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REVIEW PAPER

The role of transitory starch in C_3, CAM, and C_4 metabolism and opportunities for engineering leaf starch accumulation

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Abstract

Essentially all plants store starch in their leaves during the day and break it down the following night. This transitory starch accumulation acts as an overflow mechanism when the sucrose synthesis capacity is limiting, and transitory starch also acts as a carbon store to provide sugar at night. Transitory starch breakdown can occur by either of two pathways; significant progress has been made in understanding these pathways in C_3 plants. The hydrolytic (amylolytic) pathway generating maltose appears to be the primary source of sugar for export from C_3 chloroplasts at night, whereas the phosphorolytic pathway supplies carbon for chloroplast reactions, in particular in the light. In crassulacean acid metabolism (CAM) plants, the hydrolytic pathway predominates when plants operate in C_3 mode, but the phosphorolytic pathway predominates when they operate in CAM mode. Information on transitory starch metabolism in C_4 plants has now become available as a result of combined microscopy and proteome studies. Starch accumulates in all cell types in immature maize leaf tissue, but in mature leaf tissues starch accumulation ceases in mesophyll cells except when sugar export from leaves is blocked. Proper regulation of the amount of carbon that goes into starch, the pathway of starch breakdown, and the location of starch accumulation could help ensure that engineering of C_4 metabolism is coordinated with the downstream reactions required for efficient photosynthesis.

Key words: C_4, CAM, maltose, photosynthesis, starch.

Introduction

The evolution of C_3 into C_4 metabolism required alteration of control of carbon metabolism both spatially and in terms of the rate. While many of the required changes are known, it is unclear what changes might be needed or desirable in starch metabolism to optimize C_4 metabolism. It may be beneficial to modify transitory starch synthesis and accumulation patterns in C_4 plants to improve their utility. Leaf starch is not surrounded by a protein layer as grain starch is, and it is therefore more accessible for digestion. Therefore, increased starch content is likely to improve the quality of silage (Allen *et al.*, 2003). Modified leaf starch metabolism could also improve C_4 plants as sources of biofuels if it provided a bigger carbon sink. Starch is more easily fermented than cell wall material and, because of its location in leaves that are not part of the human diet, leaf starch, unlike grain starch, would not directly compete with food production, providing that increased leaf starch levels do not reduce grain yield.

In light of both the basic questions of transitory starch in C_4 metabolism and the possible engineering opportunities to manipulate starch accumulation in C_4 plants, the current state of knowledge of transitory starch in C_4 plants and crassulacean acid metabolism (CAM) plants is reviewed here. Since most information about transitory starch is known from studies using C_3 plants, what is known in C_3 plants is described first and then that information is put into the perspective of C_4 and CAM plants. The understanding of transitory starch metabolism in C_3 plants has increased significantly in the past decade and has been the subject of several excellent reviews (Tetlow *et al.*, 2004; Lu and Sharkey, 2006; Zeeman *et al.*, 2007; Kötting *et al.*, 2010).

What is transitory starch?

Transitory starch is formed in chloroplasts during the day as a result of photosynthesis and is broken down at night. Transitory starch is confined to leaves and other photosynthesizing tissues of the plant. While plants can live without transitory starch, they are at a disadvantage without it (Caspar *et al.*, 1985). Transitory starch is a highly conserved feature in all divisions of land plants. Transitory starch serves two functions in plants: (i) as a carbon overflow, allowing photosynthesis to proceed faster than sucrose synthesis; and (ii) as a continuous supply of carbon at night when CO_2 cannot be fixed by photosynthesis (Caspar *et al.*, 1985; Stitt and Quick, 1989; Schulze *et al.*, 1991; Huber and Hanson, 1992; Ludewig *et al.*, 1998). Transitory starch metabolism is highly regulated. Both sucrose and starch synthesis occur when carbon fixation is limited by light or CO_2 (Sharkey *et al.*, 1985; Lin *et al.*, 1988; Kruckeberg *et al.*, 1989; Neuhaus *et al.*, 1989), and the synthesis of both increases with increasing photosynthetic rate. When plotted against the CO_2 assimilation rate, the rate of starch synthesis is delayed relative to sucrose synthesis, but then increases more quickly than the sucrose synthesis rate (Fig. 1A). Therefore, starch synthesis is favoured at high rates of photosynthesis, while at low rates sucrose synthesis is favoured. This provides support to both roles for starch synthesis: on the one hand an overflow mechanism at high photosynthetic rates and on the other hand a storage mechanism even at lower rates of photosynthesis so that the plant will not starve at night.

