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Use of TILLING and robotised enzyme assays to generate an allelic series of *Arabidopsis thaliana* mutants with altered ADP-glucose pyrophosphorylase activity

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ABSTRACT

ADP-glucose pyrophosphorylase (AGPase) catalyses the synthesis of ADP-glucose, and is a highly regulated enzyme in the pathway of starch synthesis. In *Arabidopsis thaliana*, the enzyme is a heterotetramer, containing two small subunits encoded by the *APS1* gene and two large subunits encoded by the *APL1-4* genes. TILLING (*T*argeting *I*nduced *L*ocal *L*esions *IN* *G*enomes) of a chemically mutagenised population of *A. thaliana* plants identified 33 novel mutations in the *APS1* gene, including 21 missense mutations in the protein coding region. High throughput measurements using a robotised cycling assay showed that maximal AGPase activity in the *aps1* mutants varied from <15 to 117% of wild type (WT), and that the kinetic properties of the enzyme were altered in several lines, indicating a role for the substituted amino acid residues in catalysis or substrate binding. These results validate the concept of using such a platform for efficient high-throughput screening of very large populations of mutants, natural accessions or introgression lines. AGPase was estimated to have a flux control coefficient of 0.20, indicating that the enzyme exerted only modest control over the rate of starch synthesis in plants grown under short day conditions (8 h light/16 h dark) with an irradiance of 150 μmol quanta m^{-2} s^{-1}. Redox activation of the enzyme, via reduction of the intermolecular disulphide bridge between the two small subunits, was increased in several lines. This was sometimes, but not always, associated with a decrease in the abundance of the APS1 protein. In conclusion, the TILLING technique was used to generate an allelic series of *aps1* mutants in *A. thaliana* that revealed new insights into the multi-layered regulation of AGPase. These mutants offer some advantages over the available loss-of-function mutants, e.g. *adg1*, for investigating the effects of subtle changes in the enzyme's activity on the rate of starch synthesis.

Introduction

ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) catalyses the first committed step in the pathway of starch biosynthesis – the conversion of ATP and glucose 1-phosphate (Glc1P) to adenosine-5′-diphosphoglucose (ADPG) and inorganic pyrophosphate (PP_i) (Preiss, 1988). Although the reaction is freely reversible *in vitro*, hydrolysis of PP_i by the plastidial alkaline pyrophosphatase makes the reaction essentially irreversible *in vivo* (Weiner et al., 1987; Tiessen et al., 2002). In higher plants, AGPase is a heterotetrameric ($\alpha_2\beta_2$) enzyme comprised of small (~50 kDa) and large (~51 kDa) subunits, unlike most bacterial AGPases which are homotetrameric (α_4) (Morell et al., 1987; Okita et al., 1990; Ballicora et al., 2004). The small and large subunits of the plant enzyme are closely related to each other, and are thought to have evolved from a common ancestral form derived from the cyanobacterial endosymbiont that gave rise to plastids (Ballicora et al., 2004; Patron and Keeling, 2005). Although the small subunit contains the main active site of the enzyme, two of the large subunit isoforms from *Arabidopsis thaliana*, APL1 and APL2, have been shown to have residual catalytic activity (Ventriglia et al., 2008). Both subunits influence the kinetic and regulatory properties of the AGPase holoenzyme, as shown by studies of chimeric forms of the potato and maize enzymes (Cross et al., 2004).

The genome of *A. thaliana* contains two small subunit genes – *APS1* (At5g48300) and *APS2* (At1g05610). *APS2* is expressed only very weakly and the predicted APS2 protein lacks several of the

Abbreviations: ADPG, ADP-glucose; AGPase, ADP-glucose pyrophosphorylase; 3PGA, 3-phosphoglycerate; SIFT, sorting intolerant from tolerant; TILLING, targeting induced local lesions in genomes.

