A Low-Starch Barley Mutant, Risø 16, Lacking the Cytosolic Small Subunit of ADP-Glucose Pyrophosphorylase, Reveals the Importance of the Cytosolic Isoform and the Identity of the Plastidial Small Subunit

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To provide information on the roles of the different forms of ADP-glucose pyrophosphorylase (AGPase) in barley (Hordeum vulgare) endosperm and the nature of the genes encoding their subunits, a mutant of barley, Risø 16, lacking cytosolic AGPase activity in the endosperm was identified. The mutation specifically abolishes the small subunit of the cytosolic AGPase and is attributable to a large deletion within the coding region of a previously characterized small subunit gene that we have called Hv.AGP.S.1. The plastidial AGPase activity in the mutant is unaffected. This shows that the cytosolic and plastidial small subunits of AGPase are encoded by separate genes. We purified the plastidial AGPase protein and, using amino acid sequence information, we identified the novel small subunit gene that encodes this protein. Studies of the Risø 16 mutant revealed the following. First, the reduced starch content of the mutant showed that a cytosolic AGPase is required to achieve the normal rate of starch synthesis. Second, the mutant makes both A- and B-type starch granules, showing that the cytosolic AGPase is not necessary for the synthesis of these two granule types. Third, analysis of the phylogenetic relationships between the various small subunit proteins both within and between species, suggest that the cytosolic AGPase single small subunit gene probably evolved from a leaf single small subunit gene.

In the endosperm of all of the species of grasses so far investigated, there are both cytosolic and plastidial forms of the enzyme of ADP-Glc pyrophosphorylase (AGPase). However, there are indications that there may be differences between species in the relative amounts of the plastidial and cytosolic activities of AGPase in the endosperm and in the nature of the genes encoding their subunits. Studies of mutants of maize (Zea mays) show that the cytosolic AGPase accounts for >95% of the total activity of AGPase in the endosperm (Denyer et al., 1996) and that this form is essential for normal rates of starch synthesis (Tsai and Nelson, 1966; Dickinson and Preiss, 1969). These studies also show that the plastidial and cytosolic AGPase are encoded by different pairs of large and small subunit genes. For example, mutations in maize at the Bt2 locus specifically affect the cytosolic single small subunit (SSU) of AGPase and eliminate the activity of AGPase in the cytosol, but they do not affect the plastidial AGPase SSU or activity (Giroux and Hannah, 1994; Hannah et al., 2001). For barley (Hordeum vulgare), the situation is less clear, but there is evidence to suggest that barley differs from maize in the following respects.

First, the plastidial activity as a proportion of the total AGPase activity is considerably higher in barley endosperm (15%; Thorbjørnsen et al., 1996b) than in maize (<5%; Denyer et al., 1996). In maize endosperm, we calculate from published measurements of the rate of starch synthesis and total AGPase activity in maize (Singletary et al., 1997) that the plastidial AGPase activity alone is not sufficient to account for the normal rate of starch synthesis. In contrast, in barley, the plastidial activity alone is sufficient (Torbjørnsen et al., 1996b). Thus although it seems unlikely, it is possible that the cytosolic AGPase activity is not actually required for normal rates of starch synthesis in the endosperm of barley.
Second, a SSU gene in barley produces two transcripts in the endosperm (Thorbjørnsen et al., 1996a). These encode two proteins, one with and one without a transit peptide. A gene with strong sequence identity and structure to that in barley also exists in wheat (Triticum aestivum; Ta.AGP.S.1; Burton et al., 2002b). Thus, unlike maize, a single gene could encode both the cytosolic and plastidial SSU of AGPase in wheat and barley endosperms. Contrary to this, our recent work indicated that the plastids of wheat contain a SSU protein that is encoded by a gene other than Ta.AGP.S.1 (Burton et al., 2002b). Thus there may be two SSU genes in wheat, but the second has yet to be identified, and the relative contribution of the two genes to the plastidial AGPase activity is unknown.

We reasoned that these major questions about AGPase in grass species other than maize could be addressed by the discovery and characterization of mutants lacking cytosolic AGPase activity in the endosperm. Such mutants might be expected to have a lower than normal starch content. A good place to search for suitable genetic variants of a grass species other that maize is in the existing collections of barley mutants. Low-starch mutants of barley exist that are not allelic to one another. Limited genetic and biochemical characterization of these suggests that within the low-starch group of barley mutants, there are at least eight separate complementation groups. Apart from the waxy mutants that carry mutations in the gene encoding granule-bound starch synthase I (e.g. Patron et al., 2002), efforts to identify the sites of the gene encoding granule-bound starch synthase I apart from the

we have recently shown that the barley mutants Risø 17 and Notch-2 have mutations in a gene encoding isoamylase (isa1), a starch-debranching enzyme (Burton et al., 2002a).

To discover whether any of the existing low-starch barley mutants lacked cytosolic AGPase activity, we screened for altered amounts of ADP-Glc and an altered ratio of ADP-Glc to UDP-Glc in the developing endosperms. The rationale for this approach was as follows. The endosperms of wild-type grasses, in which there are both plastidial and cytosolic AGPases, typically contain larger amounts of ADP-Glc than do organs in which AGPase is confined to the plastids (Beckles et al., 2001). In addition, the ratio of ADP-Glc to UDP-Glc is closer to 1 in the endosperms of grasses than in other plant organs. Thus a high ADP-Glc content and an ADP-Glc to UDP-Glc ratio approaching 1:1 can be taken to indicate the presence of cytosolic AGPase activity (Beckles et al., 2001). From this, we reasoned that a low-ADP-Glc content and a lower than normal ADP-Glc to UDP-Glc ratio in the endosperm of a mutant barley might indicate that the cytosolic AGPase was not present or was inactive.

