1
Biosynthesis and Metabolism of Starch and Sugars

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1.1 Introduction

Regulation of photosynthetic carbon metabolism is central for plant growth and development. In plants, carbohydrates are produced in photosynthetically active tissues, primarily in the chloroplast-containing cells of source leaves. The conversion of photoassimilates into sucrose allows the transport via the phloem from these source tissues to support growth of sink tissues such as young leaves, roots, fruits or tubers which themselves are unable to produce assimilates. During development, sink to source ratios change, which implies that assimilate production must be adjusted to the changing needs of distant tissues. Research on this subject has recently undergone a renaissance, driven by the significance of photosynthetic carbon metabolism in determining crop yield. Rising demand for food and bioenergy makes it imperative to devise novel strategies based on biotechnology and conventional breeding, respectively, for increased crop yield. In order to achieve these goals, a thorough understanding of the regulatory properties of individual enzymes as well as of the regulatory networks linking entire pathways of primary photosynthetic metabolism is required. The widespread adoption of transgenic plants, the availability of plant genome information and the rise of plant functional genomics research has greatly advanced our understanding of the factors controlling the synthesis and degradation of carbohydrates and their partitioning within and between organs.

In this chapter, we summarise the current understanding of the central pathways of carbohydrate metabolism in plants, before describing approaches to exploit this knowledge for
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plant metabolic engineering. Finally, we briefly introduce the rising field of systems biology as an approach toward a holistic understanding of central carbon metabolism in plants.

1.2 Carbon Partitioning in Mesophyll Cells

During CO₂ assimilation the reductive pentose phosphate cycle (Calvin-Benson cycle) generates triose phosphate (triose-P) at the expense of energy (ATP) and reducing equivalents (NADPH) that were generated by photosynthetic light reactions. Triose-P can either be retained with the plastid in order to either regenerate the primary CO₂ acceptor ribulose-1,5-bisphosphate (RuBP) or to be fed into the synthesis of starch, respectively, or it can be exported into the cytosol to make soluble sugars (Figure 1.1). The rate of consumption

![Diagram of photosynthetic carbon metabolism in mesophyll cells of source leaves.](image)

**Figure 1.1** Photosynthetic carbon metabolism in mesophyll cells of source leaves. Dotted lines indicate the night path of carbon export from the chloroplast when starch mobilisation occurs. Abbreviations: FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; ADP-Glc, ADP-glucose; UDP-Glc, UDP-glucose; S6P, sucrose-6-phosphate; cytFBPase, cytosolic fructose-1,6-bisphosphatase; SPS, sucrose-phosphate synthase; TP, triose phosphate.
of triose-P by either of these processes has to be balanced with the momentary rate of photosynthesis to ensure that the correct portion is recycled back into the regeneration of RuBP. Therefore, communication between the stromal compartment of the chloroplast and the cytosol is necessary to adjust the rate of photosynthesis to the demands of various parts of the plant for photoassimilates. A specific transport system, the triose-P translocator (TPT), located in the inner membrane of the chloroplast envelope, mediates the export of triose-P into the cytosol in strict counter-exchange for P$_i$ (Flügge, 1998). The rate of triose-P export catalysed by the TPT is thought to be primarily regulated by the availability of P$_i$ liberated from phosphorylated intermediates by cytosolic processes, particularly sucrose synthesis. When the export of triose-P from the chloroplast is limited by the availability of P$_i$, for example, due to decreased sucrose synthesis, fixed carbon can be deposited in the chloroplast in the form of transitory starch, which is mobilised during the following dark period and used to sustain carbon export from source tissue.

Transgenic plants with reduced activity of the TPT or mutants defective in TPT function do not show a substantial growth phenotype but profound changes in the allocation of carbohydrates and the distribution of metabolites between the chloroplast and the cytosol (Riesmeier et al., 1993; Häusler et al., 1998; Schneider et al., 2002). The patterns of carbohydrate synthesis in such plants suggest that they compensate metabolically for the reduced levels of TPT by diverting assimilates into starch and thereby releasing P$_i$ required for continuous photosynthesis. This additional starch is largely absent by the end of the night, indicating that there is an increased rate of starch breakdown during the night that provides the substrates needed to sustain sucrose export to the rest of the plant. Because starch degradation preferentially uses an amylolytic pathway the leads to the formation of glucose and maltose (see discussion below), sugars not requiring TPT activity for export from the chloroplast to the cytosol, the reduced availability of carbohydrates during the day is compensated for by an increase export rate during the night. Only plants in which triose-P export and starch synthesis are inhibited simultaneously show a severe growth phenotype (Schneider et al., 2002).

### 1.3 Sucrose Biosynthesis in Source Leaves

As outlined in the previous section, triose-P leaving the Calvin-Benson cycle can be exported from the plastid into the cytosol via the TPT and is then distributed between glycolysis and amino acid, lipid and sucrose synthesis (Figure 1.1). Sucrose is the major transport form of photoassimilates in higher plants, and as such forms the interface between photosynthetically active source tissue and heterotrophic sink tissue, where it serves as an energy source for growth and provides building blocks for storage metabolism. During the light period, sucrose synthesis proceeds from triose-P and comprises seven enzymatic steps. The entry molecule is fructose-1,6-bisphosphate (FBP), which is formed by the condensation of two molecules of triose-P catalysed by the enzyme aldolase. In a subsequent reaction, a phosphate group is removed from the C1 atom of FBP by the cytosolic isoform of fructose-1,6-bisphosphatase (cytFBPase) to yield fructose-6-phosphate (F6P). This reaction is essentially irreversibly and is supposed to represent one of the key regulatory steps of sucrose biosynthesis (see below). Glucose-6-phosphate (G6P) and glucose-1-phosphate (G1P) are maintained in equilibrium with F6P by the action of phosphoglucoisomerase.
(PGI) and phosphoglucomutase (PGM), respectively. Uridine diphosphate glucose (UDP-Glc) and pyrophosphate are then formed from UTP and G1P by the action of UDP-glucose pyrophosphorylase (UGPase). UDP-Glc is then combined with F6P to form sucrose-6\(^-\)phosphate (Suc6P), catalysed by sucrose-6-phosphate synthase (SPS). Subsequently, Suc6P is hydrolysed to form sucrose and \(P_i\) by a specific sucrose phosphatase (SPP). The reaction catalysed by SPP is irreversible \textit{in vivo} and displaces the reversible SPS reaction from equilibrium into the direction of net sucrose synthesis (Stitt \textit{et al.}, 1987). SPS has been identified as the second major control point of the whole pathway, and the enzyme is subject to complex regulation on multiple levels.