The rates of starch synthesis and degradation are precisely matched to the day length and, if day length is altered, multiple regulatory processes will result in modification of the rates of starch synthesis and degradation within one day/night cycle to match the new day length (Fig. 1B, C) (Gibon *et al.*, 2004; Lu *et al.*, 2005; Graf *et al.*, 2010). This precise control of transitory starch metabolism allows sucrose synthesis and export to be spread evenly over 24 h. This not only ensures a continuous source of carbon, but also keeps the phloem active at night, providing nutrients and phloem-transported signals across the whole plant (Dinant and Lemoine, 2010).

When transitory starch metabolism is blocked through mutagenesis, the effects on the plant depend on where the block occurs. Completely blocking starch synthesis, for example by genetic inactivation of plastidic phosphoglucomutase or ADP-glucose pyrophosphorylase, results in severe stunting and delayed flowering when plants are grown in high light and a 12 h photoperiod. However, under continuous low light these mutants are indistinguishable from the wild type (Caspar *et al.*, 1985; Lin *et al.*, 1988). If starch degradation is completely blocked at the beginning of the pathway, as is the case for a null mutant for *SEX1* encoding plastidic α-glucan water dikinase (GWD), growth is stunted, and flowering is reduced and delayed under alternating light–dark growth conditions (see Supplementary Movie S1 available at *JXB* online). However, only partially blocking starch degradation at the beginning of the pathway results in a phenotype that is much less severe. This is the case in *sex4* plants missing the phosphoglucan phosphatase; these plants accumulate 50% of the starch of *sex1* plants and the diel synthesis and degradation of starch is still observed (Zeeman *et al.*, 1998). These *sex4* plants have a phenotype almost indistinguishable from that of the wild type (see Supplementary Movie S1). The phenotype of other starch-accumulating mutants such as *mex1*, which lacks the maltose transporter in the plastid inner envelope, and *dpe2*, which lacks the cytosolic

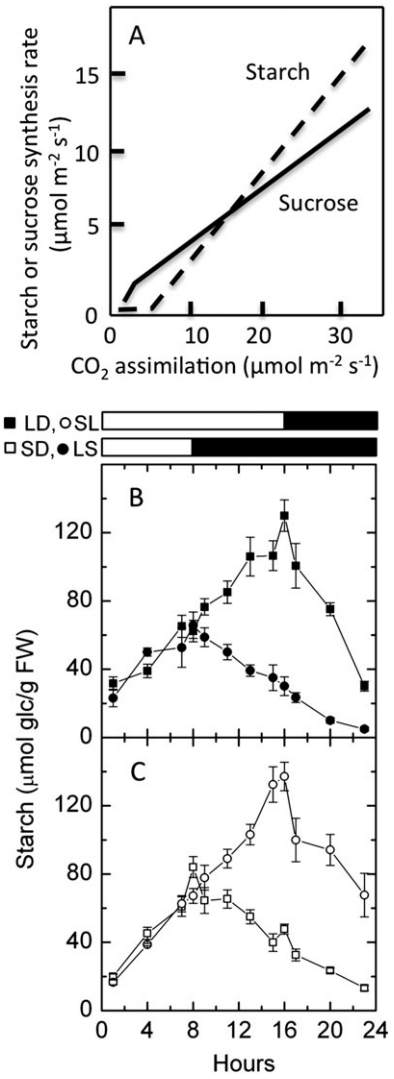

Fig. 1. Effects of photosynthetic rate (A) and day length (B, C) on starch synthesis rates. Photosynthesis was varied by changing either light or CO_2, and the amount of radioactivity fed to leaves that went into the non-ionic soluble (sucrose) or insoluble (starch) fractions was determined [redrawn from Sharkey *et al.*, 1985 (Copyright American Society of Plant Biologists, www.plantphysiol.org)]. Starch accumulated linearly in *Arabidopsis* plants grown in 16 h (b) or 8 h (c) days (square symbols). Plants shifted from long to short or short to long day (circles) adapted to the new night length on the first night. Data reprinted from Lu *et al.* (2005) (Copyright American Society of Plant Biologists, www.plantphysiol.org).

amylomaltase, is similar to, or more severe than that of *sex1* (Lu and Sharkey, 2004; Nittylä *et al.*, 2004). These mutants accumulate less starch than *sex1* or *sex4*, but the proteins blocked are further downstream in the starch degradation pathway than the GWD or phosphoglucan phosphatase. This allows a build-up of maltose and glucose, both of which are reducing sugars and both may be sensed by signalling proteins (Rolland *et al.*, 2006). The dwarf phenotype of these downstream mutants may be more a result of maltose and/or glucose sensing or toxicity (Stettler *et al.*, 2009) and less a result of absolute carbon starvation from an impaired ability to degrade starch.

