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REVIEW

Structure, function, and evolution of plant ADP-glucose pyrophosphorylase

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Abstract

Key message **This review outlines research performed in the last two decades on the structural, kinetic,regulatory and evolutionary aspects of ADP-glucose pyrophosphorylase, the regulatory enzymefor starch biosynthesis.**

Abstract ADP-glucose pyrophosphorylase (ADP-Glc PPase) catalyzes the first committed step in the pathway of glycogen and starch synthesis in bacteria and plants, respectively. Plant ADP-Glc PPase is a heterotetramer allosterically regulated by metabolites and post-translational modifications. In this review, we focus on the three-dimensional structure of the plant enzyme, the amino acids that bind the regulatory molecules, and the regions involved in transmitting the allosteric signal to the catalytic site. We provide a model for the evolution of the small and large subunits, which produce heterotetramers with distinct catalytic and regulatory properties. Additionally, we review the various post-translational modifications observed in ADP-Glc PPases from different species and tissues. Finally, we discuss the subcellular localization of the enzyme found in grain endosperm from grasses, such as maize and rice. Overall, this work brings together research performed in the last two decades to better understand the multiple mechanisms involved in the regulation of ADP-Glc PPase. The rational modification of this enzyme could improve the yield and resilience of economically important crops, which is particularly important in the current scenario of climate change and food shortage.

Keywords Allosteric regulation · Enzyme evolution · Post-translational regulation · Redox regulation · Subfunctionalization · Nucleotide-sugar synthesis

Starch, a main reserve polysaccharide in plants

Living organisms synthesize polyglucans as a strategy to store carbon and energy. The structure and intracellular location of the polysaccharide vary in organisms with different trophic and evolutionary characteristics (Gould et al. 2008; Suzuki and Suzuki 2013). For the scope of this review, it is worth considering the common scenario taking place in prokaryotes and photosynthetic eukaryotes grouped as Chlorophytes and higher plants, which use ADP-glucose (ADP-Glc) as a key intermediate to synthesize branched α-1,4 (α-1,6) polyglucans as reserve molecules. All microorganisms including Archaebacteria and Eubacteria, either heterotrophic or autotrophic (chemolithotrophic as well as photosynthetic of the type anaerobic and oxygenic, the latter named cyanobacteria) have glycogen as the main carbon storage. On the other hand, members of the Archaeplastida (formerly known as Plantae) kingdom identified as green algae (grouped as Chlorophytes) and higher plants have characteristic plastids accumulating starch as a major product of photosynthesis (Gould et al. 2008; Figueroa et al. 2016).

Starch is a mixture of two polysaccharides, amylose (mostly a linear α-1,4-polyglucan chain with a low number of α-1,6 branches) and amylopectin (α-1,4-polyglucan highly branched with α-1,6 bonds). Starch, with different ratios of

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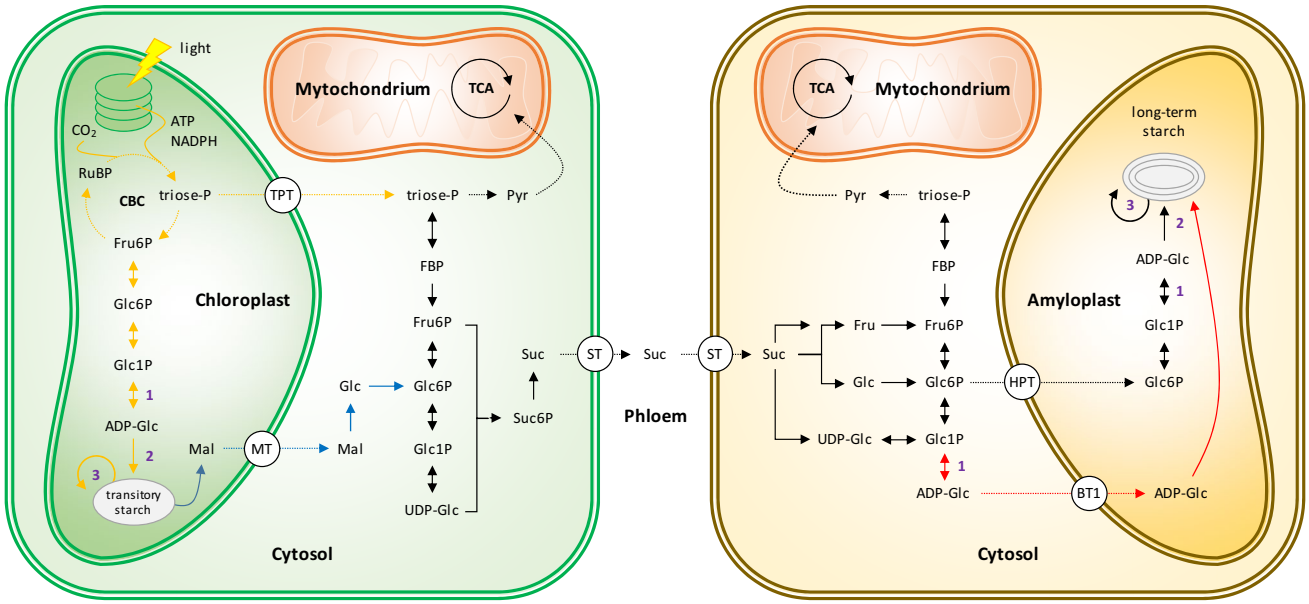

Fig. 1 Simplified scheme of carbon partitioning between photosynthetic and heterotrophic tissues. Green cell (left), photosynthetic tissue; brown cell (right), heterotrophic tissue. Reactions occurring during day and night hours are indicated by yellow and blue arrows, respectively. The pathway of starch synthesis in cereal endosperm is indicated with red arrows. Enzymes discussed in this review are indicated with violet numbers: 1, ADP-Glc pyrophosphorylase; 2, starch synthase; 3, branching enzyme. Dotted lines represent multiple enzymatic steps or transport across membranes. *CBC* Calvin-Benson cycle; *TCA* tricarboxylic acid cycle; *RuBP* ribulose-1,5-bisphosphate; *Pyr* pyruvate; *Suc* sucrose; *Mal* maltose; *TPT* triose-P transporter; *MT* maltose transporter; *ST* sucrose transporter; *HPT* hexose-P transporter; *BT1* ADP-Glc transporter (BRITTLE1). Adapted from Figueroa et al. (2016)

amylose and amylopectin, deposits as granules in plastids of various types of plant tissues and organs: leaves, shoots, roots, grains, fruits, and stems. For a detailed description regarding the chemical and physical properties of starch granules and their components, we recommend two excellent reviews (Ball and Morell 2003; Raguin and Ebenhoh 2017). The polysaccharide plays critical tasks for carbon assimilation, partitioning, and allocation in the different tissues and periods of plant development (MacNeill et al. 2017; Goren et al. 2018). Starch is synthesized in plastids, forming granules, with the semi-crystalline structure of the polymer being distinctive according to the auto- (source) or hetero- (sink) trophic nature of the tissue. The starch found in photosynthetic cells exhibits a structure known as type-B, with a morphology of long and disperse α-glucan chains arranging a hexagonal lattice able to incorporate water molecules. Non-photosynthetic tissues contain type-A starch granules, having shorter α-glucan chains that interact forming double helices, thus packing densely and excluding water. A type-C intermediate structure of the polymer is also found in many plant organs, like fruits and roots. Starch granules accumulated in plastids of plant heterotrophic cells vary in size, shape, and molecular composition (relative contents of amylose and amylopectin, proteins, and phosphate) in the different botanical species (Glaring et al. 2006).

