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The Plastidial Glucan Phosphorylase Affects the Maltooligosaccharide Metabolism in Parenchyma Cells of Potato (*Solanum tuberosum* L.) Tuber Discs

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Maltodextrin metabolism is thought to be involved in both starch initiation and degradation. In this study, potato tuber discs from transgenic lines containing antisense constructs against the plastidial and cytosolic isoforms of α-glucan phosphorylase and phosphoglucomutase were used to evaluate their influences on the conversion of externally supplied glucose-1-phosphate into soluble maltodextrins, as compared to wild-type potato tubers (*Solanum tuberosum* L. cv. Desiree). Relative maltodextrin amounts analyzed by capillary electrophoresis with laser-induced fluorescence revealed that tuber discs could immediately uptake glucose-1-phosphate and use it to produce maltooligosaccharides with a degree of polymerization of up to 30, as opposed to tubers repressing the plastidial glucan phosphorylase. The results presented here support previous indications that a specific transporter for glucose-1-phosphate may exist in both the plant cells and the plastidial membranes, thereby allowing a glucose-6-phosphate-independent transport. Furthermore, it confirms that the plastidial glucan phosphorylase is responsible for producing longer maltooligosaccharides in the plastids by catalyzing a glucosyl polymerization reaction when glucose-1-phosphate is available. All these findings contribute to a better understanding of the role of the plastidial phosphorylase as a key enzyme directly involved in the synthesis and degradation of glucans and their implication on starch metabolism.

Keywords: α-Glucan phosphorylase • Glucose-1-phosphate • Maltodextrins • Potato • *Solanum tuberosum* L

Introduction

Soluble glucans are important metabolites in plants, with maltooligosaccharides (MOSs) being an integral part of them requiring more profound analysis. Solid evidence from previous studies has indicated a fundamental role of MOSs in both the synthesis and degradation of starch (Chia et al. 2004, Malinova et al. 2014, Lin et al. 2017). During the initial stages of the starch granule initiation, MOSs are involved in the creation of priming structures (Myers et al. 2000, Cuesta-Seijo et al. 2013, Shoaib et al. 2021). However, MOSs are directly generated in the chloroplasts during starch degradation by the action of α-amylase (AMY) and isoamylase (ISA) and further metabolized by disproportionating enzyme 1 (DPE1; EC 2.4.1.25), as well as β-amylase (BAM; EC 3.2.1.2), ISA (EC 3.2.1.68) and plastidial glucan phosphorylase (PHO1; EC 2.4.1.1) (Myers et al. 2000, Hwang et al. 2016, Lin et al. 2017, Shaoib et al. 2021). Whether MOSs are only restricted to the plastids remains unknown. However, a glycan metabolism has been found to exist in the cytosol, which is associated with the maltose produced during starch degradation. The mentioned cytosolic metabolism of carbohydrates includes a pool of heteroglycans and the participation of the cytosolic glucan phosphorylase (PHO2) (Fettke et al. 2005, 2008).

Starch is accumulated as a complex granular structure made of α-glucans (α-1,4 linked and α-1,6 branched) both in the leaf cell chloroplast (transitory starch) and in the amyloplast of the plant storage tissue cell (storage starch) (Ball et al. 1996, Apriyanto et al. 2022). In potato tubers, starch is a major component and since its synthesis is confined to the amyloplasts, it relies entirely on the translocation of metabolites from the cytosol through the amyloplast envelope (Hofius et al. 2007). This process is initiated with the import of sucrose through the phloem into the sink organ and thus into parenchymal cells (**Fig. 1**) (Turgeon et al. 2005, MacNeill et al. 2017). In the cytosol, sucrose is cleaved by sucrose synthase (SuSy; EC 2.4.1.13) to produce UDP-glucose and fructose. The end products of sucrose degradation will enter the hexose phosphate pool, which consists of an equilibrium mixture of fructose-6-phosphate, glucose-6-phosphate and glucose-1-phosphate (Fru-6-P, Glc-6-P and Glc-1-P, respectively) due to the readily

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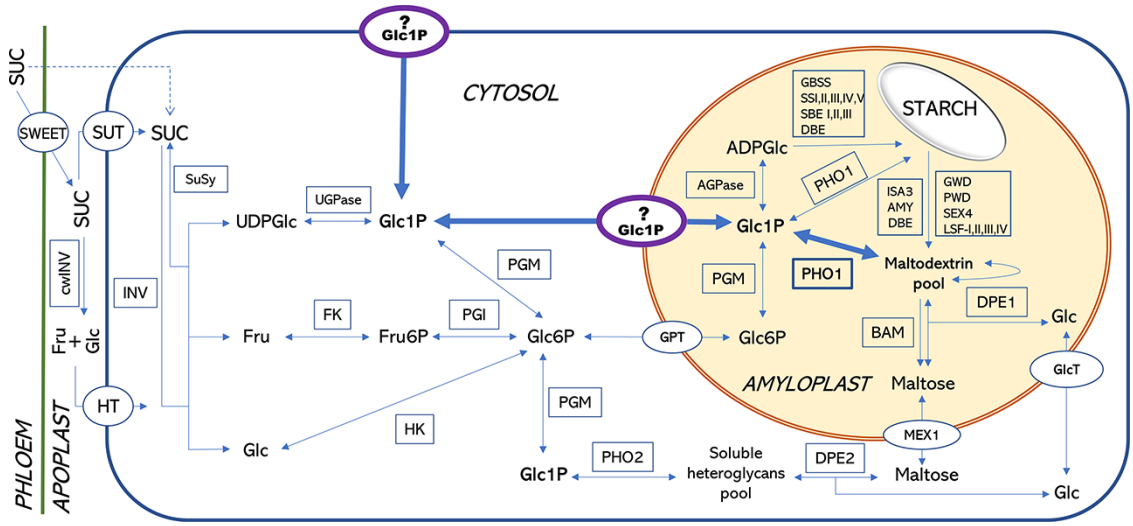