RESULTS

Identification of a Mutant with Low ADP-Glc Content

We measured the ADP-Glc and UDP-Glc contents of developing endosperms of 12 to 37 mg fresh weight of the following previously identified low-starch mutant barley lines: Risø 13, Risø 16, Risø 17, Risø 29, Risø 527, Risø 1508 (Doll, 1983), and Notch-1.

Table 1. Comparison of the maximum catalytic activities of enzymes in crude extracts of developing endosperms

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity µmol min⁻¹ g⁻¹ fresh wt</th>
<th>Bomi</th>
<th>Risø 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc synthase</td>
<td>3.44 ± 0.17 (5)</td>
<td>5.40 ± 0.82 (4)</td>
<td></td>
</tr>
<tr>
<td>UDP-Glc pyrophosphorylase</td>
<td>54.88 ± 8.56 (4)</td>
<td>96.95 ± 4.59 (4)</td>
<td></td>
</tr>
<tr>
<td>Fructokinase</td>
<td>0.21 ± 0.02 (5)</td>
<td>0.45 ± 0.05 (4)</td>
<td></td>
</tr>
<tr>
<td>Glucokinase</td>
<td>0.35 ± 0.04 (5)</td>
<td>0.71 ± 0.04 (4)</td>
<td></td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>21.05 ± 5.09 (5)</td>
<td>69.15 ± 0.61 (3)</td>
<td></td>
</tr>
<tr>
<td>Phospho-Glc isomerase</td>
<td>17.38 ± 2.99 (5)</td>
<td>26.87 ± 5.53 (4)</td>
<td></td>
</tr>
<tr>
<td>ADP-Glc pyrophosphorylase</td>
<td>4.97 ± 0.34 (5)</td>
<td>1.56 ± 0.33 (3)</td>
<td></td>
</tr>
<tr>
<td>Soluble starch synthase</td>
<td>0.18 ± 0.02 (5)</td>
<td>0.31 ± 0.01 (3)</td>
<td></td>
</tr>
<tr>
<td>Granule-bound starch synthase</td>
<td>0.45 ± 0.06 (5)</td>
<td>0.30 ± 0.06 (3)</td>
<td></td>
</tr>
<tr>
<td>Alkaline pyrophosphatase</td>
<td>7.69 ± 1.46 (5)</td>
<td>5.83 ± 0.56 (4)</td>
<td></td>
</tr>
<tr>
<td>Starch-branching enzyme</td>
<td>47.86 ± 3.03 (3)</td>
<td>31.76 ± 3.95 (3)</td>
<td></td>
</tr>
</tbody>
</table>
and Notch-2 (Balaravi et al., 1976). One of the mutants, Riso 16, had a significantly lower ADP-Glc content (84.5 ± 17.4 nmol g⁻¹ fresh weight) than two wild-type lines, Bomi and Carlsberg II (184.5 ± 19.2 and 174.5 ± 12.1 nmol g⁻¹ fresh weight, respectively). Riso 16 also had a higher UDP-Glc content (610 ± 28.3 nmol g⁻¹ fresh weight) than Bomi and Carlsberg II (289.8 ± 20.1 and 296.7 ± 30.4 nmol g⁻¹ fresh weight, respectively). Together, this resulted in a very reduced ADP-Glc to UDP-Glc ratio (0.14 in Riso 16 compared with 0.64 and 0.59 in Bomi and Carlsberg II, respectively), suggesting that the synthesis of ADP-Glc could be impaired in Riso 16. All of the above values are means ± s.e. of measurements made on a minimum of four independent extracts.

Riso 16 Has a Reduced Activity of AGPase in the Endosperm

Measurements of the activities of various enzymes in the pathway of starch synthesis in the developing endosperms of Riso 16 and the wild-type, parental line Bomi showed that Riso 16 endosperm possessed only 15% to 25% of the normal AGPase activity (Table I). The activity of starch-branching enzyme was also reduced in the mutant but not to the same extent as AGPase. The activities of the other enzymes measured were either higher than normal in the mutant or were not statistically significantly different from normal.

The Subcellular Location of AGPase Activity in Wild-Type and Riso 16 Endosperms

The low ADP-Glc content of Riso 16 endosperms, together with a decreased total AGPase activity, is consistent with the idea that this mutant lacks the cytosolic form of AGPase. To discover the location of the residual AGPase activity in Riso 16, we used three different methods. Both of the first two methods involved the isolation of a fraction that was enriched in plastids by chopping the developing endosperm tissue to release intact plastids and collecting these from the resulting homogenate by centrifugation. First, plastid fractions were deliberately contaminated with varying amounts of the supernatant fraction and these combined fractions were assayed for the activities of AGPase and marker enzymes known to be exclusively located in either the plastids or the cytosol. The relative activities of these marker enzymes and AGPase in fractions of wild-type (Bomi) or Riso 16 endosperms were then compared as shown in Figure 1. The proportion of the total AGPase activity that was attributable to the plastids is indicated by the intercept on the y axis. AGPase clearly was almost completely plastidial in the mutant, whereas only a small proportion of AGPase activity was plastidial in the wild type.

