Starch Metabolism in Green Plants

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Abstract

Starch is a substantial component of the human diet providing about 50 % of daily energy uptake, mostly through unrefined cereals. Starch and sucrose are the primary products of photosynthesis. Starch represents the main plant storage carbohydrate that provides energy during heterotrophic growth. Its synthesis and degradation have been studied deeply, reaching a good level of knowledge of the metabolism as a whole. Not only the enzymes involved but also the intracellular localization of the reactions, its regulation, its dependence on light–dark cycle, its evolution from ancestral bacteria, and its correlation with parameters of agronomic interest have been studied. In this work we have attempted a comprehensive review of the starch metabolism in *Arabidopsis thaliana* and other species of agronomic interest and the modular structures present in starch-related enzymes from *Arabidopsis thaliana*.

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1 Introduction

Polyglucans are the most important and widespread carbohydrate storage compounds found in nature, being cellulose, glycogen, and starch the most abundant forms. These two last polysaccharides are comprised of glucose chains linked by an α-1,4 bond and branched at α-1,6. Glycogen is a homogeneous water-soluble polymer with relatively uniformly distributed branches (Roach 2002) and is found in organisms such as archaea, bacteria, and certain eukaryotes (Busi et al. 2012). Starch is the main form in which plants and algae accumulate carbohydrate storage, because cellulose, the most abundant carbohydrate in plants, complies with structural functions. Starch is present in the cytosol of Glaucophyta and Rhodophyceae (red algae) (Dauvillee et al. 2009); however, it is confined to the plastid stroma (chloroplasts in green tissues and amyloplasts in reserve organs) in green algae and higher plants. Starch is essential for the carbon economy of the different organs and tissues of the plants and it can be found in large amounts in fruits, seeds, leaves, roots, and tubers (Smith and Zeeman 2006).

Starch molecules are polymers of glucose in two configurations: amylose, which is mostly unbranched, and amylopectin, which is branched. Amylose is predominantly linear and made up of α-1,4-linked glucosyl moieties, while amylopectin is branched and contains both α-1,4 and α-1,6 linkages. These branches pack together into ordered arrays to form semicrystalline starch granules (Ball et al. 1996; Grennan 2006). Starches from different plant sources vary in size, composition, and structure of amylopectin. These factors influence the physical properties and end-uses for the different natural starches (Santelia and Zeeman 2011) (Table 1).

The structural features of the starch also determine the ability to be digested. This polysaccharide can be hydrolyzed by enzymes of the human gut and its digestion occurs in the small intestine, except for a portion named resistant starch (RS) which is degraded in the large intestine (Busi et al. 2012). RS is defined as the set of starch and products of starch degradation (oligosaccharides and other sugars)

Table 1 Amylose and amylopectin properties

Property/trait	Amylose	Amylopectin
Granule weight	15–35 %	65–85 %
Molecular mass	10^4–10^5	10^7–10^8
Average chain length	3–1,000	3–50
Ramifications	<1 %	4–6 %
Degree of polymerization[a]	10^2–10^3	10^3–10^4
Iodine affinity (g/100 mg)	19–20.5	0–1.2
λ_{max}[b]	630–660 nm	530–570 nm

[a]Molecule average size, expressed as glucose units
[b]With Lugol (I_2/KI)

that are not absorbed in the small intestine but are fermented in the colon producing short-chain fatty acids such as butyrate, and it promotes the normal function of the colonocytes. RS functions as dietary fibers, including prebiotic effect on the colon microflora, improving lipid metabolism. There are at least four mechanisms by which RS is obtained: RS1, a physically inaccessible starch, usually encapsulated on indigestible tissues (encapsulated or embedded within a lipidic or proteic matrix); RS2, starch granules highly resistant to degradation, which has two subtypes, RS2a, with lower amounts of amylose (0–30 %), which typically loses its strength after cooking, and RS2b, starches with high amylose content which retains its granular structure during processing; RS3, retrograde starch which requires cooking to be released from the granules, and the starch retrograde capacity is affected by the intrinsic biosynthetic process; and finally, RS4, chemically modified starches. Although the last mechanism is the most used to produce resistant starch, there are no reports of changes in plant that can mimic those obtained by chemical methods (Topping et al. 2003; Morell et al. 2004; Busi et al. 2012).

2 Generalities About Location, Timing, and Regulation of the Synthesis and Degradation of Starch

Starch synthesis is restricted to the Archaeplastida, which is thought to be originated via an endosymbiotic event between ancestors of cyanobacteria and a heterotrophic host (Cavalier-Smith 2009), resulting in organelles called plastids that are able to perform oxygenic photosynthesis. Recently, phylogenetic studies show that the plastidial starch pathway is highly complex and made up of genes belonging from cyanobacteria and other eukaryotes (Patron and Keeling 2005; Deschamps et al. 2008a, b) and is in sharp contrast to the lower-complexity pathway of cytosolic starch synthesis found in the Rhodophyceae and Glaucophyta (Deschamps et al. 2008a, b; Busi et al. 2012).

Phylogenetic analysis of the enzymes of the starch biosynthetic pathway strongly suggests that the pathway was originally cytosolic in the common ancestor of the Archaeplastida and then redirected to plastids via three discrete steps, leaving some enzymes involved in the metabolism of malto-oligosaccharides (MOS) and amylopectin degradation in the cytoplasm (Busi et al. 2012). The three evolutionary steps involved are (i) plastidial synthesis of unbranched MOS; (ii) glycogen synthesis (including priming steps and branching activities); and (iii) plastidial starch synthesis, resulting in the eventual loss of cytosolic starch synthesis. Interestingly, the relocation of the starch synthesis pathway to plastids coincides with the evolution of light-harvesting complexes (Deschamps et al. 2008a, b; Tetlow 2011; Busi et al. 2012).

Plants live and grow in an environment where the light period (day) is followed by dark (night). The sunlight is the only source of energy for photosynthetic CO_2 assimilation and also constitutes a source of energy for other metabolic processes that occur into the cell. During the night, plants use the storage metabolites that accumulated during the day (Smith and Stitt 2007). When the plants grow, the

accumulation of starch produced during the day (in a near-linear manner) is offset by the degradation overnight, so that most of the reserve polysaccharide is depleted before the start of the new light period. This pattern is conserved under different conditions of irradiances, day lengths, nutrients, water amount, and CO_2 levels (Gibon et al. 2009; Tschoep et al. 2009; Hummel et al. 2010; Graf and Smith 2011; Stitt and Zeeman 2012), indicating that it represents a system feature maintained and adapted to large fluctuations in environmental conditions. This balance requires adequate changes in the rates of synthesis and degradation of starch (Smith and Stitt 2007; Stitt and Zeeman 2012).

Furthermore, it has been found indirect evidence for a role of the biological clock in regulating starch breakdown. Transcripts of genes involved in starch breakdown show large and coordinated diurnal changes, which are at least driven in part by the clock (Harmer et al. 2000; Smith et al. 2004; Lu et al. 2005; Usadel et al. 2008; Stitt and Zeeman 2012; Streb and Zeeman 2012).

Usadel et al. (2008) showed the response of genes involved in starch degradation based on data from Smith et al. (2005) and Zeeman et al. (2007). Most of them (PHS1, ISA3, DPE1, PHS2, DPE2, GWD, PWD, and SEX4 among others) show a marked circadian cycle showing a minimum at the end of the dark period and a peak near the end of the light period (Harmer et al. 2000; Lu et al. 2005), which is retained in a light/dark cycle. However, this increase is weakened or abolished early in an extended night when the clock is antagonized by darkness or falling carbon. This provides a potential mechanism to decrease the rate of starch breakdown when carbon stocks are decreasing in the light period, although the impact depends on the levels of proteins involved (Usadel et al. 2008).

Finally, we should note that the redox-related signals are crucial for the regulation of starch synthesis. ADP-glucose pyrophosphorylase (ADPGlc PPase), the key regulatory enzyme of starch synthesis in the plastid, is subject to posttranslational redox regulation. It was reported that the isoforms from pea leaf chloroplasts and potato tuber are activated by thioredoxin (Ballicora et al. 2000; Geigenberger et al. 2005). In addition, various enzymes involved in starch degradation have been found to be redox regulated, which may imply a coordinated regulation of starch synthesis and degradation by redox signals (Kotting et al. 2010; Geigenberger 2011).

One example of the redox regulation of the starch breakdown is the case of the glucan, water dikinase (GWD). This enzyme catalyzes the phosphorylation of the starch granules, an essential step for the initiation of starch degradation in plant leaves and tubers (Lorberth et al. 1998; Yu et al. 2001a; Ritte et al. 2002, 2004). It was shown that a major fraction of GWD is attached to the surface of the starch granule when isolated from dark-adapted plants, whereas the protein is predominantly found in a soluble form upon isolation from illuminated plants (Ritte et al. 2000). Mikkelsen et al. (2005) showed that GWD is subject to redox activation mediated by thioredoxin f and m, which is followed by the reversible reduction of a specific intramolecular disulfide bond. It was demonstrated that the redox state of GWD affects binding of the enzyme to the starch granules in a selective and reversible manner, which would promote granule association of

GWD in the dark (Mikkelsen et al. 2005). More studies are needed to clarify the in vivo relevance of this mechanism to initiate granule degradation at the beginning of the dark period. Similar to GWD, other enzymes involved in starch degradation such as β-amylase or pullulanase have been found to be activated upon reduction (Spradlin and Thoma 1970; Dauvillee et al. 2001; Schindler et al. 2001; Wu et al. 2002) or to interact with thioredoxins (Balmer et al. 2003), but whether this affects granule binding or multi-protein complex formation was not studied yet. It was suggested that the degradation of the crystalline starch granule may involve a coordinated association of several enzymes to the surface of the granule that is triggered by a decrease in the reduction state of thioredoxins. This mechanism may link starch degradation to light and the carbon status of the leaf (Geigenberger et al. 2005).

Figure 1 presents an outline of starch synthesis and degradation, indicating the main reactions and protein involved.

3 Starch Synthesis: Enzymes Involved

Starch synthesis initiates in chloroplasts when the intermediate of the Calvin cycle fructose-6-phosphate (Fru6P) is converted into glucose-6-phosphate (Glc6P) by the action of phosphoglucose isomerase (PGI). After that, a phosphoglucomutase (PGM1) converts Glc6P into glucose-1-phosphate (Glc1P) (Caspar et al. 1985; Streb et al. 2012).

On the other hand, in non-photosynthetic plastids that accumulate starch, this polysaccharide is produced from sucrose synthesized in the leaves (Comparot-Moss and Denyer 2009). Then, sucrose is catabolized to Glc6P in the cytosol, which could be imported into the plastids via specific hexose-P translocators (Neuhaus and Emes 2000). On the other hand, part of the ATP is also generated in cytosol after sucrose breakdown, transported into plastids via an adenylate transporter and used as energy source for starch synthesis. Inside the plastid, Glc6P is converted to Glc1P by a plastidial phosphoglucomutase. It was also reported that in specific situations, different substrates such as Glc1P may be imported into the chloroplast by the same hexose-P transporter to maintain starch biosynthesis (Tetlow et al. 1996; Fettke et al. 2010).