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highly conserved amino acid residues from the active site region, which most likely explains why the heterologously expressed protein had no detectable catalytic activity (Crevillén et al., 2003). These observations led to the conclusion that *APS1* encodes the only catalytically active small subunit in this species. There are four large subunit genes in *A. thaliana*: *APL1* (At5g19220), *APL2* (At1g27680), *APL3* (At4g39210) and *APL4* (At2g21590). Transcript and proteomic analyses have shown that APL1 is the predominant isoform in leaves, whereas APL3 and APL4 are mostly expressed in sink tissues (Crevillén et al., 2003, 2005). Expression of AGPase in leaves is transcriptionally induced by light and sugars (Sokolov et al., 1998; Bläsing et al., 2005; Crevillén et al., 2005), but repressed by nitrate and phosphate (Scheible et al., 1997; Nielsen et al., 1998). Such transcriptional regulation could allow the plant to adjust its accumulation of starch in response to sustained changes in its carbon and nutritional status. Expression of AGPase in starch storing tubers is also induced by sugars (Müller-Röber et al., 1990; Harn et al., 2000).

AGPase is exclusively localised in the chloroplasts in leaves, and is also restricted to the plastids (e.g. amyloplasts) in non-photosynthetic organs of dicotyledonous plants. In contrast, most (85–95%) of the AGPase activity in developing cereal grains (e.g. maize, wheat and barley) is located in the cytosol, and ADPG is imported into the amyloplasts via an adenylate transporter (Thorbjørnsen et al., 1996; Denyer et al., 1996). AGPase displays sigmoidal substrate kinetics for ATP and Glc1P, and the enzyme from leaves is allosterically activated by 3-phosphoglycerate (3PGA) and inhibited by orthophosphate (P_i) (Sowokinos, 1981; Sowokinos and Preiss, 1982; Preiss, 1988). These properties make the production of ADPG highly sensitive to changes in the availability of photoassimilate and the phosphate status of the chloroplast stroma, and help to coordinate starch synthesis with photosynthetic CO_2 fixation and sucrose synthesis (MacRae and Lunn, 2006). In heterotrophic tissues, the kinetic properties of AGPase, including its sensitivity to regulation by 3PGA and Pi, can differ significantly from the leaf enzyme (Ballicora et al., 2004; Boehlein et al., 2010). Crevillén et al. (2003) showed that the substrate affinities and allosteric properties of the *A. thaliana* AGPase are largely determined by which of the four isoforms of the large subunit is present in the holoenzyme, accounting for the different properties of the enzyme from leaves, where APL1 is the major isoform, and sink tissues in which APL3 and APL4 predominate.

In addition to transcriptional and allosteric regulation, the activity of AGPase is also subject to post-translational redox modulation. The potato tuber AGPase contains an intermolecular disulphide bridge, which links the two small subunits of the heterotetramer via their respective Cys_{12} residues, and reduction of the disulphide bridge by dithiothreitol or reduced thioredoxin was shown to activate the enzyme *in vitro* (Fu et al., 1998; Ballicora et al., 1999, 2000). The reduced monomeric small subunits (50 kDa) can be readily separated from the oxidised dimeric (100 kDa) form by SDS-polyacrylamide gel electrophoresis under non-reducing conditions, and visualised by immunoblotting (Tiessen et al., 2002). Using this approach, Hendriks et al. (2003) demonstrated that redox activation of AGPase occurs *in vivo* when leaves of *A. thaliana*, pea and potato are illuminated, or when sugar levels increase in the leaf. The potato tuber enzyme is also redox activated *in vivo* in response to higher sugar levels, providing a mechanism for coordinating the rate of starch synthesis in the tubers with the supply of photoassimilate from the leaves (Tiessen et al., 2002). Light activation of AGPase presumably depends on ferredoxin-dependent reduction of thioredoxins via ferredoxin–thioredoxin reductase, as is the case for many other enzymes involved in photosynthesis. A dual function NADPH-dependent thioredoxin reductase (NTRC) has been implicated in sugar-induced redox activation of AGPase (Michalska et al., 2009). It has also been proposed that trehalose-6-phosphate (Tre6P) is an intermediary in the sugar-induced redox activation of AGPase, both in leaves and in non-photosynthetic tissues (Kolbe et al., 2005). In support of this proposal, the redox activation state of AGPase was found to be correlated with the levels of both sucrose and Tre6P in *A. thaliana* leaves and seedlings (Lunn et al., 2006). However, many of the molecular details of the redox regulation of AGPase and the role of Tre6P *in vivo* remain to be elucidated.