Transitory starch in C_3

The major steps of the starch biosynthetic pathway are well understood, but understanding of the route by which transitory starch is broken down and converted to sucrose at night was only recently elucidated in C_3 plants. In C_3 plants there are two routes for starch degradation, a hydrolytic and a phosphorolytic pathway (Fig. 2) (Weise *et al.*, 2006). Carbon released from starch by the hydrolytic pathway is exported from the chloroplast and converted to sucrose. In contrast, products of the phosphorolytic pathway are used for internal chloroplast metabolism.

In *Arabidopsis* leaf starch, ~1 in every 2000 glucose molecules has a phosphate group attached (Zeeman *et al.*, 2007). In the hydrolytic pathway, one of the first steps in starch degradation is the sparse addition of phosphate esters to the starch granule by the GWD (Ritte *et al.*, 2002). *In vitro* studies have demonstrated that the breakdown of starch is increased 2-fold if starch is simultaneously phosphorylated by GWD (Edner *et al.*, 2007). If GWD is missing, starch degradation is abolished and starch accumulates up to 50% of dry weight (Messerli *et al.*, 2007). It was believed that the initial attack on the starch granule was by an α-amylase; however, elimination of all three α-amylases in *Arabidopsis* has no effect on starch metabolism (Yu *et al.*, 2005). The initial attack is now thought to be by β-amylases and debranching enzymes (Scheidig *et al.*, 2002; Kaplan and Guy, 2005; Delatte *et al.*, 2006). The glucose moieties are then dephosphorylated by a phosphoglucan phosphatase (Kötting *et al.*, 2009). The maltodextrins can be metabolized by further action of β-amylase, releasing additional maltose. Since β-amylase cannot work on maltotriose, a disproportionating enzyme (DPE1) is required that can take two maltotriose molecules and make one maltopentaose plus glucose (Critchley *et al.*, 2001) (Fig. 2). The maltose and glucose are transported out of the chloroplast (Weise *et al.*, 2004). In the cytosol, the maltose is acted