The function and dynamics of starch synthesis/degradation are also different in source or sink tissues (Fig. 1). The polysaccharide is actively synthesized in the chloroplast during the light period in green cells (typically in the leaf), therein serving as a short-time storage of carbon and energy (Goren et al. 2018). During the day, photoassimilated carbon is partitioned between chloroplastic starch and carbon exported from the plastid to assure demands for respiration and primary metabolism, in addition to sucrose synthesis requirements for delivery to heterotrophic cells. At night, the leaf glucan (named transient starch) is degraded to provide carbon and energy to the same cell as well as carbon export to other organs (Fig. 1). Once in the heterotrophic tissue, sucrose is partitioned into different intracellular compartments and metabolic pathways. An example of the latter is the major route of synthesis of starch in the plastid, leading the polymer to constitute a middle or long-term carbon storage molecule (MacNeill et al. 2017; Goren et al. 2018). Typically, in mature seeds of grasses, starch is the predominant reserve compound accumulated for a very long period, specifically until seed germination (Fig. 1).

Biosynthesis of starch in plants

The discovery of nucleoside-diphosphate-sugars by Leloir et al. in the 1950s untied a knot in the elucidation of the metabolic routes for oligo and polysaccharide synthesis in different organisms (Leloir 1971; Figueroa et al. 2021). The pioneering work of Leloir with mammalian cells made clear that catabolism and anabolism of glycogen involve different reactions. Furthermore, it was established that the synthesis of the polyglucan requires UDP-Glc, an activated form of Glc. Shortly after (Ballicora et al. 2004; Figueroa et al. 2021), it was identified that ADP-Glc, a nucleotide-sugar used in plants and prokaryotes, was utilized in the synthesis of α-1,4-glucan chains in soybean (Ballicora et al. 2003). The metabolism of starch initiates with Glc-1P and involves three enzymatic steps, as detailed by reactions [1–3].

$$\text{ATP} + \text{Glc-1P} \leftrightarrow \text{ADP-Glc} + \text{PPi} \quad (1)$$

$$\text{ADP-Glc} + \alpha\text{-}1,4\text{-glucan} \rightarrow \alpha\text{-}1,4\text{-glucosyl-}\ \alpha\text{-}1,4\text{-glucan} + \text{ADP} \quad (2)$$

$$\text{Elongated } \alpha\text{-}1,4\text{-glucan chain} \rightarrow \alpha\text{-}1,4\text{-}\ \alpha\text{-}1,6 \text{ branched glucan} \quad (3)$$

Reaction [1], catalyzed by ADP-Glc pyrophosphorylase (ADP-Glc PPase; ATP:α-D-glucose-1-phosphate adenylyltransferase; EC 2.7.7.27), gives rise to the formation of ADP-Glc. The reaction depends on the presence of a divalent metal ion (physiologically Mg^{2+}) and is the first committed step (and the main point of regulation) in the pathway of starch and glycogen synthesis in plants and bacteria, respectively. Although the reaction is freely reversible in vitro, it is inclined to function irreversibly in vivo because of: (i) hydrolysis of PPi by the high levels of inorganic pyrophosphatase that are present in plastids (Ballicora et al. 2004); and (ii) the use of ADP-Glc by starch synthase (SSase; ADP-α-D-glucose:(1,4)-α-D-glucan 4-α-D-glucosyltransferase; EC 2.4.1.21) to transfer the Glc moiety to the non-reducing end of an existing α-1,4-glucan chain (reaction [2]). Reaction [3], mediated by branching enzyme (BE; (1,4)-α-D-glucan:(1,4)-α-D-glucan 6-α-D-[(1,4)-α-D-glucano]-transferase; EC 2.4.1.18), completes the route by producing the α-1,6 branches in the amylose and mostly in the amylopectin polymer.

In the above-detailed route for starch biosynthesis, the first reaction is the main regulatory step (Jeon et al. 2010; Tuncel and Okita 2013; MacNeill et al. 2017; Goren et al. 2018). As extensively analyzed in previous reviews (Ballicora et al. 2003, 2004), ADP-Glc PPase is allosterically regulated by metabolites that are key intermediates of the primary carbon assimilation pathway in the different organisms. In cyanobacteria, green algae and plants, the enzyme is regulated by 3-phosphoglycerate (3-PGA) and orthophosphate (Pi) as the main allosteric activator and inhibitor, respectively (Ballicora et al. 2004). The enzyme found in certain plant tissues is also modulated by a post-translational redox mechanism involving thioredoxin (Ballicora et al. 2000; Lunn et al. 2006). In general, the levels of ADP-Glc PPase activity determine the amount of starch synthesized in a plant cell.

A variety of SSase and BE isoforms have been detected in the genomes of numerous species, ranging from the picophytoplanktonic green alga *Ostreococcus tauri* (Ral et al. 2004; Barchiesi et al. 2017; Hedin et al. 2017) to Arabidopsis (Delvalle et al. 2005) and rice (Pandey et al. 2012). Differences include the presence of starch binding domains (which critically affect affinity for substrate and kinetic properties) in both SSases and BEs (Gomez-Casati et al. 2013; Wilkens et al. 2018). There are two types of SSases: soluble (SSSase) or bound to the starch granule (GBSSase). Ten isoforms of SSases (eight SSSases and two GBSSases) were found in rice (Pandey et al. 2012). Considering sequence homology, SSSases have been classified into five groups (SSSase I, II, III, IV, and V). Some of the SSSases (for example forms I and II) may be partially associated with the starch granule. BEs are divided into two classes according to the length of the chains transferred in vitro, with BE-I relocating larger chains than BE-II. SSases and BEs are targets of post-translational phosphorylation, which exerts changes in their catalytic properties. The action of all these variant forms of SSase and BE, together with the involvement of degrading (starch debranching enzymes, isoamylase I and II) enzymes, determine the final fine structure of the polysaccharide granules (MacNeill et al. 2017; Goren et al. 2018). It has been speculated that multi-molecular complexes of all the above proteins critically affect the characteristics of the starch synthesized in the different plastids (Crofts et al. 2017). In the last few years (Seung et al. 2017, 2018; Vandromme et al. 2019), it has been evidenced that initiation of the starch granule determines the number, size, and shape of the granules. In such a priming process, non-catalytic proteins play a key role by interacting with maltooligosaccharides and specific isoforms of SSase.

ADP-Glc PPases from photosynthetic organisms

ADP-Glc PPase is absent in heterotrophic eukaryotic organisms but is found in (i) most bacteria, including photosynthetic cyanobacteria (Ballicora et al. 2003, 2004) and chemolithoautotrophic *Nitrosomonas* spp. (Machtey et al. 2012); (ii) green algae (Iglesias et al. 1994); and (iii) higher plants, both in photosynthetic and heterotrophic cells (Ballicora et al. 2003, 2004; Figueroa et al. 2021). Except for the non-regulated enzyme found in *Firmicutes* of the group *Bacillales* (Takata et al. 1997; Asencion Diez

et al. 2013; Cereijo et al. 2018), ADP-Glc PPases from other sources were characterized as exhibiting sensitivity to allosteric regulators (Ballicora et al. 2003, 2004). These regulators are main intermediates of the major route for carbon metabolism in the respective organism, relating to the carbon and energy content within a cell; with signals of high or low levels activating or inhibiting the enzymatic activity, respectively. Based on the characteristics of allosteric modulators, together with the fact that ATP is a substrate, it is clear that in bacteria and plants the enzyme activity, which initiates the synthetic pathway to build of α-1,4-glucans, is stimulated by an excess of carbon and energy, and is constrained under starving cellular conditions.

