The evolution of the starch biosynthetic pathway in cereals and other grasses

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Abstract

In most species, the precursor for starch synthesis, ADPglucose, is made exclusively in the plastids by the enzyme ADPglucose pyrophosphorylase (AGPase). However, in the endosperm of grasses, including the economically important cereals, ADPglucose is also made in the cytosol via a cytosolic form of AGPase. Cytosolic ADPglucose is imported into plastids for starch synthesis via an ADPglucose/ADP antiporter (ADPglucose transporter) in the plastid envelope. The genes encoding the two subunits of cytosolic AGPase and the ADPglucose transporter are unique to grasses. In this review, the evolutionary origins of this unique endosperm pathway of ADPglucose synthesis and its functional significance are discussed. It is proposed that the genes encoding the pathway originated from a whole-genome-duplication event in an early ancestor of the grasses.

Key words: ADPglucose pyrophosphorylase, ADPglucose transporter, Brittle1, cereal grain, endosperm, genome duplication.

Introduction

Several of our most important crop plants are grasses (for example, maize, rice, wheat, sorghum, and barley) and they are known collectively as the cereals (Evans, 1998). Grasses were first domesticated for their starch-rich seeds (grains or kernels) and the starch content has subsequently been increased by breeding. In the developing seed, starch accumulates in the endosperm, a tissue which comprises the bulk of the seed at maturity. The cells in the endosperm are very rich in starch. For example, starch accounts for 70–80% of the dry weight of the mature maize kernel (Dinges et al., 2001). Understanding the way in which starch is made in grass endosperm is of fundamental biological interest and such knowledge may also benefit agriculture by providing the means to manipulate the quantity and quality of starch in cereal seeds. In this review, a feature of starch synthesis that is unique to cereal endosperms will be described and the evolutionary origins of the genes encoding this part of the pathway will be discussed.

Although the pathway of starch synthesis in grass endosperm has features that are unique and functionally important, it also has much in common with that in the starch-synthesizing organs of other plant species. Before introducing the cereal-endosperm-specific features, the nature and proposed evolutionary origins of the pathway of starch synthesis in plant cells will first be described in general. In non-photosynthetic, starch-storing plant organs (including important crops such as legume seeds, potato tubers, and cassava roots), starch is synthesized from the products of photosynthesis (usually sucrose) imported into the developing storage organ from the leaves. Sucrose catabolism in the cytosol and mitochondria produces the substrates for starch synthesis, glucose 6-phosphate and ATP. These are imported into the plastids from the cytosol via specific translocators in the plastid envelope: namely the glucose 6-phosphate/phosphate transporter and the ATP/ADP transporter (Neuhaus and Wagner, 2000). Inside the plastid, glucose 6-phosphate is converted to glucose 1-phosphate by phosphoglucomutase and then ADPglucose is made from glucose 1-phosphate and ATP by ADPglucose pyrophosphorylase (AGPase) (Fig. 1A). The glucose 6-phosphate/phosphate transporter may also be capable of transporting glucose 1-phosphate into the plastids for starch synthesis (Tetlow et al., 1996). ADPglucose in the plastid is used by multiple, distinct isoforms of starch synthase to elongate
Fig. 1. The pathways of starch synthesis in a typical non-photosynthetic cell (e.g. from potato tuber) (A) and in a cereal endosperm cell (B) are shown. The cytosolic and plastidial compartments are indicated. Abbreviations for enzymes (italic, grey) are SuSy, sucrose synthase; UGPase, UDPglucose pyrophosphorylase; PGM, phosphoglucomutase; FK, fructokinase; PGI, phosphoglucomutase isomerase; PPase, pyrophosphatase; AGPase, ADPglucose pyrophosphorylase; SS, starch synthase; SBE, starch-branching enzyme. The transporters in the plastidial inner membrane are shown as circles: black circle, ADPglucose/ADP transporter; white circle, glucose 6-phosphate/phosphate transporter. Other abbreviations are PPI, pyrophosphate, Pi, phosphate.

the starch polymers amylose and amyllopectin. These polymers are then branched by multiple isoforms of starch-branching enzyme. The synthesis of starch granules also requires other enzymes, including isoamylase, and processes such as granule initiation and glucan turnover. The synthesis of starch in chloroplasts is similar to that in non-photosynthetic organs except that glucose 6-phosphate and ATP are made photosynthetically within the plastid. For detailed descriptions of starch-polymer and starch-granule synthesis, see Kossmann and Lloyd (2000), Ball and Morell (2003), Tomlinson and Denyer (2003), and Hannah and James (2008).

Starch may have first appeared in nitrogen-fixing, photosynthetic prokaryotes related to the present-day group V cyanobacteria (Deschamps et al., 2008a). To protect nitrogenase from damage by oxygen produced in photosynthesis, this type of cyanobacteria fixes nitrogen only at night. Storage polysaccharides synthesized via photosynthesis in the light are used to fuel nitrogen fixation in the dark. Whereas other bacteria synthesize glycogen as a storage polysaccharide, this group of cyanobacteria synthesize large, insoluble polysaccharide granules resembling starch granules (Schneegurt et al., 1997; Nakamura et al., 2005). The advantage of starch-like granules may be that, unlike glycogen, they are osmotically inert and this facilitates the accumulation of polysaccharide in the cell in large amounts.