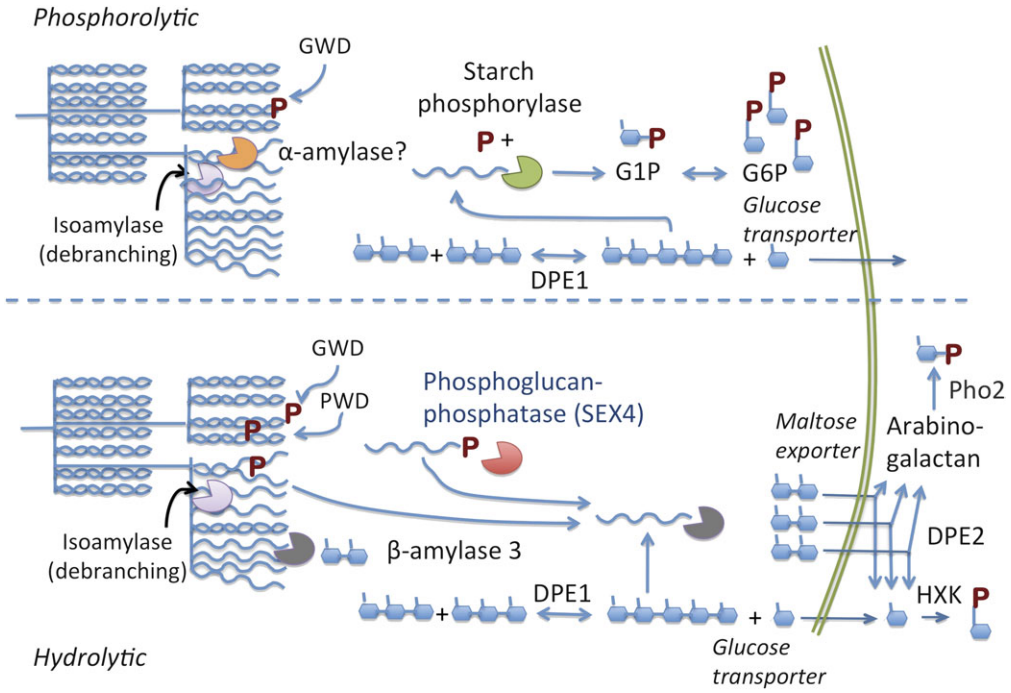

Fig. 2. Phosphorolytic (top) and hydrolytic (bottom) pathways of starch breakdown. It is assumed that the glucan water dikinase (GWD) is required for both pathways, but the involvement of phosphoglucan water dikinase (PWD) and glucan phosphatase (SEX4) in phosphorolytic starch degradation has no experimental support yet. The involvement of α-amylase in phosphorolytic starch breakdown is speculation. Both pathways produce some glucose that is exported, but with no GPT expressed in green tissue the G6P produced stays inside the chloroplast. It is speculated in this figure that β-amylase attacks both the phosphorylated starch granule and maltodextrins released from the starch granule. Exported maltose is metabolized by disproportionating enzyme 2 (DPE2) releasing one glucose and transferring the other to an arabinogalactan. The glucose on the arabinogalactan is cleaved phosphorolytically to make G1P, while the free glucose is phosphorylated to G6P by hexokinase (HXK). Each hexagon represents one glucose residue (with carbon six shown as a line).

upon by an amylomaltase and one glucose is added to an arabinogalactan (consisting of glucose, arabinose, and galactose) (Lu and Sharkey, 2004) while the other glucose is released. The glucose added to the arabinogalactan is released from the arabinogalactan by an α-glucan phosphorylase to form glucose-1-phosphate, which is used in sucrose synthesis (Lu *et al.*, 2006) (Fig. 2). The pathway from maltose to sucrose through an arabinogalactan preserves the energy in the glucose–glucose bond and avoids costly ATP use by hexokinase at night (Weise *et al.*, 2005).

At least half of the glucose that eventually becomes sucrose at night must be phosphorylated, presumably by hexokinase operating in the cytosol (Wiese *et al.*, 1999). Since hexokinase signalling affects many processes in plants (Moore *et al.*, 2003; Rolland *et al.*, 2006; Smeekens *et al.*, 2010), carbon conversion from starch to sucrose at night provides an indication of the sugar status of the plant, which could link carbon metabolism with the physiology of the plant (Sharkey *et al.*, 2004).

While it is thought that the majority of the carbon in starch is used in the hydrolytic pathway, the phosphorolytic pathway may be particularly important during the light period (Fig. 2). In C_3 plants the products of phosphorolytic starch degradation are not exported from the chloroplast because envelope glucose-6-phosphate transporters (GPTs) are not expressed in mature photosynthetic tissue. There are two GPTs in *Arabidopsis*, but expression of these is normally confined to heterotrophic tissue (Kammerer *et al.*, 1998; Niewiadomski *et al.*, 2005). An *Arabidopsis* null mutant in starch phosphorylase is not affected in plant growth and almost no impact on starch degradation can be observed (Zeeman *et al.*, 2004). It is thought that phosphorolytic starch degradation in C_3 plants is used for internal chloroplast metabolism such as the oxidative pentose phosphate, shikimic acid, or methylerythritol pathways (Stitt and ap Rees, 1979; Weise *et al.*, 2006). Nevertheless, the lack of an obvious phenotype in plastidic starch phosphorylase mutants makes it hard to understand the importance of this pathway, at least under standard laboratory growth conditions.

Transitory starch metabolism in CAM

Plants performing CAM take up CO_2 at night and fix it into malic acid. During the day when it is hot and the leaf to air gradient for water vapour loss is higher, CAM plants keep their stomata closed and CO_2 and pyruvate are generated by the decarboxylation of malic acid by $NADP^+$ malic enzyme. The CO_2 is then fixed by Rubisco in the Benson–Calvin cycle.

One hallmark of CAM is the large diel change in transitory starch and malic acid (Pucher *et al.*, 1947; Black and Osmond, 2003). There are also CAM plants that exhibit a smaller change in starch content, because they store reduced carbohydrates in the vacuole in the form of hexose, in addition to starch (Kenyon *et al.*, 1985; Christopher and Holtum, 1998; Borland *et al.*, 2009). Both starch and vacuolar hexose are converted into phospho*enol*pyruvate (PEP) at night (Christopher and Holtum, 1996). Thus transitory starch in CAM plants has the added role of providing the three-carbon CO_2 acceptor molecule PEP (through glycolysis), in addition to the roles in C_3 plants of carbon overflow and carbon supply at night (Stitt and ap Rees, 1979; Fondy and Geiger, 1982). At night, PEP is carboxylated (by PEP carboxylase) using bicarbonate imported as CO_2 through the open stomata, forming the four-carbon oxaloacetate. The oxaloacetate is reduced to malate, and malic acid is stored in the vacuole. During the day the malate is remobilized and converted to pyruvate, releasing CO_2. The pyruvate is used to regenerate starch.

