Starch biosynthesis, its regulation and biotechnological approaches to improve crop yields


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Abstract

Structurally composed of the glucose homopolymers amylose and amylpectin, starch is the main storage carbohydrate in vascular plants, and is synthesized in the plastids of both photosynthetic and non-photosynthetic cells. Its abundance as a naturally occurring organic compound is surpassed only by cellulose, and represents both a cornerstone for human and animal nutrition and a feedstock for many non-food industrial applications including production of adhesives, biodegradable materials, and first-generation bioethanol. This review provides an update on the different proposed pathways of starch biosynthesis occurring in both autotrophic and heterotrophic organisms, and provides emerging information about the networks regulating them and their interactions with the environment. Special emphasis is given to recent findings showing that volatile compounds emitted by microorganisms promote both growth and the accumulation of exceptionally high levels of starch in mono- and dicotyledonous plants. We also review how plant biotechnologists have attempted to use basic knowledge on starch metabolism for the rational design of genetic engineering traits aimed at increasing starch in annual crop species. Finally we present some potential biotechnological strategies for enhancing starch content.

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Abbreviations: ADPG, ADPglucose; AGP, ADP pyrophosphorylase; AGPP, ADP pyrophosphatase; A/N-inv, alkaline/neutral invertase; BT1, Brittle 1; F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; GBSS, granule bound starch synthase; GPT, glucose-6-phosphate translocator; GWD, glucan, water dikinase; HvBT1, Hordeum vulgare BT1; MCF, mitochondrial carrier family; MGL, microbial volatiles; MB, mitochondrial matrix; MIVOISAP, MIcrobial VOlatiles Induced Starch Accumulation Process; NTRC, NADP-thioredoxin reductase C; OPP, oxidative pentose phosphate pathway; Pi, orthophosphate; PGM, phosphoglucomutase; PGI, phosphoglucomutase; Pi, inorganic pyrophosphate; pPOLL, plastidial starch phosphorylase; RSR1, Rice Starch Regulator1; SuSy, sucrose synthase; SS, starch synthase; T6P, trehalose-6-phosphate; Trx, thioredoxin; UDPG, UDPglucose; UGP, UDPG pyrophosphorylase; WT, wild type; ZmBT1, Zea mays BT1.

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1. Introduction

Starch is the main storage carbohydrate in vascular plants. Its abundance as a naturally occurring compound of living terrestrial biomass is surpassed only by cellulose, and represents the primary source of calories in the human diet. Because starch is the principal constituent of the harvestable organ of many agronomic plants, its synthesis and accumulation also influences crop yields.

In this paper, we first review recent advances in the biochemistry of starch biosynthesis and its regulation, and provide an update on different proposed pathways of starch biosynthesis occurring in both autotrophic and heterotrophic organs. Special emphasis is given to recent findings showing that volatile compounds emitted by microorganisms promote both growth and the accumulation of exceptionally high levels of starch. Finally, we assess the progress in molecular strategies for increasing starch in annual crop species by genetic engineering approaches.

2. Proposed starch biosynthetic pathways

Starch is found in the plastids of photosynthetic and non-photosynthetic tissues. Mature chloroplasts occurring in photosynthetically active cells possess the capacity of providing energy (ATP) and fixed carbon for the synthesis of starch during illumination. By contrast, production of long-term storage of starch taking place in amyloplasts of reserve organs such as tubers, roots and seed endosperms depends upon the incoming supply of carbon precursors and energy from the cytosol. This difference between the metabolic capacities of chloroplasts and amyloplasts has led to the generally accepted view that the pathway(s) involved in starch production are different in photosynthetic and non-photosynthetic cells.

2.1. Starch biosynthesis in leaves

In leaves, a portion of the photosynthetically fixed carbon is retained within the chloroplasts during the day to synthesize starch, which is then remobilized during the subsequent night to support non-photosynthetic metabolism and growth by continued export of carbon to the rest of the plant. Due to the diurnal rise and fall cycle of its levels, foliar starch is termed “transitory starch”.

Transitory starch is a major determinant of plant growth (Sulpice et al., 2009), and its metabolism is regulated to avoid a shortfall of carbon at the end of the dark period. It is typically degraded in a near-linear manner during darkness, with only a small amount remaining at the end of the night. When changes in the day length occur that leads to a breakdown and, consequently, decreasing the rate of growth during darkness. The importance of starch turnover in plant growth and industrial needs and social demands. However, while much is known about starch metabolism, there are still major gaps in our knowledge that prevent breeders and biotechnologists from advancing in their attempts to increase starch content in a predictable way (Chen et al., 2012).

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These three enzymatic steps are reversible, but the last step is rendered irreversible upon hydrolytic breakdown of PPI by plastidial alkaline pyrophosphatase.

pPGI is strongly inhibited by light (Heuer et al., 1982) and by Calvin–Benson cycle intermediates such as erythrose-4-P and 3-phosphoglycerate (3PGA) (the latter being an indicator of photosynthetic carbon assimilation) that accumulate during the day at stromal concentrations higher than the Ki values for pPGI (Backhausen et al., 1997; Dietz, 1985; Kelly and Latzko, 1980; Zhou and Cheng, 2008). Although both these characteristics of pPGI, and the low stromal G6P/F6P ratio occurring during illumination (Dietz, 1985) would indicate that this enzyme is inactive during illumination (and thus not involved in transitory starch biosynthesis), genetic evidence showing that transitory starch biosynthesis occurs solely by the pPGI pathway has been obtained from characterization of starch-deficient pPGI mutants from various species (Jones et al., 1986; Kruckeberg et al., 1989; Kunz et al., 2010; Niewiadomski et al., 2005; Yu et al., 2000).

The classic view of leaf starch biosynthesis illustrated in Fig. 1 also implies that AGP is the sole source of ADPG, and functions as the major regulatory step in the starch biosynthetic process (Neuhaus et al., 2005; Stitt and Zeeman, 2012; Streb and Zeeman, 2012; Streb et al., 2009). This enzyme is a heterotetramer comprising two types of homologous but distinct subunits, the small (APS) and the large (APL) subunits (Crevillon et al., 2003, 2005). In Arabidopsis, six genes encode proteins with homology to AGP. Two of these genes code for small subunits (APS1 and APS2, the latter being in a process of pseudogenization) and four (APL1–APL4) encode large subunits. Whereas APS1, APL1 and APL2 are catalytically active, APL3 and APL4 have lost their catalytic properties during evolution (ventriglia et al., 2008). In Arabidopsis, the large subunits are highly unstable in the absence of small subunits (Wang et al., 1998). Therefore, APS1 T-DNA null mutants contain neither the large nor the small subunit proteins, which result in a total lack of AGP activity (Bahaji et al., 2011a; Ventriglia et al., 2008).

### Fig. 1

Classic interpretation of the mechanisms of starch and sucrose syntheses in leaves. The enzymes are numbered as follows: 1, 1′, fructose-1,6-bisphosphate aldolase; 2, 2′, fructose 1,6-bisphosphatase; 3, PPI: fructose-6-P 1-phosphotransferase; 4, 4′, pGPI; 5, 5′, pPGM; 6, UGP; 7, sucrose-phosphate-synthase; 8, sucrose phosphate phosphatase; 9, AGP; and 10, SS.

According to this view, the starch biosynthetic process resides exclusively in the chloroplast and is segregated from the sucrose biosynthetic pathway that takes place in the cytosol.

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AGP is allosterically activated by 3PGA, and inhibited by Pi (Kleczkowski, 1999). This property makes the production of ADPG highly sensitive to changes in photoassimilate (e.g. 3PGA) availability in the chloroplast, and helps to coordinate photosynthetic CO₂ fixation with starch synthesis in leaves. AGP activity is also subjected to APS redox regulation (Hendriks et al., 2003; Kolbe et al., 2005; Li et al., 2012; Michalska et al., 2009). Under oxidizing conditions the two APS subunits are covalently linked via an intermembrane disulfide bridge, thus forming a stable dimer within the heterotetrameric AGP enzyme (Fu et al., 1998; Hendriks et al., 2003). Conversely, under reductive conditions APS monomerization is accompanied by activation of AGP activity. APS dimerization markedly decreases the activity of AGP and alters its kinetic properties, making it less sensitive to activation by 3PGA and increasing its Kₘ for ATP (Hendriks et al., 2003). In turn, APS monomerization activates AGP, which is then sensitive to fine control through allosteric activation by 3PGA.

By examining the correlation between APS redox status, AGP activity and starch levels in leaves, different works have provided evidence that transitory starch accumulation is finely regulated by APS post-translational redox modification in response to environmental inputs such as light, sugars and biotic stress (Gibon et al., 2004; Hendriks et al., 2003; Kolbe et al., 2005; J. Li et al., 2011). However, using aps1 Arabidopsis plants ectopically expressing a redox-insensitive, mutated APS1 form, Li et al. (2012) and Hadrich et al. (2012) have independently shown that the rates of transitory starch accumulation in these plants are comparable to that of WT leaves. In addition, Li et al. (2012) reported WT rates of starch accumulation in aps1 plants expressing in the plastid a redox-insensitive AGP from Escherichia coli, and concluded that post-translational redox modification of APS1 in response to light is not a major determinant of fine regulation of transitory starch accumulation in Arabidopsis leaves.