Plastids are derived from cyanobacteria (Mereschkowsky, 1905). The initial endosymbiotic event is proposed to have involved a glycogen-synthesizing eukaryotic host cell and a starch-synthesizing prokaryotic symbiont, perhaps an ancestor of the present-day group V cyanobacteria (Deschamps et al., 2008a). Recent phylogenetic analyses suggest that the plastidial pathway of starch synthesis found in all extant higher plants and green algae (the Chloroplastidae) arose through a complex sequence of changes in the nature and location of storage glucan synthesis. Following the endosymbiotic event, it is proposed that there was a transfer of the genes for starch metabolism from the symbiont to the host nucleus, leading to a loss of starch synthesis from the symbiont and alteration of glucan synthesis in the host (now the cytosol) from glycogen to starch. The substrate for starch synthesis in the cytosol was probably UDPglucose: ADPglucose synthesis remained in the plastid, although the fate of ADPglucose synthesized in the plastid at this time is not clear. Subsequently, but still at an early stage in the evolution of the Chloroplastidia, the enzymatic machinery for starch metabolism was relocated back into the symbiont (now the the plastid) (Deschamps et al., 2008b, c). The starch biosynthetic enzymes that are now present in higher plants are remarkably similar in isoform type and functionality to those present in green algae (Deschamps et al., 2008a). This suggests that the pathway of starch synthesis and the genes required to encode it have remained relatively unchanged since these lineages diverged from a common ancestor.

Despite dramatic changes in the subcellular location of starch metabolism in the early stages of the evolution of the Chloroplastidia, the location of ADPglucose synthesis probably remained constant. ADPglucose was synthesized by AGPase in the free-living cyanobacterium and, after endosymbiosis, has continued to be synthesized in the symbiont/plastid only. ADPglucose in the cyanobacterium was committed to starch synthesis and it continued to be used for this purpose from the initial endosymbiotic event to the present day (Deschamps et al., 2008b). This conservation of plastidial ADPglucose synthesis contrasts with the relatively recent evolution of cytosolic ADPglucose synthesis in the grasses. Grasses (Poaceae) are monocots that evolved approximately 70 million years ago (mya) at the beginning of our current geological era, the Cenozoic. They appeared during the period of huge global environmental change that followed the end of the Jurassic period.
The pathway of starch synthesis in cereal endosperm

ADPglucose is synthesized in plastids in all plants, including cereals. However, in the developing endosperm cells of cereals, ADPglucose is synthesized in the cytosol as well as in the plastids. Different isoforms of AGPase are present in these two compartments (Fig. 1B; Table 1). The ADPglucose synthesized in the cytosol is imported into the plastid for starch synthesis and as far as we know it has no other metabolic fate in the endosperm cell. Import into the plastids occurs via an ADPglucose/ADP antiporter (ADPglucose transporter) that is found in grass endosperm only. Thus, grass endosperm cells have two separate ways of providing ADPglucose for starch synthesis, using plastidial and cytosolic isoforms of AGPase. The grass-endosperm-specific pathway via cytosolic AGPase and the ADPglucose transporter will be referred to as the ‘alternate pathway’. Evidence that the pathway of starch synthesis in cereal endosperm might be different from that in other species first appeared in the mid-1990s. Prior to this, it was accepted that AGPase was exclusively plastidial in all tissues and species. AGPase had been demonstrated to be plastidial in a range of plant organs (soybean suspension cultures: Macdonald and ap Rees, 1983; oilseed embryos: Kang and Rawsthorne, 1994; pea embryos: Denyer and Smith, 1988; pea roots: Trimming and Emes, 1993) and AGPase was widely used as a ‘plastid marker enzyme’ to quantify the distribution of stromal enzymes in cell fractionation studies. An early indication that AGPase in cereal endosperm might be cytosolic came from the work of Giroux and Hannah (1994). They used a rabbit reticulocyte system to synthesize in vitro the large and small subunits responsible for most of the AGPase activity in the maize endosperm—encoded by the Shrunken2 (Sh2) and Brittle 1 (Bt1) genes, respectively. The masses of the in vitro synthesized subunits were identical to the masses of the subunits in maize endosperm. Plastidial proteins synthesized in vitro are expected to be larger than their in vivo counterparts because transit peptides are cleaved from proteins entering plastids in vivo. Thus, this result indicated that the SHRUNKEN2 (SH2) and BRITTLE2 (BT2) proteins might not be located inside plastids in vivo. Interestingly, almost a decade earlier, similar evidence for the lack of a transit peptide in the AGPase subunits of wheat endosperm had been published (Krishnan et al., 1986). At this time, these authors dismissed the idea that AGPase in wheat endosperm might be cytosolic because of the weight of evidence that indicated that AGPase was universally plastidial.

