

A Possible Role for Pyrophosphate in the Coordination of Cytosolic and Plastidial Carbon Metabolism within the Potato Tuber¹

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The early stages of tuber development are characterized by cell division, high metabolic activity, and the predominance of invertase as the sucrose (Suc) cleaving activity. However, during the subsequent phase of starch accumulation the cleavage of Suc occurs primarily by the action of Suc synthase. The mechanism that is responsible for this switch in Suc cleaving activities is currently unknown. One striking difference between the invertase and Suc synthase mediated cleavage of Suc is the direct involvement of inorganic pyrophosphate (PPi) in the latter case. There is presently no convincing explanation of how the PPi required to support this process is generated in potato (*Solanum tuberosum*) tubers. The major site of PPi production in a maturing potato tubers is likely to be the reaction catalyzed by ADP-glucose pyrophosphorylase, the first committed step of starch biosynthesis in amyloplasts. We present data based on the analysis of the PPi levels in various transgenic plants altered in starch and Suc metabolism that support the hypothesis that PPi produced in the plastid is used to support cytosolic Suc breakdown and that PPi is an important coordinator of cytosolic and plastidial metabolism in potato tubers.

During the development of starch-accumulating storage sinks such as seeds or tubers, there is a remarkable change in the mechanism by which incoming Suc is cleaved to support biosynthesis and growth. Whereas during the early phases of sink development when the rates of cell division and metabolic activity are high, the invertase pathway is the dominate route by which Suc is metabolized, during the later phases of sink development when storage product synthesis predominates, the activity of the invertase pathway declines and is substituted by the Suc synthase (Susy) pathway. Remarkably, the emergence of the Susy pathway is highly correlated with the onset of starch biosynthesis (Quick and Schaeffer, 1996; Appeldoorn et al., 1997, 1999; Weber et al., 1997; Sturm and Tang, 1999). One of the striking differences between the invertase- and Susy-dependent Suc breakdown pathways is that whereas Suc mobilization via invertase is followed by the ATP-dependent phosphorylation of hexoses, UDP-Glc, the product of the Susy reaction, is metabolized to Glc-1-P via the inorganic pyrophosphate (PPi)-dependent enzyme UDP-Glc pyrophosphorylase (UGPase) (ap Rees and Morrell, 1990; Stitt, 1998; Fig. 1). The mechanism that is

involved in coordinating this shift in metabolism in developing sink organs is unknown, however, the potential involvement of PPi cannot be ignored. There is also presently no convincing explanation of how PPi is produced to support the Susy-dependent breakdown of Suc. Indeed the question has received virtually no attention, which is somewhat surprising given that the production of PPi could be a crucial regulatory and controlling level in the coordination of this development.

In potato (*Solanum tuberosum*) tubers it has been estimated that up to 70% of the incoming Suc is metabolized to starch, the remainder being roughly equally divided between respiration, structural polysaccharides, and other storage products (ap Rees and Morrell, 1990). Given the strong predominance of the starch biosynthetic flux in potato tubers in comparison to the flux through other metabolic pathways, there are essentially only two possibilities for the supply of the amount of PPi needed for Suc breakdown via the Susy-dependent pathway: It is either recycled from the starch biosynthesis pathway where PPi is produced in the amyloplast by ADP-Glc pyrophosphorylase (AGPase), or it is provided by a cycling process. In the latter case, two possibilities have been proposed (Taiz, 1986; ap Rees and Morrell, 1990): the PPi-dependent reaction of pyrophosphate: Fru-6-P phosphotransferase (PFP) and the tonoplast pyrophosphatase (vPPase), both of which would produce PPi by coupling to a parallel ATP consuming process (ATP-dependent tonoplast proton pump or phosphofructokinase [PFK], respectively).