ADP-Glc PPase from cyanobacteria differs in quaternary structure from that found in green algae and higher plants (Ballicora et al. 2004). The cyanobacterial enzyme is a homotetramer (as is the case for the enzyme from most bacteria); whereas in plants, the enzyme is composed by two small (S; 50–54 kDa) and two large (L; 54–60 kDa) subunits, arranging an S_2L_2 heterotetramer. Different mechanisms have been identified as involved in modulating the activity of ADP-Glc PPases from photosynthetic organisms, including small molecules exerting allosteric regulation and post-translational chemical modification (Ballicora et al. 2003, 2004). The enzyme from higher plants has 3-PGA and Pi as the main activator and inhibitor, respectively; although it also exhibits a significant sensitivity to activation by hexose-P (fructose-1,6-bisP, Glc6P, and Fru6P; Gómez-Casati and Iglesias 2002; Kuhn et al. 2013). It has been evidenced that the plant enzyme activity is finely modulated by the crosstalk established between 3-PGA and Pi. Kinetic studies performed with ADP-Glc PPase from cyanobacteria under conditions of molecular crowding (mimicking intracellular environments) pointed out that such an interaction between allosteric effectors lead to ultrasensitive behavior. With these common characteristics, four distinct regulatory patterns by the allosteric effectors have been identified (reviewed in Ballicora et al. 2004). Thus, (i) each regulator affects activity separately (3-PGA activates the enzyme by reducing the K_m, increasing the V_{max} or both, while Pi acts as inhibitor) with an interacting reversion when combined; (ii) Pi only inhibits by reverting the activation of the enzyme by 3-PGA; (iii) 3-PGA only activates the enzyme already inhibited by Pi; and (iv) the effect of 3-PGA is limited to increase the enzyme's affinity for substrates. The canonical 3-PGA activation detailed above was observed in ADP-Glc PPases from a cornucopia of plant species, even in barley (Kleczkowski et al. 1993) and wheat (Gómez-Casati and Iglesias 2002; Ferrero et al. 2018) endosperm, where 3-PGA was initially described as non-activator but reduced the $S_{0.5}$ for substrates.

Three-dimensional structure of plant ADP-Glc PPases

For a long time, the three-dimensional structure of ADP-Glc PPases was elusive, until the crystal structure of a potato tuber homotetramer was solved (Jin et al. 2005). Despite the in vivo native form of the enzyme is a heterotetramer, the information was invaluable to understand critical aspects of the structure–function relationships. After that structure was solved, other bacterial structures from heterotrophic sources (*Agrobacterium tumefaciens* and *Escherichia coli*) were also solved (Cupp-Vickery et al. 2008; Cifuente et al. 2016; Hill et al. 2019). The potato tuber ADP-Glc PPase S subunit monomer comprises an N-terminal catalytic domain and a C-terminal β-helix domain (Fig. 2B). The fold of the catalytic domain is very similar to other nucleotide-sugar pyrophosphorylases whose structures have been elucidated, despite a low similarity at the protein sequence level (Brown et al. 1999; Blankenfeldt et al. 2000; Kostrewa et al. 2001; Olsen and Roderick 2001; Sulzenbacher et al. 2001; Sivaraman et al. 2002). The catalytic domain is composed of mostly parallel but mixed seven-stranded β-sheets covered by α-helices, which is reminiscent of the Rossmann fold (Rossman et al. 1975). Despite no obvious evolutionary relationships with glycosyltransferases, the structure of ADP-Glc PPases can be cataloged as a GT-A fold, which is one of the possible folds that glycosyltransferases adopt (Coutinho et al. 2003; Lombard et al. 2014).

The catalytic domain is connected to the C-terminal β-helix domain by a long loop, making numerous interactions with the equivalent region of another subunit (Jin et al. 2005). The fold of the C-terminal domain is a left-handed β-helix of six complete or partial coils with two insertions, one of which encompasses residues 368–390 and the other comprises residues 401–431 (Jin et al. 2005). The former is unique in plant structures compared to the bacterial ones (Cupp-Vickery et al. 2008; Cifuente et al. 2016; Hill et al. 2019), creating a particular steric hindrance in the C- and N-domain interface. Considering that most residues involved in the interaction with allosteric effectors are in this interface (Cifuente et al. 2016; Hill et al. 2019; Bhayani et al. 2019), this structural motif is a possible source for shaping different allosteric regulations between photosynthetic and heterotrophic ADP-Glc PPases. The presence of this left-handed β-helix domain fold poses interesting evolutionary questions, as it has been found in the structures of different proteins (Raetz and Roderick 1995; Kisker et al. 1996; Beaman et al. 1997; Brown et al. 1999; Kanamaru et al. 2002). Functionally, the other β-helix domains are either acetyltransferases, succinyltransferases, or a 'membrane-puncturing

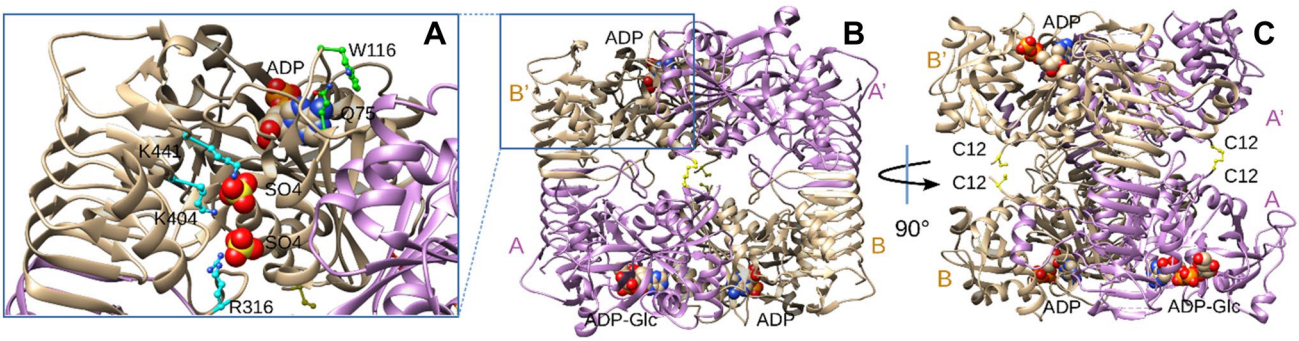

Fig. 2 Structure of the ADP-glucose pyrophosphorylase from potato tuber. In panels **B** and **C**, the structure of the small subunit homotetramer with ADP and ADP-Glc is depicted (PDB code: 1YP4). In lilac, monomomers A and A′ are shown, where in light brown are monomers B and B′. Monomers A and A′ are linked by a disulfide bridge between residues C12 from each subunit. The structure is slightly asymmetric: each monomer B and B′ bind a molecule of ADP in the active site, where only monomer A binds the product ADP-Glc. In panel **A**, the interface between the catalytic and regulatory domain is shown. There are two sulfates (SO_4) that occupy a site suggested to be the allosteric site (3-PGA and phosphate). Residues K404 and K441 (in cyan), which are involved in 3-PGA binding, are shown interacting with one of the sulfates. Residue R316 (in cyan) is a homologue of R294 in the Anabaena enzyme, which is critical for phosphate binding. Residues Q75 and W116 (in green), involved in triggering the allosteric signal are also shown in two loops near the active site

device'. In the ADP-Glc PPase, the β-helix domain is involved in allosteric regulation and contributes to the unique oligomerization structure.

The tetrameric structure can be interpreted as a dimer of dimers with each monomer labeled A, A′, B, and B′ (Fig. 2B). Monomers A and B interact predominantly by an end-to-end stacking of their β-helix domains (Fig. 2B), where the A and B′ catalytic domains also interact (as well as B and A′). Based on the identity of the residues defining the interactions, it has been suggested that a similar dimerization interface between S and L subunits is present in the heterotetrameric enzyme (Jin et al. 2005). The presence of Cys12 and a disulfide bond, responsible for redox regulation (Ballicora et al. 2000), helps predict the subunits' arrangement in the heterotetramer. Cys12 of monomer A and the equivalent residue of monomer A′ make a disulfide bond, as it would also be possible for the equivalent cysteine residues of monomers B and B′. The inter-subunit disulfide bond between S subunits (Fig. 2C) is preserved in the heterotetramer; however, there is no disulfide bond between L subunits, as Cys12 is not conserved. For this reason, this disulfide bond establishes that the S subunits would be located where A and A′ are, and the L subunits where B and B′ locate (Fig. 2C). This prediction is also in agreement with computational studies performed by Baris et al. (2009), who showed that lateral interaction between the L and S subunit (mainly mediated by hydrophobic interactions) is stronger for monomers A and B than for monomers A′ and B.