Plastidial NADP-thioredoxin reductase C (NTRC) plays an important role in protecting plants against oxidative stress (Publido et al., 2010; Serrato et al., 2004). Serrato et al. (2004) and Michalska et al. (2009) reported that Arabidopsis ntrc mutants grown at 180 µmol photons s⁻¹ m⁻² are small and their leaves accumulate low levels of starch when compared with WT leaves. Furthermore, Michalska et al. (2009) reported that ntrc leaves exhibit a decrease in the extent of APS1 activation (reduction) by light. These authors thus concluded that NTRC is a major determinant of both light-dependent APS1 redox status and transitory starch accumulation. However, Li et al. (2012) have recently found that the size of Arabidopsis ntrc mutants is similar to that of WT plants when cultured at 90 µmol photons s⁻¹ m⁻². Under these conditions, APS1 redox status and starch accumulation rates in leaves of ntrc mutants are similar to those of WT leaves, strongly indicating that NTRC plays a minor role, if any, in the control of both light-dependent APS1 redox status and transitory starch accumulation in Arabidopsis cultured under non-stressing conditions.

In vitro assays using heterologously expressed potato and pea AGP have shown that APS can be activated by plastidial thioredoxins (Trxs) f and m (Ballicora et al., 2000; Geigenberger et al., 2005), indicating that these proteins could act as major determinants of APS redox status and of fine regulation of transitory starch accumulation. However, Li et al. (2012) found no differences in the rate of starch accumulation between WT leaves and leaves of homozygous trxf1, trxf2, trxm2 and trxm3 mutants. Furthermore, Thormählen et al. (2013) reported that starch levels in trxf1 leaves were only slightly reduced when compared with WT leaves. In addition, Sanz-Barrío et al. (2013) have shown that APS redox status in high-starch tobacco leaves overexpressing Trxf and Trxm genes from the plastid genome is similar to that of WT leaves. The overall data thus indicate that, individually, plastidial Trxs are not major determinants of APS1 redox status-controlled transitory starch content.

Genetic evidence showing that transitory starch biosynthesis occurs solely by the AGP pathway has been obtained from the characterization of leaves with reduced pPGM and AGP activities. For example, leaves of APS1 and pPGM antisensed potato plants accumulate low levels of starch when compared with WT leaves (Lytovchenko et al., 2002; Müller-Röber et al., 1992; Muñoz et al., 2005). Furthermore, Arabidopsis pgm null mutants (Caspar et al., 1985; Kofler et al., 2000), adg1-1 mutants with less than 3% of the WT AGP activity (Lin et al., 1988; Wang et al., 1998), and APS1 T-DNA null mutants completely lacking AGP activity (Bahaji et al., 2011; Ventriglia et al., 2008) accumulate less than 2% of the WT starch. Further evidence supporting that AGP plays a crucial role in starch biosynthesis in leaves comes from alkaline pyrophosphatase-silenced plants of Nicotiana benthamiana, since their leaves accumulate ca. 60% of the WT starch (George et al., 2010).

### 2.1.2. Models of starch biosynthesis according to which the starch biosynthetic pathway is not linked to the Calvin–Benson cycle by means of plastidal phosphoglucose isomerase

A vast amount of biochemical and genetic data appear to support the starch biosynthetic pathway illustrated in Fig. 1 according to which (a) the whole starch biosynthetic process resides exclusively in the chloroplast, (b) pGPI links the Calvin–Benson cycle with starch biosynthesis, and (c) AGP is the sole source of ADPG linked to starch biosynthesis. However, in recent years an increasing volume of evidence has been provided that supports the occurrence of additional/alternative starch biosynthetic pathways involving the cytosolic and plastidial compartments wherein the supply of ADPG is not directly linked to the Calvin–Benson cycle by means of pGPI. Thus, Fettke et al. (2011) found that the envelope membranes of chloroplasts in mesophyll cells possess a yet to be identified G1P transport machinery enabling the incorporation of cytosolic G1P into the stroma, which is subsequently converted into starch by the stepwise AGP and SS reactions. According to these authors, such mechanism would only allow the accumulation of 1% of the WT starch, and would explain the occurrence of some trace amounts of starch in the pPGM mutants (Caspar et al., 1985; Muñoz et al., 2005; Streb et al., 2009).

Kunz et al. (2010) found that leaves of the pgI-2 null mutant impaired in pGPI activity accumulate 10% of the WT starch content, which is restricted to bundle sheath cells adjacent to the mesophyll and stomatal guard cells. This mutant exhibits high G6P transport activity as a consequence of the elevated expression of GPT2 (Kunz et al., 2010), a gene that codes for a G6P/Pi translocator (GPT) mainly operating in heterotrophic tissues where the imported G6P can be used for the synthesis of starch and fatty acids or to drive the oxidative pentose phosphate pathway (Bowsher et al., 2007; Kammerer et al., 1998; Kang and Rawsthorne, 1996; L. Zhang et al., 2008). According to Kunz et al. (2010) the unexpected high levels of starch occurring in leaves of pGPI mutants can be ascribed to high GPT2-mediated incorporation of G6P into the chloroplast of bundle sheath cells adjacent to the mesophyll and stomatal guard cells, where this hexose-phosphate can be then converted to starch by means of pPGM, AGP and SS as schematically illustrated in Fig. 2. This interpretation, however, is hardly reconcilable with previous studies carried out by the same group showing that the starch-deficient phenotype of pGPI mutants can be totally reverted to WT starch phenotype by the ectopic expression of GPT2 (Niewiadomski et al., 2005), and with the fact that leaves of the pgI1-2/pgI2 double mutant accumulate as much as 60% of the starch occurring in pgI-2 leaves (Kunz et al., 2010). Furthermore, considering that (a) most of leaf starch accumulates in the mesophyll cells, (b) microscopic analyses revealed that stomatal guard cells and bundle sheath cells adjacent to the mesophyll of pGPI mutants accumulate nearly WT starch content (Kunz et al., 2010; Tsai et al., 2009), and (c) guard cells of WT leaves possess a G6P transport machinery (Overlach et al., 1993), it is difficult to understand how guard cells and bundle sheath cells adjacent to the mesophyll of pGPI mutants can accumulate as much as 10% of the WT starch as a consequence of high GPT2 activity. Needless to say, further investigations will be necessary to understand how and where pgI mutants accumulate ca. 10% of the WT starch.

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Gerrits et al. (2001) have provided evidence that a sizable pool of sucrose in the plant cell has a plastidial localization. Consistent with the occurrence of a plastidial pool of sucrose, Murayama and Handa (2007) and Vargas et al. (2008) reported the presence of a functional alkaline/neutral invertase (A/N-inv) inside plastids. Noteworthy, Vargas et al. (2008) reported that leaves of Arabidopsis mutants impaired in A/N-inv accumulate ca. 70% of the WT starch content. The same authors hypothesized that chloroplastic sucrose plays a sensing role in a mechanism of control of carbon partitioning and sucrose/starch ratio wherein glucose and fructose generated by A/N-inv are sensed through plastidial hexokinase (pHK) (Giese et al., 2005; Olsson et al., 2003). However, a possibility cannot be ruled out that, as schematically illustrated in Fig. 3, plastidial sucrose acts as precursor for starch biosynthesis, its A/N-inv breakdown products being converted into starch by means of pHK, pPGM and AGP.

Sufficient evidence exists to support the view that a sizable pool of ADPG in leaves accumulates in the cytosol of photosynthetically competent cells. This hypothesis is compatible with the occurrence of cytosolic ADPG metabolizing enzymes such as ADPG phosphorylase (McCoy et al., 2006), glucan synthases (Tacke et al., 1991), sucrose synthase (SuSy) and ADPG hydrolases (Olejnik and Kraszewska, 2005; Rodríguez-López et al., 2000) and with the occurrence in the chloroplast envelope membranes of yet to be molecularly identified ADPG transport machineries whose activities can account for rates of starch accumulation occurring in leaves (Pozueta-Romero et al., 1991a). Genetic evidence supporting the occurrence of important ADPG source(s), other than AGP, has been obtained from different sources. First, leaves of transgenic potato and Arabidopsis plants ectopically expressing in the cytosol a bacterial ADPG hydrolase (Moreno-Bruna et al., 2001) accumulate lower ADPG levels than WT leaves, as determined by HPLC analyses (Bahaji et al., 2011a; Baroja-Fernández et al., 2004). Second, ADPG content in the leaves of AGP and pPGM mutants is comparable to that of WT leaves, as confirmed by both HPLC (Bahaji et al., 2011a; Muñoz et al., 2005) and HPLC: MS analyses (Baroja-Fernández et al., 2013). Third, HPLC:MS analyses of the triple s3/ss4/ops1 mutant impaired in AGP and SS class III and IV revealed that leaves from this mutant accumulate ca. 40-fold more ADPG than WT leaves (Ragel, 2012).