Additional support for the cytosolic location of AGPase in maize endosperm came in 1996, when studies of the brittle1 (bt1) mutant of maize showed that it accumulated more than 13 times the normal amount of ADPglucose but was unable to synthesize the normal amount of starch (Shannon et al., 1996). The reduction in starch content (to 60% normal) was not because of a defect in the pathway of starch synthesis beyond ADPglucose: activities of starch synthase and starch-branching enzyme in bt1 mutant endosperms were normal. Shannon and colleagues had shown previously that the BRITTLE1 (BT1) protein is a plastidial

Table 1. The subcellular and tissue locations of components of the alternate pathway

| Clade       | Species | Protein | Subcellular location | Tissue location | Reference       |
|-------------|---------|---------|----------------------|----------------|-----------------|
| PANT Type 1 | Arabidopsis (At) | BT1 (NP_194966) | Plastid envelope | Leaf | Kirchberger et al., 2008 |
| PANT Type 2 | Maize (Zm) | BT1 (NP_001105889) | Plastid envelope | Endosperm | Cao et al., 1995 |
| Plastidial ADP-glucose transporter | Maize (Zm) | NST1 (AAT12275) | Plastid envelope | Endosperm | Patron et al., 2004 |
| AGPase SSU type 1 | Maize (Zm) | BT2 (product of transcript a) (NP_001105038) | Cytosol | Endosperm | Tsai and Nelson, 1966; Denyer et al., 1996 |
|  | barley (Hv) | AGPS1a (product of transcript a) (AAU06190) | Cytosol | Endosperm | Thorbjørnsen et al., 1996b |
|  | rice (Os) | AGPS1b (product of transcript b) (AAU06191) | Plastid | Leaf | Rösti et al., 2006 |
| AGPase SSU type 2 | rice (Os) | AGPS2b (NP_001061603) | Cytosol | Endosperm | Lee et al., 2007 |
| AGPase LSU type 1 | rice (Os) | AGPS2a (product of cDNA AK071826) | Plastid | Endosperm | Lee et al., 2007 |
| AGPase LSU type 1 | maize (Zm) | SH2 (NP_001112104) | Cytosol | Endosperm | Tsai and Nelson, 1966 |
| AGPase LSU type 2 | rice (Os) | AGPL1 (NP_001056424) | Plastid | Endosperm | Lee et al., 2007 |
| AGPase LSU type 3 | rice (Os) | AGPL4 (NP_001059276) | Plastid | Endosperm | Lee et al., 2007 |
envelope protein related to mitochondrial ATP/ADP transporters, and that plastids isolated from bt1 endosperm have a reduced capacity to synthesize starch from exogenously-supplied ADPglucose (Li et al., 1992; Cao et al., 1995).

Together, these data suggested that ADPglucose is synthesized in the cytosol and accumulates in bt1 mutants because they lack the capacity to transport ADPglucose into the plastid. Independent evidence for a cytosolic AGPase in barley endosperm came from Thorbjørrnsen et al. (1996a) who identified two different transcripts encoded by a single AGPase small subunit gene. One of these transcripts encoded a transit peptide sequence but the other did not. Thus, the single AGPase gene was predicted to encode both a plastidial and a cytosolic AGPase small subunit. Orthologous genes encoding two different transcripts have subsequently been identified in a range of cereal species (Sikka et al., 2001; Burton et al., 2002; Johnson et al., 2003; Rösti and Denyer, 2007).

A direct demonstration that AGPase activity exists in both the cytosol and the plastids in cereal endosperm finally came from plastid isolation experiments, first in barley (Torbjørrnsen et al., 1996b) and maize (Denyer et al., 1996) and later in a range of cereal species (maize: Shannon et al., 1998; rice: Sikka et al., 2001; wheat: Tetlow et al., 2003). Importantly these experiments allowed the estimation of the relative activity of the cytosolic and plastidial forms of AGPase in developing endosperm. In all cereals tested to date, 85% (barley: Thorbjørrnsen et al., 1996b) to 95% (maize: Denyer et al., 1996) of the AGPase activity in the endosperm is cytosolic. The same kinds of experiments confirmed that AGPase activity is present in plastids but not in the cytosol in other types of starch-synthesizing organs and in cereal mutants that lack the major form of AGPase (e.g. brittle2 mutants of maize: Denyer et al., 1996).

How widespread amongst plant species is the alternate pathway?

The distribution of the alternate pathway among species was investigated by Beckles et al. (2001) who reasoned that measurement of the ADPglucose:UDPglucose ratio would provide a simple means to identify starch-storing organs likely to possess this pathway. This is because the reaction catalysed by AGPase in the cytosol is close to equilibrium, as is the reaction catalysed by cytosolic UDPglucose pyrophosphorylase (UGPase). These reactions are closely coupled: glucose 1-phosphate and inorganic pyrophosphate (PPi) are reactants in both (Fig. 1B). By contrast, the AGPase reaction in the plastid is far removed from equilibrium because ADPglucose and pyrophosphate are consumed by the effectively irreversible reactions catalysed by starch synthase and alkaline inorganic pyrophosphatase, respectively. Consequently, Beckles et al. (2001) expected the ratio of ADPglucose to UDPglucose in tissues with predominantly cytosolic ADPglucose synthesis to be very different from that in tissues with plastidial ADPglucose synthesis. Tests on tissues in which the distribution of AGPase activity was known showed this expectation to be correct: the ratio in tissues with predominantly cytosolic AGPase activity was about 1:2 whereas the UDPglucose levels were 5–100-fold higher than those of ADPglucose in tissues with exclusively plastidial AGPase. Extending these measurements to a wide range of organs in which the distribution of AGPase activity was not known, Beckles et al. (2001) established that the endosperms of all monocot species examined (all of which were Poaceae) probably synthesized ADPglucose in the cytosol as well as in the plastids, whereas all dicot organs examined (including seeds, fruits, tubers, and corms) probably synthesized ADPglucose exclusively in the plastids. Thus the alternate pathway is a feature of a particular subset of plants, including the grasses, and not a widespread feature of starch-accumulating plants in general.