Fig. 1 Starch metabolism pathway in potato tuber. For reserve starch accumulation in sink organs, photosynthesis-derived sucrose is apoplastic loaded into the phloem for tuber translocation (Braun et al. 2014). Sucrose can be unloaded in the apoplastic space facilitated by sugar transporters like SWEET and then loaded into the parenchyma cells by SUT or via hexose transport proteins (HT) after being hydrolyzed by cwINV (Koch 1996, Proels and Hückelhoven 2014, Fernie et al. 2020). Alternatively, sucrose enters symplastically the tuber parenchyma cells via plasmodesmata (dashed arrow) (Chen 2014, Julius et al. 2017, Fernie et al. 2020). Once sucrose has reached the sink cell, it is hydrolyzed by INV into glucose and fructose or degraded by SuSy into UDP-glucose and fructose. Glc-1-P is then produced either from UDP-glucose by UGPase or from Glc-6-P interconversion mediated by the cytosolic PGM. When Glc-1-P is externally supplied, it can be taken up by tuber cells and enters two paths: (i) it is metabolized in the cytosol via PHO2 by transferring the glucosyl residue to soluble heteroglycans (Fettke et al. 2008) and (ii) it directly enters the amyloplasts and, through a PHO1-dependent action, is converted into starch (Fettke et al. 2010, 2012). The route of Glc-1-P directed to maltodextrins, which, this study focuses on, is highlighted. SUC—sucrose; Fru—fructose; Glc—glucose; SUT—sucrose transporter; cwINV—cell-wall invertase; HT—hexose transporter; INV—invertase (cytosolic); UGPase—UDP-glucose pyrophosphorylase; AGPase—ADP-glucose pyrophosphorylase; GBSS—granule-bound starch synthase; SSI–V—starch synthases I–V; DBE—debranching enzyme; GWD—α-glucan, water dikinase; PWD—phosphoglucan, water dikinase; SEX4—starch excess 4; ISA1–3—isoamylases 1, 2 and 3; DPE1 and DPE2—disproportionating enzymes 1 and 2; GlcT—glucose transporter (Cho et al. 2011); MEX1—maltose exporter (Kammerer et al. 1998); HK—hexokinase; FK—fructokinase.

reversible reactions catalyzed by the cytosolic isoforms of phosphoglucoisomerase (PGI; EC 5.3.1.9) and phosphoglucomutase (PGM; EC 5.4.2.2) (Fernie et al. 2001, Hofius and Börnke 2007). In the current storage starch biosynthesis model, outlined in **Fig. 1**, Glc-6-P is imported into the amyloplast by Glc-6-P/phosphate translocator (GPT) (Kammerer et al. 1998, Knappe et al. 2003). In the amyloplast, Glc-6-P is then reconverted into Glc-1-P by the plastidial PGM. At this point, the path for Glc-1-P utilization is diverse. Either it can be converted into ADP-glucose to be used as a substrate for starch synthases (SSs; EC 2.4.1.21) or it could function as a substrate for the plastidial α-1,4 glucan phosphorylase enzyme (PHO1). The latter facilitates the addition of glucosyl units to the non-reducing end of α-glucan, thereby generating longer MOSs. In this way, the elongated glucans might be successively used for the building up of starch through a coordinated action of SSs and starch-branching enzymes (SBEs) (Tetlow et al. 2004, Lin et al. 2017, Shoaib et al. 2021).

In higher plants, two types of PHO have been observed. Both isoforms differ in their subcellular localization, protein structure, enzymatic kinetic properties and expression patterns during plant cell development (Steup 1988, Lin et al. 2017, Shoaib et al. 2021). The plastidial isoform of PHO (known as PHO1 or L-SP) acts preferentially on linear MOS, whereas the cytosolic isoform of PHO (known as PHO2 or H-SP) shows a high affinity toward highly branched glucans and cytosolic heteroglycans (Fettke et al. 2004, 2005, Lin et al. 2017). The dominance of the ADP-glucose-dependent pathway in starch biosynthesis is clear considering that transgenic *Solanum tuberosum* plants with strong repression of the plastidial PHO1 isozyme did not reflect any significant alteration on starch accumulation, neither in leaves nor in tubers (Sonnewald et al. 1995). However, when grown at low temperatures, an alteration in starch metabolism has been observed, pointing to a PHO1 involvement in starch metabolism under abiotic stress conditions (Orawetz et al. 2016).

Transgenic potato plants repressing simultaneously the plastidial and cytosolic PGM unexpectedly possessed starch levels comparable to the wild-type (WT) plants (Fernie et al. 2002), indicating that additional carbon fluxes toward starch occur. In this regard, the uptake of Glc-1-P into the plastids seems to be the most likely mechanism for alleviating the inhibition of the storage starch synthesis imposed by the simultaneous repression of the PGM isoforms (Fernie et al. 2002, Fettke et al. 2008).

In previous studies, it has been demonstrated that WT potato tuber discs fed with carbon-14 (^{14}C)-labeled Glc-1-P were able to incorporate it into the starch at a higher rate

compared to glucose, Glc-6-P and sucrose (Fettke et al. 2010). Nevertheless, when using transgenic potato lines with reduced activity of PHO1, starch labeling was significantly diminished (Fettke et al. 2010). A reduction in the PGM activity, by contrast, revealed no effect on starch labeling (Fettke et al. 2010). These results indicated that a putative Glc-1-P transporter may exist within the cell and amyloplast membranes of the parenchyma cells of potato tuber. In addition, this suggested that PHO1 played a significant role in the starch biosynthesis by using the available Glc-1-P to elongate glucan chains that were then used in starch formation or that glucosyl units were incorporated into the starch surface mediated by PHO1. Apart from the evidence indicating a direct transport of Glc-1-P, it was not clear whether the Glc1-P also affected the cellular MOS metabolism.

To date, no evidence of acceptor-free or unprimed synthesis by the typical elongating enzymes (SSs and PHO1) has been reported (Merida and Fettke 2021). For *Arabidopsis* SSs, maltose was shown to be the shortest acceptor; glucose and ADP-glucose alone could not be used as starting points for producing longer MOSs (Brust et al. 2013). Similarly, *Arabidopsis* phosphorylases also required a glucan acceptor with at least a degree of polymerization (DP) of 4 (DP4) (Fettke et al. 2005, 2006). Previous studies concerning rice proposed that PHO1 in a complex within DPE1 converted DP5 glucans into longer MOSs (Lin et al. 2017).

To gain more insights into the MOS metabolism, their location and the implication of PHO and PGM on their regulation, we analyzed the plant system by supplying external sugars and sugar phosphates to tuber discs from WT and transgenic potatoes repressing these enzymes in their different isoforms. The MOSs were extracted and characterized by capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection, and a comparative examination of transgenic and WT tubers was performed.

Results

The MOS metabolism of WT tubers was significantly affected by incubation with Glc-1-P

We first evaluated whether parenchyma cells from freshly harvested WT potato tubers incubated with Glc and Glc-6-P for 60 min revealed an altered MOS content. The relative amounts, expressed as a percentage of each DP from the total MOS detected by CE-LIF, are depicted in **Fig. 2A**. MOSs with DP10 were detected following Glc-6-P incubation as well as in the controls. However, following incubation, an increase in the relative amount of maltotriose (DP3) and maltohexaose (DP6), accompanied by a decrease in the proportion of maltotetraose (DP4) and maltopentaose (DP5), was observed. In samples incubated with glucose, MOSs with a maximal DP of 7 were detected together with a significant increase in the amount of DP3, whereas all other DPs revealed no alteration or reduced amounts. Similar alterations were also observed after 30-min incubation (**Supplementary Fig. S1**).