Three functional phosphoglucomutase proteins are present in Arabidopsis (Egli et al. 2010), one plastidial (PGM1) and two cytosolic (PGM2 and PGM3). Arabidopsis mutants with reduced PGI or PGM1 activities have significantly lower levels of leaf starch (Caspar et al. 1985; Lin et al. 1988a, b; Kofler et al. 2000); however, these plants still contain small amounts of starch in their chloroplasts, showing unaltered ADPGlc levels, suggesting the presence of another pathway that could synthesize the sugar-nucleotide precursor (Vitha et al. 2000; Munoz et al. 2005; Streb et al. 2009). One possibility to explain this situation is that as mentioned above, the Glc1P is translocated to the plastid from the cytosol. However, another possible explanation is that the ADPGlc molecule could be synthesized by alternative enzymatic activities in the mutants.

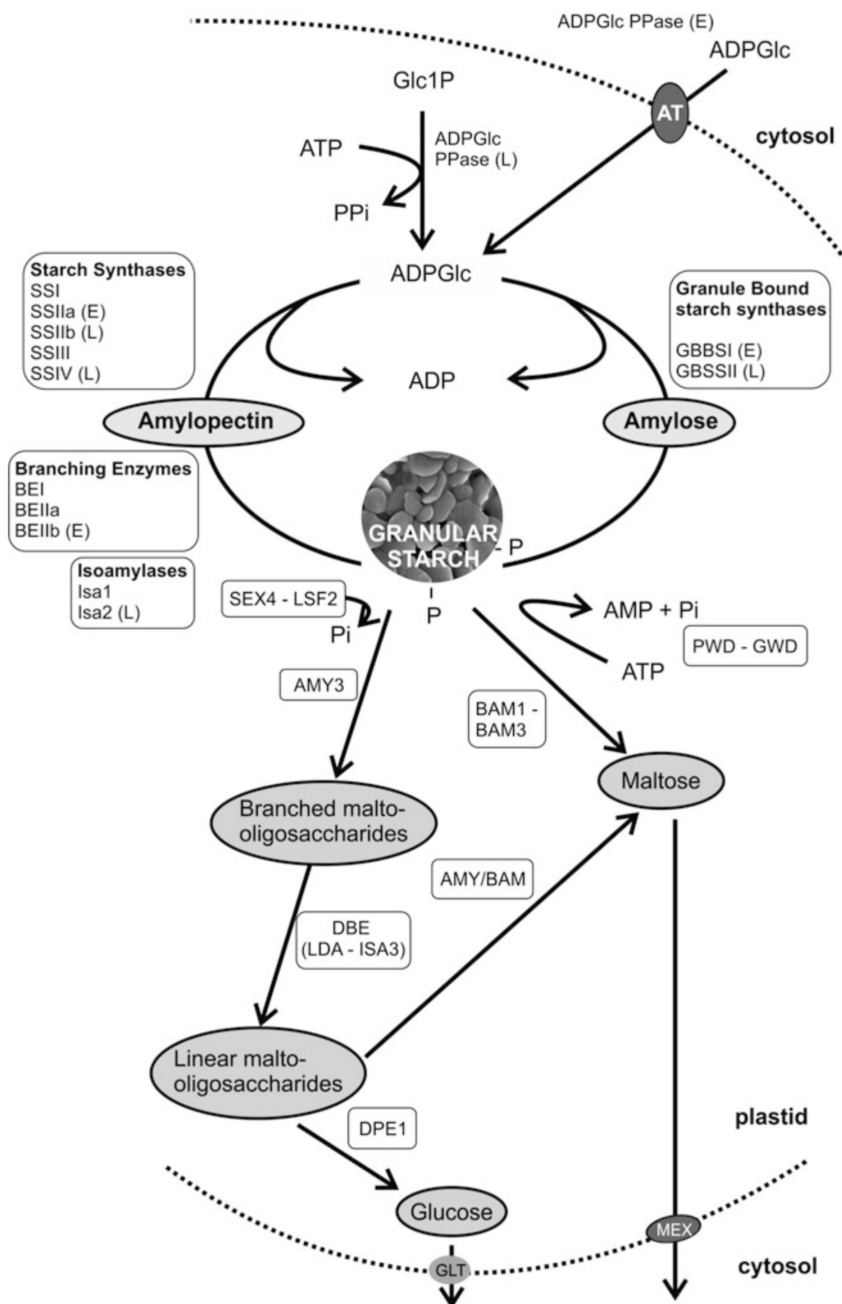

Fig. 1 Synthesis and degradation reactions of starch in plants. The scheme indicates the involvement of different isoforms in leaf (*L*) or endosperm (*E*). When not specified, the enzymes are dual localized in both compartments. All proteins concerned are listed in Tables 2, 3, 4, and 5 (AT, adenylate transporter; GLT, glucose transporter; MEX, maltose transporter)

Arabidopsis PGM2 and PGM3 single mutants present wild-type-like phenotypes; however, the loss of both cytosolic phosphoglucomutases significantly reduces the gametophyte function, probably due to a flaw on the distribution of sugars in the different metabolic pathways (Egli et al. 2010). In addition, the repression of cytosolic PGM in potato causes a significant delay in growth, a decrease in tuber number, an inhibition of sucrose synthesis in leaves, and a decrease level of sucrose and starch in tubers (Fernie et al. 2002).

The ADPGlc pyrophosphorylase (ADPGlc PPase) catalyzes the synthesis of ADPGlc from Glc1P and ATP, and this reaction constitutes the rate-limiting step for starch biosynthesis. This enzyme usually contain two catalytic (small) and two regulatory (large) subunits. Homotetrameric potato tuber ADPGlc PPase is the only PPase from plants which the 3D structure have been solved (Jin et al. 2005). The analysis of the structure show that there are two interacting domains: the N-terminal domain (catalytic) which is similar to a dinucleotide-binding Rossmann fold, whereas the C-terminal domain participates in cooperative allosteric regulation and oligomerization.

ADPGlc PPase from photosynthetic cells is tightly regulated by allosteric modulation, comprising of 3-phosphoglycerate (3PGA) activation and orthophosphate (Pi) inhibition. There is a strong evidence that the regulation by these metabolites is relevant in vivo, being the 3PGA/Pi ratio a main signal determining the starch synthesis within chloroplasts (Preiss et al. 1991; Ball et al. 1996; Preiss and Sivak 1998; Gomez-Casati et al. 1999; Gomez Casati et al. 2000; Ballicora et al. 2004). However, 3PGA can inhibit the enzymatic activity at high concentrations (Hwang et al. 2005). In contrast, whereas some ADPGlc PPases from non-photosynthetic tissues such as maize and rice endosperm and cassava roots are regulated by 3PGA/Pi, the enzymes from pea embryos, barley and wheat endosperm, and bean cotyledons are insensitive to regulation (Gomez-Casati and Iglesias 2002). In addition, as mentioned above, the small subunits of ADPGlc PPase from vascular plants are subjected to redox regulation (Ballicora et al. 2000).

All the plants analyzed contained at least three genes encoding ADPGlc PPase subunits (Deschamps et al. 2008a, b) including at least one large (L) and one small (S) subunit each. Arabidopsis mutants lacking the S or L subunit exhibit a starch-deficient phenotype (Lin et al. 1988a, b). Besides, the first maize mutant plants with altered amounts of either ADPGlc PPase S or L subunits were described almost 50 years ago. These mutant plants also showed starch-deficient phenotype, confirming a relevant function of ADPGlc PPase in starch synthesis (Tsai and Nelson 1966; Dickinson and Preiss 1969). On the other hand, transgenic plants with increased ADPGlc PPase activity triggered elevated yields in several starch-producing crops, including rice, maize, wheat, and potato (Stark et al. 1992; Greene and Hannah 1998; Huang et al. 2014).

Plant ADPGlc PPases differ in spatial and temporal expression. Cereal endosperm ADPGlc PPases are mainly located in the cytosol with only a minor amount in amyloplasts; meanwhile, they are rigorously plastidial located in other species and tissues. The differential expression of subunits in different organs results in variable degrees of sensitivity of AGPase to allosteric effectors (Wang et al. 2014).

Both cytosolic and plastidial AGPases would contribute to the production of endosperm starch (Comparot-Moss and Denyer 2009; Geigenberger 2011). Such compartmentalization requires distinctive modes of regulation and metabolic control of starch biosynthesis compared with other plants, including transport of ADPGlc and hexose-P from the cytosol into the amyloplast (Fig. 1) (Denyer et al. 1996; Thorbjornsen et al. 1996; Tetlow et al. 2003; Patron et al. 2004). Recently, it has been identified that the HvBT1 transporter in barley transports ADPGlc, the main precursor for starch biosynthesis during grain filling, from the cytosol into the amyloplasts of endospermic cells (Soliman et al. 2014). Its localization in the plastid envelop and specific expression in the endospermic barley cells indicate its significance in determining starch yield in this cereal and confirm the distinct modes of regulation between leaf and endosperm starch synthesis (Soliman et al. 2014).

Several starch synthases are thought to catalyze amylopectin synthesis, while granule-bound starch synthases (GBSS) are responsible for the biosynthesis of amylose. These enzymes are tightly bound to starch granules; for this reason they are called "granule bound" (Smith et al. 1997). *Arabidopsis thaliana* presents only one GBSS (Deschamps et al. 2008a, b), while cereals present two types of GBSS called GBSSI and GBSSII (Jeon et al. 2010). Recently, the 3D structure of the catalytic domain of rice (*Oryza sativa* japonica) GBSSI has been solved (Momma and Fujimoto 2012). It has been found that the structure was similar to those reported for the bacterial and archaeal glycogen synthases belonging to the glycosyltransferase family 5 (GT5). According to the transcripts found on the different organs, it has been shown in wheat that GBSSII is expressed predominantly in pericarp, leaf, and culm tissues, suggesting that almost all transient starch is produced by this granule-bound starch synthase (Vrinten and Nakamura 2000), whereas GBSSI (encoded by the Waxy loci (Wx) of cereals) is solely responsible or the biosynthesis of amylose in storage tissues (Smith et al. 1997). In rice it has been shown that a mutation at the Wx locus generates starch but lacking the amylose fraction, whereas a 15–30 % of amylose content is present in wild-type rice endosperm (Sano 1984). Similar results were observed in wheat (Nakamura et al. 1995). However, there is increasing evidence that GBSS is not only involved in amylose synthesis, but it is also involved in amylopectin synthesis. Studies carried out by Yoo et al. have demonstrated that there is a decreased amount of extra-long unit chains of amylopectin in Wx mutants of wheat (Yoo and Jane 2002).

SSI is one of four starch synthases present in the plastid stroma of those organisms that synthesize starch (Ball and Morell 2003). The activity of this isoform, as is the case of the other ones, depends on the organism and the tissue. For example, in developing rice endosperm, the activity of this enzyme represents the 62–66 % of the total starch synthase activity in the soluble fraction, followed by SSIII with a 26 % of the total SS activity, consistent with results obtained also in maize endosperm (Fujita et al. 2006).

SSI from barley is the only plant-soluble starch synthase of which crystallographic structures have been solved (Cuesta-Seijo et al. 2013). Unlike other SSs which present more than one isoforms in cereal species, excepting some cases like

Arabidopsis thaliana, there are no known isoforms of SSI (Deschamps et al. 2008a, b; Jeon et al. 2010). As seen on deficient SSI organisms, it was suggested that this enzyme is the major determinant for the synthesis of amylopectin, mainly involved in the biosynthesis of small outer chains (Delvalle et al. 2005).