The 'night path' of sucrose synthesis slightly differs from that operating during the light period. It is now generally accepted that the primary products of starch mobilisation appearing in the cytosol are maltose and, to a lesser extent, Glc, which are initially metabolised to G6P and then further to F6P and G1P, respectively (Smith \textit{et al.}, 2005). Thus, the night path of sucrose synthesis involves neither triose-P as intermediate nor the enzymatic step catalysed by cytFBPase (Figure 1.1; dotted lines).

### 1.3.1 Regulatory Enzymes of the Pathway

In source leaves, sucrose synthesis has to be balanced with the momentary rate of photosynthesis. If sucrose synthesis operates too quickly, photosynthesis is inhibited because intermediates are withdrawn from the Calvin-Benson cycle too rapidly and RuBP regeneration is inhibited. On the other hand, if sucrose synthesis is too slow, \(P_i\) is sequestered from the chloroplast into phosphorylated intermediates, and ATP synthesis, 3PGA production and photosynthesis are inhibited (Stitt \textit{et al.}, 1987).

Three enzymes of the pathway of sucrose synthesis are known to catalyse reactions removed from thermodynamic equilibrium \textit{in vivo}. At these sites, the flux depends on the current activity of the enzyme, and such enzymes often possess regulatory properties, including allosteric regulation and/or post-translational modification. Hence, they are viewed as potential control sites. The current view of how sucrose synthesis is regulated by coordination of these enzymatic activities is discussed below.

#### 1.3.1.1 Cytosolic Fructose-1,6-Bisphosphatase

CytFBPase irreversibly converts FBP into F6P and thus catalyses the first committed step of sucrose synthesis. The enzyme is also active in non-photosynthetic tissues where it controls the rate of F6P production in gluconeogenesis. The crucial role of cytFBPase for photosynthetic carbon partitioning has been established in mutant plants or transformants with a reduction in enzyme activity (Serrato \textit{et al.}, 2009), although differences exist in the metabolic response between species. The inhibition of cytFBPase below 20\% of the wild type activity led to an accumulation of triose-P, 3PGA and F1,6BP in source leaves of potato antisense transformants, but caused no major phenotypic effects (Zrenner \textit{et al.}, 1996). This led to a massive shift of carbon partitioning towards starch, yielding starch levels which were three times higher at the end of the light period in transgenic plants than in the wild type. The surplus of starch is efficiently remobilised during the next night when sucrose synthesis is not dependent on cytFBPase activity, thereby circumventing
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the limitation of sucrose synthesis caused by a reduced cytFBPase activity. The situation is somewhat different in rice mutants carrying a T-DNA insertion in the cytFBPase gene (Lee et al., 2008). These plants showed a marked decrease in photosynthesis, reduced growth rates and eventually died when grown in soil. Biochemical analyses revealed that the impaired synthesis of sucrose in the cytosol of the cytFBPase rice mutants cannot be compensated for by an increase in carbon partitioning towards starch (Lee et al., 2008). Obviously, the capacity to synthesise and store starch is limited in rice, and it appears that sucrose represents the major transitory carbon storage compound in rice leaves.

The regulatory properties of cytFBPase are highly complex and distinct from those described for the plastidic isoform. While the latter is mainly regulated through thioredoxin mediated thiol modification, regulation of cytFBPase involves the action of several metabolites (Daie, 1993). In the absence of effector molecules, the enzyme has a high affinity for its substrate FBP ($K_m = 4–6 \mu M$). It is strictly Mg$^{2+}$ dependent, weakly inhibited by F6P, P$_i$ and AMP, and strongly inhibited by the regulatory metabolite fructose-2,6-bisphosphate (F2,6BP). F2,6BP is effective already at nano- to micromolar concentrations and substantially lowers the affinity of cytFBPase for its substrate FBP by shifting the sigmoidal substrate saturation curve of the tetrameric enzyme. The concentration of F2,6BP is determined by the relative in vivo activities of fructose-6-phosphate,2-kinase (F6P,2-K) and fructose-2,6-bisphosphate phosphatase (F2,6BPase). Both of these activities reside on a single bifunctional polypeptide (F2KP), and the ratio between the two activities is allosterically regulated through intermediates of primary metabolism (Nielsen et al., 2004). F6P,2-K activity is enhanced by F6P and P$_i$ and, in turn, is inhibited by 3PGA and triose-P, whereas F2,6BPase activity is inhibited by F6P and P$_i$. Upon the onset of photosynthesis, rising 3PGA and triose-P and decreasing P$_i$ lead to a rapid decline in F2,6BP levels which, together with rising F1,6BP, stimulate cytFBPase activity.

The proposed role of F2,6BP in the regulation of photosynthetic carbon partitioning has gained additional support from the analysis of transgenic plants with altered expression of the bifunctional F2KP. Potato and Arabidopsis plants harbouring F2KP antisense constructs have a decreased F2,6BP content and show a two-fold increase in the sucrose to starch ratio in $^{13}$CO$_2$-labelling experiments (Draborg et al., 2001; Rung et al., 2004). In Arabidopsis, this resulted in a 20–30% higher level of sucrose and a delay in diurnal starch accumulation (Draborg et al., 2001).

1.3.1.2 SPS and SPP

The last two steps in sucrose synthesis are unique to the pathway; they are catalysed by SPS and SPP. Owing to the rapid removal of Suc6P by SPP, the reaction catalysed by SPS is considerably displaced from equilibrium in vivo (Stitt et al., 1987), and thus it is thought that SPS contributes to control of flux into sucrose. The enzyme is regulated by hierarchy mechanisms that operate at different levels and in different time frames (Huber and Huber, 1996; Winter and Huber, 2000). Allosteric regulation, involving activation by G6P and inhibition by P$_i$, allows sucrose synthesis to be immediately increased in response to increased availability of precursors. The regulation of SPS from spinach (Spinacia oleracea) has been particularly well characterised. The enzyme contains three phosphorylation sites, Ser-158, Ser-229 and Ser-424, which are involved in light/dark regulation (Huber and
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Huber, 1996), 14-3-3 protein binding (Toroser et al., 1998), and osmotic stress activation (Toroser and Huber, 1997), respectively. In darkness, phosphorylation of Ser-158 inactivates SPS by decreasing the enzyme’s affinity for its substrates and by making it less prone to activation by G6P. After the onset of photosynthesis, rising G6P and falling P levels activate SPS allosterically, and rising G6P inhibits SPS-kinase, leading to dephosphorylation and post-translational activation of SPS (Huber and Huber, 1996).