Second, the activities of AGPase and marker enzymes were determined for the homogenate, plastid-enriched pellet, and supernatant fractions of endosperms of Bomi and Riso 16 (Table II). From these values, the percentage of AGPase activity in the plastids was calculated according to Denyer and Smith (1988). This showed that in the mutant, essentially all of the AGPase activity was plastidial, whereas in Bomi, approximately 15% of the activity was plastidial. This value for Bomi was similar to that measured previously (Thorbjørnsen et al., 1996b). Using these values and the total AGPase activities given in Table I, it is clear that Riso 16 is greatly reduced in, or lacks entirely, cytosolic AGPase activity but has a plastidial AGPase activity of the same order as the wild type (Bomi, 0.74 μmol min⁻¹ g fresh weight; Riso 16, 1.50 μmol min⁻¹ g fresh weight).

The third method was based on our previous discovery that the plastidial and cytosolic forms of AGPase in developing wheat endosperm could be separated by chromatography on columns of Q Sepharose (Burton et al., 2002b). We used the same technique to separate the two forms of AGPase from barley endosperms (Fig. 2a). For extracts of wild-type endosperms, AGPase eluted as two peaks: a first, major peak (accounting for 75%-80% of the total recovered activity) and a second, more minor peak. Western blots of crude homogenate of wild-type endosperm probed with an antiserum raised against the cytosolic AGPase SSU of maize endosperm (BT2...
Characterization of AGPase in the Barley Mutant, Risø 16

Table II. The subcellular location of AGPase activity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
<th>Activity Recovered in Pellet</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Homogenate</td>
<td>Supernatant</td>
</tr>
<tr>
<td>Bomi Plastid marker enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble starch synthase</td>
<td>7.43 ± 0.30</td>
<td>5.61 ± 0.26</td>
</tr>
<tr>
<td>Alkaline pyrophosphatase</td>
<td>636 ± 77</td>
<td>513 ± 48</td>
</tr>
<tr>
<td>Cytosolic marker enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>179 ± 23</td>
<td>176 ± 18</td>
</tr>
<tr>
<td>UDP-Glc pyrophosphorylase</td>
<td>6,280 ± 408</td>
<td>6,300 ± 398</td>
</tr>
<tr>
<td>AGPase</td>
<td>686 ± 108</td>
<td>612 ± 107</td>
</tr>
<tr>
<td>Riso 16 Plastid marker enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble starch synthase</td>
<td>7.03 ± 0.61</td>
<td>3.19 ± 0.52</td>
</tr>
<tr>
<td>Alkaline pyrophosphatase</td>
<td>662 ± 64</td>
<td>281 ± 23</td>
</tr>
<tr>
<td>Cytosolic marker enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>205 ± 26</td>
<td>195 ± 27</td>
</tr>
<tr>
<td>UDP-Glc pyrophosphorylase</td>
<td>6,760 ± 609</td>
<td>6,670 ± 728</td>
</tr>
<tr>
<td>AGPase</td>
<td>73.1 ± 8.3</td>
<td>34.8 ± 4.8</td>
</tr>
</tbody>
</table>

antiserum) showed the expected doublet of proteins (Fig. 2b). The higher and lower $M_r$ proteins correspond to the cytosolic and plastidial SSU proteins, respectively (Thorbjørnsen et al., 1996b). Western blots of the fractions from the Q Sepharose column probed with the BT2 antiserum (Fig. 2b) showed that the cytosolic SSU was largely confined to the first peak, whereas the plastidial SSU was detected primarily in the second, minor peak. Similar analysis of extracts of Riso 16 endosperms showed that the first peak was absent. Only the second peak was observed with extracts of the mutant. Western blots showed that the second peak contained the plastidial SSU only. No cytosolic SSU was detected in any of the fractions of the mutant examined. This suggests that, as in wheat, the first peak of AGPase activity represents cytosolic AGPase, and the second the plastidial AGPase. For wild-type barley endosperms, the relative activities of the cytosolic and plastidial AGPase calculated from the areas under the peaks broadly agree with the values obtained in the experiments with isolated plastids described in Figure 1 and Table II.

Riso 16 Lacks the Cytosolic But Not the Plastidial AGPase SSU Protein

When western blots of homogenates of endosperms of the mutant were probed with the BT2 antiserum, the small subunit of the cytosolic AGPase could not be detected. To determine whether there was any change in this pattern of small subunit proteins during endosperm development, we examined the abundance of AGPase SSUs in endosperms of different ages (Fig. 3a). For the wild-type endosperms, the amounts of both proteins per endosperm increased with developmental age. With Riso 16 endosperms, at all developmental stages examined, the cytosolic SSU protein was absent. The plastidial SSU protein increased in amount per endosperm with developmental age as in the wild type.

We examined the SSU proteins in leaves, pericarps, roots, and embryos of mutant and wild-type plants using the BT2 antiserum (data not shown). Only a single protein band of the same size as the plastidial protein in endosperms was observed, and this was present in organs of mutant and wild-type plants. This showed that the cytosolic SSU protein is present only in the endosperm and that in all organs examined, the plastidial AGPase proteins were not affected by the mutation.

We examined the LSU proteins in endosperms using antisera raised against the LSUs of endosperms of maize or barley (maize SH2 [Giroux and Hannah, 1994] and barley LSU [Kleczkowski et al., 1993]). We observed no consistent differences between mutant and wild-type endosperms in the proteins that cross-reacted with these antisera (data not shown). However, both antisera gave numerous, presumably nonspecific cross-reacting bands. It was not possible, therefore, to identify specifically the cytosolic and plastidial LSUs.