Studies on SSI-deficient rice surprisingly shows normal seed morphology, seed weight, amount of endosperm starch, and morphology and crystallinity of endosperm starch granule. On the other hand, the structure of amylopectin is affected by this mutation. SSI prefers short glucan chains as substrate, and they propose that when the chain becomes long enough, this isoenzyme becomes inactive and actually it is entrapped inside the starch granule during the glucan deposition by others SSs (Commuri and Keeling 2001). When SSI becomes deficient, there was a reported increase of chains with degree of polymerization (DP) 6–7 and 16–19, while those of DP 8–12 decreased, indicating that SSI generates chains ranging from DP 8–12 (Fujita et al. 2006). Similar studies have been done on *Arabidopsis thaliana* and the results were in agreement with what was observed in rice. In Arabidopsis, the SSI deficiency leads to a dramatic reduction in the amount of chains with a DP of 8–12, while the ones of DP 17–20 increased (Delvalle et al. 2005). From these results it was suggested that SSI functions from early to late stages of endosperm development. In addition, the complete absence of SSI, despite being a major starch synthase isoform in endosperm, had no effect on the morphology of the starch granules, indicating that the other SS isoenzymes could partially complement SSI deficiency, leading to the modification of the length of the chains (Fujita et al. 2006). In summary, this study strongly suggested that amylopectin chains are synthesized by the coordinated actions of different SS isoforms such as SSI, SSIIa, and SSIIIa.

The second soluble starch synthase from plants, SSII, plays an essential role synthesizing the mid-length amylopectin chains. In general, SSII deficiency in different species results in increased amounts of short glucan chains (DP6 to DP11) within amylopectin, decreased abundance of DP12 to DP25 chains, and in most instances an elevated amylose to amylopectin ratio (Zhang et al. 2008). SSII deficiency in Arabidopsis showed a decreased of DP12 to DP18 chains of amylopectin, while DP6 to DP10 were significantly increased. The starch granules from *Arabidopsis* SSII mutant exhibit distorted morphology and the amount of amylose produced in leaves was increased, presumably by an increased activity of GBSS (responsible of amylose synthesis) in these mutants (Zhang et al. 2008).

In dicots only one SSII is present, in contrast to monocots that contains more than one isoform. SSII in rice presents three isoforms named osSSIIa, osSSIIb, and osSSIIc. The reason for the requirement of multiple isoforms is unknown at the present (Nakamura et al. 2005). The expression analysis of rice SSII isoforms showed that osSSIIa is expressed in endosperm, leaves, and roots, while osSSIIb is primarily expressed in leaves and osSSIIc mainly in endosperm (Jiang et al. 2004). Even though the three SSII have similar structures and the same function, it has been demonstrated that actually they are not redundant. For example, osSSIIa deficiency in endosperm leads to the increase of short chains of

amylopectin, suggesting that this isoform (and not osSSIIc) is responsible for the differences on the rice starch structure and properties. The higher content of amylopectin short chains could explain the different quality of this starch such as an easier disintegration in alkali and lower gelatinization temperature (Umemoto et al. 2002).

In maize and wheat, there are also three SSII present. In these cases, the deletion of SSIIa leads to an increase in amylose content, a decrease of longer chains of amylopectin (DP13 to DP25), and an increase of shorter chains (DP6 to DP11). Thus, it could be postulated that SSIIa has a major role, while SSIIc has a minor function in starch synthesis in endosperm (Yan et al. 2009a).

Taking in account the intron and exon arrangements of the SSII genes of both mono- and dicots, it seems that the development of the SSII genes predates the separation of these two classes (Jiang et al. 2004) and the SS gene duplication in starchy seeds species indicates that this duplication benefits storage starch synthesis in seeds (Yan et al. 2009a).

SSIII has been proposed as the responsible for the synthesis of longer amylopectin "spacers" chains extending between clusters (Maddelein et al. 1994; Wang et al. 2014) and has been proposed to perform a regulatory function in starch biosynthesis (Zhang et al. 2005; Valdez et al. 2008; Wayllace et al. 2010). However, this isoform also performs other functions that overlap with SSII to generate single-cluster chains.

Arabidopsis, wheat, and potato have only one SSIII gene (Wang et al. 2014); on the other hand, two genes are responsible for the expression of SSIII in the endosperm and leaves of rice and maize (Hirose and Terao 2004; Dian et al. 2005). The analysis of Arabidopsis *ssiii* mutants has shown that SSIII deficiency produces a starch-excess phenotype and an increase in total SS activity (Zhang et al. 2005). Loss of SSIII activity in potato tubers leads to a significant reduction of total SS activity and alterations in starch structure and physical properties (Edwards et al. 1999). Starches from SSIII-deficient maize and rice mutants show a decrease in long-branch ($DP > 30$) proportion in addition to altered granule morphology (Gao et al. 1998). In addition, it was also documented that loss of SSIII in the maize endosperm causes decreased activity of SBEIIa and an increase in other SS activities (Boyer and Preiss 1981).

SSIII from higher plants contains two regions: an N-terminal non-catalytic SSIII-specific domain and a C-terminal domain, the catalytic domain (CD) common to all SS isoforms (Dian et al. 2005; Busi et al. 2008). It has been demonstrated that the N-terminal region of SSIII contains three starch-binding domains (SBD) that show starch-binding capacity (Palopoli et al. 2006; Busi et al. 2008; Valdez et al. 2008). These N-terminal SBDs have also a regulatory role since they modulate the catalytic properties of SSIII (Valdez et al. 2008; Wayllace et al. 2010).

Current experimental evidences provided by Gamez-Arjona et al. (2014) show that Arabidopsis SSIII exhibits a localization pattern close to the surface of the starch granule and suggest that this specific localization is mediated by its three N-terminal starch-binding domain (Gamez-Arjona et al. 2014).

Besides, we previously demonstrated the presence of protein–protein interactions between the SBDs and the catalytic domain of SSIII from *A. thaliana*. The starch-binding capacity of the SBDs and the interactions between the N- and C-terminal domains are critical in the modulation of the catalytic activity of the protein (Wayllace et al. 2010). Furthermore, it was described that the N-terminal region of maize endosperm SSIII and the central SSIII region each bind to different components of the starch biosynthetic pathway (Hennen-Bierwagen et al. 2008). Specifically, in vivo protein–protein interaction studies demonstrate that the amino terminal portion of SSIII can bind to a branching enzyme (BEIIa), whereas the SSIII central domain binds to SSI (Hennen-Bierwagen et al. 2008). Suggested potential functions for the starch synthesizing machinery complexes include substrate channeling and/or effects on the crystallization of the linear chains of amylopectin to form crystalline lamellae (Hennen-Bierwagen et al. 2008, 2009).

Meanwhile, Arabidopsis possesses just one SSIV isoform; two isoforms of SSIV exist in rice, potato and wheat, which are expressed differentially in endosperm and leaves (Dian et al. 2005; Deschamps et al. 2008a, b; Leterrier et al. 2008). Arabidopsis SSIV mutant presents just 1–2 starch granules per chloroplast and the core of these granules has a different structure from normal granules (Roldan et al. 2007). These observations indicate that SSIV is involved in starch granule initiation in Arabidopsis, granule number control, and production of short branches, although the mechanisms involved remain unclear (Roldan et al. 2007; Szydlowski et al. 2009). On the other hand, no SSIV-deficient mutant has yet been characterized in cereal plants at the present (Roldan et al. 2007; Jeon et al. 2010).

Recently, Gamez-Arjona et al. (2014) described that SSIV displays a different localization from other starch synthases. This enzyme is located in specific areas of the thylakoid membrane and interacts with fibrillin 1a (FBN1a) and 1b (FBN1b), which are mainly located in plastoglobules. The localization pattern of SSIV is mediated through its N-terminal region, which contains two long coiled-coil motifs (Gamez-Arjona et al. 2014). The localization of this isoform in specific areas of the thylakoid membrane suggests that starch granules are originated at specific regions of the chloroplast.

A summary of the enzymes involved in starch synthesis in Arabidopsis as well as in other species are shown in Tables 2 and 3.

4 Starch Degradation Pathway

Leaf starch breakdown occurs at night via the coordinated actions of a suite of enzymes constituting a network of reactions rather than a linear pathway. Many of the enzymes involved are represented by numerous isoforms. The chloroplastic starch degradation pathway is summarized and illustrated in Fig. 1 with the different *A. thaliana* isoforms implicated listed in Table 4 and the isoforms from other species in Table 5.

As it is generally known, starch is a water-insoluble polymer whose surface is inaccessible to most enzymes; thus, the starch granule surface reversible

Table 2 Arabidopsis proteins involved in starch synthesis and coding loci

Name	Locus	Localization/mutant phenotype	References
Glc6P < = > Glc1P			
PGM1	At5g51820	Plastidial, mutant near-starchless	Caspar et al. (1985), Streb et al. (2009)
PGM2	At1g70730	Cytosolic; functionally redundant with PGM3	Egli et al. (2010)
PGM3	At1g23190	Cytosolic; functionally redundant with PGM2	Egli et al. (2010)
Glc1P + ATP < = > ADPGlc + PPi			
ADG1	At5g48300	Plastidial/major catalytic subunit, (m) low starch phenotype	Lin et al. (1988a)
APS2	At1g05610	Plastidial/likely nonfunctional	Crevillen et al. (2003)
ADG2	At5g19220	Plastidial, major regulatory subunit in leaf/ catalytic, (m) low starch phenotype	Lin et al. (1988b)
APL	At1g27680	Plastidial, minor regulatory subunit in leaf/ catalytic	Ventriglia et al. (2008)
APL3	At4g39210	Plastidial, regulatory subunit in root,	Ventriglia et al. (2008)
APL4	At2g21590	Plastidial, regulatory subunit in root,	Ventriglia et al. (2008)
(1,4-α-D-Glc) (n) + ADPGlc = > (1,4-α-D-Glc)(n + 1) + ADP			
GBSS	At1g32900	Plastidial, mutants lacks amylose	Yoo and Jane (2002), Deschamps et al. (2008a, b)
SSI	At5g24300	Plastidial, amylopectin have decreased amount of DP8-12 chains	Delvalle et al. (2005), Brust et al. (2014)
SSII	At3g01180	Plastidial, Amylopectin have decreased amount of DP12-18 chains	Zhang et al. (2008)
SS3	At1g11720	Plastidial/ (m) amylopectin have increased amount of DP > 60 chains, starch-excess phenotype, and starch with higher phosphate content	Zhang et al. (2005), Szydlowski et al. (2009)
SS4	At4g18240	Plastidial, in plastoglobules/ (m) present only one large granule	Szydlowski et al. (2009), Roldan et al. (2007), Gamez-Arjona et al. (2014)
Transfers a segment of a (1→4)-α-D-glucan chain to a primary hydroxy group in a similar glucan chain			
BEI	At3g20440	Arrest of embryo development at heart stage	Wang et al. (2010)
BEII	At5g03650	Plastidial, single mutant have no apparent phenotype	Dumez et al. (2006), Brust et al. (2014)
BEIII	At2g36390	Plastidial, single mutant have no apparent phenotype	Dumez et al. (2006), Brust et al. (2014)

m, mutant phenotype

phosphorylation probed to be a crucial process for the successful completion of the breakdown process (Blennow et al. 2002; Kotting et al. 2010; Mahlow et al. 2014).