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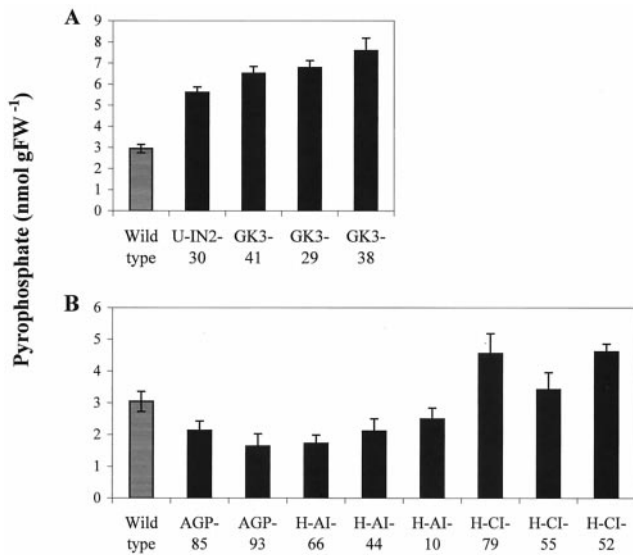


Figure 2. PPI concentration in transgenic tubers. Developing tuber samples were taken from 12-week-old plants grown in 2.5-L pots in the greenhouse. The PPI concentration was measured in TCA extracts by an enzyme-coupled test. A, Wild-type and transgenic potato lines U-IN2-30 and GK3. B, Wild-type and transgenic potato lines AGP, H-AI, and H-CI. The error bars represent the SE for determinations on six individual plants per line.

expressing lines are due to independent events, we analyzed transgenic plants containing combinations of both genetic modifications. As shown in Figure 2, the PPI level increased significantly in comparison to the AGPase background (AGP-93) when an invertase was additionally expressed in the cytosol (H-CI-lines). However, the PPI levels were below those found in the single transgenic lines expressing the invertase in the cytosol (U-IN2-30).

To ensure that the changes seen as a result of the expression of the invertase are due to its localization in the cytosol, we analyzed transgenic tubers where the invertase was targeted to the apoplast in the

AGP-93 background (H-AI-lines). These lines did not differ significantly in PPI content from the AGPase inhibited parental lines (AGP-93; Fig. 2).

Changes in Nucleotide Concentrations Do Not Always Correlate with Changes in PPI Levels

We decided to analyze the levels of nucleotides and nucleotide sugars in the same extracts used for the PPI measurements. Nucleotides are directly or indirectly involved in all PPI-dependent reactions. They are substrates and/or products in both the UGPase and AGPase reactions. Furthermore they are substrates for reactions occurring in parallel to PPI-dependent reactions such as the ATP-dependent phosphorylation of Fru-6-P by PFK (paralleling PFP) or the vacuolar proton-pumping ATPase (paralleling the vPPase). Nucleotide contents in the transgenic lines are presented in Table I. ATP, UTP, and GTP contents in the U-IN2-30 and all GK3 lines were significantly higher than in the wild type. The UTP concentration was elevated by up to 2-fold; the ATP content rose by 40% in U-IN2-30 and by 68% in GK3-38 in comparison to the wild type. These lines do not show strong changes in the ADP, UDP, and GDP contents, only in the GK3 lines was a significant increase in the UDP content observed. There was a tendency for an increase in both the ATP to ADP and UTP to UDP ratios in the U-IN2-30 and GK3-lines. These results are in agreement with the already published data on these transgenic lines (Trethewey et al., 1998), although it was important to confirm in this study that the expected changes and trends actually occurred in the samples used for the PPI analysis.

Changes in the nucleotide contents could also be observed in the AGPase antisense plants and in the H-CI and H-AI lines (Table II). There was a tendency toward higher nucleosidetriphosphate contents in the AGP and H-CI lines, whereas a reduction was

Table I. Nucleotide concentrations in transgenic tubers

Developing tuber samples were taken from 12-week-old plants grown in 2.5-L pots in the greenhouse. Nucleotide concentrations were measured in TCA extracts by HPLC. All data represent the means \pm SE of measurements on six independent plants. Asterisks represent values significantly different (*t* test $P < 0.05$) to the wild-type level. Nos. in bold highlight values significantly different (*t* test $P < 0.05$) to the parental line.