SuSy catalyzes the reversible conversion of sucrose and NDP into fructose and the corresponding NDP-glucose, where N stands for uridine, adenosine, guanosine, cytidine, thymidine or inosine. Although UDP is generally considered to be the preferred nucleoside diphosphate for SuSy, ADP also serves as an effective substrate to produce ADPG (Baroja-Fernández et al., 2003, 2012; Cumino et al., 2007; Delmer, 1972; Nakai et al., 1998; Porchia et al., 1999; Silvius and Snyder, 1979; Zervosen et al., 1998). SuSy is highly regulated at both transcriptional and post-transcriptional levels (Asano et al., 2002; Ciereszko and Kleczkowski, 2002; Déjardin et al., 1999; Fu and Park, 1995; Hardin et al., 2004; Koch et al., 1992; Pontis et al., 1981; Purcell et al., 1998; for a review see Kleczkowski et al., 2010). Although this sucrolytic enzyme is very active in reserve organs, it also expresses in the mesophyll cells of source leaves (Fu et al., 1995; Wang et al., 1999), its activity in potato and Arabidopsis leaves greatly exceeding the minimum needed to support normal rate of starch accumulation during illumination (Baroja-Fernández et al., 2012; Muñoz et al., 2005). Accordingly, a metabolic model of transitory starch biosynthesis has been proposed wherein (a) both sucrose and starch metabolic pathways are tightly interconnected by means of cytosolic ADPG producing enzymes such as SuSy (acting when cytosolic sucrose transiently accumulates during illumination), and by the action of an ADPG translocator located at the chloroplast envelope membranes, and (b) both AGP and pPGM play an important role in the scavenging of glucose units derived from starch breakdown occurring during starch biosynthesis and during the biogenesis of the starch granule (Bahaji et al., 2011a; Baroja-Fernández et al., 2005; Muñoz et al., 2005, 2006) (Fig. 4).

Thus, the net rate of starch accumulation is determined by the balance between the rates of SuSy-mediated ADPG synthesis in the cytosol, import of

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cytosolic ADPG to the chloroplast, starch synthesis, starch breakdown, and by the efficiency with which starch breakdown products can be recycled back to starch via the coupled reactions of pPGM and AGP. Accordingly, this view predicts that the recovery toward this process can be recycled back to starch via the coupled reactions of pPGM and AGP. Accordingly, this view predicts that the recovery toward starch biosynthesis of the glucose units derived from the starch breakdown will be inefficient in pPGM and AGP mutants, resulting in a parallel decline of starch accumulation. 

The occurrence of a substrate or “futile” cycle as a result of the simultaneous synthesis and breakdown of starch in leaves is not surprising since pulse-chase and starch–preloading experiments using isolated chloroplasts (Fox and Geiger, 1984; Stitt and Heldt, 1981), intact leaves (Scott and Kruger, 1995; Walters et al., 2004), or cultured photosynthetic cells (Lozovaya et al., 1996) have shown that the illuminated chloroplasts can synthesize and mobilize starch simultaneously. Furthermore, enzymes involved in starch breakdown such as SEX1 and BAM1 are redox-activated during the light under different environmental conditions (Mikkelsen et al., 2005; Sparla et al., 2006; Valerio et al., 2010). Futile cycles lead to a net consumption of ATP, which in some instances can be estimated at about 60% of the total ATP produced by the cell (Alonso et al., 2005; Hill and ap Rees, 1994). Although these cycles appear to waste energy without any apparent physiological reason, they form part of the organization of the plant central metabolism, and contribute to its flexibility (Ronteltap et al., 2002). As presented above, starch acts as a major integrator in the plant growth that accumulates to cope with temporary starvation imposed by the environment (Sulpice et al., 2009). It is thus conceivable that highly regulated starch futile cycling may entail advantages such as sensitive regulation and rapid metabolic channeling toward various pathways in response to physiological and biochemical needs of the plant. Therefore, due to the importance of starch in the overall plant metabolism and growth, it is tempting to speculate that both redundancy of ADPG sources and starch futile cycling have been selected during plant evolution to warrant starch production and rapid connection of starch metabolism with other metabolic pathways.

The postulated starch biosynthetic pathways involving the plastidial and cytosolic compartments are consistent with still enigmatic results obtained from experiments carried out more than 50 years ago showing that $^{14}$C in the glucose moiety of sucrose, starch, UDP-glucose, and hexose-Ps is asymmetrically distributed in green leaves exposed to $^{14}$CO$_2$ for a short period of time (Gibbs and Kandler, 1957; Havir and Gibbs, 1963; Kandler and Gibbs, 1956). Unlike the “classic” view on transitory starch biosynthesis (Fig. 1) predicting that green leaves exposed to $^{14}$CO$_2$ for a short period of time should synthesize starch with $^{14}$C symmetrically distributed in the glucose moiety, the proposed mechanisms of starch synthesis illustrated in Figs. 2–4 provide a possible explanation for solving the above enigma. As shown in Fig. 4, triose-Ps produced in the Calvin–Benson cycle are exported to the cytosol where they can be channeled into the oxidative pentose-P pathway (OPPP), thereby leading to a randomization of the carbons giving rise to the asymmetric $^{14}$C distribution observed in hexose-Ps, nucleotide-sugars and sucrose upon exposure to $^{14}$CO$_2$ for a short period of time. Cytosolic G1P, G6P, sucrose and/or ADPG will be then transported into the chloroplast and utilized as precursors for the synthesis of starch molecules possessing glucose molecules with asymmetric $^{14}$C distribution.

Genetic evidence consistent with the occurrence of a link between sucrose and transitory starch metabolism can be obtained from sucrose-phosphate-synthase and cytosolic PGM deficient plants (Fernie et al., 2002; Strand et al., 2000), since their leaves contain low levels of both sucrose and starch as compared with WT leaves. Further genetic evidence can be obtained from sedoheptulose-1,7-bisphosphate overexpressing plants (Lefebvre et al., 2005; Miyagawa et al., 2001), since their leaves are characterized by having high levels of both sucrose and starch when compared with WT leaves. Finally, genetic evidence indicating that leaf sucrose and starch metabolic pathways are linked by SuSy has been
obtained from SuSy-overexpressing potato plants whose leaves accumulate higher ADPG and starch contents than WT leaves (Muñoz et al., 2005). Further endeavors based on the characterization of mutants totally lacking SuSy will be necessary to confirm (or refute) the involvement of SuSy in starch biosynthesis in leaves.

2.2. Starch biosynthesis in heterotrophic organs

Sucrose produced in leaves is imported by the heterotrophic organs and used as carbon source for energy production and starch synthesis in the amyloplast. As in the case of leaves, various mechanisms have been proposed to describe starch biosynthesis in the heterotrophic organs, which together with the mechanisms of sucrose unloading, will be presented and discussed below.

2.2.1. Mechanisms of sucrose unloading in heterotrophic organs

Unloading of sucrose arriving from aerial parts of the plant into sink organs occurs either symplastically (moving via plasmodesmata) or apoplastically. The route depends on the species, organ or tissue. In the apoplastic delivery of sucrose to rapidly growing sinks, apoplastic sucrose that has been released into the extracellular space can be split by cell wall-bound invertases into glucose and fructose for subsequent uptake into the cell by hexose transporters. Alternatively, sucrose can be taken up by plasma membrane-bound sink specific sucrose transporters of non-dividing storage sinks (Sauer, 2007). Sucrose is then transported via yet-to-be-identified tonoplast sucrose transporters to the vacuole, which serves as temporary reservoir. Apoplastic sucrose can also be taken up by a sucrose-induced endocytic process, and transported to the central vacuole of heterotrophic cells (Etzberria et al., 2005a, 2005b; for a review see Etzberria et al., 2012). Endocytic uptake of extracellular sucrose and subsequent transport to the vacuole is not in conflict with transport through membrane-bound carriers given that cell homeostasis can be better maintained if both mechanisms operate in parallel (Etzberria et al., 2005a). Consistent with this view, Baroja-Fernández et al. (2006) reported that carrier-mediated sucrose import in starved cells of sycamore (Acer pseudoplatanus L.) plays an important role in the overall sucrose–starch conversion process during the initial period of sucrose unloading, whereas endocytosis may play an important role in transporting the bulk of sucrose to the vacuole and its subsequent conversion into starch after prolonged period of sucrose unloading.

2.2.2. Proposed mechanisms of sucrose–starch conversion in heterotrophic cells

Sucrose entering the heterotrophic cell by any of the mechanisms described above must be metabolized to molecules that can be transported to the amyloplast for subsequent conversion into starch. Because both chloroplasts and amyloplasts are ontogenically related, and because chloroplasts possess a very active triose-P translocator connecting the plastidial and cytosolic compartments, it was originally assumed that cytosolic triose-Ps entering the amyloplast by means of a triose-P translocator act as precursors for starch biosynthesis (Boyé, 1985). However, NMR spectroscopy experiments of starch biogenesis in wheat and maize grains using 13C NMR (Hatzfeld and Stitt, 1990; Keeling et al., 1988) demonstrated that a C-6 molecule, not a triose-P (C-3), is the precursor of starch biosynthesis in amyloplasts. Further investigations of the enzyme capacities of amyloplasts (Entwistle and ap Rees, 1988; Frehner et al., 1990) supported the view that C-6 molecules entering amyloplasts are the precursors of starch biosynthesis. Depending on the nature of the C-6 molecule entering the amyloplast...
(G6P or ADPG), and depending on the enzymes involved in ADPG synthesis, various mechanisms have been proposed to explain the sucrose–starch conversion process in heterotrophic cells.

