Regulation of sucrose to starch conversion in growing potato tubers

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Abstract
Growing potato tubers have been used as a model system to investigate the regulation of starch synthesis. Results indicate that sucrose degradation and starch synthesis are controlled via regulatory signals in response to sucrose and oxygen availability. (i) Sucrose leads to a coordinated up-regulation of sucrose synthase and ADP-glucose phosphorylase at the transcriptional and post-transcriptional level. Transcriptional regulation of ADP-glucose phosphorylase leads to rapid changes in transcript levels, but relatively slow changes in protein levels. The rapid regulation of this enzyme in response to sucrose is mediated by a novel mechanism, involving redox-activation of ADPGlc pyrophosphorylase. Sucrose synthase is regulated via transcriptional regulation, but again the resulting changes in enzyme activity occur relatively slowly. More rapid changes in the flux of this enzyme follow due to rapid changes in the levels of uridine nucleotides. (ii) Internal oxygen concentrations fall to low levels in growing tubers, triggering a restriction of respiration, a decrease in the adenylate energy status, and a widespread decrease in metabolic and biosynthetic activity. These metabolic adaptations will allow oxygen consumption to be decreased and prevent the tissue from becoming anoxic. It will be discussed how these factors interact at different levels and different time-scales of control to regulate tuber metabolism in response to physiological and environmental inputs.

Key words: Potato, regulation, starch synthesis, sucrose degradation, oxygen, sucrose, nucleotides.

Introduction
Starch is the most widespread carbon reserve stored in plants and is of considerable industrial significance for food and non-food uses. Despite recent advances in the genetic analysis of starch biosynthesis, the regulation of this important process is still poorly understood. Attempts to improve starch yield were mainly hampered by the lack of a complete understanding of the factors and mechanisms regulating this pathway (Nelson and Pan, 1995; Fernie et al., 2002a).

To investigate the regulation of sucrose to starch conversion in more detail, growing potato tubers have been used as a model system. The reasons are that (i) unlike many other tissues the entry of sucrose into metabolism is relatively simple, in that it is unloaded symplastically from the phloem (Oparka and Prior, 1988; Viola et al., 2001) and degraded via sucrose synthase (Susy) (Morell and ap Rees, 1986; Zrenner et al., 1995), (ii) the pathway for the conversion of sucrose to starch is known (Fig. 1), the enzymes mediating it are well characterized and most of the genes that encode them have been cloned (Kruger, 1997), and (iii) transgenic plants can be readily produced (Fernie et al., 2002a). In the following it will be discussed how conversion of sucrose to starch is regulated in potato tubers, and what are the major physiological and environmental factors affecting this process.

Transcriptional regulation of starch synthesis
Various transgenic lines have been generated in which the activities of most of the individual enzymes involved in the pathway of sucrose to starch have been independently modulated. A 2–3-fold decrease in AGP-glucose pyrophosphorylase (AGPase) (Müller-Röber et al., 1992) or SuSy activity (Zrenner et al., 1995) in antisense transformants
had no significant effect on tuber yield or starch synthesis. In analogous studies, a 30% decrease in plastidial phosphoglucomutase (Fernie et al., 2001a), or a more than 80% decrease of UDP-glucose pyrophosphorylase (Zrenner et al., 1993), sucrose-phosphate synthase (SPS) (Geigenberger et al., 1999), hexokinase (Veramendi et al., 1999, 2002), starch synthase (Marshall et al., 1996; Fulton et al., 2002), or branching enzyme (Safford et al., 1998) had no significant effect on starch accumulation in growing tubers. These studies show that the expression levels of individual enzymes of the pathway largely do not limit the flux from sucrose to starch, and demonstrate the need for co-ordinated mechanisms regulating starch synthesis at the transcriptional and post-transcriptional level.