The alternate pathway has subsequently been found in cereals from different branches of the grass family (e.g. rice: Sikka et al., 2001; wheat: Tetlow et al., 2003; maize: Denyer et al., 1996) suggesting that it evolved in a monocot ancestor of the grasses. Starch-rich endosperms are found in a subset of monocot species, in the commelinid clade. This clade includes the grasses and their close relatives the rushes and sedges, and also the gingesters and bananas. Other monocots (including lilies, orchids, pineapples and palms) have endosperm that contains little starch. Our investigations of the origins of the genes encoding the alternate pathway (see below) lead us to conclude that it is restricted to the grasses, and not found in other commelinids or in monocots generally. The whole-genome duplication that enabled the evolution of the alternate pathway is thought to have occurred after the grass lineage separated from those of the other commelinids. If correct, then the origins of the pathway do not coincide with the appearance of starchy endosperm, but rather with the later evolution of the grasses.

The functional significance of the alternate pathway

The importance of the alternate pathway in cereals in supplying ADPglucose for starch synthesis has been revealed by investigations of mutants that lack particular components of the pathway. Seeds of mutants that lack either cytosolic AGPase activity (maize: Tsai and Nelson, 1966; Denyer et al., 1996; barley: Johnson et al., 2003; rice: Lee et al., 2007) or the ADPglucose transporter (maize: Tobias et al., 1992; barley: Patron et al., 2004) have reduced starch contents and are shrivelled or shrunken at maturity. In the barley mutant lacking cytosolic AGPase activity, the activity of the plastidial AGPase is approximately the same as in normal barley (Johnson et al., 2003), but the starch content is reduced to 44% of that in the parent line (Tester et al., 1993). Thus, the plastidial AGPase alone is not sufficient to support the normal rate of starch synthesis. This analysis of mutants confirms that, in maize and barley, and therefore probably in many other grass species as well,
most of the flux of carbon to starch in the endosperm is supported by the alternate pathway.

The dominance of the alternate pathway is a feature of the endosperms of wild grasses as well as domesticated cereals. This is indicated by measurements of the ADPglucose to UDPglucose ratios in a range of wild and domesticated grasses (Beckles et al., 2001), and has been confirmed recently by our direct measurement of plastidial and cytosolic AGPase activities. We found in both wild grasses (Hordeum murinum, Hordeum spontaneum, Triticum dicoccoides) and the domesticated cereals, barley (Hordeum vulgare) and wheat (Triticum aestivum) that the activity of cytosolic AGPase was higher than that of plastidial AGPase, although the precise ratio varied between species and with growth conditions (Rösti, 2006).

The conservation and dominance of the alternate pathway throughout the grass family, and during domestication of the cereals, suggest that it has a selective advantage. Our hypothesis is that the synthesis of ADPglucose in the cytosol is more energy-efficient than its synthesis in the plastids. Energy efficiency is important because starch synthesis is a major metabolic activity in starch-rich plant organs such as grass endosperm. For every molecule of ADPglucose synthesized via AGPase, one molecule of ATP is consumed. For ADPglucose synthesis in non-photosynthetic plastids, this ATP has to be imported from the cytosol via the ATP/ADP transporter in the plastid envelope. For potato tubers, in which ADPglucose synthesis is exclusively plastidial, there is good evidence that this adenylate transporter exercises strong control over the rate of starch synthesis. Transgenic potatoes with reduced levels of the transporter make less starch than normal potatoes and, conversely, tubers with elevated levels of the transporter actually make more starch (Tjaden et al., 1998).

Direct manipulation of ATP levels in the plastid – through changing the activity of plastidial adenylate kinase – also strongly affects the rate of starch synthesis (Regierer et al., 2002). Direct measurements of the ATP:ADP ratio in subcellular compartments of tubers showed that the cytosolic ratio is more than five times greater than the plastidial ratio (Farre et al., 2001). In cereal endosperm, as in potato tubers, the availability of ATP for starch synthesis may be greater in the cytosol than in the plastid. The alternate pathway may offer a selective advantage by drawing upon the relatively abundant cytosolic pool of ATP rather than the more limited pool of ATP in the plastid.

