Analysis of Cytosolic Heteroglycans from Leaves of Transgenic Potato (Solanum tuberosum L.) Plants that Under- or Overexpress the Pho 2 Phosphorylase Isozyme

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During starch degradation, chloroplasts export neutral sugars into the cytosol where they appear to enter a complex glycan metabolism. Interactions between glycans and glucosyl transferases residing in the cytosol were studied by analyzing transgenic potato (Solanum tuberosum L.) plants that possess either decreased or elevated levels of the cytosolic (Pho 2) phosphorylase isozyme. Water-soluble heteroglycans (SHGs) were isolated from these plants and were characterized. SHG contains, as major constituents, arabinose, rhamnose, galactose and glucose. Non-aqueous fractionation combined with other separation techniques revealed a distinct pool of the SHG that is located in the cytosol. Under in vitro conditions, the cytosolic heteroglycans act as glucosyl acceptor selectively for Pho 2. Acceptor sites were characterized by a specific hydrolytic degradation following the Pho 2-catalyzed glucosyl transfer. The size distribution of the cytosolic SHG increased during the dark period, indicating a distinct metabolic activity related to net starch degradation. Antisense inhibition of Pho 2 resulted in increased glucosyl and rhamnosyl contents of the glycans. Overexpression of Pho 2 decreased the content of both residues. Compared with the wild type, in both types of transgenic plants the size of the cytosolic glycans was increased.

Keywords: Carbohydrate metabolism — Cytosolic heteroglycans — Overexpression — Phosphorylase isozymes — Solanum tuberosum L. — Starch metabolism.

Introduction

Starch is a water-insoluble storage carbohydrate that almost ubiquitously occurs in plants. In higher plants, starch is synthesized inside the plastids. However, the biochemistry of the starch varies depending on the differentiation of the organelle: in non-green plastids, the reserve starch granules are continuously synthesized over weeks, whereas in chloroplasts the transitory starch pool exhibits large diurnal fluctuations and the rate of net starch synthesis during light appears to be precisely coordinated with that of starch mobilization during the night (Gibbon et al. 2004, Smith et al. 2005). The pathways of both chloroplastic starch biosynthesis and degradation are still incompletely known and regulatory mechanisms, especially those controlling the degradative process, remain to be elucidated. The higher plant cell possesses a large number of chloroplasts, all of which are capable of degrading starch and exporting the degradation products into the cytosol. Therefore, it is reasonable to assume that the metabolic control of starch degradation is exerted at the level of the entire cell rather than at that of the individual organelle. If so, mechanisms are required which coordinate the initiation of starch degradation at many subcellular sites, i.e. in the entire cellular plastid population, and to adapt this multisite process to the actual cytosolic demand for starch-derived carbohydrates. Currently, such cellular control mechanisms are largely unknown.

During the last years, novel starch-related proteins and glycan structures have been identified that appear to be relevant in this context. Among the newly discovered proteins are the maltose transporter MEX1 of the chloroplast envelope membrane (Niittylä et al. 2004), the glucan, water dikinase (EC 2.7.9.4; Yu et al. 2001, Rütte et al. 2002), the phosphoglucan, water dikinase (EC 2.7.9.5; Baunsgaard et al. 2005, Köthting et al. 2005) and the transglucosidase (synonymous designations: glucanotransferase, amylomaltase or disproportionating enzyme; DPE2; EC 2.4.1.25; Chia et al. 2004, Lloyd et al. 2004, Lu and Sharkey 2004). The two dikinases reside in the chloroplast stroma whereas the transglucosidase is restricted to the cytosol (for the localization of DPE2 in Solanum tuberosum L. — Starch metabolism.

Abbreviations: DPE2, amylomaltase, glucanotransferase or transglucosidase (EC 2.4.1.25); FFF, field flow fractionation; FITC, fluorescein isothiocyanate; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; MALLS, multiangle laser light scattering; SHG, water-soluble heteroglycans (including monosaccharides and disaccharides); SHG 1, water-soluble heteroglycans (large size fraction); SHG 2, water-soluble heteroglycans (small size fraction); SHG 3, water-soluble heteroglycans (total fraction).

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L. see below). DPE2 appears to be similar to the maltase Q (MalQ) gene product that is involved in bacterial maltose metabolism (Boos and Shuman 1998). The phenotypic characterization of transgenic Arabidopsis or Solanum plants revealed that the deletion of any of these novel proteins results in elevated levels of transitory starch and, therefore, each of these proteins fulfills an important starch-related function. In contrast, elimination of some (iso)enzymes that are traditionally attributed to starch degradation failed to have any noticeable impact on the transitory starch levels in Arabidopsis leaves. The deletion of either the plastidial α-amylases (EC 3.2.1.1; Tetlow et al. 2004, Yu et al. 2005) or the phosphorylase (Pho 1; EC 2.4.1.1; Zeeman et al. 2004) did not significantly impede starch degradation and, therefore, these classical ‘starch-metabolizing enzymes’ do not exert any metabolic function that cannot be bypassed or taken over by other enzymes. However, in rice leaves, a plastidial α-amylase isoform has been reported to be indispensable for starch degradation (Asatsuma et al. 2005).

Of special interest is the phenotype of Arabidopsis mutants that lack a functional cytosolic transglucosidase, DPE2. These mutants possess extremely high levels of maltose, an elevated starch content and a significantly retarded growth (Chia et al. 2004, Lu and Sharkey 2004). Thus, DPE2 appears to be involved in the cytosolic conversion of the starch-derived maltose and this process is likely to be essential for both metabolism and growth of the plant. Based on these data, Zeeman et al. (2004) and Smith et al. (2005) postulated a complex cytosolic maltose metabolism in which glucosyl residues are transferred from maltose to so far unidentified cytosolic acceptors. DPE2 effectively transfers glucosyl residues to glycogen when assayed under in vitro conditions. However, glycogen is a homoglucon prepared from non-plant sources and appears to act as a non-physiological substitute of some high molecular weight glycans residing in the cytosol of the plant cell.

Recently, cytosolic water-soluble heteroglycans (SHGs; designated as subfraction I) have been identified in Pisum sativum L. and Arabidopsis thaliana L. that contain, as major constituents, arabinose, galactose and glucose and possess a highly complex pattern of glycosidic linkages. By several physicochemical and biochemical criteria, these glycans can be distinguished from water-soluble cell wall-related polysaccharides (designated as subfraction II): they are present in isolated mesophyll protoplasts, and during non-aqueous fractionation they co-distribute with cytosolic marker proteins but not with crystalline cellulose (Fettke et al. 2004, Fettke et al. 2005). As revealed by field flow fractionation (FFF), the size distribution of the cytosolic glycans differs from that of apoplastic heteroglycans and, unlike the latter, they do not react with the β-glucosyl Yariv reagent. Under in vitro conditions, the cytosolic heteroglycans (but not the cell wall-related glycans) act as acceptor of α-1,4-linked glucosyl residues that are transferred by the cytosolic phosphorylase isoform (Pho 2). Although the formation of the cytosolic heteroglycans does not require a functional starch biosynthesis (Fettke et al. 2005), it is conceivable that these glycans are involved in the postulated complex cytosolic metabolism of starch degradation products.

The studies mentioned above clearly demonstrate that in vitro the cytosolic glycans exert a priming function for the phosphorylase isoform (Pho 2). However, currently no evidence is available for this type of heteroglycan-phosphorylase interactions occurring in vivo. In this communication, we have analyzed heteroglycans from transgenic potato plants that possess an altered expression of the cytosolic phosphorylase isoform. Pho 2 expression was lowered by using an antisense approach (Duwenig et al. 1997). For overexpression, potato plants were transformed with a construct encoding the Pho 2 isoform from Vicia faba L.

Unlike proteins, polysaccharides usually possess a polydispersed size distribution and often they cannot be selectively quantified by monitoring any biospecific action of the macromolecules. Especially for complex carbohydrates, it is difficult to obtain unequivocal evidence for either homogeneity or inhomogeneity of the glycans preparation under investigation and, therefore, in many cases a considerable effort is needed vigorously to exclude a possible interference by contaminating compounds. Contaminations derived from the plant cell wall are particularly relevant as it possesses a highly complex structure and consists of several types of major polysaccharides, such as cellulose, hemicellulose and pectin. As a further complication, the cell wall chemistry varies greatly depending upon plant species and organ (Reiter 2002).

In this study, we present evidence that in S. tuberosum L., the glycans that selectively interact with Pho 2 reside in the cytosol (in a strict sense). The Pho 2-related priming capacity is an intrinsic property of the entire cytosolic glycans pool. For various independent lines of transgenic potato plants, heteroglycans and other carbohydrate pools were quantified during both the light and the dark period. The data clearly show that an altered expression of Pho 2 results in in vivo changes in the cytosolic heteroglycans. To the best of our knowledge, this is the first in vivo indication for the physiological carbohydrate substrate of the cytosolic phosphorylase isoforms.