Non-covalent allosteric regulation of ADP-Glc PPase

According to their affinity for the allosteric regulators, ADP-Glc PPases were separated into nine classes, where classes 8 and 9 belonged to the enzyme from organisms performing oxygenic photosynthesis (Ballicora et al. 2004). Class 8 includes enzymes from cyanobacteria, green algae and plants, all activated by 3-PGA and inhibited by Pi. Although 3-PGA is ascribed as the main regulator, the ratio 3-PGA/Pi actually controls the ADP-Glc PPase activity (Kleczkowski 1999). Instead, class 9 comprises ADP-Glc PPases from plant non-photosynthetic tissues (maize, barley and wheat endosperm), insensitive to 3-PGA alone and inhibited by Pi, ADP, and Fru-1,6-bisP. Such inhibition can be reversed by 3-PGA and Fru6P (Ballicora et al. 2003, 2004). The fine-tuned regulation of plant ADP-Glc PPases is important from an agroeconomic point of view, given its impact on the starch content (Ballicora et al. 2004; Hannah and James 2008; Ferrero et al. 2018). In all cases, the importance of the ADP-Glc PPase allosteric phenomenon emerges and leads to its understanding from a metabolic/physiological perspective.

Since the last reviews by Ballicora et al. (2003, 2004) several efforts were made to further understand the ADP-Glc PPase allostery and its mechanisms. Advances implied identifying critical amino acidic residues involved in either binding and/or responding to effectors. For example, Boehlein et al. (2008) studied the impact of other effectors

rather than the "canonical activator" 3-PGA on the maize ADP-Glc PPase. They characterized the activation by metabolites such as Glc6P, 6-phosphogluconate, phospho-*enol*pyruvate, sucrose-6P, and Fru6P. Also, glycerol-P and ribose-5P were described as new effectors for a plant ADP-Glc PPase. All these metabolites increased the enzyme activity, some to the same degree as 3-PGA, although with higher $A_{0.5}$ values. An important observation was that the analyzed molecules, either activator or inhibitor of the maize endosperm ADP-Glc PPase, stabilized the enzyme towards thermal treatment (42 °C). Curiously, Pi elicited the best protective effect. In addition, amongst the different metabolites, the lowest K_d was determined for Pi, which was in the micromolar range. Further studies combining effector, inhibitor, and binding assays led to the hypothesis that 3-PGA and Pi share a putative binding site, which is different from the one for substrates ATP and ADP-Glc. The authors sustained this idea because a higher 3-PGA concentration in the medium required increased levels of Pi to reach inhibition. They proposed that, instead of inhibiting the enzyme *per se*, Pi acts diminishing 3-PGA activation. In addition, the Pi behavior in the presence of Glc6P or Fru6P showed $I_{0.5}$ values suggesting that these effectors are intertwined (Boehlein et al. 2008). The main contribution of this work relies in widening the attention to other sugar phosphates, ranging from 3 to 12 carbons. The study also revealed the combination of effectors, as further activation could be achieved, thus remarking the importance of ADP-Glc PPase regulation by complex metabolic scenarios, such as those presented in heterotrophic tissues.

From the solved structure of the potato tuber ADP-Glc PPase (Jin et al. 2005), Boehlein et al. (2010) could identify arginine residues whose side chains were in contact with sulfate ions. Previously, R294 in the *Anabaena* enzyme was found to be critical for the binding of Pi. This result was corroborated by the crystal structure of the potato tuber enzyme, where the analog amino acid (R316) binds a sulfate ion (Sheng and Preiss 1997; Fig. 2A). This residue was also important to determine the inhibitor selectivity (Frueauf et al. 2002). Then, the corresponding Arg residues in both the small (R77, R107, and R340) and large (R104, R116, R146, and R381) subunits of maize endosperm ADP-Glc PPase were analyzed by alanine mutagenesis, to test their binding properties and roles in the allosteric response. All mutations affected the enzyme allosteric properties, although with diverse particularities depending on the mutated Arg. For instance, all the analyzed mutants showed weaker apparent binding of 3-PGA than the native enzyme, with $A_{0.5}$ values up to 20-fold higher. Curiously, the same mutants behaved differently for the activators Glc6P and Fru6P and, additionally, they exhibited altered Pi binding. The authors observed that a single mutation (R104A or R107A in the large and small subunits, respectively) in the ADP-Glc PPase from maize could create allosteric properties emulating an ADP-Glc PPase from a bacterial source, since they obtained a higher sensitivity towards Fru6P. The effect was a decrease in the affinity towards 3-PGA, thus making the hexose-P more efficient. Then, the authors proposed that plant ADP-Glc PPases are under positive evolutionary selection for 3-PGA activation. This agrees with a later work where it was suggested that a widespread promiscuity is ancestral and intrinsic to the enzyme family (Kuhn et al. 2013). This may have been an efficient evolutionary mechanism to accommodate the ADP-Glc PPase regulation to different metabolic needs. Overall, results with the mutated Arg in the maize ADP-Glc PPase further sustain previous kinetic results (Boehlein et al. 2008), supporting the idea that the effector binding sites belong to both subunits in the heterotetrameric conformation. Those binding sites might be intertwined and function in a concerted manner.

Based on previous studies performed with the cyanobacterial and potato tuber enzymes (Charng et al. 1994; Ballicora et al. 1998), Figueroa et al. (2018) hypothesized that Lys404 and Lys441 from the small subunit of the potato tuber ADP-Glc PPase were involved in the binding of 3-PGA through its phosphate moiety. They observed that Lys404 is highly conserved in the ADP-Glc PPases from cyanobacteria and plants (in both subunits, small and large); while Lys441 is mostly conserved in the enzymes from cyanobacteria and the plant small subunits, this residue is less conserved in the plant large subunits (Ballicora et al. 1998; Jin et al. 2005). When the authors analyzed the ADP-Glc PPase from *O. tauri*, they found that both Lys residues are present in the small subunit (Lys406, Lys443). Still, only one residue (Lys429, analogous to Lys404 in the potato tuber small subunit) is found in the large subunit of the *O. tauri* ADP-Glc PPase (Figueroa et al. 2018). The other Lys is replaced by an arginine (Arg466). Then, the K443R and R466K mutants were produced, respectively, for the small and the large subunit from the *O. tauri* ADP-Glc PPase. The mutated proteins were expressed together or with their wild-type large or small counterparts, respectively.

Results presented by Figueroa et al. (2018) showed that restoration of the Lys residue in the *O. tauri* large subunit increased the affinity towards 3-PGA. In contrast, the mutation to an Arg in the small subunit decreased the activator's affinity. On the other hand, Pi inhibition analysis showed a lower effect on the small subunit K443R mutant, and slight changes were found in the analysis of the R466K large subunit mutant. The emerging scenario indicates that the small subunit displays a leading role in the inhibition by Pi. This statement is strengthened with different kinetics combining both activator and inhibitor. Then, 3-PGA activation was analyzed in the presence of 1 mM Pi, boosting the kinetic effect produced by the mutations in both subunits from the

O. tauri ADP-Glc PPase. The presence of Pi in the reaction mixtures increased the $A_{0.5}$ for 3-PGA for either the wild-type or the mutant enzymes. The highest differences were observed for those heterotetramers containing the R466K mutation in the large subunit. Then, results sustained the idea that introducing a Lys residue in the large subunit of *O. tauri* ADP-Glc PPase related to the 3-PGA binding site produces a highly 3-PGA sensitive and, concomitantly, more recalcitrant to Pi inhibition heterotetramer. Regarding Pi inhibition, kinetics depicted similar low $I_{0.5}$ values for those enzymes portraying the wild-type small subunit and higher values in the conformations containing the mutated K443R small subunit.

