

# Differential Processing of Homologues of the Small Subunit of ADP-Glucose Pyrophosphorylase from Barley (*Hordeum vulgare*) Tissues

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ADP-glucose pyrophosphorylase (AGPase), a two-gene-encoded enzyme, is the key component of starch synthesis in all plants. In the present study, we have used an *E. coli* expression system for the (over)production of proteins derived from both full length and specifically truncated cDNAs encoding small subunits of AGPase from seed endosperm (AGPase-B1) and leaves (AGPase-B2) of barley (*Hordeum vulgare*). Based on immunoblot analyses, the molecular mass of the expressed AGPase-B1 (52 kD) was similar to that from endosperm extracts, whereas the expressed AGPase-B2 (56 kD) was larger than that in barley leaves (51 kD). Expression of truncated cDNAs for both the seed and leaf proteins has allowed for a direct verification of molecular masses that were earlier proposed for mature AGPases in barley tissues. The data suggest that seed AGPase-B1 does not undergo any post-translational proteolytic processing in barley, whereas the leaf homologue is processed to a smaller protein. Possible implications of these findings are discussed.

## Introduction

ADP-glucose pyrophosphorylase (AGPase) is an important enzyme of starch biosynthesis in all plants (Kleczkowski *et al.*, 1991; Nelson and Pan, 1995; ap Rees, 1995). Mutations or antisense studies that eliminate most (or all) of AGPase activity cause a severe reduction in starch content, indicating that the enzyme is the major, if not the only, route for generating ADP-glucose for starch formation (Müller-Röber *et al.*, 1992; Martin and Smith, 1995; Nelson and Pan, 1995). The enzyme is encoded by two genes, corresponding to small (AGPase-B) and large (AGPase-S) subunits, respectively. In barley, several genes for the large subunit and one gene for the small subunit have been mapped (Kilian *et al.*, 1994), and several cDNAs corresponding to either of the subunits have been characterised (Villand *et al.*, 1992a,b; Thorbjørnsen *et al.*, 1996b; Eimert *et al.*, 1997). The expression of the small subunit gene is now known to be differentially regulated, possibly in-

volving two different promoters, resulting in the production of two transcripts encoding AGPase-B homologues from seeds and leaves (AGPase-B1 and -B2, respectively) (Thorbjørnsen *et al.*, 1996b). Although the homologues differ only in their *N*-termini (40 and 81 amino acids for AGPase-B1 and -B2, respectively), this may have important implications for their intracellular targeting in barley (Villand *et al.*, 1994; Thorbjørnsen *et al.*, 1996b).

AGPase from any plant species has been assumed to be located exclusively in plastids [*e.g.* (ap Rees 1995)], but this has been the subject of controversy [reviewed in (Kleczkowski, 1996)]. Recent studies on subcellular location of AGPase activity in cereal seeds have suggested, based on subcellular fractionation approaches, that the major form of AGPase is confined to the cytosol rather than to plastid compartment (Thorbjørnsen *et al.*, 1996a; Denyer *et al.* 1996). Also, whereas the cDNA-derived amino acid sequence of leaf AGPase-B2 contains a putative processing site at its *N*-terminus for transport into plastid stroma, the corresponding sequence of seed AGPase-B1 bears no defined cleavage site (Thorbjørnsen *et al.*, 1996b). In the present study, the question of proteolytic processing of AGPase-B *in vivo* has been addressed by using a heterologous expression sys-

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tem to produce AGPase-B homologues of defined molecular masses for a direct comparison of the sizes of the expressed proteins with those present in barley preparations.

## Materials and Methods

### Plant material

Barley (*Hordeum vulgare*), cv. Bomi, plants were greenhouse-grown. Crude extracts of barley leaf and endosperm proteins were isolated in SDS-buffer (Kleczkowski *et al.*, 1993a,b). Leaf AGPase was purified as in (Kleczkowski *et al.*, 1993b), except that purification on Cibacron Blue 3GA agarose column was omitted from the procedure.