**Results**

Soluble heteroglycans from leaves of Solanum tuberosum L. act selectively as glucosyl acceptor for Pho 2 from plants

From leaves of S. tuberosum L., SHGs were isolated and their priming capacity was tested using either a recombinant Pho 2 from V. faba L. or the rabbit muscle phosphorylase (Table 1). In this particular experiment, the entire SHG preparation (designated as SHG<sub>0</sub>; for details see Fettke et al. 2004) was used. SHG<sub>0</sub> contains all extractable glycans including all water-soluble low molecular weight compounds. The Pho 2 preparation clearly catalyzes a glucosyl transfer to primers present in SHG<sub>0</sub> but the mammalian phosphorylase does not. Although both phosphorylases possess high affinity towards branched polyglucans (Steup and Schächtele 1981, Shimomura
Table 1  

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glycan (0.8 mM glucose-equivalent)</th>
<th>$^{14}$C Incorporation (dpm)</th>
</tr>
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<tr>
<td><em>Vicia faba</em> Pho 2 (3.3 nkat)</td>
<td>SHG&lt;sub&gt;0&lt;/sub&gt;</td>
<td>3,420</td>
</tr>
<tr>
<td><em>Vicia faba</em> Pho 2 (3.3 nkat)</td>
<td>SHG&lt;sub&gt;0&lt;/sub&gt;</td>
<td>110</td>
</tr>
<tr>
<td>Rabbit muscle Pho a (3.3 nkat)</td>
<td>SHG&lt;sub&gt;0&lt;/sub&gt;</td>
<td>88</td>
</tr>
<tr>
<td>Rabbit muscle Pho a (33 nkat)</td>
<td>SHG&lt;sub&gt;0&lt;/sub&gt;</td>
<td>207</td>
</tr>
<tr>
<td>Rabbit muscle Pho a (33 nkat)</td>
<td>Glycogen</td>
<td>32,542</td>
</tr>
<tr>
<td>Rabbit muscle Pho a (33 nkat)</td>
<td>SHG&lt;sub&gt;0&lt;/sub&gt;</td>
<td>86</td>
</tr>
<tr>
<td>Rabbit muscle Pho a (33 nkat)</td>
<td>Glycogen + SHG&lt;sub&gt;0&lt;/sub&gt;</td>
<td>33,106</td>
</tr>
</tbody>
</table>

SHG<sub>0</sub> was incubated with recombinant Pho 2 from *V. faba* L. (3.3 nkat/250 µl) or rabbit muscle phosphorylase a (3.3 or 33 nkat in 250 µl) and [U-14C]glucose 1-phosphate. For comparison, glycogen was added instead of or in addition to SHG<sub>0</sub>. After 10 min, the reaction was terminated by adding ethanol/potassium chloride and the $^{14}$C content of the precipitate was determined.

et al. 1982), essentially no transfer of $^{14}$C-labeled glucosyl residues to SHG<sub>0</sub> was observed even when the activity of the rabbit muscle enzyme was increased 10-fold. The addition of SHG<sub>L</sub> to the glycen-containing mixture did not affect the glycen elongation catalyzed by the mammalian phosphorylase and, therefore, any inhibitory effect exerted by SHG<sub>S</sub> can be ruled out. Instead, the data compiled in Table 1 indicate that, unlike the mammalian muscle enzyme, the plant-derived phosphorylase isomorph is capable of using primers present in SHG<sub>S</sub>.

Due to heterogeneity, it is not meaningful to quantify SHG<sub>S</sub> and to compare this value with the size of other carbohydrate fractions of the leaf. In order to diminish heterogeneity, all compounds having a size below 1 kDa (such as sucrose, glucose, fructose and sugar phosphates) were removed from SHG<sub>L</sub> by exhaustive dialysis. The retentate that is referred to as SHG<sub>L</sub> was monitored following acid hydrolysis and estimated to be approximately 0.5–1 mg SHG<sub>L</sub> g<sup>-1</sup> FW of leaf material depending upon growth conditions and the age of the plant material. In these experiments, leaves had consistently been harvested in the middle of the light period. In potato leaves, the total amount of the SHG<sub>L</sub> is in the same order of magnitude as that of sucrose and in the lower range of the starch content (see Table 7). It should, however, be noted that the SHG<sub>L</sub> fraction contains several glycans pools that differ in both physicochemical and biochemical properties (see below).

By using membrane filtration, SHG<sub>S</sub> was separated into a small size (SHG<sub>L</sub>) and a large size (SHG<sub>S</sub>) fraction. In potato leaves, the SHG<sub>L</sub> fraction comprises approximately 75% of the total monomer content of SHG<sub>L</sub>. The monomer patterns of SHG<sub>L</sub>, SHG<sub>S</sub> and SHG<sub>S</sub> as revealed by acid hydrolysis and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) are shown in Fig. 1A. SHG<sub>L</sub> contains, as most prominent constituents, arabinose, rhamnose, galactose and glucose, whereas fucose, xylose and mannose are very minor compounds. The monomer patterns of SHG<sub>S</sub> and SHG<sub>L</sub> differ significantly: in SHG<sub>S</sub>, rhamnose and glucose are the two predominant monosaccharides, whereas in SHG<sub>L</sub> arabinose and galactose are the two most abundant constituents. SHG fractions have also been prepared from growing potato tubers. Although the tuber starch content by far exceeds that of the leaves, the relative glucose levels in the tuber-derived SHG<sub>L</sub>, SHG<sub>S</sub> and SHG<sub>S</sub> were not increased (data not shown). Thus, the starch content of the starting material does not noticeably affect the glucose content observed in the heteroglycan fractions and, therefore, the isolation procedure established yields a SHG<sub>L</sub> that lacks detectable amounts of starch-derived contamination. The same conclusion had been reached recently for heteroglycans isolated from either wild-type or starch-deficient mutants of *Arabidopsis thaliana* L. (Fettke et al. 2005).

Because of the high rhamnose content, the monomer pattern of SHG<sub>S</sub> from *S. tuberosum* L. (Fig. 1A) differs significantly from all low molecular weight heteroglycans studied so far. In contrast, the monomer pattern of SHG<sub>L</sub> from potato leaves is very similar to that of leaves from *A. thaliana* L. or *P. sativum* L. (Fettke et al. 2004, Fettke et al. 2005).

Despite the low glucose content, SHG<sub>L</sub> was effectively used as primer for the recombinant cytosolic (Pho 2) isoform from *V. faba* L. (Fig. 1B). Following incubation with the recombinant Pho 2 and glucose 1-phosphate, the >10 kDa glycans were subjected to acid hydrolysis and analyzed by HPAEC-PAD. Due to the Pho 2-catalyzed glucosyl transfer, the glucose content was significantly increased. As controls, two reaction mixtures lacking either the recombinant Pho 2 or the glycogen (Fig. 1B) were incubated for the same period of time and were then processed identically. In the absence of the SHG<sub>L</sub>, HPAEC-PAD did not reveal any carbohydrates indicating that the recombinant Pho 2 does not contain noticeable amounts of any endogenous primer. When SHG<sub>L</sub> was incubated in the absence of Pho 2, no increase in the glucose content was observed. Remarkably, the priming capacity of SHG<sub>L</sub> was not effective when Pho 2 was replaced by an equal amount of the rabbit muscle phosphorylase a. Under these conditions, the monosaccharide patterns derived from the complete reaction mixture and the phosphorylase a-deficient reaction mixture (Fig. 1B) are indistinguishable. In a further control experiment, the plant-derived heteroglycan was replaced by maltoheptaose under otherwise identical conditions. In contrast to SHG<sub>L</sub>, maltoheptaose was effectively elongated by the mammalian phosphorylase under the assay conditions used (Fig. 1C). Thus, despite its preference for highly branched polyglucans the rabbit muscle enzyme can utilize maltodextrins as substrate but not any constituent of SHG<sub>L</sub>.

Low molecular weight heteroglycans (SHG<sub>S</sub>) also act as primers for the Pho 2-catalyzed glucosyl transfer. Pho 2-dependent priming capacity was observed when SHG<sub>S</sub> was incubated with the recombinant plant phosphorylase and glucose 1-phosphate. At intervals, aliquots of the reaction mixture were withdrawn and the reaction was terminated by heat treat-
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Subsequently, the oligoglycans were analyzed by HPAEC-PAD (Fig. 1D). The complexity of the oligoglycan patterns increased with time. After 120 min incubation, >20 additional oligoglycans were resolved that were eluted from the column between 60 and 80 min. As indicated by the chromatograms of the two controls, formation of these additional glycans strictly depends upon both SHG<sub>S</sub> and Pho 2. As has been observed with SHG<sub>L</sub> (Fig. 1B), no glucosyl transfer was detectable when SHG<sub>S</sub> was incubated with the phosphorylase from rabbit muscle (data not shown). Thus, neither SHG<sub>L</sub> nor SHG<sub>S</sub> act as a primer for the mammalian glycogen phosphorylase.
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The oligosaccharide pattern of SHG<sub>L</sub> is complex and heterogeneous. In Arabidopsis, some of the oligoglycans have been shown to be associated with the microsomal fraction and are presumably related to cell wall biosynthesis, whereas others reside in the cytosol in a strict sense (Fettke et al. 2005). Thus, the glycan that constitute SHG<sub>L</sub> are both functionally and biochemically inhomogeneous and it is likely that the Pho 2-related priming capacity is restricted to relatively few constituents of the entire oligoglycan pattern shown in Fig. 1D. Until now, these glucosyl-accepting molecules have not been identified. However, the in vitro experiments shown in Fig. 1D strongly suggest that the cytosolic heteroglycan metabolism includes glucosyl transfer reactions between both low and high molecular weight compounds.