Glucan phosphorylation is the only known modification of starch that occurs in vivo with steady-state starch phosphate levels varying between species and

Table 3 *Zea mays*, *Triticum aestivum*, *Solanum tuberosum*, and *Oryza sativa* best characterized genes coding for proteins involved in starch synthesis

Name	Specie	Locus	Localization/mutant phenotype	References
Glc6P < = > Glc1P				
PGM1	*Zea mays*	ZMU89341	Cytosolic	Manjunath et al. (1998)
PGM2	*Zea mays*	ZMU89342	Cytosolic	Manjunath et al. (1998)
PGM	*Triticum aestivum*	CAC85913	Cytosolic	Davies et al. (2003)
PGM1	*Solanum tuberosum*	NP_001275333	Cytosolic, alterations in plant morphology and in carbon partitioning between sink and source organs	Fernie et al. (2002)
PGM2	*Solanum tuberosum*	NP_001275281	Plastidial/(m) near-starchless	Tauberger et al. (2000)
PGM	*Oryza sativa*	Os10g0189100	Plastidial	Tanaka et al. (2008)
PGM2	*Oryza sativa*	Os03g0712700	Cytosolic	Tanaka et al. (2008)
Glc1P + ATP < = > ADPGlc + PPi				
Bt2	*Zea mays*	NP_001105038	Bt2a cytosolic–Bt2b plastidial, endosperm/(m) starch-deficient kernel, (OE) enhanced seed weight, and starch content	Teas and Teas (1953), Bae et al. (1990), Hannah et al. (2001)
AGPSEMZM	*Zea mays*	NP_001105178	Plastidial, embryo and endosperm/(m) 50 % decreased starch content	Hannah et al. (2001), Huang et al. (2014)
AGPSLZM	*Zea mays*	DAA49237	Plastidial, leaves/(m) reduced starch content	Prioul et al. (1994), Hannah et al. (2001), Slewinski et al. (2008)
Sh2	*Zea mays*	DAA58443	Cytosolic, endosperm/(m) starch-deficient kernel; (OE) enhanced seed weight and starch content	Tsai and Nelson (1966), Bhave et al. (1990)
AGPLEMZM	*Zea mays*	NP_001105717	Plastidial, leaves and embryo	Giroux and Hannah (1994), Giroux et al. (1995)
AGPL3	*Zea mays*	DAA60137	Plastidial, low transcript level	Yan et al. (2009b)

(*continued*)

Table 3 (continued)

Name	Specie	Locus	Localization/mutant phenotype	References
AGPLLZM	*Zea mays*	NP_001106017	Plastidial, leaves and endosperm/(m) reduced starch content	Yan et al. (2009b), Huang et al. (2014)
OsAGPS1	*Oryza sativa*	Os09g12660	Plastidial, endosperm	Lee et al. (2007)
OsAGPS2a	*Oryza sativa*	Os08g25734	Plastidial, leaves/(m) reduced transitory starch content	Lee et al. (2007)
OsAGPS2b	*Oryza sativa*	Os08g25734	Cytosolic, endosperm/(m) reduced starch biosynthesis in seeds	Lee et al. (2007)
OsAGPL1	*Oryza sativa*	Os05g50380	Plastidial, endosperm	Lee et al. (2007)
OsAGPL2	*Oryza sativa*	Os01g44220	Cytosolic, endosperm/(m) reduced starch biosynthesis in seeds	Lee et al. (2007)
OsAGPL3	*Oryza sativa*	Os03g52460	Plastidial, leaves	Lee et al. (2007)
OsAGPL4	*Oryza sativa*	Os07g13980	Plastidial, leaves and endosperm	Lee et al. (2007)
(1,4-α-D-Glc)(n) + ADPGlc = > (1,4-α-D-Glc)(n + 1) + ADP				
GBSSI	*Zea mays*	AY109531	Plastidial, amylose-free endosperm and pollen grains	Vrinten and Nakamura (2000)
GBSSI	*Oryza sativa*	AB425323	Amylose-free endosperm	Sano (1984)
GBSSI	*Triticum aestivum*	X03935	Amylose-free endosperm	Nakamura et al. (1995)
GBSSIIa	*Zea mays*	EF471312	Plastidial	Yan et al. (2009b)
GBSSIIb	*Zea mays*	EF472248	Plastidial	Yan et al. (2009b)
SSI	*Zea mays*	AF036891	Plastidial	Knight et al. (1998)
SSI	*Oryza sativa*	AY299404	Plastidial, amylopectin have decreased amount of DP8–12 chains	Bertoft (1991), Fujita et al. (2006)
SSI	*Triticum aestivum*	AF091803	Plastidial, decrease chains of DP8–12 in amylopectin	McMaugh et al. (2014)
SSIIa	*Zea mays*	AF019296	Increased content of amylose starch and decrease chains of DP13–25 in amylopectin	Zhang et al. (2004), Yan et al. (2009a, b)

(*continued*)

Table 3 (continued)

Name	Specie	Locus	Localization/mutant phenotype	References
SSIIb	*Zea mays*	AF019297	Plastidial	Hennen-Bierwagen and Myers (2013)
SSIIc	*Zea mays*	EU284113	Plastidial	Yan et al. (2009a, b)
SSIIa	*Oryza sativa*	AF419099	Higher proportion of short chains within clusters observed in amylopectin	Umemoto et al. (2002)
SSIIb	*Oryza sativa*	AF395537	Plastidial	Yan et al. (2009b)
SSIIc	*Oryza sativa*	AF383878	Plastidial	Yan et al. (2009b)
SSIIa	*Triticum aestivum*	CAB86618	Increased content of amylose starch and decrease chains of DP13–25 in amylopectin	Yan et al. (2009b), Yamamori et al. (2000)
SSIIb	*Triticum aestivum*	AB201446	Plastidial	Li et al. (1999)
SSIIc	*Triticum aestivum*	AB201447	Plastidial	Yan et al. (2009b)
SS3a(du1)	*Zea mays*	AAC14014	Plastidial/(m) increased amylose content, reduced proportion of long amylopectin chains, SSI activity increased	Wang et al. (1993a, b), Gao et al. (1998), Cao et al. (1999)
SS3b1	*Zea mays*	ABP35815		Valdez et al. (2011)
SS3b2	*Zea mays*	ABP35816		Valdez et al. (2011)
SS3	*Solanum tuberosum*	NP_001274802	Plastidial/(m) reduced amylopectin synthesis short length of amylopectin chains (DP25-35) (m) altered granule morphology	Edwards et al. (1999) Abel et al. (1996), Marshall et al. (1996)
SS3-1	*Oryza sativa*	AF432915	Plastidial, mainly expressed in leaves	Dian et al. (2005)
SS3-2	*Oryza sativa*	AY100469	Plastidial, endosperm/ (m) high amylose content, decreased long chains of amylopectin, and altered granules morphology. Increased SSI and GBSSI levels	Dian et al. (2005) Fujita et al. (2007)

(*continued*)

Table 3 (continued)

Name	Specie	Locus	Localization/mutant phenotype	References
SS3	*Triticum aestivum*	AAF8799	Plastidial, endosperm	Li et al. (2000)
SS4	*Zea mays*	NP_001123603		Schnable et al. (2009)
SS4a	*Oryza sativa*	AY373257	Plastidial, mainly expressed in endosperm	Dian et al. (2005)
SS4b	*Oryza sativa*	AY373258	Plastidial, mainly expressed in leaves	Dian et al. (2005)
Transfers a segment of a (1- > 4)-α-D-glucan chain to a primary hydroxy group in a similar glucan chain				
BEI	*Zea mays*	ZMU17897	No apparent phenotype	Jeon et al. (2010)
BEI	*Oryza sativa*	EF122471	Amylopectin have decreased amount of DP12-21 chains	Jeon et al. (2010)
BEI	*Triticum aestivum*	AF286317	Plastidial	Regina et al. (2005)
BEIIa	*Zea mays*	ZMU65948	Slight reduction in the short-chain content of leaf starch	Jeon et al. (2010)
BEIIb	*Zea mays*	AF072725	High amylose starch	Jeon et al. (2010)
BEIIa	*Oryza sativa*	AB023498	Slight reduction in the short chain content of rice endosperm starch	Jeon et al. (2010), Sawada et al. (2014)
BEIIb	*Oryza sativa*	D16201	Amylopectin have decreased amount of DP8–12 chains	Nishi et al. (2001), Sawada et al. (2014)
BEIIa	*Triticum aestivum*	AF338432	Increase in amylose content	Botticella et al. (2011)
BEIIb	*Triticum aestivum*	AY740401	Plastidial	Regina et al. (2005)

m, mutant phenotype; OE, overexpression phenotype

tissues (Blennow et al. 2000; Kotting et al. 2009). The levels of starch phosphate reported varied from a relatively high amounts in potato (*Solanum tuberosum*) tuber starch (3.4–5.8 nmol phosphate per μmol glucose) and intermediate levels in *A. thaliana* (0.8–1.4 nmol phosphate per μmol glucose) to almost undetectable amounts in cereal starches.

Phosphorylation of superficial glucosyl moieties at their C6 position by glucan, water dikinase (GWD) is the essential signal that triggers starch catabolism (Ritte et al. 2006; Mahlow et al. 2014). Subsequently, C6 phosphorylation triggers phosphorylation in position C3 through the activity of phosphoglucan, water

Table 4 Arabidopsis proteins involved in starch degradation and coding loci

Name	Locus	Localization/phenotype	References
Glucan phosphorylating enzyme – 1,4 α-D-Glc + H2O + ATP → phospho-1,4-α- D-Glc + AMP + Pi			
GWD1	At1g10760	Chloroplastic. KO/KD: *sex*, strongly reduced starch phosphorylation, plants compromised in growth	Yu et al. (2001a), Ritte et al. (2002), Baunsgaard et al. (2005), Kotting et al. (2005), Ritte et al. (2006)
PWD1	At5g26570	Chloroplastic. KO/KD: *sex*, reduced level of C3 phosphorylation of glucosyl residues, minor effect on plant development	Baunsgaard et al. (2005), Kotting et al. (2005)
GWD2	At4g24450	Cytosolic. KO: growth and starch/ sugar levels similar to the wild type	Glaring et al. (2007)
Phosphoglucan phosphatase – removes a phosphate group from phosphoglucosyl residues within amylopectin			
SEX4	At3g52180	Chloroplastic. KO: *sex*, leaf accumulation of linear phosphorylated oligosaccharide, stunted plants	Zeeman et al. (1998), Kotting et al. (2009), Hejazi et al. (2010)
LSF1	At3g01510	Chloroplastic. KO: *sex*, comparable growth and rosette morphology to wild-type plants	Comparot-Moss et al. (2010), Silver et al. (2014)
LSF2	At3g10940	Chloroplastic. KO: normal starch levels, increased starch phosphorylation of glucosyl residues at C3 position	Santelia et al. (2011)
β-amylase – from the nonreducing end, catalyzes the hydrolysis of the second α(1,4)-glycosidil-bond cleaving off maltose			
BAM1	At3g23920	Chloroplastic. KO: reduced total beta-amylase activity	Sparla et al. (2006), Fulton et al. (2008)
BAM2	At4g00490	Chloroplastic. KO: not distinguishable from wild-type plants	Fulton et al. (2008)
BAM3	At4g17090	Chloroplastic. KO: *sex*, lower nighttime maltose levels. Reduced total beta-amylase activity	Fulton et al. (2008), Lao et al. (1999), Kaplan and Guy (2005).
BAM4	At5g55700	Chloroplastic. Inactive. KO: *sex*	Fulton et al. (2008)
BAM5	At4g15210	Cytosolic. KO: almost complete loss of beta-amylase activity	Laby et al. (2001)
BAM6	At2g32290	Cytosolic	Smith et al. (2004)
BAM7	At2g45880	Nuclear. BZR1-BAM transcription factor	Reinhold et al. (2011)
BAM8	At5g45300	Nuclear. BZR1-BAM transcription factor	Reinhold et al. (2011)
BAM9	At5g18670	Cytosolic	Chandler et al. (2001)