In another series of incubation experiments including a solution containing an equal amount of Glc-1-P (**Fig. 2B**), longer MOSs with DP30 were detected. When comparing the various DPs, a drop in the relative amounts of DP3–DP5 was determined following Glc-1-P incubation, as compared to Glc-6-P, glucose and control. Most likely, these shorter glucans were elongated and formed the observed extended MOS. Furthermore, following the incubation with Glc-1-P, a significant increase in the total amount of MOSs was detected (**Fig. 2C**), whereas for Glc-6-P and glucose incubation, no alteration was observed compared to control.

The plastidial phosphorylase was responsible for the longer and higher amounts of MOSs following Glc-1-P incubation

Following incubation with Glc-1-P, we observed more and longer MOSs. As Glc-1-P is the substrate for the phosphorylases, we tested whether the reduction in the phosphorylase activities would have an influence on the observed alteration of the MOS metabolism. As it was shown that Glc-1-P was taken up by the parenchyma cells and directly transported into the amyloplasts (Fettke et al. 2010, 2011), we included both isoforms in our analysis. Therefore, we incubated Glc-1-P with transgenic tuber discs repressing either the PHO1 or the PHO2 (**Fig. 3A**, see also Fettke et al. 2011) and WT discs for 60 min (**Fig. 3B**). For each genotype, the difference between the relative amount of MOSs for each DP following incubation with Glc-1-P and the controls, where hexose phosphates were omitted, was graphed as the difference in percentage.

In PHO1-repressed tuber discs, the MOS content did not change over time, except for DP5, where an increase was seen (**Fig. 3B**). Nevertheless, unlike WT tubers, this transgenic line already contained maltodextrins with a higher DP irrespective of Glc-1-P incubation (**Fig. 3D**). PHO2 repression, in contrast, did not affect the capability of the tuber cells to form longer MOSs, as similar distribution of the MOS in WT was observed (**Fig. 3B**). Alterations were detected mainly for short glucan chains (DP3–DP7). Furthermore, longer incubations (120 min) revealed a very similar MOS pattern, pointing to a shifted MOS metabolism (**Supplementary Fig. S2A**, see **Supplementary Fig. S2B** for 30-min incubation) with a slight reduction in the amount of MOSs with DP7–DP10 and thus an increase in the proportion of MOSs with higher DP13. Additionally, an increased amount of DP3 was found after 120 min.

To evaluate if the uptake of Glc-1-P was in some way restricted to a specific physiological state of the potato tubers at the time the experiments were run, we also utilized discs from potato tubers that were stored for 3 months after harvesting, which contained visible sprouts. As seen in **Fig. 3C**, for PHO1-repressed discs, again, nearly no differences were estimated for each DP, meaning that incubation with Glc-1-P did not affect the MOS pattern in this genotype. In WT and PHO2-repressed tuber discs, a very similar proportion of glucans with