The ice plant *Mesembryanthemum crystallinum* is a facultative CAM plant switching from C_3 to CAM under drought or salt stress. When in CAM mode, the oscillation in starch levels between day and night is 20% greater than in C_3 mode (Dodd *et al.*, 2003). Because transitory starch is critical for CAM in the ice plant, inactivation of starch synthesis by mutation in chloroplastic phosphoglucomutase results in the inability to operate in CAM mode (Cushman *et al.*, 2008*a*). Feeding glucose to these mutants restored nocturnal acidification. These experiments prove that starch provides the substrate for malic acid synthesis. More interestingly, the metabolic pathway of starch degradation changes when the ice plant switches from C_3 to CAM. When undergoing C_3 metabolism, leaf starch is degraded to maltose and exported from the chloroplast with little hexose phosphate exported. However, upon switching to CAM, export switches from maltose to glucose-6-phosphate, indicative of induction of the phosphorolytic pathway (Fig. 3) (Neuhaus and Schulte, 1996).

Transcripts for both the starch phosphorylase and hexose phosphate transporter are increased 14- and 71-fold, respectively, when ice plants switch from C_3 to CAM (Cushman *et al.*, 2008*b*). In addition to an increase of β-amylase transcripts, there is an 8-fold increase in α-amylase transcript when the ice plant is in CAM mode (Cushman *et al.*, 2008*b*). α-Amylase has been found to play little, if any, role in

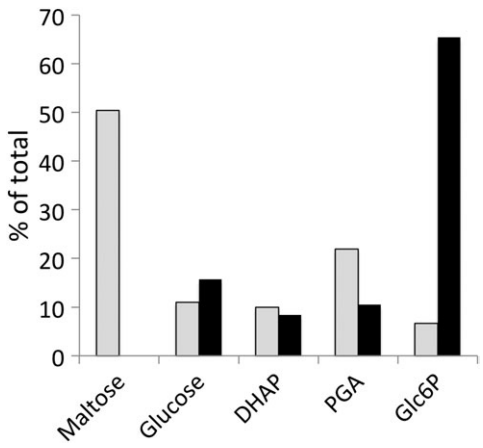

Fig. 3. Proportion of carbon coming out of *Mesembryanthemum crystalinum* chloroplasts isolated from plants operating in C_3 mode (light grey bars) or in CAM mode (black bars). DHAP, dihydroxyacetone phosphate; PGA, 3-phosphoglycerate. Data redrawn from Neuhaus and Schulte (1996).

transitory starch degradation in C_3 *Arabidopsis* plants (Yu *et al.*, 2005). However, in CAM plants, α-amylase may serve to increase the overall rate of starch degradation or may provide longer glucan chain substrates that are more amenable to degradation by plastidic starch phosphorylase (Shimomura *et al.*, 1982). The work with the ice plant demonstrates a switch from hydrolytic to phosphorolytic transitory starch metabolism and the expression of GPTs (Fig. 4). This switch does not utilize any genes unique to CAM plants, but rather a change in expression level, timing, and location of genes for proteins needed for starch degradation that already exist in C_3 plants. The switch to hexose phosphate export at night has an advantage over maltose or glucose export in that it reduces the ATP cost as starch is converted to PEP, making starch conversion to malate a net source of one ATP per two molecules of malate (Fig 5).

Hexose phosphate export from ice plant chloroplasts is correlated with CAM, which demands more transitory starch metabolism. It is not known why hexose phosphate export from chloroplasts at night is not seen in C_3 plants. Presumably there is some cost to hexose phosphate export, perhaps in terms of metabolic control, but the cost of hexose phosphate export is unknown. It can be speculated that regulatory control of the Benson–Calvin cycle is compromised if hexose phosphates leave the chloroplast during the day. Perhaps the lower overall rate of photosynthesis observed in many CAM plants (Osmond *et al.*, 1982) coupled with environmental selective pressures may offset such regulatory issues.

