencing kit. The complete sequences of the clones with the longest inserts were determined and analysed with a VAX computer using the Genetics Computer Group Sequence Analysis Software Package (GCG, 1994). Sequence comparisons were performed using BLAST (Tatusova and Madden, 1999)

Southern and Northern blot analysis

For genomic Southern blot analysis, 10 µg DNA were digested, electrophoresed on a 0.8% agarose gel, blotted and hybridised as described (Memelink *et al.*, 1994). For Northern blot analysis, 10 µg total RNA were electrophoresed on a 1.5% formaldehyde gel, blotted and hybridised as described (Memelink *et al.*, 1994). As probes, randomly labelled cDNA inserts were used. Blots were washed with 0.1xSSPE, 0.1%SDS at 65°C.

In situ mRNA hybridisation

In situ hybridisation was performed essentially as described by Cox and Goldberg (1988). RNA probes were labelled with digoxygenin-uridine 5'-triphosphate (Boehringer Mannheim) according to the manufacturer's manual. Hybridisation was performed for 16 h at 42°C. After RNase A treatment and four washings in RNase A buffer for 5, 10, 15 and 20 min, respectively, a high stringency wash was carried out in 0.2 x SSC, 1 mM DTT at 60°C. Detection involved a short wash in washing buffer (100 mM Tris/HCl, 150 mM NaCl, pH 7.5), a 30 min incubation in 0.5% (w/v) blocking agent (Boehringer Mannheim) and a 30 min incubation in 1% BSA, 0.3 % Triton, both in the same washing buffer. Slides were incubated for 2 h in a moist chamber with a fresh 1000x dilution of anti-DIG-AP FAB fragments (Boehringer Mannheim). The bound AP was visualised by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium as substrate in high pH buffer. Colour development was stopped by washing the slides in 1xPBS, 0.1% Tween, 20 mM EDTA.

Antibody production

For cloning of wheat *Rgp1* and *Rgp2* coding regions in pET expression vectors (Novagen), the sequences 5' of the ATG start codons were modified by PCR to obtain *Nde*I restriction sites. The following primers were used for PCR reactions: SP91 (sense) and SP90 (antisense) for wheat *Rgp1*, SP80 (sense) and SP79 (antisense) for wheat *Rgp2* (Table 1). His-tag fusions were constructed as follows. The *Nde*I-*Sac*I PCR fragment containing the 5' region of *Rgp1* was cloned in pET16h. This vector is a derivative of pET16b containing the *Bam*HI-*Bgl*II polylinker fragment of pIC20H in its *Bam*HI site. The coding region was completed by addition of the 3' *Sac*I-*Kpn*I *Rgp1* fragment. The 5' region of wheat *Rgp2* was cloned as *Nde*I-*Kpn*I PCR fragment in pET16b. The coding region was completed by addition of the 3' *KpnI-XhoI* fragment. Sequence analysis confirmed that mutations had not been introduced during the PCR reactions.

His-tagged RGP1 and RGP2 were expressed in *E.coli* Bl21(DE3)pLysS for antibody production. Fusion proteins were affinity purified on His-Bind^{tm} resin (Novagen) according to the manufacturers instructions. His-tagged RGP1 and RGP2 were further purified by SDS-PAGE. The desired band was cut from the gel and electro-eluted as described (Hunkapiller *et al.*, 1983). These proteins were freeze-dried and used to raise antibodies in rabbits (performed by Eurogentec).

Two-dimensional Electrophoresis

Fifty mg endosperm were ground in 300 µl IEF lysis buffer (9.5M urea, 2% CHAPS (v/v), 0.8% Pharmalyte pH 3-10 (v/v), 1% DTT and Protease Inhibitor Complete Cocktail (Boehringer Mannheim, concentrations according to the manufacturer's recommendations). After 3 times 10 sec sonification, the lysate was centrifuged at 14,000 g for 45 sec. A 7 µl sample mixed with 83 µl IEF buffer was run on a ureum tube gel, as described by O'Farrell *et al.* (1975). After separation in the first dimension, the tube gel was equilibrated in 2% SDS, 10% glycerol (v/v), 5% β-mercaptoethanol (v/v) and 62.5 mM Tris/HCl, pH 6.8, for 1.5 h, placed and run in a 10% SDS polyacrylamide gel for separation in the second dimension, and blotted as described under Western blot analysis.

Western blot analysis

Ten mg of isolated tissue (endosperm, embryo, pericarp, leaf, root) or starch were ground in 60 µl of 1x sample buffer (Laemmli, 1970). Samples were boiled for 5 min and 10 to 15 µl were run on a 10% SDS polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane by semidry blotting, essentially as described by Towbin *et al.* (1979) and Kyhe-Andersen *et al.* (1984). After blotting, the nitrocellulose membranes were blocked in PBS containing 0.05% Tween 20 (PBST) plus 1% BSA for 30 min, followed by a 16 h incubation with a polyclonal antibody against RGP1 or RGP2 in the same buffer in a 1:20,000 dilution. Subsequently, the membranes were washed with PBST and incubated for 1 h with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase (AP). After washing (4x with PBST, then once with distilled water), the bound AP was visualised by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium as substrate in high pH buffer. Colour development was stopped by washing the nitrocellulose membranes with distilled water.

Immunolocalisation

For immunolocalisation, paraffin sections were used, which were prepared according to Cox and Goldberg (1988). Leaf tissue was, in contrast to wheat grain, fixed in a 1:1 methanol/acetone mixture at -20°C for 28 h, via 4°C gradually transferred to room temperature and embedded in paraffin. After removal of the paraffin by xylol, sections were hydrated and incubated for 15 min in 0.05M glycine in 1% PBS. Then sections were blocked for 30 min in BSA-c buffer (0.1% BSA-c (Aurion, The Netherlands), 0.9% NaCl, and 0.1% BSA in 20 mM Tris-HCl, pH 8.2), followed by an overnight (16 h) incubation with the rabbit polyclonal antibody against RGP1 or RGP2 at 4°C (1:2000 dilution). Sections were washed in BSA-c buffer and incubated for 1 h with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase. After a second wash, the bound alkaline phosphatase was visualised by the addition of 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium as a substrate in high pH buffer. Colour development was stopped by washing the sections with 1x PBS, 20 mM EDTA, 0.1% Tween 20.

Starch staining

Starch grains were stained with a IKI solution containing 5.7 mM iodine and 43.4 mM potassium iodine in 0.2 N HCl for a few minutes at room temperature.

Results

cDNA clones encoding RGP homologues from wheat and rice

In order to isolate putative protein candidates for a self-glucosylating starch primer, cDNAs homologous to published self-glycosylating proteins, termed RGPs, were cloned (Delgado *et al.*, 1998; Dhugga *et al.*, 1997). *Rgp* cDNAs and rice ESTs were compared, and primers designed on conserved DNA sequences were used to produce a PCR probe. A cDNA library made on 10-dpa wheat endosperm was screened with this PCR probe. Twenty positive clones were sequenced from the 5' and 3' ends and were found each to encode the same protein. By comparison of the rice EST sequences, two classes could be identified, one of which is homologous to the RGP1 clone described by Dhugga *et al.* (1997). Each of the isolated wheat cDNA clones was homologous to the other class, here designated RGP2. To obtain cDNA clones corresponding to RGP1, a new specific antisense primer was designed. Using this primer, an *Rgp1* homologous PCR probe was obtained and used to isolate *Rgp1*

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TaRGP1    MAGTVTVPGSSVPSTPLLKDELDIVI-PTIRNLDFLEM-WRPFFQPYHLIIVQDGDPSKVIKVP
OsRGP1    ........SA...............................................T.T.R..
StRGP1              MAAA.............................................I....
PsRGP1           MASLPKP..............................EQ..................
AtRGP1    MVEPANTV.LP.NP.............................L...............K.H..
ZmRGP1    ...........T.............................A...............T.T....
VuRGP1                                         .......QP.............T....
TaRGP2               MSL-EVHDS.V....AALQP..TSFFQA.Q...SRFDI.V.K.PELAADLQI.
OsRGP2               MSL-EIQDS.V....AALQP..TTFFEA.....SRF.I.V.K.P.HAEELQI.
AtRGP2               MSLAEIN.N.V....GALNA..TQFLTS.....SGF...V.K.PELKEELNI.

                                                    hhhxhDXDxh
TaRGP1    EGFDYELYNRNDINRILGPKASCISFKDSACRCFGYMVSKKKYVFTIDDDCFVAKDPSGKDI
OsRGP1    ..............................................................
StRGP1    ...........................................IY.................
PsRGP1    ...........................................IY............T.HE.
AtRGP1    ...........................................I................AV
ZmRGP1    ...........................................IY.................
VuRGP1    ...........................................IY.................
TaRGP2    S..NVKV.TKS..DGL..--.TT.N.SGHS..Y...L..R....IS...N.LP....A.MT.
OsRGP2    T...LKV.TKS.M-GV..--.TS.D.SGHS..Y...L..R....IS...N.L....NG.MTV
AtRGP2    ....VDV.SKT.MEKVV.-ASNSTM.SGYS..Y...L......IVS.....VP....K.-FL

                                           ∇
TaRGP1    NALEQHIKNLLSPSTPFFFNTLYDPYREGADFVRGYPFSLREGAPTAVSHGLWLNIPDYDAP
OsRGP1    ............................................K.................
StRGP1    ...........C....H..........D...........M......................
PsRGP1    ..........I..................T................................
AtRGP1    ...........C..S.............................S.................
ZmRGP1    ............................................H.................
VuRGP1    ...........C.A................................................
TaRGP2    D.VT..M...KT.A.............K...............VECML.C....HNA...PM
OsRGP2    D.VA..MS..KT.A...........F.K...............VECML.C....HNA...PM
AtRGP2    D.VT..V...EN.A..L..........C.............S.V.C.A.C.....LA.L...

TaRGP1    TQMVKPRERNSRYVDAVLTIPKGTLFPMCGMNLAFDRQLIGPAMYFGLMGDGQPIGRYD---
OsRGP1    .................M.V.................D........................
StRGP1    ..L...H...T......M...................D........................
PsRGP1    ..L...H...T.F......I...S...........N.E........................
AtRGP1    ..L...K...T........N.................D........V...............
ZmRGP1    ..L...K...E..........................D........................
VuRGP1    ..L.......T..........................E........................
TaRGP2    .HV..RNQ..TN.....M.V.L.AM..VS.I.V..N.EVL..V.FP..RIRKEGKH.W.TLE
OsRGP2    .HV..RNQ..TT.....M.V.L.AMM.VS.I.V..N.EVL..V.FPA.RLRKEGKH.W.TLE
AtRGP2    ..AL.TEK..TA.....M.V.AKAML.IS.I.I..N.E.V...LVPA.RLA.EGKV.WETLE

TaRGP1    DMWAGWCVKVICDHLSLGVKTGLPYLWHSKA-SNPFLNLKKEYKGIFWQEDIIPFFQNATIP
OsRGP1    .......M............................V......................SLS
StRGP1    .......T.......G..I......I..........V......N......E......A..L.
PsRGP1    .......I.......GY........I..........V.............E......A..LS
AtRGP1    .......I.................IY.........V.............E.........LS
ZmRGP1    .........................I..........V.....................V...
VuRGP1    ...............G.........I..........V.............E....S......
TaRGP2    .I.N.L.A..V..S.GY........VMR.D.EAGKALESL..WE.VKVMD.VL...ESLKLS
OsRGP2    .V.N.L.A..V..R.GY........VMR.D.EAGKALESL..WE.VKVMDVVL...ESLKLS
AtRGP2    .V.C.M.L.H.S...GY........V.RNERGDAVESLR..-WE.MKLM.KSV...DSLKL.

TaRGP1    KECDTVQKCYISLSEQVKEKLGKIDPYFVKLADAMVTWIEAWDELNPSDTVVAAGNGKAAAK
OsRGP1    ..........L..A...R................................TAA.EN.KAK
StRGP1    ...T..Q..LE..K...K..SS.....T..GE.............LLG.TWLSCLSPMVQQRLKSRCY
PsRGP1    .D.TS.....E..K.....T....I............T.V....I.NNKSEETTSTKASEVAATK
AtRGP1    ..AV...Q...E..KM.....SSL....D...............PAASGKSLRAV
ZmRGP1    .D........Y..G.......T.......G...............STPAAANGK.K
VuRGP1    ...T.......E..K.......AV....N................NTSEQTSSKKAGQWGCQVNQSNIAFQSCGL
TaRGP2    RTAV..DD.VKE.AGI..Q..APKNAI.A.A..V.EE.TKL.KSHGAQNA
OsRGP2    STSV..ED.VKE.TSI......PQNAI.A.A..A.EE.TKL.KSHGAQ.A
AtRGP2    ETALK.ED.V.E.AKA...Q..SD..A.TQA.....K.VQL.NSV.S.A
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	TaRGP1	OsRGP1	ZmRGP1	StRGP1	PsRGP1	VuRGP1	AtRGP1	TaRGP2	OsRGP2
OsRGP1	94 (97)								
ZmRGP1	93 (96)	90 (93)							
StRGP1	90 (95)	91 (94)	90 (95)						
PsRGP1	89 (95)	86 (94)	89 (93)	89 (95)					
VuRGP1	92 (96)	88 (95)	89 (93)	92 (96)	90 (94)				
AtRGP1	87 (92)	87 (92)	87 (90)	90 (93)	89 (94)	90 (94)			
TaRGP2	47 (68)	47 (68)	46 (67)	46 (68)	47 (69)	47 (68)	50 (70)		
OsRGP2	47 (66)	47 (66)	46 (66)	46 (66)	48 (68)	48 (67)	49 (68)	87 (93)	
AtRGP2	49 (69)	49 (69)	49 (69)	49 (69)	51 (71)	51 (71)	51 (71)	61 (76)	63 (76)

Table 2. Homology between RGP1 and RGP2 proteins of different species. For name abbreviations, see Figure 1. The percentages identity and similarity (between brackets) are given.

clones. The complete sequence of the longest clone from each class was determined.

To obtain full-length rice cDNAs from both classes, rice cDNA libraries from etiolated shoot and 7-days old somatic embryo were screened with two EST clones corresponding to both classes. From both classes, one cDNA clone containing the complete coding region was sequenced. The deduced amino acid sequences of both cDNA clones from wheat and rice are shown in Figure 1 in comparison with the amino acid sequences of homologous proteins from some other plant species. Both RGPs from the first class have a deduced molecular weight of 41 kD and an isoelec-

Figure 1. Sequence comparison of RGP protein sequences from wheat (*Triticum aestivum*), rice (*Oryza sativa*), maize (*Zea mais*), potato *(Solanum tuberosum)*, pea (*Pisum sativum*), cowpea *(Vigna unguiculata)* and *Arabidopsis thaliana*. Wheat RGP has been used as a reference. Dots indicate identical amino acids and dashes indicate gaps. The DXD motif flanked by hydrophobic residues (Wiggins and Munro, 1998) is indicated and the arginine that was shown to become glucosylated (Singh *et al.*, 1995) is indicated with an arrowhead. Database accession numbers of the sequences used for the comparison are: Y18626 (TaRGP1), Y18624 (OsRGP1), AJ223252 (StRGP1), U31565 (PsRGP1), AF013627 (AtRGP1), U89897 (ZmRGP1), AF005279 (VuRGP1), Y18625 (TaRGP2), Y18623 (OsRGP2), AB005242 (AtRGP2).

tric point of 6.1. The proteins from the other class have a molecular weight of 39 kD and isoelectric points of 6.6 for the wheat protein and 6.4 for the rice protein.

The region around the arginine that was determined to be the glucosylation site (Singh *et al.*, 1995) is highly conserved in all proteins. The overall homology between the proteins is given in Table 2. These data clearly show that the sequences fall into two classes. The class consisting of the RGP1 proteins is 87-93% identical (90-96% similarity), whereas the RGP2 class is 63-88% identical (69-91% similarity). Proteins from different classes are less homologous and 47-51% identical (57-61% similarity). The proteins do not seem to contain membrane spanning domains (Von Heijne, 1994) or N-terminal protein sorting signals (Claros *et al.*, 1997). The so-called DXD motif, which consists of two aspartates flanked by hydrophobic residues and was suggested to have a catalytic role (Wiggins and Munro, 1998), seems to be conserved among RGPs. A diverse range of glycosyltransferase families was shown to contain this motif, although in some families the second aspartate is lacking (Wiggins and Munro, 1998).

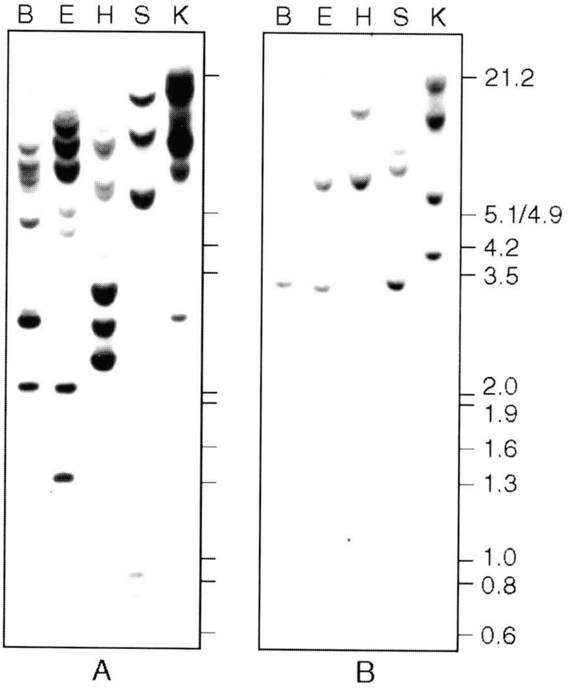

Figure 2. Southern blot analysis of *Rgp1* and *Rgp2*. Wheat genomic DNA was digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Sac*I (S) and *Kpn*I (K), electrophoresed, blotted and hybridised with *Rgp1* (A) or *Rgp2* (B) cDNA inserts. Positions and sizes in kb of *Eco*RI and *Hin*dIII digested lambda DNA fragments are indicated.

Copy number analysis

We performed Southern blot analysis to estimate the number of genes in wheat that are homologous to *Rgp1* (Figure 2A) and *Rgp2* (Figure 2B). Multiple DNA fragments were hybridising with *Rgp1* indicating a gene family. Probing of the same blot with *Rgp2* resulted in 2-4 bands in each lane. Considering the hexaploidy of wheat, we assume that *Rgp2* is present as a single copy gene per haploid genome.