(*continued*)

Table 4 (continued)

Name	Locus	Localization/phenotype	References
α-amylase – hydrolyzates α-1,4 linkages at random location yielding glucose, maltose, maltotriose, or limit dextrin			
AMY1	At4g25000	Secreted from cell. KO: starch degradation is not altered	Yu et al. (2005), Doyle et al. (2007)
AMY2	At1g76130	KO: starch degradation is not altered	Yu et al. (2005)
AMY3	At1g69830	Chloroplastic. KO: starch degradation is not altered	Yu et al. (2005), Streb et al. (2012)
α-1,6-glucosidase/starch debranching enzyme – acts on α-1,6 linkages releasing oligosaccharides			
ISA3	At4g09020	Chloroplastic. KO: sex. Increased abundance of short chains in amylopectin. Slower rate of starch breakdown than wild-type plants	Wattebled et al. (2005), Delatte et al. (2006)
LDA	At5g04360	Plastidic, KO: indistinguishable from the wild type	Delatte et al. (2006)
α-Glucan phosphorylase – ((**1,4**)-**α-D-glucosyl**)(**n**) + **Pi** ↔ ((**1,4**)-**α-D-glucosyl**)(**n-1**) + **α-D-glucose 1-phosphate**			
PHS1	At3g29320	Chloroplastic. KO: starch degradation is not altered	Zeeman et al. (2004)
PHS2	At3g46970	Cytosolic. KO: Increase in nighttime maltose	Lu et al. (2006b)
4-α-glucanotransferase/disproportionating enzyme – transfers glucose/ α-1,4-linked glucan moiety from a donor glucan to an acceptor releasing the nonreducing end glucose/glucan moiety (**reversible**)			
DPE1	At5g64860	Plastidial. KO: sex. Large accumulation of malto-Oligosaccharides, increased amylose to amylopectin ratio, lower amounts of starch synthesized and degraded	Critchley et al. (2001)
DPE2	At2g40840	Cytosolic. KO: maltose excess in leaves. Increased maltose moved from the shoots to the roots	Lu et al. (2006a)

KO knockout, *KD* knockdown, *OE* overexpression

dikinase (PWD) (Ritte et al. 2006). C6 phosphorylation overlaps the non-phosphorylated structure of the amylopectin structure, but C3 phosphorylation imposes a marked steric effect that leads to a conformational shift. C6 phosphorylation by GWD, then, promotes the local hydration of the crystalline lamella and C3 phosphorylation by PWD induces a deformation of the helix. This deformation renders the substrate more accessible to the glucan hydrolytic enzymes.

Both starch-phosphorylating enzymes are effectively the first step on the degradation pathway and so they are attractive candidates for the control of flux through

Table 5 *Solanum tuberosum*, *Oryza sativa*, *Solanum lycopersicum*, *Triticum aestivum*, and *Hordeum vulgare* proteins involved in starch degradation and coding loci

Name	Specie	Locus	Localization/phenotype	References
Glucan phosphorylating enzyme – 1,4 α-D-Glc + H2O + ATP → phospho-1,4-α- D-Glc + AMP + Pi				
StGWD	*Solanum tuberosum*	AFH8838	Plastidic; KD: *sex*, drastically reduced phosphate content in leaf and tuber starches	Lorberth et al. (1998)
OsGWD1	*Oryza sativa*	Os06g0498400	KO: *sex* in leaves but limited effects on vegetative growth, 20–40 % grain yield reduction	Hirose et al. (2013)
LeGWD	*Solanum lycopersicum*	ACG69788	KO: sex in leaves, pollen germination arrest, and male sterility	Nashilevitz et al. (2009)
GWD	*Triticum aestivum*	ADG27838	KD: decrease in starch phosphate content and increase in grain size and vegetative biomass	Ral et al. (2012)
Phosphoglucan phosphatase – removes a phosphate group from phosphoglucosyl residues within amylopectin				
HvSEX4	*Hordeum vulgare*	AID16302	Chloroplastic	Ma et al. (2014)
β-amylase – from the nonreducing end, catalyzes the hydrolysis of the second α(1,4)-glycosidil-bond cleaving off maltose				
PCT-BMY1	*Solanum tuberosum*	NP_001275172	KD: *sex* in leaves	Scheidig et al. (2002)
Os BAM 1-9	*Oryza sativa*	–	OsBAM2 and OsBAM3 are plastid-targeted active β-amylase	Hirano et al. (2011)
α-amylase – hydrolyzates α-1,4 linkages at random location yielding glucose, maltose, maltotriose, or limit dextrin				
OsAMY1-1	*Oryza sativa*	P17654	Chloroplastic. KO: sex. seed germination and seedling growth markedly delayed	Huang et al. (1990) Asatsuma et al. (2005)
TaAMY3	*Triticum aestivum*		Plastidic. OE: minimal effect on the overall grain weight or the starch content	Barrero et al. (2013), Whan et al. (2014)
α-1,6-glucosidase/Starch debranching enzyme – Acts on α-1,6 linkages releasing oligosaccharides				
StISA3	*Solanum tuberosum*	AAN15319	ISA-type DBE. KO: sex in leaves	Hussain et al. (2003)
OsISA3	*Oryza sativa*	AEV92948	ISA-type DBE. Plastidial. KO: sex	Yun et al. (2011)

(*continued*)

Table 5 (continued)

Name	Specie	Locus	Localization/phenotype	References
ZPU1	*Zea mays*	O81638	Pleomorphic plastids and starch granules LD/PUL-type DBE. KO: *sex* in leaves Decreased rate of starch degradation Decreased rate of cotyledon growth	Beatty et al. (1999), Dinges et al. (2003)
HvLD	*Hordeum vulgare*	O48541	LD/PUL-type DBE Amyloplastic in aleurone cells	Kristensen et al. (1999), Vester-Christensen et al. (2010)
α-Glucan phosphorylase – ((**1,4**)-**α**-D-**glucosyl**)(**n**) + **Pi** ↔ ((**1,4**)-**α**-D-**glucosyl**)(**n-1**) + **α**-D-**glucose 1-phosphate**				
HvPHO1	*Hordeum vulgare*	AFP19106	Plastidic. KD: not lead to any visible phenotype, no dramatic alterations in the structure of the starch	Ma et al. (2013)
HvPHO2	*Hordeum vulgare*	ACV72276	Cytosolic	Ma et al. (2013)
SP	*Zea mays*	–	Plastidic	Mu et al. (2001), Yu et al. (2001b)
TaPHO1	*Triticum aestivum*	ACC5920	Plastidic	Tickle et al. (2009)
4-α-glucanotransferase/disproportionating enzyme – **transfers glucose/ α-1,4-linked glucan moiety from a donor glucan to an acceptor releasing the nonreducing end glucose/glucan moiety** (**reversible**)				
StDPE1	*Solanum tuberosum*		KO: slightly *sex*	Takaha et al. (1993), Lutken et al. (2010)
StDPE2	*Solanum tuberosum*		Cytosolic. KO: large amounts of maltose and starch	Lutken et al. (2010)

KO knockout, *KD* knockdown, *OE* overexpression

starch degradation. Both enzymes and their expression profile possess properties consistent with modulation of their activities over the day/night cycle (i.e., redox regulation and transcript levels shows large daily changes) (Yu et al. 2001a; Smith et al. 2004; Baunsgaard et al. 2005; Kotting et al. 2005). However, recent work presented begun to tackle this point by establishing that translational control and redox activation are of limited relevance for the control of starch turnover in *Arabidopsis* leaves (Skeffington et al. 2014).

It is worth mentioning that for an efficient digestion of the starch molecule, a glucan phosphatase activity is required to avoid the accumulation of either phosphorylated starch or phosphorylated intermediates from starch breakage (Kotting et al. 2009). This role is played mainly by the starch-excess 4 (SEX4) glucan phosphatase. Primary structure of SEX4 includes an N-terminal chloroplast transit peptide, a dual-specificity phosphatase domain (DSP), and a carbohydrate-binding module (CBM) belonging to the family 48 group (Kotting et al. 2009; Vander Kooi et al. 2010). The glucan-free SEX4 crystal structure indicated that its CBM and DSP domains interact to form binding pocket that carry out two functions: glucan binding and dephosphorylation (Vander Kooi et al. 2010). SEX4 mutations lead to an important increase of starch accumulation in *A. thaliana* leaves due to a decrease in the degradation rate and an increase in the levels of soluble phospho-oligosaccharides (Zeeman et al. 1998; Sokolov et al. 2006; Kotting et al. 2009). Besides catalyzing the dephosphorylation of amylopectin, recombinant SEX4 is able to dephosphorylate crystalline maltodextrins (Hejazi et al. 2010), starch granules isolated from *A. thaliana* leaves and phospho-oligosaccharides (Kotting et al. 2009). While phosphorylation at positions C6 and C3 is performed by two different dikinases, SEX4 is able to dephosphorylate both positions but preferentially acts on C6 position (Kotting et al. 2009; Hejazi et al. 2010). Recently, the phosphoglucan-bound crystal structure of SEX4 was resolved and the authors described its possible catalytic mechanism. In addition, the identification of two DSP domain residues involved in SEX4 site-specific activity leads to the construction of a SEX4 mutant that reversed the specificity from the C6 to the C3 position (Meekins et al. 2014).

Two SEX4-homolog enzymes have also been characterized in Arabidopsis and named as LSF1 (for Like SEX4 1)and LSF2 (for Like SEX4 2) (Comparot-Moss et al. 2010; Santelia et al. 2011). Although plants lacking LSF2 possess normal levels of starch, *lsf2/sex4* double mutants show a more severe starch-excess phenotype respect to *sex4* alone, suggesting that LSF2 has a partial overlapping function in starch dephosphorylation. Indeed, LSF2 exclusively dephosphorylate the C3 position of amylopectin in vitro (Santelia et al. 2011); however, unlike other starch-binding enzymes, including SEX4, LSF2 does not possess a CBM. Instead, LSF2 possesses a unique DSP active site which has a dual function: a glucan binding site and phosphatase activity (Meekins et al. 2013).

LSF1 is also a chloroplast enzyme needed for efficient starch degradation: lsf1 mutants, like sex4 mutants, contain more quantities of starch in their leaves than wild type (wt) plants during the day (Comparot-Moss et al. 2010). However, genetic and transcription profile analyses as well as biochemistry studies indicate that its function would be different from SEX4 since it is mentioned as an inactive phosphatase although it shares similar primary structure to SEX4 and contains all the essential residues required for catalysis (Comparot-Moss et al. 2010). Thereby, LSF1 functions in starch metabolism, although its precise role is unknown; however, it is possible that LSF1 may recruit starch degradative enzymes to the granule surface.

As a consequence, during starch degradation, the granule surface is destabilized by phosphorylation and then simultaneously degraded by glucan hydrolytic enzymes and dephosphorylated by SEX4 and LSF2 (Stitt and Zeeman 2012). Precisely how GWD, PWD, SEX4, and LSF2 might be regulated to limit the ATP consumption of the system while permitting an appropriate flux through the starch degradation pathway remains to be discovered.