2.2.2.1. Models of sucrose–starch conversion in heterotrophic cells according to which AGP is the sole source of ADPG linked to starch biosynthesis. It is widely assumed that AGP is the sole source of ADPG in heterotrophic organs of mono- and dicotyledonous plants. Maximal in vitro AGP activity greatly exceeds the minimum required to support the normal rate of starch accumulation in starch storing organs (Denyer et al., 1995; Li et al., 2013; Weber et al., 2000). This would indicate that AGP is not a rate-limiting step in the sucrose–starch conversion process in many heterotrophic organs. In fact, the flux control coefficient of AGP has been estimated to be as low as 0.08 in some heterotrophic organs (Denyer et al., 1995; Rolletschek et al., 2002; Weber et al., 2000). As presented above, AGP is a highly regulated enzyme. However, whereas evidence has been provided that starch synthesis is regulated by post-translational redox modification of AGP and 3PGA/Pi balance in potato tubers (Tiessen et al., 2003), AGP in developing barley, wheat, pea and bean seeds is insensitive to 3PGA and Pi regulation (Gómez-Casati and Iglesias, 2002; Hylton and Smith, 1992; Kleczkowski et al., 1993; Weber et al., 1995).

In heterotrophic organs of dicotyledonous plants, sucrose entering the cytosolic compartment of the heterotrophic cell is broken down by SuSy to produce fructose and UDPglucose (UDPG), the latter being converted to G1P and Pi by UDPG pyrophosphorylase (UGP). G1P is subsequently metabolized to G6P by means of the cytosolic phosphoglucomutase. Cytosolic G6P then enters the amyloplast, where it is converted to starch by the sequential activities of pPGM, AGP and SS (Fig. 5A). Unlike chloroplasts, amyloplasts are unable to photosynthetically generate ATP and therefore, cytosolic ATP must enter the amyloplast to produce ADPG by means of AGP. The presence of an ATP/ADP translocator in the envelope membranes of amyloplasts has been firmly established by different laboratories (Pozueta-Romero et al., 1991b; Tjaden et al., 1998), and evidence about its relevance in the starch biosynthetic process has been provided by Tjaden et al. (1998) and Geigenberger et al. (2001) who reported that potato tubers with decreased plastidic ATP/ADP transporter activities exhibit reduced starch content.

Genetic evidence demonstrating the importance of SuSy in the sucrose–starch conversion process in heterotrophic organs of

![Fig. 5](https://example.com/fig5.png)

**Fig. 5.** Classic interpretation of the mechanisms of sucrose–starch conversion in (A) heterotrophic cells of dicotyledonous plants and (B) cereal endosperm cells according to which AGP is the sole source of ADPG linked to starch biosynthesis. Note that in heterotrophic cells of dicotyledonous plants (a) AGP is exclusively localized in the plastid, (b) the cytosolic carbon substrate compound entering the amyloplast for subsequent conversion into starch is G6P, and (c) cytosolic UGP is involved in the sucrose–G6P conversion process. Plastidial and cytosolic G1P pools are likely connected by means of a yet to be identified G1P translocator occurring in amyloplasts (Fettke et al., 2010). Note also that in cereal endosperm cells (a) most of AGP has a cytosolic localization, and (b) the cytosolic carbon substrate compound entering the amyloplast for subsequent conversion into starch is ADPG. Numbering of enzyme activities is the same as in legends of Figs. 1–4.

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dicotyledonous plants comes from the reduced levels of starch in potato tubers and carrot roots exhibiting low SuSy activity (Tang and Sturm, 1999; Zrenner et al., 1995). In addition, genetic evidence showing that starch biosynthesis in heterotrophic organs involves the incorporation of G6P and its subsequent conversion into ADPG by means of pPGM and AGP has been obtained from the characterization of Vicia narbonensis embryos expressing antisense GPT (Rolletschek et al., 2007) (these embryos, however, expressed lower levels of starch-related genes such as AGP and SuSy encoding genes), genetically engineered pea seeds and potato tubers with reduced pPGM (Harrison et al., 1998; Tauberger et al., 2000) and from V. narbonensis seeds and potato tubers with reduced AGP activity (Müller-Röber et al., 1992; Weber et al., 2000). These organs all exhibit reduced starch content when compared with their corresponding WT organs.

Using disks from WT and pPGM antisensed potato tubers incubated with radiolabeled G1P, Fettke et al. (2010) reported that G1P can be efficiently taken up by potato tuber parenchyma cells and converted to starch. Moreover, these authors reported that exogenously added G1P-dependent starch synthesis was diminished in tuber disks of potato plants with low plastidial starch phosphorylase (pSP) activity. Fettke et al. (2010) thus proposed that G1P can be taken up by amyloplast by a yet to be identified transporter to be subsequently used as substrate for pSP- and/or AGP-mediated starch biosynthesis. However, although this hypothesis can explain the results obtained using potato tuber disks, it conflicts with previous reports using pSP antisensed potato tubers showing that, in planta, the lack of pSP did not exert any effect on starch accumulation (Sonnewald et al., 1995). Furthermore, this hypothesis is hardly reconcilable with the strong reduction of starch content in tubers of pPGM antisensed potato tubers, indicating that plastidial G6P is linked to starch biosynthesis (Tauberger et al., 2000).

Unlike dicotyledonous plants where AGP is exclusively localized in the plastidial compartment, most of AGP in cereal endosperm cells has a cytosolic localization (Beckles et al., 2001; Denyer et al., 1996; Johnson et al., 2003; Kleczkowski, 1996; Thornbjörnsen et al., 1996a, 1996b; Villand and Kleczkowski, 1994). Consistently, most of ADPG has a cytosolic localization in cereal endosperm cells (Liu and Shannon, 1981; Tiessen et al., 2012). Import studies using amyloplasts isolated from maize and barley endosperms of WT plants and starch-deficient/ADPG-excess maize Zmbt1 and barley Hvbt1 mutants provided genetic evidence that Zea mays bristl1 (BT1) (Zmbt1) and Hordeum vulgare BT1 (Hvbt1) are membrane proteins that can incorporate cytosolic ADPG into the amyloplast (Liu et al., 1992; Möhlmann et al., 1997; Patron et al., 2004; Shannon et al., 1996, 1998). Furthermore, cell fractionation studies revealed that most of ADPG accumulating in “high-ADPG” developing endosperms of the Riso 13 Hvbt1 mutant has a cytosolic localization (Tiessen et al., 2012). The overall data thus indicate that, in cereal endosperm cells, BT1 proteins facilitate the transfer of cytosolic ADPG into the amyloplast for starch biosynthesis and are rate-limiting steps in this process. Based on this evidence, a starch biosynthesis model has been proposed for cereal endosperms wherein the stepwise reactions of SuSy, UGP and AGP take place in the cytosol to convert sucrose into ADPG, which enters the amyloplast by means of BT1 to synthesize starch by the action of the action of an ADPG translocator, and (b) pPGM and AGP play an important role in the scavenging of the glucose units derived from the starch breakdown (Fig 6). Accordingly, these models preview that the net rate of starch accumulation in heterotrophic cells is determined by the balance between the rates of ADPG synthesis in the cytosol, import of cytosolic ADPG to the amyloplast, starch synthesis and starch breakdown, and by the efficiency with which starch breakdown products can be recycled back to starch via the coupled reactions of pPGM and AGP. Also, these models predict that the recovery toward starch biosynthesis of starch breakdown products will be deficient in pPGM and AGP mutants, resulting in a parallel decline of starch accumulation.

The occurrence of the sucrose–starch conversion pathway illustrated in Fig 6A implies that neither UDPG produced by SuSy, nor cytosolic hexose-Ps derived from the action of UGP on UDPG, are involved in starch biosynthesis in heterotrophic organs of dicotyledonous plants. This view is consistent with the unexpected WT starch content phenotype of UGP antisensed potato tubers (Zrenner et al., 1993), and with the WT G6P and G1P contents of starch-deficient SuSy antisensed potato tubers (Baroja-Fernández et al., 2003). Also, this view is coherent with the unexpected starch-deficient phenotype of transgenic potato
tubers heterologously expressing in the cytosol either yeast invertase plus glucokinase (Trethewey et al., 1998) or bacterial sucrose phosphorylase (Trethewey et al., 2001), since the high sucrorytic activity occurring in these tubers leads to drastic reduction of cytosolic sucrose levels, thus limiting ADPG-producing SuSy activity.

Genetic evidences demonstrating the significance of ADPG-producing SuSy in the sucrose–starch conversion process in heterotrophic cells of both mono- and dicotyledonous plants are the same as those presented in Section 2.2.2.1.

3. Microbial volatiles promote both growth and accumulation of exceptionally high levels of starch in mono- and dicotyledonous plants

Microbes synthesize and emit many volatile compounds. Volatile emissions from rhizobacterial isolates of *Bacillus* spp. promote growth in *Arabidopsis* plants by facilitating nutrient uptake, photosynthesis and defense responses, and by decreasing glucose sensing and abscisic acid levels (Ryu et al., 2003, 2004; H. Zhang et al., 2008, 2009). In contrast, volatiles from *Pseudomonas* spp., *Serratia* spp. and *Stenotrophomonas* spp., and from some fungal species inhibit growth in *Arabidopsis* plants (Splivallo et al., 2007; Tarkka and Piechulla, 2007; Vespermann et al., 2007). Given the lack of knowledge on how microbial volatiles (MVs) affect reprogramming of carbohydrate metabolism in plants, Ezquer et al. (2010a) explored the effect on starch metabolism of volatiles released from different microbial species ranging from Gram-negative and Gram-positive bacteria to different fungi. Toward this end, the authors measured the starch and soluble sugar contents in leaves of plants cultured in the presence or in the absence of adjacent microbial cultures. Noteworthy, Ezquer et al. (2010a) reported that, in the absence of physical contact between plant and microbe, all microbial species tested (including plant pathogens and microbes that normally do not interact with plants) emitted volatiles that promoted enhancement of the intracellular ADPG levels and a rapid accumulation of exceptionally high levels of starch in leaves of both mono- and dicotyledonous plants. Levels of starch reached by MV-treated leaves were comparable to those occurring in heterotrophic organs such as tubers and cereal seeds. This phenomenon, designated as MIVOISAP (for Microbial VOLatiles Induced Starch Accumulation Process), was also accompanied by strong promotion of growth. Therefore, MIVOISAP cannot be ascribed to utilization

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of surplus photosynthates and/or ATP for starch biosynthesis that would occur under growth arrest conditions.