### Bacterial strains and plasmid construction

Throughout all procedures standard DNA techniques (Sambrook *et al.*, 1989) were used. The *E. coli* strain DH5 $\alpha$  was used as a host for cloning foreign DNA. For all cloning steps the identity of the constructed DNA was verified by sequencing. cDNAs encoding full open reading frames (ORFs) of barley AGPase-B1 and -B2 and those corresponding to specifically truncated proteins were amplified by polymerase chain reaction (PCR). The following forward PCR primers were used: (a) 5'-CGGGATCCATGGATGTACCTTTGGCA-3'; (b) 5'-ATAAGACCATGGCGAAGCATGCA-3'; (c) 5'-CTCGGGATCCACCTCCATGGCGATG-3'; and (d) 5'-GCCGTGACCATGGCCGCGTCC-3'. The reverse PCR primers were: (e) 5'-CGGGA-TCCTTATTTATTTATATGACTGTTCCACTAG-3'; and (f) M13 reverse primer. Primers (a) and (e) were used for amplification of *bepsF1*, the cDNA encoding full ORF of seed AGPase-B1 (Thorbjørnsen *et al.*, 1996b); primers (b) and (f) for a 27 amino acids truncated AGPase-B1; primers (c) and (f) for amplification of *blps14*, the cDNA encoding full ORF of leaf AGPase-B2 (Thorbjørnsen *et al.*, 1996b); and primers (d) and (f) for a 24 amino acids truncated AGPase-B2. The forward primers were designed to contain *Nco*I and *Bam*HI restriction sites. Amplified PCR products were cleaved with *Bam*HI and inserted into the *Bam*HI site of pBluescript (Stratagene). Following partial digestion with *Nco*I and complete digestion with *Bam*HI, the cDNAs for full length AGPase-B1 and -B2 and for truncated AG-

Pase-B1 were inserted between *Nco*I and *Bam*HI sites of pET3d (Novagen) expression vector. The fragment corresponding to truncated AGPase-B2 was inserted into pET23d (Novagen) vector.

### Expression and preparation of recombinant proteins

The resulting pET plasmids containing cDNAs for full length and truncated AGPase-B1 and -B2 were transformed into *E. coli* strains BL21(DE3) or BL21(DE3)lysS (Studier *et al.*, 1990). All cells, except those containing cDNA for truncated AGPase-B2 in pET23d vector, were grown at 37 °C until an OD<sub>600</sub> of ca. 0.5 was reached. The expression was induced by addition of different concentrations of isopropylthio- $\beta$ -D-galactoside (IPTG) (see the Results). Two hours later the cells were collected by centrifugation at 6000 rpm for 10 min, and the pellet was resuspended in one tenth of the original volume with 10 mM Tris (pH 8.0). This suspension was then mixed with loading SDS-buffer. Cells containing the pET23d vector with DNA for 24 amino acids truncated AGPase-B2 were incubated and induced at room temperature. The IPTG was added when cells reached OD of ca. 1.0 and the induction was carried out for 24 h. Other details were the same as for cells bearing pET3d vectors.

### Electrophoresis and immunodetection

SDS-PAGE was carried out as in (Laemmli, 1970). The relative abundance of bands corresponding to AGPase was estimated using computer-linked scanner (Pharmacia). The SDS-PAGE resolved proteins were transferred onto nitrocellulose membrane (Bio-Rad) using a Bio Rad protein transfer system. Immunodetection of AGPase-B proteins was carried out using rabbit antibodies against barley AGPase-B1 (Thorbjørnsen *et al.*, 1996a) and donkey anti-rabbit secondary antibodies linked to horseradish peroxidase (Amersham); the peroxidase activity was detected using ECL fluorescence reagents (Amersham). Protein content was determined using the Bio-Rad Protein Assay, with bovine serum albumin as standard.

## Results and Discussion

cDNAs corresponding to homologues of AGPase-B from seed endosperm and leaves of barley

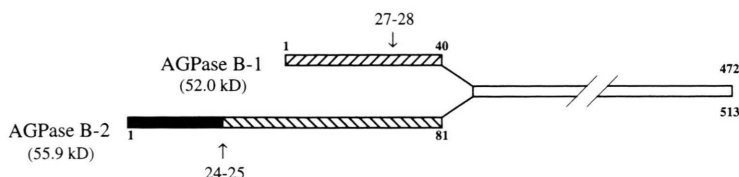


Fig. 1. Comparison of the relative length of the derived amino acid sequences of *N*-termini of the small subunits of AGPase from endosperm (AGPase-B1) and leaves (AGPase-B2) of barley. Numbers refer to respective amino acids in AGPase-B proteins. Arrows refer to *N*-termini of truncated AGPase-B proteins expressed in *E. coli* in the present study. A segment painted black corresponds to a putative transit peptide for leaf AGPase-B2 (Thorbjørnsen *et al.*, 1996b). Starting with amino acids numbered 41 (AGPase-B1) and 82 (AGPase-B2), both proteins share identical amino acid sequence (Thorbjørnsen *et al.*, 1996b).