Riso 16 Is Deficient in Starch Synthesis throughout Endosperm Development

Previous measurements of mature seeds of Riso 16 show that they have 44% of the starch contents of normal seeds and 72% of the normal weight (Tester et
To determine whether the low starch content and seed weight were attributable to a decreased rate of starch accumulation or to a reduced period of starch synthesis, we measured the starch contents of the endosperm and the seed weights throughout development (Fig. 3b).

For both lines, the starch content and seed weight increased with age, with the maximal rates of starch synthesis occurring at 16 to 22 DPA. At all developmental stages, both seed weight and starch content were lower in Risø 16 than in Bomi. No large differences between Bomi and Risø 16 in either the timing of the onset of starch accumulation or its cessation were observed. These data suggested that Risø 16 has a reduced capacity for starch synthesis in the endosperm and that this deficiency occurred throughout the grain-filling period.

The A-Type Starch Granules in Risø 16 Are Misshapen

Examination of mature seeds of Risø 16 by scanning electron microscopy showed that most of the A-type starch granules were smaller than normal and were misshapen (Fig. 4). The B-type granules were also smaller than normal but there were no obvious differences in their shape. The A-type granules are thought to grow by the expansion of an equatorial zone beginning at one side of an initially spherical granule and growing in two plates or lobes around the circumference to meet eventually at the opposite side (Evers, 1971). The plates are separated by an equatorial groove. In Risø 16, the equatorial plates are thickened at their outer edges relative to those of Bomi granules, and there are frequently depressions in the surfaces of the granules. The equatorial grooves are very distinct. Some of the granules appear to be incomplete in that the equatorial plates do not completely meet resulting in kidney-shaped granules. The equatorial plates occasionally appear to spiral and slightly overlap resulting in snail shell-shaped granules. These morphological defects are probably not specifically attributable to the lack of cytosolic AGPase but to the reduced rate of starch synthesis. Similar defects, although usually not so severe, were observed in other low-starch mutants of barley, e.g. Risø 1508 and Risø 13, which are not allelic to Risø 16 (data not shown).

Analysis of the Gene Encoding the AGPase Small Subunit, Hv.AGP.S.1

A single gene thought to encode both the plastidial and cytosolic AGPase SSUs of barley has been de-

scribed previously (AGPaseB; P55238; Thorbjørnsen et al., 1996a) The gene structure is shown diagrammatically in Figure 5. A gene almost identical at the nucleotide level was identified in wheat and named Ta.AGP.S.1 (Burton et al., 2002b). We will refer to the gene from barley as Hv.AGP.S.1. The genes from both wheat (Burton et al., 2002b) and barley (Thorbjørnsen et al., 1996a) encode two distinct transcripts with different 5′ exons (1a or 1b) and identical 3′ regions. These genes, therefore, each potentially encode two different SSU proteins that differ only at their N termini. The deduced amino acid sequence of the two putative proteins showed the presence of a transit peptide in one but not the other. Thus these transcripts, which we will refer to in barley as Hv.AGP.S.1a and Hv.AGP.S.1b, encode putative cytosolic and plastidial proteins, respectively.

To determine whether the mutation responsible for the low starch-phenotype of Riso 16 directly affects Hv.AGP.S.1, and if so, in what way, we used PCR to compare the AGPase SSU genes in Riso 16 and the wild type. Primers were designed to amplify various segments of the coding region of Hv.AGP.S.1 (Fig. 5). PCR showed that fragments of the expected size could be amplified from the wild-type Bomi but not from the mutant Riso 16. However, fragments of the same size and with identical sequences were observed for mutant and wild type using primers that amplified the 3′-untranslated region (-UTR) of the gene. These data suggest that a large proportion of the coding region of the gene Hv.AGP.S.1 is missing in Riso 16. The presence of a large deletion in Riso 16 is consistent with the fast-neutron bombardment that was used to create the mutant population from which it was selected (Doll, 1983).

The lack of cytosolic AGPase activity and a functional Hv.AGP.S.1 gene in Riso 16 shows that this gene encodes the cytosolic AGPase SSU. The cytosolic SSU of AGPase protein in wheat endosperm is similarly encoded by one of the two transcripts produced from Ta.AGP.S.1 (Burton et al., 2002b). However, Riso 16 has a normal plastidial AGPase activity. Thus these data suggest very strongly that Hv.AGP.S.1 does not encode the major plastidial AGPase in this tissue. In wheat endosperm, the plastidial AGPase SSU was also shown to be encoded by a gene other than Ta.AGP.S.1 (Burton et al., 2002b). We accordingly attempted to discover the gene encoding the plastidial AGPase SSU in barley.

**Figure 4.** Scanning electron microscopy of barley grains. Mature grains were fractured to reveal the starch granules within the endosperm cells and viewed in a scanning electron microscope. The magnifications are indicated by the scale bars.