Higher sequence conservation in the small subunit has been described compared to the large subunit in heterotetrameric ADP-Glc PPases, suggesting relaxed evolutionary constraints for the latter (Georgelis et al. 2007; Figueroa et al. 2018). The hypothesis is that, after the gene duplication produced the ADP-Glc PPase small and large subunits, mutations were introduced in the 3-PGA binding site of the large subunit, with no impact on the overall function of the heterotetramer. The overlapped roles from both subunits produced a redundancy leading to subfunctionalization (described below). In this process, the small subunit conserved its integrity regarding the 3-PGA binding domain and allowed the large subunit to introduce (deleterious) mutations in its allosteric site. Considering this hypothesis, the authors proposed that during evolution the affinity towards activator(s) in the ADP-Glc PPase large subunit(s), or the interaction between the small and different large subunits, was modified to produce a population of enzymes with varied allosteric behaviors. Then, the subfunctionalization process could be ascribed as an "evolutionary tool" that enables this tuning. The authors concluded that the ADP-Glc PPase large subunit from green algae and plants accumulated mutations in the regulatory site, probably to modulate the sensitivity towards the specific metabolic environment present in an organelle, cell and/or tissue (Figueroa et al. 2018).

In the last decade, it was established that two specific (Trp and Gln, or WQ) residues are critical for triggering the activation of ADP-Glc PPases. First described in the *E. coli* enzyme, both studied residues are highly conserved among enzymes from different sources and are located in loops adjacent to the ATP binding site (Figueroa et al. 2011). This "allosteric trigger" was also analyzed by site-directed mutagenesis in the ADP-Glc PPase from model organisms performing oxygenic photosynthesis (*Anabaena* and *Solanum tuberosum*; Figueroa et al. 2013). The activation by 3-PGA of the Q58A and W96A mutants of the *Anabaena* ADP-Glc PPase was lower than for the wild-type enzyme, although their $A_{0.5}$ values for 3-PGA were similar to that of the wild-type enzyme. Then, the activation effect was significantly reduced rather than abolished, as in the case of Fru-1,6-bisP in the *E. coli* ADP-Glc PPase (Figueroa et al. 2011). Thus, loops containing the mutated WQ residues in the *Anabaena* ADP-Glc PPase participate in the 3-PGA activation mechanism in a similar fashion than the homologous amino acids for the Fru-1,6-bisP activation of the *E. coli* enzyme (Figueroa et al. 2011, 2013).

There are homologous WQ residues present in the structure of the potato tuber enzyme (Fig. 2A). These residues, involved in the allosteric trigger, were also replaced with alanine in the small (*Stu*S) and large (*Stu*L) subunits of the potato tuber ADP-Glc PPase (Figueroa et al. 2013). The concomitant Q mutation in both subunits led to unstable structures, and the following proteins were characterized regarding their respective regulatory sensitivity: *Stu*SQ75A/*Stu*L, *Stu*SW116A/*Stu*L, *Stu*S/*Stu*LQ86A, *Stu*S/*Stu*LW128A, and the double mutant *Stu*SW116A/*Stu*LW128A. The study showed that all mutants depicted higher $A_{0.5}$ values for 3-PGA than the wild-type enzyme. Response to 3-PGA activation for WQ mutated in the catalytic subunit (*Stu*SQ75A/*Stu*L, and *Stu*SW116A/*Stu*L) was equivalent to the mutations in the *Anabaena* ADP-Glc PPase. However, the *Stu*S/*Stu*LQ86A mutant was activated to a similar extent as the wild-type enzyme. In the same study, all the potato tuber ADP-Glc PPase WQ mutants displayed Pi inhibition kinetics with higher $I_{0.5}$ values (5- to 8-fold) than for the wild-type enzyme. The Pi-inhibition curves for the wild-type enzyme when 3-PGA was present depicted remarkably higher (70-fold) $I_{0.5}$ values. Instead, the Pi/3-PGA crosstalk was tenfold lower for the WQ mutants, thus confirming a disrupted propagation of the allosteric activation. Importantly, experiments with the WQ mutants from the *S. tuberosum* enzyme confirmed that both subunits share the mechanism for 3-PGA activation. The proposed WQ model agrees with the hypothesis where the residues locate at mobile loops involved in triggering the allosteric activation for ADP-Glc PPases. In the model, the activation sensitivity depends on the loop dynamics. This model was assayed using two ADP-Glc PPases from photosynthetic organisms but only regarding 3-PGA and/or Pi, leaving a question unanswered whether the allosteric trigger is the same for other activators, such as Fru6P or Glc6P.

The relative sensitivity towards the different effectors activating the ADP-Glc PPase from some photosynthetic organisms was also characterized (Kuhn et al. 2013). In that work, the activation promiscuity towards 3-PGA, Fru-1,6-bisP, Fru6P, and Glc6P of the ADP-Glc PPases from *Anabaena* PCC 7120, *O. tauri*, and potato tuber were analyzed. The authors defined a "specificity constant", as the ratio between the activation fold and the $A_{0.5}$ value (thus being analog to the catalytic efficiency). Results show that the specificity constant for 3-PGA is significantly higher than for other activators in the case of ADP-Glc PPases from *Anabaena* (homotetramer), *O. tauri* and potato tuber

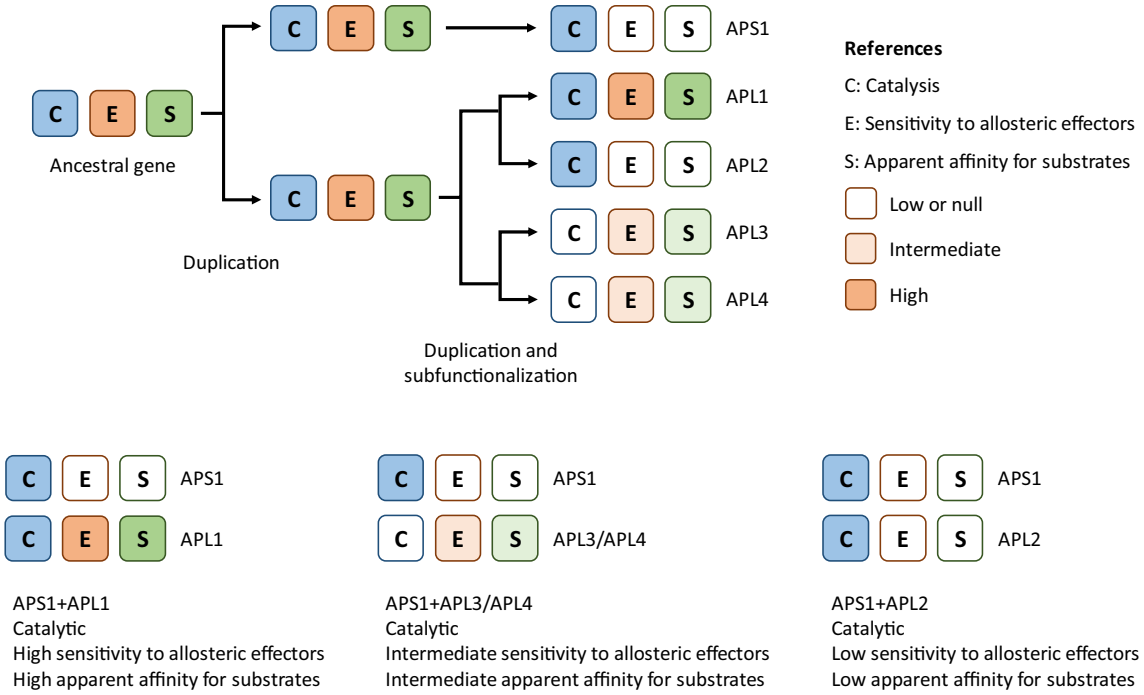

Fig. 3 Subfunctionalization of the different ADP-Glc PPase subunits found in Arabidopsis. The functions already present in the ancestral gene were inherited by the duplicates, which underwent subfunctionalization (APS1) or duplication and subfunctionalization (APL1-4). The combination of the small subunit (APS1) with different large subunits (APL1-4) produces catalytically active heterotetramers with particular kinetic and regulatory properties. Adapted from Kuhn et al. (2009) using data reported by Crevillén et al. (2003)