were introduced into pET vectors for expression in *E. coli*. Previously, a similar heterologous system was used to express cDNAs for AGPase from maize (Giroux and Hannah, 1994) and potato (Ballicora *et al.*, 1995). In the present study, both full length cDNAs as well as those truncated at specific sites were used to yield proteins of different sizes. The 5'-end truncated cDNAs for endosperm and leaf AGPase-B lacked 81 and 72 bp, respectively, of their coding regions, corresponding to proteins lacking 27 and 24 amino acids from their *N*-termini (Fig. 1). Calculated molecular masses of proteins derived from full length and truncated cDNAs for seed AGPase-B1 were 52.0 and 49.0 kD, respectively, whereas the respective molecular masses for leaf AGPase-B2 were 55.9 and 53.7 kD (Thorbjørnsen *et al.*, 1996b).

The bacterial system overproduced both of the AGPase-B homologues, as found following SDS-PAGE and staining for the resolved proteins (Fig. 2). Scanning of the gels and measuring band intensities have allowed us to estimate the abundance of AGPase-B1 and -B2 proteins at 35 and 17%, respectively, when compared to the total amount of crude protein loaded on the gels (data not shown). Lowering of the induction temperature from 37 °C to 22 °C led to a dramatic reduction of the expression (data not shown).

The production of barley AGPase-B1 and -B2 in *E. coli* was studied in more detail on immunoblots, using antibodies raised against AGPase-B1 (Thorbjørnsen *et al.*, 1996a). In these experiments, AGPase-B1 from crude endosperm extracts served as a useful reference marker for comparison of the size of expressed proteins. The expression of AGPase depended, to some extent, on IPTG concentration, with 0.01 mM IPTG appa-

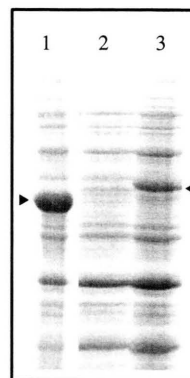


Fig. 2. SDS-PAGE of crude extracts of *E. coli* expressing full length cDNAs corresponding to AGPase-B1 (lane 1) and -B2 (lane 3) from barley. The control (lane 2) contained crude proteins from untransformed *E. coli*. Approximately 10 µg proteins were loaded onto each lane of the 12% SDS-PAGE gel. Arrows refer to AGPase-B bands.

rently sufficient for maximal expression of all but truncated AGPase-B1, which required an order of magnitude higher IPTG concentration (Fig. 3).

The molecular masses of AGPase-B proteins expressed in *E. coli* have been directly compared with those from crude and partially purified protein extracts from barley endosperm and leaves, respectively (Fig. 4). AGPase-B1 which was derived from full length cDNA had similar molecular mass (52 kD) as its counterpart in crude endosperm extracts. Truncation of 27 amino acids from its *N*-terminal resulted in a protein which had noticeably lower molecular mass (Fig. 4). In fact, although the calculated molecular masses of all of the expressed proteins were in a rather narrow range of 49.0 to 55.9 kD, their relative positioning on SDS-PAGE gels corresponded directly to their