Unlike the cytosolic phosphorylase isozyme, Pho 2, the plastidial phosphorylase isozymes are unable to interact with SHG<sub>L</sub>. This conclusion was reached in 14C labeling experiments in which leaf-derived protein fractions were incubated with SHG<sub>L</sub> and 14C-labeled glucose 1-phosphate. Two Pho 1-type phosphorylases (designated as Pho 1a and Pho 1b) exist in leaves of S. tuberosum L. Both genes are highly homologous except for the large insertion (Albrecht et al. 1998). The quaternary structure of the two plastidial gene products is complex: in leaves, Pho 1a occurs in both a homodimeric and a heterodimeric state (Pho 1a–Pho 1b) whereas the level of the homodimeric Pho 1b is usually below the limit of detection (Albrecht et al. 1998). For the 14C labeling experiments, buffer-soluble proteins were extracted from leaves of wild-type and various transgenic potato plants. In three independently generated lines (designated as A, B, and C), the expression of both Pho 1a and Pho 1b was depressed by using two antisense constructs (Duwenig 1996). In another line, the expression of the cytosolic phosphoglycanase isoform was selectively inhibited (line 1; see below). For two reasons, all leaf extracts were subjected to a precipitation with ammonium sulfate (35–55% saturation) prior to use. First, low molecular weight compounds (such as orthophosphate) that possibly interfere with the [14C]glucosyl transfer reaction were removed. Secondly, the hydrolytic activities that are capable of degrading SHG<sub>L</sub> were reduced. The phosphorylase patterns of the five protein preparations, as revealed by non-denaturing PAGE in a glycogen-containing separation gel, are shown in Fig. 2. Due to the high affinity towards glycogen, the cytosolic phosphorylase (Pho 2) is essentially immobile whereas both the homodimeric and the heterodimeric Pho 1-type isoforms are mobile. In the transgenic lines A–C, the Pho 2 activity is indistinguishable from that of the wild type whereas the plastidial isoforms are strongly diminished. Antisense inhibition was most effective in line C. In contrast, leaves from line 1 possess an essentially unchanged level of the Pho 1-type isoforms but expression of Pho 2 is strongly inhibited.

Using the same protein preparations (see Fig. 2), the incorporation of 14C-labeled glucosyl residues into SHG<sub>L</sub> was monitored (Table 2). Incorporation into the heteroglycans observed with protein fractions from lines A–C was almost the same as that with the wild-type protein fraction. Thus, the low levels of the Pho 1-type isoforms do not significantly affect the glucosyl transfer to SHG<sub>L</sub>. In contrast, the reduction of the Pho 2 activity (line 1) clearly decreases the labeling of SHG<sub>L</sub> to that of the control assay in which no SHG<sub>L</sub> was added. This indicates that SHG<sub>L</sub> selectively acts as glucosyl acceptor for Pho 2.

### Table 2

<table>
<thead>
<tr>
<th>Line</th>
<th>14C Incorporation (nmol gle mg&lt;sup&gt;-1&lt;/sup&gt; protein)</th>
</tr>
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<tbody>
<tr>
<td>Wt</td>
<td>0.146 ± 0.014</td>
</tr>
<tr>
<td>A</td>
<td>0.132 ± 0.002</td>
</tr>
<tr>
<td>B</td>
<td>0.134 ± 0.002</td>
</tr>
<tr>
<td>C</td>
<td>0.135 ± 0.011</td>
</tr>
<tr>
<td>I</td>
<td>0.013 ± 0.003</td>
</tr>
<tr>
<td>Control</td>
<td>0.012 ± 0.007</td>
</tr>
</tbody>
</table>

SHG<sub>L</sub> was incubated with [U-14C]glucose 1-phosphate and a protein fraction (buffer-soluble proteins pelletable between 35 and 55% saturation with ammonium sulfate) prepared from leaves of wild-type potato plants or various transgenic lines. In three independent lines (A–C), expression of both Pho 1a and Pho 1b is inhibited. Line 1 contains a Pho 2 antisense construct. After 15 min incubation, the reaction was terminated and the incorporation into SHG<sub>L</sub> was monitored. As a control the SHG<sub>L</sub> was omitted and the sample was processed identically.

Fig. 2 Native PAGE of a protein fraction prepared from leaves of wild-type potato plants or from various transgenic lines. Protein preparations from wild type (wt), lines A–C (antisense inhibition of both Pho 1a and 1b) and line 1 (antisense inhibition of Pho 2) were applied to a native slab gel that contained 0.25% (w/v) glycogen in the separation gel. A 20 µg aliquot of protein was applied per lane. Following electrophoresis (3 h at 250 V constant voltage; 4°C), the separation gel was equilibrated with 0.1 M citrate-NaOH (pH 6.5) at room temperature for 30 min and then incubated in a mixture of 20 mM glucose 1-phosphate and 100 mM citrate-NaOH (pH 6.5) for 120 min at 37°C. Following incubation, the gel was stained with iodine. The open arrows labeled I and II mark the position of the homodimeric [(Pho 1a)<sub>2</sub>] and heterodimeric [Pho 1a–Pho 1b] plastidial phosphorylase isoforms, respectively. The closed arrow marks the position of the cytosolic isozyme, Pho 2.
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It should, however, be noted that the accuracy of the labeling assays as shown in Table 2 is, to some extent, limited by plant-derived hydrolases that act on SHG L. Interference by enzymatic hydrolysis was minimized by using a protein fraction enriched in the phosphorylase isoforms (rather than a crude extract from leaf tissue), relatively short incubation times, low protein concentrations and high glycan concentrations.

For a further in vitro characterization of the Pho 2-related glucosyl acceptors, a two-step protocol was established. In the first step, the Pho 2-catalyzed glucosyl transfer elongated the SHG L preparation. Subsequently, the elongated glycans were deprived of low molecular weight compounds and were then subjected to an enzymatic degradation using either pullulanase (EC 3.2.1.69) or an endo-α-1,5-arabinanase (EC 3.2.1.99). Pullulanase from Klebsiella pneumoniae is a type I debranching enzyme that specifically hydrolyzes α-1,6-D-glycosidic linkages of α-homoglucan-like structures. To ensure the reliability of the data, several controls were included that lacked defined compounds but were otherwise treated identically.

In the first step, SHG L (100 µg) was reacted with recombinant Pho 2 (0.167 nkat phosphorolytic activity) and glucose 1-phosphate for 2 h. As a control, the same amount of SHG L was incubated in the citrate/glucose 1-phosphate mixture but Pho 2 was omitted. Subsequently, both mixtures were heated, deprived of low molecular compounds and were then incubated with pullulanase. The degradation products were separated into compounds having a size below or above 10 kDa. The low molecular weight compounds were resolved by HPAEC-PAD (Fig. 3A). Following the Pho 2-dependent glucosyl transfer, pullulanase released a series of oligosaccharides ranging from DP 1 to 6, with glucose being the most prominent compound. Another major constituent (DP X; Fig. 3A) that has not yet been identified eluted between glucose and maltose. Enzymatic digestion of SHG L without a Pho 2-dependent elongation resulted in the liberation of small amounts of glucose and DP X. Thus, SHG L contains α-1,6 linked glucosyl residues that are accessible to pullulanase.

Using the same experimental conditions, glycogen (50 µg) was reacted with the same amount of pullulanase (Fig. 3B). The pattern of the oligosaccharides released ranged from DP 2 to approximately 10, with maltotriose being most prominent. This oligosaccharide pattern is typically observed after exhaustive pullulanase treatment of glycogen. Thus, the size distributions of the side chains that are liberated by pullulanase from SHG L or glycogen differ significantly.