**Purification and Sequencing of AGPase from Wild-Type Endosperm**

To provide sequence information for the plastidial and cytosolic SSUs of AGPase, we purified these proteins from wild-type barley endosperm (Table III). The purification scheme was the same as that designed to purify these proteins from wheat endosperm (Burton et al., 2002b). After separation on Q Sepharose, the cytosolic (peak 1) and plastidial (peak 2) fractions were individually applied to a Sephadex S-200 gel filtration column. AGPase activity eluted from this column as a single peak with an estimated mass of approximately 250 kD (calculated from the elution times of molecular mass standards; data not shown). Fractions from the gel-filtration column were subjected to SDS-PAGE and selected protein bands were submitted for matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) and quadrupole time-of-flight (Q-TOF) analysis.

A gel showing AGPases from a typical purification from wild-type endosperms is shown in Figure 6. The major proteins labeled a through e (Fig. 6) were subjected to amino acid sequencing by Q-TOF. The amino acid se-
quences of fragments of the cytosolic large and small subunits confirmed that these proteins were encoded by previously identified genes. However, the sequences of fragments of the plastidial protein did not match any previously identified genes. The sequences of the plastidial SSU protein fragments specifically differed from the predicted sequence of the putative plastidial SSU encoded by Hv.AGP.S.1 in five of 49 amino acids (Table IV).

Identification of a Novel Plastidial AGPase Small Subunit cDNA

To identify the gene encoding the novel plastidial SSU of AGPase, we searched for novel AGPase SSU expressed sequence tags (ESTs). A barley EST (accession no. AL505815) was identified that was similar but not identical (94% identity in the amino acid level) to the equivalent region of Hv.AGP.S.1. Oligonucleotides to this EST were used to amplify a full-

Table III. Plastidial and cytosolic forms of AGPase were simultaneously purified from 400 endosperms each of approximately 15 mg fresh wt

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total Activity</th>
<th>Recovery of Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Purification Relative to Crude Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mL</td>
<td>μmol min⁻¹</td>
<td>% crude homogenate</td>
<td>mg</td>
<td>μmol min⁻¹ mg⁻¹ protein</td>
<td>-fold</td>
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<tr>
<td>Crude homogenate</td>
<td>6</td>
<td>24.78 ± 2.77</td>
<td>100</td>
<td>349 ± 18.5</td>
<td>0.071 ± 0.013</td>
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<tr>
<td>Q Sepharose (peak 1,</td>
<td>20</td>
<td>15.92 ± 1.15</td>
<td>64.2</td>
<td>7.60 ± 0.73</td>
<td>2.09 ± 0.56</td>
<td>29.5</td>
</tr>
<tr>
<td>cytosolic)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q Sepharose (peak 2,</td>
<td>15</td>
<td>1.82 ± 0.14</td>
<td>7.33</td>
<td>0.280 ± 0.037</td>
<td>6.49 ± 1.15</td>
<td>91.4</td>
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<tr>
<td>plastidial)</td>
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<tr>
<td>Sephades 200</td>
<td>9</td>
<td>12.56 ± 0.87</td>
<td>50.7</td>
<td>1.03 ± 0.19</td>
<td>12.26 ± 2.85</td>
<td>173</td>
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<tr>
<td>(peak 1, cytosolic)</td>
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</tr>
<tr>
<td>Sephades 200</td>
<td>9</td>
<td>0.72 ± 0.07</td>
<td>2.89</td>
<td>0.062 ± 0.007</td>
<td>11.62 ± 3.64</td>
<td>164</td>
</tr>
<tr>
<td>(peak 2, plastidial)</td>
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</table>
be detected in any of the examined organs of the Risø 16 plants. In the wild type, Hv.AGP.S.1 was expressed most strongly in the endosperms and less strongly in the embryos, and the expression was stronger in younger endosperms than in old endosperms.

The Hv.AGP.S.1 (Fig. 7a) and the Hv.AGP.S.2 (Fig. 7b) transcripts could be seen in both mutant and wild-type endosperms and embryos. No expression of Hv.AGP.S.2 could be detected in the leaves of either genotype even after an extended exposure (data not shown). Thus the expression of Hv.AGP.S.2 is not affected by the mutation in Risø 16. Unlike Hv.AGP.S.1, there were no large differences between young and old endosperms in the amounts of Hv.AGP.S.2 transcript. However, the size of the Hv.AGP.S.2 transcript observed in older endosperms was smaller than that seen in younger endosperms. The transcript in older endosperms corresponds in size to the truncated form of Hv.AGP.S.2 observed in the PCR experiments described above. In embryos, both truncated and full-length transcripts were detected by the Hv.AGP.S.2 probe. It would appear that the Hv.AGP.S.2 transcript in different tissues is subject to various degrees of posttranscriptional processing. The physiological significance of this is unknown at present.

Phylogenetic Analysis of the AGPase SSUs of the Grasses

Phylogenetic analysis of the coding sequences of the currently known AGPase SSUs of the grass family (Fig. 8) shows that there are at least two distinct subgroups or clades. One clade (type 1 genes) consists of cDNAs encoding the cytosolic SSUs of cereal endosperms such as Ta.AGP.S.1a and Hv.AGP.S.1a. It also includes the putative plastidial transcript Hv.AGP.S.1b (barley 1b) for which no protein has yet been identified. This transcript is identical to Hv.AGP.S.1a (barley 1a) except for the region corresponding to exon 1. The second clade (type 2 genes) includes the plastidial SSUs of cereal seeds and is statistically significant (62% bootstrap support). This clade consists of cDNAs encoding embryo and endosperm plastidial AGPase SSU such as our novel barley endosperm SSU. This suggests that, in the grass family, a single gene encodes the plastidial SSUs of both the embryos and endosperms. The SSUs of different species within a clade are more similar to one another than are SSUs within a species. This suggests that the type 1 and 2 AGPase small subunit genes existed before the divergence of these species within the grass family.