There is growing evidence that higher plants contain more than one gene encoding SPS. An inspection of the Arabidopsis genome revealed the presence of four genes putatively encoding SPS enzymes, all of which appear to be both transcribed (Lunn and MacRae, 2003) and enzymatically active (F. Börnke, unpublished data). Phylogenetically, the four SPS sequences from Arabidopsis and all those known from other dicot species fall into three families: A, B and C (Lunn and MacRae, 2003). Recently, it was shown that monocot species contain an additional D family that probably arose after monocots and dicots diverged (Castleden et al., 2004). The number of SPS genes in plants raises the question about functional specialisation of particular isoforms. A-family members have been the subject of most expression studies and most of the expressed sequence tags (ESTs) examined belong to the A-family, implying that A-family genes are more abundantly expressed than those belonging to other families. Antisense repression of SPS A in Arabidopsis led to a reduction of SPS activity of about 60–70%. While this led to an inhibition of sucrose synthesis in leaves, photosynthetic carbon partitioning was surprisingly not altered towards starch (Strand et al., 2000). This approach targeted only one of the SPS gene families, and the extent to which other SPS genes were targeted or may have been up-regulated to compensate has not been investigated. Expression analysis of SPS family members in tobacco (Nicotiana tabacum) revealed that the A and C family members constitute the major SPS isoforms expressed in source leaves (Chen et al., 2005a). Specific down-regulation of tobacco SPSA or SPSC by RNA-interference in transgenic plants affected overall SPS activity only slightly, indicating that during the day A and C can be mutually substituted by each other or, alternatively, by SPS B. However, transgenic tobacco plants with reduced SPS C displayed dramatically increased starch content in their leaves. Further analysis revealed that starch accumulation in NtSPSC silenced plants was not due to an increased partitioning of carbon into starch, but rather showed that starch mobilisation was impaired. The transgenic plants were unable to mobilise their transitory leaf starch efficiently during a prolonged period of darkness, and accumulated maltose as a major intermediate of starch breakdown. The NtSPSC mRNA level increased appreciable during the dark period, while transcript levels of the other isoforms showed no diurnal changes (Chen et al., 2005a). Together, these results suggest that NtSPSC is specifically involved in the synthesis of sucrose during starch mobilisation in the dark, and future studies will have to investigate differences in the kinetic and regulatory properties of the different SPS isoforms to understand the biochemical basis for the functional specification of this protein family.

The role of SPP in the regulation of photosynthetic carbon partitioning has been relatively neglected. A recent study on transgenic tobacco plants with reduced SPP expression revealed that SPP is abundant in source leaves and does not exert significant control over sucrose synthesis under standard growth conditions (Chen et al., 2005b). The SPP activity in those transformants could be reduced to 10% of the wild-type value before any effect on sucrose synthesis and photosynthetic carbon partitioning was observed.
1.4 Starch Metabolism in Source Leaves

1.4.1 Starch Synthesis within the Chloroplast

Starch is the major higher plant storage carbohydrate and is made up of glucose molecules that are linked in two different forms. Amylose is an essentially linear polymer in which the glucose moieties are linked end-to-end by \( \alpha(1\rightarrow4) \) linkages. Amylopectin is a much larger branched molecule, in which about 5% of the glucose units are joined by \( \alpha(1\rightarrow6) \) linkages. Despite its simple composition, starch forms complex semi-crystalline structures, starch granules, in plastids.

In leaves, as much as half of the triose-P produced during photosynthesis is partitioned at a linear rate into starch during the day (Zeeman and ap Rees, 1999). Leaf starch represents a transient store for assimilates, which is mobilised during the following night also at a linear rate to support leaf metabolism, and continued synthesis and export of sucrose. Initially, starch was viewed as an overflow product that is synthesised when the rate of photosynthesis exceeds the rate of other end products such as sucrose. However, the amount of starch synthesised appears to be precisely regulated to match the length of the dark period and is affected by developmental and sink/source effects, as well as by environmental factors including light intensity and photoperiod. If plants are grown in a short photoperiod enabling less photosynthesis per day, a larger proportion of photosynthate is temporarily stored as starch (reviewed by Smith and Stitt, 2007). This prevents carbon limitation at the end of a prolonged dark period, which can have serious consequences for plant growth.

Starch synthesis requires three consecutive enzymatic reactions catalysed by ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS) and starch branching enzyme (SBE). The first committed step in the pathway of starch synthesis is the formation of ADP-glucose (ADP-Glc) from G1P and ATP in a reaction that is catalysed by AGPase and liberates pyrophosphate. In chloroplasts, ATP is derived from photosynthesis, and G1P can be supplied by the Calvin-Benson cycle via the plastidial isoforms of PGI and PGM. The reaction catalysed by AGPase is reversible under physiological conditions, but the high activity of plastidial alkaline pyrophosphatase (hydrolysing pyrophosphate to \( P_i \)) is assumed to drive the reaction towards ADP-Glc production.

AGPase in higher plants is a heterotetramer that consists of two ‘regulatory’ subunits (AGPS; \( \sim 51 \text{kDa} \)) and two slightly smaller ‘catalytic’ subunits (AGPB; \( \sim 50 \text{kDa} \)) (Okita et al., 1990). AGPase activity is subject to allosteric regulation, with 3PGA acting as an activator and \( P_i \) as an inhibitor (Preiss, 1988). The ratio of these two metabolites changes according to supply of photoassimilates and the demand for them. For example, feedback regulation of sucrose synthesis in the cytosol will lead to the accumulation of phosphorylated intermediates, depletion of \( P_i \) and activation of AGPase by the rising 3PGA:3Pi ratio in the chloroplast, eventually resulting in a compensatory stimulation of starch synthesis.