The >10 kDa glycan fraction (that is retained after Pho 2-catalyzed elongation and subsequent pullulanase treatment) was analyzed following acid hydrolysis (Fig. 3C). As revealed by HPAEC-PAD, arabinose, rhamnose, galactose and glucose were the major constituents. Compared with the Pho 2-deficient control, the Pho 2-catalyzed glycan elongation resulted in a significant increase in the glucose content of the >10 kDa...
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Thus, a significant proportion of the glucosyl residues transferred by Pho 2 are not released by pullulanase. In another series of experiments, SHG<sub>L</sub> was elongated by Pho 2 as described in Fig. 3 but in the subsequent hydrolase treatment pullulanase was replaced by a fungal endo-β-1,5-arabinanase. This hydrolase cleaves internal β-1,5-linkages between arabinose residues. The oligoglycans (<10 kDa) released from SHG<sub>L</sub> by arabinanase were analyzed by HPAEC-PAD (Fig. 4A). A series of oligoglycans was eluted between 15 and 25 min, but these compounds were detectable only following the Pho 2-catalyzed elongation (see Fig. 4A + Pho 2 + arabinanase and – Pho 2 + arabinanase). Furthermore, the occurrence of these glycans strictly depends upon the treatment of SHG<sub>L</sub> with arabinanase. When the SHG<sub>L</sub> was treated with arabinanase without a preceding Pho 2-dependent elongation, the pattern of the oligosaccharides released was restricted to some early eluting (5–12 min) compounds. As a further control, an equal amount of glycogen was incubated with the same amount of arabinanase using exactly the same sample processing procedure. Under these conditions, no oligosaccharides were released (Fig. 4B). This indicates that, under the conditions used, the fungal arabinanase does not hydrolyze inter-glucose linkages.

Following the arabinanase treatment of the elongated SHG<sub>L</sub>, the >10 kDa glycans were also analyzed. The monosaccharide patterns of the high molecular weight fraction are shown in Fig. 4C. Three controls were included. The arabinanase treatment of the elongated SHG<sub>L</sub> resulted in a significant decrease in the contents of arabinose and galactose. In contrast, the decrease in glucose and rhamnose was less. When arabinanase was reacted with SHG<sub>L</sub> without a preceding Pho 2-catalyzed elongation, the arabinose and galactose levels of the >10 kDa glycans were lowered but the levels of both rhamnose and glucose were essentially unchanged. Thus, the cleavage of inter-arabinose linkages releases both arabinosyl and galactosyl residues irrespective of a preceding elongation. As the arabinanase treatment after the Pho 2-dependent elongation decreased the glucose content of the >10 kDa fraction (Fig. 4C), some of the glucosyl acceptor sites used by Pho 2 are susceptible to the arabinanase. However, as the elongated >10 kDa glycans still contain more glucose than the respective control glycans, the arabinanase treatment is not capable of releasing all of the glucosyl residues that have been transferred by Pho 2.

These conclusions were confirmed by a detailed analysis of the oligosaccharides that were released by arabinanase from a Pho 2-elongated SHG<sub>L</sub> preparation (see Fig. 4A). In this particular experiment, 50 µg of SHG<sub>L</sub> was incubated with 0.167 nkat of the recombinant Pho 2 for 150 min. Following termination of the reaction, low molecular weight compounds were removed and the >10 kDa glycans were treated with arabinanase. Subsequently, the oligosaccharides released were resolved by HPAEC-PAD and were collected as two separate pools eluting during 18–21 min (pool a; Fig. 5A) and during 21–24 min (pool b). Glycans from both pool a and b were hydrolyzed and the monomer patterns were determined by HPAEC-PAD (Fig. 5B). As expected from the chromatogram, the relative glucose content of pool b was higher than that of the earlier eluting pool a. However, it is important to note that...
both pools contained, in addition to glucose, arabinose, galactose, xylose, mannose and traces of rhamnose as well. For phosphorylases, the non-reducing ends of (side) chains function as glucosyl acceptor sites. Thus, some of the glucosyl acceptor sites used by Pho 2 form the non-reducing ends of chains that are susceptible to the arabinanase treatment. As these chains contain several non-glucosyl residues, such as xylose, mannose, arabinose and galactose, they represent heterooligosaccharyl constituents of SHG_L.

In summary, the in vitro elongation and hydrolysis experiments shown in Fig. 3–5 clearly indicate that the recombinant Pho 2 utilizes biochemically heterogeneous acceptor sites in SHG_L.

Physicochemical and biochemical heterogeneity of the SHG_L fraction isolated from potato leaves was demonstrated by using several approaches. First, the SHG_L fraction possesses an asymmetric size distribution when subjected to FFF multangle laser light scattering (FFF-MALLS-RI; Fig. 6A). One part of the glycans (designated as subfraction I) is eluted between 4 and 8 min (Fig. 6A), but higher molecular weight compounds were removed by filtration and the glycans retained were hydrolyzed. Monosaccharides released were analyzed by HPAEC-PAD. Sub I/II + Pho 2, complete reaction mixtures; Sub I/II – Pho2, during incubation Pho 2 was omitted.

Fig. 6 Separation of SHG_L into subfraction I and II. (A) FFF-RI elution profiles of the entire SHG_L fraction, of the β-glucosyl Yariv reagent reactive glycans (precipitate) and the Yariv reagent non-reactive glycans (supernatant). (B) In a final volume of 40 µl, subfraction I (Sub I) and II (Sub II; 10 µg each) were incubated with the recombinant Pho 2 (0.167 nkat) for 60 min at 37°C. Following incubation, low molecular weight compounds were removed by filtration and the glycans retained were hydrolyzed. Monosaccharides released were analyzed by HPAEC-PAD. Sub I/II + Pho 2, complete reaction mixtures; Sub I/II – Pho2, during incubation Pho 2 was omitted.
Yariv reagent-reactive glycans was essentially the same as that of subfraction II, whereas that of the non-reactive glycans is indistinguishable from that of subfraction I (data not shown). Thus, subfraction I and II are essentially equivalent to the Yariv-non-reactive and -reactive glycans, respectively. Similar results have been obtained for SHG-L from *P. sativum* L. and *A. thaliana* L. (Fettke et al. 2004, Fettke et al. 2005). However, the ratio between subfractions I and II varies strongly depending upon both the plant species and plant organ. Compared with *A. thaliana* L. and *P. sativum* L., leaves of *S. tuberosum* L. possess a higher proportion of subfraction I. In potato tubers, the relative content of subfraction I is even higher and accounts for >90% of SHG-L (data not shown).

Both subfraction I and II from potato leaves were resolved by FFF and were then tested for priming capacity for Pho 2. To minimize cross-contamination, subfraction I contained only those glycans that eluted between 4 and 7 ml, and subfraction II contained those eluting between 9 and 15 ml (Fig. 6A). Following incubation with recombinant Pho 2 and glucose 1-phosphate, the monosaccharide patterns of both glycans were determined by HPAEC-PAD (Fig. 6B). As indicated by the increased glucose content, subfraction I clearly acted as glucosyl acceptor for Pho 2 whereas subfraction II did not.

The monosaccharide pattern of the entire subfraction I (Fig. 6) clearly deviates from that of the chains released by arabinanase treatment (Fig. 5B). Whilst arabinose and galactose are the most prominent compounds of the entire subfraction I, the patterns of the chains which are released by arabinanase and which act as glucosyl acceptor sites for Pho 2 are dominated by xylose and mannose.

The data shown in Fig. 6 clearly demonstrate that subfraction I possesses a Pho 2-related priming capacity. However, the question remains as to whether the entire subfraction I acts as glucosyl acceptor or, alternatively, this biochemical function is restricted to a distinct subpopulation. Biochemical homogeneity or heterogeneity of subfraction I was tested by using extended periods of reaction time (24 or 48 h). Following incubation, the heteroglycans were analyzed by FFF (Fig. 7A). The Pho 2-catalyzed glucosyl transfer resulted in a shift of the entire subfraction I into the higher molecular weight region with a concomitant disappearance of the original subfraction I peak (Fig. 7A). This indicates that essentially the entire subfraction I did act as glucosyl acceptors and, therefore, participates in the Pho 2-catalyzed elongation.

For a further characterization of the elongated glycans, the eluate of the FFF was collected in distinct samples as indicated in Fig. 7A (eluate samples I–X). All samples were subjected to acid hydrolysis and the monomer patterns were analyzed by HPAEC-PAD. For a quantitative evaluation, three non-glucose monomers were estimated by HPAEC-PAD and were plotted against the respective eluate sample (Fig. 7B). Due to the Pho 2-catalyzed glucosyl transfer, the non-glucose constituents of the glucosyl acceptor(s) participate in the shift towards the higher molecular weight region. It should, however, be noted that following massive glycan elongation, a part of subfraction I is insoluble and was removed by centrifugation prior to separation by FFF.

Taken together, these data clearly indicate that the Pho 2-related priming capacity is an intrinsic property of subfraction I. Furthermore, the data clearly confirm that the Pho 2-related glucosyl acceptors are heteropolysaccharides.

**Subcellular localization of subfraction I and II**

For two reasons, the subcellular distribution of the heteroglycans in potato leaves was studied in more detail: first, the monomer composition of SHG_I and SHG_S (Fig. 1) deviates
from that previously described for leaves of *A. thaliana* L. (Fettke et al. 2005). Secondly, the DPE2 enzyme from potato leaves has been claimed to be a plastidial protein (Lloyd et al. 2004, Lloyd et al. 2005) whereas in *A. thaliana* L. this enzyme has been attributed to the cytosol (Chia et al. 2004, Fettke et al. 2005). If in *S. tuberosum* L. this enzyme is restricted to the plastids, its biochemical function remains obscure unless the subcellular distribution of soluble carbohydrate pools has been elucidated for this plant species.