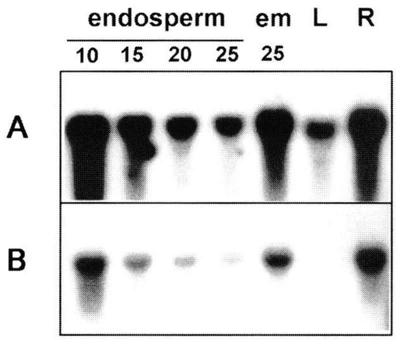

Figure 3. *Rgp1* and *Rgp2* expression in wheat tissues. Gel blots containing total RNA from endosperm of 10 to 25 dpa (lanes 1-4), embryo of 25 dpa (em, lane 5), leaf (L, lane 6) and root (R, lane 7) of two-week-old seedlings were hybridised with *Rgp1* (A) or *Rgp2* (B).

Localisation of RGP mRNAs

In order to find indications about the function of RGP1 and RGP2 in polysaccharide biosynthesis, we performed Northern blot analysis using total wheat RNA from endosperm of 10 to 25 dpa, embryo of 25 dpa and leaf and root of two-weeks-old seedlings (Figure 3). Rgp1 mRNA is very abundant in all tissues analysed. During endosperm development, gene expression decreased in time. In leaf, expression was lower than that in root. Expression of Rgp2 was also relatively high in 10-dpa endosperm and decreased during development. In 25-dpa embryo, expression was high compared to that in endosperm of the same age. In leaf, expression could not be detected. In root, expression was comparable to that in embryo, however, the expression level was at least ten-fold lower compared to that of Rgp1 in root. Rgp1 and Rgp2 transcripts were both about 1.7 kb in size.

Because priming of starch synthesis occurs early in development of wheat endosperm, we also wanted to study the expression of *Rgp2* in endosperm younger than 10 dpa. Since the isolation of very young endosperm is difficult, we performed *in situ* RNA hybridisations on cross-sections of developing wheat grains. An *Rgp2* antisense probe showed the presence of *Rgp2* mRNA in 7-dpa endosperm (Figure 4A). An ADP-glucose-pyrophosphorylase (AGPase) antisense probe was used as a positive control, expression of which was visible in the endosperm (Figure 4B). Since AGPase supplies the cell with ADP-glucose, the substrate for starch biosynthesis, the presence of AGPase mRNA indicates that the tissue is preparing for or performing starch synthesis.

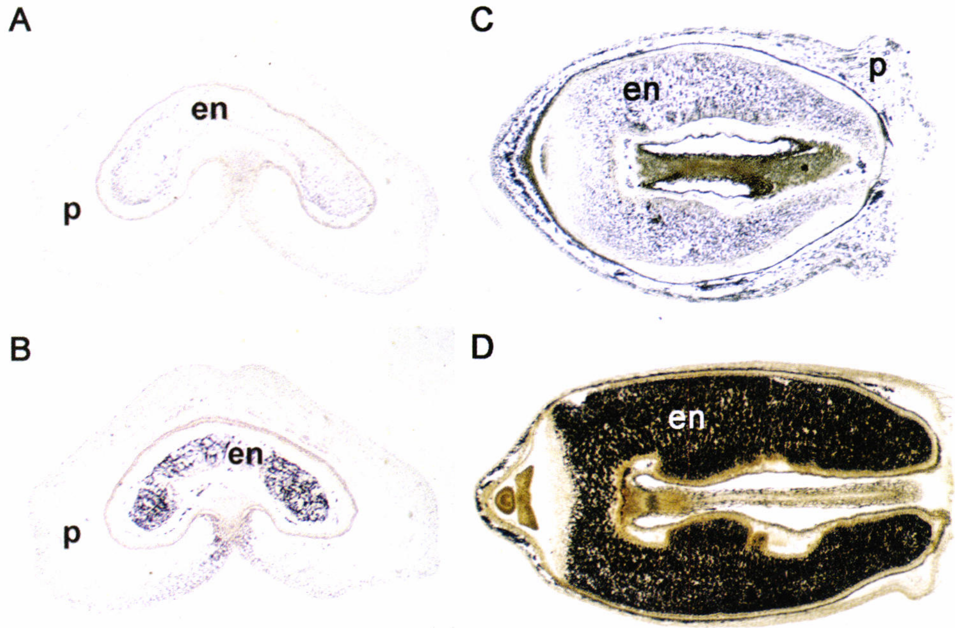

Figure 4. Localisation of mRNA by *in situ* hybridisation (A-B) and starch stained with an IKI solution (C-D) in sections of developing wheat grains. (A) *Rgp2* mRNA in transverse section of 6-dpa wheat grain. (B) large subunit of *AGPase* mRNA in transverse section of 7 dpa. (C) Starch, 10 dpa. (D) starch, 14 dpa. en, endosperm; p, pericarp.

RGP antibodies

As judged from their deduced amino acid sequences, the calculated isoelectric points of wheat RGP1 and RGP2 are 6.1 and 6.6, respectively. This difference in pI was used to test the specificity of the RGP2 antibodies. After separation of RGP1 from RGP2 by two-dimensional electrophoresis and Western blotting with RGP2 antibodies (Figure 5A), only one clear spot was detected in wheat endosperm protein extract, at the expected pI and size values of RGP2 (arrow). When the RGP1 antibodies were used on the same blot, a clear spot appeared at the expected pI of

RGP1 (arrowhead, Figure 5B). These results demonstrated that the RGP2 antibodies specifically recognised RGP2 protein. Furthermore, RGP1 antibodies did not recognise the RGP2 protein in tobacco plants overexpressing RGP2, and the RGP1 antibodies only recognised the *E.coli* RGP2 protein when this protein was present at a very high concentration (Langeveld and de Pater, unpublished), showing that both antibodies were useful for further studies.

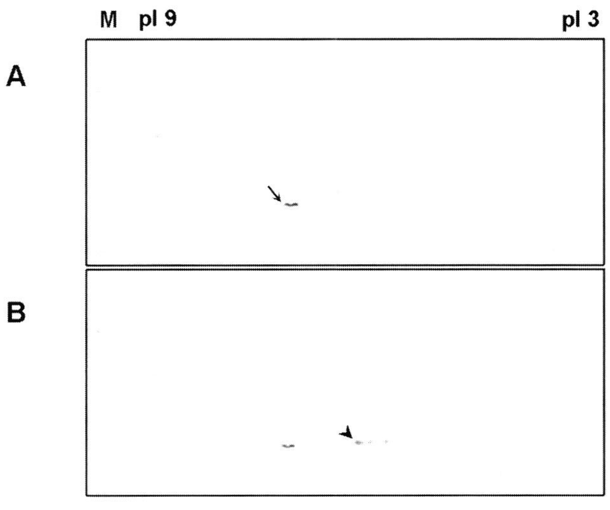

Figure 5. RGP1 and RGP2 differ in pI. (A) Proteins extracted from 10-dpa endosperm were separated on a two-dimensional gel, blotted onto nitrocellulose and RGP2 protein was detected with polyclonal RGP2 antibodies (arrow). (B) The same blot was rehydrated and blocked and RGP1 protein was detected with polyclonal RGP1 antibodies (arrowhead).

Localisation of RGP proteins and starch

The specific polyclonal antibodies were used to study the localisation of the RGP1 and RGP2 gene products. Gels for Western blot analysis were loaded based on tissue fresh weight. A Coomassie-stained gel is shown as a control for protein content (Figure 6C). On Western blots, RGP1 antibodies recognised a 41 kD protein present throughout endosperm development from 6 dpa until 18 dpa (Figure 6A). RGP2 antibodies recognised a 39 kD protein, which was detected in developing endosperm of 6 dpa. After 8 dpa, the amount of RGP2 was gradually decreasing.

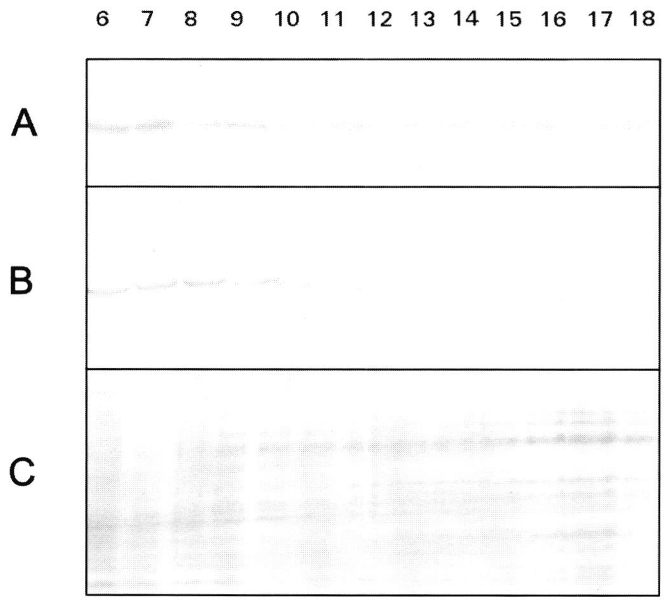

Figure 6. Western blots showing the presence of RGP1 (A), RGP2 (B) and total protein (C) in developing endosperm of wheat from 6-18 dpa.

During embryo development RGP1 protein was abundantly present in 17-, 19- and 25-dpa embryo's (Figure 7). However, at 30 dpa the RGP1 protein could hardly be detected in the embryo. The overall level of RGP2 protein was lower compared to that of RGP1, but showed hardly any reduction in 30-dpa embryo's. Both RGP1 and RGP2 were abundant in pericarp (seed coat) tissue (6-16 dpa). RGP1 was detected in leaf and root but not in isolated starch. In accordance with the mRNA localisations, RGP2 was not detected in leaf and was barely present in root. RGP2 was also not detectable in isolated starch.

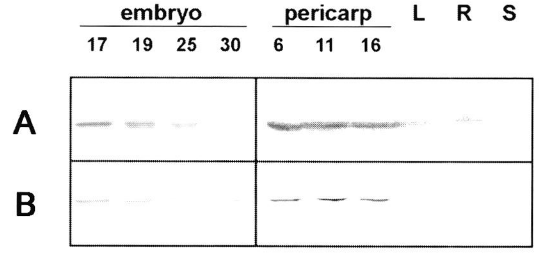

Figure 7. Western blots showing the presence of RGP1 (A) and RGP2 (B) in developing embryo of 17 to 30 dpa, pericarp from 6 to 16 dpa, leaf (L) and root (R) from two-week-old seedlings and isolated starch from 12-dpa endosperm (S).

Immunolocalisation studies confirmed the presence of RGP1 and RGP2 protein in the endosperm and pericarp of 8-dpa wheat grains (Figures 8A and 8C, respectively). Sections incubated with pre-immune serum were used as a control and showed no staining (Figures 8B and 8D). In cross sections of young leaf only RGP1 was detected in parenchyma cells (Figure 8E). Sections challenged with RGP2 antibodies did not show any additional staining compared to pre-immune serum-treated sections (Figures 8F and 8H). These immunolocalisation studies are consistent with the Western blot data.

In order to correlate the accumulation of RGP proteins with starch localisation in wheat grains, iodine staining was performed, which specifically detects starch granules. Figures 4C and D show the presence of starch in the endosperm and pericarp of 10 dpa (Figure 4C) and 14-dpa wheat grains (Figure 4D).

Discussion

In order to study the function of reversibly glycosylated polypeptides, we have isolated RGP-like cDNAs from wheat and rice. Comparison with related sequences from other species showed that two classes of RGP proteins can be distinguished, RGP1 and RGP2. The RGP described by Dhugga *et al.* (1997) belongs to the first class and is designated RGP1. The other class is referred to as RGP2.

In a first characterisation of these RGPs, we studied the localisation of their gene products. Northern blot analysis, *in situ* hybridisation, Western blot analysis and immunolocalisation experiments showed the presence of *Rgp1* mRNA in all tissues examined, which correlates with a general function such as cell wall synthesis. *Rgp2* mRNA and protein were detected in young endosperm, pericarp, root and developing embryo. In all these tissues, starch is present. Black *et al.* (1996) showed that in the embryo starch accumulates from about 20 dpa to reach a maximum at 35 dpa. *Rgp2* mRNA and protein were not detected in leaf tissue, which only contains so-called transitory starch. Taken these results together, we may conclude to a correlation between the presence of RGP2 and the starch-storing function of plant tissues. In pericarp tissue however, that is broken down during grain development to make space for the expanding endosperm, both RGP1 and RGP2 are abundantly present. Around 8 dpa, cell death can be visualised in pericarp by viability staining (Young and Gallie, 1999), and at about 10 dpa the small starch granules start to disappear

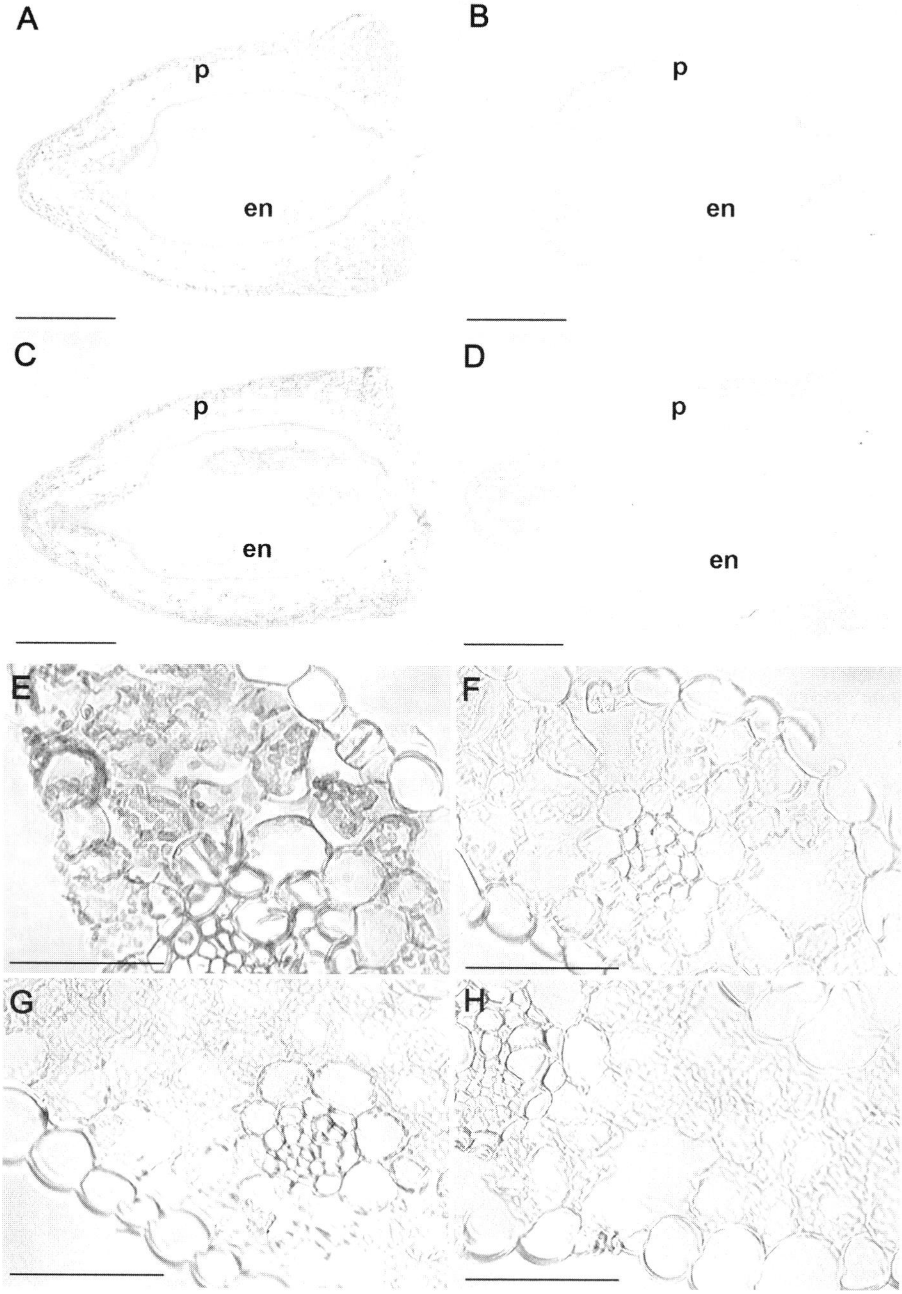

Figure 8. Localisation of RGP1 and RGP2 in 10-dpa wheat grain (A-D) and leaf (E-H). (A) and (E) RGP1, (B) and (F) pre-immune serum RGP1, (C) and (G) RGP2, (D) and (H) pre-immune serum RGP2. en, endosperm; p, pericarp. Bars in A-D represent 1 mm; bars in E-H represent 50 μm.

(Duffus, 1979). The large amounts of RGP1 and RGP2 found in pericarp seem to be in conflict with the proposed role of RGPs in polysaccharide synthesis.

RGP2 does not seem to contain a signal peptide for import into amyloplasts and is not detected in isolated starch. RGP1 in *Arabidopsis* was found to be present in the soluble and microsomal fractions (Delgado *et al.*, 1998) and was localised to the Golgi apparatus in pea (Dhugga *et al.*, 1997). These observations are consistent with the more recent idea that RGPs function in transport of UDP-sugars from the cytoplasm towards membranes (Delgado *et al.*, 1998), as an intermediate in the transfer of a single sugar residue from a nucleotide-sugar to an acceptor molecule (Saxena and Brown Jr, 1999).

The RGP1 clones are very conserved, even between monocots and dicots. Probably they play an essential role in the plant, which does not allow extensive mutations in the amino acid sequence. Parts of the RGP2 proteins are different from the RGP1 proteins but the amino acid changes are conserved among the different RGP2 proteins. This suggests that these amino acids are important for the function of RGP2 proteins. The fact that RGP1 and RGP2 proteins each are more homologous to other members of the class to which they belong than to members of the other class suggests that they may have different activities and functions in plants.