Among the hydrolytic enzymes we found β-amylases, α-amylases, debranching enzymes, disproportionating enzymes, and α-glucan phosphorylases. The five enzymes play a concerted role in the liberation and metabolism of malto-oligosaccharides in the stroma, being β-amylases and debranching enzymes the most important.

β-amylases (BAM) are exoamylases that catalyze the hydrolysis of α1-4 D-glucosidic linkages in polysaccharides, successively removing maltose units from the nonreducing ends of the chains. BAMs cannot hydrolyze nor act close to α-1,6 branch points neither can act over phosphorylated glucans. That is the reason why dephosphorylating enzymes (discussed above) as well as debranching enzymes (DBEs, discussed later) are critical for correct starch degradation. Plant genomes encode multiple β-amylase-like proteins. In Arabidopsis, there are nine genes, which have been designated BAM1 to BAM9 to provide a unifying nomenclature (Smith et al. 2004). Of all such genes, only BAM1 and BAM3 have been directly implicated in starch degradation playing a central role in the breakdown of leaf starch (Kaplan and Guy 2005). The loss of BAM2 has little effect in all genetic backgrounds assayed and no discernible function has been assigned yet (Fulton et al. 2008). Other data suggest that BAM4 facilitates or regulates starch breakdown and operates independently of BAM1 and BAM3 revealing unexpected complexity in terms of the specialization of protein function within the β-amylase gene family (Fulton et al. 2008; Li et al. 2009; Francisco et al. 2010). The complexity of β-amylase gene family was more recently exemplified by the finding of BAM7 and BAM8 as nuclear β-amylases involved in transcriptional activation (Soyk et al. 2014).

DBEs, also called isoamylases (ISAs), hydrolyze α1-6 D-glucosidic branch linkages in amylopectin and their β-limit dextrins releasing oligosaccharides into the stroma. There are two classes of DBE in higher plants, and they are referred to as pullulanase-type DBE (PUL) and isoamylase-type DBE (ISA) (Ball et al. 1996; Beatty et al. 1999). Four genes coding for DBE proteins are conserved in higher plants, one for a PUL protein and three for ISA proteins, designated as ISA1, ISA2, and ISA3 (Deschamps et al. 2008a, b). They play a dual role in the synthesis and degradation of starch in higher plants. ISA1 and ISA2 proteins exist together in a heteromeric complex, function together, and are strongly implicated in amylopectin synthesis (Fujita et al. 1999; Hussain et al. 2003; Bustos et al. 2004; Delatte et al. 2005; Utsumi and Nakamura 2006; Takashima et al. 2007; Zeeman et al. 2010). On the other hand, PU1 and ISA3 are not strongly associated within a multimer and may function as a monomer, both preferring substrates with short outer chains, such as β-limit dextrins, suggesting that their role is primarily in starch degradation. Consistently, mutating these

genes in Arabidopsis causes a sex phenotype (Delatte et al. 2006; Streb et al. 2008; Wattebled et al. 2008).

Although BAM3 and ISA3, the major enzymes that hydrolyze the α-1,4 and 1,6 linkages of the Glc polymers that constitute the starch granule, are inhibited by oxidation in vitro (Glaring et al. 2012), there is no evidence about the importance of these proteins on the modulation or control of starch degradation in vivo.

The starch degradative scenario is completed by α-amylases (an endoamylase that hydrolyze α 1–4 D-glucosidic linkages in polysaccharides containing three or more α-1,4-linked D-glucose units), α-glucan phosphorylases (act on the nonreducing end of α-1,4-linked glucose giving Glc P using polysaccharide and phosphate as substrate), and the disproportionating enzymes.

Alpha-amylases are endo-hydrolases belonging to the GH13 family (Majzlova et al. 2013). The roles and number of isoforms vary across the plant kingdom. In Arabidopsis, three isoforms have been described: AtAMY1, AtAMY2, and AtAMY3. AtAMY3 is the only plastidic alpha-amylase, whereas AtAMY1 and AtAMY2 do not have any chloroplast targeting peptide (Yu et al. 2005).

The importance of AMY3 in transitory starch catabolism was demonstrated when AMY3 knockout mutants were combined with knockout ones of debranching enzyme and/or limit dextrinase-deficient lines. AMY3 could participate in starch breakdown in wild type leaves; however, other enzymes can compensate its deficiency. In addition, neither of the three AMY proteins are required for normal rates of starch degradation, and starch degradation is normal even when all three AMY genes are disrupted (Yu et al. 2005). Recently, Streb et al. (2012) suggested that AMY3 acts on starch granules leading to short branched glucans within the chloroplast stroma. Thus, these glucans undergo a rapid conversion into maltose and glucose by debranching enzymes (Streb et al. 2012).

In Arabidopsis, α-amylase does not have a major role in starch degradation. However, in germinating cereal seeds, different amylase isoforms are secreted from the aleurone cells and play an important role in the degradation of storage starch in the nonliving starchy endosperm (Asatsuma et al. 2005; Whan et al. 2014).

Another enzyme involved in starch breakdown is α-glucan phosphorylase (PHS). This is the only phosphorolytic enzyme involved in starch metabolism and catalyzes a reversible glucosyl transfer reaction either in the direction of glucan synthesis from Glc1P in the presence of glucan primer liberating orthophosphate (Pi) or, if Pi is abundant, in the direction of glucan phosphorolysis forming Glc1P. In both cases, the nonreducing end(s) of the glucan primer act(s) as glucosyl acceptor and donor, respectively.

The metabolic function of PHS has been a great deal of debate. One widely accepted idea, according to which the physiological function of PHS1 is to degrade rather than to synthesize glucans, is based on the observations that PHS1 has a low affinity for Glc1P and that low levels of Glc1P and high levels of inorganic phosphate are found in plant cells (Kruger and Ap Rees 1983; Preiss and Sivak 1998). On the other hand, it has been demonstrated that PHS homologs are involved in starch biosynthesis in rice endosperm and potato tuber (Satoh et al. 2008; Fettke et al. 2010; Hwang et al. 2010; Nakamura et al. 2012).

In this direction, PHS plays a probed role in the capacity of the leaf lamina to endure a transient water deficit; however, it is not required for the degradation of plant starch: complete loss of the enzyme does not cause a significant change in the overall accumulation of starch during the day or its remobilization at night in healthy plants (Zeeman et al. 2004).

Finally, we have to mention the disproportionating enzymes (DPE). DPE is an α-1,4 glucanotransferase which catalyzes the cleavage of α-1,4 glucosidic bonds of polyglucans (maltotriose or larger), transferring the glucosyl groups to the nonreducing end of another glucan chain or free glucose and releasing either glucose or a glucan chain depending on the cleavage site. The *Arabidopsis* genome encodes two 4-α-glucanotransferase-like proteins: DP1 and DP2. DP1 is a chloroplastic enzyme responsible for the metabolism of the maltotriose or short malto-oligosaccharide products of starch degradation at night, presumably generated by chloroplastic β-amylolysis since DPE1 mutants accumulate maltotriose during starch breakdown at night (Critchley et al. 2001). The other DPE protein, DPE2, is cytosolic (Lutken et al. 2010). Independent mutant lines deficient in DPE2 show a decreased capacity for both starch synthesis and degradation in leaves. Thus, DPE2 is an essential component of the pathway from starch to sucrose and cellular metabolism in leaves at night despite its cytosolic localization. Probably, its role is related with the metabolization of maltose exported from the chloroplast (Chia et al. 2004; Lu and Sharkey 2006).

In summary, the main route of starch degradation in *Arabidopsis* is via GWD and PWD, which introduce some disorder at the starch granule surface, and are followed by the action of the β-amylases, debranching enzymes, and glucan phosphatases (SEX4 and LSF2). These enzymes act progressively and in combination to produce maltose, maltotriose, and longer malto-oligosaccharides (MOS) as the major products of the initial steps of starch degradation. α-amylases, DBE, PHS1, and DPE1 complement starch degradation to produce finally maltose and glucose, the two products of starch breakdown that are exported from the chloroplast to the cytosol via distinctive specific transporters (see Fig. 1). Despite its importance for productivity, we do not yet know how the rate of starch degradation is controlled.

5 Starch-Related Enzymes in *Arabidopsis thaliana* Preserve a Modular Structure

Enzymes that catalyze the reactions of glycosidic bonds share a modular organization that includes a catalytic domain and one or more carbohydrate binding module (CBM) connected by a loosely structured chain (Henrissat and Davies 1997).

Catalytic domains are often classified with an EC number according to the International Union of Biochemistry and Molecular Biology (IUBMB) recommendations and their substrate specificities (Barrett 1995), but this classification may fail to describe structural, evolutionary, and mechanistic similitude between these enzymes (Henrissat and Davies 1997). Sequence similarity classification can take into account these aspects because they are defined by the primary sequence alone

(Henrissat and Davies 2000) and could provide a more descriptive classification. CAZy classification includes these approaches (structural and mechanistic based on sequence similarity) to create a database of all known carbohydrate-associated enzymes. However, this system often grouped enzymes of different substrate specificity into a single “poly-specific” group. Nevertheless, a family can only be defined when one of its members is characterized biochemically (Valdez et al. 2008). Still, catalytic residues could be identified in both position and function for one member of a family, and then, they can easily be inferred for all members of the family (Henrissat and Davies 2000). This classification is available on the CAZy website (http://www.cazy.org) (Lombard et al. 2014).

This common modular structuration is also conserved in numerous *Arabidopsis thaliana* starch metabolism enzymes (Table 6). A batch of starch-associated enzymes were analyzed with the NCBI Conserved Domain-search tools (Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2009, 2011), and the output was organized according to detected domain superfamily. Of the 40 proteins analyzed, we found 19 independent domains, many of them repeated in several degradation and synthesis enzymes.abcdefg

Table 6 shows that some domains tend to be exclusive of degradative (alpha-amyl_C2, CBM20, PPDK_N, PTPc, Glyco_hydro_77 and PDZ) or synthetic (alpha-amylase C, CBM 53, GT-A, LbH, phosphohexomutase, MRP-L20, RAP1, and tropomyosin_1) enzymes and others are present in both types of enzymes (AmyAc_family, CBM 48 and GT-B).

The domains present in the AmyAc_family are the most frequent glycoside hydrolase (GH) domain with the majority of enzymes acting on starch, glycogen, and related oligo- and polysaccharides (Janecek 1997; Janecek et al. 1997) catalyzing the transformation of α-1,4 and α-1,6 glucosidic linkages with retention of the anomeric center. In the *Arabidopsis thaliana* starch metabolic pathway, these modules are present in degradative enzymes such as ß-amylases (BAM1-9), pullulanase, and isoamylase (ISOA) as well as in synthesizing enzymes such as starch branching enzymes (SBE3, SBE2.2, and SBE2.1). These latter proteins also have an alpha/beta-barrel domain present in eight stranded α/ß barrel, interrupted by a calcium-binding domain, and a C-terminal Greek key beta-barrel domain (Strobl et al. 1998).

Alpha-amyl_C2 domains are associated to an O-glycosyl hydrolase (EC 3.2.1.) activity, which are found in the C-terminal end in numerous α-amylases and fold into a five-stranded antiparallel ß-sheet structure (Kadziola et al. 1998). They are actually present in three *A. thaliana* α-amylases (AMY1, AMY2, and AMY3) catalyzing the hydrolysis of α-1,4-glucosidic bonds of starch (Lloyd et al. 2005). The branching enzymes (BEs) catalyze the formation of α-1,6 branch points in either glycogen or starch by cleavage of the α-1,4 glucosidic linkage yielding a more reactive glycogen with an increased number of nonreducing ends.