The problem of ATP supply for starch synthesis is compounded by the relatively low oxygen levels in dense, bulky tissues such as potato tuber and cereal endosperm (van Dongen et al., 2004; Rolletschek et al., 2002). An adaptive response to low oxygen involves the co-ordinated restriction of both ATP production (via respiration) and ATP consumption (via a range of biosynthetic processes) (Geigenberger, 2003). Low oxygen concentrations lead to a reduction in the rate of starch synthesis in potato tubers and also to the induction of energy-conserving pathways (such as sucrose degradation via sucrose synthase rather than via invertase).

The alternate pathway in cereal endosperm is, in theory, an energy-conserving pathway due to the conservation of pyrophosphate. In the AGPase reaction, some of the energy of the ATP molecule that is consumed is conserved in the bond of the pyrophosphate that is produced. The cytosol lacks a pyrophosphatase (Weiner et al., 1987), hence pyrophosphate produced by AGPase in the cytosol could be used to drive other reactions such as glucose 1-phosphate production by UDPglucose pyrophosphorylase (Kleczkowski, 1996) and the pumping of protons across the tonoplast into the vacuole (Rea and Sanders, 1987). By contrast, pyrophosphate produced by AGPase in the plastids is immediately hydrolysed to phosphate by the plastidial alkaline inorganic pyrophosphatase (Gross and ap Rees, 1986; Weiner et al., 1987).

The presence of the alternate pathway in grass endosperm may therefore, enable the production of starch in a more energy-efficient fashion than is possible in tissues in which ADPglucose is produced exclusively in the plastids. Whether increased energy efficiency was sufficient to drive selection of the alternate pathway in grasses millions of years ago is difficult to investigate. The increase in fitness due to the alternate pathway may be very small, and may occur at some stages of development only or in some environments only. Very small differences in fitness of this sort may bring about evolutionary changes over long periods of time, but may be impossible to detect and assess in an experimental time-frame and with access to modern grass species only. The same selection pressure towards increased energy efficiency presumably applies to other species with dense, non-photosynthetic starch-storing organs. The reason why a more efficient mechanism for ADPglucose synthesis has not evolved in the plant kingdom more than once is not clear, although opportunities for the contemporaneous evolution of multiple novel genes are likely to have been limited.

The origin of the genes encoding the alternate pathway in grasses

The alternate pathway requires three genes: two to encode the large and small subunits of the cytosolic AGPase and a third to encode the ADPglucose transporter. Homologous genes are present in a wide range of cereals and grasses, so are likely to be a general feature of the Poaceae. To shed light on the origin of these three genes, their phylogenetic relationships to other genes in the Poaceae, and in higher plants generally, were investigated (Fig. 2). For this work, the grass species for which full genome sequences were available (rice, Brachypodium, Sorghum) are particularly useful as all homologues can be identified.

The ADPglucose transporter

The best-characterized plastidial ADPglucose transporters are those of maize and barley. These are encoded by the Bt1 (Cao et al., 1995; Sullivan and Kaneko, 1995) and NST1 (Patron et al., 2004) genes, respectively. This type of transporter exchanges ADPglucose for ADP (or AMP)
Fig. 2. Phylogenies of the ADPglucose transporters (A), small (B) and large (C) subunits of AGPase and the gene structure of the AGPase SSU (D). Figure 2D was redrawn with permission from Thorbjørnsen et al. (1996a, Biochemical Journal 313, 149–154, © the Biochemical Society, http://www.biochemj.org). For (B) the first exon sequences were excluded. The trees in (A–C) were generated using a parsimony algorithm (Program PROTPARS; Phylip version 3.67 software package). Numbers are bootstrap values as a percentage of 100 bootstrap replicates. Species names are abbreviated as follows: At, Arabidopsis thaliana; Bd, Brachypodium distachyon; Hv, Hordeum vulgare; Os, Oryza sativa; Pt, Populus trichocarpa; Sb, Sorghum bicolor; St, Solanum tuberosum; Ta, Triticum aestivum; Vv, Vitis vinifera; Zm, Zea mays. The Bd sequence data were produced by the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/. The names of the proteins are those published previously or they are taken from the name of the clade. The protein sequences or the DNA sequences (or Unigene assemblies) from which these were predicted are: (A) VvPANT1, CAO44212; PtPANT1, LG_VI000313; AtBT1, NP_194966; SbBT1, ABA81858; BdPANT1, Bradi2g34270; TaPANT1, BT009587; SbBT1-2, NP_001054775; SbPANT1, Sb09g005250; ZmBT1, NP_001105889; SbBT1, Sb04g007010; OsBT1-1, NP_001046230; HvNST1, AAT12275; TaBT1, BT008958; BdPANT2, Bradi1g36670; HvPANT2, Hv.2334; TaPANT2, Ta.10612; OsBT1-3, NP_001058009; ZmPANT2, AY104855; SbPANT2, Sq01g023470. (B) SbAPS1, Sb07g012320; ZmL2, AAK69628; SbBT1, NP_001105038; TaAPS1, CAA46879; HvAPS1, CAA48679; BdAPS1, Bradi3g22330; OsAPS1, NP_001061603; OsAPS2, NP_001062808; ZmAGP2, NP_001105178; SbAPS2, Sb02g020410; TaAPS2, AAU50665; HvAPS2, AAO16183; BdAPS2, Bradi4g27570. (C) BdAPL1, Bradi1g09540; OsAPL1, NP_001051184; SbAPL1, Sb01g008940; ZmAPL1, NP_00106017; OsAPL2, NP_001043654; SbAPL2, ABK97530; ZmSH2, NP_001121104; BdAPL3, Bradi2g14970; OsAPL3, NP_001056424; SbSH2, AAB94012; ZmAPG1, NP_001105717; BdAPL4, Bradi1g53500; OsAPL4, NP_0011059276; SbAPL4, Sb02g007310; ZmAPL4, NP_001106058.
across the plastid envelope (Möhlmann et al., 1997; Bowsher et al., 2007; Kirchberger et al., 2007). In maize (Cao et al., 1995) and barley (Patron et al., 2004) endosperm, the ADPglucose transporter is one of the most abundant proteins in the plastid envelope.