Parameter	Wild Type	U-IN2-30	GK3-41	GK3-29	GK3-38
			<i>nmol g⁻¹ fresh wt</i>		
ATP	47.0 \pm 2.5	66.3 \pm 5.3*	66.3 \pm 8.2*	77.6 \pm 7.5*	78.9 \pm 9.0*
UTP	25.2 \pm 1.5	41.2 \pm 2.3*	45.9 \pm 5.0*	51.7 \pm 4.5*	56.1 \pm 5.9*
GTP	11.7 \pm 1.1	14.5 \pm 1.4*	15.2 \pm 1.3*	16.7 \pm 1.7*	18.2 \pm 2.0*
ADP	20.2 \pm 1.1	19.5 \pm 1.9	25.8 \pm 2.9	27.2 \pm 3.8	23.6 \pm 3.1
UDP	7.5 \pm 0.9	9.1 \pm 0.5	13.4 \pm 1.6*	11.8 \pm 1.2*	12.4 \pm 0.6*
GDP	6.2 \pm 0.3	3.5 \pm 0.9*	4.8 \pm 0.6	5.7 \pm 1.5	7.1 \pm 1.1
ATP/ADP	2.3 \pm 0.1	3.5 \pm 0.2*	2.5 \pm 0.4	2.9 \pm 0.3	3.5 \pm 0.4*
UTP/UDP	3.5 \pm 0.3	4.6 \pm 0.2	3.5 \pm 0.7	4.5 \pm 0.6	4.6 \pm 0.6
ADP-Glc	1.4 \pm 0.3	2.3 \pm 0.5	1.9 \pm 0.5	2.4 \pm 0.8	1.3 \pm 0.3
Glc-1-P	16.6 \pm 0.8	47.0 \pm 4.2*	65.2 \pm 4.0*	54.4 \pm 3.2*	56.3 \pm 4.1*

Table II. Nucleotide concentrations in transgenic tubers

Developing tuber samples were taken from 12-week-old plants grown in 2.5-L pots. Nucleotide concentrations were measured in TCA extracts by HPLC. All data represent the means \pm SE of measurements on six independent plants. Asterisks represent values significantly different (t test $P < 0.05$) to the wild-type level. Nos. in bold highlight values significantly different (t test $P < 0.05$) to the parental line.

Parameter	Wild Type	AGP-85	AGP-93	H-AI-66	H-AI-44	H-AI-10	H-CI-79	H-CI-55	H-CI-52
	<i>nmol g⁻¹ fresh wt</i>								
ATP	33.4 \pm 2.2	44.7 \pm 7.2	50.3 \pm 6.5*	23.2 \pm 3.8	30.0 \pm 1.7	25.5 \pm 1.2*	47.3 \pm 5.9	52.4 \pm 8.2	42.0 \pm 3.1
UTP	15.1 \pm 1.6	18.0 \pm 2.8	19.3 \pm 2.9	14.2 \pm 2.0	12.7 \pm 1.9	9.3 \pm 0.4*	33.9 \pm 4.7*	22.5 \pm 3.1	18.6 \pm 1.4
GTP	7.5 \pm 0.5	8.4 \pm 1.3	11.4 \pm 1.5*	5.8 \pm 0.3*	7.4 \pm 0.7	6.2 \pm 0.3	10.5 \pm 1.1*	12.4 \pm 1.6*	10.7 \pm 0.5*
ADP	17.6 \pm 0.4	17.8 \pm 3.0	17.6 \pm 2.0	10.2 \pm 0.5*	12.6 \pm 1.1*	9.9 \pm 0.4*	22.4 \pm 1.5*	17.8 \pm 1.1	17.4 \pm 1.4
UDP	9.1 \pm 1.0	7.7 \pm 1.2	9.3 \pm 1.3	5.7 \pm 0.3*	6.0 \pm 0.7*	5.8 \pm 0.5*	9.3 \pm 0.9	7.4 \pm 0.8	7.5 \pm 0.8
GDP	4.6 \pm 0.1	3.7 \pm 1.0	2.9 \pm 1.0*	3.2 \pm 1.5	3.0 \pm 0.6*	2.8 \pm 0.2*	4.6 \pm 0.6	5.2 \pm 0.2	5.3 \pm 0.5
ATP/ADP	1.9 \pm 0.1	2.5 \pm 0.2*	2.9 \pm 0.3*	2.3 \pm 0.4	2.5 \pm 0.2	2.6 \pm 0.1*	2.1 \pm 0.3	2.9 \pm 0.4	2.5 \pm 0.2*
UTP/UDP	1.8 \pm 0.3	2.5 \pm 0.2	2.2 \pm 0.3	2.6 \pm 0.4	2.3 \pm 0.5	1.7 \pm 0.2	3.6 \pm 0.3*	3.0 \pm 0.1*	2.5 \pm 0.2

observed in the H-AI-lines. We found similar levels of nucleosidediphosphates in the AGP and H-CI lines in comparison to the wild type, but a reduction in the H-AI-lines where there was a decrease in ADP and UDP content of around 35% and 30%, respectively. The ATP to ADP ratio in the AGP-85 and AGP-93 lines was found to be significantly higher than in the wild type.