Sequence comparison indicated that RGP1 has a putative nucleotide sugar-binding region that is conserved in cellulose synthase and other β-glycosyltransferases (Saxena and Brown Jr, 1999) and was suggested to represent a non-processive β-glycosyltransferase. However, in RGP2 proteins from rice and wheat the second of the two aspartic acid residues in the DXD motif is replaced by an asparagine. Moreover, several other amino acid residues conserved among RGP1 and cellulose synthase are not conserved in RGP2 proteins (Saxena and Brown Jr, 1999). This suggests that RGP1 and RGP2 proteins may indeed have different activities.

Alternatively, RGP1 and RGP2 may be isoforms, exhibiting a similar enzymatic reaction in different tissues and/or different developmental stages. However, in general, isoforms show a higher homology than found for *Rgp1* and *Rgp2*. For example, several isoforms of the sucrose synthase enzyme have been identified, such as the potato sucrose synthase genes *Sus3* and *Sus4*, which share 92% amino acid identity and are differentially expressed (Fu and Park, 1995). More recently, a second granule bound starch synthase (GBSSII) in wheat was identified, which was specifically expressed in leaf and pericarp and showed 66% identity to GBSSI at the amino acid level (Vrinten and Nakamura, 2000).

Finally, RGP1 and RGP2 may be subunits of larger complexes. For example the enzyme ADP-glucose-pyrophosphorylase (AGPase), an enzyme involved in starch biosynthesis, consists of two small and two large subunits. The homology between these small and large subunits is about 50% (maize: 52% (Smith-White and Preiss, 1992); wheat 48% (Ainsworth *et al.*, 1995); pea 54% identity (Burgess *et al.*, 1997); tomato 47-52% (Chen *et al.*, 1998), which correlates well with the 47-51% identity found for RGP1 and RGP2. Chapter 5 of this thesis will describe a study of putative complexes in which RGP1 and RGP2 could participate.

In summary, the exact functions of RGP1 and RGP2 remain obscure. Subcellular localisation experiments may indicate whether RGP1 and RGP2 have similar or divergent functions in polysaccharide metabolism.

Acknowledgements

We thank Dr Takuji Sasaki (MAFF DNA Bank) for the two rice RGP clones from the Japanese Rice Genome Research Program of the National Institute of Agrobiological Resources in Japan, Dr Annemarie Meijer for the rice cDNA libraries, and Dr Johan Memelink for vector pET16h.

Chapter 4

Subcellular localisation of reversibly glycosylated polypeptides in wheat endosperm

Sandra MJ Langeveld, Ringo van Wijk, Jan W Kijne and Sylvia de Pater

Abstract

Reversibly glycosylated polypeptides (RGPs) have been implicated in polysaccharide biosynthesis. In order to get an indication about the role of the two classes of RGPs in plants, the subcellular localisation of RGP1 and RGP2 was studied in endosperm of developing wheat seeds, a storage tissue for polysaccharides. Differential centrifugation and confocal laser scanning microscopy studies of endosperm cells showed the presence of both RGP1 and RGP2 in the cytoplasm and showed them to be associated with small and large organelles. Results of a co-expression study using plastid-localised protein excluded the possibility that RGP1 and RGP2 are associated with amyloplasts. Neither RGP1 nor RGP2 were found to be localised at the plasma membrane. From membrane association studies, it was concluded that RGP1 and RGP2 both are peripheral membrane proteins. Taken together, these results support the hypothesis that both classes have a function in transporting UDP-sugars, although their exact role remains to be established.

Introduction

Polysaccharides are important constituents of the plant cell, serving functions in structures such as the cell wall and in short- and long-term storage of carbohydrates. Reversibly glycosylated polypeptides (RGPs) have been implicated in polysaccharide biosynthesis (Delgado *et al.*, 1998). Whether RGPs are involved in cell wall synthesis or starch biosynthesis is still a matter of debate.

Dhugga *et al.* (1991) identified a 40 kD doublet in pea membranes, which could be glycosylated by radiolabelled UDP-glucose. The label rapidly disappeared when unlabeled UDP-glucose, UDP-xylose or UDP-galactose was added, the sugars of which xyloglucan is composed (Hayashi, 1989). Moreover, the ratio of UDP-glucose, -xylose and -galactose present in xyloglucan (1: 0.75: 0.25) was found to be similar to the steady state glycosylation of PsRGP1 (10:7:3). Together with immunolocalisation experiments that showed the presence of PsRGP1 in the trans-Golgi dictyosomal cisternae, this suggested the involvement of RGP1 in synthesis of xyloglucan and possibly of other hemicelluloses (Dhugga *et al.*, 1997).

An auto-glucosylating protein is also thought to be involved in starch synthesis. Although considerable effort has been put in isolation and characterisation of enzymes involved in elongation and ramification of the α-1,4-glucan chain, little is known about the mechanism of *de novo* biosynthesis of starch. In mammalian systems, a 38 kD self-glucosylating protein was identified as the primer for the soluble α-1,4-glucan polymer glycogen (Lomako *et al.*, 1988). This so-called glycogenin was able to incorporate radiolabelled UDP-glucose in an α-1,4-glucan chain covalently linked to the polypeptide through a single tyrosine residue in a Mn^{2+}-dependent reaction (Alonso *et al.*, 1995). A protein with similar characteristics designated UDP-glucose:protein transglucosylase (UPTG) was isolated from potato (Lavintman and Cardini, 1973). This 38 kD self-glucosylating protein was suggested to be the protein primer for starch synthesis (Moreno *et al.*, 1987). In analogy with glycogenin, a self-glucosylating protein in maize was called amylogenin (Singh *et al.*, 1995). Sequence analysis of this protein revealed a high homology with RGP1. Molecular cloning of the UPTG from potato also showed a high homology (86-93% identity at the amino acid level) with RGP1 from several plant species, e.g., pea and *Arabidopsis* (Bocca *et al.*, 1999).

After comparison of wheat and rice RGP clones with other known RGPs, it was concluded that two classes of RGPs can be distinguished (Chapter 3, this Thesis) The RGP1 described by (Dhugga *et al.*, 1997) belongs to the first class. The other group, designated RGP2, was shown to be localised in storage tissue. Interestingly, RGP1 and RGP2 proteins were abundantly present in pericarp tissue, which is apoptotic during grain development. These findings suggested a possible role of both proteins in transport of nucleotide sugars. We investigated whether the subcellular localisation of RGP2 in comparison with RGP1 supported this working hypothesis.

Material and methods

Plant material

Wheat plants, cultivar Minaret, were grown as described in Langeveld *et al.* (2000).

Differential centrifugation

Whole wheat seeds (10 days post anthesis (dpa)) were ground in liquid nitrogen. Four ml of extraction buffer (20 mM Hepes, pH 7.0; 13.5% sucrose (w/v); 10 mM potassium acetate; 1 mM DDT; 0.5 mM PMSF and 1 mM EDTA) were added to 2 g of plant tissue. This homogenate was called total protein (tot). Total protein was centrifuged at 25,000 g and 100,000 g for 30 min at 7°C. The resulting pellets (P25 and P100, respectively) were resuspended in extraction buffer. The supernatant of the 100,000 g centrifugation (S100) was retained. The protein concentration in each fraction was quantified and analysed by Western blotting (Chapter 3, this Thesis)

Membrane association

Membrane fractions were isolated by centrifugation of protein preparations extracted from isolated endosperm at 500 g at 7°C, filtration of the supernatant through nylon mesh and centrifugation of 100 µl portions in a microfuge at 150,000 g for 10 min. The pellets were washed with extraction buffer, resuspended and incubated in buffer containing 1 M NaCl, 0.1 M sodium carbonate, 0.5 or 2.0 M urea or 1% Triton X-100 for 1 h on ice. A control fraction was resuspended and incubated in extraction buffer only. After centrifugation at 150,000 g, the pellets were washed twice with extraction buffer and resuspended in equal amounts of loading buffer. Protein in the pellets was analysed by Western blotting.

Confocal laser scanning microscopy

Confocal laser scanning microscopy was basically performed as described by Langeveld *et al.* (2000). Pactin-CFP was constructed as Pactin-YFP using the pECFP from Clontech. RGP1 and RGP2 clones (Chapter 3, this Thesis) were modified by PCR, introducing *NcoI* sites at both ends and deleting the stopcodons at the 5' ends. Pactin-RGP1-CFP, Pactin-RGP2-CFP, Pactin-RGP1-YFP and Pactin-RGP2-YFP constructs were produced by inserting the RGP1 and RGP2 sequences as *NcoI* fragments in Pactin-CFP and Pactin-YFP, respectively. The orientation was checked by restriction analysis. Pactin-TP-CFP was constructed as Pactin-TP-YFP (Langeveld *et al.*, 2000).

Results and Discussion

To test whether RGP2 is soluble or membrane-associated, fractionation experiments were performed. Total protein from wheat seeds was subjected to differential centrifugation. The pellets obtained after centrifugation at 25,000 g (P25) and 100,000 g (P100) and the supernatant of the latter treatment were analysed by Western blotting (Figure 1). Our results show that RGP2 is mainly present in the soluble fraction (S100) but also in the membrane-containing fraction P100. Delgado *et al.* (1998) obtained the same results with RGP1 in cell-suspension derived protoplasts from *Arabidopsis*.

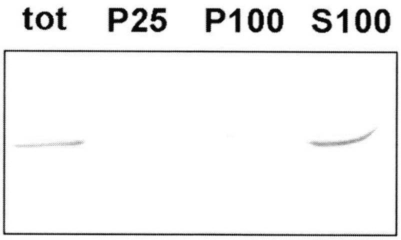

Figure 1. Differential centrifugation of RGP2. The presence of RGP2 in total protein (tot), pellet fractions after centrifugation at 25,000 g (P25) and 100,000 g (P100) and soluble fraction after 100,000 g centrifugation (S100), was analysed by Western blotting.

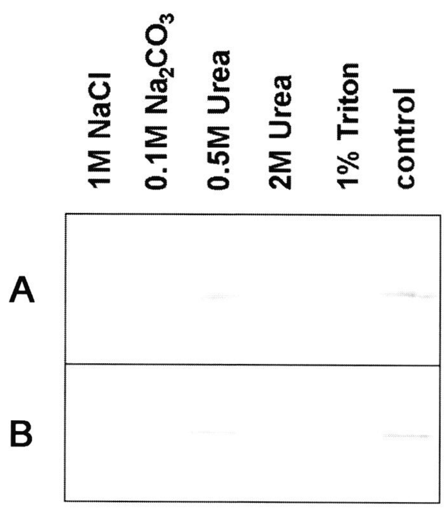

Figure 2. Membrane association of RGP1 **(A)** and RGP2 **(B)**. Pellet fractions after centrifugation at 150,000 g were washed, subjected to treatment in 1M NaCl, 0.1M Na_2CO_3, 0.5M and 2M urea, 1% Triton X-100 in extraction buffer and extraction buffer (control), respectively and analysed by Western blotting.

To further investigate the nature of the association of the protein with membranes, we challenged 150,000 g pellets with solutions each containing different chemicals. After the different treatments, the membranes were recovered by centrifugation at 150,000 g and washed in buffer. Protein that was still associated to the membranes was analysed by Western blotting (Figure 2). Non-ionic detergent

(1% Triton X-100), alkaline (0.1M Na_2CO_3) and high salt (1M NaCl) treatment released RGP1 as well as RGP2 from the membranes. High urea levels (2M) eluted part of the RGP1 and RGP2 proteins, whereas low levels of urea (0.5 M) and neutral buffer left both RGP1 and RGP2 associated with the membranes. These results are similar to the findings of Delgado *et al.* (1998) for AtRGP1, indicating that both proteins are peripheral membrane proteins.

To further study the subcellular localisation, RGP1 and RGP2 constructs fused to CFP or YFP were made under control of the rice actin promoter. These constructs were introduced into wheat endosperm of 10 dpa by particle bombardment. The transiently transformed endosperm cells were analysed by confocal laser scanning microscopy. Figure 3 shows one endosperm cell transformed with both the Pactin-RGP1-CFP (Figure 3A) and Pactin-RGP2-YFP (Figure 3B) constructs. RGP1-CFP as well as RGP2-YFP is detected in the cytoplasm, in small organelles (arrow) and in large organelles (v), in support of the results obtained by differential centrifugation and membrane-association experiments. Moreover, the overlay (Figure 3C) shows that RGP1 and RGP2 are present at exactly the same subcellular locations, suggesting a similar function.

To test whether the large organelles are amyloplasts, wheat seeds were bombarded with a mixture of Pactin-RGP2-YFP and Pactin-TP-CFP, the latter specifically labelling the plastid fraction by the presence of a transit peptide, (Langeveld *et al.*, 2000). Figure 3D shows that RGP2 (red) is not associated with the small plastids (green) present in this cell. This is consistent with a lack of a targeting sequence, as observed earlier for RGP1 and RGP2 (Chapter 3, this Thesis).

Both RGP1 and RGP2 were found to be present in the cytoplasm and associated with small and large organelles. The small organelles might represent small vesicles and/or Golgi membranes. Since the large organelles were not always observed, they may represent storage vacuoles resulting from the high expression of the fusion proteins after the transformation procedure (see Kavakli *et al.*, 2000).

The hypothesis postulated by Delgado *et al.* (1998) who suggested that RGP1 functions as a carrier of UDP-sugars from the cytoplasm to membranes seems plausible for RGP2 as well. Since RGP2 is also mainly present in the cytoplasm where UDP-sugars are found and since a small amount of RGP2 is associated with endomembranes, RGP2 and RGP1 may have the same function. Considering the partial homology with cellulose synthase and other β-glycosyltransferases, Saxena and Brown Jr (1999) suggested that RGPs may be non-processive β-glycosyltransferases. Activity tests will be necessary to determine the exact function of RGP1 and RGP2.

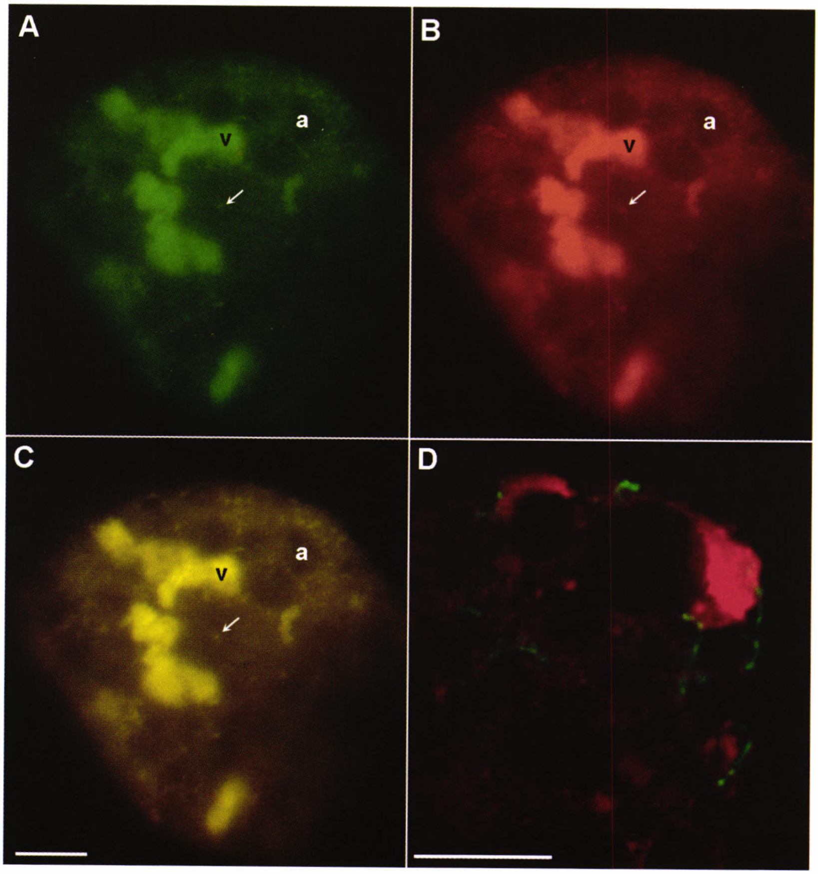

Figure 3. Localisation of RGP1 and RGP2 fusion proteins in wheat endosperm. Half seeds were transiently transformed using particle bombardment and visualised by confocal laser scanning microscopy. **(A)** pActin-RGP1-YFP, **(B)** pActin-RGP2-CFP in the same cell as (A), **(C)** overlay of (A) and (B) showing identical localisation of RGP1 and RGP2 in small organelles (arrow) and large organelles, possibly storage vacuoles (v). RGP1 and RGP2 are not found in amyloplasts (a). **(D)** localisation of pActin-RGP2 (red) and pActin-TP-CFP (green) showing that RGP2 is not associated with the small plastids in this cell. Bars indicate 10 µm.

Acknowledgements

We thank Gerda Lamers and Nico Stuurman for help with the CLSM.

Chapter 5

Glucosylation activity and complex formation of reversibly glycosylated polypeptides

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Abstract

Glucosylation activity of two classes of reversibly glycosylated polypeptides (RGPs) from wheat and rice was studied. After separate expression of *Rgp1* and *Rgp2* in *E.coli*, only RGP1 showed self-glucosylation. In wheat endosperm Superose 12 fractions, a polypeptide with a molecular weight of about 40 kD is glucosylated by UDP-[^{14}C]glucose. Chase experiments showed that this protein reacts with unlabeled UDP-sugars such as UDP-glucose, UDP-xylose or UDP-galactose, but not with ADP-glucose. Since RGP1 and RGP2 activity could not be distinguished in wheat endosperm extracts, transgenic tobacco plants overexpressing either wheat *Rgp1* or *Rgp2* were generated. Subsequent glucosylation assays revealed that in RGP1-containing tobacco extracts as well as in RGP2-containing tobacco extracts UDP-glucose is incorporated, indicating that an RGP2-containing complex is active. Gel filtration experiments with wheat endosperm extracts and extracts from transgenic tobacco plants overexpressing either wheat *Rgp1* or *Rgp2* showed the presence of RGP1 and RGP2 in HMW complexes. Yeast two-hybrid studies showed that each of the RGP classes is able to bind to both RGPs. Screening of a cDNA library using the yeast two-hybrid system and purification of the complex by an antibody affinity column did not reveal the presence of other proteins in the RGP complexes. Taken together, these results suggest the presence of active RGP1 and RGP2 homo- and hetero-multimers in wheat endosperm.