Transitory starch metabolism in C_4

The C_4 metabolic pathway for CO_2 uptake is almost identical to that of CAM except that CO_2 uptake and fixation in C_4 species are separated in space rather than time. Because the three-carbon CO_2 acceptor PEP is generated during the day, CO_2 fixation is not tied to starch metabolism as it is in CAM. Transitory starch metabolism in C_4 plants is thought to function in a similar manner to C_3 but, because very few enzymatic studies, or genetics studies, regarding C_4 starch metabolism have been carried out, our knowledge of transitory starch metabolism in C_4 is lacking. However, recent proteome analysis of isolated chloroplasts from differentiated maize bundle sheath cells and mesophyll cells, as well as proteome analysis of leaf and bundle sheath sections along the maize leaf developmental gradient have provided a qualitative and quantitative overview of accumulation patterns of starch metabolic enzymes (Majeran *et al.*, 2005, 2010; Friso *et al.*, 2010). Moreover, extensive electron microscopic studies of developing maize leaf at the beginning and end of the light period has provided more insight into accumulation of starch granules (Majeran *et al.*, 2010). These recent studies, combined with several previous observations (Spilatro and Preiss, 1987; Voznesenskaya *et al.*, 1999, 2005), clearly show that the majority of enzymes in starch metabolism, as well as starch granules, preferentially accumulate in the bundle sheath cell chloroplasts in mature photosynthetic tissue in C_4 plants.

The starch accumulation pattern is different in the sink–source transition zone where cells have not completed their C_4 differentiation and development. Here transient starch accumulates in both mesophyll cells and bundle sheath cells, with even higher amounts of starch in the mesophyll cell chloroplasts than bundle sheath cell chloroplasts (Majeran *et al.*, 2010). The *SEX1* gene in C_3 *Arabidopsis* encodes a GWD and is necessary for transitory starch degradation (Kötting *et al.*, 2005). Reduced expression of the maize

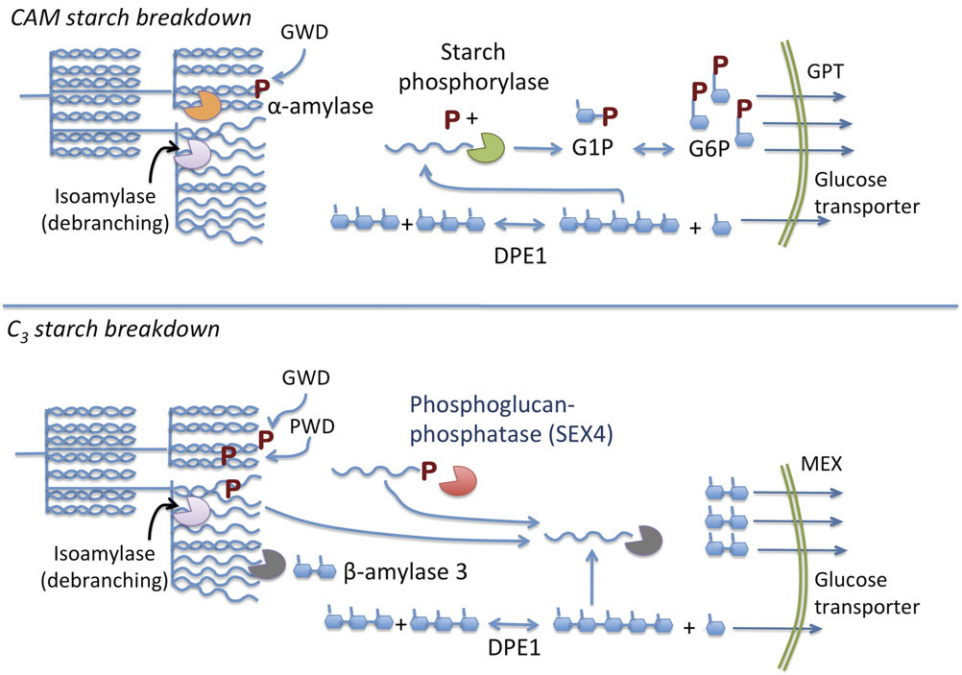

Fig. 4. Presumed pathway of starch breakdown and carbon export from chloroplasts for plants in CAM mode (top) or C_3 (mode) (bottom). Based on ideas in Fig. 2 and data in Fig. 3 MEX is the maltose exporter.

homologue of *SEX1* through RNA interference (RNAi) results in leaf starch levels that are four times higher than those of the corresponding control line; starch was localized to the bundle sheath cells similar to control plants (Fig 6).

