Molecular Cloning and Spatial Expression of an *ApL1* cDNA for the Large Subunit of ADP-Glucose Pyrophosphorylase from *Arabidopsis thaliana*

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Carbon Partitioning, 3′-RACE, Starch Synthesis, Transit Peptide

A cDNA, *ApL1a*, corresponding to a homologue of the large subunit of ADP-glucose pyrophosphorylase (AGPase), has been isolated/characterised by screening a cDNA library prepared from leaves of *Arabidopsis thaliana*, followed by rapid amplification of cDNA 3′-ends (3′-RACE). Within the 1685 nucleotide-long sequence (excluding polyA tail), an open reading frame encodes a protein of 522 amino acids (aa), with a calculated molecular weight of 57.7 kDa. The derived aa sequence does not contain any discernible transit peptide cleavage site motif, similarly to two other recently sequenced full-length *Arabidopsis* homologues for AGPase, and shows *ca.* 58–78% identity to homologous proteins from other plants/tissues. The corresponding gene was found to be expressed in all tissues examined (rosette and stem leaves, stems, flowers and fruits). The ubiquitous expression of the gene is consistent with its critical role in starch synthesis in *Arabidopsis.*

Introduction

ADP-glucose pyrophosphorylase (AGPase) is the key enzyme of starch biosynthesis in all plants [reviewed in (Preiss, 1978; Kleczkowski *et al.*, 1991)]. In plants, the enzyme is composed of two subunit types, encoded by different genes (Olive *et al.*, 1988; Lin *et al.*, 1988; Nelson and Pan, 1995). Studies with AGPase mutants (Lin *et al.*, 1988; Nelson and Pan, 1995) and using "antisense" technology (Müller-Röber *et al.*, 1992) have established that AGPase plays a pivotal role in providing carbon skeletons for starch synthesis in plants. AGPase from several tissues (*e.g.* leaves) has been shown to be potently regulated by metabolic effectors (Preiss, 1978; Kleczkowski *et al.,* 1993b), and this in turn may control the rate of starch synthesis. Effector regulation of AGPase has frequently been considered as a model in studies on control of carbon metabolism in plants (Kleczkowski, 1994). Apart from its possible regulation by metabolic effectors, AGPase is also regulated at the transcriptional level, most notably by sugars (Müller-Röber *et al.*, 1990; Nakata and Okita, 1995; Sokolov *et al.*, 1998) and/or osmotica (Sokolov *et al.*, 1998), suggesting the involvement of complex signal transduction mechanisms that mediate gene expression. In several plants, at least two isozymes of AGPase have been described that may differ in their molecular mass (M_r), kinetic/regulatory properties and/or intra- and inter-cellular compartmentation (Kleczkowski *et al.,* 1993a, b; Villand and Kleczkowski, 1994; Nelson and Pan, 1995; Weber *et al.*, 1996; Denyer *et al.*, 1996; Kleczkowski, 1996; Chen and Janes, 1998).

Previously, we have used a polymerase chain reaction (PCR) technique to amplify several partial [*ca.* 540 nucleotide (nt)-long] cDNAs corresponding to small and large subunits of AGPase from various plant tissues (Villand *et al.*, 1992a, 1993). These PCR products have proven to be very useful as gene-specific probes to isolate full length cDNAs, genomic clones and for expression studies (Villand *et al.*, 1992a, b, 1993; Thorbjørnsen *et al.*, 1996; Eimert *et al.*, 1997). In *Arabidopsis*, a model plant species, at least three genes for the large subunit and one gene for the small subunit have been identified (Villand *et al.*, 1993). One of the large subunit genes (*ApL1*) corresponds to the large subunit of AGPase that is considered to be critical for starch synthesis, based on mutant studies (Lin

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et al., 1988; Wang *et al.*, 1997). A mutant lacking ApL1 protein had greatly reduced AGPase activity and its starch levels were at 40% when compared to wild-type plants (Lin *et al.*, 1988). Expression of the *ApL1* gene is strongly dependent on osmotic pressure, as found by exposing excised *Arabidopsis* leaves to specific sugars and general osmotics (Sokolov *et al.*, 1998), suggesting the involvement of an osmoticum-mediated signal transduction pathway.