Introduction

Reversibly glycosylating polypeptides (RGPs) have been implicated in polysaccharide biosynthesis. Since RGP1 from pea was localised to trans-Golgi dictyosomal cisternae and could be glycosylated by UDP-sugars in a ratio as found in xyloglucan, it was argued that RGP1 is involved in xyloglucan synthesis (Dhugga *et al.*, 1997). Previously, a structurally homologous protein from maize designated UDP-glucose:protein transglucosylase (UPTG) or "amylogenin" was thought to function as a primer for starch synthesis (Rothschild and Tandecarz, 1994; Singh *et al.*, 1995). Sequence analysis of UPTG from potato together with biochemical properties almost identical to those of RGP1 indicated that UPTG, amylogenin and RGP1 are homologous proteins, and suggested a similar function in cell wall synthesis (Bocca *et al.*, 1999). Gel filtration experiments with potato suggested an oligomeric structure for UPTG in its native active state (Bocca *et al.*, 1999), estimated to consist of five or six 38 kD subunits. Sequence homology studies with β-glycosyl transferases such as cellulose synthases suggested that RGP1 is a non-processive β-glycosyltransferase (Saxena and Brown Jr, 1999). In contrast to processive glycosyltransferases, only a single sugar residue from a nucleotide sugar is transferred to an acceptor molecule. In this case, the acceptor molecule may be RGP1 itself or another protein (Saxena and Brown Jr, 1999).

Molecular cloning of RGPs from wheat endosperm showed that two classes of RGPs can be distinguished, designated RGP1 and RGP2. The RGP1 proteins share about 90% sequence identity at the amino acid level, whereas the class of RGP2 proteins shows 63-88% identity. RGP1 and RGP2 proteins are less homologous and share about 50% identity (Chapter 3, this Thesis). Both RGP1 and RGP2 were found to be peripheral membrane proteins and located in the cytoplasm as well as associated with membranes (Delgado *et al.*, 1998, Chapter 4, this Thesis). This suggested that RGPs have a function in transport of UDP-sugars from the cytoplasm towards membranes (Delgado *et al.*, 1998). However, the fact that several amino acid residues conserved among RGP1 and cellulose synthase are not conserved in RGP2 proteins (Chapter 3, this Thesis), suggests that RGP1 and RGP2 proteins may have different activities.

A 80 kD inhibitor protein (IP) of UPTG was isolated from maize endosperm and was found to be capable of inhibiting UPTG activity in 5-day old maize seedlings as well as in potato tuber preparations (Rothschild *et al.*, 1996). Sequence analysis of two internal peptides showed 60-91% identity with sucrose synthases from several plant sources. Anti-sucrose synthase antibodies were able to neutralise sucrose synthase as well as IP activities. Moreover, UPTG activity is not inhibited in the maize mutant *shrunken-1*, which lacks the Sucrose Synthase-1 (SS1) isoform, suggesting that the SS1 isoform is the best candidate for representing the inhibitor protein (Wald *et al.*, 1998).

The present study aimed at characterisation of RGP1 and RGP2 from wheat and rice. Glucosylation assays were performed with extracts from *E.coli* and tobacco, each overexpressing *Rgp1* or *Rgp2*. Furthermore, the presence of RGP complexes in wheat endosperm was studied by Superose 12 gel filtration experiments. The ability of RGP proteins to form dimers was tested using a yeast two-hybrid system. The presence of other proteins in the complex was studied by affinity purification.

Materials and methods

Constructs and expression in E.coli

Wheat and rice *Rgp1* and *Rgp2* were cloned in pET29b (Novagen) for purification and performance of glucosylation activity tests. The sequences 5' of the ATG start codons were modified by PCR to obtain *Nde*I restriction sites. The following primers were used for PCR reactions: SP91 (sense) and SP90 (antisense) for wheat *Rgp1*, SP80 (sense) and SP79 (antisense) for wheat *Rgp2*, SP91 and SP79 for rice *Rgp1*, SP93 (sense) and SP79 for rice *Rgp2* (Table 1). The 5' region of wheat *Rgp1* was cloned in pET29b as *Nde*I-*Sac*I PCR fragment. The 3' region was cloned into the resulting plasmid as *Xho*I fragment. The 5' region of wheat *Rgp2* was cloned as *Nde*I-*Kpn*I PCR fragment in pET29b and the 3' region as *Kpn*I-*Xho*I fragment. The 5' region of rice *Rgp1* was cloned in pET29b as *Nde*I-*Bam*HI PCR fragment and the 3' region as *Bam*HI-*Xho*I fragment. The 5' region of rice *Rgp2* was cloned as *Nde*I PCR fragment in pET29b and the 3' region as *Kpn*I fragment. Sequence analysis confirmed that mutations had not been introduced during the PCR reactions. The His-tag of pET29b was not fused to the *Rgp1* and *Rgp2* coding regions, because the stop codons of the introduced coding regions were not removed. The constructs containing a His-tag were made as described in Chapter 3 (this Thesis).

SP79	GGCCGAGCTCATCACAGCATCCACATAC
SP80	CCGGCCATGGAGTAACATATGTCTTTGGAGGTTCAC
SP90	GCCACAGGCCGTGAG
SP91	GATCGGTACCATATGGCAGGGACGGTGAC
SP93	CCGGCATATGTCTTTGGAAATTCAGG

Table 1. Nucleotide sequences of primers used for PCR reactions. *Nde*I restriction sites are underlined.

The resulting plasmids were introduced into *E.coli* CGSC4954(DE3) (Alonso *et al.*, 1994). For protein production, overnight cultures grown in LB medium containing 50 µg kanamycin were diluted 20 times in the same growth media and incubated with shaking at 37°C until the OD_{600} reached a value of 0.6. IPTG was added to a final concentration of 1 mM and incubation was continued for 3 h. Cells were harvested by centrifugation, washed with 50 mM Tris/HCl, pH 7.5, and resuspended in 1/10 of the culture volume cold 50 mM Tris/HCl, pH 7.5, containing Complete^{tm} proteinase inhibitor cocktail (Boehringer). Cells were sonicated two times for 4 sec and centrifuged 10 min at 4°C in an Eppendorf centrifuge. Protein concentrations in supernatants were determined by the method of Bradford (Bradford, 1976).

Protein analysis

Crude protein extracts were analysed by SDS-PAGE followed by staining with Coomassie Brillant Blue, silver-staining or by Western blotting as described (Memelink *et al.*, 1994). Glucosylation activity was tested with 40-60 µg protein extract or 1-5 µg purified protein and 2 µM UDP-[^{14}C]glucose or ADP-[^{14}C]glucose as a substrate in 50 mM Tris/HCl, pH 7.5, 5 mM $MnCl_2$, for 30 min at 30°C. After separation of the mixtures by SDS-PAGE, gels were fixed in 25% isopropanol, 10% acetic acid, soaked in Amplify (Amersham), dried and exposed for fluorography.

Superose 12 gel filtration

Total protein was extracted in buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 0.2 mM PMSF) containing Complete^{tm} proteinase inhibitor cocktail (Boehringer) for 15 min on ice. Extracts were centrifuged two times for 5 min at 4°C, the second time through a 0.22 µm filter.

Total protein extract (200 µl) was fractionated on a Superose 12 HR 10/30 column at a flow rate of 0.5 ml/min using 50 mM Tris, pH 7.5, 2 mM EDTA, generating 200 µl fractions. Fractions were taken after 20 min. Aliquots of each fraction (20 µl) were analysed by Western blotting and compared with 5 µl of the total protein extract.

Tobacco transformation

For tobacco transformation, a *Bam*HI-*Eco*RV fragment containing the coding region of *Rgp1* and a *Sal*I-*Hind*III fragment encoding *Rgp2* were separately cloned in vectors containing the double 35S promoter (van der Fits and Memelink, 1997) and the *RbcS*-3C terminator. The vectors were transferred to *Agrobacterium tumefaciens* strain MOG101 (Hood *et al.*, 1993). Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) was transformed with the MOG101 derivatives by the leaf disc transformation method (Horsch *et al.*, 1985).

Yeast two-hybrid

Wheat *Rgp1* and *Rgp2* were separately cloned in pAS2-1 containing the GAL 4 DNA binding domain and in pACTII, containing the GAL 4 activation domain (Clontech). pET16h-RGP1 (Chapter 3, this Thesis) was digested with *Nde*I. For cloning in pACTII, blunt ends were produced using the Klenow fragment of DNA polymerase I. The linear plasmids were digested with *Bam*HI and the *Rgp1* encoding cDNA fragments were cloned in pAS2-1 and pACTII digested with *Nde*I-*Bam*HI or *Sma*I-*Bam*HI, respectively. A *Nco*I site was introduced at the ATG of *Rgp2* by PCR and the stopcodon was replaced by a *Nco*I site using a linker. The *Nco*I fragment was cloned into the *Nco*I sites of pAS2-1 and pACTII. The orientations were checked by restriction analysis.

pAS2-1-RGP1 or pAS2-1-RGP2 and pACTII-RGP1 or pACTII-RGP2 were introduced in yeast strain CG1945 according to Gietz *et al.* (1992). The transformed yeast was tested for growth on plates lacking Trp, Leu and His. Positive colonies were transferred to plates lacking Leu and containing cycloheximide. Loss of the pAS2-1 vector was checked by growing the strains on plates lacking Leu and Trp, Leu and His, and Leu only. Colonies only growing on the latter were re-transformed with pAS2-1-RGP1 or pAS2-1-RGP2 to test whether growth was dependent on RGP1 or RGP2, respectively.

To isolate other proteins that bind to RGP2, a 10-dpa endosperm cDNA library was cloned into λACTII (Memelink, 1997) using the *Eco*RI and *Xho*I restriction sites. Screening of the cDNA library was performed in the yeast strain PJ69-4a (James *et al.*, 1996).

Affinity purification

An ImmunoPure IgG Orientation Kit (Pierce) was used to covalently bind anti-RGP1 antibodies to protein A-coated beads. Superose 12 fractions (600 μl) were incubated overnight at 4°C with 100 μl beads in buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40) containing Complete^{tm} proteinase inhibitor cocktail (Boehringer) and 0.8 mM PMSF. Beads were consecutively washed six times with 1 ml and two times with 0.2 ml of buffer. Bound proteins were eluted with 200 μl 0.1M glycine, pH 2.8.

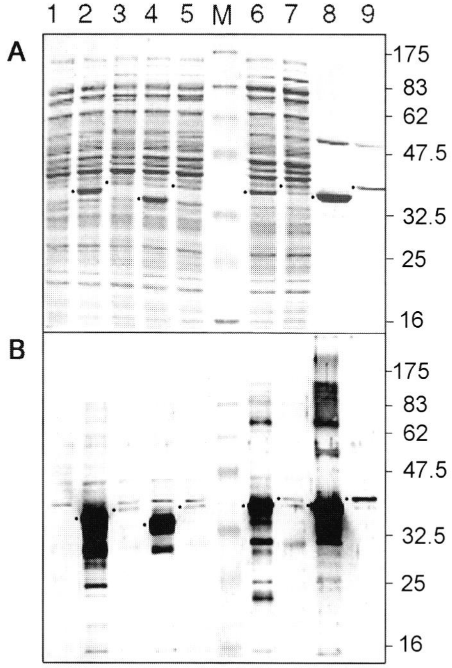

Figure 1. RGP1 and RGP2 produced in *E.coli*. Soluble proteins were isolated from *E.coli* expressing RGP1 or RGP2 from wheat or rice and separated by SDS-PAGE. Proteins were stained with Coomassie Brillant Blue (A) or blotted onto nitrocellulose and analysed for the presence of recombinant protein with anti-RGP2 antiserum (B). The following protein preparations were used: control extract containing plasmid pET29b (lane 1), extracts expressing wheat RGP2 (lane 2), wheat RGP1 (lane 3), rice RGP2 (lane 4), rice RGP1 (lane 5), wheat His-RGP2 (lane 6), wheat His-RGP1 (lane 7), purified wheat His-RGP2 (lanes 8) and purified wheat His-RGP1 (lanes 9). Positions and sizes in kD of prestained molecular weight marker proteins (Biolabs; lane M) are indicated. Dots at the left side of the lanes indicate the RGPs.

Results

Glucosylation activity

RGPs from several plant species have been shown to react with UDP-sugars in an autocatalytic manner (Delgado *et al.*, 1998; Dhugga *et al.*, 1997). To test whether RGP1 and RGP2 from wheat and rice are autocatalytic self-glucosylating proteins, these proteins were separately produced in *E.coli* using the pET expression system. After induction, crude extracts were analysed by SDS-PAGE (Figure 1A) for the presence of the cloned gene products. The soluble extracts from *E.coli* expressing *Rgp1* contain proteins of the correct size, but these were present in very small amounts (Figure 1A, lanes 3 and 5). An appropriate band was visible in gels loaded with the soluble protein extracts containing RGP2 from wheat or rice (Figure 1A, lanes 2 and 4, respectively). A large amount of RGP2 was present in the insoluble fractions, whereas RGP1 was not detected in the insoluble fractions (results not shown).

Additionally, the extracts were analysed by Western blotting using anti-RGP2 antiserum (Figure 1B). A cross-reactive polypeptide was detected in all extracts, including the control extract not expressing *Rgp1* or *Rgp2*, indicating that the antiserum cross-reacted with an *E.coli* protein. However, the overexpressed RGP2 proteins were clearly detected at the correct mass value. Only faint bands were visible in the lanes containing RGP1, even after overnight incubation of the blot with the antiserum and a prolonged staining reaction, most probably as a result of the weak reaction of the antiserum with RGP1.

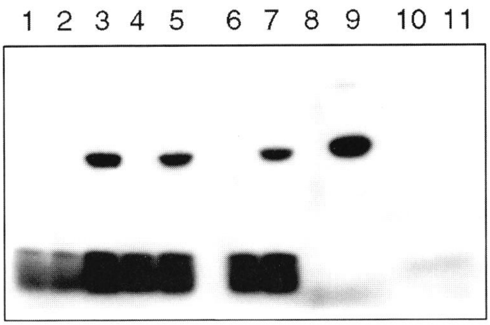

Figure 2. Glucosylation of RGP1 and RGP2 produced in *E.coli*. For testing of glucosylation activity, soluble proteins were incubated with UDP-[^{14}C]glucose (lanes 1-9) or ADP-[^{14}C]glucose (lanes 10-11) prior to SDS-PAGE and fluorography. The same protein preparations were used as shown in Figure 1. For lanes 10 and 11, purified wheat His-RGP2 and purified wheat His-RGP1 were used, respectively.

Subsequently, the extracts were tested for glucosylation activity with UDP-[^{14}C]glucose (Figure 2). Control extract contained radiolabelled material, but this was running with the bromophenol blue tracking dye and is smaller than the 16.5 kD polypeptide of the molecular weight marker (Figure 2, lane 1). The extracts containing wheat or rice RGP1 clearly showed a radiolabelled polypeptide of the correct size (Figure 2, lanes 3 and 5, respectively). In contrast, the extracts containing wheat or rice RGP2 did not show a polypeptide that was labelled by UDP-

[^{14}C]glucose (Figure 2, lanes 2 and 4), although RGP2 was present in considerably larger amounts than was RGP1.

To test whether RGP1 is an autocatalytic protein that is not using components from *E.coli* and that glucosylation of RGP2 was not inhibited by *E.coli* components, wheat *Rgp1* and *Rgp2* were expressed as His-tag fusions and purified on His-Bind^{tm} resin. The extracts and purified proteins were analysed by SDS-PAGE and Western blotting (Figure 1A and B). Both His-RGP1 and His-RGP2 were present in the soluble extracts. Purification on His-Bind^{tm} resin resulted in almost pure preparations. One additional polypeptide of about 55 kD was detected in both preparations. This polypeptide was not purified from control extract (results not shown). Probably, it interacted with RGP1 and RGP2 and not with the His-Bind^{tm} resin. These extracts and purified proteins were used for glucosylation assays. Both the crude extract containing His-RGP1 and the purified His-RGP1 preparation showed self-glucosylation (Figure 2, lanes 7 and 9). In contrast, glucosylation was not observed with His-RGP2 (Figure 2, lanes 6 and 8).

Since it is possible that RGP2 uses a substrate other than UDP-glucose, glucosylation assays were performed with ADP-[^{14}C]glucose, the substrate for starch synthases. Crude *E.coli* control extract incorporated all ADP-[^{14}C]glucose in high molecular weight material that does not enter the running gel (results not shown). Therefore, purified His-RGP2 and His-RGP1 were used in this assay. Neither protein incorporated ADP-[^{14}C]glc (Figure 2, lanes 10 and 11).

To test the possibilities that RGP2 produced in *E.coli* is either not folded correctly, needs to be incorporated in membrane or should be post-transcriptionally modified for its activity, yeast was used to generate both proteins. Both RGP1 and RGP2 were produced, but only preparations containing RGP1 incorporated UDP-[^{14}C]glucose (results not shown). This suggests that RGP2 does not have glucosylating activity or needs to be expressed in plant cells for its activity. Therefore, we extended the study of RGP activity to plants.

Total protein extract from wheat endosperm did not incorporate radiolabelled UDP-glucose (Figure 4). Because an inhibitor might interfere with RGP activity, total protein extract from wheat endosperm was partly purified on a Superose 12 gel filtration column and eluted fractions were analysed by Western blotting (Figure 3). RGP1 appeared to be present in all fractions examined, but more abundantly in the high molecular weight fractions (Figure 3, upper panel). RGP2 was present as a monomer as well as part of a larger complex (Figure 3, lower panel). Several fractions of this wheat endosperm extract were tested for glucosylation activity with UDP-[^{14}C]glucose, and they all show a radioactive polypeptide of the correct size (Figure 4). Chase experiments were performed in which unlabeled UDP-glucose (Figure 5, lane 2), UDP-xylose (Figure 5, lane 3), UDP-galactose (Figure 5, lane 4) or ADP-glucose (Figure 5, lane 5) was added to the radioactive labelled proteins. The label was chased by each UDP-sugar, but not by ADP-glucose.

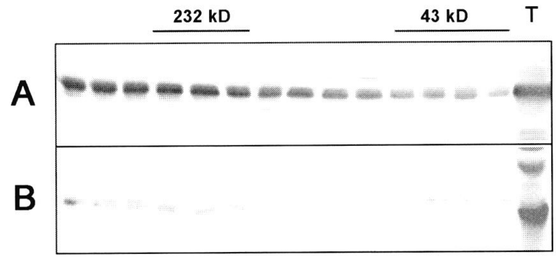

Figure 3. Western blot analysis of wheat endosperm fractions obtained by Superose 12 gel filtration. Fractions 2-15 and total protein (T) were used. (A) anti-RGP1 antibodies. (B) anti-RGP2 antibodies. Estimated size of HMW and LMW fractions is indicated.