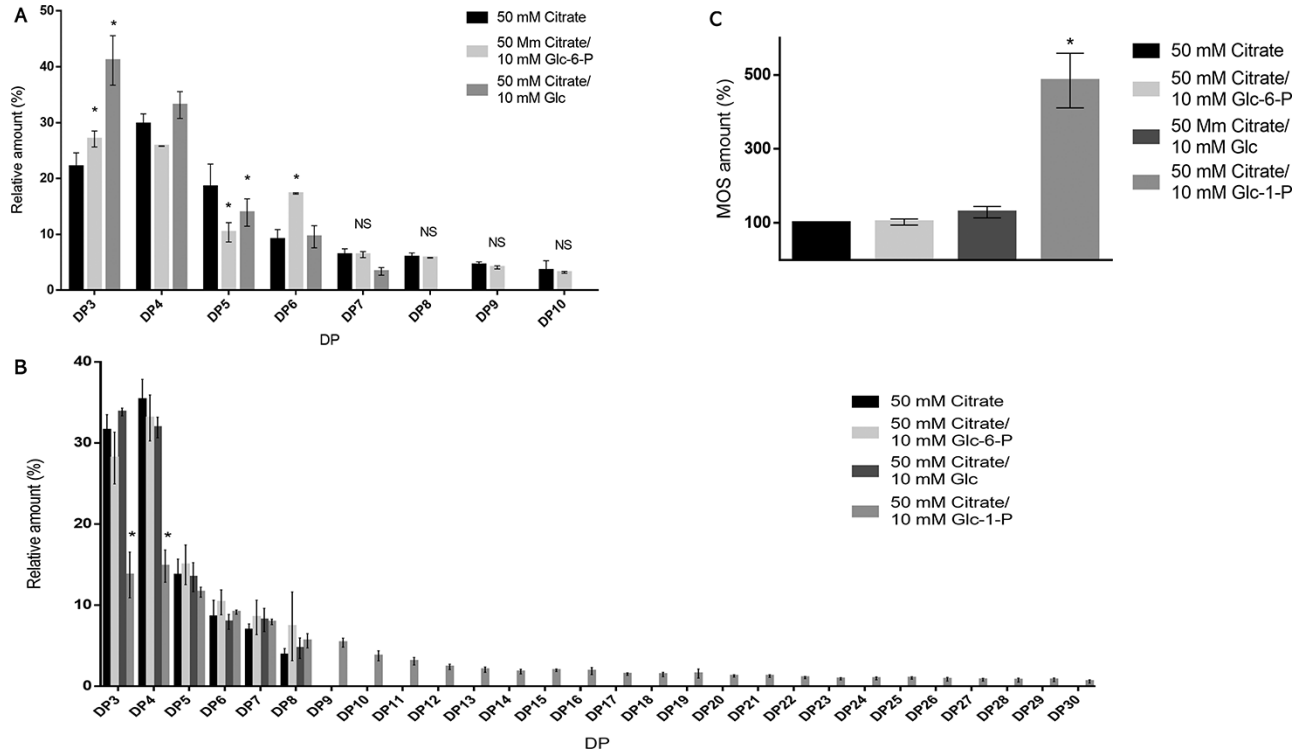

Fig. 2 (A) Maltodextrins in potato tubers following incubation with various sugars and sugar derivatives. Relative amount (%) of MOSs in potato WT tuber discs after 60-min incubation in 50 mM citrate (control solution) and two treatment solutions, one supplemented with 10 mM Glc-6-P and the other with 10 mM glucose (Glc). The effect of Glc and Glc-6-P on the MOS metabolism was evaluated by incubating discs from freshly harvested WT tubers in the mentioned solutions. After incubation, soluble glucans were extracted in 20% (v/v) ethanol, concentrated and analyzed by CE-LIF. The mean of three biological replicas with standard deviation is presented. Dunnett's multiple comparison test against control was performed for statistical analysis ($\alpha = 0.05$). (B) Relative amount (%) of MOSs in potato WT tuber discs after 60-min incubation in 50 mM citrate (control solution), Glc-6-P, Glc and Glc-1-P. In a second incubation experiment, where a treatment solution supplemented with Glc-1-P was included, an extensive proportion of MOSs with higher DP30 was detected. The mean of three biological replicas with standard deviation is presented. Two-way ANOVA was used for statistical analysis ($\alpha = 0.05$). (C) Total amount of CE-LIF detected MOSs in WT potato tuber discs after 60-min incubation in 50 mM citrate (control solution), Glc-6-P, Glc and Glc-1-P. The mean of the total MOS amount obtained following incubation in the control solution was set as 100%. Thus, the increased amount in treatment solutions was calculated with respect to control. The mean of three biological replicas with standard deviation is presented. One-way ANOVA was used for multiple comparisons ($\alpha = 0.05$).

a DP between 12 and 30 resulted after 60-min Glc-1-P incubation. A similar MOS pattern following 30-min incubation was observed (**Supplementary Fig. S2B**). For shorter MOSs, a reduced amount was observed again in the PHO2-repressed tuber discs. These data indicate that the utilization of the externally supplied Glc-1-P by the plastidial phosphorylase for MOS elongation was conducted regardless of the physiological state of the potato tubers.

In addition, besides PHO2-repressed tuber discs not being affected in the MOS formation capability, they consistently showed an even higher activity of the PHO1 isoform (**Fig. 3A**). Based on these observations, it was suggested that the decrease in shorter MOSs was related to the rapid elongation and thus to a stronger shift of the MOS distribution for longer MOSs during the same incubation time. Therefore, experiments with WT and PHO2-repressed tuber discs incubated with Glc-1-P for shorter periods were conducted (**Fig. 4A**, see **Supplementary Fig. S3** for longer incubation time). Following a 5-min incubation, an extensive MOS generation was detected in both lines, showing that the Glc-1-P uptake and the generation of MOSs are fast processes. Significant differences between WT and PHO2-repressed lines were observed in the relative proportion of DP6, DP9 and DP10. In the PHO2-repressed line, a lower percentage of DP6 and a higher proportion of DP9 and DP10 were observed, as compared to WT (**Fig. 4A**). The total amount of MOSs generated during Glc-1-P incubation was significantly higher in PHO2-repressed discs, as compared to WT (**Fig. 4B**). In addition, in tuber discs repressing PHO2, the MOS content increased more rapidly when comparing different incubation times (**Fig. 4B**). All these observations were likely a consequence of the observed higher activity of PHO1 in the tubers with antisense inhibition of PHO2, as compared to the WT control (see **Fig. 3A**).

PGM had no impact on the usage of Glc-1-P for MOS metabolism

Since the PGM catalyzes the interconversion of Glc-1-P and Glc-6-P, we used a transgenic potato line simultaneously