Two key enzymes involved in sucrose to starch conversion are known to be subject to transcriptional regulation, SuSy and AGPase. SuSy, the first step in the pathway from sucrose to starch, catalyses the conversion of sucrose and UDP to UDPGlc and fructose via a readily reversible reaction (Geigenberger and Stitt, 1993), its expression being increased by sucrose, anaerobiosis and wounding (Salanoubat and Belliard, 1989; Zeng et al., 1998). AGPase catalyses the first committed step of starch biosynthesis in the plastid by converting Glc1P and ATP to ADPGlc and P Pi. Its expression is increased by sucrose (Müller-Röber et al., 1990; Sokolov et al., 1998) and decreased by nitrate (Scheible et al., 1997) and phosphate (Nielsen et al., 1998). The levels of transcripts for AGPB, AGPS and SUS1 are high in growing tubers, and both decrease rapidly after an interruption of assimilate supply, i.e. after detaching tubers (Ross and Davis, 1992; Tiessen et al., 2002). There are also parallel changes in AGPase and SuSy expression in response to diurnal changes of sucrose in tubers, the levels of both transcripts being high at the end of the light period and decrease at the end of the night (Geigenberger and Stitt, 2000). These results indicate a co-ordinated up-regulation of SuSy and AGPase in potato tubers in response to sucrose supply. The sensing and signalling mechanisms mediating this process are unknown. The finding that sucrose-induction of sucrose

Fig. 1. Pathway of sucrose to starch conversion and its subcellular compartmentation in potato tubers. (1) Sucrose synthase, (2) UDP-glucose pyrophosphorylase, (3) sucrose-phosphate synthase, (4) fructokinase, (5) cytosolic phosphoglucomutase, (6) phosphoglucoisomerase, (7) hexose-phosphate translocator, (8) triose-phosphate translocator, (9) plastidial phosphoglucomutase, (10) ADP-glucose pyrophosphorylase, (11) soluble starch synthase, (12) granule-bound starch synthase, (13) branching enzyme, (14) adenylate translocator.
Regulation of starch synthesis by changes in the levels of phosphorylated intermediates

A premium in the regulation of carbon metabolism in leaves is to maintain metabolite concentrations, which allow the fixation of carbon in the chloroplast. This requires rapid metabolic regulation in response to changes in light, CO₂ supply or sucrose export. Regulation is therefore occurring via regulatory circuits acting on key enzymes of photosynthetic metabolism in response to changes in metabolite levels (Stitt et al., 1987). In this context, a rising 3PGA/Pi ratio acts as a signal that fixed carbon is available beyond that required for sucrose synthesis. Starch synthesis is then stimulated, because AGPase is exquisitely sensitive to allosteric regulation, being activated by 3PGA and inhibited by Pi (Preiss, 1988).

AGPase from potato tubers resembles the leaf enzyme in being allosterically activated by 3PGA and inhibited by Pi (Sowokinos and Preiss, 1982). In contrast to leaves, however, the levels of phosphorylated intermediates in potato tubers are remarkably constant. There are only marginal changes in phosphorylated intermediates in the diurnal time-frame (Geigenberger and Stitt, 2000) or during tuber development (Merlo et al., 1993). The remarkable constancy of metabolite levels in tubers is mainly due to the operation of metabolic cycles in which the reversible reactions catalysed by SuSy and PFP play an important role (Hatzfeld and Stitt, 1990; Geigenberger and Stitt, 1993; Fernie et al., 2001b). These metabolic cycles allow large and rapid changes in the net rate of sucrose breakdown in response to the demand in the cell, even though the steady-state concentrations of metabolites hardly change.

During environmental perturbations like wounding (Hajirezaei and Stitt, 1991), water stress (Geigenberger et al., 1997), high temperature (Geigenberger et al., 1998), and hypoxia (Dixon and ap Rees, 1980; Geigenberger et al., 2000) this balance is disturbed and, consequently, large changes in metabolite levels occur in the tubers. During water stress, SPS is activated via protein phosphorylation and the subsequent stimulation of sucrose resynthesis leads to a decrease in metabolite levels including 3PGA (Geigenberger et al., 1997, 1999). A similar decrease in 3PGA levels takes place when respiration is stimulated in response to elevated temperatures (Geigenberger et al., 1998). In both cases the drop in 3PGA levels is accompanied by an inhibition of starch synthesis. The strong correlation between the levels of 3PGA and the levels of ADPGlc under these conditions provide evidence for the importance of the allosteric properties of potato AGPase for the regulation of starch synthesis in vivo. They operate to link the rate of starch synthesis to short-term changes in the balance between sucrose breakdown and respiration in potato tubers.