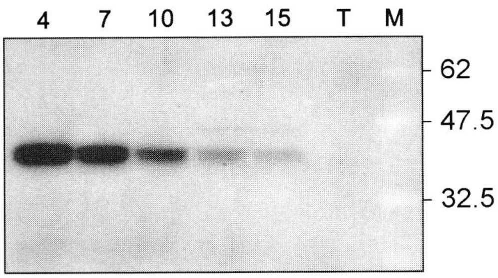

Figure 4. Glucosylation of wheat endosperm fractions 4, 7, 10, 13 and 15 obtained by Superose 12 gel filtration. T, total protein extract. Positions and sizes in kD of prestained molecular weight marker proteins (Biolabs; lane M) are indicated.

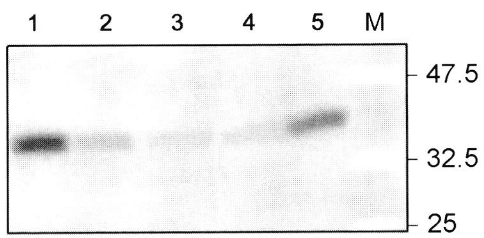

Figure 5. Chase of glucosylated high molecular weight fractions of wheat endosperm (lane 1) with unlabeled UDP-glucose (lane 2), UDP-xylose (lane 3), UDP-galactose (lane 4) and ADP-glucose (lane 5). Positions and sizes in kD of prestained molecular weight marker proteins (Biolabs; lane M) are indicated.

Since both RGP1 and RGP2 are present in wheat endosperm and they could not be distinguished by their molecular masses, their separate activities could not be identified. Because in tobacco leaf endogenous RGP2 is not detected (not shown), tobacco was transformed with either wheat *Rgp1* or *Rgp2*. Leaf tissue extracts from wildtype SR1, RGP1- and RGP2-transformed tobacco plants were partly purified on a Superose 12 gel filtration column and the eluted fractions were analysed by Western blotting (Figure 6). In tobacco wildtype SR1 plants, RGP1 and RGP2 were not detected (Figure 6B and 6D). In tobacco plants overexpressing *Rgp1* or *Rgp2*, the respective proteins were present in low as well as in high molecular weight fractions (Figure 6A and 6C). Several fractions containing the RGP2 proteins were subjected to glucosylation tests using UDP-[^{14}C]glucose and compared to similar fractions from SR1 plants. The low molecular weight fractions 13 and 15 of RGP2 plants hardly show glucosylation, suggesting that RGP2 monomers are inactive. In contrast, high molecular weight fractions of RGP2 plants did show glucosylation, whereas only light-radioactive bands were detected in fractions 4 and 7 of the SR1 plants (Figure 7). These results show that RGP2-containing complexes are active.

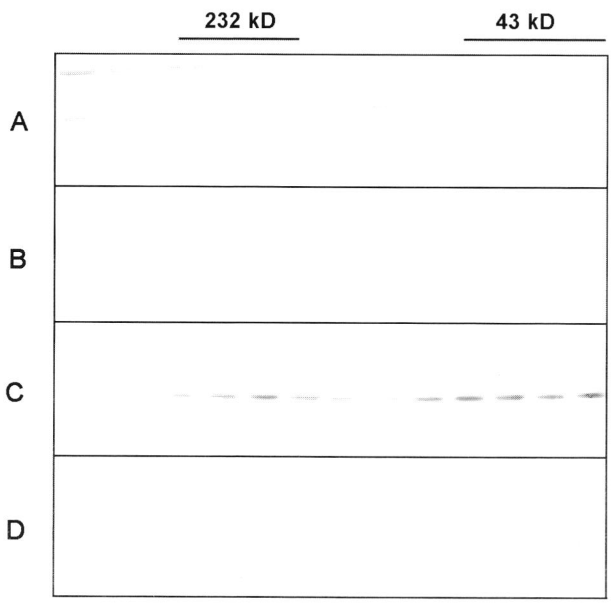

Figure 6. Western blot analysis of tobacco leaf fractions 2-15 obtained by Superose 12 gel filtration. Extracts of tobacco plants overexpressing *Rgp1* (panel A; anti-RGP1 antibodies) or *Rgp2* (panel C; anti-RGP2 antibodies) were compared with extract from wildtype SR1 plants, which were challenged with anti-RGP1 antibodies (panel B) and anti-RGP2 antibodies (panel D).

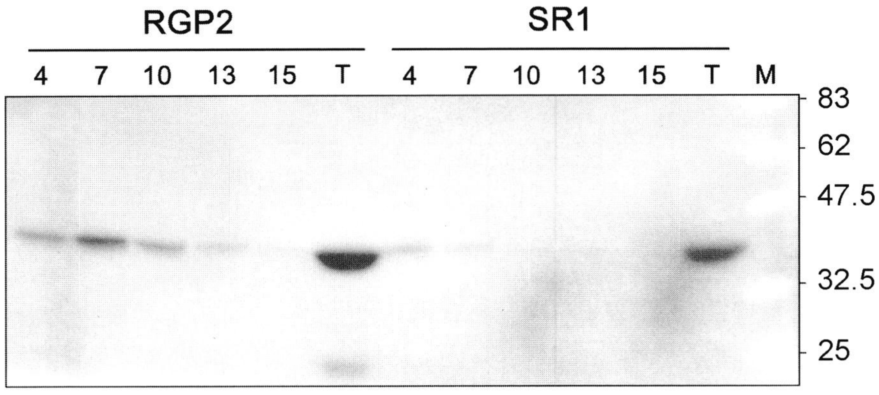

Figure 7. Glucosylation of Superose 12 fractions 4, 7, 10, 13 and 15 and total protein (T) by leaf extracts from tobacco plants overexpressing *Rgp2* and from wildtype SR1 tobacco plants.

Complex formation

Since gel filtration experiments with wheat endosperm as well as tobacco leaf extracts suggested the presence of RGP1 and RGP2 in high molecular weight complexes, our research was extended to complex formation of RGPs and the analysis of these complexes *in vivo*. The ability of RGP1 and RGP2 to form dimers was analysed using a yeast two-hybrid system. Specific complex formation of RGPs was studied by expression of RGP1 and RGP2 fusion proteins with the GAL4 DNA binding domain (BD) or activation domain (AD) in yeast. Table 2 shows that RGP1 as well as RGP2 are able to form homo-dimers. Moreover, when RGP1 was used as bait, RGP2-AD was able to activate yeast growth, indicating that RGP1 and RGP2 can form hetero-dimers.

To reveal the putative presence of other proteins in RGP complexes, a wheat endosperm cDNA library was screened using RGP2 as bait in the yeast two-hybrid system. Of the 100,000 colonies tested, 117 were found to be positive when tested for the three different reporter genes. Of the 25 clones which expression was dependent on RGP2, nine were identified as RGP1 and three as RGP2. Three clones were identified as prohibitin. Prohibitin has been described to be a part of a complex present in mitochondria (Nijtmans *et al.*, 2000). Since RGP2 and prohibitin are localised in different cellular compartments, prohibitin was discarded as a possible candidate for the RGP2 complex. Eight clones contained 3' non-coding regions, one was possibly contaminated with a vector and all were considered to be artefacts.

binding domain	activation domain	growth
RGP1	-	-
-	RGP1	-
RGP1	RGP1	+
RGP2	-	-
-	RGP2	-
RGP2	RGP2	+
RGP1	RGP2	+
RGP2	RGP1	+

Table 2. Yeast two-hybrid analysis of RGP1 and RGP2 proteins. Binding domain of pAS2-1 and activation domain of pACTII were each fused to the coding regions of *Rgp1* and *Rgp2*. Growth was tested in yeast strain CG1945 on plates lacking Trp, Leu and His.

The native wheat complex was analysed using an antibody affinity column. Polyclonal RGP1 antibodies were covalently linked to immobilised protein A. The column material was incubated with different Superose 12 endosperm fractions. After several wash steps, the bound proteins were eluted and non-bound, wash and elution fractions were analysed by SDS-PAGE and Western blotting (Figure 8). The latter technique showed that mainly RGP1 (Figure 8B), but also RGP2 proteins (Figure 8C) were detected in the eluted fractions. The silver-stained gels showed bands of 38-40 kD, 47 kD and 64 kD in the eluted fractions. The 47 kD band binds to the RGP1 antibodies and is therefore not considered to be part of the complex. Since the 64 kD band is present in all lanes it was considered to be an artefact. The 38-40 kD doublet corresponds to RGP1 and RGP2. Taken together, these results suggest that RGP complexes in wheat endosperm do not seem to contain other proteins besides RGP1 and/or RGP2. Since RGP1 and RGP2 are able to form hetero-dimers in yeast, both proteins may be present in the same complex.

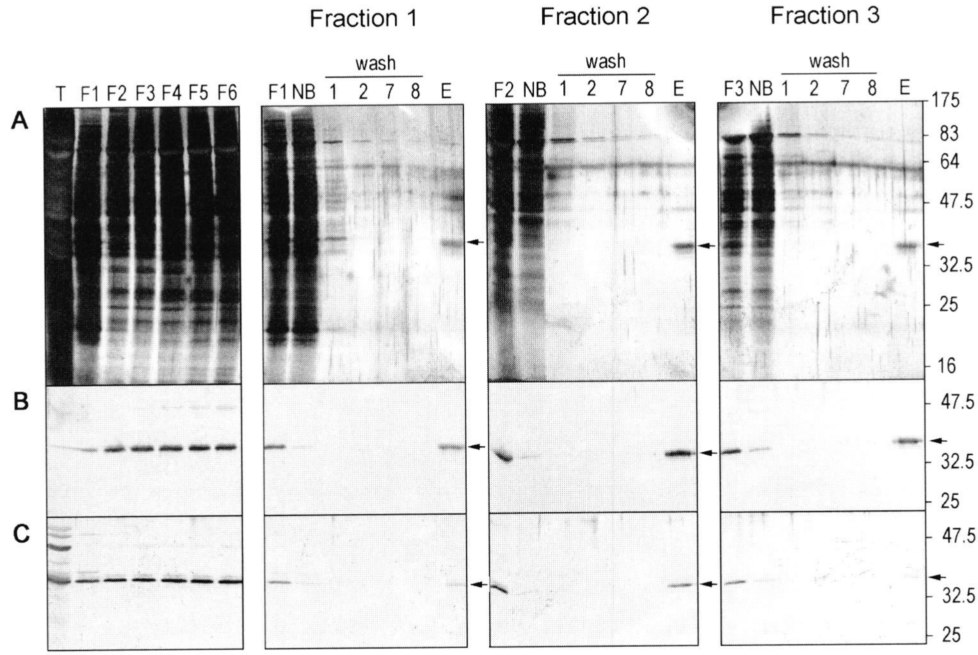

Figure 8. Affinity purification of the wheat RGP1 complex using RGP1 antibodies covalently linked to protein A-coated beads. Total wheat endosperm protein (T) and Superose 12 fractions 1-6 (F1-F6) were analysed by silver staining (A) or blotted onto nitrocellulose and analysed for the presence of recombinant protein with anti-RGP1 antiserum (B) or anti-RGP2 antiserum (C) (left panel). Superose 12 fractions 1-3 were subjected to several purification steps (washes 1-8; elution), analysed by silver staining (A) and Western blotting, challenged with anti-RGP1 antibodies (panel B) and anti-RGP2 antibodies (panel C). NB, non-bound protein; E, eluted proteins. Positions and sizes in kD of prestained molecular weight marker proteins (Biolabs) are indicated. Eluted RGPs are indicated with arrows.

Discussion

To test whether RGP1 and RGP2 have glucosylation activity, both genes were expressed in *E.coli* and extracts as well as isolated protein were subjected to glucosylation tests. Only RGP1 incorporated radio-labelled UDP-glucose, whereas we could not detect covalent linkage of RGP2 with UDP-[^{14}C]glucose or with ADP-[^{14}C]glucose. Superose 12 gel filtration experiments revealed the presence of RGP1 as well as RGP2 in high molecular weight complexes in wheat endosperm. The Superose 12 fractions could be glucosylated by UDP-[^{14}C]glucose, after which the label could be chased with unlabeled UDP-glucose, UDP-xylose or UDP-galactose, but not with ADP-glucose. Interestingly, in contrast to the Superose 12 fractions, total protein extracts did not show glucosylation, suggesting the presence of an inhibitor protein in wheat endosperm, as previously described by Rothschild *et al.* (1996) and Wald *et al.* (1998).

The glucosylation activity detected in the HMW Superose 12 fractions is probably the result of the presence of a mixture of RGP1 and RGP2 since both proteins are present in these fractions. In order to determine putative activity of RGP2 in plants, transgenic tobacco plants were generated overexpressing *Rgp1* or *Rgp2*. Neither of the antibodies detected endogenous RGP proteins in the Superose 12 fractions of untransformed tobacco plants, whereas in the RGP1-transformed plants as well as in the RGP2-transformed plants the respective protein was detected in high as well as in low molecular weight fractions. This shows that also in transgenic tobacco plants, RGP1 and RGP2 are present in HMW complexes. In subsequent glucosylation assays with HMW fractions from tobacco plants overexpressing *Rgp2*, a polypeptide of the expected size was glucosylated by UDP-[^{14}C]glucose, suggesting that RGP2 only seems to be active when it is present in a complex. Alternatively, the presence of RGP2 may stimulate the endogenous RGP1 activity, since low RGP1 activity is present in the wildtype SR1 background.

Screening of a 10-dpa wheat cDNA library using RGP2 as a bait in the yeast two-hybrid system resulted in the identification of only RGP1 and RGP2 clones as possible RGP partners. Purification of the wheat endosperm RGP complex using an antibody affinity column and subsequent analysis of the proteins on a silver-stained gel revealed bands of 38-40 kD, 47 kD and 64 kD with the 38-40 kD doublet corresponding to the RGP proteins. Although the detection of RGP2 proteins in the eluted fractions may reflect the weak binding of RGP2 to the beads coated with RGP1 antibodies, it is conceivable that RGP1 and RGP2 are present in the same complexes. Since the subcellular localisation experiments showed that RGP1 and RGP2 are present in exactly the same organelles (chapter 4, this Thesis), it is well possible that RGP hetero-multimers exist *in vivo*. We estimate the RGP1 complexes as well as the RGP2 complexes to consist of about six subunits. This is consistent with the findings of Bocca *et al*, (1997), who predicted the RGP1-complex in potato to be a pentamer or a hexamer.

Interestingly, affinity purification of the complex nor yeast two-hybrid analysis did reveal the presence of a 80 kD sucrose synthase-like inhibitor protein as described by Rothschild *et al.* (1996) and Wald *et al.* (1998). The former authors reported separation of the inhibiting activity from glucosylating activity by FPLC-Mono Q column chromatography (0.18 M KCl). If a physical interaction between sucrose synthase and RGP exists, this interaction is considered to be weak. Alternatively, it is possible that not the sucrose synthase itself but the produced unlabelled UDP-glucose is the cause of the "inhibitory effect" observed in maize endosperm.

In conclusion, results obtained with a yeast two-hybrid system, as well as affinity purification of the RGP complexes suggest that RGP1 and RGP2 can form homo- and hetero-dimers.

Acknowledgements

We thank Dr WJ Whelan (Miami) and his lab members for their hospitality and advice on the activity tests.

Chapter 6

Analysis of potato tubers overexpressing two classes of reversibly glycosylated polypeptides

Sandra MJ Langeveld, Luc CJM Suurs, Gwenaël MDJ-M Gaussand, Marco Vennik, Jan W Kijne, Richard GF Visser and Sylvia de Pater

Abstract

To study the function of reversibly glycosylated polypeptides (RGPs) in plant polysaccharide biosynthesis, transgenic potato lines were produced overexpressing wheat *Rgp1* or *Rgp2.* Tubers containing wheat RGP1 showed a significant decrease in starch granule size for both harvests examined. In the smallest tubers of the first harvest, this effect was accompanied by appearance of aberrantly shaped starch granules, decreased amounts of endogenous RGP2 and patatin, and increased amounts of free glucose and fructose. Transgenic lines containing relatively more RGP2 than RGP1 did not show an effect on starch granule size or morphology. The observed phenotype suggests a competition of RGP1 and RGP2 for UDP-glucose. The RGP1/RGP2 ratio may influence the carbon flux into cell wall polysaccharides and starch synthesis, respectively.

Introduction

Reversibly glycosylated polypeptides (RGPs) have been implicated in polysaccharide synthesis. Because of their binding affinity for UDP-sugars and their Golgi-specific localisation, it was assumed that RGPs are involved in plant cell wall synthesis (Dhugga *et al.*, 1997). Claims for the involvement of a protein designated UDP-glucose:protein transglucosylase (UPTG) in the priming of starch synthesis seemed inaccurate when was shown that UPTG and RGP were biochemically similar and structurally homologous proteins (Bocca *et al.*, 1999). Besides their localisation and affinity for UDP-sugars, indications for the function of RGPs are lacking.

Previously it has been found that two classes of RGPs can be distinguished, designated RGP1 and RGP2 (Chapter 3, this Thesis). In wheat endosperm, both types of RGPs are present in the cytoplasm as well as bound to membranes (Chapter 4, this Thesis). Expression of both RGPs in *E.coli* and subsequent activity tests showed that RGP1 was actively binding UDP-glucose, in contrast to RGP2. However, gel filtration experiments with extracts from tobacco plants overexpressing *Rgp1* and *Rgp2* indicated that RGP2-containing complexes were able to incorporate UDP-glucose (Chapter 5, this Thesis).

Localisation experiments with wheat showed that RGP1 is present in source as well as in sink tissue, whereas RGP2 was not found in leaves (Chapter 3, this Thesis). This localisation pattern suggested that RGP1 and RGP2 have different functions possibly related to carbon partitioning. In maize endosperm, a similar phenomenon has been found for the sucrose synthase isozymes SS1 and SS2. SS1 encoded by *Shrunken1* was shown to be critical for cell wall integrity, whereas SS2 encoded by *Sucrose synthase1* was found to generate precursors for starch synthesis (Chourey *et al.*, 1998). Moreover, UPTG activity was shown to be inhibited by SS1 in wildtype maize endosperm, whereas in the mutant lacking SS1 UPTG activity was not inhibited (Rothschild *et al.*, 1996; Wald *et al.*, 1998).