For subcellular localization, two approaches were chosen: in the first approach, potato leaf material was subjected to non-aqueous fractionation. Following centrifugation, the apolar gradient was divided into five fractions and equal aliquots of each fraction were withdrawn for the quantification of marker proteins or enzyme activities. The residual of each apolar fraction served as the starting material for the heteroglycan isolation. The pellic fraction is designated as P. The four other fractions and marker enzyme distributions using a three-compartment calculation program. The subcellular distribution was calculated by comparing the glycan and marker enzyme distributions using a three-compartment calculation program.

<table>
<thead>
<tr>
<th>Compound/marker</th>
<th>Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>AGPase (plastidal)</td>
<td>1.9</td>
</tr>
<tr>
<td>GWD (plastidal)</td>
<td>1.4</td>
</tr>
<tr>
<td>Pho 2 (cytosolic)</td>
<td>6.2</td>
</tr>
<tr>
<td>Subfraction I</td>
<td>7.5</td>
</tr>
<tr>
<td>Glucosidase (vacuolar)</td>
<td>11.8</td>
</tr>
<tr>
<td>Mannosidase (vacuolar)</td>
<td>8.8</td>
</tr>
<tr>
<td>Subfraction II</td>
<td>40.1</td>
</tr>
</tbody>
</table>

The subcellular distribution was calculated by comparing the glycan and marker enzyme distributions using a three-compartment calculation program.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Distribution (%)</th>
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<tbody>
<tr>
<td>Subfraction I</td>
<td>Subfraction II</td>
</tr>
<tr>
<td>Vacuole/Apoplast</td>
<td>12</td>
</tr>
<tr>
<td>Plastid</td>
<td>0</td>
</tr>
<tr>
<td>Cytosol</td>
<td>88</td>
</tr>
</tbody>
</table>

The data shown in Fig. 8 clearly indicate a cytosolic location of the potato DPE2 and are consistent with the localization of the enzyme reported for *Arabidopsis* leaves (Chia et al. 2004). They do, however, not concur with the recently proposed plastidial location of this enzyme (Lloyd et al. 2004). During aqueous fractionation of potato leaves, we have consistently observed that DPE2 tends to bind to particulate α-glucans, such as starch granules, and the particulate starch often co-separates with intact chloroplasts. Therefore, the binding of DPE2 to starch granules that occurs during or after tissue homogenization may result in an association with the plastidial compartment. By using the technique of non-aqueous tissue fractionation, these artifacts can be avoided.

Subfractions I and II are distributed differently in the apolar gradient: subfraction I possesses the same distribution as cytosolic marker enzymes such as Pho 2 and DPE2 (Table 3). For a more quantitative approach, the subcellular distribution of subfraction I and II was calculated according to Riens et al. (1991). Using this approach, most (88%) of subfraction I but 0% of subfraction II was attributed to the cytosol (Table 4).
Based on the data shown in Tables 3 and 4, the precise subcellular location of subfraction II remains ambiguous. In Arabidopsis leaves, the distribution of apoplastic markers, such as crystalline cellulose, does not differ from that of vacuolar constituents (Fettke et al. 2005). Therefore, subfraction II could be located either in the vacuole or in the apoplast (or in both). However, in isolated intact mesophyll protoplasts prepared from leaflets of P. sativum L., subfraction I but not subfraction II was retained and, therefore, a vacuolar location of the latter was excluded (Fettke et al. 2004). Probably, this is also the case for S. tuberosum L. However, the precise subcellular location of subfraction II is not the focus of this communication as these glycans (as opposed to subfraction I) do not participate in the Pho 2-catalyzed glucosyl transfer reactions.

The non-aqueous fractionation does not permit a distinction to be made between the cytosol (in a strict sense) and minor compartments such as mitochondria, peroxisomes or (intra-)cellular membranes that reside in the cytosol but constitute distinct subcellular sites. The distinction between the cytosolic compartment in a strict and a wide sense is especially relevant as the SHGs possess some similarity with cell wall-related carbohydrates (see also Table 6) and the possibility remains that the glycans attributed to the cytosol (Tables 3 and 4) occur associated with membranes, such as the Golgi apparatus or the plasmalemma. If so, they would not necessarily be accessible for cytosolic proteins. In order to test this possibility, intact microsomes or organelles (mitochondria plus peroxisomes) were isolated from potato leaves and were then pelleted. For the microsome preparation, UDPase and the luminal Hsp70 chaperone BIP (Alder and Johnson 2004) were used as marker proteins whereas citrate synthase and catalase served as markers for mitochondria and peroxisomes, respectively. In both preparations, cytosolic contaminations were quantified by monitoring Pho 2 (Table 5). The distribution of SHG\textsubscript{T} isolated either from the pelleted microsomes/organelles or from the respective supernatants was the same as that of Pho 2. From these data, it can be concluded that the heteroglycans present in the microsomal or mitochondria/peroxisome preparations are due to cytosolic contaminations rather than to any membrane or organelle association.

Glycosidic linkages of the Pho 2-related heteroglycans

SHG\textsubscript{T} was resolved into subfraction I and II by FFF. The glycosidic linkages of both subfractions were analyzed and quantified (Table 6). A high number of different glycosidic linkages was detected in both glycans. The by far most prominent linkage is 3,6-Gal\textsubscript{p} that represents 25 and >40% of the

<table>
<thead>
<tr>
<th>Table 5 Characterization of the microsome and organelle preparations</th>
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<tbody>
<tr>
<td><strong>Microsome preparation</strong></td>
</tr>
<tr>
<td>Latent UDPase</td>
</tr>
<tr>
<td>BIP</td>
</tr>
<tr>
<td>Pho 2</td>
</tr>
<tr>
<td>SHG\textsubscript{T}</td>
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</tbody>
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<table>
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<tr>
<th><strong>Organelle preparation</strong></th>
<th><strong>Organelles</strong></th>
<th><strong>Supernatant</strong></th>
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</thead>
<tbody>
<tr>
<td>Citrate synthase</td>
<td>67.9</td>
<td>32.1</td>
</tr>
<tr>
<td>Catalase</td>
<td>59.5</td>
<td>40.5</td>
</tr>
<tr>
<td>Pho 2</td>
<td>1.1</td>
<td>98.9</td>
</tr>
<tr>
<td>SHG\textsubscript{T}</td>
<td>1.9</td>
<td>98.1</td>
</tr>
</tbody>
</table>

Distribution of the marker enzymes is given as a percentage. Carbohydrate amounts are given as a percentage of the sum of the glycans derived from the pellet and the supernatant. UDPase, UDP phosphatase; BIP, binding protein; Pho 2, cytosolic phosphorylase; n.d., not detected. Latent UDPase was determined according to Schaller and DeWitt (1995) using the Malachite Green method to estimate the release of orthophosphate.

<table>
<thead>
<tr>
<th>Table 6 Glycosidic linkage analysis of subfractions I and II of SHG\textsubscript{T}</th>
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<tbody>
<tr>
<td><strong>Sugar and linkage(s)</strong></td>
</tr>
<tr>
<td>Arabinose</td>
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<tr>
<td>Galactose</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>Mannose</td>
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<tr>
<td>Xylose</td>
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<td>Rhamnose</td>
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<td></td>
</tr>
<tr>
<td>Fucose</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

All sugars and linkages are given in mol% (nomenclature according to Selvendran and O’Neill 1987). P, pyranose; f, furanose.
Cytosolic heteroglycans in *Solanum tuberosum* L. 1998

Linkages observed in subfraction I and II, respectively. Taking into account that in subfraction I some of the glucosyl and mannosyl linkages also indicate branching points, it is obvious that both subfractions are highly branched polysaccharides. Next to 3,6-Galp, three other galactosyl residues (3-Galp, 6-Galp and terminal galactose) and terminal arabinose as well are recovered as the most prominent linkages. However, their relative frequencies differ in subfraction I and II. Furthermore, subfraction I contains more glucose, xylose, mannose and fucose than subfraction II and, compared with the latter, the linkages related to these four monosaccharides possess a higher diversity (Table 6). In addition, subfraction I possesses more compounds that could not be identified by their mass spectrometry spectra. In summary, all these data indicate a higher structural complexity of subfraction I compared with subfraction II.