In the present study, isolation of a cDNA encoding full open reading frame (ORF) of *Arabidopsis* ApL1 protein is reported, along with characterisation of its expression in *Arabidopsis* tissues. The nucleotide sequence of *ApL1a* has been deposited in GenBank under accession number AF117570.

Materials and Methods

Isolation and characterisation of ApL1a

The characterisation of *ApL1a* cDNA was carried out in two steps. First, a 1669 nt-long *ApL1-1* clone was isolated from an *Arabidopsis* cDNA library by probing with an earlier isolated (Villand *et al.*, 1993) 540-nt-long *ApL1* cDNA. The *ApL1-1* clone encoded a full ORF for the large subunit of AGPase, but lacked polyA tail, indicating that it corresponded to a 3′-end truncated mRNA. Subsequently, a putative composite full-length cDNA sequence (*ApL1a*) was obtained by rapid amplification of cDNA 3′-ends (3′-RACE) using specific primers. For this purpose, an aliquot of 5 μg of total RNA was isolated from leaves of one-month-old *Arabidopsis* (see below) and reversed to cDNA with oligo dNTPs by avian myeloblastosis virus (AMV) reverse transcriptase, following manufacturers′ instruction (Promega). A specific forward primer (5′-CTCGGAGGGTATACAAGAAGCAGAT-3′) and an adapter primer (5′-CTGATCAAGTTCAG$[T]_{15}$-3′) were used for reverse transcriptase PCR; the adapter primer contained 15 oligo dT plus 14 nt random nucleotides to balance the antisense primer's annealing temperature. The specific PCR product was cloned by TA-cloning (Invitrogen). A 196 nt-long clone isolated by this approach fully aligned within the corresponding sequences with the *ApL1-1* cDNA and allowed for determination of the complete 3′-end nt sequence of the transcript. All cDNAs were sequenced on both strands using custom-made primers (fmol DNA Sequencing, Promega, Madison, USA). The nt and aa sequence comparisons were performed against GenBank non-redundant databases (www.ncbi.nlm.nih.gov), using the basic local alignment search tool (BLAST).

Northern blot analyses

Arabidopsis thaliana (L.) Heynh., ecotype Columbia, plants were grown in a chamber with 10 h white light (125 μE m^{-2} s^{-1}, 22 °C) and 14 h darkness (18 °C) photoperiod regime. Total RNA was isolated from various tissues of 6–7 week-old mature plants in the end of the 14 h dark period, using a modification of the guanidine thiocyanate-based method, as described in (Sokolov *et al.*, 1998), and aliquots of 15 μg of total RNA were electrophoretically resolved on 1.2% agarose-formaldehyde gels. Following Northern blot transfer, Hybond-N^+ (Amersham) membranes with the blotted/immobilised RNA were hybridised with *ApL1-1* probe. Hybridisations and subsequent washes were performed at 65 °C, following standard procedures (Sambrook *et al.*, 1989).

Results and Discussion

Nucleotide and deduced aa sequence

The nt sequence of *ApL1a*, a cDNA encoding large subunit (ApL1) of AGPase, was obtained from *ApL1-1* clone isolated after screening an *Arabidopsis* cDNA library with an earlier characterised (Villand *et al.*, 1993) 540 nt-long *ApL1* clone, followed by 3′-RACE. The 1685 nt-long (excluding polyA tail) *ApL1a* encodes an ORF of 522 aa (Fig. 1). Based on nt sequence, *ApL1a* is the most homologous (78–81% identity) to large subunits of AGPase from leaves of tomato (GenBank accession # U85497) (Chen *et al.*, 1998) and barley (acc. # U66876) (Eimert *et al.*, 1997), and to a large subunit homologue from potato (acc. # S53992). Identities of over 50% have been found between *ApL1a* and all the corresponding cDNAs for the large subunit of AGPase from other plants and tissues [*e.g.* (Olive *et al.*, 1988; Weber *et al.*, 1995; Chen *et al.*, 1998; Park and Chung, 1998)].