It is now apparent that AGPase activity is also redox-regulated (Fu et al., 1998) in both non-photosynthetic (Tiessen et al., 2002) and photosynthetic (Hendriks et al., 2003) tissues. The mechanism involves a redox-regulated formation of an intermolecular Cys bridge between the two small AGPB subunits of the AGPase heterotetramer. Reduction of the enzyme breaks the bridge and results in activation. In chloroplasts, redox-activation is probably mediated by the ferredoxin/thioredoxin system, allowing the Calvin-Benson cycle and starch synthesis to be coordinately regulated (Hendriks et al., 2003). In addition to light,
redox-activation of AGPase is sensitive to metabolic changes. Activation of AGPase and hence increased starch synthesis can be triggered by high levels of glucose, sucrose or trehalose (Tiessen et al., 2002; Hendriks et al., 2003; Kolbe et al., 2005). It appears that sugars signal redox-activation of AGPase through different, apparently independently operating pathways. Kolbe et al. (2005) provided evidence that sucrose-dependent activation might be mediated by the newly identified signalling metabolite trehalose-6-phosphate (Tre6P) and additionally involves SnRK1 (sucrose-non-fermenting-1-related protein kinase). The treatment of isolated chloroplast with Tre6P led to a complete reductive activation of AGPase (Kolbe et al., 2005). The factors controlling Tre6P levels in plants are currently unclear. In Arabidopsis, it has been demonstrated that Tre6P amounts increase at the onset of the light period in parallel to a redox-activation of AGPase (Lunn et al., 2006). The increase in Tre6P content was even greater when the dark period was extended by 6h. An extended night leads to carbon starvation towards the end of the dark period which considerably affects growth (Gibon et al., 2004). The elevation of Tre6P level at the start of the following day is preceded by a rapid rise in sucrose level which together is assumed to lead to redox-activation of AGPase (Lunn et al., 2006). This results in an increased partitioning of photoassimilate into starch as compared with that in the previous light period. Thus a single night during which starch supply was not adequate to meet demand could trigger a mechanism ensuring that a greater reserve of starch is built up during the subsequent light period, sufficient to allow for an extension of the subsequent night (Lunn et al., 2006).

Recently, Michalska et al. (2009) demonstrated that NADP-thioredoxin reductase C (NTRC) mediates NADPH-dependent reduction of AGPase in vitro. NTRC is an unusual plastid-localised bifunctional protein that contains a NADP-thioredoxin reductase (NTR) and a thioredoxin domain in a single enzyme (Serrato et al., 2004). Leaves of an Arabidopsis NTRC knock-out mutant showed a decrease of both in the extent of AGPase redox-activation and in the enhancement of starch synthesis either in the light or after treatment with external sucrose (Michalska et al., 2009). The authors propose that NTRC serves as an alternative system to transferring reducing equivalents to AGPase in leaves, thereby enhancing starch synthesis. In the light, NTRC is mainly linked to photoreduced ferredoxin via ferredoxin-NADP reductase. In the dark or under conditions in which light reactions are impaired, NTRC is primarily linked to sugar oxidation via the initial reactions of the oxidative pentose phosphate pathway and regulates the redox-status of AGPase in this way, independently of the ferredoxin/thioredoxin system (Michalska et al., 2009).

The ADP-Glc produced by AGPase is used as a substrate by SSs which catalyse the formation of new glycosidic linkages by transferring the glucose residue of ADP-Glc to the non-reducing end of an existing α-1,4-linked glucan chain, thereby elongating it. Higher plant SSs are encoded by five gene classes, designated GBSS (for granule bound starch synthase), SSI, SSII, SSIII and SSIV. GBSS is found exclusively bound to, and buried within, the starch granule. It is responsible for the synthesis of the linear amylose fraction of starch and is thought to elongate either soluble malto-oligosaccharides or the side-chains of amyllopectin. The SS families SSI, SSII and SSIII are localised in the plastid stroma and appear to all be involved in the elongation of the amyllopectin chains. The function of the SSIV class has not been established, although this isoform might be involved in starch-granule initiation (Roldán et al., 2007).

The branching of amyllopectin proceeds concurrently with chain elongation. SBE forms the α(1→6) linkages found in amyllopectin via a glucosyltransferase reaction in which an
existing α(1→4) linked chain is cut and a glucan segment of six or more glucose residues is transferred to the same, or an adjacent chain. Higher plant SBEs fall into two classes, designated class I and II. Class I enzymes preferentially transfer longer chains than class II enzymes (for a recent review, see Zeeman et al., 2007).

In addition to SSs and SBEs, other glucan-modifying enzymes participate in the starch biosynthetic process. Debranching enzymes (DBEs; α-1,6-glucanohydrolase), which cleave branch points, are important determinants of amylopectin morphology and structure. Mutations affecting particular classes of DBEs result in the complete replacement of semi-crystalline starch granules by soluble phytoglycogen. Thus DBE activity has been proposed to be necessary for crystallisation (Zeeman et al., 2007).

Although the enzymatic steps within the starch biosynthetic pathway succeeding the AGPase reaction were regarded as unregulated, there is increasing evidence for the occurrence of multi-enzyme complexes comprising SSs, SBEs and other enzymes (Kötting et al., 2010). The functional significance of complex formation and whether it occurs in all starch-synthesising organs is not clear yet, but it could influence both enzyme properties and the product of their combined action.

1.4.2 Starch Breakdown in Leaves and Metabolism of its Degradation Products in the Cytosol

For a long time our understanding of how transitory starch is remobilised at night and how these processes are regulated considerably lagged behind that concerning the complex metabolic network regulating photoassimilate partitioning between the different pathways during the photoperiod. During the last decade, the identification and analysis of Arabidopsis mutants defective in key enzymes of starch degradation have introduced a radically new picture of the pathway resulting in nocturnal degradation of leaf starch. These so-called starch-excess (sex) mutants accumulate starch in their leaves over repeated diurnal cycles and thus can be identified in large populations with relative ease by simple iodine staining. This approach constitutes a prime example of how Arabidopsis genetics and genomics have revolutionised pathway discovery also in a relatively mature research field such as photosynthetic primary metabolism (Stitt et al., 2010).