Transcriptome, metabolite content and enzyme activity analyses of potato leaves exposed to volatiles emitted by the plant pathogen Alternaria alternata revealed that starch over-accumulation was accompanied by enhanced 3PGA/Pi ratio and up-regulation of SuSy, acid invertase inhibitors, SSIII and SSIV, starch branching enzyme, GPT2, and synthesis of phosphatidlyinositol-phosphates implicated in processes such as endocytosis and vesicle traffic (Ezquer et al., 2010a). MIVOISAP was also accompanied by down-regulation of acid invertase, plastidial Trxs, plastidial \( \beta \)-amylase and pSP, and proteins involved in the conversion of plastidial triose-phosphates into cytosolic G6P. No changes were found neither in the expression levels of AGP encoding genes nor in the redox status of APS1. Furthermore AGP-antisensed potato leaves accumulated exceptionally high levels of starch in the presence of MVs. The overall data thus indicated that potato MIVOISAP is the consequence of transcriptionally and post-transcriptionally regulated metabolic reprogramming involving (a) up-regulation of ADPG-producing SuSy, SSIII and SSIV, and proteins involved in the endocytic uptake and traffic of sucrose, and (b) down-regulation of acid invertase and starch breakdown enzymes. During potato MIVOISAP there also occurs a down-regulation of ATP-consuming functions involved in internal amino acid provision such as proteases and enzymes involved in de novo synthesis of amino acids. Ezquer et al. (2010a, 2010b) thus hypothesized that, under conditions of limited ATP-consuming protein breakdown occurring during exposure to MVs, the surplus ATP and carbon are diverted from protein metabolism to starch biosynthesis.

Time-course analyses of starch and malate contents in illuminated Arabidopsis leaves of plants exposed to volatiles emitted by A. alternata revealed that both starch synthesis and \( \beta \)-amylase-dependent starch degradation were enhanced upon MV treatment, although the final balance was positive for synthesis over breakdown (J. Li et al., 2011). The increase of starch content in illuminated leaves of MV-treated hy1/cry1, hy1/cry2 and hy1/cry1/cry2 Arabidopsis mutants was many-fold lower than that of WT leaves, indicating that Arabidopsis MIVOISAP is subjected to photo-receptor-mediated control. Transcriptomic analyses of Arabidopsis leaves exposed to A. alternata volatiles revealed changes in the expression of genes involved in multiple processes. However, unlike potato, no changes could be observed in the expression of starch related genes (J. Li et al., 2011). Also, unlike potato plants, Arabidopsis MIVOISAP is accompanied by 2-3-fold increase of the levels of reduced (active) form of APS1. Using different Arabidopsis knockout mutants J. Li et al. (2011) observed that the magnitude of the MV-induced starch accumulation was low in mutants impaired in SSIII, SSIV and NTRC. Unlike WT leaves, no changes in the redox status of AGP occurred in ntrc mutants, providing evidence that MV-promoted monomerization (activation) of AGP is mediated by NTRC. The overall data thus strongly indicated that Arabidopsis MIVOISAP involves a photocontrolled, transcriptionally and post-transcriptionally regulated network wherein photoreceptors and post-translational changes (e.g. changes in the redox status) of plastidial enzyme(s) such as AGP, SSIII and SSIV play important roles. Consistent with the possible involvement of changes in redox status of SSs in MIVOISAP, Glaring et al. (2012) have recently reported that SS1 and SSIII are reductively activated enzymes.

4. Biotechnological approaches for increasing starch content in heterotrophic organs

Despite the monumental progress made in understanding the genetic and biochemical mechanisms of starch biosynthesis in plants, up to the late 2000s relatively little had been achieved in terms of increasing starch content and yield using biotechnological approaches, particularly in heterotrophic organs of cereal crops (Smith, 2008). However, within the last 5 years, a series of major developments has produced significant results that indicate the likelihood of enhancing starch production. We next assess the progress made in molecular strategies, both successful and unsuccessful, for improving starch accumulation in heterotrophic organs of plants of agronomic interest. Finally we will present some potential strategies for enhancing starch content.

4.1. Enhancement of AGP activity

Most attempts to increase starch accumulation have focused on enhancing AGP activity. The focus on AGP arose originally from the belief that this enzymatic reaction catalyzes a “rate-limiting step” in the process of starch synthesis and thus, increases in its activity would concomitantly result in enhanced starch accumulation.

One strategy for enhancing AGP activity in plants consisted of heterologously expressing \( \text{g} \text{l} \text{g} c 16 \) (a mutant variant of the E. coli \( \text{g} \text{l} \text{g} c \) gene) that encodes an allosterically insensitive AGP form. Recent studies, however, have shown that the allosterically sensitive \( \text{g} \text{l} \text{g} c \) equally serves the same purpose, since ectopic \( \text{g} \text{l} \text{g} c \) expression restores WT starch content in \( \text{a} \text{p} \text{s} 1 \) Arabidopsis mutants (Li et al., 2012). First attempts to increase AGP activity in plants were made in potatoes through expression of \( \text{g} \text{l} \text{g} c 16 \) under the control of the constitutive 35S promoter. Using this approach, Stark et al. (1992) reported that when the product of \( \text{g} \text{l} \text{g} c 16 \) was targeted to plastids, tubers from the transgenic lines accumulated more starch than control tubers, although there was no correlation between starch levels and AGP activity in different lines. Subsequent attempts to reproduce these results on a different cultivar of potato expressing \( \text{g} \text{l} \text{g} c 16 \) under the control of a tuber-specific promoter did not render tubers with increased levels of starch and ADPG, although they showed increased levels of AGP activity (Sweetlove et al., 1996). Ectopic expression of allosterically insensitive E. coli AGP has also been used in other crops such as rice (Nagai et al., 2009; Sakukingsharaj et al., 2004), maize (Wang et al., 2007) or cassava (Ihemere et al., 2008). In these cases enhanced AGP activity in seeds resulted in enhanced biomass, but there was no increase in the starch content on a fresh weight basis.

Other attempts at achieving increased starch content in cereal endosperms have used modified variants of the AGP large subunit, which contributes little to the catalytic activity of the enzyme but is important in determining its regulatory properties. Groux et al. (1996) obtained maize lines expressing Sh2r6hs (a mutated variant of the AGP large subunit gene), which encodes an enzyme that is allosterically insensitive. The starch content of these lines was higher than in control lines; however there was no increase in starch as a percentage of seed weight and, instead, these lines had significantly heavier seeds (Groux et al., 1996). Ectopic expression of Sh2r6hs has been conducted in maize, rice and wheat (Meyer et al., 2004; Smidansky et al., 2002, 2003). In these cases enhanced AGP activity in seeds resulted in increased individual seed weight and seed yield per plant.

Most recently N. Li et al. (2011) have shown that ectopic expression of either the BT2 gene coding for the small subunit maize AGP, or SH2 gene coding for the maize AGP large subunit, results in enhanced seed weight and starch content.

4.2. Increasing supply of starch precursors to the amyloplast

As indicated above, synthesis of storage starch in the amyloplast requires the incorporation of ATP and carbon skeletons from the cytosol. Tjaden et al. (1998) and Geigenberger et al. (2001) provided evidence that increasing the supply of ATP to the plastid in potato tubers could be a strategy to enhance tuber starch content, since transgenic potato tubers ectopically expressing a plastidic ATP/ADPG transporter from Arabidopsis under the control of the cauliflower mosaic virus 35S promoter accumulated 2-fold more ADPG and 16–36 more starch per gram fresh weight than control tubers. Although these results would suggest that starch content is very sensitive to changes in the transport machinery that supplies ATP, more recent studies using transgenic potato plants ectopically expressing the Arabidopsis ATP/ADPG transporter under the control of a tuber specific promoter did not result in increased...

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levels of starch (L. Zhang et al., 2008). Furthermore, these studies showed that increasing G6P import to the amyloplast by ectopic expression of a pea GPT had no effect on tuber starch content or tuber yield. By contrast, double transformants simultaneously expressing the pea GPT and the Arabidopsis ATP/ADP transporter exhibited an enhanced tuber yield and starch content, the overall data indicating that both ATP/ADP and GPT share control of the starch content and tuber yield, most probably by co-limiting substrate (ATP and hexose-P) supply to AGP.

Indirect evidence that increasing ATP supply to the amyloplast is a strategy for increasing starch content comes from the studies carried out by Regierer et al. (2002) who reported that constitutive overexpression of the ADPG and starch contents of potato tubers (see also patent WO 02/097101 A1). These changes were accompanied by increases in the total adenylate pools (including ATP), although not by changes in the adenylate energy charge or by changes in developmental phenotype. Regierer et al. (2002) thus hypothesized that adenylate kinase acts in the ATP-consuming direction, competing for the same ATP pool with AGP. The increase in starch content (2 fold) is the largest so far reported for any targeted manipulation of starch synthesis.