Based on aa sequence, the derived protein has calculated M_r of 57,674, which is slightly higher than M_r estimations of *ca.* 54 kDa for the large subunit of AGPase in *Arabidopsis* (Lin *et al.*, 1988;

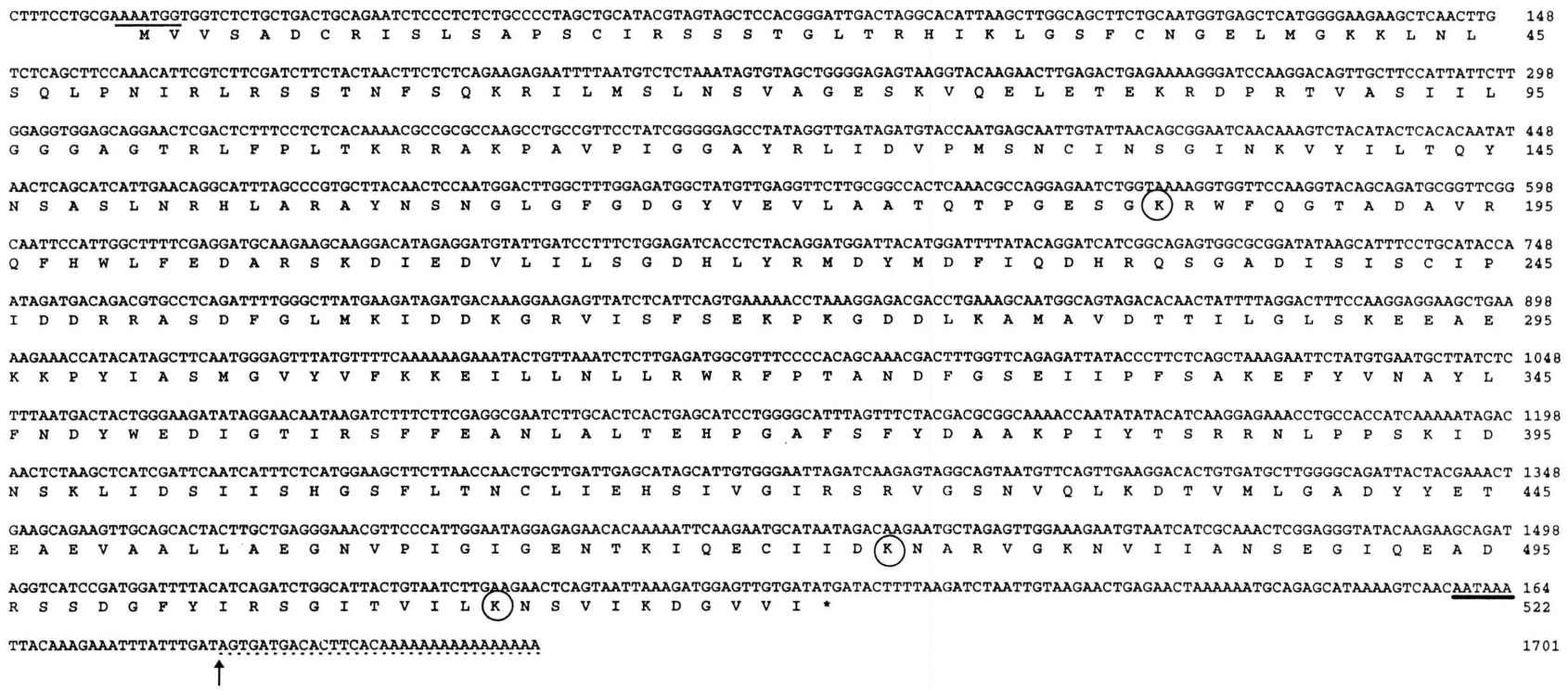

Fig. 1. Nucleotide and derived aa sequences of *ApL1a* (GenBank acc. # AF117570) from *Arabidopsis*. A nt sequence consensus motif to the translation initiation site is underlined by thin line, and that defining the termination signal for polymerase II (polyadenylation signal) is underlined by thick line. An arrow denotes the 3′-end of *ApL1–1* clone, whereas the dotted line underlines nt sequence that was obtained by 3′-RACE amplification. The original earlier reported *ApL1* cDNA clone (Villand *et al.*, 1993) is denoted by nt sequence # 920-1428. Lysine residues (Lys^{184}, Lys^{475}, Lys^{512}) which are homologous to those previously shown for spinach AGPase to bind pyridoxal-P (Ball and Preiss, 1994), an analog of activators of the enzyme, are circled.