(these two, heterotetramers). On the other hand, the homotetramer composed of the S subunit from *O. tauri* depicted higher promiscuity since the specificity constant is similar for 3-PGA and Fru-1,6-bisP. Further analyses using *O. tauri* heterotetramers with mutated and wild-type S and L subunits, respectively, demonstrated a critical role for the *O. tauri* L subunit as a 3-PGA specifier, thus supporting the subfunctionalization process that occurred after gene duplication (see below). This behavior was not observed for the L subunit from the potato tuber enzyme. This phenomenon is important to understand the allosteric regulation according to the different metabolic environments to which ADP-Glc PPases are exposed in plant tissues. Indeed, a certain degree of allosteric promiscuity would be beneficial to adapt to different heterotrophic scenarios. Recently, Ferretti et al. 2021 revisited the effect of Glc6P and Fru6P in the *Anabaena* ADP-Glc PPase. The latter increased the activity 14-fold ($A_{0.5}$ 0.9 mM), with almost no impact on the substrates' affinity; whereas the former increased the activity fivefold ($A_{0.5}$ 1.4 mM) and produced a decrease in the relative affinity for Glc1P of one order of magnitude. Then, since the regulation by hexose-P of ADP-Glc PPase from photosynthetic organisms is now characterized from prokaryotic to plant sources, an evolutionary thread might be traced in this enzyme family.

Subfunctionalization of ADP-Glc PPase subunits

As previously mentioned, ADP-Glc PPases from eukaryotic organisms (green algae and plants) are heterotetramers (Ballicora et al. 2004). A recombinant small subunit can be expressed alone, thus rendering a homotetramer. Conversely, the large subunit must be co-expressed with the small subunit; otherwise, no activity is recovered (Iglesias et al. 1993; Crevillén et al. 2003; Kuhn et al. 2009). The only exception reported so far seems to be the L3 isoform from tomato, a large subunit that, when recombinantly expressed in *E. coli* in the absence of the small subunit, was recovered as an active monomer (Petreikov et al. 2010).

It has been suggested that the genes encoding both subunits evolved from a common ancestor by duplication followed by subfunctionalization (Ballicora et al. 2005). The subfunctionalization of genetic processes has been reviewed extensively (Lynch 2006). On the other hand, the literature has not focused enough on biochemical examples with protein-structure relationships (D'Ovidio et al. 2004; Ballicora et al. 2005; Tocchini-Valentini et al. 2005). The ADP-Glc PPase is a good case study of this important molecular evolutionary process (Fig. 3). To better understand this process, Kuhn et al. (2009) analyzed

the recombinant ADP-Glc PPase from the green algae *O. tauri*, one of the most ancient species that diverged from the common ancestor of green algae and plants (Derelle et al. 2006). Interestingly, the large subunit showed catalytic activity when co-expressed with a mutant, non-catalytic small subunit. The apparent affinities of this construct for Glc1P and 3-PGA were strikingly high (in the low micromolar range). However, the large subunit could only be expressed in the presence of the small subunit, as previously observed for the plant enzyme (Iglesias et al. 1993; Crevillén et al. 2003). Based on these results, the authors proposed different subfunctionalization paths for the enzymes from green algae and plants. After duplication, some properties became suboptimal in one subunit, while the other one complemented them. For example, the small subunit of the *O. tauri* enzyme retained the solubility function, while the large subunit retained the catalytic and regulatory roles. In the case of the potato tuber ADP-Glc PPase, the small subunit maintained the catalytic and solubility properties, while the large subunit kept the regulatory function (Kuhn et al. 2009).

The number of genes encoding the small and large subunits varies in different species. The small subunit is generally expressed in most tissues, whereas the large subunits show a tissue-specific expression pattern. For instance, Arabidopsis has two genes encoding the small subunit (*APS1* and *APS2*, although the latter seems to be a pseudo-gene) and four genes encoding different isoforms of the large subunit (*APL1-4*). The *APS1* gene is expressed in all tissues, while the *APL1-4* genes are differentially expressed in various tissues. APL1 is mainly expressed in source tissues, while APL3 is the main isoform in sink tissues (Crevillén et al. 2005). The heterotetramers resulting from the combination of different large subunits (APL1-4) with the small subunit (APS1) produce isoforms with particular regulatory properties: the heterotetramer composed by APS1 and APL1 showed the highest apparent affinities for substrates and sensitivity to allosteric effectors; those arranged by APS1 and APL3/4 showed intermediate levels of apparent affinities for substrates and sensitivity to allosteric effectors; and the heterotetramer composed by APS1 and APL2 displayed the lowest apparent affinities for substrates and sensitivity to allosteric effectors (Fig. 3). This pattern of expression and kinetic parameters strongly suggests that starch synthesis is modulated in a tissue-specific manner (Crevillén et al. 2003).

This scenario is complex because some large subunits from plant ADP-Glc PPases are both regulatory and catalytic, as previously observed for the *O. tauri* enzyme (see above). Ventriglia et al. (2008) showed that APL1 and APL2 contain a pair of residues (Arg102 and Lys112, according to the APL1 sequence) normally found in catalytically active small subunits but absent in several large subunits, such as APL3-4 and the large subunit of the potato tuber enzyme. The physiological relevance of this finding was tested by complementing the starchless Arabidopsis *aps1* mutant with an inactivated form of APS1. The resulting transgenic line recovered the wild-type phenotype, indicating that APL1 and APL2 have catalytic activity and might contribute to starch synthesis in vivo. Thus, it seems reasonable that the capability to perform catalysis was retained in some isoforms (APL1-2) but lost in others (APL3-4; Fig. 3). Previously, Ballicora et al. (2005) resurrected the ancestral catalytic role of the large subunit of the potato tuber ADP-Glc PPase by introducing the Arg and Lys residues found in catalytic subunits. To test the resurrected large subunit activity, it was necessary to abolish the catalytic activity of the small subunit because the large subunit can only be expressed in combination with the small subunit (Iglesias et al. 1993). Interestingly, the mutant heterotetramer was active and displayed higher apparent affinities for Glc1P and 3-PGA than the wild type (Ballicora et al. 2005), as observed for the *O. tauri* enzyme (Kuhn et al. 2009). In a different study, the solubility of the large subunit of the potato tuber enzyme was resurrected by introducing a single mutation (S302N), which produced a functional homotetramer (L_4). This variant displayed 100-fold lower activity than the small subunit homotetramer (S_4) and showed no activation by 3-PGA nor Pi inhibition; however, the mutant large subunit increased the solubility of the corresponding heterotetrameric (S_2L_2) enzyme (Hwang et al. 2008).

The resurrection of properties lost in different subunits supports the subfunctionalization mechanism to evolve new roles. To expand the evolutionary landscape, the presence of heteromers allow the subunits to relax the evolutionary constraints on specific roles ("subfunctions") because the opposite subunit could retain it in case of a mutation (Fig. 3). That is why some of the S or L subunits may look "defective" in a particular role when analyzed isolated. Another example of this was the resurrection of regulatory roles in the L subunit from *O. tauri* (Figueroa et al. 2018). As stated above, sequence identity between small subunits from different species is higher than the identity between large subunits. The higher divergence observed in large subunits could be attributed to increased evolutionary constraints in the small subunit. Georgelis et al. (2007) showed that the small (BRITTLE2) and large (SHRUNKEN2) subunits of the maize endosperm ADP-Glc PPase are equally predisposed to mutations altering amino acids important for enzyme activity when expressed in *E. coli* with a single complementary subunit. Thus, these authors proposed that the small subunit has more evolutionary constraints *in planta* than the large subunit because the former is expressed in different tissues, is less redundant, and must form heterocomplexes with different large subunits.