No Increase Was Found in the ADP-Glc Content of the Invertase Expressing Lines

No significant change was found in the ADP-Glc content of line U-IN2-30 with respect to the wild type (Table I). However, the Glc-1-P content of tubers of U-IN2-30 was found to be around three times higher than in the wild type. The presence of a bacterial glucokinase in the GK3 lines led to a further increase in the Glc-1-P level in comparison to the parental line U-IN2-30, but no change in the ADP-Glc levels could be found in the GK3 lines (Table I).

DISCUSSION

The aim of this work was to determine whether transgenic potato tubers altered in Suc and starch metabolism could provide evidence of a regulatory role for PPI in the coordination of tuber carbohydrate metabolism. Further, through evaluation of PPI levels, nucleotides, nucleotide sugars, and consideration of what is known about the metabolism in the lines studied (Trethewey et al., 1998, 1999a, 1999b), we aimed to draw some conclusions about the source of PPI for Suc metabolism in potato tubers.

A Reduced AGPase Activity in Transgenic Tubers Leads to a Decrease in the PPI Level

As described in "Results," antisense inhibition of the AGPase led to a reduction in the steady-state PPI levels in the transgenic tubers. In principle this reduction could be due either to an increased consumption of PPI or to a decreased production of PPI.

An increase in PPI consumption could be achieved by (Fig. 1): (a) an increase in UDP-Glc breakdown via UGPase; (b) an increase in PFP activity in the glycolytic direction; and (c) an increase in the activity of the vPPase.

With respect to the first possibility, the AGPase antisense lines demonstrate no changes in the respiration rate (Geigenberger et al., 1999; Sweetlove et al., 1999). This fact together with the inhibition of starch synthesis makes it unlikely that there is an increase in the net rate of Glc-1-P production and therefore of PPI usage via UGPase in these transgenic tubers. The second possibility, that there is an increase in the net flux through PFP in the glycolytic direction in the transgenic tubers is also unlikely for the same reasons; no increase in the glycolytic flux was found in experimentation by two separate groups (Geigenberger et al., 1999; Sweetlove et al., 1999). The third possibility is more complex to evaluate. There is an increase in the soluble sugar content of tubers from the AGPase antisense lines, and this might represent sugars predominantly stored in the vacuole. If this were the case, then there would be an increased demand for transport of sugar across the tonoplast membrane, a process that would require energy. This energy could be supplied by the vPPase, although the increase in the ATP to ADP ratio in these lines might indicate that the ATP-dependent tonoplast proton pump would be just as likely to participate in the enhanced energization of the tonoplast. Further, the absolute increase in the storage of sugars in the tonoplast is likely to be insignificant in comparison to the reduction in flux through the pathways of Suc mobilization and starch synthesis (ap Rees and Morrell, 1990), and it is therefore questionable whether changes in vPPase activity could have a significant impact on the steady-state level of PPI in a growing potato tuber. Taking these arguments together, we believe that it is unlikely that the decreased content of PPI in the AGPase antisense lines is due to any of the possible explanations centered on an increased consumption of PPI.

We therefore favor the possibility that the observed decrease in PPi in the AGPase antisense lines is due to a decreased production. The most predominant PPi producing reaction in growing potato tubers is the formation of ADP-Glc catalyzed by AGPase (ap Rees and Morrell, 1990; Fig. 1). The reduction in AGPase activity in the antisense lines would directly lead to a reduced production of PPi in the plastid. It is a generally accepted dogma that the AGPase reaction is effectively irreversible due to the action of an alkaline pyrophosphatase in plastids (Gross and ap Rees, 1986; Weiner et al., 1987; ap Rees and Morrell, 1990). However, there is currently no *in vivo* evidence to support the hypothesis that PPi produced in the amyloplast is immediately cleaved into the component phosphate groups. Therefore, we regard the possibilities that significant levels of PPi are maintained in the amyloplast, or that PPi is rapidly channeled back to the cytosol to be hypotheses worthy of further experimentation. In the case of the latter possibility there is a single report of a PPi transporter located in the spinach chloroplast membrane (Lunn and Douce, 1993). We believe that these two hypotheses represent the most plausible explanations for the reduction in PPi found in the AGPase antisense lines.