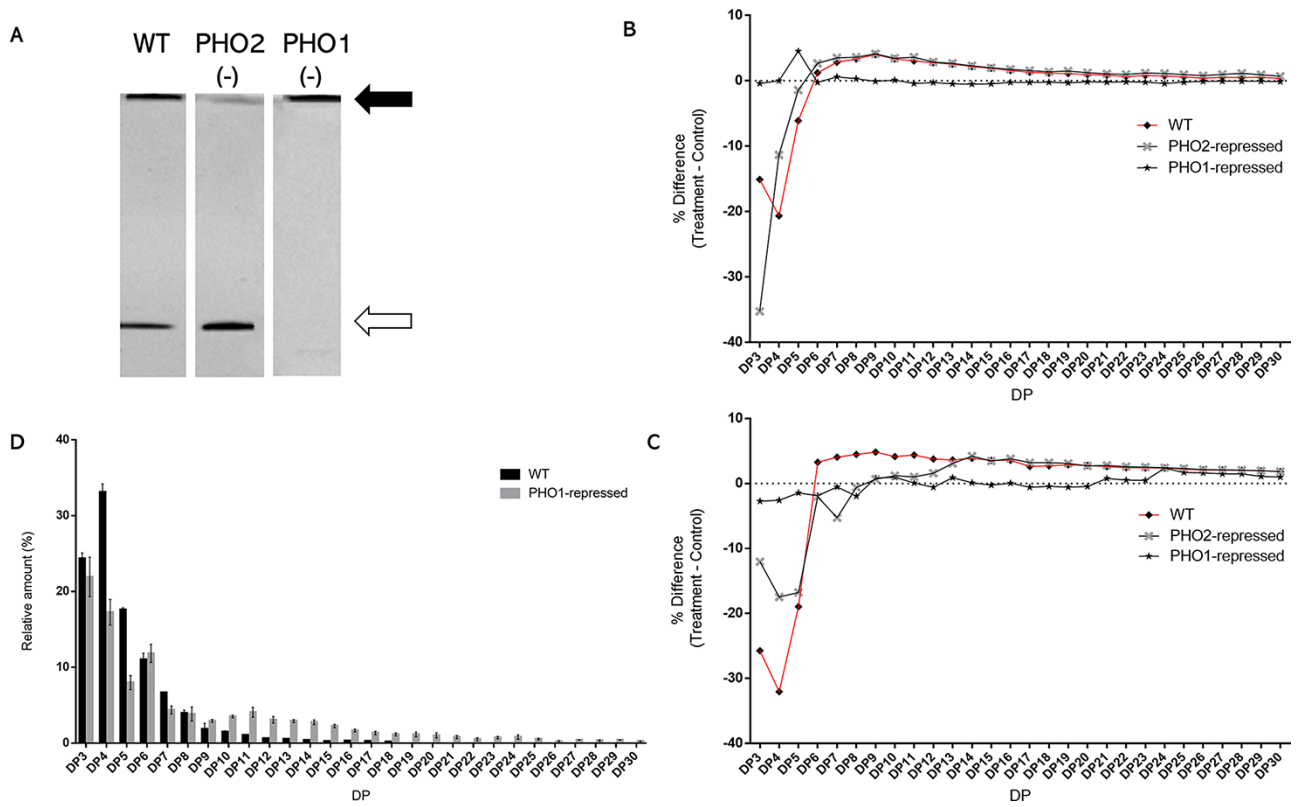

Fig. 3 Maltodextrin metabolism in transgenic potato tubers with reduced phosphorylase activities. (A) Native PAGE separation of buffer-soluble proteins extracted from potato WT, plastidial (PHO1) and cytosolic (PHO2) phosphorylase repressing tubers following the procedure described by Fettke et al. (2010) for phosphorylase activity detection. In each lane, 20 μg of protein was loaded. The separation gel contained 0.2% (w/v) glycogen. Following electrophoresis, the separation gel was equilibrated in 100 mM citrate-NaOH (pH 6.5) at room temperature for 10 min and then incubated for 30 min in a 100 mM citrate-NaOH (pH 6.5) plus 20 mM Glc-1-P solution. Subsequently, the separation gel was washed with water and then stained with a solution containing 0.23% (w/v) of KI (potassium iodide) and 0.13% (w/v) I_2 (iodine). The black arrow indicates the position of cytosolic PHO2; the white arrow indicates the position of plastidial PHO1. (B) MOS difference comparison between WT tuber discs and potato lines repressing the cytosolic (PHO2) and the plastidial (PHO1) phosphorylase after 60-min incubation in control and 10 mM Glc-1-P solution. Freshly harvested tuber discs from each line were used. Through the CE-LIF analysis, the relative amount (%) of MOSs was estimated and the difference for each DP in treatment minus control incubation was calculated and graphed as the difference (%). Values are the difference between the means of three biological replicates used for treatment and control incubation. (C) MOS difference comparison in tuber discs from WT, PHO2-repressed and PHO1-repressed sprouting tubers (stored for 3 months after harvested) after 60-min incubation in control and Glc-1-P solution. Values are the difference between the means of three biological replicates used for treatment and control incubation. (D) Relative amount of MOSs in tuber discs from WT and transgenic lines repressing the plastidial PHO1 incubated for 60 min in control solution lacking hexose phosphate. The numbers are based on triplicates with bars representing the standard deviation.

repressing the cytosolic and plastidial PGM activity to investigate whether a reduced turnover of the acquired Glc-1-P in the parenchyma cells would have an impact on the MOS formation. The antisense inhibition of PGM was followed via native polyacrylamide gel electrophoresis (PAGE) and activity staining (**Fig. 5A**). The transgenic line revealed a strong reduction in the overall PGM activity (**Fig. 5A**, see also Fettke et al. 2008). Following a 5-min Glc-1-P incubation of PGM-repressed and WT tuber discs, the PGM-repressed line revealed an MOS pattern similar to the WT controls. However, a closer look revealed a lower amount in MOSs with DP7–DP9, as compared to WT (**Fig. 5B**, see **Supplementary Fig. S4** for other incubation times). Comparing the total MOS amount in all the evaluated genotypes following Glc-1-P incubation, in the PGM-repressed line, the MOS content increased by 34.6%, as compared to its control, based on the estimation of the reducing sugar amount (**Table 1**). Such an increase was considerably higher, as compared to WT, PHO2-repressed and PHO1-repressed lines (11%, 8% and 0%, respectively).

Regarding the intrinsic MOS content of all lines and thus the MOS without any incubation, the potato line with antisense inhibition of PGM contained the highest MOS amount, followed by the PHO1-repressed line (**Fig. 6**). In WT and PHO2-repressed lines, the MOS content showed no significant difference and was the lowest.

Overall, the experiments clearly showed that the uptake of Glc-1-P was directly used by PHO1 for MOS metabolism, and no detectable amount was transferred into Glc-6-P, which could,

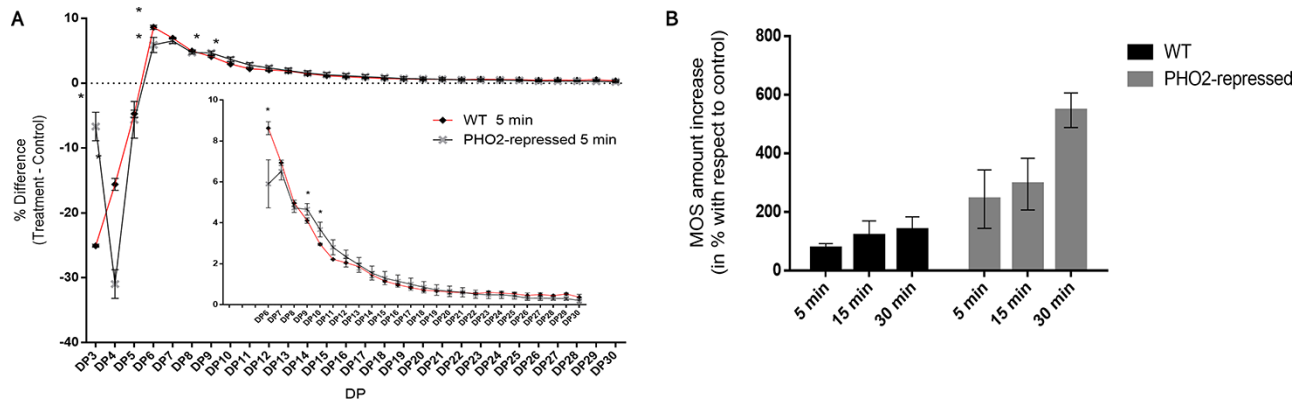

Fig. 4 Comparison of the maltodextrins of WT and PHO2-repressed tuber discs. (A) MOS amount difference in WT and PHO2-repressed tuber discs after 5-min incubation in control and Glc-1-P solution. For each genotype, 1 g (10–12 potato tuber discs) tuber discs were incubated for 5 min in control solution with no hexose phosphate and in treatment solution containing Glc-1-P. Relative MOS amount (given in percentage) was evaluated by CE-LIF. The difference in the relative MOS amount obtained after treatment minus control solution for each genotype is depicted. The numbers are based on triplicates. Bars represent standard deviations. Multiple *t*-tests were used for statistical analysis ($\alpha = 0.05$). (B) MOS amount increase (%) in WT and PHO2-repressed tuber discs after Glc-1-P incubation. The total amount of MOSs detected by CE-LIF after control incubation was used to determine the increased proportion of MOSs after Glc-1-P incubation.

therefore, reduce the availability of Glc-1-P. Therefore, a linear utilization of Glc-1-P was demonstrated, including the uptake of Glc-1-P into the apoplasts and the direct metabolism via the plastidial phosphorylase into MOS metabolism.