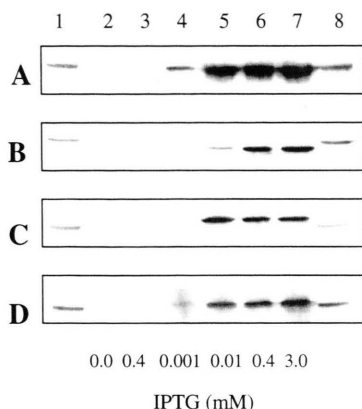


Fig. 3. Immunoblot detection of the IPTG-induced expression of barley AGPase-B proteins in *E. coli*. Bacterial cells were transformed with cDNAs corresponding to: **(A)** full length AGPase-B1, **(B)** truncated AGPase-B1, **(C)** full length AGPase-B2, and **(D)** truncated AGPase-B2. The cells were treated with a given IPTG concentration (lanes 3–7) for 24 h. Crude bacterial proteins were loaded onto 12% SDS-PAGE gel (*ca.* 5 µg protein per lane). AGPase-B proteins were detected by immunoblotting (see Methods). Lanes 1 and 8, crude protein extract from barley endosperm; lanes 2 and 4–7, pET-3d construct containing respective cDNA for AGPase-B; lane 3, pET-3d construct without the insert.

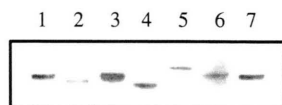


Fig. 4. Comparison of molecular masses of barley AGPase-B proteins expressed in *E. coli* from full length and truncated cDNAs. Proteins (*ca.* 5 µg per lane) were resolved by SDS-PAGE (12% gel). AGPase-B bands were detected by means of immunoblotting. Lanes 1 and 7, crude protein extract from barley endosperm; lane 2, barley leaf preparation; lane 3, full length AGPase-B1; lane 4, the 27 amino acids truncated AGPase-B1; lane 5, full length AGPase-B2; lane 6, the 24 amino acids truncated AGPase-B2.

sizes, indicating that differences of as little as 1.0 kD (*ca.* 10 amino acids) can be resolved by this method. Concerning AGPase-B2 from leaf extracts, its detection was greatly obscured by the abundance of the large subunit of Rubisco, which has a similar molecular mass as AGPase subunits (Akazawa and Hara-Nishimura, 1985), and thus we had to partially purify the leaf protein prior to immunoblot analyses. AGPase-B2 derived from

full length cDNA had considerably higher molecular mass (55.9 kD) than the corresponding protein from leaves (51 kD). Removal of 24 amino acids yielded a protein which was still larger (53.7 kD) than the leaf counterpart (Fig. 4). Previously, we have proposed a tentative processing site in the cDNA-derived amino acid sequence of AGPase-B2 [between amino acids number 24 and 25 (Thorbjørnsen *et al.*, 1996b)], which corresponded to the truncation of 72 nt introduced to the cDNA in the present study. However, the size of the protein in barley is approximately 2 kD smaller than that determined for the truncated homologue (Fig. 4), suggesting that the processing site is localized *ca.* 20 amino acids downstream from the proposed site. This region does not contain any obvious processing site, as determined by examining cDNA-derived amino acid sequence (Thorbjørnsen *et al.*, 1996b). The lack of a defined processing site should not be seen as surprising, however, since *ca.* 30% of all nucleicoded and plastid-localised proteins do not contain any obvious processing motifs in their immature sequences (Bairoch, 1992).

Based on recent subcellular fractionation studies (Thorbjørnsen *et al.*, 1996b), barley endosperm contains two isozymes of AGPase that are localised in plastids (minor form) and the cytosol. The plastidial location of the minor form implies that it must, as any other nucleicoded and plastid-localised protein (Keegstra *et al.*, 1989), be proteolytically processed *in vivo* for an efficient transport into plastid stroma. Such a processing has been confirmed in the present study for the AGPase-B2 homologue (Fig. 4). Since the transcript for AGPase-B2 has been found in leaves and seed endosperm, whereas the one for AGPase-B1 is confined exclusively to seed endosperm (Thorbjørnsen *et al.*, 1996b), we believe that the AGPase-B2 homologue corresponds to the same plastidic AGPase isozyme in both leaves and seeds of barley. On the other hand, we have failed to detect any proteolytic processing for the AGPase-B1 protein (Figs 3 and 4), which is consistent with our earlier proposal (Villard and Kleczkowski, 1994; Kleczkowski, 1994; Thorbjørnsen *et al.*, 1996b) that it corresponds to the cytosolic isozyme. The presence of cytosolic (in the endosperm) and plastidic (in the endosperm and leaves) isozymes of AGPase should have impor-



tant implications for biotechnological approaches aimed at manipulation of starch levels in plants through an introduction of suitably engineered AGPase proteins that are targeted to appropriate cell compartments.

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