One of the surprising findings during the last years was that starch degradation requires the prior incorporation of phosphoryl groups into the polyglucan (Figure 1.2) (reviewed by Fettke et al., 2009). Relevant to starch phosphorylation is a glucan-water dikinase (GWD, formerly known as R1; Lorberth et al., 1998; Ritte et al., 2002) that transfers the β-phosphate of ATP to the C6 position of a glycosyl residue of amylopectin (Ritte et al., 2002). Antisense suppression of GWD activity in potato substantially lowers the phosphorylation level of amylopectin and leads to a starch excess phenotype in leaves, but also prevents cold-induced starch degradation in tubers (Lorberth et al., 1998). Similarly, loss of GWD in Arabidopsis (the sex 1 mutant) leads to a very severe sex phenotype (Yu et al., 2001). The activity of GWD was recently found to be dependent on the reduction by thioredoxin in a reaction that also alters its starch-binding capacity (Mikkelsen et al., 2005).

A second glucan-water dikinase was identified in Arabidopsis (PWD, phosphoglucan water dikinase) that phosphorylates the C3 position of glucosyl residues and is also required for starch degradation, as judged from the weak sex phenotype of Arabidopsis pwd
knock-out mutants (Baunsgaard et al., 2005; Köttig et al., 2005). This enzyme uses a phosphorylated glucan as a substrate and thus requires the prior action of GWD. Recent evidence suggests that phosphorylation disrupts the crystalline structure of amyllopectin and thus mediates its transition into a soluble state (Hejazi et al., 2008). This is required for downstream enzymes to further degrade the glucan polymer. Several independent studies
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identified a glucan-binding phosphatase in Arabidopsis leaves (Kerk et al., 2006; Niittylä et al., 2006; Sokolov et al., 2006). A loss-of-function mutant (sex4) has decreased rates of starch degradation and accumulates soluble phospho-oligosaccharides during the night (Kötting et al., 2009). Recombinant glucan-binding phosphatase can dephosphorylate semi-crystalline amylopectin, acting on phosphate groups either on C3 or C6 positions (Hejazi et al., 2010). Taken together, this suggests that removal of the phosphate groups added by GWD and PWD is also necessary for complete starch degradation.

Further hydrolysis of the glucan chains is catalysed primarily by β-amylases that act as exo-amylases, releasing maltose from exposed non-reducing ends of chains. β-amylases cannot hydrolyse α(1→6) linkages or act close to them. Thus, the complete degradation of amylopectin requires DBEs that release linear malto-oligosaccharides (MOSs). These are probably further metabolised by β-amylases to yield maltose and maltotriose, with the latter being too short to act as a substrate for further degradation by β-amylases. It appears that maltotriose is further metabolised by the disproportionating enzyme (DPE1), which preferentially transfers a maltosyl residue from maltotriose to an acceptor glucan, generating glucose and longer glucan that can be further degraded by β-amylases (Zeeman et al., 2007). The Arabidopsis genome encodes for nine β-amylases (BAM1–9), four of which have been shown to be plastid localised (BAMs 1–4) (Fulton et al., 2008). BAM1 and BAM3 are active enzymes and appear to have overlapping functions as bam1 and bam3 mutants have a wild-type and a mild sex phenotype, respectively, while the bam1/bam3 double mutant has a strong sex phenotype (Fulton et al., 2008). Interestingly, BAM4 has no apparent catalytic activity but the bam4 mutant has a sex phenotype. Thus, a regulatory role for BAM4 in starch degradation has been proposed (Fulton et al., 2008).

Alternatively to being broken down hydrolytically, linear glucans can potentially also be metabolised by the chloroplast-localised α-glucan phosphorylase that liberates G1P (Beck and Ziegler, 1989). The precise role and importance of phosphorolysis in starch breakdown is yet not clear. Potato plants with reduced plastid localised phosphorylase activity have a wild-type phenotype (Sonnewald et al., 1995).

There is now strong evidence that maltose is the major metabolite exported from chloroplasts during the night. Arabidopsis lacking a plastidic maltose transporter (mex1) accumulate high levels of maltose in their leaves, are severely impaired in growth and display a starch excess phenotype (Niittylä et al., 2004). In addition to the maltose produced by β-amylases, glucose accumulates to a lesser extent during starch breakdown, produced from the disproportionation of maltotriose by DPE1. A specific transporter that could mediate glucose export from the plastid into the cytosol has been identified in several plant species (Weber et al., 2000), although the importance of this protein for starch breakdown has not yet been established.

In the cytosol, maltose and glucose undergo a series of reactions that finally lead to the formation of sucrose, substrates for cellular respiration, or building blocks for biosyntheses. In Arabidopsis, maltose is further metabolised by transglucosidase named DPE2 (Chia et al., 2004). DPE2 catalyses the transfer of one of the glucosyl moieties of maltose to a soluble heteroglycan and releases the other as glucose (Fettke et al., 2009). Arabidopsis dpe2 mutants accumulate high levels of maltose and show impaired starch breakdown, suggesting that metabolism via DPE2 is the major route for maltose metabolism in the cytosol (Chia et al., 2004). While it is clear that glucose released from maltose is subsequently channelled into the hexose-P pool via the action of hexokinase,
of heteroglycan is much less well understood. Recent evidence from work with transgenic potato indicates that the cytosolic isoform of starch-phosphorylase (Pho2/PHS2) catalyses the reversible glucosyltransfer of glucosyl residues to the heteroglycans. Hence, the enzyme can utilise orthophosphate as an acceptor and the heteroglycan as donor, yielding the formation of G1P (Fettke et al., 2009).

1.5 Sucrose to Starch Conversion in Storage Organs

The pathway of starch synthesis in developing storage organs is relatively well understood (Figure 1.3). In all organs apart from cereal endosperms, sucrose entering the storage parenchyma is converted to G6P in the cytosol. In the case of the potato tuber, sucrose delivered by the phloem from source tissue can be metabolised in different ways. It can either be hydrolysed by apoplastic or cytosolic invertase, respectively, resulting in glucose and fructose, or converted into UDP-Glc and fructose by sucrose synthase (SuSy). The prevailing route of sucrose cleavage depends upon the developmental stage of the tuber. At the onset of tuberisation when cell division takes place, hydrolytic degradation of sucrose

![Figure 1.3](image)