Mutants deficient in *Rgp* or transgenic plants containing *Rgp* constructs in either sense or antisense orientation have not yet been reported. In our earlier studies, RGP-containing tobacco plants did not show a phenotype possibly caused by the lack of a starch-storage organ. Although transformation and regeneration procedures for wheat are available (e.g., Becker *et al.*, 1994), the procedures involved are labour-intensive and inefficient. Since transformation and regeneration of potato by *Agrobacterium tumefaciens* are relatively simple procedures (Visser, 1991), potato was chosen as a model plant. This study describes the results of the transformation of potato and the subsequent analysis of tubers overexpressing wheat *Rgp1* and *Rgp2*.

Material and methods

Plant material

Potato plants (*Solanum tuberosum* L., cv. Karnico) were grown in the greenhouse with temperatures of 20°C (day) and 18°C (night), 80% humidity and a 16h photoperiod. Primary transformants were allowed to form tubers during the late summer of 1999 and were harvested the beginning of November. A second batch of transgenic potato tubers was formed from plants sprouted from several large transgenic tubers from the first harvest. This batch was harvested at the end of May, when most tubers were 1.5 cm in diameter. Production of *in vitro* tubers was performed as described by Hovenkamp-Hermelink *et al.* (1988).

Transformation procedure

Potato plants were transformed according to Visser (1991), with 35S-*Rgp1* and 35S-*Rgp2* constructs described in Chapter 5 of this Thesis.

Protein analysis

Transformed potato tubers were analysed for RGP1 and RGP2 content essentially as described in Chapter 3 (this Thesis), except that gels were loaded based on protein concentration. Per lane, 5 μg protein were analysed. Relative intensities were determined using Image J (National Institutes of Health, USA).

Sugar analysis

Ten mg freeze-dried tissue of each potato tuber tested were boiled in 1 ml 80% methanol for 15 min at 75°C. After removal of the methanol by vacuum centrifugation, the samples were resuspended in 1 ml water and shaken on ice during 1h. The extracts were centrifuged over a 0.22 μm filter (15,000g, 5 min) to remove undissolved residue, and subsequently the supernatants were analysed using a Dionex HPLC system equipped with a 4 x 250 mm CarboPac PA1 column. Eluted sugars were identified and quantified by comparison with metabolite standards.

Starch isolation

Starch from greenhouse-grown tubers was isolated according to Visser *et al.* (1997). Pieces of microtubers frozen in liquid nitrogen were treated overnight with 1% Rapidase (Gist-Brocades) in 0.2 M NaAc/Na_2SO_3, pH 5.5, at 30°C. Starch was pelleted by centrifugation at high speed for 2 min and cell wall remnants were removed using tweezers. Starch was washed three times with 30 mM phosphate buffer, pH 7.0, to remove patatin and other proteins. After two successive washes with water and acetone, respectively, the starch was dried at 30°C.

Starch granule size distribution

Size distribution of >50,000 starch granules per sample was determined by Coulter Counter analysis, as described in Visser *et al.* (1997).

Statistical analysis

Independent *t*-tests were performed to test whether obtained results were significantly different.

Results

Potato was transformed with the wheat 35S-*Rgp* constructs described in Chapter 5 (this Thesis). The smallest tubers of each line with diameters ranging from one to three centimetres were tested for the presence of RGP proteins by Western blotting (Figure 1). Analysis of the wildtype tuber shows that the antibodies generated against the wheat RGP1 and RGP2 recognise the endogenous potato RGP1 and RGP2, respectively. Intensities of the bands were determined and the ratio RGP1/RGP2 was calculated (Table 1). Two different tubers of line RGP1-17 were assayed which differed in size and were designated RGP1-17a (1 cm in diameter) and RGP1-17b (3 cm in diameter). Because the tubers of the different lines differed considerably in specific gravity, the gels were loaded based on protein concentration rather than fresh weight. The amounts of protein loaded were visualised by CBB staining (Figure 1C). Figure 1A and Table 1 show that the tubers from lines RGP1-4, RGP1-10 and both tubers from line RGP1-17 contain relatively more RGP1 protein than does the wildtype, whereas line RGP1-3 shows an amount of RGP1 equal to that of the wildtype. The amount of RGP2 in RGP2-7 is comparable to the amount of endogenous RGP2 in the wildtype, although a third, smaller band is present representing the wheat RGP2. RGP2-24 contains an increased amount of RGP2 protein, whereas lines 5 and 26 show a decreased level of RGP2 protein compared to the wildtype (Figure 1B). PCR analysis showed that RGP2-26 contains the transgene (not shown), whereas RGP2 protein was not detected. This absence is possibly caused by co-suppression of the endogenous gene. Interestingly, all lines overexpressing *Rgp1* show a reduced amount of endogenous RGP2 protein. Moreover, the amount of the major storage protein patatin (Figure 1C, arrow) seems to be correlated with the RGP1/RGP2 ratio. In all lines exhibiting an RGP1/RGP2 ratio of 2.8 or higher the amount of patatin is low (Table 1; Figure 1C).

The same tubers were used to study other effects of transgenesis. It was noticed that the analysed tubers from the lines RGP1-17 and RGP2-26 had developed hollow heart disorder, whereas RGP1-10 and a second tuber from line RGP1-17 had a waxy appearance. Starch from the lines RGP1-4 and RGP1-17

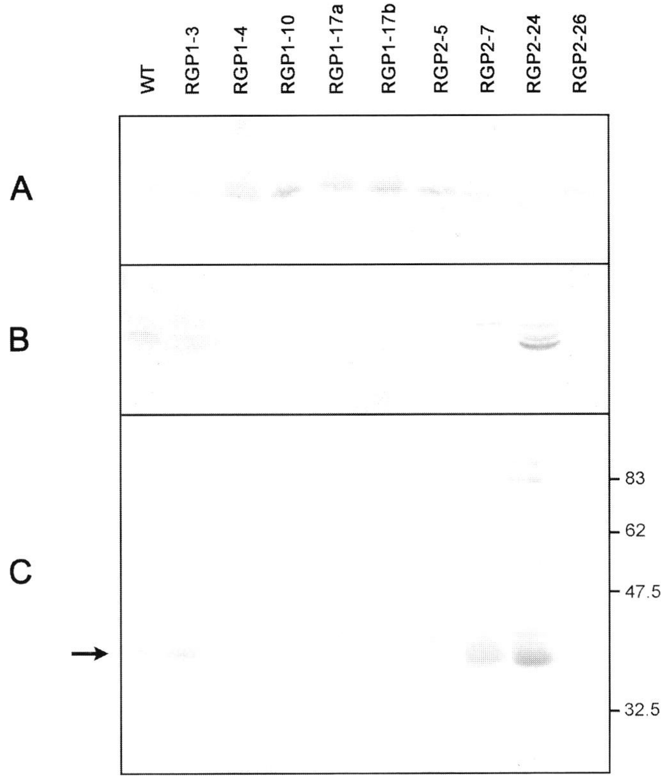

Figure 1. Protein analysis of transgenic potato tubers. Proteins were isolated from potato tubers expressing wheat *Rgp1* or *Rgp2* and separated by SDS-PAGE. Proteins were blotted onto nitrocellulose and analysed for the presence of recombinant protein with anti-RGP1 antiserum (A), anti-RGP2 antiserum (B) or stained with Coomassie Brillant Blue (C). RGP1-17a and RGP1-17b are two different potato tubers from the same line. Positions and sizes in kD of prestained molecular weight marker proteins (Biolabs; lane M) are indicated. WT, wildtype.

	int. RGP1	int. RGP2	RGP1/RGP2	int. patatin
Wildtype	100	100	1.0	100
RGP1-3	97	56	1.7	137
RGP1-4	211	30	7.0	50
RGP1-10	245	27	9.1	38
RGP1-17a	289	45	6.4	63
RGP1-17b	195	18	10.8	25
RGP2-5	117	42	2.8	39
RGP2-7	85	101	0.8	135
RGP2-24	126	372	0.3	354
RGP2-26	101	22	4.6	41

Table 1. Relative intensities of RGP1, RGP2 and patatin bands from Figure 1 and the ratio RGP1/RGP2. The wildtype values for RGP1, RGP2 and patatin were set at 100%.

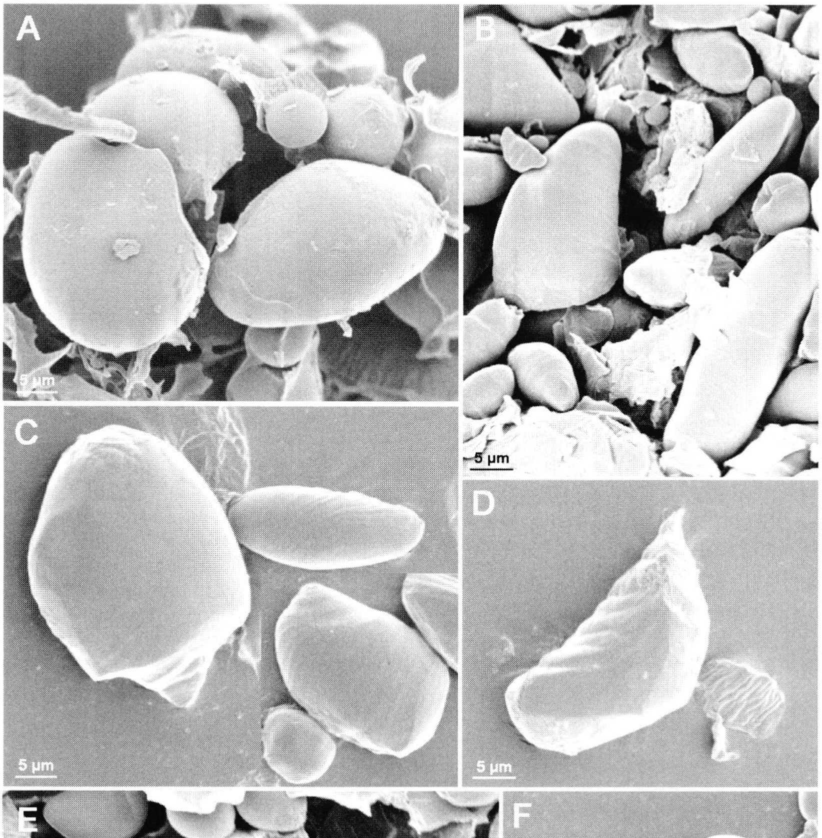

Figure 2. Scanning Electron Microscope analysis of starches from small tubers expressing wheat *Rgp1* or *Rgp2*. **A.** RGP2-24. **B.** RGP1-4. **C-D.** RGP1-17. **E-G.** RGP2-26. Bars represent 5 µm.

(Figure 2B-D), contained aberrantly shaped starch granules, ranging from rod-shape, to hooked conical structures. The phenotype of line RGP1-10 resembled that of line RGP1-17 (not shown). The lines RGP2-5, RGP2-7 and RGP2-24 show a phenotype comparable to that of the wildtype (not shown), whereas in line RGP2-26 besides shell-shaped structures, protrusion-like starch granules were found (Figure 2E-F). The frequencies of aberrantly shaped starch granules were determined by counting 100 starch granules per line in duplo (Figure 3).

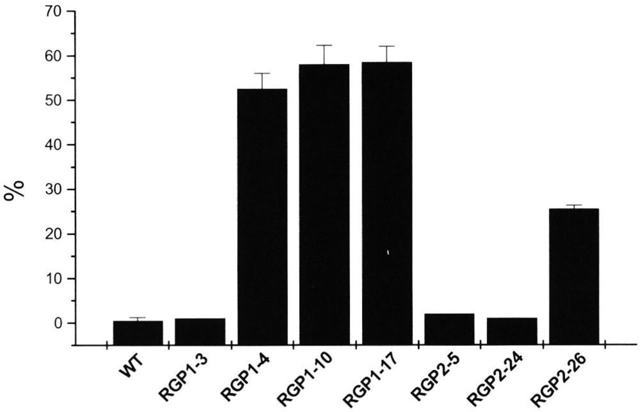

Figure 3. Percentage of aberrantly shaped starch granules in small tubers expressing wheat *Rgp1* or *Rgp2*.

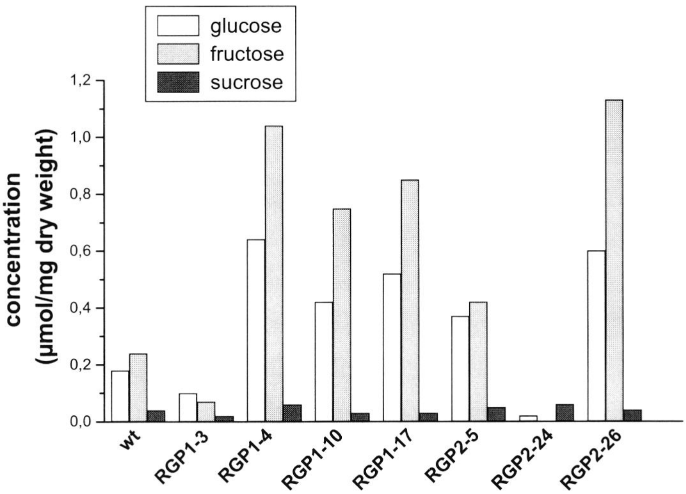

Figure 4. Sugar analysis of potato tubers expressing wheat *Rgp1* or *Rgp2*. Glucose, fructose and sucrose concentrations were measured in methanol extracts prepared from wildtype (WT) and transgenic tubers of the first harvest. Values represent single tuber measurements.

Extracts from the same tubers were used to determine free sucrose, glucose and fructose concentrations (Figure 4). The lines RGP1-4, RGP1-10 and RGP1-17, overexpressing wheat *Rgp1*, accumulated increased levels of glucose and fructose per mg dry weight. Moreover, line RGP2-26, in which RGP2 was not detected, showed the same accumulation of hexoses, whereas in line RGP2-24, which contained a high amount of RGP2, glucose and fructose were hardly detectable. This suggests that a high ratio of RGP1 and RGP2 is correlated with a high free hexose concentration.

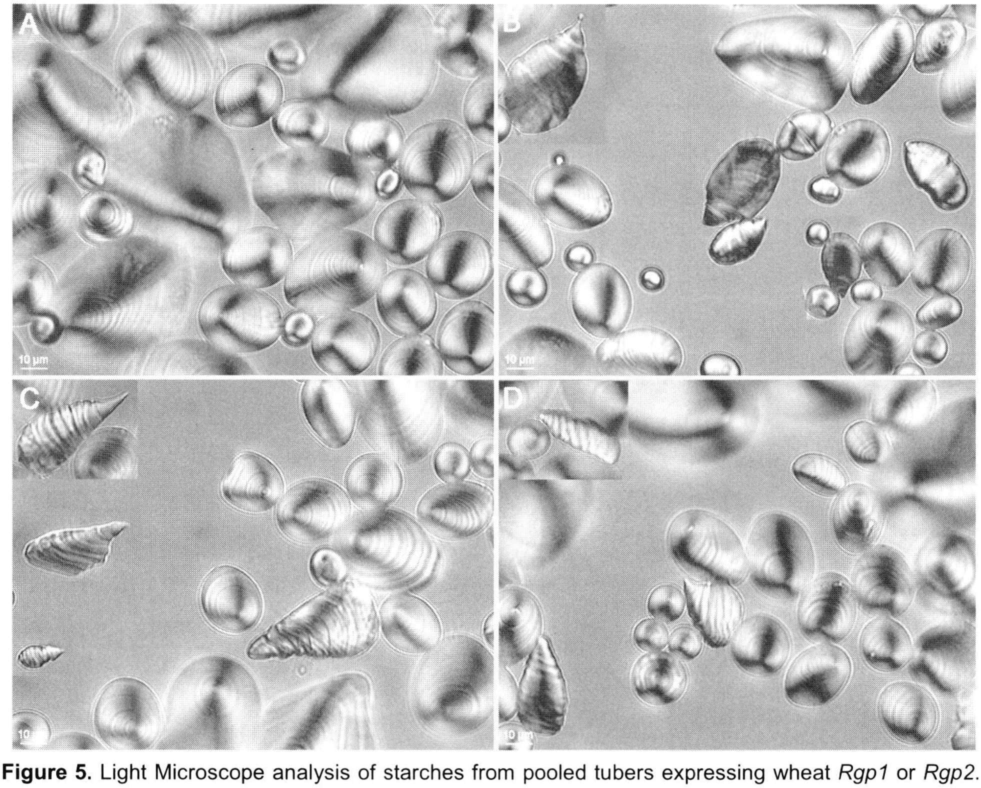

Figure 5. Light Microscope analysis of starches from pooled tubers expressing wheat *Rgp1* or *Rgp2*. **A.** Wildtype. **B.** RGP1-4. **C.** RGP1-17. **D.** RGP2-26.

Other, larger, tubers from the transgenic lines were used for starch isolation. Aberrantly shaped starch granules were also present in larger tubers from RGP1-lines (Figure 5), albeit at a frequency of about 7% (not shown). Additionally, the starch granules in these tubers appeared smaller (Figure 5). Coulter Counter analysis was performed to study the size distribution of the isolated starches. All lines showed a shift towards smaller starch granules. The lines RGP1-3, RGP1-4, RGP1-10, RGP1-17 and RGP2-7 showed the largest shift towards smaller starch granules compared to the wildtype (Figure 6). For the lines RGP2-24 and RGP2-26 this shift was less distinct. Gelatinisation temperatures of the different starches were assayed in triplo by Differential Scanning Calorimetry (DSC). DSC values differed per line, but were not related to the presence of RGPs. Starch content, amylose content, opacity and chain length distribution of the different starches showed no differences (not shown).

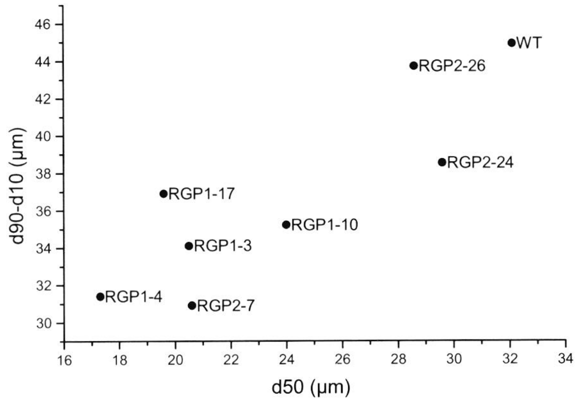

Figure 6. Size distribution of starch granules isolated from wildtype and transgenic RGP potato tubers plotted as d50 (median) against d90-d10 (d90: 90% of the starch granules is smaller than the indicated size; d10: 10% of the starch granules is smaller than the indicated size).