There is, at present, only one leaf SSU sequence (type 3 gene) available for the grass family, and this shows a closer phylogenetic relationship to the cytosolic SSUs (type 1) than to the seed-plastidial SSUs (type 2). The statistical significance of the cytosolic

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Figure 6. SDS PAGE of cytosolic (C) and plastidial (P) AGPase purified from developing endosperms of Bomi. AGPase was purified and subjected to SDS-PAGE on a 4% to 12% gradient gel as described in “Materials and Methods.” The tracks are peak fractions from separations on Sephadex 200 (see Table III). Protein bands labeled a through e were excised from the gel and subjected to MALDI-TOF analysis. The protein identities determined by analysis of the MALDI-TOF data are indicated.

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length cDNA that was cloned and sequenced (accession no. AF537363). We will refer to this cDNA and the gene encoding it as Hv.AGP.S.2. This cDNA is 82% identical to the Hv.AGP.S.1b transcript encoding a putative plastidial SSU (accession no. Z48563). The cereal AGPase with which it shares most identity (89%) is the rice (Oryza sativa) seed SSU (accession no. Y028315; Sikka et al., 2001). The predicted amino acid sequence of Hv.AGP.S.2 matches exactly with the sequence determined from the purified plastidial SSU protein (Table IV). This cDNA therefore encodes the SSU of the plastidial form of AGPase in barley endosperm.

The full-length cDNA was obtained by reverse transcriptase-PCR from RNA purified from endosperms of less than 20 mg. In older endosperms, a smaller transcript derived from HV.AGP.S.2 was present (Fig. 7). When sequenced, this smaller transcript was found to lack nucleotides 36 to 239 (inclusive), which encode the bulk of the predicted transit peptide and the beginning of the mature SSU protein. This transcript may be the result of aberrant processing of the HV.AGP.S.2 pre-mRNA. However, the protection of the Hv.AGP.S.2 transcript in different tissues is subject to various degrees of posttranscriptional processing. The physiological significance of this is unknown at present.
clade is low because of the close relationship of the maize endosperm cytosolic cDNA to the maize leaf cDNA. This high similarity resulted in a proportion (though not significant) of the 100 trees used to make this consensus tree, grouping these two maize genes together as a separate clade (as described by Hannah et al. [2001]).

In most eudicots for which information is available, as in grasses, there are multiple AGPase SSU genes. An exception is Arabidopsis, for which we have found only one type of SSU gene (Burton et al., 2002b). It is therefore possible that at least two SSU genes existed before the divergence of monocots and eudicots. Within the grasses, the seed-plastidial genes are the likely descendants of one of these genes and the maize leaf gene the descendant of the other, because in the tree, these two main branches are closest to the eudicot outgroup (Arabidopsis SSU gene).

**DISCUSSION**

**The Plastidial and Cytosolic SSUs of Barley Seeds Are Encoded by Two Separate Genes**

Comparison of wild-type and Risø 16 barley clearly shows that there are two genes encoding different AGPase SSUs in barley seeds. Hv.AGP.S.1 (identified by Thorbjørnsen et al. [1996b]) encodes the cytosolic SSU, and Hv.AGP.S.2 (identified in this paper) encodes the plastidial SSU. In Risø 16, the Hv.AGP.S.1 gene is substantially deleted and therefore inactive, and the mutant therefore lacks the cytosolic SSU and cytosolic AGPase activity. However, the Hv.AGP.S.2 gene is not affected in Risø 16. Normal amounts of transcript are produced from Hv.AGP.S.2 in the mutant, and it has a normal plastidial AGPase activity.

Torbjørnsen et al. (1996b) showed that Hv.AGP.S.1, in addition to producing the transcript encoding the cytosolic SSU, also produces a second transcript encoding a putative plastidial SSU. However, in our experiments, this transcript contributed very little, if at all, to the total SSU protein in the plastid. We have not examined in detail all stages of endosperm development or all growth conditions. It is possible that
Hv.AGP.S.1 may contribute more substantially to the pool of plastidial SSU protein at some stages of seed development or in some environmental conditions.

Plastidial AGPase Alone Cannot Support the Normal Rate of Starch Synthesis

The Riso 16 mutant of barley resembles the bt2 mutant of maize in that both lack the cytosolic SSU of AGPase, have no cytosolic AGPase activity, and have a reduced starch content. In barley, the plastidial activity of AGPase was estimated to be greater than the rate of starch synthesis, and hence it was suggested that the cytosolic AGPase activity could potentially be redundant (Thorbjørnsen et al., 1996a). However, the reduced starch content of the Riso 16 mutant shows that the plastidial AGPase activity alone is not sufficient to catalyze the normal rate of starch synthesis. Therefore, as in maize endosperm, the cytosolic AGPase activity makes an important, possibly major contribution to starch synthesis. No cereal mutants lacking plastidial AGPase activity in the endosperm have yet been identified. This means that we cannot yet determine the importance of the plastidial activity in supporting the normal rates of starch synthesis.