The Cytosolic Expression of a Yeast Invertase in Potato Tubers Leads to an Increase in PPi Concentration

It has been shown that in the lines U-IN2-30 and GK3 an intense Suc cycling occurs (Trethewey et al., 1999b; Fig. 3). Suc is cleaved by the yeast invertase to hexoses, which are subsequently phosphorylated by hexose kinases prior to conversion to UDP-Glc and PPi via the action of phosphoglucomutase, phosphoglucoisomerase, and UGPase. UDP-Glc is finally re-incorporated into Suc via Suc-P synthase. This cycling, which results in a net production of PPi, is likely to be the explanation for the increase in PPi seen in these lines. Further evidence for this view comes from potato tubers that express a bacterial Suc phosphorylase. These lines also contain a PPi-independent Suc breakdown pathway leading to Glc-1-P and Fru and have been found to contain elevated PPi levels (data not shown). All of these lines have a reduced starch accumulation; however, the reduced flux through the starch biosynthetic pathway is likely to be masked in influence on the PPi levels by the very active nature of the cytosolic Suc cycling (Trethewey et al., 1998, 1999b).

Invertase Expressing Lines Exhibit an Increase in Glc-1-P, ATP, and 3-P-Glycerate Levels, However, ADP-Glc Levels Remain Unchanged

The increase in Glc-1-P, ATP (Table I), and 3-P-glycerate levels (Trethewey et al., 1998) in the invertase expressing line U-IN2-30 and the GK3 lines does not lead to a parallel increase in the ADP-Glc level

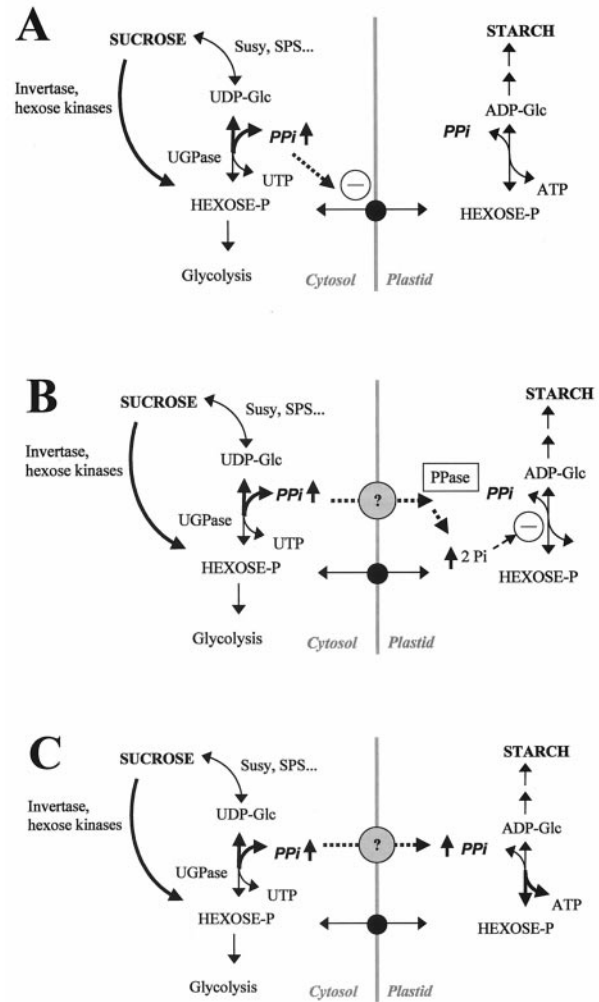


Figure 3. Possible mechanisms through which an increase in PPi leads to a reduction in starch accumulation in potato tubers that express a yeast invertase in the cytosol. The elevated PPi level may impede plastidial transport process (A); lead to an increase in plastidial Pi, which would inhibit the AGPase reaction (B); and/or an increase in plastidial PPi concentration may influence the rate of the AGPase reaction (C).