The CAZy glycoside hydrolase family 77 contains a 4-α-glucanotransferase activity (disproportionating activity) that comprise the transfer of a (1 → 4)- α-D-glucan segment to a new position in an acceptor, which may be glucose or another (1 → 4)-α-D-glucan (Pazur and Okada 1968). This domain is present in the DPE1

Table 6 Summary of domains presents in *Arabidopsis thaliana* starch metabolism enzymes

Protein name	Description	Locus ID	UniProt ID	From	To	Domain	Function
AMY1	Alpha-amylase 1 (AMY1)	At4g25000	Q8VZ56	362	422	Alpha-amyl_C2	Degradation
AMY3	Alpha-amylase 3 (AMY3)	At1g69830	Q94A41	826	885	Alpha-amyl_C2	Degradation
AMY2	Alpha-amylase 2 (AMY2)	At1g76130	Q8LFG1	355	413	Alpha-amyl_C2	Degradation
SBE3 (BE1)	1,4-alpha-glucan-branching enzyme 3	At3g20440	D2WL32	799	895	Alpha-amylase_C	Synthesis
SBE2.2	1,4-alpha-glucan-branching enzyme 2-2	AT5G03650	Q9LZS3	707	801	Alpha-amylase_C	Synthesis
SBE2.1 (BE3)	1,4-alpha-glucan-branching enzyme 2-1	At2g36390	O23647	742	800	Alpha-amylase_C	Synthesis
BAM3	Beta-amylase 3 (BAM3, CT-BMY, BMY8)	At4g17090	O23553	1	548	AmyAc_family	Degradation
BAM4	Beta-amylase 4 (BAM4, BMY6)	At5g55700	Q9FM68	1	531	AmyAc_family	Degradation
BAM6	Beta-amylase 6 (BAM6)	At2g32290	Q8L762	63	573	AmyAc_family	Degradation
BAM5	Beta-amylase 5 (BAM5, BMY1, RAM1)	At4g15210	P25853	1	498	AmyAc_family	Degradation
PULA	Pullulanase 1	At5g04360	Q8GTR4	332	796	AmyAc_family	Degradation
ISOA	Isoamylase 3	At4g09020	Q9M0S5	220	659	AmyAc_family	Degradation
BAM1	Beta-amylase 1 (BAM1, TR-BAMY)	At3g23920	Q9LIR6	106	541	AmyAc_family	Degradation
BAM7	Beta-amylase 7 (BAM7)	At2g45880	O80831	247	678	AmyAc_family	Degradation
BAM2	Beta-amylase 2 (BAM2, BMY9)	At4g00490	O65258	95	525	AmyAc_family	Degradation
BAM8	Beta-amylase 8 (BAM8)	At5g45300	Q9FH80	254	683	AmyAc_family	Degradation
BAM9	Beta-amylase 9 (BAM9, BMY3)	At5g18670	Q8VYW2	77	503	AmyAc_family	Degradation
SBE2.1 (BE3)	1,4-alpha-glucan-branching enzyme 2-1	At2g36390	O23647	309	724	AmyAc_family	Synthesis
SBE2.2	1,4-alpha-glucan-branching enzyme 2-2	AT5G03650	Q9LZS3	274	689	AmyAc_family	Synthesis
SBE3 (BE1)	1,4-alpha-glucan-branching enzyme 3	At3g20440	D2WL32	381	780	AmyAc_family	Synthesis
AMY1	Alpha-amylase 1 (AMY1)	At4g25000	Q8VZ56	28	372	AmyAc_family	Degradation

AMY3	Alpha-amylase 3 (AMY3)	At1g69830	Q94A41	497	836	AmyAc_family	Degradation
AMY2	Alpha-amylase 2 (AMY2)	At1g76130	Q8LFG1	27	365	AmyAc_family	Degradation
SS3	Starch Synthase 3	At1g11720	F4IAG2	328	424	CBM 53	Synthesis
SS3	Starch Synthase 3	At1g11720	F4IAG2	495	584	CBM 53	Synthesis
SS3	Starch Synthase 3	At1g11720	F4IAG2	153	238	CBM 53	Synthesis
DPE2	4-alpha-glucanotransferase	At2g40840	Q8RXD9	20	120	CBM20	Degradation
DPE2	4-alpha-glucanotransferase	At2g40840	Q8RXD9	164	261	CBM20	Degradation
PWD	Phosphoglucan water dikinase (PWD1, GWD3)	At5g26570	Q6ZY51	73	164	CBM20	Degradation
PULA	Pullulanase 1	At5g04360	Q8GTR4	791	963	DUF3372	Degradation
BAM7	Beta-amylase 7 (BAM7)	At2g45880	O80831	65	229	DUF822	Degradation
BAM8	Beta-amylase 8 (BAM8)	At5g45300	Q9FH80	96	245	DUF822	Degradation
ISOA	Isoamylase 3	At4g09020	Q9M0S5	91	240	CBM 48 (E_set)	Degradation
PULA	Pullulanase 1	At5g04360	Q8GTR4	206	307	CBM 48 (E_set)	Degradation
SBE2.2	1,4-alpha-glucan-branching enzyme 2-2	AT5G03650	Q9LZS3	176	270	CBM 48 (E_set)	Synthesis
SBE2.1 (BE3)	1,4-alpha-glucan-branching enzyme 2-1	At2g36390	O23647	212	305	CBM 48 (E_set)	Synthesis
DSP4	Starch-excess 4 (SEX4, DSP4)	At3g52810	Q9FEB5	256	337	CBM 48 (E_set)	Degradation
LSF1	Like Sex four 1 (LSF1)	At3g01510	F4J117	456	536	CBM 48 (E_set)	Degradation
SBE3 (BE1)	1,4-alpha-glucan-branching enzyme 3	At3g20440	D2WL32	118	184	CBM 48 (E_set)	Synthesis
SBE3 (BE1)	1,4-alpha-glucan-branching enzyme 3	At3g20440	D2WL32	334	377	CBM 48 (E_set)	Synthesis
DPE1	4-alpha-glucanotransferase	At5g64860	Q9LV91	56	576	Glyco_hydro_77	Degradation
APL2	Glucose-1-phosphate adenylyltransferase large subunit 2	At1g27680	P55230	88	349	GT-A	Synthesis
APL3	Glucose-1-phosphate adenylyltransferase large subunit 3	At4g39210	P55231	91	352	GT-A	Synthesis

(*continued*)

Table 6 (continued)

Protein name	Description	Locus ID	UniProt ID	From	To	Domain	Function
GLGL	Probable glucose-1-phosphate adenylyltransferase large subunit	At2g21590	Q9SIK1	93	354	GT-A	Synthesis
ADG2	Glucose-1-phosphate adenylyltransferase large subunit 1	At5g19220	P55229	93	353	GT-A	Synthesis
APS1	Glucose-1-phosphate adenylyltransferase small subunit	At5g48300	P55228	101	350	GT-A	Synthesis
ADG2	Glucose-1-phosphate adenylyltransferase large subunit 1	At5g19220	P55229	212	310	GT-A	Synthesis
APS1	Glucose-1-phosphate adenylyltransferase small subunit	At5g48300	P55228	213	271	GT-A	Synthesis
PHS1	Alpha-glucan phosphorylase 1	At3g29320	Q9LIB2	97	956	GT-B	Degradation
PHS2	Alpha-glucan phosphorylase 2	At3g46970	Q9SD76	33	835	GT-B	Degradation
SS1	Starch synthase 1	At5g24300	Q9FNF2	143	643	GT-B	Synthesis
GBSS1	Granule-bound starch synthase 1	At1g32900	Q9MAQ0	86	582	GT-B	Synthesis
SS2	Starch synthase 2	At3g01180	Q9MAC8	302	785	GT-B	Synthesis
SS4	Starch synthase 4	At4g18240	Q0WVX5	544	1026	GT-B	Synthesis
SS3	Starch Synthase 3	At1g11720	F4IAG2	595	1039	GT-B	Synthesis
APS1	Glucose-1-phosphate adenylyltransferase small subunit	At5g48300	P55228	388	514	LbetaH	Synthesis
ADG2	Glucose-1-phosphate adenylyltransferase large subunit 1	At5g19220	P55229	390	516	LbetaH	Synthesis
APL2	Glucose-1-phosphate adenylyltransferase large subunit 2	At1g27680	P55230	386	512	LbetaH	Synthesis
APL3	Glucose-1-phosphate adenylyltransferase large subunit 3	At4g39210	P55231	389	515	LbetaH	Synthesis

GLGL	Probable glucose-1-phosphate adenylyltransferase large subunit	At2g21590	Q9SIK1	391	517	LbetaH	Synthesis
SS3	Starch synthase 3	At1g11720	F4IAG2	254	302	MRP-L20	Synthesis
LSF1	Phosphoglucan phosphatase LSF1	At3g01510	F4J117	76	118	PDZ	Degradation
PGMC	Probable phosphoglucomutase	At1g23190	O49299	7	583	phosphohexomutase	Synthesis
PGMC	Probable phosphoglucomutase	At1g70730	Q9SGC1	9	585	phosphohexomutase	Synthesis
PGMP	Phosphoglucomutase	At5g51820	Q9SCY0	70	623	phosphohexomutase	Synthesis
PWD	Phosphoglucan water dikinase (PWD1, GWD3)	At5g26570	Q6ZY51	870	1193	PPDK_N	Degradation
GWD1	α-glucan water dikinase (GWD1, SEX1)	At1g10760	Q9SAC6	1089	1397	PPDK_N	Degradation
GWD2	α-glucan water dikinase (GWD2)	At4g24450	Q9STV0	970	1276	PPDK_N	Degradation
LSF2	Phosphoglucan phosphatase LSF2	At3g10940	Q9SRK5	100	237	PTPc	Degradation
DSP4	Starch excess 4 (SEX4, DSP4)	At3g52810	Q9FEB5	101	235	PTPc	Degradation
LSF1	Phosphoglucan phosphatase LSF1	At3g01510	F4J117	293	420	PTPc	Degradation
SBE2.1 (BE3)	1,4-alpha-glucan-branching enzyme 2-1	At2g36390	O23647	34	204	RAP1	Synthesis
SS4	Starch synthase 4	At4g18240	Q0WVX5	344	461	Tropomyosin_1	Synthesis

Script curated CD-search output (Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2009; 2011) was used to construct this table. See text for domain description. Domains that do not have a specific function annotated are annotated as "DUF" families (domain of unknown function). Protein name refers to the most common name for a specific protein. "From" and "to" refers to the domain specific position in the amino acidic sequence. "Synthesis" and "degradation" attempt to summarize the specific role of a protein in the starch metabolism, although a protein may be playing a role in both pathways

(disproportionating enzyme) of *Arabidopsis thaliana* and folds into a (β/α) 8-barrel with a strand β4-aspartic acid (catalytic nucleophile), a β5-glutamic acid (proton donor), and a β7-aspartic acid (transition-state stabilizer) critical for the catalytic machinery (Jespersen et al. 1993). At this moment, the three-dimensional structures have been solved for only five members of the family (Cantarel et al. 2009; Levasseur et al. 2013).