Post-translational regulation

Redox

Early reports from Preiss and coworkers (Fu et al. 1998) showed that ADP-Glc PPase from potato tuber is redox-regulated by forming a disulfide bridge between Cys12 of both small subunits. The enzyme is activated not only by DTT but also by reduced thioredoxin (TRX) *f* and *m* from spinach leaves. It is important to note that, in the reduced form, the enzyme is more sensitive to 3-PGA than in the oxidized form, particularly at a low (micromolar) concentration of the allosteric activator. Thus, reductive activation of the enzyme could fine tune the effect of 3-PGA (Ballicora et al. 2000). Tiessen et al. (2002) later showed that ADP-Glc PPase from detached tubers was oxidized, in agreement with inhibition of starch synthesis, even though the levels of substrates (ATP and Glc1P) and the 3-PGA/Pi ratio were higher than in the control tubers. Interestingly, these authors showed that the small subunit of the enzyme was present as a mixture of reduced (50 kDa band) and oxidized (100 kDa band) in growing tubers but became completely oxidized in detached tubers, suggesting that redox regulation was physiologically relevant, even in amyloplasts. Similarly, the enzyme from pea, potato, and Arabidopsis leaves was shown to be redox regulated; moreover, the dimerization status was dependent on the level of soluble sugars and time of the day (i.e. the enzyme was more active in the light when sucrose levels are higher; Hendriks et al. 2003). These observations are consistent with the so-called "positive feedback regulation" of starch synthesis by sucrose levels, postulating that sucrose accumulation in the cytosol activates the synthesis of the polysaccharide in the chloroplast (Stitt et al. 2010). Then, Hädrich et al. (2012) demonstrated that mutation of Cys81 from the small subunit of the Arabidopsis ADP-Glc PPase (equivalent to Cys12 in the small subunit of the potato tuber enzyme) prevented dimerization of the small subunit *in planta*. However, the *adg1* mutant complemented with the redox insensitive C81S mutant of the small subunit (APS1) showed only minor changes in the turnover of transitory starch in Arabidopsis leaves. Analysis of this mutant line showed that redox modulation is less important than allosteric regulation for the adjustment of leaf starch synthesis in plants grown under different photoperiods (Mugford et al. 2014).

Michalska et al. (2009) showed that a plastid-localized NADP-thioredoxin reductase C (NTRC), which contains both a NADP-thioredoxin reductase (NTR) and a TRX domain in a single polypeptide, was capable of activating ADP-Glc PPase in vitro after NADPH supply. Moreover, Arabidopsis *ntrc* mutants showed decreased redox activation of ADP-Glc PPase and lower levels of starch in photosynthetic (leaves) and heterotrophic (roots) tissues. These results were further validated by Lepistö et al. (2013), who demonstrated that Arabidopsis *ntrc* mutants displayed retarded growth under short-day conditions due to the inability of the plants to synthesize proper amounts of starch during light hours, necessary to sustain growth in the following night. In addition to NTRC, TRX *f* seems to be important for regulating ADP-Glc PPase in leaves. Arabidopsis *trxf1* mutants showed decreased light reductive activation of ADP-Glc PPase, accompanied by reduced starch and increased sucrose content. However, these changes were not significant enough to affect overall plant growth (Thormählen et al. 2013).

The Cys residue located at the N-terminal domain of the small subunit subject to redox regulation in enzymes from dicot species (Cys12 from the potato tuber enzyme) is absent in the cytosolic isoform expressed in the endosperm of rice grains. However, the reduced ADP-Glc PPase purified from rice endosperm, and the corresponding recombinant enzyme, showed higher sensitivity to the allosteric activator (3-PGA) than the non-reduced forms. Truncation of the N-terminal domain of the large subunit decreased the affinity towards 3-PGA and abolished the response to redox agents. Site-directed mutagenesis of conserved Cys residues located at the N-terminal domain of the large subunit (also present in the large subunit of ADP-Glc PPases from other grasses, such as maize, sorghum, barley, and wheat) showed that Cys47 and Cys58 (but not Cys12) are responsible for the redox regulation of the enzyme. Redox regulation of the large subunit from the rice endosperm enzyme seems independent of a disulfide bridge formation (Tuncel et al. 2014).

Establishing a disulfide bridge between the two small subunits of the potato tuber heterotetrameric ADP-Glc PPase is intrinsically related to its heat stability (Ballicora et al. 1995; Fu et al. 1998). However, the enzyme from cereal endosperms is heat-labile; thus, several authors have tried (successfully) to enhance the stability of ADP-Glc PPases from different grasses to increase the synthesis of starch in plants exposed to heat stress during the grain-filling period. The addition of a QTC motif (present in the small subunit of thermostable ADP-Glc PPases) at the N-terminal domain of the small subunit from the maize endosperm enzyme increased the stability of the heterotetrameric protein when incubated at 58 °C, although kinetic parameters for substrates (ATP and Glc1P) and effectors (3-PGA and Pi) were almost unaffected. The heat-stability increment was related to the formation of a disulfide bridge between the mutant small subunits (Linebarger et al. 2005). Similarly, the addition of the QTC motif at the N-terminal domain of the small subunit of the rice endosperm ADP-Glc PPase allowed the formation of a disulfide bridge (absent in the

wild-type enzyme), with the concomitant increase in heat stability (Hwang et al. 2019).

Using the potato ADP-Glc PPase as a model, Seferoglu et al. (2014) demonstrated that mutation of Tyr378 to Cys in the small subunit renders an enzyme with increased heat stability. The same group showed that Glu370 from the small subunit is also relevant for obtaining a heat-stable enzyme (Seferoglu et al. 2016). The action of these residues is directly linked to the interface interaction for the heterotetrameric assembly of the enzyme. These studies complemented works performed with the maize ADP-Glc PPase, where mutations introduced in either allosteric sites or the subunit interfaces produced enzyme variants with increased stability to temperature (Boehlein et al. 2008, 2014, 2015). In general, these mutated variants of the maize enzyme were screened for intended assets with impact on grain yield (such as activity at higher temperatures or a diminished thermal denaturation). In a recent work, Ferrero et al. (2018) characterized a hybrid ADP-Glc PPase composed of the potato small subunit and the large subunit from the wheat enzyme, which yielded a heat-stable enzyme with minor modifications. Indeed, they found that the hybrid proteins were more stable than the wheat endosperm wild-type enzyme, retaining more activity after the heat treatment. In the study of the hybrid proteins (obtained by combining small and large subunits from wheat and potato ADP-Glc PPases), they observed that constructs harboring the small subunit from the potato tuber enzyme retained more than 80% of activity after treatment for 5 min at 55 °C. This result reinforces the relevance of Cys12 in the small subunit of the potato tuber enzyme for heat stability, not only in the native enzyme but also in hybrid heterotetramers that could be produced by arrangement of different ADP-Glc PPase subunits from agriculturally important crops, including those with mutated residues related to thermal stability.

Phosphorylation

In the last decade, phospho-proteomic studies showed that ADP-Glc PPases from different species and tissues are phosphorylated (Nakagami et al. 2010; Rose et al. 2012). Particularly, several works performed with samples from grasses described the phosphorylation of ADP-Glc PPase subunits. Dong et al. (2015) analyzed germinating wheat seeds by differential gel electrophoresis and found that the small subunit of the plastidial ADP-Glc PPase was phosphorylated, although the phosphorylation site was not determined. Similarly, the small subunit of maize endosperm (also known as BRITTLE2, encoded by the *Bt2* gene) was found phosphorylated in a protein extract enriched in phosphorylated proteins using Phos-tag agarose, followed by proteomic analysis. In this study, authors showed by iTRAQ that the small subunit of maize endosperm ADP-Glc PPase was phosphorylated at Ser10, Thr451, and Thr462. Moreover, in-gel activity assays with alkaline phosphatase-treated samples showed that the enzyme activity is lower than the phosphorylated control (Yu et al. 2019). Recently, Ferrero et al. (2020) showed that ADP-Glc PPase from wheat endosperm is phosphorylated in the large subunit. In this work, the authors demonstrated that phosphorylation of the enzyme increased with grain development and was positively correlated with ADP-Glc PPase activity and starch content in endosperm extracts. In vitro experiments using recombinant ADP-Glc PPase from wheat endosperm and calcium-dependent protein kinases established that phosphorylation mainly occurred at the large subunit.