Starch synthesis is severely compromised in mutants that have either lost one of the AGPase subunits completely or have greatly decreased activity, e.g. *brittle2* and *shrunken2* in maize, *rb* in pea, and the *adg1* and *adg2* mutants in *A. thaliana* (Tsai and Nelson, 1966; Dickinson and Preiss, 1969; Lin et al., 1988a,b; Smith et al., 1989; Wang et al., 1997, 1998). Antisense repression of AGPase in transgenic potatoes strongly inhibited starch synthesis in the leaves, and essentially abolished starch synthesis in the tubers (Müller-Röber et al., 1992), confirming the importance of AGPase for starch synthesis.

Neuhaus and Stitt (1990) used the *adg2* mutant to assess the contribution of AGPase to control flux through the pathway of starch synthesis in *A. thaliana* leaves. In the homozygous state (*adg2*/*adg2*) this mutant lacks a functional APL1 large subunit, and retains only 4% of the small subunit protein and 5–7% of the AGPase activity found in WT leaves (Lin et al., 1988a,b; Neuhaus and Stitt, 1990). The remaining activity could be due to homotetrameric forms of the residual APS1 protein, or heterotetrameric complexes with the other large subunit isoforms (APL2-4), which are present at very low levels in leaves (Crevillén et al., 2005). In contrast, the heterozygous mutant (*adg2*/*ADG2*) retains about 50% of WT activity. From comparison of the rates of starch synthesis with AGPase activities in homozygous, heterozygous and WT plants, AGPase was estimated to have a flux control coefficient (C_j) of 0.28 under light-limiting conditions (75 μmol quanta m^{-2} s^{-1}) and 0.64 in saturating light (600 μmol quanta m^{-2} s^{-1}). These estimates indicated that AGPase does make a substantial contribution to control of flux through the pathway of starch synthesis, but that control is shared to a greater or lesser extent with other enzymes in the pathway depending on the prevailing light conditions. A later study comparing the *adg1* and *adg2* mutants reported somewhat different levels of residual AGPase activity in the mutants, but confirmed that the effect of decreased AGPase activity on starch synthesis was strongly dependent on irradiance and CO_2 concentration (Sun et al., 1999).

A major drawback of using loss-of-function mutants for metabolic control analysis is that the decrease in enzyme activity is usually quite severe, and flux control coefficients must be estimated by extrapolation between a limited number of data points. Flux control coefficients can be determined more precisely if plants with a range of only moderately decreased enzyme activities are available (Kacser and Burns, 1973). A PCR-based approach known as TILLING (*T*argeting *I*nduced *L*ocal *L*esions *IN* *G*enomes) allows a mutagenised population of plants to be rapidly screened for mutations in a particular gene (Greene et al., 2003; Till et al., 2003), and has been used to identify novel starch mutants in *Lotus japonicus* (Vriet et al., 2010). In this paper we describe a TILLING screen to identify a large allelic series of *A. thaliana* plants with mutations in the *APS1* gene. The mutants were analysed using a robot-based platform (Gibon et al., 2004) to identify those with altered maximal activities of AGPase, and to measure their starch content. This approach tested the concept of using the robotised platform for high throughput screening of a large numbers of mutants or natural accessions for differences in enzyme properties, as proposed by Rogers and Gibon (2009). Further analyses were carried out to test if any of the mutations affected the redox status of the AGPase, and to identify potential binding sites for Tre6P on the APS1 protein, with the aim of revealing new details of the molecular mechanism of redox activation.