Redox-regulation of starch synthesis

Several situations have been reported in which changes in the rate of starch synthesis could not be explained by allosteric regulation or changes in expression of AGPase, including tuber detachment from the mother plant (Geigenberger et al., 1994), feeding sucrose to tuber discs (Geiger et al., 1998), diurnal changes in sucrose supply (Geigenberger and Stitt, 2000), and ectopic expression of sucrose phosphorylase in the cytosol of transgenic tubers (Trethewey et al., 2001). In all of these cases starch synthesis changed independently of overall AGPase activity, and reciprocally to the levels of phosphorylated intermediates, especially 3PGA.

Recent studies clarified that, under these conditions, starch synthesis is regulated by a novel mechanism, which involves post-translational redox-modification of AGPase (Tiessen et al., 2002). The site at which regulation occurs was clarified by analysis of the subcellular level of each metabolite between sucrose and starch using a non-aqueous fractionation technique (Farré et al., 2001), indicating AGPase as the only step whose substrates rise and mass-action ratios falls after detaching tubers. This was confirmed by substitution of higher plant AGPase with a heterologous bacterial AGPase in transgenic tubers, providing genetic evidence that the inhibitory mechanism requires the presence of a native AGPase.

The missing mechanism was subsequently clarified by the separation of extracts in non-reducing SDS gels revealing that the small subunit of the heterotrimeric AGPase (AGPB) was present as a mixture of monomers and dimers in growing tubers, and becomes completely dimerized in detached tubers (Tiessen et al., 2002). When activity was measured using a modified protocol omitting dithiothreitol in buffer preparations and allowing rapid
extraction and analysis, dimerization was accompanied by a decrease in enzyme activity due to changes in the kinetic properties of the enzyme, including a decrease in substrate affinities and sensitivity to allosteric effectors. Dimerization could be reversed and AGPase reactivated by incubating extracts \textit{(in vitro)} or tuber slices \textit{(in vivo)} with dithiothreitol. A similar activation of AGPase in response to dithiothreitol has been observed \textit{in vitro} using heterologously over-expressed potato AGPase (Fu \textit{et al.}, 1998). In this case it could be shown that the enzyme is activated by the reduction and opening of an intermolecular disulphide bridge between the Cys12 of the two small subunits.

Redox-modulation of AGPase provides a novel mechanism that combines with allosteric and transcriptional control to co-ordinate AGPase activity in a network that allows starch synthesis to respond across a range of time-scales to a variety of physiological and environmental inputs (Fig. 2). Allosteric control by 3PGA and Pi operates in a time-frame of seconds to adjust the rate of starch synthesis to the balance between sucrose breakdown and respiration. Post-translational redox-modulation leads to changes in AGPase activity in a time-frame of about 30–60 min. Activation occurs in response to factors directly or indirectly related to an increased sucrose availability, and leads to stimulation of starch synthesis and a decrease in glycolytic metabolite levels (Tiessen \textit{et al.}, 2002). The signalling components leading to redox-modulation of AGPase are still unknown and may involve thioredoxins (Ballicora \textit{et al.}, 2000) as well as putative sugar sensors (Smeekens, 2000). Transcriptional regulation in response to changes of sucrose allows more gradual changes in AGPase activity, which requires several days to develop.