4.3. Increasing supply of sucrose to heterotrophic cells

The occurrence of high levels of sucrose transporters in heterotrophic organs where apoplastic sucrose unloading occurs (e.g. developing rice, maize, barley, faba bean, and wheat seeds) has led to the conclusion that sucrose transporters may play a role in sucrose uptake into endosperm cells for the synthesis of storage compounds during grain filling (Aoki et al., 1999, 2002, 2003; Harrington et al., 1997; Hirose et al., 1997; Sivitz et al., 2005; Weschke et al., 2000). Supporting this hypothesis, Scofield et al. (2002) reported that antisense inhibition of the rice sucrose transporter OsSUT1 leads to impaired grain filling, the final grain weight being reduced. Toward the aim of evaluating the potential of improved sucrose uptake capacity on wheat grain quality and yield Weichert et al. (2010) produced transgenic wheat plants ectopically expressing the barley sucrose transporter HvSUT1 under the control of an endosperm-specific promoter. The authors reported that, although protein content in transgenic grains was higher than in WT grains, starch concentration was unchanged.

In very early stages of potato tuber development, i.e. during the elongation phase of stolon growth, apoplastic sucrose unloading predominates over symplastic unloading. Subsequent tuberization process involves a switch from apoplastic to symplastic phloem unloading (Viola et al., 2001). Although this would apparently imply that sucrose transporters play a minor role in sucrose unloading in developing tubers and subsequent uptake of sucrose in parenchymatic cells, Kühn et al. (2003) reported that reduction of SsSUT1 expression in antisensed potato plants leads to reduced yield during early stages of tuber development, indicating that in this phase SsSUT1 plays an important role for sugar transport. To evaluate whether it is possible to improve potato plant performance by overexpressing a sucrose transporter Leggewie et al. (2003) produced transgenic potato plants heterologously expressing a spinach sucrose transporter under the control of the 35S promoter. These plants produced tubers with WT levels of starch content and yield. Thus, the overall data obtained using both mono- and dicotyledonous crops would indicate that up-regulation of sucrose transport is not a satisfactory strategy for increasing starch content in heterotrophic organs.

4.4. Enhancement of sucrose breakdown within the heterotrophic cell

Inspired on the widely accepted view that cytosolic hexose-Ps are precursors for starch biosynthesis in heterotrophic organs of dicotyledonous plants (see Fig. 5A), attempts to direct more carbon into starch production in potato tubers consisted of heterologously expressing in the cytosol enzymes such as yeast invertase plus bacterial glucokinase, bacterial sucrose phosphorylase or fructokinase, that facilitate the conversion of sucrose or sucrose breakdown products (i.e. glucose and fructose) into cytosolic hexose-Ps. However, contrary to expectations, transgenic tubers expressing in the cytosol bacterial sucrose phosphorylase, fructokinase or yeast invertase plus bacterial glucokinase all exhibited reduced starch content (Davies et al., 2005; Trehweley et al., 1998, 2001). Despite extensive research, the mechanisms leading to these unexpected results remain obscure (see Section 2.2.2.2).

More successful attempts to promote sucrose–starch conversion have centered on enhancing SuSy activity. The focus on SuSy arose from the belief that this sucrolytic enzyme is a major determinant of sink strength and starch accumulation in both mono- and dicotyledonous plants. First attempts to increase SuSy activity were conducted in potatoes through expression of SsSUS4 under the control of the constitutive 35S promoter. Baroja-Fernández et al. (2009) reported that UDPG, ADPG and starch contents in SuSy overexpressing tubers were higher than in WT tubers. Furthermore, starch yield per plant was shown to be higher in SuSy-overexpressing plants than in WT plants, under both greenhouse and open field conditions (Baroja-Fernández et al., 2009). “High starch” SuSy-overexpressing tubers exhibited a trend-wise increase of AGP activity. Furthermore, they exhibited reduced acid invertase activity, which together with previous studies showing that down-regulation of SuSy is accompanied by a concomitant reduction of starch levels and enhancement of acid invertase activity (Zrenner et al., 1995) indicates that (a) SuSy, but not acid invertase, is involved in the sucrose–starch conversion process, (b) SuSy and acid invertase compete for the same substrate (sucrose), and (c) the balance between SuSy- and acid invertase-mediated sucrolytic pathways is a major determinant of starch accumulation in potato tubers. Interestingly enough SuSy overexpressing tubers exhibited other characteristics of agronomic interest such as substantial increase in antioxidant activity and resistance to enzymatic browning when compared to control tubers. Protection against browning was ascribed to the involvement of SuSy in the production of UDPG necessary for glycosylation and stabilization of polyphenols (patent WO 2010/055186 A1).

Most recently Li et al. (2013) produced transgenic maize plants ectopically expressing SsSUS4 under the control of the constitutive UBI promot- er. The five independent transgenic lines characterized produced seeds exhibiting higher SuSy activity, higher ADPG content and ca. 10% more starch than WT seeds at the mature stage. Furthermore, SsSUS4 expressing seeds contained a higher amylose/amyllopectin balance that WT seeds. SuSy (and AGP) activity in WT seeds greatly exceeded the minimum required to support normal rate of starch accumulation in developing maize endosperms (Li et al., 2013). Consequently, SuSy overexpression should not lead to enhancement of starch content. To explain the observed positive effect of SuSy over-expression on starch content, the authors argued that the rate of starch accumulation is the result of the balance between SuSy–AGP–ADPG transporter–SS-mediated starch synthesis, and amylose- and/or SP-mediated starch breakdown. Therefore, up-regulation of SuSy results in a concomitant increase of the balance between starch synthesis and breakdown, thereby leading to enhancement of starch accumulation. The enhancement of ADPG content in SuSy over-expressing lines (also occurring in AGP over-expressing rice endosperms, Nagai et al., 2009) would indicate that one or more reactions constrain carbon flux from cytosolic ADPG into starch. Li et al. (2013) thus hypothesized that the transport of ADPG is perhaps the more important limiting factor in starch synthesis in SuSy over-expressing maize endosperms.

The overall data thus indicate that enhancement of SuSy activity represents a useful strategy for increasing starch accumulation and yield in heterotrophic organs of both mono- and dicotyledonous crops.

4.5. Enhancement of expression of starch synthase class IV

Five distinct classes of SsSs are known in plants: granule-bound SS (GSSS), which is responsible for the synthesis of amylase, and soluble
SS classes I, II, III, and IV (SSI, SSII, SSIII, and SSIV, respectively), which are responsible for the synthesis of amylopectin. Using developing wheat endosperms exposed to SS inactivating temperatures, Keeling et al. (1993) reported that SSs have a starch biosynthesis flux control coefficient approaching to unity. This and the fact that maximum catalytic activity of SS is close to metabolic flux through starch biosynthesis in several starch storing organs would indicate that SS is a major site of regulation of starch synthesis, and therefore, a good target for the production of genetically engineered “high-starch” plants. However, ectopic expression of SS genes has hardly ever been used to increase the accumulation of starch. This is mainly due to the presence of different classes of SSS in plants interacting with each other in a protein complex and displaying a specific role in the synthesis of the final architecture of the starch granule (Hennen-Bierwagen et al., 2008). Consequently, changes in the expression of a single SS may lead to profound and unpredictable effects not only on starch structure, but also on starch content, revealing that SS isoforms have specific but interdependent roles in starch polymer synthesis. This problem is well exemplified by the results obtained using transgenic potato plants expressing the E. coli glycogen synthase (an enzyme that catalyzes the same reaction as SS) whose tubers accumulated less starch than WT tubers. Furthermore, the starch from the transgenic tubers had altered structural properties (Shewmaker et al., 1994).

Roldán et al. (2007) reported that SSIV T-DNA mutants accumulate only one large starch granule in Arabidopsis chloroplasts. In addition, Szydłowski et al. (2009) reported that Arabidopsis SSIII/SSIV double T-DNA mutants accumulate very reduced starch levels, the overall data indicating that SSIV plays a key role both in the starch granule initiation process and in starch accumulation, being mandatory to render the regular number of starch granule found in WT plants. That elimination of SSIV results in both reduction of number of starch granules and starch content in Arabidopsis leaves suggested that the number of starch granules could represent a regulatory step in the control of starch accumulation (D’Hulst and Mérida, 2010). Confirming this hypothesis Gámez-Arjona et al. (2011) reported that SSIV overexpression results in enhanced levels of transitory starch in Arabidopsis leaves. Noteworthy, high levels of starch accumulated in the end of the day were completely mobilized during the night in SSIV overexpressing plants, allowing them to grow at a higher rate than WT plants. Gámez-Arjona et al. (2011) also reported that ectopic expression of SSIV results in increased levels of reserve starch and yield in tubers of transgenic potato plants cultured both under greenhouse and open field conditions. Interestingly enough, SSIV overexpression was accompanied by pleiotropic increase in AGP and SuSy activities, reduction of acid invertase activity and enhancement of ADP glucose content. Gámez-Arjona et al. (2011) thus hypothesized that enhanced starch accumulation in SSIV overexpressing tubers would be the result of enhanced SSIV, AGP and SuSy activities and reduced acid invertase. Further investigations will be necessary to determine whether ectopic expression of SSIV represents a useful strategy for increasing starch content and yield in other crops of agronomic interest such as cereal crops.