Lack of the Cytosolic AGPase Leads to Many Pleiotropic Effects

We observed changes in activities of enzymes other than AGPase in developing endosperms of Riso 16. The cause of this is not known, but such changes are common in starch mutants of grasses. For example, we observed decreases in starch-branching enzyme and alkaline pyrophosphatase and increases in soluble starch synthase and AGPase activities in a mutant of barley lacking activity of the starch-debranching enzyme, isoamylase (Riso 17; Burton et al., 2002a). Multiple changes in enzyme activities were also observed in the endosperm of the shx mutant of barley, although the molecular basis for the lesion in this low-starch mutant is not yet known (Schulman and Ahokas, 1990). Similar pleiotropic effects on enzyme activities (Singletary et al., 1997) and amounts of transcripts (Giroux et al., 1994) were seen in low-starch mutants of maize. Some of the pleiotropic effects on enzyme activities may be attributable to the increase in sugar contents seen in low-starch mutants (e.g. Riso barley mutants 29, 527, and 1508; Kreis and Doll, 1980) and the effect of this on gene expression, mRNA stability, or enzyme activity. This phenomenon makes the biochemical identification of the primary site of a lesion in a cereal starch mutant more
difficult because a decrease in the activity of an enzyme alone is not sufficient to indicate that the lesion lies in the gene encoding that enzyme.

Cytosolic AGPase Activity Is Not Required for the Synthesis of A- or B-Type Granules

The Risø 16 mutant makes both A-type and B-type starch granules, showing that neither the cytosolic AGPase nor a supply of ADP-Glc from the cytosol is necessary for the synthesis of these two granule types. The shape and size of the A-type starch granules is more profoundly affected than that of the B-type granules (this paper; Tester et al., 1993). However, the number and weight of B-type granules per endosperm are reduced more in Risø 16 relative to wild-type barley than those of A-type granules (Tester et al., 1993).

The Cytosolic AGPase SSU Gene Probably Evolved from a Leaf SSU Gene

From the phylogenetic relationships shown in Figure 8 and the assumption that close sequence identity reflects recent sequence duplication, we propose that the cytosolic SSU genes (type 1) of the grass family derive from an ancestral leaf-SSU gene (type 3) rather than from a plastidial seed-SSU gene (type 2). This idea is supported by the closer phylogenetic relationship between the type 1 and type 3 genes than between the type 1 and type 2 genes.

Whether the type 1 genes evolved once or several times during the evolution of the grasses is difficult to determine given the paucity of type 3 genes currently available. If the duplication event leading to the evolution of a cytosolic AGPase SSU occurred only once during the evolution of the grass family, then all of the cytosolic genes would be descendants of the same ancestral, duplicated leaf-SSU gene. However, as discussed by Hannah et al. (2001), the first exons of the cytosolic SSU genes of the grasses are very dissimilar. Exon 1 of maize cytosolic SSU (Bt2) is unlike exon 1 of the other type 1 genes. Thus we cannot rule out the possibility that the acquisition of exon 1 took place twice. Exon 1 could have been acquired once, after the divergence of the ancestors of maize from the other members of the grass family and again, independently, after this divergence but before the speciation of wheat, barley, and rice. It is possible that the two alternate exon 1s in the wheat and barley AGPS1 genes represent the incomplete or aberrant divergence of the duplicate leaf-SSU gene. One of them may have evolved directly from exon 1 of the duplicate leaf-SSU gene, and the other may represent a separately and more recently acquired cytosolic exon 1. The addition to the tree of sequences encoding leaf genes from other grass species will be necessary to resolve these questions.

MATERIALS AND METHODS

Plant Material

Barley (Hordeum vulgare) lines were from the John Innes Germplasm Collection. Plants were grown in individual pots in a greenhouse at a minimum temperature of 12°C, with supplementary lighting in winter to give 16-h days. Tissues were used immediately or harvested directly into liquid nitrogen and stored at −80°C before use.

Extraction and Assay of ADP-Glc and UDP-Glc

Duplicate samples of endosperm were isolated from grain by squeezing or rapid dissection and were immediately frozen in liquid nitrogen. The frozen endosperm samples were extracted by grinding in perchloric acid, extracts were neutralized, and ADP-Glc and UDP-Glc were assayed by HPLC on a Partisil-10-SAX column (Hichrom Ltd., Reading, UK) as described by Beckles et al. (2001). To assess the recovery of nucleotide sugars from the tissue, 15 nmol of either ADP-Glu or UDP-Glu was added to one of each pair of duplicate samples after extraction. Each compound was identified by comparison of retention times of and chromatography with pure samples. The amount of each compound was quantified by reference to a calibration curve, which was determined before each set of samples was run. The recoveries of added ADP-Glu or UDP-Glu varied between 70% and 135% of that added, suggesting that there had been no substantial losses of metabolites during extraction or assay.

Enzyme Activities

AGPase activity was assayed as in Smith (1990; assay 2b). The assay contained, in a volume of 1 mL, 100 mM Hepes (pH 7.9), 5 mM MgCl2, 0.4 mM NAD, 1 mM ADP-Glc, 1.5 mM NaPi, 5 units of NAD-linked Glc-6-phosphate dehydrogenase, 5 units of phosphoglucomutase, and extract. The activity reported was dependent upon the presence in the assay of all of the appropriate substrates and cofactors and also upon extract concentration within the range used to make the measurements. The concentrations of components of each of the assays and their pH values were optimized to give the maximum rate. The rate of the reaction was linear with respect to time for at least 4 min. All other enzymes were assayed as described by Burton et al. (2002a).