**Figure 1.3** Principle pathway leading to the formation of starch in storage organs. The alternative route of ADPGlc via generation within the cytosol and subsequent uptake into the amyloplast, as it occurs in the endosperm cells of graminaceous species, is shown by dotted arrows. Susy, sucrose synthase; UDPGlc, UDP-glucose; F6P, fructose-6-phosphate; FRK, fructokinase; UGPase, UDP-glucose pyrophosphorylase; PGI, phosphoglucose isomerase; PGM, phosphoglucose mutase; G1P; glucose-6-phosphate; AGPase, ADP-glucose pyrophosphorylase; ADPGlc, ADP-glucose; SSS, starch synthase; BE, branching enzyme; DBE, debranching enzyme.
by invertases dominates. Later at the beginning of the storage phase of the developing tuber, a switch to SuSy mediated sucrose degradation occurs (Appeldoorn et al., 1997). The products of sucrose cleavage enter metabolism by the concerted action of UGPase and fructokinase or hexokinase in the case of the SuSy or invertase pathways, respectively. As a result, the end products of sucrose degradation will enter the hexose-P pool, which consists of an equilibrium mixture of F6P, G6P and G1P, because of readily reversible reactions catalysed by the cytosolic isoforms of PGI and PGM (Fernie et al., 2002).

The crucial role for Susy in sucrose breakdown during the storage phase has been established from transgenic potato plants expressing a Susy antisense construct driven by the CaMV 35S promoter (Zrenner et al., 1995). In transgenic tubers a reduction in Susy activity of up to 95% resulted in a reduction in starch and storage protein content of mature tubers, but surprisingly the sucrose levels remained unchanged. There was, however, a significant increase in the levels of hexoses which was paralleled by a 40-fold increase in invertase activity. The fact that the induction of invertase activity could not compensate for the loss in Susy activity argues for metabolic channelling of sucrose via the Susy-dominated pathway into starch, and thus indicates that Susy plays a predominant role in determining potato tuber sink strength (Zrenner et al., 1995). Accordingly, expression of a yeast-derived invertase either in the apoplast or in the cytosol of transgenic tubers did not result in increased sink strength in these tubers, despite their higher sucrolytic capacity (Sonnewald et al., 1997).

Since starch synthesis in potato tubers is confined to amyloplasts, it entirely relies on the translocation of metabolites from the cytosol through the amyloplast envelope. It now seems clear that carbon for starch synthesis enters the amyloplast in the form of G6P (Tauberger et al., 2000). Uptake is mediated by specialised glucose-6-phosphate/phosphate translocator (GPT) residing in the inner envelope membrane of amyloplasts (Kammerer et al., 1998). Following uptake of carbon into the tuber amyloplast, starch synthesis proceeds via the concerted action of plastidial PGM, AGPase and the polymerising reactions already described for the synthesis of transitory starch in chloroplasts (see above). The important role of AGPase for starch synthesis in potato tubers has been proven by antisense studies in which a reduction of AGPase activity led to a dramatic reduction in the level of starch (Müller-Röber et al., 1992). Concerning its regulatory properties, potato tuber AGPase resembles the leaf enzyme (Tiessen et al., 2002).

Whereas in chloroplasts the ATP necessary for starch synthesis can be readily provided through photosynthesis, potato tuber amyloplasts have to import ATP from the cytosol via an ATP/ADP transport protein (NTT) located on the inner-envelope membrane (Neuhaus and Emes, 2000). Tjaden et al. (1998) showed that a relatively small decrease in ATP/ADP transporter activity leads to a reduced level of total starch content and a lower amylose to amylopectin ratio. In contrast, increased transporter activity correlated with higher starch contents and a higher amylose to amylopectin ratio. In total, these observations indicated that the rate of ATP import exerts considerable control on the rate of starch synthesis and affects the molecular composition of starch in potato tubers.

Although the later polymerising reactions of starch synthesis catalysed by the various isoforms of SSs, SBEs and DBEs do not appear to play a role in the control of starch accumulation, they are crucial in determining the structure of starch (Kossmann and Lloyd, 2000; Tetlow et al., 2004).
A recent comparative analysis of gene expression in potato leaves and tubers revealed that transient and storage starch biosynthesis are strikingly similar in respect to the expression of particular isoforms of starch biosynthetic genes (Ferreira et al., 2010).

Starch synthesis in endosperms of graminaceous species, including cereals, differs from that in potato tubers in that the synthesis of ADP-Glc occurs largely in the cytosol (Figure 1.3), via a cytosolic isoform of AGPase. ADP-Glc is imported into the plastid by a specific sugar nucleotide transporter (Tomlinson and Denyer, 2003).

1.6 Metabolic Engineering of Carbohydrate Metabolism

Photosynthetic primary metabolism is a classical field of plant biochemical research, and many fundamental insights into plant function have been obtained in this field over the past decades. Due to the fact that photosynthesis and post-photosynthetic processes are supposed to be the main drivers of crop yield, there is continued interest in further unravelling the underlying regulatory mechanisms and networks. It is hoped that this will lead to novel knowledge-based strategies to engineer carbohydrate metabolism towards altered crop composition using biotechnology. Starch and sugar accumulation have been targeted by metabolic engineering in transgenic plants since the advent of plant biotechnology (Lytovchenko et al., 2007; Smith, 2008), and examples from this area of metabolic engineering are highlighted below.

1.6.1 Increasing Starch Content

Besides its importance as a staple in human and animal diets, starch is also used as a renewable raw material for a wide range of industrial purposes (Jobling, 2004). The source for industrial starch is mainly corn, but significant amounts are also extracted from a range of other species, including rice, wheat, cassava and potato. Starches from different species differ in their polymer composition (e.g. amylose to amylopectin ratio) and structure. These characteristics determine the functional properties and thus the range of applications for which a given starch is used. The modification of starch metabolism in crops could be beneficial to increase starch accumulation in harvestable organs, to prevent or increase starch degradation, or to modify starch structure to enhance or diversify its functionality for industrial uses.

To increase the efficiency of the pathway and thus to increase starch accumulation in crop plants, molecular strategies have initially concentrated on AGPase, the enzyme assumed to catalyse the rate-limiting step of starch synthesis. In an early attempt to increase the activity of the starch biosynthetic pathway in potato tubers, Stark et al. (1992) over-expressed a deregulated bacterial AGPase in the potato variety Russet Burbank. Overall, the transformed lines were reported to have an average of 35% more tuber starch than the controls. However, this effect was lost upon transformation of a different potato cultivar (Sweetlove et al., 1996). In the latter case starch degradation was up-regulated in addition to starch synthesis, resulting in no net change in starch accumulation.