(Table I). At first sight this is surprising if the reaction catalyzed by AGPase is irreversible due to the removal of PPi by the inorganic pyrophosphatase, and the consumption of ADP-Glc by starch synthases can be assumed to be reduced given the decrease in starch accumulation in these lines. One possible explanation for this observation is that the increase in total Glc-1-P and ATP in the tuber is not reflected in the amyloplast, due to a disturbance of plastidial transport processes by PPi, as has been shown for some photosynthetic systems (Fig. 3A; Heldt and Rapley, 1970; Woldegiorgis et al., 1985; Bölter et al., 1999). An alternative explanation, building on the arguments in the previous section, would be that a higher PPi production in the cytosol leads to an elevated steady-state level of PPi in the amyloplast and this in turn influences either directly or indi-

rectly the activity of the AGPase. An elevated plastidial PPI level may result in an increase in plastidial orthophosphate levels via the action of the inorganic pyrophosphatase, which in turn would lead to a decreased 3-P-glycerate to orthophosphate ratio and a decrease in AGPase activity (Fig. 3B; Sowokinos and Preiss, 1982). Alternatively if the high cytosolic PPI concentration leads to an increase in the plastidial level of PPI, PPI could have a direct effect on the net flux through AGPase by influencing the equilibrium of the reaction (Fig. 3C). All of the hypotheses discussed in this section (Fig. 3) could account for the hitherto unexplained reduction in starch content in the GK3 and U-IN2-30 lines (Sonnewald et al., 1997; Trethewey et al., 1998).

The Decrease in PPI Levels in the AGPase Antisense Lines and the Increase Found in the Cytosolic Invertase Lines Probably Reflect Two Independent Mechanisms

The combined reduction in AGPase activity and expression of a yeast invertase in the cytosol of the H-CI transgenic lines also results in a doubling of the tuber PPI content with respect to the parental line (AGP-93). However, no change in PPI levels was seen when the yeast invertase was expressed in the apoplast in combination with reduced AGPase activity (H-AI-lines). This strongly suggests that the increase in PPI seen in the U-IN2-30 and GK3 lines is indeed due to cytosolic reactions and not to metabolism in the amyloplast, e.g. starch cycling. On the other hand the reduction in PPI content seen in the antisense AGPase lines is probably due to plastidial reactions and not to changes in the cytosolic metabolism.

Hypothesis: PPI Might Integrate the Susy-Dependent Breakdown of Suc, Starch Synthesis, and Glycolysis in Starch-Storing Sinks Like Potato Tubers

Taking all the evidence presented and reviewed here together, we propose the following model to describe the situation in starch-storing sinks. During the early phases of development invertases are active, resulting in an accumulation of PPI. This high PPI level would, by an as-yet-unidentified mechanism (see previous discussion), inhibit starch synthesis and activate glycolysis via the PPI-dependent PFP. This mechanism might be the same one that leads to the reduced starch accumulation found in the U-IN2-30 and GK3 transgenic lines. The switch from an invertase-dependent to a Susy-dependent Suc breakdown during normal tuber development would lead to a decrease in PPI levels following an increased use of PPI by the Susy pathway of Suc mobilization. The subsequent reduction in tuber PPI content would lead to the observed activation in the flux through the starch biosynthetic pathway. The question of where the PPI is generated to support continued Suc mobilization by the Susy pathway has

been indirectly addressed in this study and, based upon the results from the analysis of transgenic tubers, we propose that the most likely source of PPI is the AGPase reaction. This proposal runs against the current dogma, although the hypothesis that the PPI level provides a link between catabolic and anabolic reactions has already been proposed in the context of cytosolic metabolism in plant cells (Taiz, 1986). Indeed, a link between the supply of PPI for the cleavage of Suc by the Susy pathway and the production of PPI by the AGPase has been proposed from mainly theoretical considerations for endosperm sink tissue (Doehlert, 1990; Kleczkowski, 1994). Further, the recent discovery that AGPase is localized in the cytosol in endosperms (Denyer et al., 1996; Thorbjørnsen et al., 1996; Shannon et al., 1998) strongly supports the hypothesis that the activities of the AGPase and UGPase enzymes are coupled via the PPI level in these tissues.