Sokolov *et al.*, 1998). The ApL1 protein shares 77–78% identity with the tomato, barley and potato large subunits of AGPase. The identity to a host of homologous sequences from other plants and/or tissues is at *ca.* 58–67%, including a full-length homologue from *Arabidopsis* (acc. # Y18432) [corresponding to *ApL3* (Villand *et al.*, 1993)]. The derived aa sequence of *ApL1a* contains three highly conserved lysine (Lys) residues (Lys^{184}, Lys^{475}, Lys^{512}) (Fig. 1), which are thought to be critical for binding of pyridoxal-P, an analog of AGPase activators (Ball and Preiss, 1994). The two Lys residues located close to *C*-terminus are positioned near or at an allosteric site of the leaf enzyme. A similar positioning of Lys residues occurs in a host of other homologous large subunit sequences of AGPase [*e.g.* (Eimert *et al.*, 1997)], but not in barley seed AGPase large subunit which lacks the lysines in positions corresponding to Lys^{184} and Lys^{475} (Villand *et al.*, 1992b). The latter enzyme has been shown to be remarkably insensitive to effector regulation (Kleczkowski *et al.* 1993a, b).

Motifs

In the vicinity of the first ATG codon from the 5′-end of the *ApL1a* cDNA clone, a sequence homology to the translation initiation region [(A/G)CCATGG] (Kozak motif) has been identified (Fig. 1). In this sequence, the positions of cytidine bases are less conserved than those of other nucleotides. In addition, the 3′-untranslated region of *ApL1a* contains a motif characteristic of the termination signal for polymerase II (polyadenylation signal) (AATAAA).

Concerning derived aa sequence, we have failed to locate any transit peptide motif characteristic of a plastid-targeted nuclear-encoded proteins. Leaf AGPases are supposed to be located exclusively in plastids (Preiss, 1978) and their immature aa sequences should contain a peptide that is cleaved upon transport to chloroplast stroma. Based on empirical rules summarised in (Bairoch, 1992), the criteria for such a transit peptide in ApL1 protein are not met due to the shortage of G, A, S, T and C residues in the first 15 aa of the sequence, as well as the lack of a consensus cleavage site (I/V–X–A/C– ↓–A) within first 80 aa of the *ApL1a*-derived protein. Interestingly, derived full length aa sequences for *Arabidopsis* ApL3 (acc. # Y18432) and ApS (acc. # U70616) proteins also do not have such an obvious motif for a transit peptide cleavage site. The significance of this is not entirely clear; whereas most nucleus-encoded chloroplast-located proteins do have such a discernible motif, *ca.* 30% proteins do not conform to this rule (Bairoch, 1992). Thus, the evidence derived from aa sequence alone is not sufficient to assign an either chloroplastic or cytosolic location for a given protein. Were *Arabidopsis* AGPase cytosol-located, this would imply that ADP-glucose formed in the cytosol would have to be exported to plastids for starch synthesis, similarly to the situation in cereal seeds (Villand and Kleczkowski, 1994; Denyer *et al.* 1996; Kleczkowski, 1996). In contrast to the *Arabidopsis* homologues, barley leaf AGPase subunits do contain discernible transit peptide cleavage sites, as found both by analyses of derived cDNA sequences (Thorbjørnsen *et al.*, 1996; Eimert *et al.*, 1997) and by expression in a heterologous system of full-length and specifically truncated cDNAs (Luo *et al.*, 1997; Luo and Kleczkowski, 1998).