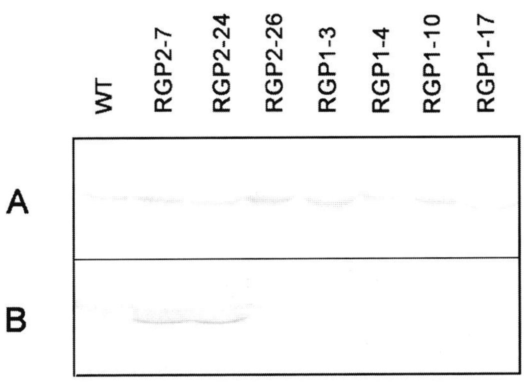

Figure 7. Protein analysis of the second batch of transgenic potato tubers. Proteins were isolated from potato tubers expressing wheat *Rgp1* or *Rgp2* and separated by SDS-PAGE. Proteins were blotted onto nitrocellulose and analysed for the presence of recombinant protein with anti-RGP1 antiserum (A), and anti-RGP2 antiserum (B).

Since the aberrantly shaped starch granules were primarily observed in small tubers, we checked whether the observed phenotype was related to the developmental stage of the tubers. Therefore, transformed tubers were planted under greenhouse conditions and allowed to form tubers, which were harvested when they were about 1.5 cm in diameter. Of each transgenic line one tuber was tested for starch granule shape and for the presence of RGP proteins by Western blotting (Figure 7). In none of these tubers aberrantly shaped starch granules were observed (not shown). No differences were detected in the amount of RGP1 between the different lines (Figure 7A), whereas only RGP2-7 and RGP2-24 accumulated RGP2. Line RGP2-5 was not assayed. The amount of RGPs did not seem to be correlated with the presence of patatin. These results show that aberrantly shaped starch granules are not always found in small tubers.

	SuSy activity nmol.mg $DW^{-1}.min^{-1}$ (SD)
wildtype	85 (22)
RGP1-10	32 (20)
RGP1-17	52 (40)
RGP2-24	32 (38)
RGP2-26	80 (35)

Table 2. Sucrose synthase activity in microtubers expressing wheat *Rgp1* or *Rgp2*. SD values indicate standard deviations of 3-4 microtubers.

In order to synchronise tuber development, the transgenic lines were grown *in vitro* to produce microtubers. Three to four microtubers per transgenic line were analysed for starch granule shape, fresh weight, sugar content and sucrose synthase activity. Starch granules from all transgenic lines appeared similar to wildtype starch granules (not shown). Although all plants were transferred to induction medium at the same time, the fresh weights of the tubers of lines RGP2-7 and RGP2-24 were reduced compared to the other lines (not shown). Glucose, fructose and sucrose levels as well as sucrose synthase activity were determined for all tubers. No significant differences in sugar content (not shown) were found. The variation in sucrose synthase activity (Table 2) within the transgenic lines was considerable, resulting in large standard deviations. Although two out of four lines showed a decreased SuSy activity, this is not convincingly related to the presence of RGP. From all lines, starch was isolated and the size distribution was determined by Coulter Counter analysis. The median diameters (d50) of granules from the RGP1 lines 10 and 17 and RGP2 lines 7 and 24 were smaller compared to the median diameter of those of the wildtype (Figure 8). However, the size distribution of line RGP2-26 was similar to the size distribution of the wildtype. Taken together, since in both harvests the transgenic RGP1 lines showed a decrease in starch granule size, these findings indicate that the effect of *Rgp1* overexpression on granule size is consistent.

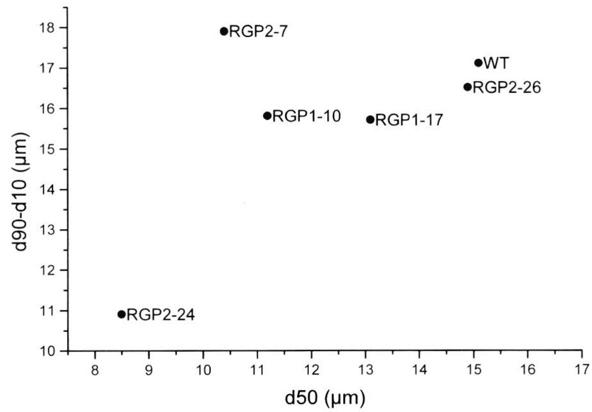

Figure 8. Size distribution of starch granules isolated from wildtype and transgenic RGP potato microtubers plotted as d50 (median) against d90-d10 (d90: 90% of the starch granules is smaller than the indicated size; d10: 10% of the starch granules is smaller than the indicated size).

Discussion

To study the function(s) of two classes of RGPs, several transgenic potato lines were produced overexpressing either a wheat *Rgp1* or *Rgp2* construct. The effect of additional RGP1 or RGP2 was studied for three consecutive harvests, including *in vitro* grown microtubers. The clearest phenotype was observed in the smallest tubers from the primary greenhouse-grown plants in which the RGP1/RGP2 ratio seemed to be the determining factor. All lines with a RGP1/RGP2 ratio of 4.6 or higher showed aberrantly shaped starch granules. This includes line RGP2-26 in which RGP2 was not detected and the amount of RGP1 was not altered, suggesting that a high RGP1/RGP2 ratio results in aberrant starch granule formation. In all lines with a RGP1/RGP2 ratio of 2.8, and higher the amounts of patatin, one of the major soluble proteins in potato tubers, were reduced. Additionally, tubers with a

RGP1/RGP2 ratio of 4.6 and more exhibited high levels of free glucose and fructose, whereas line RGP2-5 with a ratio of 2.8 showed an intermediate phenotype. Although the natural variation in sugar content of wildtype tubers is thought to be large, the correlation found is remarkable.

All lines containing the wheat *Rgp1* construct, including line RGP1-3, produced starch granules that were significantly smaller than the wildtype starch granules. This effect was still present in the *in vitro* grown microtubers. The presence of additional RGP2 did not seem to have an effect on starch granule size. Although in the second batch the starch granules were smaller in lines RGP2-7 and RGP2-24, this was correlated with the size and developmental stage of the microtubers of these lines.

Although the size-effect consisted in the microtubers, aberrant starch granules were neither detected in a consecutive batch of small greenhouse-grown tubers nor in *in vitro* microtubers. As determined by Western blot analysis of the consecutive batch of small greenhouse-grown tubers, the differences in the amount of RGP1 between the different lines were less clear. The lines RGP2-7 and RGP2-24 exhibited elevated levels of RGP2, whereas the amount of RGP2 in the *Rgp1* overexpressing lines as well as in line RGP2-26 was comparable to the amount of RGP2 in the wildtype. These results suggest that the RGP1/RGP2 ratio in this batch stays below a threshold value after which starch granule biosynthesis is impaired. This implies that the imbalance between the two types of RGPs is being repaired after propagation. Thus, the lack of aberrantly shaped starch granules and the not significantly altered hexose levels in microtubers may be the result of an unknown repair mechanism, although the amounts of RGP1 and RGP2 were not determined. Alternatively however, these findings may be explained by the fact that these *in vitro* tubers are produced on medium with a high sucrose concentration.

The aberrantly shaped starch granules are thought to be an indirect effect since RGPs lack targeting information for plastids and have not been found to be present in the amyloplast (Chapter 4, this Thesis). It is conceivable that the RGP1/RGP2 ratio influences the concentration of free carbohydrates since RGPs, most notably RGP1, are able to bind UDP-glucose. Lack of substrate may be the cause of the aberrant shape of the starch granules. Since aberrant starch granules were neither detected in a consecutive batch of small greenhouse-grown tubers nor in *in vitro* microtubers, it is unlikely that this phenomenon is caused by the developmental stage of the potato tubers. The aberrant starch granule shape may be related to hollow heart disorder, which was observed in the smallest tubers of lines RGP1-17 and RGP2-26. Although the causes of this physiological disorder remain elusive, it is often associated with rapid tuber growth preceded by a period of moisture or nutritional stress (Rex and Mazza, 1989). However, under greenhouse conditions the watering of the plants is controlled and therefore hollow heart disorder is rarely encountered. Since the sugar signalling pathway has been shown to interact with the signalling pathway(s) involved in stress responses (reviewed in Roitsch, 1999; Smeekens, 2000), it is possible that the transgenic RGP1 potato tubers are more susceptible to stress. Whether this results in, or is the result of the elevated hexose level remains to be tested.

The *Rgp1* overexpressing lines exhibited a decreased level of endogenous RGP2. This together with the low amount of patatin may be the result from indirect downregulation of these genes by a change in free carbohydrate concentrations. It has been shown that a class I patatin promoter fragment is inducible by high levels of sucrose (Rocha-Sosa *et al.*, 1989) and glucose (Martin *et al.*, 1997). In addition, the expression of many genes is regulated by sugars (reviewed in Koch, 1996). Our preliminary results indicate that in rice suspension cells expression of both *Rgp1* and R*gp2* is induced by sucrose and glucose (not shown). Interestingly, expression of *Rgp2* is repressed by the glucose analogs mannose and 2-deoxyglucose, whereas this was not observed for expression of *Rgp1* (not shown), indicating that *Rgp1* and *Rgp2* are differentially regulated.

The observed phenotype in small tubers shows similarities to the phenotype of antisense sucrose synthase (Susy) potato tubers (Zrenner *et al.*, 1995). As in *Rgp1* overexpressing tubers, the antisense Susy tubers accumulated high levels of glucose and fructose, which was accompanied by an increase in invertase activity and a decrease in the amount of patatin. In addition, starch accumulation was impaired in these lines, causing a decrease in total tuber dry weight. When RGP1 functions in cell wall synthesis, it is conceivable that an excess of RGP1 has similar effects as antisense Susy, since both will result in a decrease of availability of UDP-glucose for starch synthesis. The fact that the lines overexpressing *Rgp2* did not show impaired starch granule formation, suggests a distinct function for RGP2.

In conclusion, in contrast to RGP2, the presence of additional RGP1 in potato seems to affect starch granule size and, under certain conditions, starch granule shape. These results, together with the changed concentrations of free sugars, present the first indications that RGP1 and RGP2 may compete for the available UDP-glucose for cell wall synthesis and starch synthesis, respectively, which suggests a role for RGPs in carbon partitioning. Further analysis of the cell walls in *Rgp1* and *Rgp2* overexpressing lines should verify this hypothesis.

Acknowledgements

We thank Andre Wijfjes for help with the Dionex system and Dirk Jan Huigen for growing the potato tubers and microtubers.

Chapter 7

General and summarising discussion

Starch granule size

Initially this study aimed at identification of factors involved in the formation of large A-type and small B-type starch granules in endosperm of wheat. Therefore, the formation of small B-type granules in amyloplasts was studied, using transmission electron microscopy and confocal laser scanning microscopy (CLSM). Both techniques showed the presence of B-type granules in protrusions emanating from the A-type containing amyloplasts. The non-destructive nature of CLSM facilitated the visualisation of movements of these structures.

The presence of small granules may be related to the high starch yields obtained from wheat, since small granules are able to fill spaces which are created by packing large starch granules only. Modification of wheat in order to reduce the number of B-type granules may, therefore, result in a lower yield. Nevertheless, this may be acceptable for industries in which the presence of B-type granules causes problems. One option to reduce the number of B-type starch granules is the modification of the protrusions in which these starch granules are formed. However, little is known about the mechanism involved in the formation of these tubular structures. Since B-type granules are formed later in development compared to the A-type starch granules, another possibility to manipulate the number of B-type granules was expected to be feasible by changing the number of primer molecules at a specific developmental stage. Therefore, a putative primer for starch synthesis designated UDP-glucose:protein transglucosylase (UPTG) or "amylogenin" (Rothschild and Tandecarz, 1994; Singh *et al.*, 1995) was selected as a primary candidate for further study.

Reversibly glycosylated polypeptides

The localisation of reversibly glycosylated polypeptides (RGP) in trans-Golgi dictyosomal cisternae shown by Dhugga (1997), and the observed homology of RGP with UPTG (Bocca *et al.*, 1999), shifted the proposed function of UPTG (or now RGP) from a primer in starch synthesis to a function in the synthesis of the cell wall polysaccharide xyloglucan (Dhugga *et al.*, 1997). However, isolation of two classes of RGP cDNA clones (Chapter 3) were consistent with the identification of two classes of sucrose synthase in maize endosperm of which one was shown to be essential for cell wall synthesis (*Shrunken1)*, whereas the other (*Sucrose synthase1*) was shown to be involved in starch synthesis (Chourey *et al.*, 1998). These findings led to the working hypothesis that different RGPs are involved in cell wall or in starch synthesis.

Subcellular localisation experiments showing that RGP2 is localised in the cytoplasm as well as in small and large organelles, but not in amyloplasts (Chapter 4), questioned the direct involvement of RGP2 in the priming of starch synthesis. However, the possibility remains that glucan-primers for starch synthesis are produced in the cytoplasm and transported into the amyloplasts. The fact that RGP1 was found to be localised in all tissues examined and RGP2 mainly in starch-storing tissues (Chapter 3) supported our working hypothesis. However, the large amount of RGP1 as well as RGP2 in degenerating pericarp tissue pointed towards a more general role such as in transport of UDP-sugars, as hypothesised by Delgado *et al.* (1998).

Partial homology with cellulose synthase and other β-glycosyltransferases suggested that RGPs may be non-processive β-glycosyltransferases, functioning as intermediates in the transfer of a single sugar residue from a nucleotide-sugar to an acceptor molecule (Saxena and Brown Jr, 1999). Using hydrophobic cluster analysis (HCA) (Gaboriaud *et al.*, 1987), processive β-glycosyltransferases were shown to contain two conserved domains, designated domain A and domain B, whereas non-processive glycosyltransferases carry only domain A (Saxena *et al.*, 1995). Domain A usually consists of alternating α-helices with β-strands, the latter containing the DXD motif. This motif consists of two aspartic acids, flanked by hydrophobic residues. Mutations of the two aspartic acids eliminated enzymatic activity in α-1,3-mannosyltransferase of *Saccharomyces cerevisiae* (Wiggins and Munro, 1998). However, in other families of glycosyltransferases such as the chitin synthases and N-acetyl-galactosaminyltransferases, the second aspartic acid is replaced by glycine or histidine, respectively. This suggests that substitution of one of the aspartates does not imply incompatibility with enzyme activity, although sufficient compensating changes elsewhere in a protein are thought to be required (Wiggins and Munro, 1998). In addition to domain A, processive β-glycosyltransferases contain domain B, a single conserved aspartate together with the motif QXXRW (Saxena *et al.*, 1995).

Domain A and B can be subdivided in 6 regions (Kamst and Spaink, 1999). The DXD motif can be found in region 3 and the QXXRW motif in region 6 (Figure 1).

RGP1, RGP2, starch synthases from wheat and *Arabidopsis* and glycogenin from rabbit muscle, the primer for glycogen synthesis, were subjected to hydrophobic cluster analysis, assayed for regions 1-6 and compared with NodC, a chitin-oligosaccharide synthase (Figure 1).

	Domain A			Domain B		
	region 1	region 2	region 3	region 4	region 5	region 6
TaRGP1	Q-LIIVQDGD	ELYNRN	YVFTIDDD	Y--GADFVR	YVDAVLT	QPIGRY
TaRGP2	R-IIVVKDPE	KVYTKS	YVISIDDN	Y--GADFVR	YVDAVMT	EGKHRW
AtRGP2	G-LIVVKDPD	DVYSKT	YIVSIDDD	Y--GADFVR	YVDAVMT	EGKVRW
TaSSI	R-IVFVTGEA	RYLNGS	DWVFVDHP	Y--GDNQFR	YRDSRST	SQGYSW
AtSSS	N-LVFVTSEA	RYLNGT	DWVFVDHK	Y--GDNQFR	YKDARSI	SQGYAW
OcGlg	K-ALVLGSSL R--DILDSGDS	RLAVLTTPQV TLMKRP	KCVFMDAD	Y--GANAKVV	GHDPTMT	ERKERW
RmNodC	K-QVYVVDDGS	RFNFI--GKR	LILNVDSD	GPCAMYR	GEDRHLT	RQQLRW
consensus	Q-β-XDG R K	R-β-XKR K KS PQ	βDXD	GXXXXR	YXDXXXT G	QXXRW
proposed function in NodC	NDP-binding		donor saccharide-binding		acceptor saccharide binding	product binding

Figure 1. Amino acid sequences of domains A and B (Saxena *et al.*, 1995) and regions 1-6 (Kamst and Spaink, 1999) of α- and β-glycosyltransferases. Database accession numbers of the sequences used for the comparison are: Y18626 (TaRGP1), Y18625 (TaRGP2), AB005242 (AtRGP2), AJ292521 (TaSSI; wheat Starch Synthase I), AB006701 (AtSSS; *Arabidopsis* Soluble starch synthase), L01791 (OcGlg; rabbit muscle Glycogenin) X01649 (RmNodC; *Rhizobium melitoti* NodC). Bold amino acids indicate the consensus for processive β-glycosyltransferases. β-sheets are underlined.

The regions 1 and 2 are known as the Walker-B and Walker-A motifs respectively, both consisting of a β-sheet preceded by an arginine or lysine and shown to be involved in mono- or di-nucleotide binding (Rossmann *et al.*, 1975). Region 1 was only clearly observed in RGP1, whereas all RGP2 proteins lack the glycine that is thought to function in the binding of a phosphate group in β-polysaccharide synthases. Since none of the α-glycosyltransferases such as the starch synthases and glycogenin contain the DG motif, this may be specific for β-glycosyltransferases. Region 2 is more difficult to identify in the analysed proteins due to the large variation in amino acids. However, all proteins contain a β-sheet that seems to be flanked by amino acids capable of interacting with phosphate groups.