Attempts to increase starch contents through manipulation of AGPase in cereal seeds have made use of a variant of the maize AGPase gene (shrunken2) whose gene product is less sensitive to inhibition by phosphate when compared with the wild-type protein.
Smidansky and colleagues (Smidansky et al., 2002, 2003, 2007) have shown that maize, rice and wheat plants expressing this AGPase allele in the endosperm and grown under controlled conditions display an increase in individual seed weight as well as in seed yield per plant. However, in field trials, transgenic wheat plants only showed a yield enhancement under conditions of minimal inter-plant competition and optimal water supply (Meyer et al., 2007).

A second general approach to increase starch accumulation in storage organs is to increase sucrose catabolism, thereby diverting more carbon from sucrose to starch. Expression of yeast invertase within the apoplast of transgenic tubers led to a dramatic reduction in tuber sucrose contents and a corresponding large increase in glucose content. Although an increase in size was observed in the transgenic tubers, an increase in yield was largely compensated for by a simultaneous reduction in the number of tubers per plant (Sonnewald et al., 1997). To further stimulate hexose utilisation in invertase-expressing plants, a bacterial glucokinase was co-expressed along with invertase. Despite the massive accumulation of hexose-phosphates the transgenic tubers showed no increase in starch synthesis, but were rather characterised by an induction of glycolysis and a massive partitioning of carbon into respiration (Trethewey et al., 1998). The reason for this dramatic change in partitioning remains mysterious, but it clearly demonstrates the flexibility of plant metabolism and highlights the necessity for a detailed understanding of the underlying factors that regulate it.

Starch content in potato tubers is very sensitive to manipulation of the plastidial adenylate transporter providing the ATP necessary for the AGPase reaction. Overexpression of an adenylate transporter from Arabidopsis in potato tubers resulted in 16–36% more starch per gram fresh weight, indicating that ATP supply to the plastid limits starch synthesis (Tjaden et al., 1998; Geigenberger et al., 2001). Recently, a further increase in potato tuber starch content was achieved by the simultaneous overexpression of a GPT from pea and an Arabidopsis adenylate translocator (NTT). Double transformants exhibited an increase in tuber yield of up to 19% in addition to an increase in starch content of 28%, when compared with control plants (Zhang et al., 2008). Both effects taken together led to a calculated increase in potato tuber starch of up to 44%. The authors concluded that starch synthesis in potato tubers is co-limited by the ATP supply as well as by the import of carbon skeletons into the amyloplast (Zhang et al., 2008). Further evidence for an energy limitation of starch synthesis in potato tubers comes from transgenic plants with reduced expression of plastidial adenylate kinase (ADK) (Regierer et al., 2002). In this study a strong negative influence of ADK activity on starch accumulation was found, suggesting that ADK normally competes with starch synthesis for plastidial ATP.

Taken together, successful attempts to increase starch content through metabolic engineering are scarce. The analyses so far suggest that in potato tubers considerable control of starch synthesis lies outside of the linear pathway, as both the adenylate transporter as well as the plastidial ADK appear to exert higher control over the pathway than AGPase, the enzyme widely believed to be rate-limiting (Geigenberger et al., 2004).

### 1.6.2 Altering Starch Quality

As stated above, one of the major factors influencing starch quality is the amylose to amylopectin ratio. Due to their contrasting physico-chemical properties, it is advantageous if starches for industrial applications are composed mainly of either amylose or amylopectin.
The synthesis of amylose is accomplished through the activity of a particular isoform of starch synthase, the GBSS, and antisense inhibition of this gene has led to amylose-free potato starch (Visser et al., 1991). No penalties in starch content have been observed and thus these plants are seemingly suited for commercialisation. Amylose-free potato starch has improved paste clarity and stability and can be expected to find application in both the food industry and in paper manufacture. Although produced almost 20 years ago, it was not before the year 2010 that a transgenic amylose-free potato variety received approval for commercial cultivation in Europe. Meanwhile, a non-functional mutant GBSS from a non-transgenic amylose-free mutant potato line, originally identified in a diploid variety (Muth et al., 2008), was bred into a commercial cultivar, and amylose-free starch is produced from that novel variety on a commercial scale.

Also high-amylose starches have been of great interest although they are much more difficult to obtain (Jobling, 2004). Recently, in an innovative approach to increase amylose content by the inhibition of starch branching enzyme A (SBE A) activity, the enzyme responsible for introducing α(1→6) linkages into amyllopectin has been reported (Jobling et al., 2003). The authors of this study expressed a single-chain antibody targeted against the active centre of SBE A, thereby neutralising its activity. They found that immunomodulation increased the amylose content of starch granules from about 20% in wild-type tubers up to 74% in the best transgenic line, exceeding the levels of amylose achieved by conventional antisense strategies (Jobling et al., 2003).

1.7 Engineering Soluble Sugars

For several crop species, soluble sugar content is much more important than that of starch. This is either because soluble sugars such as sucrose are major reserve carbohydrates (e.g. in sugar cane and sugar beet), or, as in fruit-bearing species, because sugar is an important component of taste. An increase in sugar content in strawberry has been achieved through fruit-specific antisense repression of AGPase. Transgenic strawberry fruits showed a decrease in starch content of approximately 50% and an increase in total soluble solids of up to 37% (Park et al., 2006). In general, relatively little is known at the biochemical or genetic level about the factors that control the rate of sucrose storage in sugar beet taproots or sugar cane nodes. This reflects the intractability of both crops using genetics and the difficulties in assessing storage metabolism at the biochemical level. Factors controlling sucrose accumulation in storage tissues are photoassimilate partitioning on the whole plant level, but also the control of phloem unloading, the nature and kinetics of sugar transporters in storage tissues, and the control of futile cycling of sucrose in the cytosol of storage cells (Smith, 2008). One of the rare examples of a successful increase in soluble sugar content was recently reported for sugar cane. Wu and Birch (2007) introduced a bacterial sucrose isomerase (SI) gene tailored for vacuolar compartmentation into transgenic sugar cane. SI activity converts sucrose into its non-metabolisable isomer isomaltulose (IM) and transgenic SI-expressing lines accumulated substantial amounts of IM in their culm. Remarkably, this was not at the expense of sucrose levels, resulting in a total sugar concentration of up to double in harvested juice. The reason for this boost in sugar concentration is not understood, but it has been hypothesised that IM accumulation in the culm leads to enhanced sink strength which fosters import of additional carbon from source tissues (Wu and Birch,
2007). It remains to be shown whether this strategy allows increasing total sugar content in other sucrose-storing crops such as sugar beet.