In transgenic potato plants, the alteration of the expression of Pho 2 affects the size distribution of SHG subfraction I but not subfraction II. In transgenic potato plants, expression of Pho 2 was lowered by using an antisense construct (Duwenig et al. 1997). Pho 2-overexpressing transgenic plants were generated by transformation with a cDNA that encodes, under the control of the 35S promoter, the Pho 2 from *V. faba* L. In these plants, the expression of the endogenous Pho 2 isoform was essentially unaffected. This is indicated by Northern blotting experiments performed with probes that selectively interact with the Pho 2 transcripts from either *Solanum* or *Vicia* (Fig. 9A). The leaf phosphorylase patterns of two lines each of underexpressing and overexpressing potato plants are shown in Fig. 9B. In this particular experiment, the separation gel did not contain any carbohydrate and, therefore, the cytosolic phosphorylase isoforms possess a higher mobility than the plastidial isozymes (cf. Fig. 2). Both the transgenic and the endogenous Pho 2 do not differ in electrophoretic mobility and form a single band of activity. All four transgenic lines and the wild-type plants possess approximately the same level of the plastidial phosphorylase (Pho 1) but the level of the Pho 2 activity varied, covering a wide range. In the most efficient overexpressing potato plants.
For a phenotypical characterization of the transgenic plants, various carbohydrate pools and the heteroglycans were analyzed. Under the growth conditions applied, the starch content of both the under- and overexpressing plants did not differ significantly from that of the wild-type control (Table 7). Similarly, the levels of neutral mono- and disaccharides (glucose, fructose and sucrose) were essentially unchanged. However, the levels of both glucose 6-phosphate and fructose 6-phosphate were higher in the Pho 2 antisense plants when harvested in the light period, whereas the glucose 1-phosphate content was not significantly altered. Overexpressing plants did not differ from the wild type in any of the three hexose phosphates (Table 8).

\[
\begin{array}{ccccccc}
\text{Line} & \text{Time of harvest} & \text{Carbohydrate (mmol g}^{-1}\text{FW)} & \text{Starch (g-equivalent)} \\
\hline
\text{Wild type} & \text{a} & 0.5 \pm 0.2 & 0.9 \pm 0.3 & 2.0 \pm 0.5 & 3.0 \pm 0.8 \\
 & \text{b} & 1.0 \pm 0.6 & 1.2 \pm 0.3 & 1.8 \pm 0.3 & 28.7 \pm 6.7 \\
 & \text{c} & 0.7 \pm 0.1 & 1.3 \pm 0.2 & 6.5 \pm 0.6 & 86.1 \pm 14.2 \\
\text{1} & \text{a} & 0.7 \pm 0.3 & 1.0 \pm 0.2 & 1.4 \pm 0.3 & 2.7 \pm 1.0 \\
 & \text{b} & 1.2 \pm 0.2 & 1.4 \pm 0.3 & 2.5 \pm 0.8 & 25.2 \pm 9.4 \\
 & \text{c} & 0.9 \pm 0.2 & 1.7 \pm 0.4 & 6.0 \pm 1.3 & 75.6 \pm 9.8 \\
\text{2} & \text{a} & 0.8 \pm 0.3 & 0.5 \pm 0.1 & 1.3 \pm 1.0 & 4.1 \pm 1.3 \\
 & \text{b} & 1.6 \pm 0.6 & 0.9 \pm 0.4 & 2.1 \pm 0.6 & 24.0 \pm 4.3 \\
 & \text{c} & 1.0 \pm 0.3 & 2.0 \pm 0.4 & 5.6 \pm 0.7 & 72.0 \pm 11.0 \\
\text{3} & \text{a} & 0.9 \pm 0.1 & 1.5 \pm 0.2 & 2.1 \pm 0.3 & 1.8 \pm 0.5 \\
 & \text{b} & 0.9 \pm 0.3 & 1.4 \pm 0.6 & 2.2 \pm 0.7 & 25.5 \pm 3.8 \\
 & \text{c} & 0.8 \pm 0.1 & 1.3 \pm 0.1 & 5.1 \pm 0.4 & 76.5 \pm 15.8 \\
\text{4} & \text{a} & 0.9 \pm 0.3 & 0.7 \pm 0.3 & 2.4 \pm 0.3 & 4.1 \pm 1.6 \\
 & \text{b} & 0.9 \pm 0.4 & 1.0 \pm 0.1 & 1.9 \pm 0.5 & 20.7 \pm 3.2 \\
 & \text{c} & 0.7 \pm 0.1 & 1.2 \pm 0.3 & 5.2 \pm 1.2 & 95.2 \pm 8.1 \\
\end{array}
\]

Table 7 Carbohydrate contents from leaves of wild-type, Pho 2 antisense (1 and 2) and overexpressing (3 and 4) lines

Starch and soluble sugars were extracted from leaves that were harvested at the end of the dark period (a), after 4 h in the light (b) and at the end of the light period (c), respectively. Data are given as averages with the SD (n = 5).

SHG\textsubscript{4} was isolated from the transgenic lines and the wild-type control as well. Subsequently, the SHG\textsubscript{4} preparations were analyzed by FFF-MALLS. In addition, aliquots of SHG\textsubscript{4} subjected to acid hydrolysis and the monosaccharide patterns of the hydrolysates were analyzed by HPAEC-PAD (Fig. 10A, B). The following effects were consistently observed.

Another phenotypic difference that presumably is functionally relevant is related to the dynamics of subfraction I. Wild-type leaves were harvested at four different time points during the light–dark cycle and used for the isolation of SHG\textsubscript{4}. Subsequently, the preparations were analyzed by FFF-MALLS and the size distribution of subfraction I and II was determined (Fig. 10C). Whilst subfraction II was essentially unchanged throughout the entire light–dark regime, the size distribution of subfraction I increased during the dark period. In samples prepared from illuminated leaves, subfraction I had an average molar mass of 4x10\textsuperscript{10} Da. When leaves were harvested during darkness, this value increased to approximately 5x10\textsuperscript{10} Da. These dynamics strongly suggest that subfraction I, as opposed to subfraction II, is a metabolically active polyglycan pool of the leaf cells and responds to the intracellular carbon fluxes that are altered during the light–dark regime. In both types of transgenic potato plants (that either underexpress or overexpress
Cytosolic heteroglycans in *Solanum tuberosum* L. 2000

Pho 2), the size distribution of subfraction I is increased throughout the light–dark cycle. In Fig. 10D, this is shown for leaf samples harvested during the middle of the light period. When the leaves were harvested during the dark period, the size distribution of subfraction I from both types of transgenic plants increased to 8–9×10^5 Da (Fig. 10E). Remarkably, the molecular weight of subfraction I from the antisense plants is somewhat higher than that from the overexpressing plants. In contrast, the size distribution of subfraction II is unaffected in all transgenic plants. In Fig. 10C–E, the size distributions of subfraction I and II are documented for only one line from each type of transformant. However, the same effects were observed in four independently generated lines (data not shown).

**Discussion**

In this communication, we provide evidence that in leaves of *S. tuberosum* L. the cytosolic phosphorylase isoform (Pho 2) interacts with distinct pools of cytosolic heteroglycans both in vitro and in vivo. The carbohydrate analyses are complicated by the fact that, in addition to the cytosolic glycans, SHGs (designated as subfraction II) that contain both arabinose and galactose occur outside the plant cell. Presumably, these glycans are constituents of the complex extracellular matrix formed by various non-cellulose polysaccharides (Burton et al. 2005). Despite some similarities in the glycosidic linkage patterns, these glycans possess a higher degree of polymerization and, therefore, can be separated by FFF. Alternatively, they react with the β-glucosyl Yariv reagent allowing a selective precipitation of the apoplastic SHGs. Using in vivo 14CO₂-dependent labeling in *Arabidopsis* leaves, the cytosolic and apoplastic heteroglycans have been shown to possess different labeling kinetics and, therefore, appear to be placed in different pathways (Fettke et al. 2005). It is highly unlikely that the heteroglycan fractions described here contain, to any noticeable extent, covalently bound peptide or protein. All attempts failed to detect any peptide or protein that is released by established chemical deglycosylation procedures (Sojar and Bahl 1987, Rademaker et al. 1998; data not shown). Under in vitro conditions, the cytosolic heteroglycans act as glucosyl acceptor for Pho 2 and a massive Pho 2-catalyzed elongation results in a

![Fig. 10](image-url) Characterization of heteroglycans from leaves of transgenic and wild-type potato plants. (A) Monomer composition of SHG₄ derived from the wild type (wt), line 1 (underexpressing) or line 4 (overexpressing) of transgenic potato plants. Leaves were harvested in the middle of the light period. All chromatograms were normalized to galactose. (B) Glucosyl (Glu; insert) and rhamnosyl (Rha; insert) contents of SHG₄. (C) Molar mass distributions of subfraction I and II as revealed by FFF-MALLS-RI. SHG₄ was isolated from leaves of wild-type plants harvested at four time points of the light or dark period. (D and E) Molar mass distributions of subfraction I and II from leaves of transgenic plants (line 4, overexpressing Pho 2; line 1, underexpressing Pho 2) and wild type. Leaves were harvested at the middle of the light (D) or dark (E) period.
gradual disappearance of the original heteroglycan population and the appearance of a peak having a higher molecular weight (Fig. 7A). The glycan elongation is accompanied by a shift of the non-glucose constituents, such as arabinose, rhamnose and galactose, towards the high molecular weight region (Fig. 7B). These data confirm and extend earlier results obtained by gel filtration using a heteroglycan preparation from pea tissues. However, in the earlier study, an alternative isolation procedure had been used. Furthermore, the cytosolic heteroglycans had not been separated from the apoplastic glycans and, therefore, not all glycans participated in the Pho 2-catalyzed elongation process (Yang and Steup 1990). The data presented in Fig. 7 are fully consistent with the distribution of the Pho 2-dependent priming capacity as determined for Arabidopsis leaves by using non-aqueous fractionation. As the priming capacity closely co-distributes with the entire subfraction I, the conclusion was reached that priming is not restricted to a proportion of the subfraction I glycans but rather is an intrinsic property of the cytosolic glycans (Fettke et al. 2005).