Regions 3 and 4 are thought to be involved in the recognition of the saccharide in the donor-substrate. The DXD motif (region 3) is present in all proteins analysed. However, in RGP2 from wheat and rice as well as in the starch synthases from wheat and *Arabidopsis*, the second aspartate is replaced. In region 4 the arginine of RGP1 in maize was shown to be glucosylated upon addition of UDP-[^{14}C]-glucose (Singh *et al.*, 1995). In glycogenin, which does not contain the conserved arginine, the tyrosine preceding region 4 was shown be glucosylated (Alonso *et al.*, 1994).

Region 5, suggested to be involved in binding of the saccharide acceptor (Kamst and Spaink, 1999), is present in all proteins examined. Since the glycine found in NodC is substituted by a tyrosine in RGPs and starch synthases this is another indication for the acceptor specificity of this region. However, since RGP1 is thought to be a non-processive self-glucosylating protein, an acceptor binding site seems unnecessary.

The QXXRW motif or region 6 may interact with the growing chain and facilitates the translocation of the chain upon elongation. RGP1 does not contain this motif as was observed by Saxena (1999). However, RGP2 as well as glycogenin contain an RW motif. Since glycogenin is known to be a processive glucosyltransferase, this suggests that RGP2 might be able to transfer more than one glucose moiety to an acceptor molecule. Because of the observed similarities of RGP2 with NodC, which is a chitin oligosaccharide synthase, it is tempting to speculate about the priming activity of RGP2 in starch synthesis by generating oligosaccharides. However, a mechanism for transporting the oligosaccharides into the amyloplast is unknown.

Glucosylation activity of RGPs

Glucosylation activity studies showed that the product of *Rgp1* expressed in *E. coli*, yeast, wheat endosperm and tobacco binds UDP-glucose, whereas RGP2 was reluctant to show glucosylating activity (Chapter 5). Although tobacco plants overexpressing *Rgp2* showed enhanced glucose-labelling of a 40 kD protein, (suggesting that RGP2 is only folded into a functional tertiary structure when produced in plant cells), the possibility remains that RGP2 merely enhances RGP1 activity. This is possible since RGP1 and RGP2 show identical subcellular localisation patterns (Chapter 4). Moreover, analysis of the RGP complex in wheat endosperm and binding studies indicated that RGP1 and RGP2 are present in homo- and hetero-multimers (Chapter 5). However, in transgenic potato tubers containing relatively more RGP1 compared to RGP2, aberrantly shaped starch granules and a decrease in starch granule size were observed, although the overall starch content was not affected (Chapter 6). This suggests that RGP1 is not involved in the carbon flux into starch, but merely competes for the substrate, possibly directing it into cell wall synthesis. The role of RGP2 is less clear since RGP2 overexpressing lines did not show aberrantly shaped starch granules. Moreover, the effect on starch granule size was not consistent (Chapter 6). In the first harvest starch granules from line RGP2-24 showed a size distribution similar to that of the wildtype, whereas the starch granules from line RGP2-7 were significantly smaller. The size distribution of starch granules isolated from RGP2-7 and RGP2-24 microtubers together with the reduced mean fresh weight of the microtubers, indicated a retarded tuber development. The latter may suggest that RGP2 overexpression results in reduced tuber growth. However, the starch yield from the primary greenhouse-grown tubers was not significantly affected in these lines.

The occurrence of aberrantly shaped starch granules in the *Rgp1* overexpressing lines is remarkable, since such an effect is not common in mutants or transgenic lines with aberrant carbohydrate metabolism. However, pear-shaped and conical starch granules have been reported in the past (reviewed in Jenner, 1982). Moreover, high amylose genotypes of maize, barley, pea and potato were shown to contain aberrantly shaped starch granules (Banks *et al.*, 1974; Schwall *et al.*, 2000). However, these high amylose starch granules did not resemble the conical, shell-shaped starch granules found in *Rgp1*-overexpressing lines, and amylose content was not increased in the RGP1 lines. Variation in the substrate supply for starch synthesis causing lamellae formation was hypothesised by Buttrose (1962). In wheat and barley, shells are not detected in plants grown under constant environmental conditions, but can be induced by imposing a regime of alternating light and darkness (Buttrose, 1960; Buttrose, 1962). However, this is not the case for potato, which displays lamellae independent of the light regime. The shell-shaped starch granules observed in the *Rgp1*-overexpressing potato tubers may be caused by the lack of substrate for starch synthesis. The shell-shape of the starch granules in the *Rgp1* overexpressing potato tubers suggests that lamellae formation is prematurely aborted, possibly caused by a lack of substrate or downregulation of enzymes involved in lamellae formation.

Since the effects of *Rgp1* overexpression are thought to be related to the supply of substrate, the possibility exists that the aberrantly shaped starch granules are related to the subcellular localisation of ADP-glucose. Preliminary results suggested that the localisation of AGPase is influenced by environmental factors. In a primitive species of barley, *Hordeum murinum*, 70%-100% of the AGPase activity resided in the plastid when the plant was growing poorly, whereas under vigorously growing conditions 70% of the AGPase activity appeared to be cytosolic (Beckles *et al.*, 1998). This is in agreement with the observation that in young endosperm (until 10 dpa) AGPase is localised in the plastid, whereas in endosperm older than 10 dpa AGPase is predominantly found in the cytoplasm (Ainsworth *et al.*, 1997). A barley mutant affected in the plastidial ADP-glucose transporter showed reduced starch accumulation (Denyer *et al.*, 2000), indicating that the plastidial AGPase is not generating sufficient substrate to maintain starch granule biosynthesis. Although direct evidence about the cytosolic localisation of ADP-glucose in potato is lacking, it was shown that isolated potato amyloplasts are able to transport ADP-glucose across the amyloplast envelope (Naeem *et al.*, 1997). Therefore a cytosolic localisation of ADP-glucose in potato tubers should not be excluded. Moreover, the symptoms of hollow heart disorder observed in several tubers affected in starch granule shape (Chapter 6), indicates the presence of stress in these tubers. Thus, stress caused by extra RGP1 may result in a switch from the use of cytosolic ADP-glucose to plastidial ADP-glucose, the latter being limiting for starch granule synthesis. Why in potato this results in an aberrant shape of the starch granules instead of normal but limited starch accumulation is unclear.

Overexpression of wheat *Rgp1* and *Rgp2* in tobacco did not lead to an obvious phenotype, possibly caused by the lack of a specialised starch storage organ in this species (Chapter 5). Overexpression of rice RGP1 and RGP2, as well as antisense expression of RGP2 in rice did not cause a change in starch granule size distribution nor caused an aberrant granule shape (not shown). It should be noted, however, that in rice endosperm multiple starch granules are formed within one amyloplast, resulting in small, polygonal shaped starch granules (Buttrose, 1960).

Preliminary results from Differential Scanning Calorimetry experiments showed significant differences in gelatinisation temperature in the transgenic rice starches indicating a change in starch granule structure. Starch from one line overexpressing *Rgp1* and one line containing an antisense *Rgp2* construct gelatinised at a temperature 1.5°C higher than that of the wildtype, whereas starch from an *Rgp2*-overexpressing line gelatinised at a 1.5°C-lower temperature. Since only a single rice line per construct was tested and this effect was not found in starch from transgenic potato (Chapter 6), these DSC values might be specific for the transgenic rice lines.

Hypotheses

Based on the obtained results, two hypotheses are proposed both in which RGPs function as carriers of UDP-glucose. In the first hypothesis, RGP2 is merely an activator of RGP1 in starch-storing tissues and both RGPs are involved in directing UDP-glucose into cell wall synthesis. In competition with starch synthesis for substrate in starch-storing tissues, RGP1 co-operates with RGP2. Since leaf tissue lacks a permanent starch storing function, RGP2 is hypothesised not to be necessary in this tissue. However, based on the structural homology of RGP2 with RGP1 and other glycosyltransferases revealed by hydrophobic cluster analysis, RGP2 is expected to have glucosylating activity. Moreover, this model does not clearly explain the observed phenotypes of *Rgp1*- and *Rgp2*-overexpressing potato lines, since similar phenotypes would be expected for the *Rgp1*- and *Rgp2*-overexpressing lines. Nevertheless, it remains possible that an excess of RGP2 does not further enhance RGP1 activity and results in an unaltered metabolism.

The other hypothesis is based on the analogy of the situation with the cell wall-specific and starch-specific sucrose synthases in maize endosperm (Chourey *et al.*, 1998). According to this hypothesis, RGP1 and RGP2 transport UDP-glucose produced by the respective sucrose synthases, thereby facilitating the carbon flux into cell wall and starch, respectively. RGP1 may preferably bind UDP-glucose produced by *Shrunken1*, whereas RGP2 may be dependent for its UDP-glucose on *Sucrose synthase1*. Remarkably, a sucrose synthase-like protein has been reported to inhibit RGP1 activity (Wald *et al.*, 1998). Since in the maize mutant shrunken-1 RGP1 activity was not inhibited it was concluded that Shrunken-1 is able to inhibit RGP1 activity. However, the "inhibition" may be explained by the production of unlabelled UDP-glucose by starch synthase. The fact that sucrose synthase was not isolated in a yeast-two hybrid screening of a cDNA library, suggested that RGP and sucrose synthase do not form a stable complex. However, sub-optimal binding conditions cannot be ruled out. Thus, in young leaf tissue, in which only RGP1 is detected (Chapter 3), RGP1 homo-multimers may bind UDP-glucose to direct this substrate into cell wall synthesis. In starch-storing tissue, such as wheat endosperm and potato tuber, RGP1 and RGP2 may compete for the available UDP-glucose in hetero- and homo multimers, directing UDP-glucose into cell wall and starch synthesis, respectively. In contrast to the first hypothesis, this model is consistent with the phenotype found for transgenic *Rgp1* and *Rgp2* potato lines. Since AGPase is considered to be the key regulatory enzyme in starch biosynthesis (Smith *et al.*, 1997), an increased amount of RGP2 will not lead to a higher rate of starch synthesis and thus will not affect starch granule biosynthesis.

Analysis of cell walls in RGP-overexpressing tubers and crossings between lines altered in the amount of certain sucrose synthases and RGPs may give additional information about the function of RGPs in starch synthesis.

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Samenvatting

Zetmeel is een belangrijk onderdeel van onze voeding. Het geeft ons de benodigde energie voor onze dagelijkse activiteiten. Ook voor de plant is zetmeel belangrijk. In tijden van overvloed worden suikers die geproduceerd zijn in de bladeren vervoerd naar de opslagorganen zoals aardappelknollen en tarwezaden. Daar worden de suikers aan elkaar geknoopt en opgeslagen in de vorm van zetmeelkorrels. Hoewel zetmeel erg belangrijk voor ons is, is er nog maar weinig bekend over hoe de zetmeelkorrels precies worden gemaakt. Het eiwit dat een suikermolecuul aan een bestaande suikerketen kan knopen (zetmeel synthase) is bekend, maar welk eiwit de eerste twee suikers aan elkaar koppelt (de zgn. "primer") is onduidelijk. Ook over de daaropvolgende vorming van de suikerketen tot een zetmeelkorrel, zijn de wetenschappers het nog niet eens.

Vorm en grootte van de zetmeelkorrels verschillen bovendien per plantensoort. Aardappelzetmeel heeft vrij grote zetmeelkorrels (zo'n 0,1 mm), rijst daarentegen heeft hele kleine korrels (0,005 mm). Tarwe- en gerstzaden bevatten zowel grote als kleine zetmeelkorrels. Tijdens de groei van het zaad worden de grote korrels het eerst gevormd, een paar dagen later volgen de kleintjes. De hoeveelheid grote en kleine zetmeelkorrels varieert ook weer per tarwe- of gerstras en is belangrijk voor de kwaliteit van het zetmeel. Bij het brouwen van bier, bijvoorbeeld, kunnen de kleine korrels bepaalde filters verstoppen. Bierbrouwers zijn daarom gebaat bij gerstzaden met weinig kleine zetmeelkorrels. Dit proefschrift beschrijft de zoektocht naar factoren die de hoeveelheid kleine zetmeelkorrels kunnen beïnvloeden.

Omdat nog weinig bekend was over de vorming van de kleine zetmeelkorrels in tarwezaad, heb ik hier eerst onderzoek naar gedaan. Hoofdstuk 2 laat zien dat de kleine zetmeelkorrels zich bevinden in uitstulpingen van zgn. amyloplasten. Amyloplasten zijn de compartimenten van de cel waarin zetmeel wordt gevormd en opgeslagen. Dit onderzoek is uitgevoerd met twee verschillende microscopen. De elektronen microscoop is een conventionele microscoop waarbij het materiaal (in dit geval ontwikkelende tarwekorrels) eerst gefixeerd en geplastificeerd moet worden. Bij confocale laser scanning microscopie (CLSM) daarentegen kan met behulp van een laser levend materiaal bekeken worden. Opnames met deze microscoop laten zien dat de amyloplasten en hun uitstulpingen niet stilzitten, maar bewegen. Hiervan

is een filmpje te zien op wwwimp.leidenuniv.nl/tno.html.

De rest van dit proefschrift beschrijft de speurtocht naar de zgn. primer van de zetmeelsynthese. De hypothese was dat een verandering in de hoeveelheid van deze primer, de hoeveelheid kleine zetmeelkorrels kan beïnvloeden. Immers, als er op het juiste moment minder van deze primer aanwezig is kunnen er waarschijnlijk ook minder van deze kleine zetmeelkorrels gevormd worden. Hoofdstuk 3 beschrijft het kloneren (=zoeken, vinden en isoleren) van twee genen die, toen we met het onderzoek begonnen, mogelijk codeerden voor een zetmeelprimer. Echter, tijdens dit onderzoek verscheen er een artikel waarin een eiwit, vergelijkbaar met "onze" zetmeelprimer, in verband werd gebracht met celwandsynthese. Dit eiwit, Reversibly Glycosylated Polypeptide (RGP) genaamd, kan suikermoleculen binden en ook weer loslaten. Net als zetmeelkorrels bestaan ook celwanden uit suikerketens, de suikers worden alleen op een andere manier aan elkaar geknoopt. Argumenten dat RGP bij celwandsynthese betrokken zou zijn, waren de plaats van het eiwit in de cel (daar waar celwand materialen worden gemaakt) en de specifieke suikers die dit eiwit kunnen binden. Ons onderzoek liet zien dat er twee klassen van dit soort eiwitten bestaan. Eiwitten in de eerste klasse (RGP1) komen voor 90% overeen met het beschreven "celwand eiwit". Bij eiwitten in de tweede klasse (RGP2) is dit maar 50%. Om meer duidelijkheid over de functies van deze twee RGP klassen te krijgen, hebben we gekeken naar de weefsels waarin RGP1 en RGP2 aanwezig zijn. RGP1 bleek in alle onderzochte weefsels aanwezig te zijn, overeenkomend met een functie in celwandsynthese, terwijl RGP2 niet detecteerbaar was in blad. Dit leidde tot de hypothese dat RGP2 betrokken zou kunnen zijn bij zetmeelsynthese.

In Hoofdstuk 4 wordt dieper ingegaan op de lokalisatie van de twee RGP's. Met behulp van de CLSM laten we zien dat RGP1 en RGP2 op precies dezelfde plaatsen in de cel aanwezig zijn. Bovendien wordt duidelijk dat RGP2 niet aanwezig is in de amyloplasten. Deze observatie maakt een mogelijke functie als zetmeelprimer onwaarschijnlijk, omdat aangenomen wordt dat de gehele zetmeelsynthese in deze amyloplasten plaatsvindt.

Hoofdstuk 5 beschrijft het onderzoek naar de glucosyleringsactiviteit (suikerkoppelingsactiviteit) van RGP1 en RGP2. De genen werden naar verschillende organismen overgebracht (bacteriën, gist en tabak), die vervolgens de bijbehorende RGP-eiwitten produceerden. Vervolgens werd getest of deze RGP's gemerkte suikers konden binden. Uit deze proeven bleek dat RGP1 van alle organismen een bepaalde suiker, UDP-glucose, bond, terwijl RGP2 dit alleen in planten deed. Ook werd duidelijk dat zowel RGP1 als RGP2 zich in een complex van meerdere eiwitten bevinden. Analyse van het complex en bindingsproeven toonden aan dat dit complex hoogstwaarschijnlijk uitsluitend uit RGPs bestaat.

Hoofdstuk 6 beschrijft het effect van de aanwezigheid van tarwe-RGPs in aardappel. In aardappelen van de eerste oogst die veel tarwe-RGP1 produceren, werden zetmeelkorrels met een afwijkende vorm gevonden. Deze knolletjes bevatten ook meer glucose en fructose in vergelijking met een gewone aardappel. Deze effecten werden niet meer bij volgende oogsten waargenomen. Wel waren bij alle oogsten de zetmeelkorrels in de planten met extra RGP1 kleiner dan normaal. De planten met extra tarwe-RGP2 waren normaal. Dit wijst erop dat RGP1 en RGP2 verschillende functies hebben. Het is mogelijk dat in opslagorganen RGP2 de functie van RGP1 versterkt. Een andere verklaring is dat RGP1 en RGP2 competeren voor de beschikbare suikermoleculen. Het kan zijn dat RGP1 de suikermoleculen bindt ten behoeve van de vorming van celwanden, terwijl RGP2 dit doet voor de opslag van zetmeel. Verder onderzoek naar de celwanden in deze planten zou hier meer duidelijkheid over kunnen geven.

Curriculum vitae

Sandra Mariska Jeanette Langeveld werd geboren op 27 november 1970 in Schiedam. Na het behalen van het VWO diploma aan de RSG Winkler Prins te Veendam werd in 1989 begonnen met de studie Plantenveredeling aan de Landbouw Universiteit Wageningen. Tijdens deze studie werden afstudeervakken gedaan bij de Vakgroep Erfelijkheidsleer, de Vakgroep Moleculaire Biologie en de Vakgroep Plantenveredeling, waar onder leiding van prof. dr. Richard Visser de basis werd gelegd voor het zetmeelonderzoek. Aan de Cornell University in Ithaca, New York, werd een stage van 5 maanden gelopen. Van juli 1996 tot januari 2001 was zij werkzaam als promovenda in het Centrum voor Fytotechnologie UL-TNO onder leiding van dr. Sylvia de Pater en prof. dr. Jan Kijne. Op dit moment is zij werkzaam als postdoc in de sectie Schimmelgenetica van het Instituut voor Moleculaire Plantkunde (IMP) in de groep van prof. dr. Kees van den Hondel.

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