Discussion

MOS metabolism of parenchyma cells was specifically and significantly altered following Glc-1-P uptake

Despite the relevance of MOSs as constituents for starch formation and as degradation products, little focus has been placed on understanding their distribution, regulation and implications. Previous studies have already demonstrated that potato tuber discs and purified amyloplasts could more effectively utilize Glc-1-P, as compared to other externally supplied sugars or sugar derivatives (such as glucose, sucrose, maltose or Glc-6-P) (Naeem et al. 1997, Fettke et al. 2005, 2010), but the flux of the carbon source was monitored only toward starch. With the use of CE-LIF analysis of MOSs isolated from potato tuber disc samples, we confirmed that Glc-1-P was taken up into the parenchyma cells with more efficiency in comparison with glucose or Glc-6-P, and it was further incorporated into MOSs. Glc-1-P revealed a significant effect on MOS metabolism that could be observed in both the increased amount of MOSs following incubation and the massively altered chain length distribution (**Fig. 2A, B**). Even though a limited incorporation into starch has been found using ^{14}C-labeled glucose and Glc-6-P (Naeem et al. 1997, Fettke et al. 2010), the incubation with these metabolites had no conspicuous effect. Therefore, we concluded that either the MOS metabolism might be in some way restricted to Glc-1-P or in the normal parenchyma cell system, regulation mechanisms for high Glc-1-P levels are not well established. The latter seems to be a more plausible explanation considering that the Glc-1-P levels under normal conditions in the potato tuber cells are much lower than glucose or Glc-6-P (Tauberger et al. 2000, Fernie et al. 2002, Lytovchenko et al. 2002) and we used relatively high amounts of Glc-1-P. Despite the missing data regarding the concentration of Glc-1-P in the cell following the uptake, it is highly likely that this was significantly increased. If we consider that the Glc-1-P content in potato WT developing tubers is around 12 nmol/g of fresh weight (Tauberger et al. 2000, Fernie et al. 2002), we can then estimate that quantities in the order of 21.1–28.6 nmol of Glc-1-P were incorporated and used in the formation of the longer MOS following 5–30-min incubation, respectively.

Furthermore, a significant turnover of Glc-6-P into Glc-1-P and vice versa was excluded; otherwise, a similar alteration of MOS metabolism would be expected following incubation of tuber discs with Glc-6-P. In this regard, the thermodynamic equilibrium of the conversion of the glucose phosphates is on the side of Glc-6-P (Atkinson et al. 1961, Tewari et al. 1988), and therefore, it is expected that at least a portion of Glc-1-P is converted into Glc-6-P. That a specific effect of Glc-1-P was observed on the MOS metabolism further indicated that a high amount of Glc-1-P entered the cells.

MOSs were localized in the amyloplast, and their metabolism was affected by PHO1

We tested the capability of potato tubers repressing either the plastidial PHO1 or the cytosolic isoform PHO2 to convert externally supplied Glc-1-P into MOS, in comparison with WT tubers. PHO1-repressed tubers were unable to generate glucans since almost no difference was obtained when subtracting the relative amount obtained from treatment samples incubated with Glc-1-P minus control samples (**Fig. 3B, C**).

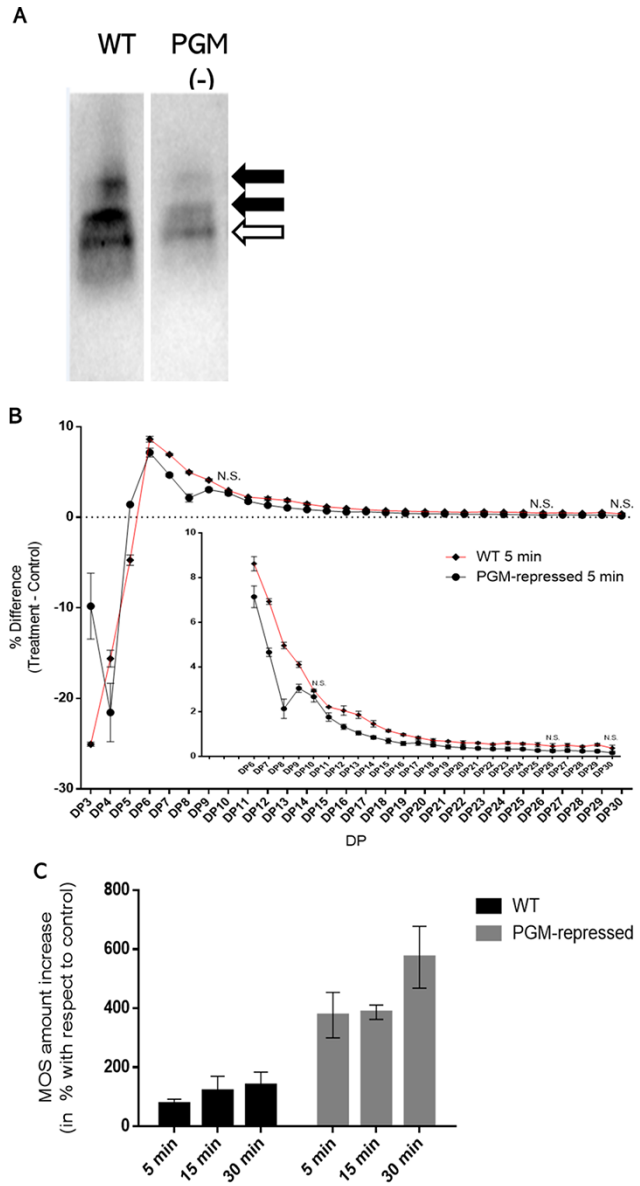

Fig. 5 Maltodextrin metabolism in transgenic plants with reduced PGM activity. (A) Native PAGE separation of buffer-soluble proteins extracted from potato WT and transgenic tuber with antisense inhibition of both cytosolic and plastidial PGM (Lytovchenko et al. 2002). PGM activity was detected following the procedure described by Egli et al. (2010). In each lane, 20 μg of protein was loaded per sample. The black arrows indicate the position of cytosolic PGM; the white arrow indicates the position of plastidial PGM. (B) MOS amount difference in WT and PGM-repressed tuber discs after 5-min incubation in control and Glc-1-P solution. N.S.: no significant difference. (C) MOS amount increase (%) in WT and PGM-repressed tuber discs with respect to control.

Nevertheless, control samples from PHO1-repressed tuber discs already contained more and longer MOSs with DP30 (**Figs. 2A**, **6**). This inherent MOS content could be explained as an accumulation of highly polymerized glucans in the amyloplast in this transgenic line and points to the function of PHO1 in potato tubers in a depolymerizing direction. The same effect

Table 1 MOS increase after 30-min incubation in Glc-1-P (%). For each genotype, the increase in the MOS amounts following incubation of tuber discs with Glc-1-P with respect to control samples was estimated using the reducing sugar assay.