Acetylation

Recently, Zhu et al. (2018) showed that both subunits of the ADP-Glc PPase from developing wheat grain were acetylated. The cytosolic form of the small subunit was acetylated at Lys365, whereas the large subunit was acetylated at Lys205 and Lys318. Interestingly, acetylation of Lys318 was reduced in samples from drought-stressed plants, which also showed reduced ADP-Glc PPase activity and smaller starch granules. Therefore, the authors suggested that deacetylation of Lys318 could be important for reducing starch synthesis under unfavorable conditions.

Subcellular localization in plants

It is widely accepted that ADP-Glc PPase locates in plastids (Ballicora et al. 2004). The only exception seems to be the cytosolic isoform found in cereal endosperms. In the last two decades, research confirmed the early reports that suggested the existence of a major cytosolic isoform (and a minor plastidial isoform) in cereal endosperms (Beckles et al. 2001; Burton et al. 2002; Tetlow et al. 2003). Rösti et al. (2006) showed that both the cytosolic and plastidial isoforms of the small subunit of the ADP-Glc PPase from barley were produced from only one gene (*Hv.AGP.S.1*) through alternative splicing. The transcript *Hv.1a* encodes the cytosolic small subunit in the endosperm, while the transcript *Hv.1b* encodes the plastidial small subunit found in leaves. The minor plastidial small subunit isoform found in the barley endosperm seems to be encoded by the *Hv.AGP.S.2* gene.

In rice, the small and large subunits of the ADP-Glc PPase are encoded by two (*OsAGPS1-2*) and four (*OsAGPL1-4*) genes, respectively. Subcellular localization studies using GFP-tagged proteins showed that all isoforms expressed in leaves are plastidial, including the leaf-specific isoform *Os*APGS2a. Contrarily, the seed-specific proteins *Os*AGPS2b and *Os*AGPL2 were located in the cytosol. It is important to note that *Os*APGS2a and *Os*APGS2b are produced from the

same gene by alternative splicing (Lee et al. 2007), as observed for the 1a and 1b isoforms of the ADP-Glc PPase small subunit from barley (see above). Both *osagps2* and *osagpl2* mutant plants displayed shrunken endosperms as a consequence of reduced starch content. Immunodetection studies confirmed that *Os*AGPS2a is the main small subunit present in leaves (consistent with the reduced transitory starch accumulation in the *osagps2* mutant), whereas *Os*AGPS2b and *Os*AGPL2 are the major isoforms found in the endosperm (Lee et al. 2007). In maize, the cytosolic and plastidial small subunits are encoded by two paralogous genes (*Bt2* and *L2*, respectively), which arose as a result of the tetraploidization of the maize genome. After duplication, *Bt2* and *L2* underwent subfunctionalization; i.e., they adopted specific functions already present in the ancestral gene (as shown in Fig. 3 for the different Arabidopsis heterotetramers). Indeed, *Bt2* encodes the cytosolic isoform present in the endosperm (BRITTLE2), while *L2* encodes the plastidial isoform found in leaves (Rösti and Denyer 2007). More recently, Huang et al. (2014) showed that mutation of two genes encoding plastidial isoforms of the small and large subunits of the ADP-Glc PPase displayed a minor reduction of starch in endosperm, thus confirming that starch synthesis in this tissue largely depends on the activity of the cytosolic enzyme.

ADP-Glc synthesized in the cytosol of cereal endosperms has to be transported to the amyloplast to synthesize starch. Shannon et al. (1998) showed that the maize adenylatetranslocator BRITTLE1 is involved in ADP-Glc transport into the amyloplast stroma. The importance of this translocator for starch synthesis in the endosperm was demonstrated by the low-starch phenotype of the *bt1* mutant kernels. Similarly, mutation of the *Hv.Nst1* gene (which encodes the barley ortholog of maize BRITTLE1) reduced the amount of starch in the endosperm, suggesting that transport of ADP-Glc produced in the cytosol is necessary for normal synthesis of starch in the amyloplast (Patron et al. 2004). Then, Bowsher et al. (2007) showed that ADP-Glc is transported into wheat amyloplasts using reconstituted amyloplast envelope proteins (in a proteoliposome-based system) or isolated organelles. Transport of ADP-Glc depended on counter-exchange of adenylates (AMP or ADP); interestingly, the rate of ADP efflux from the plastid was consistent with that of ADP-Glc utilization for starch synthesis. The transporter responsible for the counter-exchange of these molecules seems to be a 38 kDa protein located in the plastid envelope. However, there is no clear evidence that such a transporter is the BRITTLE1 homolog.

Remarks and perspectives

ADP-Glc PPase plays a central function in the metabolism of photosynthetic organisms, catalyzing the first and rate-limiting step for starch biosynthesis. For years, the enzyme was a research target concerning its kinetic, regulatory, and structural properties. It was initially established that ADP-Glc PPase is regulated by critical metabolic intermediates, specifically 3-PGA and Pi in higher plants. In the last two decades, significant advances have been reached in the field of structure to function and evolutionary relationships. A critical one was the elucidation of the crystallographic structure that could be integrated with the identification of substrate- and regulator-binding sites, as well as domains involved in triggering and propagating the allosteric signal for activity modulation. Besides that, the evolution of the enzyme was rationalized to understand the development of varied heterotetrameric forms with distinctive cell and tissue localization plus particular kinetic, regulatory, and stability properties. Mechanisms implicated in the post-translational chemical modification are critical issues that emerged from specific proteomic approaches constituting an area requiring further experimental research.

In recent years, a significant progress was made by many groups to understand the structural basis of the allosteric regulation of this enzyme. However, there are several questions that remain to be answered. A complete description of the allosteric site is needed to understand whether more residues are involved in the binding of 3-PGA and the selectivity for this effector. The structural explanation for the role of different subunits is also a very important aspect to understand the regulation determined at the quaternary structure level. Crystal structures of heterotetramers in presence of the activator or analogs will help to answer those questions. However, this information may not be enough to have a complete picture of the allosteric mechanism. Evidence from crystal structures in bacterial homologues (Cupp-Vickery et al. 2008; Cifuente et al. 2016; Hill et al. 2019; Asencion Diez et al. 2020) show that differences between the structures with or without activators/inhibitors are very subtle. It is quite possible that the allosteric mechanism in this enzyme family relies on the protein dynamics and the available crystal structures may not be able to fully describe it. This is an area that needs to be explored in the future, not only in bacteria, but also in plants. In addition, tracing the dynamic signal that leads to the activation will be important to understand the phenomenon. This type of studies has been initiated in the *E. coli* enzyme (Hill et al. 2015), but it needs to be studied in plant homologues.

Currently there is vast information on ADP-Glc PPase and its association with other enzymes, proteins, and metabolites involved in carbohydrate metabolism in the intracellular environment. This gathered knowledge is useful for understanding variations in the regulation of starch synthesis in different plant tissues. The relevance of starch as a major product determining plant productivity combined with current advances in the field establish new exciting opportunities for research ahead. There is a high potential to designing

genetically improved crops (in quantity and quality) with application in multiple industries. Several products will benefit from these advances in technology, including food, bioplastics, and biofuels. For that reason, this technology will constitute a promising tool to cope with the current severe climate change crisis.

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