4.6. Blocking starch breakdown

As discussed above (see Section 2.2.2.2), substantial evidence has been provided showing the occurrence of simultaneous synthesis and breakdown of starch in some starch accumulating heterotrophic cells. It is thus highly conceivable that starch accumulated by heterotrophic organs with active starch turnover is the result of the net balance between starch synthesis and breakdown (Baroja-Fernández et al., 2003, 2009; Li et al., 2013; Muñoz et al., 2006). Consequently, down-regulation of starch breakdown functions should be a viable means of increasing starch content in heterotrophic organs.

It is generally accepted that α-amylase plays a central role in endosperm starch degradation. This activity is high in developing seed endosperms during the process of starch accumulation (Hakata et al., 2012; Li et al., 2013). On the other hand, genes encoding glucan, water-dikinases (GWD) involved in starch phosphorylation required for amylose mediated starch breakdown are expressed in starch storing organs. Supporting the above hypothesis that reducing starch breakdown would be a viable means of increasing starch content in storage organs (see above), Ral et al. (2012) recently reported that RNAi-mediated down-regulation of GWD in wheat endosperm under the control of an endosperm-specific promoter resulted in an increase in grain yield determined by increases in seed weight, tiller number, spikelets per head and seed number per spike (see also patent WO 2009/067751 A1). These changes were not accompanied by changes in the percentage of starch per total dried weight, which is a phenomenon comparable to that occurring in AGP overexpressing plants (see above). The work of Ral et al. (2012) provided a promising mechanism for enhancing biomass and grain yield in wheat, with potential application to other crops. Further evidence supporting that down-regulation of starch degradation may contribute to enhance starch content in developing seeds has been provided by Hakata et al. (2012), who reported that suppression of α-amylase genes improves the quality of rice grain when plants are cultured under conditions of high temperature.

4.7. Altering the expression of global regulators

SnRK1 (for Sucrose non-fermenting-1-related kinase-1) is a serine/threonine protein kinase that forms a complex with sucrose non-fermenting (SNF1) and AMP-activated protein kinase (AMPK). It acts as a central integrator of transcription networks in plant stress and energy signaling, and as such, plays a role in regulating metabolism in response to carbon availability (Baena-González and Sheen, 2008; Baena-González et al., 2007).

Upon sensing the energy deficit associated with stress or nutrient deprivation, SnRK1 triggers extensive transcriptional changes that contribute to restoring homeostasis. In general, SnRK1 activates genes involved in nutrient remobilization and represses those involved in biosynthetic processes and storage, thus providing alternative sources of energy through catabolism of amino acids and starch during nutrient starvation. Thus, SnRK1 positively regulates the expression of starch breakdown genes, since antisense expression of SnRK1 reduces α-amylase gene expression in cultured wheat and rice embryos during sugar starvation (Laurie et al., 2003; Lu et al., 2007). Intriguingly, although the attributed role of SnRK1 is to repress functions involved in biosynthetic processes and storage, SnRK1 has been shown to be necessary for starch synthesis, since (a) it is required for SuSy gene expression in potato tubers (Debatt et al., 2011; Purcell et al., 1998), and (b) McKibbin et al. (2006) reported that tubers of SnRK1 overexpressing potato plants accumulate up to 30% more starch than control tubers. As discussed by McKibbin et al. (2006), starch content enhancement resulting from SnRK1 overexpression can be ascribed to up-regulation of SuSy and AGP activities occurring in the SnRK1 overexpressing lines. Although more research using different crop species is necessary to investigate the role of SnRK1 in the control of starch metabolism, the work of McKibbin et al. (2006) provided evidence that SnRK1 overexpression represents a useful strategy for increasing starch accumulation and yield in heterotrophic organs.

Genome-wide co-expression analyses conducted to identify candidate regulators for starch biosynthesis in rice revealed that Rice Starch Regulator1 (RSR1), an APETAL2/ethylene-responsive element binding protein family transcription factor, negatively co-regulates the expression of many starch synthesis genes including those coding for AGP, SSS, GBSS, branching enzymes and starch debranching enzymes (Fu and Xue, 2010). RSR1 highly expresses in rice organs and tissues with reduced starch content such as roots, seedlings and leaves, and expresses to much lesser extent in developing seed endosperms. Although starch content and amylose/amylpectin balance in RSR1 overexpressing seeds were shown to be comparable to those of WT seeds, seeds of rsr1 mutants displayed increased amylose content as a consequence of up-regulation of GBSS expression (Fu and Xue, 2010); had
WT starch content and were larger than WT seeds, a typical phenotype of AGP over-expression (see above). It thus appears that down-regulation of RSRI is a good strategy for increasing starch yield in rice.

She et al. (2010) identified FLO2 as a gene highly expressing in developing rice seeds coincident with starch accumulation whose mutation results in both reduced rice grain size and reduced amylase/amyllopeptic balance. The same authors reported that FLO2 positively co-regulates the expression of genes coding for many starch synthesis enzymes such as AGP (AGP1-4, AGP51, AGPS2a and AGPS2b), SuSy (SuSy1 and SuSy2), SSS (SSIIb, SSIIIb, SSIVa), GBSS and branching enzymes (BE1 and BEIIb), and enzymes involved in starch breakdown such as α-amylase (Amy3C, Amy3D and Amy3E), isoamylases (ISA1 and ISA2) and pullulanase. Importantly, FLO2 overexpressing rice lines produce larger and heavier grains compared with WT plants, providing evidence that FLO2 overexpression is a good strategy for increasing starch content and yield in rice (She et al., 2010). Noteworthy, FLO2 belongs to a family of rice genes whose orthologs widely exist in plants. Provided the function of FLO2 orthologs encoded proteins is similar to that of FLO2, it is likely that up-regulation of FLO2 orthologs in other plant species may result in enhanced starch content and yield. Further research will be necessary to confirm or refute this hypothesis.

4.8. Enhancing trehalose-6-phosphate content

Trehalose-6-phosphate (T6P) is a sugar signal of emerging significance that has been suggested to regulate starch biosynthesis via Trx-mediated posttranslational redox activation of AGP in response to sucrose in leaves, reporting on metabolite status between cytosol and chloroplast (Kolbe et al., 2005; Lunn et al., 2006; Schleupmann et al., 2003). Leaves of transgenic plants with enhanced T6P have increased redox activation of AGP and increased starch levels, whereas leaves of plants with reduced T6P show the opposite effect (Kolbe et al., 2005). The occurrence of a positive correlation between intracellular T6P levels, redox AGP activation and rates of starch synthesis in Arabidopsis (Kolbe et al., 2005; Lunn et al., 2006), and the promotion of SnRK1-dependent redox activation of AGP by trehalose or sucrose feeding to potato tuber disks (Tiessen et al., 2003) led to the hypothesis that T6P is a signal of sucrose status that promotes starch accumulation in a SnRK1-mediated process involving redox AGP activation (Geigenberger et al., 2005; Tiessen et al., 2003). However, this proposal conflicts with recent findings showing that T6P is a strong inhibitor of SnRK1 (Martínez-Barajas et al., 2011; Y. Zhang et al., 2009) and with the fact that genes normally induced by SnRK1 are repressed by T6P and vice-versa (Y. Zhang et al., 2009). To investigate the role of T6P signaling in starch biosynthesis in heterotrophic organs Debast et al. (2011) generated transgenic potato plants with high levels of T6P as a consequence of the heterologous expression of E.coli OtsA (which encodes a T6P synthase). They also characterized transgenic potato plants with low levels of T6P as a consequence of the ectopic expression of E. coli OtsB (which encodes a T6P phosphatase). These authors reported that tubers from transgenic lines with elevated T6P levels displayed reduced starch content. On the other hand, tubers from lines with reduced T6P accumulated WT starch content, although they showed a strongly reduced yield as a consequence of down-regulation of cell proliferation and growth. Transcriptional profiling of “low T6P” tubers revealed that SnRK1 target genes are strongly up-regulated, which confirms the inhibitory effect of T6P on SnRK1 activity. Although Debast et al. (2011) concluded that T6P plays an important role for potato tuber growth, their results indicated that altering T6P intracellular levels is not a good strategy for increasing starch content in heterotrophic organs of plants.

4.9. Potential strategies for increasing starch content in heterotrophic organs

4.9.1. Over-expression of BT1

As pointed out in Section 2.2.2.1, data on ADPG content in seeds of Zmbt1 maize and Hvbt1 barley mutants, and on ADPG transport capacities of amyloplasts isolated from Zmbt1 and Hvbt1 seeds indicated that, in cereal endosperms, BT1 proteins facilitate the transfer of cytosolic ADPG into the amyloplast for starch biosynthesis and are rate-limiting steps in this process. This has led to the proposal that up-regulation of BT1 function would be a strategy for increasing starch content in cereal seeds (Chen et al., 2012; Slattery et al., 2000). BT1 proteins belong to the mitochondrial carrier family (MCF) of proteins that are involved in the transport of metabolites across the mitochondrial membrane (Haferkamp, 2007; Millar and Heazlewood, 2003). Although MCF proteins are all presumed to be targeted to the mitochondrial inner membrane (Millar and Heazlewood, 2003), some of them occur in other subcellular compartments. Kirchberger et al. (2007, 2008) for instance provided evidence that BT1 is exclusively localized to the inner plastidial envelope membranes of plastids. However, more recent studies have shown that BT1 proteins are dually targeted to mitochondria and plastids (Bahaji et al., 2011c).