Potato line	MOS increase following Glc-1-P incubation (%)
WT	11 ± 1.5
PHO1-repressed	0 ± 2
PHO2-repressed	8 ± 1.7
PGM-repressed	34.6 ± 8.6

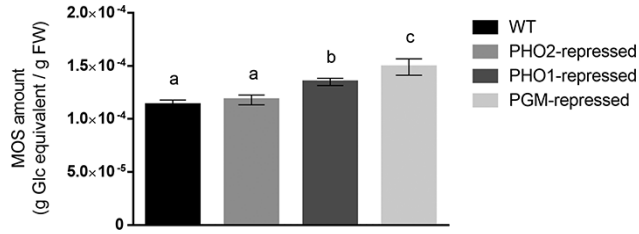

Fig. 6 MOS amount in wild-type and transgenic tubers repressing PHO1, PHO2 and PGM enzymes. The MOS content (equivalent to grams of Glc per gram of fresh weight) was estimated based on the procedure established by Waffenschmidt and Jaenicke (1987) for reducing sugar amount determination using Glc as standard. The mean of three replicates with standard deviation is depicted. One-way ANOVA was used for multiple comparisons ($\alpha = 0.05$). Different letters represent significant differences.

was observed by Malinova et al. (2014) in *Arabidopsis thaliana phs1a* knockout plants, lacking the plastidial phosphorylase, in which maltodextrins with DP10 were observed, as compared to WT plants, where maltodextrins whose DP exceeded 4 were scarcely detectable. In that case, the transitory starch metabolism was analyzed, and thus, both starch synthesis and degradation processes occurred repeatedly. However, as disc samples were generated from growing potato tubers, storage starch synthesis mainly occured in this case, the biochemical significance of the increased and longer MOS for the metabolism is not clear. A possible explanation is that the observed MOSs were the result of the initiation of new starch granules or partial degradation of starch granules during synthesis (e.g. during the trimming of the amylopectin molecules). Since PHO1 activity was repressed in this transgenic line, such trimmed MOSs could remain intact. Overall, this was fascinating but needs further research.

We found that PHO2-repressed tuber discs incubated in Glc-1-P were able to produce MOSs at a comparable level to WT (**Figs. 3**, **4**). For that reason, a PHO2 connection with the MOS metabolism can be practically excluded. Under these circumstances, we can assume that the detected MOSs were localized exclusively in the amyloplasts.

In both WT and PHO2-repressed samples, a decreased proportion of short MOSs (DP3–DP5) was detected following incubation, while the proportion of longer MOSs was increased This observation also referred to PHO1 as the responsible enzyme for the detected alterations, as this isoform revealed primer favoritism for short glucans and, specifically in potato,

a preference for DP5 has been reported (Kitaoka et al. 2002, Kadokawa 2018). Interestingly, the PHO2-repressed potato tubers revealed an increased PHO1 activity as observed from native PAGE assays of potato tuber soluble proteins (**Fig. 3A**). A possible explanation for this could be that higher expression levels of the plastidial phosphorylase existed in the PHO2-repressed line as a compensation mechanism to its repression in the cytosolic isoform. By this mechanism, Glc-1-P could be utilized or generated in the other compartment, in this case in the amyloplast, and exchanged over the plastidial membranes to prevent the lack/reduction of Glc-1-P in the cytosol. However, the biological function is not clear. The effect of this higher PHO1 activity in the PHO2-repressed lines was clearly detectable in the MOS metabolism following Glc-1-P incubation. Therefore, with increasing incubation time, more MOSs were accumulated in the transgenic tuber discs, as compared to WT (**Fig. 4B**), and the MOS revealed small alteration in the chain length (**Fig. 4A**). In particular, shorter chains (DP4–DP6) were reduced in amount, as compared to WT, and middle long chains (DP9– DP13) were increased; thus, it may be that the shorter chains had been used for elongation by PHO1 during the Glc-1-P incubation. This agreed with the known substrate specificity of PHO1 that preferred short linear MOSs (Kitaoka and Hayashi 2002).

Furthermore, glucosylation mediated through the plastidial phosphorylase immediately occurred once the Glc-1-P was available, as seen after 5-min incubation. This result supports the increasing evidence that a specific transporter for Glc-1-P must exist in the plastidial membranes, so this metabolite could be exchanged between the cytosol and plastid. Recently, Malinova et al. (2020) indicated that two transporters expressed in the membrane, previously described as UDP-galactose/UDP-rhamnose, were able to transport Glc-1-P in *A. thaliana*. Based on these observations, the existence of homologous transporters in *S. tuberosum* as well as in other plant species seems highly probable. It is known from the previous starch-labeling experiments that the transport efficiency of this hexose phosphate is higher in potato tubers compared to *Arabidopsis* (Fettke et al. 2010, 2011). This could stem from the possibilities that either the transporters are highly expressed or they perform more efficiently. Therefore, some additional Glc-1-P transporters could possibly exist in the heterotrophic cell of potato tubers. With all this evidence, an alternate route including a direct transport of Glc-1-P into the amyloplasts of the sink organs should be considered beyond the established GPT. However, evaluation of this Glc-1-P uptake capacity in different plant species would provide wider information concerning this putative-specific transporter.

Simultaneous repression of both plastidial and cytosolic PGM did not influence the MOS formation capability

Since PGM is an enzyme directly responsible for the regulation of Glc-1-P in the carbohydrates metabolism of plants by catalyzing the interconversion of Glc-1-P and Glc-6-P, we hypothesized that a repressed activity of both isoforms of PGM should not influence the observed effects on the MOS, since the alteration in the MOS content was attributted to the direct transport of the supplied Glc-1-P throughout the cell and amyloplast membrane and further utilization by PHO1. Tuber discs of a transgenic line simultaneously repressing the plastidial and cytosolic PGM revealed that the capacity to generate maltodextrins was not affected (**Fig. 5B**). Only small alterations in the chain length distribution were detected. Therefore, following incubation with Glc-1-P, a lower percentage of DP7–DP9 was present in the PGM-repressed tuber discs, as compared to WT. This could be explained by a lack of conversion of applied Glc-1-P into Glc-6-P, thus increasing amounts of the former in the amyloplasts causing an augmented action of PHO1, even though PHO1 activity is unaltered in PGM-repressed plants under normal conditions (**Supplementary Fig. S6**). The results here presented using the transgenic PGM potato line were in accordance with the findings of Fernie et al. (2002), who analyzed the content of starch, Glc-1-P and Glc-6-P in the same potato line repressing the plastidial and cytosolic PGM, resulting in little difference with respect to WT. However, the tubers of this transgenic potato revealed the highest MOS amount independent of any incubation (**Fig. 6**). The reason remains unclear, but a possibility could be the lack of conversion of Glc-6-P into Glc-1-P following the equilibrium, and therefore, more Glc-1-P was available and used for MOS metabolism. Again, a starch metabolism alteration was more likely occurring due to the disruption caused by the inability of PGM to regulate the physiological levels of the hexose phosphates in both the cytosol and amyloplast compartments, and hence, a massive flux of Glc-1-P into the cytosol and into the amyloplast was allowed. As a consequence, starch initiation, the inner starch structure or degradation could be massively altered. In addition, the fluxes and the involved metabolites may have played a role, even when the estimated starch amount was similar to WT (Fernie et al. 2002).