Starch synthesis does not have to occur in the same cell as CO_2 fixation by Rubisco. In the case of *Aristrida* with altered Kranz anatomy containing two layers of bundle sheath cells, starch is largely confined to the outer bundle sheath cells while most of the Rubisco is in the inner bundle sheath cells (Voznesenskaya *et al.*, 2005). There are several reports of maize leaf starch accumulation occurring in response to alterations in carbohydrate export. Examples are the *sxd1* mutant with blocked plasmodesmata resulting in disrupted sucrose transport (Russin *et al.*, 1996). In the *tdy1* mutant, *TDY1* encoding an uncharacterized protein necessary for phloem loading (Ma *et al.*, 2009) was disrupted, and the *sut1* mutant lacks expression of *SUT1* encoding a sucrose transporter necessary for phloem loading (Slewinski *et al.*, 2009). Each of these mutants has a dwarf phenotype and the leaf regions in which carbohydrate transport is altered are chlorotic or accumulate high levels of anthocyanins. An almost identical phenotype can be generated by cold-girdling wild-type leaves, which blocks phloem transport out of the leaf (Slewinski *et al.*, 2009). In all cases, high starch levels are observed in both the bundle sheath cells and mesophyll cells, suggesting that the starch metabolic enzymes can be expressed in both cell types, but are differentially controlled in 'normal' maize plants.

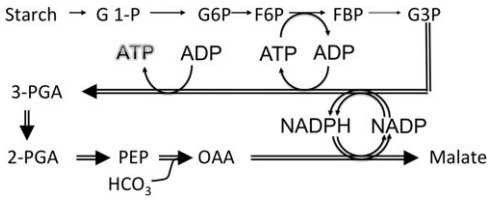

Fig. 5. Conversion of starch to phospho*enol*pyruvate (PEP) at night and subsequent metabolism to malate. This pathway results in a net gain of one ATP at night. G6P export allows all ATP and NADPH generation and consumption to occur in the cytosol. If triose phosphates were exported, ATP would be required inside the chloroplast but generated outside the chloroplast.

A better understanding of cell type-specific expression, accumulation, and regulation of starch metabolic enzymes is needed when engineering C_4 photosynthesis into a C_3 plant such as rice, to ensure a C_3 starch metabolic system is compatible with the C_4 photosynthetic system. Two possibilities could be envisaged concerning the connection between photosynthesis, yield, and starch when transforming a C_3 plant to C_4. Since most C_4 plants have a higher photosynthetic rate than C_3 plants, starch may play a vital role as an over flow mechanism helping to avoid a triose phosphate limitation, thereby allowing photosynthesis to proceed faster than sucrose synthesis and export. Therefore, it may be prudent to engineer an increased capacity for starch metabolism or the full potential of C_4 photosynthesis may not be realized. On the other hand, C_4 plants generally have higher capacity for sugar export and transport compared with C_3 plants (Leonardos and Grodzinski, 2000). Importantly, sucrose synthesis in the mesophyll, rather than the bundle sheath, provides an additional mechanism to avoid negative feedback on the bundle sheath-localized steps of the Benson–Calvin cycle (Majeran *et al.*, 2010). Therefore, starch may play a more minor role, only necessary to support respiration at night and other housekeeping metabolic activities. In this scenario it may be necessary to down-regulate starch metabolism and modify cell-specific activity of sucrose synthesis when switching from C_3 to C_4 metabolism.

Engineering leaf starch metabolism

C_4 rice

Starch metabolism in C_4 grasses appears to be spatially segregated even if both bundle sheath cells and mesophyll cells are capable of starch synthesis. Maximizing the yield of rice, if it is to be converted to a successful C_4 plant, will probably require optimization of leaf starch metabolism, in