Glycosyltransferases (GTs) are enzymes that synthesize oligosaccharides, polysaccharides, and glycoconjugates by transferring the sugar moiety from an activated nucleotide-sugar donor to an acceptor molecule (oligosaccharide, a lipid, or a protein). Based on the stereochemistry of the donor and acceptor molecules, GTs could be classified as either retaining or inverting enzymes (Lairson et al. 2008).

It has been described that glycosyltransferases adopt two different folds, named GT-A and GT-B (Bourne and Henrissat 2001; Breton et al. 2001). The GT-A includes diverse families of glycosyl transferases that folds into two closely neighboring β/α/β Rossmann domains, one for nucleotide binding and the other for the binding of the acceptor molecule (Charnock and Davies 1999).

The GT-B fold is present in other families (GT-3 and GT-5 families) including about glycogen or starch synthases from different organisms (Gomez-Casati et al. 2013). The first protein whose structure was described in this group of GTs was the glycogen synthase from *Agrobacterium tumefaciens* (Buschiazzo et al. 2004). It was reported that the overall fold and the architecture of the active site of the protein are remarkably similar to those of glycogen phosphorylase (Buschiazzo et al. 2004). GTB proteins have distinct N- and C-terminal β/α/β Rossmann domains that face each other and have high structural homology despite minimal sequence homology. The large cleft that separates the two domains includes the catalytic center and permits a high degree of flexibility (Lairson et al. 2008).

In *Arabidopsis thaliana*, those GT-A domains are associated mainly with the starch synthesis Glc1P adenylyltransferase enzymes (APL2, APL3, GLGL, ADG2, APS1, and ADG2). Some of the mentioned GT-A present a left-handed parallel beta-helix (LbetaH or LbH) domain, which is related with enzymes showing as diverse activity as acyltransferase, ion transport, or translation initiation (Raetz and Roderick 1995). In addition, the GT-B domains are associated with both degradative enzymes such as α-glucan phosphorylase (PHS1 and PHS2) and synthesizing enzymes such as starch synthases (SS1, SS2, SS3, SS4, and GBSS1). Interestingly, the *Arabidopsis* SS3 protein presents a domain that resembles the mitochondrial ribosomal protein subunit L20 (MRP-L20) of fungi (Garcia-Cantalejo et al. 1994), but has no assigned function in the *A. thaliana* enzyme.

The PTPc domain superfamily is defined by a dual-specificity phosphatase protein that catalyzes the dephosphorylation of phosphotyrosine peptides and oligosaccharides. This domain is present in *A. thaliana* DSP4 (SEX4), LSF1, and LSF2 and mediates the reversible phosphorylation of starch at the C6 and C3 positions of glucose moieties (Comparot-Moss et al. 2010; Meekins et al. 2014). Starch phosphorylation is the only known natural modification and is the key regulatory mechanism controlling its diurnal breakdown in plant leaves (Meekins et al. 2014). The PDZ domain is present in LSF1 and it is believed that LSF1 may be

responsible for specific protein–protein interactions through this N-terminal beta-strand structure (Harris and Lim 2001).

The phosphoglucan, water dikinase (PWD1) and the α-glucan, water dikinase (GWD1, GWD2) proteins share a domain classified in the PPDK_N superfamily which is mainly characterized by enzymes showing a pyruvate phosphate dikinase, a PEP/pyruvate binding, and a reversible conversion of ATP to AMP activity (Herzberg et al. 1996). However, in these enzymes this domain are implicated in the phosphorylation of starch polymers, being the first step on the pathway of starch degradation in Arabidopsis leaves at night (Skeffington et al. 2014).

Phosphoglucomutase enzymes play an important and diverse role in carbohydrate metabolism in organisms from bacteria to humans. The *A. thaliana* phosphoglucomutase PGMC, PGMC, and PGMP (Table 6) have a phosphohexomutase domain (superfamily) that catalyze a reversible intramolecular phosphoryl transfer of Glc1P and Glc6P via a Glc1,6bisP intermediate (Levin et al. 1999).

Finally, the SBE2.1 and SS4 strikingly present some unusual domains, such as RAP1 (rhoptry-associated protein 1) (RAP-1) and a tropomyosin_1, respectively. The first domain belongs to a family that consists of several rhoptry (parasite secretory organelle)-associated proteins specific to *Plasmodium falciparum* (Moreno et al. 2001), and the second domain (tropomyosin_1) resembles to a tropomyosin-like protein.

Because the starch granule structure exposes very few chain ends, starch-associated enzymes must have a "processive" or "multiple-attack" activity that can perform many hydrolytic events without releasing the polysaccharide chain (Henrissat and Davies 1997). Some proteins are equipped with carbohydrate-binding modules (CBMs), which are able to fulfill this task. A CBM is defined as the contiguous amino acid sequence in an active enzyme involved in carbohydrate metabolism, with the ability to bind carbohydrates (Shoseyov et al. 2006). CBMs contain between 30 and 200 amino acids and may exist as simple, double, or triple repeats in a protein. These domains are located at the N- or C-terminal end of a protein and occasionally can be found in the middle of the polypeptide chain. Currently, more than 300 putative sequences in over 50 different species have been identified and the binding domains have been classified in 71 families, based on their amino acid sequence, substrate binding specificity, and structure (see CAZY http://www.cazy.org) (Boraston et al. 2004; Lombard et al. 2014). By driving the catalytic process through a strong physical association with substrates, CBMs can increase the rate of enzymatic reactions. When CBMs are present in proteins with no hydrolytic activity, they constitute an organizing subunit of catalytic domains generating cohesive multienzymatic complexes that lose enzymatic activity when CBMs are removed from the structure (Shoseyov et al. 2006).

Based on the CAZy classification, we found three carbohydrate-binding modules families, CBM53, CBM20, and CBM48 (E_set). CBM53 and CBM20 which show a starch-binding function (Mikami et al. 1999; Valdez et al. 2008). The CBM48 family is often found in enzymes containing glycosyl hydrolase, family 13, catalytic domains and is mainly described in enzymes that degrade branched substrates as glycogen (Katsuya et al. 1998; Hudson et al. 2003).

Among the CBMs, we can highlight the starch-binding domains (SBD), which have acquired the evolutionary advantage to break the structure of their substrate when compared to the CBD, due to the presence of two binding sites for the polysaccharide (Southall et al. 1999). So far, no CBM 3D structure containing such binding sites has been reported. In view of this, such domains are unable to break down the polysaccharide structure as efficiently as the SBD do (Tormo et al. 1996; Southall et al. 1999).

While SSIII has three in tandem CBM in its N-terminal domain that belongs to the CBM53 family, the degradative enzymes (LSF1, SEX4, as well as the proteins encoded by At5g39790 and At1g27070) have only one CBM in their C-terminal domain belonging to the CBM48 family. It is worth mentioning that there is a clear evolutionary relatedness of CBM48, CBM53, and CBM20. Moreover, the alignment of the amino acid sequences of CBM20 (including some mammalian proteins, such as laforin, involved in the regulation of glycogen metabolism), CBM21, CBM48, and CBM53 has revealed only subtle differences in the polysaccharide-binding sites, showing a high degree of conservation (Christiansen et al. 2009).

6 Concluding Remarks

Starch is the most important higher plant storage carbohydrate and is made up of the glucose long chains amylose and amylopectin. Plants use starch as an energy source during the night, when leaves cannot generate sugars by photosynthesis. The polysaccharide forms complex semicrystalline structures called starch granules which accumulate within the plastids. Its metabolism involves the concerted and controlled actions of many enzymes. In most cases, multiple genes encode different isoforms of each enzyme, which may have slightly different roles depending on plant species and tissue (Kotting et al. 2010).

We have attempted a comprehensive review of the existing information on the different enzymes involved in *Arabidopsis thaliana* and other species of agronomic interest. From our point of view, to address biotechnologically starch metabolism is essential to expand the knowledge of the enzymes involved in this process. Tables 2, 3, 4, and 5 brought together the main works on this subject, describing not only the native proteins but also the plant phenotypes obtained by overexpression or deletion of specific genes. Furthermore, previous work was done in vivo where different groups have obtained modified starches (quantity and quality) through the action of different carbohydrate-binding modules of degradative microbial enzymes. Indeed, we also described the existence of such modules in some starch biosynthetic enzymes in plants. Therefore, and considering the modular structure of many enzymes involved in the metabolism of this polysaccharide, we have a section dedicated to review this point.

Recent work has focused on the role of starch as a carbon storage polymer without demonstrated regulatory activities. It is more likely that regulators of starch metabolism or signals derived from starch act as integrators of plant metabolism and growth. Gibon et al. (2009) have demonstrated a strong correlation between

starch turnover, protein content, and biomass when *Arabidopsis thaliana* Col0 plants were grown at different photoperiods (Gibon et al. 2009). Although the biomass was only very weakly correlated with individual metabolites in a recombinant Arabidopsis population, a highly significant prediction was obtained when multivariate analysis was used on the entire metabolite profile. Meyer et al. (2007) confirmed the negative link between biomass and starch (except in very short days). The simplest explanation for this negative relationship would be that large accessions maximize growth at the expense of their carbon reserves. Such a strategy would be an advantage when an excess of carbon is available, but not when carbon is in small amounts (Sulpice et al. 2009).

Moreover, starch represents an important nutrient for humans and animals and a raw material for many industrial applications. For the industry, starch represents a cheap and renewable material, with differential physicochemical properties that make it increasingly exploited in the agricultural and food sectors and in manufacturing processes (Blennow et al. 2002; Delcour et al. 2010). Starch is also used as a feedstock for first generation bioethanol production (e.g., corn, sugar cane, and cassava). The use of major food crops for energy purposes has led to the study of their applications and potential applications to obtain energy from cellulose present in plant cell walls, in addition to the study of different strategies to increase the levels of starch in plants as well as the production of starches with new attributes, in accordance with the present industrial requirements (Santelia and Zeeman 2011).

Finally, the importance of a variety of renewable biofuels has been renovated due to the increase of petroleum fuel costs and the consequences resulting from greenhouse emissions (Cheng et al. 2011). This has enhanced the interest in the study of photosynthetic algae (microalgae and macroalgae) as a possible biofuel resource because some algae species have a higher rate of biomass production compared to terrestrial plants (Dismukes et al. 2008). Graham et al. (2000) have postulated that land plants evolved from green algae belonging to the Charophyceae (Graham et al. 2000). Starch biosynthesis is unique to the Archaeplastida supergroup, comprising Chloroplastida (green algae and land plants), Rhodophyceae (red algae), and a minor group called the glaucophytes. Differences in the starch biosynthetic pathways between the archaeplastidal lineages have arisen during subsequent evolution whose starting point is the ancestral bacteria capable of synthesizing glycogen (Zeeman et al. 2010). Most notably, in green plants, starch is synthesized in the plastid compartment, whereas in red algae and in glaucophytes, its synthesis occurs in the cytosol (Shimonaga et al. 2008). Plant genomes are usually large and complex, having gene redundancy, duplications, and transposable elements among other features (Derelle et al. 2006; Armisen et al. 2008; Plancke et al. 2008). Interestingly, in the last years, several nuclear and organelle algae genomes have been sequenced (Busi et al. 2014). Because these genomes have proven to be simple, and due to the genomic, molecular, and physiological characteristics of these organisms, the unicellular green algae have become highly suitable for the study of numerous biological processes related with starch metabolism, and thus, these studies could help clarify the unknown mechanisms of starch synthesis in higher plants.

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