Phylogenetic analysis has shown that the ADPglucose transporter is a member of the Mitochondrial Carrier protein Family (MCF) (Patron et al., 2004; Tjaden et al., 2004). The MCF is a large family of proteins that shuttle a wide variety of metabolites (e.g. ATP/ADP, basic amino acids, carnitine, dicarboxylic acids, tricarboxylic acids, and phosphate) mostly across the inner mitochondrial membrane, although a few proteins in the family are targeted to other organelles (Picault et al., 2004; Palmieri, 2004). The family includes the well-characterized human mitochondrial ATP/ADP transporters AAC1, AAC2, and AAC3 (Palmieri, 2004). The ADPglucose transporter has no significant similarity at the DNA or protein levels to the nucleotide-sugar transporters of the Golgi/ER that belong to the drug/metabolite transporter superfamily (Patron et al., 2004).

Within the MCF, the ADPglucose transporter clade is closely related to two other clades of transporters that are known or assumed to be plastidial adenine nucleotide transporters (PANTs) (Fig. 2A; Table 1). One of these clades (PANT1) contains both dicot and monocot transporters whilst the other clade (PANT2) contains monocot transporters only. Unlike the ADPglucose transporter genes that are expressed strongly only in cereal endosperm tissues, the PANTs in Arabidopsis (Kirchberger et al., 2008) and rice (Toyota et al., 2006) are expressed at low levels in several tissues.

The PANT1s from Arabidopsis (Kirchberger et al., 2008), potato (Leroch et al., 2005), and rice (Kirchberger et al., 2007) have been functionally tested. They are uniporers and they can carry AMP, ADP, and ATP but, despite similarity in sequence to ADPglucose transporters, they cannot carry ADPglucose at a significant rate. Analysis of Arabidopsis plants with reduced activity of PANT1 (known as BT1 or SHS1) indicates that its function is to allow the net export of adenylates from their site of synthesis in the plastid to the cytosol, and thence to other cellular compartments (Inan et al., 2007; Kirchberger et al., 2008). Strong reduction of expression of the Arabidopsis PANT1 drastically reduces growth, a phenotype that can be partially compensated by supplying adenosine to the plastidial adenine nucleotide sugar transporter superfamily (Patron et al., 2004).

The monocot-specific PANT2 and the ADPglucose transporter genes are located in colinear segments of the rice and sorghum genomes (data not shown) suggesting that they arose by segmental or whole-genome duplication. One of the PANT2 proteins, that in rice encoded by OsBT1-3, has been functionally tested. Like the PANT1s, this PANT2 protein transports adenine nucleotides but is unable to transport ADPglucose (Kirchberger et al., 2007). By extension, it is assumed that all of the PANT1 and PANT2 proteins transport adenine nucleotides but not ADPglucose.

Our phylogenetic analysis (Fig. 2A; data not shown) suggests that the PANTs evolved from a transporter of the MCF class prior to the separation of the monocot and dicot lineages. Later, in the monocots but not in the dicots, there was a duplication which gave rise to the PANT1 and PANT2 clades. Later still, and specifically in the grasses (see below for evidence), the ADPglucose transporter gene evolved from a duplicate of the ancestral PANT2 gene. Diversification of the ADPglucose transporter gene from its PANT2 progenitor involved a change in pattern and level of expression as well as a change in transport properties. Given the very wide range of metabolites transported by members of the MCF, the modifications to the coding sequence required for the change in substrate specificity from adenine nucleotide to adenine nucleotide sugar were probably minimal. The change from uniporter to antiporter may also require only trivial changes in sequence. Some members of the MCF transporter family can switch from one transport mode to the other after treatment with reducing agents suggesting that minor modification of thiol groups are sufficient to induce changes in transport direction (Dierks et al., 1990). It is not yet clear what differences between the PANT2 and ADPglucose transporter genes account for their differences in expression.

The large and small subunits of AGPase

Both plastidial and cytosolic AGPases are composed of small and large subunits (SSU and LSU) that are encoded by different nuclear genes. The active protein is a tetramer of two LSU and two SSU (Jin et al., 2005). The genes for plastidial AGPases encode transit peptides to target the newly-synthesized subunits to the plastids. The cytosolic AGPase subunits have no requirement for targeting sequences. Thus, a gene encoding a cytosolic AGPase subunit could, in theory, evolve from a gene encoding a plastidial AGPase subunit if the transit peptide sequence was lost or became dysfunctional. To test whether this scenario is essentially correct, small and large subunit genes and protein sequences were compared (Fig. 2B, C).