\textbf{Relevance of nucleotide cofactors for sucrose to starch conversion and respiration}

Attempts to understand the regulation of metabolism and to engineer metabolic fluxes have been dominated by investigations of the expression levels of enzymes and the role of their kinetic properties. Less attention has been paid to the possibility that metabolic fluxes may also be restricted by the levels of nucleotide cofactors. Sucrose degradation via the reversible reaction of SuSy requires uridine nucleotides (Geigenberger and Stitt, 1993). The \textit{in vivo} concentration of UDP determined in the cytosol of potato tubers using non-aqueous fractionation (52–58 $\mu$M; Farre\textit{ et al.}, 2001; Tiessen \textit{et al.}, 2002) is well below the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig2.png}
\caption{ADP-glucose pyrophosphorylase regulates starch synthesis at different time scales and different levels of control in growing potato tubers (modified from Tiessen \textit{et al.} (2002)).}
\end{figure}
$K_m$ of potato tuber SuSy for UDP (100–700 μM; Avigad, 1982), indicating that UDP levels might co-limit the rate of sucrose degradation. Recent experiments using potato tuber slices showed that sucrose degradation and starch synthesis are indeed stimulated when the overall uridine nucleotide pool is increased by feeding orotate, an intermediate of the de novo pathway of purine synthesis (Loef et al., 1999).

Conversion of sucrose to starch via fructokinase and AGPase requires ATP. The ATP required for the AGPase reaction is imported into the plastid via an ATP/ADP transport protein located on the inner-envelope membrane (Heldt, 1969; Kampfenkel et al., 1995). Recent studies with transgenic potato tubers showed that a relatively small decrease in ATP/ADP transporter activity leads to reduced levels of ADPGlc and total starch content and a lower amylase:amylopectin ratio, whereas increased transporter activity had the opposite effect (Tjaden et al., 1998; Geigenberger et al., 2001). These findings demonstrate that the rate of ATP import exerts considerable control on the rate of starch synthesis (control coefficient of c. 0.7) and also affects the molecular composition and morphology of starch granules in potato tubers. It also indicates that AGPase is ATP limited in vivo. This conclusion is further strengthened by subcellular analysis of the plastidial concentrations of ATP in growing wild-type potato tubers (80–179 μM; Farré et al., 2001; Tiessen et al., 2002), which are in the range of the $K_m$ of potato tuber AGPase for ATP (120–190 μM; Sowokinos and Preiss, 1982; Ballicora et al., 1995). Recently, it was shown that when adenine is fed to tuber discs, there is an increase in ATP level and a stimulation of starch synthesis (Loef et al., 2001).

These studies show that sucrose breakdown and starch synthesis are restricted by the levels of adenine and uridine nucleotide cofactors. Increased adenine nucleotides, but not uridine nucleotides also lead to a stimulation of respiration, probably due to elevated levels of ADP (Loef et al., 2001). During the rapid starch accumulation phase in bulking tubers, increased expression of SuSy is accompanied by a marked increase of uridine nucleotides, but not of adenine nucleotides (Merlo et al., 1993; Loef et al., 1999). This allows sucrose mobilization to be increased, without a parallel and potentially counterproductive stimulation of respiration (see discussion below). Similar to the expression of SuSy, the increase in uridine nucleotide levels during tuber bulking could be linked to sucrose. There is a rapid decrease of uridine nucleotide levels in potato tuber slices incubated for 2 h in buffer, which can partially be prevented by adding sucrose (Loef et al., 2001; Geigenberger and Stitt, 2000). In intact tubers, diurnal changes in sucrose supply are accompanied by changes in uridine nucleotide levels, leading to increased levels of UDPGlc, UTP and UDP and an increased ratio of UDP/UTP in tubers when sucrose is high at the end of the light period (Geigenberger and Stitt, 2000). The rapidity of these changes in the uridine nucleotide levels indicate that post-transcriptional mechanisms are involved.

**On the role of oxygen and sucrose as regulators of tuber metabolism**

The results presented so far suggest a pivotal role of sucrose in regulating tuber metabolism at the transcriptional and post-transcriptional level to stimulate sucrose degradation and starch biosynthesis in response to sucrose supply (Fig. 3A). Recent studies, however, show that this concept has to be extended. By using oxygen microelectrodes it could be shown that oxygen falls to low levels (4–8% versus ambient levels of about 21%) inside bulking potato tubers (Geigenberger et al., 2000), which is in agreement with older studies summarized in Stiles (1960). The internal oxygen concentration decreases with increasing tuber size. There are marked gradients of oxygen inside growing tubers, with typical values being 8–10% in the periphery and 2–5% in the centre of the tuber. The decrease in oxygen tension from the periphery towards the centre of the tuber is accompanied by a decrease in the ATP/ADP ratio and the cellular adenylate energy charge indicating an inhibition of respiration (Geigenberger et al., 2000), as well as by a decrease in starch level indicating an inhibition of starch biosynthesis (P Geigenberger, AR Fernie, unpublished results). Crucially, this decrease in the adenylate status is not accompanied by lactate accumulation, and occurs at oxygen concentrations that are 100-fold above the $K_m$ ($O_2$) of cytochrome oxidase.