Extraction and Assay of Starch

Starch content of developing seeds was determined as described by Smith (1988), with the following adaptations. For each individual sample, five seeds were taken from the middle of an ear. The seeds were weighed, and then the endosperms were removed and frozen at −20°C. Frozen endosperms were crushed in a mortar and then incubated for 10 min in 5 mL of 80% (v/v) aqueous ethanol at 90°C. Samples were centrifuged, and the pellet was re-extracted twice more with ethanol. Ethanol was allowed to evaporate from the final pellet, and the tissue was ground in 3 mL of distilled water. Duplicate samples of 1 mL were autoclaved for 45 min at 120°C and 0.2 MPa and assayed for α-glucan as described by Burton et al. (2002a).

Isolation of Plastids from Developing Barley Endosperm

Plastids were isolated from developing barley endosperm of approximately 10 mg fresh weight as described for wheat by Burton et al. (2002b).

Localization of AGPase Activity in Barley Endosperm

The AGPase activity of wheat endosperm was localized to the plastid or extraplastidial compartments essentially according to the method of Denyer and Smith (1988) except that the extraplastidial compartment was the cytosol rather than the mitochondria. The method relies on the isolation of plastids with varying degrees of cytosolic contamination. Plastids were isolated as described above, and varying amounts of the supernatant from
the plastid isolation procedure were added back to aliquots of the plastids. Marker enzymes for the plastid (soluble starch synthase and alkaline pyrophosphatase) and cytosol (alcohol dehydrogenase and Suc synthase) and AGPase were assayed in each fraction, and the activities of AGPase in the plastidial and cytosolic compartments were calculated.

Other Methods

SDS-PAGE and immunoblotting, purification of cytosolic and plastidial AGPase from developing barley endosperm, and identification of AGPase subunits by MALDI-TOF and Q-TOF were as described by Burton et al. (2002b). Scanning electron microscopy was as described by Burton et al. (2002a) for wheat.

Isolation of Total RNA and cDNA Synthesis

RNA was purified from endosperm, embryo, and leaf tissues using Concert Plant RNA reagent (Invitrogen, Paisley, UK) as described by the manufacturer. cDNA was synthesized from 10 μg of total RNA using an oligo(dT) primer at 58°C with Thermoscript reverse transcriptase (Invitrogen) or at 42°C with Superscript reverse transcriptase (Invitrogen).

Isolation of Genomic DNA and PCR

DNA was extracted from 0.1-g samples of young barley leaves with the DNeasy plant mini kit (Qiagen, Hilden, Germany). PCR with five sets of primers (2′-5′×GCACACTCTACCAACAATCCA-3′, and 8′-5′×CCAAAGAACGCTTTCCATAGAC-3′; 1′-5′×GGAGATTGTGGCTAT- GCCGCT-3′, and 2′-5′×CTGACTGTCACCCAGACAGAAG-3′; 10′-5′×CTTTCCTCTCTCCCACGTG-3′, and 2′-17′-5′×TCAATGTTG-GACTCGGTCTTCCT-3′; 6′-5′×GTAATGAGGTTGTTGAGAGG-3′; 18′-5′×GCCAAGAGACGAGCTTCTGTCGT-3′, and 19′-5′×GCATCGAAGGTTGCAAGAACCACATT-A-3′) was done using 5 units of AmpliTaq polymerase (Roche Diagnostics, Lewes, UK). PCR conditions were optimized for each primer pair.

Amplification of a cDNA Encoding a Novel AGPase SSU

Total RNA was purified from non-photosynthetic tissues of grains of 20 to 40 mg fresh weight and used to synthesize cDNA from a poly(T) primer using the Gene Racer Kit for RACE (Invitrogen). Forward (5′×AGACGCGCC-GAGAATCTCCATACT-3′) and 5′-TACTGGGCTTTTGATCTCGAG-3′) reverse (5′×AGTTAGGATCTCCGGCCTT-3′ and 5′×GAACCTGCGTCAG- CACATAGATC-3′) primers were designed to EST AL505815 for the 3′- and 5′-RACE. All RACE products were cloned into pGEM-T Easy vector and sequenced.

Northern Analysis

Total RNA (10 μg) was separated on a 1% (w/v) agarose denaturing gel with size standards (Promega, Madison, WI). RNA was visualized with BioMax-MS film and a BioMax intensifying screen (Eastman Kodak, Rochester, NY).

Characterization of AGPase in the Barley Mutant, Risø 16

Phylogenetic Analysis

The coding regions of AGPase SSU cDNAs were aligned using PILEUP from the Wisconsin GCG v10.1 package (Genetics Computer Group, Madison, WI) on a Unix platform. The multiple sequence file was converted for use in the Phylop v3.6 package. Statistical support was given by the SEQBOOT program using 100 bootstraps, and the resulting datasets were analyzed using DNAPARS with 10 jumbles. The CONSENSE program was used to make the consensus tree.

ACKNOWLEDGMENTS

We thank Dr. Alison M. Smith and Dr. David Laurie (John Innes Centre, Norwich, UK) for support, encouragement, and useful discussions throughout the course of this work and for constructive criticism of the manuscript. We are very grateful to Prof. Curt Hannah (University of Florida, Gainesville) for the gifts of the BT2 and SH2 antisera and to Dr. Tine Thorbjørnsen (laboratory of Odd-Arne Olsen, Agricultural University of Norway, Ås) for the gift of the barley LSU antisera.

Received August 22, 2002; returned for revision September 25, 2002; accepted October 21, 2002.

LITERATURE CITED