The Pho 2-catalyzed glycan elongation leads to the formation of oligosaccharyl chains that are released by either pullulanase (Fig. 3) or arabinanase (Fig. 4, 5). The oligosaccharides released by both hydrolyses differ: pullulanase liberates glucans having a lower degree of polymerization than the chains released from glycogen (Fig. 3). This is consistent with the fact that neither subfraction I nor II possesses detectable amounts of typical starch-like interglucose linkages. However, it concurs with the occurrence of 6-Glcp (Table 6). Arabinanase that is ineffective with glycogen is capable of releasing glycan chains having a higher molecular weight and possessing a monomer composition that strongly deviates from that of the entire glycan preparation. Thus, it is very unlikely that the heteroglycans are composed of a few highly repetitive carbohydrate repeats. The observed action of arabinanase is consistent with the presence of 5-Ara in the glycosidic linkage pattern (Table 6). In addition to subfraction I, the SHG₁ also contain oligoglycans that clearly act as Pho 2-dependent primers (Fig. 1D). Similarly, SHG₃ operates as glucosyl donor when incubated with Pho 2 in the presence of orthophosphate rather than glucose 1-phosphate (data not shown). Due to the high rhamnose content, this glycan fraction deviates from that of P. sativum L. and A. thaliana L. In addition, a Pho 2-related priming capacity was not found with the respective pea glycan (Fettke et al. 2004, Fettke et al. 2005). It is, however, conceivable that at least some Pho 2-dependent glucosyl acceptor sites can be generated by very few glucosyl transfer reactions and, therefore, a non-priming glycan can be converted into a priming one. Once an acceptor site is established, Pho 2 can use it for repetitive glucosyl transfer reactions. Due to the complex intracellular location of SHG₃, these glycans are difficult to analyze. However, it is likely that some of these low molecular weight compounds participate in the cytosolic heteroglycan metabolism.

In the transgenic potato plants, the expression of the target protein (Pho 2) was altered. As revealed by immunocytochemistry, the alteration was effective in the same compartment in which subfraction I resides. Unlike starch and the main neutral sugars (such as sucrose, glucose and fructose), subfraction I is affected in vivo by altered levels of the cytosolic phosphorylase isozyme. Antisense inhibition of Pho 2 expression has several effects: both the relative glucose and rhamnose contents are elevated and the size distribution is increased. It should, however, be noted that the latter is not due to the observed change in the two monomer levels (Fig. 10). As both glucose and rhamnose are minor constituents of subfraction I, the increase in molecular mass as observed in the transgenic plants also requires a massive incorporation of the major constituents, arabinose and galactose.

Based on the diversity of the glycosidic linkages (Table 6) and following the “one enzyme one linkage” rule, it has to be postulated that the biosynthesis of the cytosolic heteroglycans requires >20 different glycosyl transferases most of which are totally unknown. Currently, the cytosolic phosphorylase isozyme and the transglucosidase (DPE2) have been identified as the only candidates for enzymes involved in this part of the primary metabolism. Two observations are relevant in this context: first, under in vitro conditions, both Pho 2 and DPE2 act on glycogen which forms a non-physiological substitute for the cytosolic heteroglycans. Secondly, in leaves of the Arabidopsis mutant deficient in DPE2, the Pho 2 level is increased 4-fold whereas that of the plastidial phosphorylase isozyme remained unchanged (Chia et al. 2004). Currently, we are studying the carbohydrate specificities of the two cytosolic transferases in more detail.

Arabidopsis insertion mutants that are deficient in Pho 2 have been reported to be lethal (Weise et al. 2004). In contrast, potato plants having a reduced level of the target protein (due to an antisense construct) did not differ significantly in their starch or sucrose content (Table 7). Presumably, this points to overlapping biochemical functions that are exerted by various cytosolic glucosyl transferases. However, some Pho 2-dependent transfer reactions appear to be irreplaceable.

In potato leaves, the molar mass of subfraction I increased during the dark period (Fig. 10). These changes in size are consistent with a cytosolic glycan metabolism that has recently been postulated by several groups (Zeeman et al. 2004, Lloyd et al. 2005, Smith et al. 2005). According to this view, starch-derived maltose is exported into the cytosol and metabolized by a series of glucosyl transfer reactions that include high molecular weight glycans acting as acceptors. As the diurnal fluctuation of the transitory starch pool by far exceeds that of the heteroglycan subfraction I, it is expected that the latter possesses a much higher turnover rate. In the transgenic potato plants possessing altered levels of Pho 2, the diurnal changes in the size distribution of the cytosolic heteroglycans are retained. However, they are shifted towards the high molecular weight region. Thus, in both types of transgenic plants, the cytosolic...
heteroglycans possess a higher degree of polymerization both during the light and in the dark period (Fig. 10D, E). Based on these phenotypes, we propose that, in addition to Pho 2, many other cytosolic glycosyl transferases are involved in the turnover of the fraction I heteroglycans. Therefore, altered levels of Pho 2 are expected to affect more strongly the molecular properties of the heteroglycans than the carbon fluxes through this polysaccharide pool.

Materials and Methods

Plant material

Potato (S. tuberosum L. cv Desiree) tubers were purchased from Saatzucht Fritz Lange KG (Bad Schwartau, Germany). Potato plants were grown under controlled conditions [16 h light period (300 µE m\(^{-2}\) s\(^{-1}\)), 20°C and 8 h darkness, 17°C]. At the beginning and the end of the light period, illumination was lowered to 150 µE m\(^{-2}\) s\(^{-1}\) for 30 min each time. Throughout the light-dark regime, the relative humidity was kept at 50%.

Isolation of the SHG and carbohydrate quantification

Leaves of S. tuberosum L. were harvested during the light period except where stated. Leaves were deprived of mid veins, frozen in liquid nitrogen. The leaf material (5 g FW) was resuspended in 10 ml of 20% (v/v) ethanol and carefully mixed (in a vortex). Tuber tissue (30 g FW) was cut into small pieces and was further homogenized in 60 ml of 20% (v/v) ethanol using a Waring blender. Subsequently, SHG\(_1\) was isolated as previously described (Fettke et al. 2004). For quantification, glycans were hydrolyzed and the monosaccharides were monitored according to Fettke et al. (2004). Using the procedure described, the yield of the glycan preparation is approximately 0.5–1 mg of SHG\(_1\) g\(^{-1}\) FW of potato leaf material, depending upon the age of the leaves and the growth conditions of the plants. Similarly, the monosaccharide patterns of the SHG preparations exhibited minor variations. Therefore, in each experiment, all controls were performed using the same SHG preparation and exactly the same conditions. Within a wide range of material (3–35 g FW) and using a ratio of 3 ml of 20% (v/v) ethanol g\(^{-1}\) FW, the same (fresh weight based) SHG\(_1\) yield was obtained. Similarly, neither yield nor monomer composition of SHG\(_1\) was affected if the heating period was varied from 3 to 15 min (data not shown). Furthermore, extraction of the leaf material (5 g) with 60 ml of 20% (v/v) ethanol resulted in essentially the same SHG\(_1\) yield (data not shown). All these data indicate that the SHG\(_1\) is quantitatively extracted from the leaf material.

For quantification of starch, sucrose, fructose and glucose, leaf discs (70–100 mg FW) were extracted twice (20 min each) in 0.5 ml of 80% (v/v) ethanol at 80°C. The supernatants were combined and used for the estimation of the monosaccharides and disaccharides according to Stitt et al. (1989). For starch determination, the residue was washed with 1 ml of ice-cold water and was then homogenized in 0.4 ml of 200 mM KOH. The homogenate was incubated for 1 h at 95°C and was then neutralized by adding approximately 70 µl of 1 M acetic acid. Following centrifugation (10 min at 20,000 g), 25 µl of the supernatant was reacted with 12 nkat amyloglucosidase dissolved in 50 µl of 50 mM sodium acetate pH 4.7, for 1 h at 55°C. Glucose released was estimated according to Stitt et al. (1989).

For quantification of hexose monophosphates, leaf material (1–2 g FW) was frozen in liquid nitrogen. Subsequently, samples were homogenized using a mortar and the homogenate was extracted using 10% (v/v) perchloric acid. Samples were processed and analyzed according to Stitt et al. (1989).