These results were confirmed and extended in an independent approach in which tuber discs were incubated in various oxygen concentrations from 0–40% using premixed gases (Geigenberger et al., 2000). There was a continuous decrease in energy state and glycolytic flux when external oxygen was decreased from 40% to 1%. Also respiration rates were shown to decrease when external oxygen fell from 21% (ambient level) to 4% or 8%. Labelling studies demonstrated that starch synthesis and other biosynthetic fluxes were inhibited at 4–8% oxygen. This was, however, not due to fermentation, since there was no increase of lactate under these oxygen concentrations. Oxygen had to be decreased below 1% before glycolysis was stimulated and lactate accumulated due to the onset of fermentation. The responses to low oxygen and anoxia are, therefore, diametrically opposed. Similar oxygen gradients and changes in response to external oxygen have been reported to occur in seeds from Arabidopsis (Porterfield et al., 1999; Gibon et al., 2002) and pea (Rolletscheck et al., 2002).

It is an interesting question why plants, unlike multicellular animals, did not evolve specialized circulation systems to allow a more efficient transport of gases to internal tissues. However, the above studies suggest that
falling internal oxygen tensions trigger metabolic adaptations, including a restriction of respiration, a decrease in adenylate levels and a widespread decrease in metabolic and biosynthetic activity, which will allow oxygen consumption to be decreased and prevent tissue from becoming anoxic (Fig. 3B). Less is known about the mechanisms involved in the scenario. It is highly unlikely that cytochrome oxidase ($K_m$ for oxygen is 0.01%) is directly limited by oxygen in this concentration range (4–40%), which suggests that an oxygen sensor may be involved. More studies are needed to investigate how oxygen is sensed in plants, and what are the factors leading to the adaptive changes in metabolism.

This premium on biochemical adaptations to conserve energy and allow oxygen consumption to be decreased may explain the unusual use of PPi as an alternative energy donor in plants (Sonnewald, 1992; Stitt, 1998). Whereas breakdown of a molecule of sucrose via invertase requires 2 molecules of ATP, breakdown of sucrose via SuSy and UGPase requires only 1 molecule of PPi. In seeds and tubers, invertase is typically expressed during the early stages and SuSy during the later stages of development (Appeldoorn et al., 1997; Weber et al., 1997). This switch correlates with a transition from cell division to cell expansion and storage and has initially been interpreted in terms of regulation by sugar-related signals (Borisjuk et al., 1998). An alternative explanation would be that it is related to the decrease in oxygen tension that develops as seeds and tubers grow. As observed in maize roots (Zeng et al., 1999) and potato tubers (K Bologa, AR Fernie, P Geigenberger, unpublished results) low oxygen repress invertase and induce specific SuSy genes.

Intriguingly, ectopic expression of invertase (Trethewey et al., 1998) or sucrose phosphorylase (Trethewey et al., 2001) to bypass the endogenous sucrose synthase route in potato tubers leads to a decrease in tuber sucrose levels, but also to a large stimulation of respiration, a fall in the internal oxygen levels down to almost zero and a decrease in the cellular energy state (Fernie et al., 2002b; K Bologa, AR Fernie, P Geigenberger, unpublished data). These
results indicate that the inhibition of starch synthesis in invertase and sucrose phosphorylase expressing tubers is attributable to two different factors: (i) the decreased levels of sucrose (see above) and (ii) the increased energy consumption due to introduction of an alternative pathway of sucrose degradation. This also implies that sucrose degradation via sucrose synthase is important to maintain a relatively large sucrose pool and to minimize ATP consumption required for normal metabolic function in the wild type.

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