Spatial expression and the role of ApL1 protein

Expression of the *ApL1* gene was studied using RNA fractions collected from various tissues of intact *Arabidopsis* (Fig. 2). The gene was expressed predominantly in rosette and stem leaves

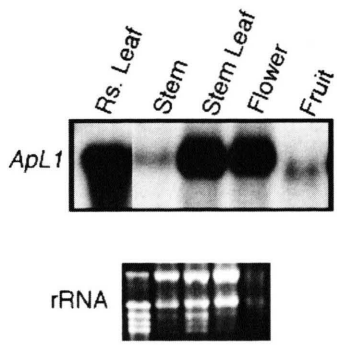

Fig. 2. Expression of the *ApL1* gene in *Arabidopsis* tissues. Total RNA from rosette (Rs.) leaves, stems, stem leaves, flowers and fruits of mature *Arabidopsis* plants was separated on agarose gel, followed by Northern transfer. The membrane was hybridised with the *ApL1–1* cDNA as a probe (see Materials and Methods for other details). The bottom panel represents an ethidium bromide-stained gel demonstrating the relative amounts of RNA loaded.

as well as in flowers. Some expression was also detected in stems and in fruits (Fig. 2). In the latter case, we were limited by the low level of total RNA due to contamination with polysaccharides (see rRNA levels in Fig. 2), so the actual comparative expression of *ApL1* there is certainly higher than shown. The expression in these various tissues was observed both during dark and light conditions (data not shown), suggesting that the gene is ubiquitously expressed in intact *Arabidopsis*, plants, albeit its expression level may vary depending on conditions and/or organ studied. All the tissues that were investigated in the present study contain a considerable pool of internal starch, as found by iodine staining tests (K. Eimert, unpublished).

The ubiquitous expression of the *ApL1a* transcript in *Arabidopsis* is consistent with the role of the corresponding gene as one of the major determinants of starch production in this species. The *ApL1* gene encodes a protein that is absent in leaves of a starch-deficient *adg2* mutant of *Arabidopsis* (Wang *et al*, 1997). This mutant does express the mutated *ApL1* transcript, but its translation is impaired resulting in a greatly reduced AGPase activity and in starch deficiency. The data, apart from underlying the major role for *ApL1* with respect to starch synthesis, suggest also that *ApL2* and *ApL3* genes can not functionally compensate for the lack of ApL1 protein in *adg2*. One possibility is that these genes encode proteins that confer regulatory properties for native AGPase that are distinct from those where ApL1 protein is present. Large subunits represent critical determinants of the regulatory response of the enzyme (Ball and Preiss, 1994) and, when substituting for ApL1, they could confer a degree of effector (in)-sensitivity to the native AGPase, limiting starch production in the mutant. Other possibility is that these genes are perhaps expressed in some non-mesophyll leaf cells only (*e.g.* guard cells) (Sokolov *et al.,* 1998). For further evaluation of the roles of individual AGPase genes in *Arabidopsis*, *in situ* expression approaches and studies using transgenic plants with "antisense" inhibition of either *ApL2* and *ApL3* will be necessary.

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Bairoch A. (1992), PC/GENE User and Reference Manual: Release 6.7, IntelliGenetics Inc.

Ball K. and Preiss J. (1994), Allosteric sites of the large subunit of the spinach leaf ADP-glucose pyrophosphorylase. J. Biol. Chem. **269**, 24706–24711.

Chen B. Y. and Janes H. W. (1998), Multiple forms of ADP-glucose pyrophosphorylase from tomato leaf. Physiol. Plant. **103**, 491–496.

Chen B. Y., Janes H. W. and Gianfagna T. (1998), PCR cloning and characterization of multiple ADP-glucose pyrophosphorylase cDNAs from tomato. Plant Sci. **136**, 59–67.

Denyer K., Dunlap F., Thorbjornsen T., Keeling P. and Smith A. M. (1996), The major form of ADP-glucose pyrophosphorylase in maize (*Zea mays* L.) endosperm is extra-plastidial. Plant Physiol. **112**, 779–785.

Eimert K., Luo C., Déjardin A., Villand P., Thorbjørnsen T. and Kleczkowski L. A. (1997), Molecular cloning and expression of the large subunit of ADP-glucose pyrophosphorylase from barley (*Hordeum vulgare*) leaves. Gene **189**, 79–82.