Phylogenetic analysis of the AGPase subunits showed that the grasses have two types of SSU (Type 1 and Type 2; Fig. 2B) and four types of LSU (Types 1–4; Fig. 2C). In barley, both types of SSU gene encode plastidial proteins. The major plastidial SSU in the leaves is encoded by the Type 1 gene (Rösti et al., 2006) and that in the seeds by the Type 2 gene (Johnson et al., 2003). The Type 1 SSU gene also encodes the cytosolic SSU in the endosperm of barley (Thorbjørnsen et al., 1996b); thus it produces two transcripts encoding proteins expressed in different tissues and targeted to different subcellular compartments (Fig. 2D). It does so by the use of an alternate first exon that does not specify a transit peptide (Thorbjørnsen et al., 1996a). The Type 1 SSU genes in other cereals also produce two transcripts with predicted functions similar to those in barley (Rösti and Denyer, 2007). This suggests that the gene encoding the cytosolic SSU in cereals evolved from a duplicate of an ancestral gene encoding a plastidial SSU.
by acquisition of the alternate first exon and not by loss of the transit peptide sequence. The sequence of the alternate first exon is not significantly similar to any other known sequence and so its evolutionary origin is unknown.

Of the four types of LSU, one is presumed to be cytosolic and the other three to be plastidial. In maize endosperm, the Type 2 LSU, SH2 is required for the major AGPase activity (Tsi and Nelson, 1966), which is now known to be cytosolic (Denyer et al., 1996). The Type 2 LSU in rice is also cytosolic (Lee et al., 2007). Therefore the Type 2 LSUs in all grasses are assumed to be cytosolic. The three other types of LSU in rice are all plastidial and are expressed in different organs: Type 1 in the leaves, Type 3 in the seeds (in both the endosperm and the embryo), and Type 4 at a low level in both seeds and leaves (Ohdan et al., 2005; Lee et al., 2007). The subcellular locations of these LSUs in other grasses are not proven, but they are assumed to be plastidial. Consistent with this, transit peptide sequences are predicted for some of the putative plastidial LSUs. For example, a transit peptide of 47 amino acids is predicted for the maize Type 3 LSU encoded by Agp1.

Our analysis (Fig. 2C) shows that the Type 2 cytosolic LSUs are most closely related to the Type 3 LSUs. To test whether the Type 2 cytosolic LSUs evolved from the Type 3 plastidial LSUs by loss of a transit peptide, the gene structures and the N-terminal protein sequences were compared. In rice and sorghum, for example, the Type 3 (plastidial) and Type 2 (cytosolic) proteins are similar in length and the genes encoding them all have 15 exons. This suggests that the cytosolic LSU gene evolved from a duplicate of an ancestral gene encoding a Type 3 plastidial LSU by loss-of-function of the transit peptide cleavage site and not by loss of the transit peptide sequence.

**The role of the grass whole-genome duplication**

As shown above, each of the three genes required for the alternate pathway arose via duplication of an existing gene. These duplication events could have occurred separately but another possibility is that they occurred all at the same time, as the result of a whole-genome-duplication event. Whole-genome duplications provide opportunities for extensive evolutionary changes and are thought to have played an important role in the evolution of many plant and animal lineages (Eckhardt, 2001; 2004). Various genomic analyses in rice, sorghum, and maize have provided evidence for an ancestral whole-genome duplication that predated the divergence of the different grass lineages (Paterson et al., 2004; Wang et al., 2005; Yu et al., 2005; Wei et al., 2007). As a result, grass genomes consist predominantly of pairs of duplicated chromosomal segments (e.g. rice; Fig. 3) and many of today's diploid species, such as rice, are, in fact, paleopolyploids (Eckhardt, 2001; Blanc and Wolfe, 2004).

Examination of the chromosomal locations of the three genes encoding the alternate pathway shows that each is embedded in one of the duplicated segments of DNA produced by the whole-genome-duplication event (Fig. 3). The progenitor genes (the adenine nucleotide transporter gene and the plastid-targeted small and large subunit genes of AGPase) are located in the three corresponding co-linear segments. This finding suggests the following scenario for the evolution of the alternate pathway. Immediately after duplication of the genome, there would have been two sets of identical genes, i.e. identical pairs of adenine nucleotide transporter genes and plastidial small and large subunit genes. This redundancy meant that one gene in each pair was free to diverge in sequence (enabling acquisition of a new function) whilst the other gene retained its original function.

By comparing the sequences of all of the duplicate genes in rice, it has been possible to date the whole-genome-duplication event to between 60 and 70 mya (Paterson et al., 2004; Wang et al., 2005). This date is well after the divergence of the monocots and dicots (200 mya; Wolfe et al., 1989) and later than, or coincident with, the appearance of the grass lineage itself (which is variously estimated at 55–70 mya; Kellogg, 2001 or 80–90 mya; Janssen and Bremer, 2004). This makes it very unlikely that the alternate pathway exists in any non-grasses, including other Commelinids. This issue may be resolved when the banana genome sequence is completed (http://www.musageneomics.org). Then it will be possible to compare this Commelinid genome and its AGPase and PANT genes with those of the grasses.