High-performance anion-exchange chromatography and field flow fractionation

Monosaccharides or oligosaccharides were separated by anion-exchange chromatography essentially as described elsewhere (Fettke et al. 2004). However, oligosaccharides were eluted using a linear gradient of sodium acetate (5–500 mM in 30 min), followed by an elution with 500 mM sodium acetate dissolved in 100 mM NaOH (10 min). Alternatively, the oligosaccharides were eluted in a sodium acetate gradient (5–500 mM in 110 min). Throughout the chromatography, the flow rate was 1 ml min\(^{-1}\). FFF-MALLS and FFF-MALLS-RI analyses of SHG\(_1\) were performed according to Fettke et al. (2004). However, a constant channel flow (1 ml min\(^{-1}\)) and a linear cross-flow gradient (0–5 min, 3 ml min\(^{-1}\); 20 min, 0.5 ml min\(^{-1}\)) were applied.

Glycan precipitation with β-glucosyl Yariv reagent

Glycans were reacted with β-glucosyl Yariv reagent [1, 3,5-tris(4-β-D-glycopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene; Biosupplies Australia] as previously described (Fettke et al. 2004).

Non-aqueous fractionation, organelle and microsome preparations and determination of marker enzyme activities

Leaf material was harvested during the light period and was frozen in liquid nitrogen. Non-aqueous fractionation was performed as previously described (Fettke et al. 2005). Isolation of intact organelles, microsomes and determination of marker enzyme activities were performed as described elsewhere (Fettke et al. 2005).

Generation of transgenic plants with altered Pho2 expression and heterologous expression of Pho 2

The generation of potato plants expressing an antisense construct directed against Pho 2 has been described elsewhere (Duwenig et al. 1997). Expression of both Pho 1a and Pho 1b was reduced by antisense constructs as reported by Duwenig (1996). For generation of Pho 2-overexpressing plants, the cDNA encoding Pho 2 from V. faba L. was cloned into the binary pBinAR vector (Höflgen and Willmitzer 1990). For transformation of S. tuberosum L. cv. Desirée, Agrobacterium tumefaciens was used (Rocha-Sosa et al. 1989). The recombinant cytosolic (Pho 2) phosphorylase isoform from V. faba L. was expressed in Escherichia coli and was purified as previously described (Fettke et al. 2004).

Northern blotting

RNA was isolated according to Logemann et al. (1987). Following electrophoresis in agarose gels (Farrell 1993) and blotting to nylon membranes (Sambrook et al. 1989), Pho 2 transcripts were detected using digoxigenin-labeled probes that had been generated using the DIG PCR Probe Synthesis Kit purchased from Roche (Mannheim, Germany).

Immunocytochemistry

In situ localization of Pho 2 using indirect immunofluorescence was performed essentially as described by Schächtele and Steup (1986).

\(^{14}\)C Labeling assays of SHG\(_s\) or SHG\(_l\)

SHG\(_s\). In a total volume of 250 µl, the reaction mixture contained 50 mM MES-NaOH; pH 6.0, 18,500 Bq of [U-\(^{14}\)C]glucose 1-phosphate and a total glucose 1-phosphate concentration of 20 mM. Glycans were added as indicated. Following pre-incubation at 30°C for 5 min, glucosyl transfer was initiated by adding recombinant Pho 2 or rabbit muscle phosphorylase a (Sigma, Taufkirchen, Germany) as indicated. After 10 min, aliquots (2×50 µl each) of the assay mixture were withdrawn and transferred into 1 ml of a mixture of 75% (v/v)
ethanol and 1% (w/v) KCl. To ensure quantitative precipitation, 250 µg of glycogen were added to each mixture. Following incubation for 20 min at room temperature, the samples were centrifuged for 5 min at 20,000×g. The pelleted carbohydrates were dissolved in 75 µl of water and precipitation was repeated. Finally, the pellet was dissolved in 300 µl of water, and 3 ml of scission mixture (Rotisint Mini; Roth, Karlsruhe, Germany) was added. Radioactivity was monitored using a Beckman liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA).

\[ \text{SHG}_{i} \]

In a final volume of 30 µl, \( \text{SHG}_{i} \) (50 µg each) and protein fractions (20 µg each) from leaves of wild-type potato plants or from various transgenic lines (line 1 and lines A–C) were incubated with \([U-1^{14}C]\text{glucose 1-phosphate (20 mM glucose 1-phosphate; specific radioactivity 8.6 Bq nmol}^{-1}\) at 37°C. As a control, \( \text{SHG}_{i} \) was omitted. After 15 min, the reaction was terminated by heating (3 min at 95°C). Low molecular weight compounds were removed by filtration (MWCO 10 kDa) and repeated washing with water. Finally, the retentate was resolved in 300 µl of water and radioactivity was monitored (see above).

\[ \text{Glycan elongation assays} \]

High molecular weight glycans were divided into two equal parts, one of which was used in an elongation assay whereas the other one served as a Pho 2-deficient control. Both parts were mixed with an equal volume of citrate-buffered glucose 1-phosphate (200 mM citrate-NaOH, 40 mM glucose 1-phosphate; pH 6.5) and were pre-incubated for 5 min at 37°C. Subsequently, recombinant Pho 2 was added to one mixture as stated, whereas an equal volume of water was added to the control mixture. Incubation was continued at 37°C and was terminated by heating (5 min at 90°C). Low molecular weight compounds were removed by filtration using a 10 kDa filter (Ultrafree-4-MC, Millipore, MA, USA) and washing with water. The retentate was used for hydrolysis with trilfluoroacetic acid, enzymatic degradation or FFF. Low molecular weight glycans (SHG) were incubated with 0.09 nkat Pho 2 at 37°C. At intervals, aliquots of the reaction mixture were withdrawn, heated for 5 min at 90°C and proteins were removed by filtration. As controls, either the recombinant Pho 2 or SHG were omitted and the mixtures were incubated for 2 h at 37°C. Subsequently, the two controls were processed as described.

\[ \text{Enzymatic glycan degradation} \]

Glycans freed of low molecular weight compounds by filtration (see above) were mixed with an equal volume of 50 mM sodium acetate buffer (pH 5.5). For pullulanase (EC 3.2.1.69) treatment, an enzyme from Klebsiella pneumoniae was added (0.167 nkat per 50 µl) and incubated for 12 h at 37°C. A fungal endo-α-1,5-arabinanase (EC 3.2.1.99) was kindly provided by Dr. Kirk Matthew Schnorr, Novozymes, Denmark. The authors are indebted to Ms. Silke Göpp for excellent technical assistance, to Erik Höchel for providing plant-derived protein fractions, to Ms. Julia Schönfeld for carrying out the GC–MS experiments, to Ms. Andrea Mohrenweiser for kindly providing the recombinant cytosolic phosphorylase (Pho 2), and to Ms. Carola Kuhn for performing the immunocytochemical localization.

\[ \text{Phosphorylolytic activity} \]

Phosphorylolytic activity was monitored using a continuous spectrophotometric assay (Steup 1990). Assays were performed at 30°C using saturating levels of soluble starch (Merck No. 1.01252).

\[ \text{Buffer-soluble protein fractions} \]

Leaves were harvested during the light period and were frozen in liquid nitrogen. The leaf material was broken in a mortar and the homogenate was then extracted with extraction buffer containing 100 mM HEPES-NaOH; pH 7.5, 2 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was passed through a nylon net and the filtrate was cleared by centrifugation (12 min at 20,000 × g). In the supernatant, proteins were pelleted between 35 and 55% saturation by adding solid ammonium sulfate. The protein fraction collected by centrifugation (20 min at 20,000 × g) was dissolved and passed through a PD10 column and subsequently through a membrane filter.

\[ \text{Native PAGE, SDS–PAGE and Western blotting} \]

For native PAGE, a discontinuous gel system (Steup 1990) was applied using a 7.5% (w/v) total monomer concentration and 0.2 (w/v) glycogen in the separation gel, except where stated. Following electrophoresis, the separation gel was stained for phosphorylase activity by incubating in a mixture of 20 mM glucose 1-phosphate and 100 mM citrate-NaOH pH 6.5 at 37°C for 4 h except where stated. For staining of the DPE2 activity, the incubation mixture contained 20 mM maltose and 100 mM citrate-NaOH pH 6.5, and the gel was incubated for 4 h at 37°C. SDS–PAGE and Western blotting were performed as previously described (Fettke et al. 2004).

\[ \text{Glycosidic linkage analysis} \]

Glycosidic linkages of the various glycans were analyzed as previously described (Fettke et al. 2004).

\[ \text{Acknowledgments} \]

Financial support by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 429 ‘Molecular Physiology, Energetics, and Regulation of Primary Metabolism’ TP B2 and B7) and a fellowship of the Land Brandenburg to J.F. are gratefully acknowledged. The arabinoxylanase preparation was a generous gift from Dr. Kirk Matthew Schnorr, Novozymes, Denmark. The authors are indebted to Ms. Silke Göpp for excellent technical assistance, to Erik Höchel for providing plant-derived protein fractions, to Ms. Julia Schönfeld for carrying out the GC–MS experiments, to Ms. Andrea Mohrenweiser for kindly providing the recombinant cytosolic phosphorylase (Pho 2), and to Ms. Carola Kuhn for performing the immunocytochemical localization.

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(Received August 26, 2005; Accepted October 6, 2005)