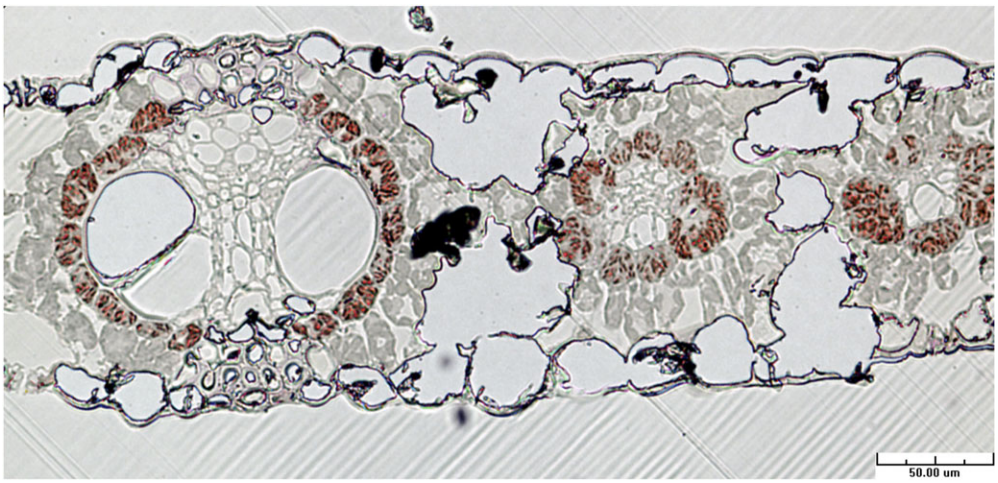

Fig. 6. Section of a leaf of *Zea mays* stained with IKI showing starch accumulation in bundle sheath cells only in this plant in which RNAi reduced GWD. The leaf was taken for fixation at 11:00 AM (SEW and TDS, unpublished).

particular bundle sheath- and mesophyll-specific expression patterns. At present nearly everything known about leaf starch metabolism in C_4 plants is by analogy with C_3 plants. There is evidence that CAM plants use starch metabolism pathways differently from C_3 plants. A better understanding of leaf starch metabolism in C_4 plants could prove useful for engineering C_4 rice plants.

Biofuels

Manipulation of starch metabolism presents an opportunity potentially to increase the yield of non-food parts of the plant for biofuel production. Producing biofuel from leaf or stem material that is not normally eaten reduces the food versus fuel competition. This is an idea that Smith called a more radical possibility (Smith, 2008), but rapid progress in genetic engineering and understanding of starch metabolism makes this a near-term possibility.

Plants make many sugars and polysaccharides that are easily fermented to ethanol, but in most cases only cell walls are left in the parts of the plant not used for food at harvest. Cell walls contain cellulose, hemicelluloses, and lignin. This mixture can be difficult to convert to liquid fuels. Any carbon that can be stored and retained in the leaf as a more easily digestible polysaccharide, such as starch, has the potential to increase the yield of biofuel. Starch fermentation currently is more complete than cell wall fermentation so, even if a slight reduction in non-food biomass occurred, starch accumulation in leaves might be a desirable trait. If biomass is burned for co-firing in conventional power plants, motivated by improved battery technology and capped net carbon dioxide emissions (Ohlrogge *et al.*, 2009), then total biomass yield will be important.

Blocking leaf starch breakdown after the plant has developed could allow accumulation of significant amounts of starch without reducing overall yield. In fact, it might be possible to increase overall yield if leaf starch acts as an additional sink for photosynthate. If starch breakdown and hexokinase signalling contribute to accelerated senescence (Sharkey *et al.*, 2004; Pourtau *et al.*, 2006; Wingler *et al.*, 2006) then blocking starch breakdown late in the plant's development could extend the growth season in plants growing in temperate climates. Further, it is predicted that blocking starch breakdown at the earliest steps will prevent build-up of potentially toxic levels of glucose and maltose (Stettler *et al.*, 2009). Preliminary results with *Arabidopsis* have confirmed that it is possible to express RNAi constructs to turn off leaf starch degradation in mature plants and that this results in starch accumulation with little or no yield penalty (SEW and TDS, unpublished).

It has been suggested that CAM plants might be well suited to exploit marginal lands for biofuel production (Borland *et al.*, 2009). Fructans are the source of much of the carbon in ethanol produced from *Agave*. It is not known what opportunities there might be to increase yield by increasing the accumulation of starch in leaves.

Biofuel production currently, and probably in the future, mainly involves C_4 plants (e.g. sugar cane, maize, switchgrass, *Miscanthus*) because of their high productivity, and water and nitrogen use efficiencies. It is hypothesized that the general enzymatic steps in starch degradation in C_4 are similar to those in C_3. If this is the case, the key enzymes that could be blocked to generate high leaf starch are already known. However, specific adaptation of the enzymes to cell-specific concentrations of substrates, allosteric factors, and redox states may be required, for example K_m, k_{cat}, and V_{max} (for discussion, see Sage, 2004; Majeran *et al.*, 2010). It has been possible to block the maize homologue to the *SEX1* gene, producing maize plants with high leaf starch without a yield penalty (SEW and TDS, unpublished). Given that it is possible to synthesize starch in the mesophyll cells in addition to the bundle sheath cells, a better understanding of regulation of starch synthesis in the mesophyll may present additional storage capacity for starch. The same reasons that make C_4 an attractive choice for food production, such as greater heat tolerance and lower nitrogen requirements, also make it an ideal choice for a biofuel crop in a globally warmer world.

Supplementary data

Supplementary data are available at *JXB* online.

Movie S1. Movie of *Arabidopsis* growth.

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