More accurate dating of the appearance of the alternate pathway comes from consideration of evolutionary
The role of the maize whole-genome duplication

Examination of the genes encoding small subunits of AGPase in maize reveals a more complex evolutionary picture than for any other grass species. Modern maize has three genes encoding small subunits of AGPase, whereas other cereals and grasses have two (Rösti and Denyer, 2007). The maize L2 gene (also called AgpsLzm) encodes the small subunit found in leaf chloroplasts (Prioul et al., 1994), Bt2 encodes the major, cytosolic small subunit in the endosperm (Tsai and Nelson, 1966; Bae et al., 1990; Denyer et al., 1996), and Agp2 (also called Agpsemzm) encodes the minor (presumably plastidial) small subunit in the seed (Giroux and Hannah, 1994).

There is evidence that the Bt2 and L2 genes are paralogues (Rösti and Denyer, 2007), produced during a maize-specific whole-genome duplication that occurred approximately 50 mya (Wilson et al., 1999; Swigoňová et al., 2004a; Wei et al., 2007). This is after maize diverged from its close relative sorghum, 12 mya (Swigoňová et al., 2004b). Examination of the regions of the genome containing Bt2 and L2 showed extensive co-linearity and careful examination of the structure of the L2 and Bt2 genes shows that they are both Type 1 SSU genes with two alternate first exons (Rösti and Denyer, 2007).

Following duplication, L2 and Bt2 diverged in function. Only one of the alternate first exons of L2 is functional: that encoding the plastidial SSU that is expressed in the leaves. The other exon contains a single base deletion (inducing a frameshift). By contrast, the Bt2 gene has two functional alternate first exons and produces the expected two mRNAs that encode cytosolic and plastidial SSUs. However, the plastidial transcript encoded by Bt2 appears to contribute very little to the leaf AGPase activity since a mutant lacking Bt2 has a near-normal AGPase activity in its leaves (Rösti and Denyer, 2007). Thus, the two Type 1 paralogous SSU genes in maize has each taken one of the two functions of the ancestral gene; a process known as sub-functionalization. This divergence of function of L2 and Bt2 has occurred in less than 5 million years and it may not yet be complete: one of the two transcripts of Bt2 is functionally redundant and it may in time be lost entirely.

Immediately following duplication of the maize genome, there would have been a pair of identical Type 2 SSU genes. The fact that modern maize contains only one Type 2 SSU gene, Agp2, suggests that its parologue has been lost or is dysfunctional—a common occurrence following whole-genome duplication (Eckhardt, 2001; Wang et al., 2005). The other genes encoding the alternate pathway in maize have not yet been examined in sufficient detail to discover whether their function was also affected by the maize whole-genome-duplication event.

The evolution of the alternate pathway

It is intriguing to speculate about the order of evolution of the genes required to encode the cytosolic AGPase and the ADPglucose transporter. It is difficult to imagine that there would be any selective advantage in having a cytosolic AGPase in the absence of an ADPglucose transporter and vice versa. However, the ancestor of the grasses may already have possessed a protein in the plastid envelope capable of transporting ADPglucose. Excess ADPglucose did not significantly reduce the transport of labelled ADP by potato (Leroch et al., 2005) and Arabidopsis (Kircherberger et al., 2008) PANTs heterologously in E. coli. However, to our knowledge, the ability of the PANTs to transport ADPglucose has not been tested directly. The possibility that they have (or had when the alternate pathway evolved) a low capacity to transport ADPglucose cannot be ruled out. Thus ADPglucose produced in the cytosol may have been able to contribute to starch synthesis in the plastid prior to the evolution of a specific ADPglucose transporter.

Acquisition of a cytosolic small subunit of AGPase alone may have been sufficient to allow some ADPglucose production in the cytosol. The small subunit of AGPase from some species is active in the absence of the LSU. When the potato tuber (Ballicora et al., 1995; Salomone et al., 2000) and barley endosperm (Doan et al., 1999) SSUs are expressed heterologously, both have high specific activity. Although a large number of barley mutants with low starch content have been identified and studied, including one lacking the cytosolic SSU (Johnson et al., 2003), no mutant lacking the cytosolic LSU has been identified. Thus it remains possible that the cytosolic SSU of some species, including barley, is active in vivo in the absence of the LSU.

Taken together, these results suggest that, in the ancestor of the grasses, the initial event in the evolution of the
alternate pathway may have been the acquisition of the cytosolic small subunit of AGPase. This involved the insertion, upstream of an existing SSU gene, of a small sequence that was capable of acting as an alternate first exon. Some of the ADPglucose produced by the cytosolic SSU may have been transported into the plastids via a PANT. Provided that this flux conferred a selective advantage to the plant (perhaps in terms of energy efficiency, as suggested above), there would have been a selection pressure for an increase in the capacity both to synthesize ADPglucose in the cytosol and to transport ADPglucose into the plastids. The redundant PANT and AGPase LSU genes, produced by the whole-genome-duplication event, were available to provide this additional capacity.

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