1.8 Production of Novel Carbohydrates in Transgenic Plants

In addition to attempts aiming at manipulating the contents and properties of endogenous carbohydrates, there have been several successful approaches for the production of novel carbohydrates in transgenic plants. By expressing enzymes that act on sucrose or sucrose biosynthetic intermediates, novel compounds can theoretically accumulate to high levels.

Fructans, or polyfructosylsucroses, are alternative storage carbohydrates that are highly soluble and are stored within the vacuole as opposed to the plastid: they are present in approximately 15% of all flowering plants (Hellwege et al., 2000). Fructan synthesis is initiated by sucrose:sucrose 1-fructosyltransferase (SST) which catalyses the fructosyltransfer from one sucrose molecule to another, resulting in the trisaccharide 1-kestose. In subsequent steps fructosyltransferase (FFT) catalyses the reversible transfer of fructosyl residues from one fructan to another, producing a mixture of fructans with different chain lengths (Ritsema and Smeekens, 2003). One of the simplest fructans is inulin, which consists of β(1→2)-linked fructose residues, while fructans of the levan type are β(2→6)-linked fructose polymers.

From a biotechnological viewpoint, interest in fructans has continued to increase as they have been recognised as beneficial food ingredients. As part of the human diet, they are considered to be prebiotics as they selectively promote the growth of beneficial intestinal bacteria. Furthermore, fructans are assumed to have anti-cancer activity, promote mineral absorption, decrease cholesterol levels and decrease insulin levels. Fructans are normally isolated from plants with low agronomic value, such as the Jerusalem artichoke (*Helianthus tuberosus*) and chicory. Thus, attempts have been made to produce transgenic plants with higher fructan yield or making fructans with specific properties. Transformation of sugar beet with an SST gene from Jerusalem artichoke resulted in the conversion of 90% of the vacuolar sucrose into fructan (Sevenier et al., 1998); since the sugar beet accumulates to concentrations approaching 600 mM sucrose, this represents a massive fructan yield. Weyens et al. (2004) introduced a pair of FFTs, namely sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 6-G-fructosyltransferase (6G-FFT) from onion into transgenic sugar beet. Expression of these two enzymes resulted in high-level accumulation of onion-type fructans in transgenic taproots without affecting storage capacity. Potato, as another crop naturally not accumulating fructans, was used to express plant fructosyltransferases. The SST and FFT enzymes from globe artichoke were engineered into potato and led to the accumulation of the full range of fructans found in globe artichoke itself (Hellwege et al., 2000).

The production of isomaltulose (IM) in transgenic sugar cane expressing a bacterial sucrose isomerase (SI) has already been introduced (see section above). IM is an excellent sucrose substitute in foods as it shares many physico-chemical properties with sucrose, but is non-cariogenic and has a low calorific value. IM is produced on an industrial scale from sucrose by an enzymatic rearrangement using immobilised bacterial cells expressing a SI. Expression of a SI gene from *Erwinia rhapontici* within the apoplast of transgenic potato tubers led to a nearly total conversion of sucrose into IM (Börnke et al., 2002). Despite
the soluble carbohydrates having been altered within the tuber, growth of SI-expressing transgenic potato plants was indistinguishable from wild-type plants. This example, together with the study on SI-expressing sugar cane (Wu and Birch, 2007), demonstrates that transgenic plants provide a valid platform for high-level IM production in storage tissues.

1.9 Network Analysis of Carbohydrate Metabolism

Traditionally, metabolism has been divided into discrete pathways. However, it has become increasingly apparent that metabolism operates as a highly integrated network (Sweetlove et al., 2008). Synthesis of a metabolite usually requires the operation of many pathways, and metabolites are not synthesised in isolation from each other; rather, large sets of metabolites must often be synthesised simultaneously. Analysis of metabolic networks at a systems level depends upon the integration of data obtained from more than one level of molecular entity (that is DNA, RNA, proteins, metabolites, organelles, cell types, organs, etc.). The expanding development of high-throughput data generation technologies (so-called “omics” such as genomics, transcriptomics, proteomics, metabolomics, etc.) made it possible to measure (profile) most or even all components of one class (e.g. transcripts, proteins, etc.) in a highly parallel way. These high-throughput profiling technologies provide a rich source of quantitative biological information that allows researchers to move beyond a reductionist approach by both integrating and understanding interactions between multiple components in cells and organisms (Yuan et al., 2008; Fukushima et al., 2009; Stitt et al., 2010). At the simplest level, correlation networks can be used to identify which components might be functionally related based on the ‘guilt by association’ principle. Gene-to-metabolite networks, for instance, define the interactions amongst genes and metabolites and are typically constructed using multivariate analysis, i.e. a statistical approach used to analyse more than one variable at a time, or data mining of gene expression profiling and metabolite profiling data under different conditions or in different genetic backgrounds, respectively. The outputs from statistical analyses are often visualised based upon the distance calculated among genes and metabolites according to their profiling patterns. If a gene is determined as being ‘close’ to a metabolite, it might be implicated in the synthesis or regulation of the latter (Yuan et al., 2008). A recent example of the correlative approaches was provided by Sulpice et al. (2009) who used the combination of molecular phenotyping techniques, including metabolite- and transcriptional profiling, to reveal correlations between molecular markers and biomass in 92 Arabidopsis accessions. By using a multivariate regression approach, the authors found that starch content at the end of the light period and the protein content negatively correlate with leaf biomass. This analysis prompted the hypothesis that fast-growing accessions utilise their starch more efficiently for growth, and that decreased synthesis of protein is one of the factors that contributes to this increase in the use of carbon. In addition, from the results of an integrative approach with transcript profiling and detailed genotyping of leaf samples, two candidate genes associated with increased biomass production were identified which might represent candidate lead genes with the potential to increase biomass production (Sulpice et al., 2009).

Given the increasing number of publications dealing with correlative approaches (Fukushima et al., 2009), it appears highly likely that these kinds of studies will greatly enhance our understanding of the connectivity that underpins the regulatory network of
carbon metabolism and the relative importance of varying environments or different genetic backgrounds, respectively.

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References


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