For the observed in situ alterations of the MOS metabolism, in planta data are still lacking. First indications for changes in MOS metabolism exist (**Supplementary Fig. S5**). Thus, it would be interesting to observe whether, under specific conditions, the MOS metabolism would be affected in a similar way. It has been shown, for instance, that repression of PHO1 did affect starch parameters at low temperatures in maize (Satoh et al. 2008) and potato (Orawetz et al. 2016). In these cases, it is very probable that the MOS metabolism was also influenced. As Glc-1-P has also been detected in the apoplast fraction (Malinova et al. 2020), a transport of this metabolite within the plant was provided and could have a potential biotechnology application in altering the starch or MOS properties in specific tissues or organs. In this regard, targeting enzymes implicated in carbohydrate metabolism, like the here evaluated PHO1 or the still uncharacterized Glc-1-P transporters, could be useful to produce, for instance, potato raw material with a high maltodextrin content or modified starch for specific industrial or dietary purposes.

The relevance of our findings relies in that an alternative route to the Glc-6-P transport via the amyloplast membranes

exist in potato tubers linked to the MOS and the starch metabolic pathway. We also showed the strong implication that the plastidial phosphorylase has on the regulation of MOSs, being able to catalyze the synthesis of glucans with a high DP when Glc-1-P is presented in high concentrations.

From the overall data, we concluded that (i) a Glc-1-P transporter must exist within the plasma membrane as well as in the amyloplast membrane; (ii) when the Glc-1-P/Pi ratio was high, simulated by the addition of Glc-1-P, synthesis of MOSs catalyzed by PHO1 occurred, which could successively impact starch metabolism, and (iii) a Glc-6-P independent pathway, and thus also independent of PGM action, could naturally occur to produce maltodextrins with a high DP.

Materials and Methods

Plant material

Potato WT plants (*Solanum tuberosum* L. cv. Desiree), transgenic lines expressing an antisense construct directed against both plastidial phosphorylase isozymes (PHO1a and b; for details, see Fettke et al. 2005) or possessing a reduced expression of the cytosolic phosphorylase isozyme (PHO2; Fettke et al. 2005) and transgenic lines with a lowered expression of both cytosolic and plastidial PGM (for details, see Lytovchenko et al. 2002, Fettke et al. 2008) were grown under controlled conditions [12-h light period (300 μE m^{-2} s^{-1}), 12-h darkness, 22°C, 30% relative humidity] in a growth chamber. Potato tubers were harvested after 3 months and immediately used for conducting the experiments, except where otherwise indicated.

Extraction of buffer-soluble proteins

Peeled tuber material (250 mg) was homogenized in 0.3 ml of precooled grinding buffer [100 mM HEPES-NaOH, pH 7.5, 1 mM EDTA, 2 mM DTE, 10% (v/v) glycerol] using an Ultra Turrax for 10 s. After centrifugation (20,000×*g*, 10 min, 4°C), the supernatant containing the soluble proteins was collected and used for quantification and native PAGE.

Quantification of protein

Buffer-soluble proteins were quantified using the Bradford (1976) micro-assay with bovine serum albumin as standard.

Native PAGE

Before conducting the incubation experiments, the repressed activity of the targeted enzymes in the transgenic plants was confirmed. For phosphorylase activity detection, 20 μg of buffer-soluble proteins was separated through a native PAGE using a discontinuous gel at a 7.5% (w/v) total monomer concentration containing 0.2% (w/v) glycogen in the separation gel, as described by Fettke et al. (2005). Following electrophoresis, the separation gel was incubated in a mixture of 20 mM Glc-1-P/100 mM citrate-NaOH (pH 6.5) for 30 min and then stained with iodine (Steup 1990). For the PGM activity, a native PAGE was performed following the procedure described by Egli et al. (2010).

Incubation of potato tuber discs in hexose and hexose phosphate solutions

Tuber discs (13-mm diameter, c. 2 mm thick) were washed 3× with 300 ml of cold distilled water. Then, 1 g (10–12 discs) was incubated per triplicate in different treatment solutions, all containing 50 mM citrate (pH 6.5) and supplemented with 10 mM Glc-1-P, 10 mM Glc-6-P or 10 mM Glc. In control solution, the sugars were omitted. Experimental incubation periods were 5, 15, 30, 60 and 120 min. After removing the solution, discs were frozen in liquid nitrogen before being homogenized with Ultra Turrax for 20 s in 3 ml of 20% (v/v) cold ethanol. Following centrifugation (12,000×*g*, 10 min, 4°C), the supernatant containing the soluble glucans was collected and heated (10 min, 99°C). A centrifugation (20,000×*g*, 10 min) was performed to pellet the proteins, and 1 ml of supernatant was concentrated in vacuum.

Estimation of carbohydrates by reducing ends

The amount of reducing glucans was estimated according to Waffenschmidt and Jaenicke (1987) using glucose as standard.

CE-LIF analysis

A total of 100 nmol of soluble glucans based on the reducing end amount were derivatized with 1 μmol of 8-Aminopyrene-1,3,6-trisulfonic acid trisodium salt (APTS) dissolved in 2.0 μl of 1 M sodium cyanoborohydride in tetrahydrofuran and 1.5 μl of 1.2 M citric acid. The samples were incubated for 1.5 h at 55°C and then diluted with 96 μl of ultrapure water. Samples were diluted 5× with ultrapure water prior to injection in a Beckman Coulter PA800-plus Pharmaceutical Analysis System (Beckman Coulter, Brea, CA, USA) equipped with a silica capillary (inner diameter: 50 μm; outer diameter: 360 μm; length: 60 cm). The capillary was rinsed with 25 mM lithium acetate buffer (pH 4.75 adjusted with acetic acid) containing 0.4% (w/v) polyethylene oxide for separation of the MOS.

The separation was performed at 30 kV during 20 min. The detection was made with a 488-nm solid-state laser module and an LIF detector.

Supplementary Data

Supplementary data are available at *PCP* online.

Data Availability

All relevant data can be found within the manuscript and its supporting materials. The transgenic potato lines can be provided by the authors following request.

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Disclosures

The authors have no conflicts of interest to declare.

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