Kirchberger et al. (2008) reported that homozygous Atbt1 Arabidopsis mutants displayed an aberrant growth and sterility phenotype, which was ascribed to impairments in export of newly synthesized adenylates from plastids to the cytosol. However, Bahaji et al. (2011b) have recently shown that the aberrant growth and sterility phenotype of Atbt1 mutants could be reverted to WT phenotype by specific delivery of AtBT1 to mitochondria, indicating that AtBT1 may play crucial roles, other than transport of newly synthesized adenylates from plastids to the cytosol, that are connected to mitochondrial metabolism. Taking into account that (a) similar to AtBT1, ZmbT1 is dually targeted to plastids and mitochondria, (b) similar to homozygous Atbt1 Arabidopsis mutants, homozygous Zmbt1 mutant seeds germinate poorly and produce plants with low vigor (Mangelsdorf, 1926), (c) mitochondrial do not synthesize starch, and (d) there are many “low starch” maize mutant seeds that germinate and produce plants that grow normally, Bahaji et al. (2011a) hypothesized that (a) the aberrant growth and sterility phenotype of homozygous Zmbt1 mutants would not be ascribed to starch deficiency and/or to reduced ADPG transport activity in endosperm amyloplasts, and (b) similar to AtBT1, ZmbT1 would be involved in yet to be identified processes occurring in mitochondria, other than ADPG transport, that are important for normal growth and fertility that indirectly influence starch accumulation. Needless to say further studies are necessary to confirm or refute the latter hypothesis and to know whether up-regulation of BT1 constitutes a valuable strategy for increasing starch content and yields.

4.9.2. Blocking the activity of ADPG breakdown enzymes

An alternative approach to enhancing starch content in crops would be to reduce the rate of ADPG degradation. Plants possess a widely distributed enzymatic activity, designated as ADPG pyrophosphatase (AGPP), that catalyzes the hydrolytic breakdown of ADPG to G1P and AMP (Rodríguez-López et al., 2000). Two different protein entities are responsible for this activity: “Nudix” hydrolases and nucleotide pyrophosphatase/phosphodiesterases (NPP). Nudix hydrolases have been suggested to act as “housecleaning” enzymes that prevent accumulation of reactive nucleoside diphosphate derivatives, cell signaling molecules or metabolic intermediates by diverting them to metabolic pathways in response to biochemical and physiological needs (McLennen, 2006). AGPPs belonging to this family have been found both in the plastidial and cytosolic compartments (Muñoz et al., 2008; Olejnik and Krzeszewska, 2005). Up-regulation of the plastidial AGPP Nudix hydrolase in transgenic potato plants results in reduced levels of starch content in both leaves and tubers, indicating that this enzyme has access to the pool of ADPG linked to the biosynthesis of both transitory and reserve starch in leaves and tubers, respectively (Muñoz et al., 2008). The control of the intracellular levels of ADPG linked to starch production likely represents an important, though still poorly investigated point of control of starch metabolism. Continued molecular genetic analysis will be needed to determine whether AGPPs play a crucial role in preventing metabolic flux toward starch in heterotrophic
organisms of plants, and whether down-regulation of the AGPP function constitutes a good strategy for enhancing starch content and yield in major crops.

4.9.3. Ectopic expression of SuSy in the plastid

As presented in Section 2.1.2., a sizable pool of sucrose has a plastidial localization (Gerrits et al., 2001; see Section 2.1.2.). SuSys from cyanobacteria are highly specific for ADP and have high affinity for this nucleotide (Curatti et al., 2000; Figueroa et al., 2013; Porchia et al., 1999). Thus, a more radical possibility for increasing the starch content of crop plants would be to express SuSy (especially from cyanobacterial origin) in the plastid to produce ADPG.

4.9.4. Blocking the synthesis of storage proteins

During development of some heterotrophic organs such as tubers and seed endosperms, cell division is followed by cell elongation, differentiation and storage. When cell division ceases, a sizable pool of carbon and ATP is mainly utilized to produce massive amount of storage starch and proteins. We must emphasize that a similar phenomenon has been observed in bacterial cultures entering the stationary phase, where ATP and carbon are diverted from processes required for cell division and growth to glycogen production (Montero et al., 2009; Rahimpour et al., 2013).

Storage protein production is expensive in terms of carbon and ATP costs and it is highly likely that both storage protein and starch biosynthetic processes compete for the same ATP and carbon pools. Therefore, blocking storage protein synthesis during the latter phase of development could be a potential strategy for increasing starch content in heterotrophic organs. One way to do it would consist in altering the expression of factors globally controlling the production of storage proteins. This, however, is difficult since it has been reported that the expression of genes involved in storage starch and protein synthesis is highly coordinated by the same factors (Giroux et al., 1994; She et al., 2010). One alternative would consist in moderately down-regulating the expression of genes directly or indirectly involved in the synthesis of amino acids by using promoters that are specifically expressed during the latter part of the heterotrophic organ development. In this respect we must emphasize that accumulation of exceptionally high levels of starch in leaves of plants exposed to MVs was accompanied by reduction of the levels of amino acids as a consequence of down-regulation of genes directly or indirectly involved in amino acid biosynthesis (see Section 3, Ezquer et al., 2010a).

5. Conclusions and main challenges

The discrepancies between the different proposed models of starch biosynthesis in leaves (illustrated in Figs. 1–4) and heterotrophic organs (Figs. 5 and 6), and the unexpected failures of some molecular strategies aimed at improving storage starch content in plants denotes that our knowledge of the starch biosynthetic process is still at a rudimentary level. Better understanding of the regulation of starch synthesis will require additional research, either at the biochemical, cell biological and molecular levels, which in turn will allow plant breeders and biotechnologists to advance in their attempts to increase starch in a predictable way. Additional knowledge on resource allocation within the plant, sucrose uptake in heterotrophic cells, sources of ADPG, mechanisms of ADPG transport in plastids, interaction of the plant with both biotic and abiotic environmental factors, and metabolic interaction of the plastidial compartment with other cellular compartments will be crucial. In this last respect we must emphasize that recent studies have demonstrated the occurrence of strong, but unexplored metabolic links between plastids and endomembrane organelles. Thus, Nanjo et al. (2006) and Kitajima et al. (2009) have shown that α-amylase and NPPs traffic between endoplasmic reticulum-Golgi and the plastidial compartment. Furthermore, Wang et al. (2013) have recently shown that autophagy contributes to starch degradation in N. benthamiana leaves, cytosolic starch granule-like structures being sequestered by autophagic bodies and delivered to vacuoles for their subsequent breakdown. Needless to say, further studies will be necessary to evaluate the relevance of such links in the overall starch metabolism. Although the study of endocytosis in higher plants remained dormant because of the notion that this mechanism would not work against the high turgor pressure in plant cells, mounting evidence has been compiled during the last years showing that endocytosis is essential for many processes in plants (Müller et al., 2007; Samaj et al., 2005), including carbohydrate metabolism. We now know that in heterotrophic organs where apoplastic sucrose unloading occurs endocytosis may play an important role in transporting the bulk of sucrose from the apoplast to the vacuole and its subsequent conversion into starch (see Section 2.2.1). Thus, further investigations will be necessary to understand the molecular and signaling mechanisms involved in this process.

Starch biosynthesis is a highly regulated process, both at transcriptional and post-transcriptional level that is interconnected with a wide variety of cellular processes and metabolic pathways. Its regulation involves a complex and an as yet not well defined assembling of factors that are adjusted to the physiological status of the cell. However, to date most studies on genetic regulation of starch metabolism have focused on the effects of single genes on starch biosynthesis, and analyses of the effect of global regulatory factors on starch metabolism, as well as systematic studies of the regulatory mechanisms of starch biosynthesis are still scanty. Evidence has been provided that, similar to the bacterial system where glycogen synthesis and breakdown genes are co-expressed and co-regulated with genes involved in diverse biological processes (Montero et al., 2011), starch metabolism genes are co-expressed and coordinated with other functions such as amino acid and fatty acid biosynthesis (Fu and Xue, 2010; Giroux et al., 1994; She et al., 2010; Tsai et al. 2009). Systematic analyses-based characterization of the interactome of starch metabolism-related genes and proteins with genes and proteins involved in other biological processes, and the identification of global factors controlling the expression of core starch metabolism functions will be crucially important to rationally design molecular strategies aimed at enhancing storage starch content in heterotrophic organs of main crops. In this respect, we must emphasize that genome-wide co-expression analyses, which are based on the assumption that genes with similar expression patterns are more likely to be functionally related, have proven to be a powerful tool to identify regulatory factors in transcriptional networks regulating starch metabolism in crop species such as rice and potato (Ferreira et al., 2010; Fu and Xue, 2010). Similar type of comparative transcriptome and proteome analyses between different plant organs of WT plants and mutants with altered starch content should be conducted using a wide range of species of agronomic interest to identify major determinants and global regulators of starch biosynthesis.

MV-promoted accumulation of exceptionally high levels of starch is a recently discovered mechanism for the elicitation of plant carbohydrate metabolism by microbes that still needs to be investigated in more detail before it can be fully understood and used as a source of ideas and tools for strategies to complement breeding and biotechnology programs aimed at improving crop yields. At short run, further efforts and research will be necessary to identify the volatiles and sensing mechanisms involved in MIVOISAP.

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