The proposed link between PPI and the coordination of cytosolic Suc and plastidial starch metabolism might also explain some discrepancies observed in potato plants expressing an *Escherichia coli* pyrophosphatase in the cytosol. Initial experiments with these transgenic tubers showed an inhibition of Suc breakdown and a reduction in starch accumulation (Jelitto et al., 1992; Sonnewald, 1992). However, in subsequent experiments an increase in starch content was reported (Geigenberger et al., 1998). The authors argue that differences in the age of the plants or in the growth conditions between the experiments may be the reason for these conflicting results. We propose that an "optimal" PPI level might exist at which Suc breakdown is unrestricted and the AGPase reaction in the plastid is promoted. Such an optimal concentration may have been reached in the circumstances of the later experiments.

In the model presented here we propose that there is a tight integration of cytosolic and plastidial metabolism via PPI. The hypothesis described here is based upon whole tissue measurements of PPI; the achievement of subcellular measurements of PPI is an important task to confirm the model. However, although still speculative in nature, we believe that the model is the only one that can account for all the recent data obtained from transgenic potato tubers while also offering an explanation for the source of PPI to support the continued breakdown of Suc. Finally, the model has the potential to explain the strong correlation seen in all starch-storing sinks between the unloading of Suc, starch biosynthesis, and glycolysis.

MATERIALS AND METHODS

Plant Material

Potato (*Solanum tuberosum* L. cv Desirée) plants (Saat-zucht Lange AG, Bad Schwartau, Germany), along with the transgenic lines U-IN2-30, GK3-29, -38, and -41 (Sonnewald

et al., 1997; Trethewey et al., 1998, 1999b), AGP-85 and 93 (Müller-Röber et al., 1992; Trethewey et al., 1999a), H-AI-66, -44, and -10 (Trethewey et al., 1999a), and H-CI-79, -55, and -52 (Trethewey et al., 1999a) were grown in the greenhouse under a 16-h light, 8-h dark regime with supplementary light to ensure a minimum of 250 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ at 22°C. The term developing tubers is used for tubers over 10 g fresh weight harvested from healthy 2- to 3-month-old plants. U-IN2-30 was the parent line used for transformation with a bacterial glucokinase to generate the GK3 lines (Trethewey et al., 1998). Line AGP-93, expressing an antisense construct targeted against the small subunit of the AGPase under the control of the 35S promoter (Müller-Röber et al., 1992), was transformed with the yeast-derived invertase gene (*suc2*) under the control of the patatin promoter giving rise to double transgenic lines with an invertase localized either in the cytosol (H-CI-79, -55, -52; Trethewey et al., 1999a) or in the apoplast (H-AI-66, -44, -10; Trethewey et al., 1999a).

Chemicals

All enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), with the exception of the PFP from *Propionobacterium freudenreichii shermanii*, which was obtained from Sigma-Aldrich (Darmstadt, Germany). Chemicals were obtained from either Sigma or Merck (Darmstadt, Germany).

Biochemical Analysis

Metabolic intermediates were determined in trichloroacetic acid (TCA) extracts exactly as described by Trethewey et al. (1998). Nucleotides and ADP-Glc were measured in TCA extracts by HPLC (Trethewey et al., 1998). PPi was determined using a coupled enzymatic assay based upon Weiner et al. (1987). The assay contained 50 mM Tris-acetate (pH 7.5), 2 mM MgCl_2 , 1 mM EDTA, 20 μM NADH, 0.2 mM Fru-6-P, 0.7 unit mL^{-1} aldolase, 7 units mL^{-1} triose-P isomerase, and 2.8 units mL^{-1} glycerol-3-P-dehydrogenase (all enzymes from rabbit muscle). The reaction was started with the addition of 0.6 unit mL^{-1} PFP from *P. freudenreichii shermanii*. Pseudoextracts (without tissue) were also prepared to confirm the absence of significant PPi contamination in all the solutions and vessels used in the procedure. The reliability of the extraction procedure and assay protocol was confirmed using recovery experiments (e.g. Trethewey et al., 1998; Veramendi et al., 1999); in the case of PPi the recovery was found to be $105\% \pm 1\%$ (SE, $n = 6$).

Statistical Analysis of Data

t Tests were performed using the algorithm integrated into Microsoft Excel 7.0 (Microsoft, Seattle). The word "significant" is used in the text only when the change in question has been confirmed to be statistically significant ($P < 0.05$) with the *t* test.

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