Kleczkowski L. A. (1994), Inhibitors of photosynthetic enzymes/carriers and metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45**, 339–367.

Kleczkowski L. A. (1996), Back to the drawing board: redefining starch synthesis in cereals. Trends Plant Sci. **1**, 363–364.

Kleczkowski L. A., Villand P., Lönneborg A., Olsen O.-A. and Lüthi E. (1991), Plant ADP-glucose pyrophosphorylase: recent advances and biotechnological perspectives. Z. Naturforsch. **46c**, 605–612.

Kleczkowski L. A., Villand P., Lüthi E., Olsen O.-A. and Preiss J. (1993a), Insensitivity of barley endosperm ADP-glucose pyrophosphorylase to 3-phosphoglycerate and orthophosphate regulation. Plant Physiol **101**, 179–186.

Kleczkowski L. A., Villand P., Preiss J. and Olsen O.-A. (1993b), Kinetic mechanism and regulation of ADP-glucose pyrophosphorylase from barley (*Hordeum vulgare*) leaves. J. Biol. Chem. **268,** 6228–6233.

Lin T.-P., Caspar T., Somerville C. R. and Preiss J. (1988), A starch deficient mutant of *Arabidopsis thaliana* with low ADPglucose pyrophosphorylase activity lacks one of the two subunits of the enzyme. Plant Physiol. **88**, 1175–1181.

Luo C. and Kleczkowski L. A. (1998), Expression of barley ADP-glucose pyroposphorylase in *Escherichia coli*: processing and regulatory considerations. Phytochemistry **50**, 209–214.

Luo C., Déjardin A., Villand P., Doan D. N. P. and Kleczkowski L. A. (1997), Differential processing of homologues of the small subunit of ADP-glucose pyrophosphorylase from barley (*Hordeum vulgare*) tissues. Z. Naturforsch. **52c**, 807–811.

Müller-Röber B. T., Koßmann J., Hannah L. C., Willmitzer L. and Sonnewald U. (1990), One of two different ADP-glucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. Mol. Gen. Genet. **224**, 136–146.

Müller-Röber B., Sonnewald U. and Willmitzer L. (1992), Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J. **11**, 1229–1238.

Nakata P. A. and Okita T. W. (1995), Differential regulation of ADP-glucose pyrophosphorylase in the sink and source tissues of potato. Plant Physiol. **108**, 361–368.

Nelson O. and Pan D. (1995), Starch synthesis in maize endosperm. Annu. Rev. Plant. Physiol. Plant Mol. Biol. **46**, 475–496.

Olive M. R., Ellis R. J. and Schuch W. W. (1989), Isolation and nucleotide sequences of cDNA clones encoding ADP-glucose pyrophosphorylase polypeptides from wheat leaf and endosperm. Plant Mol. Biol. **12**, 525–538.

Park S.-W. and Chung W.-I. (1998), Molecular cloning and organ-specific expression of three isoforms of tomato ADP-glucose pyrophosphorylase gene. Gene **206**, 215–221.

Preiss J. (1978), Regulation of adenosine diphosphate glucose pyrophosphorylase. In: Advances in Enzymology and Related Areas of Molecular Biology (Meister A., ed.), pp. 317–381, John Wiley & Sons, Inc.

Sambrook J., Fritsch E. F. and Maniatis T. (1989), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sokolov L. N., Déjardin A. and Kleczkowski L. A. (1998), Sugars and light/dark exposure trigger differential regulation of ADP-glucose pyrophosphorylase genes in *Arabidopsis thaliana*. Biochem. J. **336**, 681–687.

Thorbjørnsen T., Villand P., Kleczkowski L. A. and Olsen O.-A. (1996), A single gene encodes two different transcripts for the ADP-glucose pyrophosphorylase small subunit from barley (*Hordeum vulgare*). Biochem. J. **313**, 149–154.

Villand P., Aalen R., Olsen O.-A., Lüthi E., Lönneborg A. and Kleczkowski L. A. (1992a), PCR-amplification and sequences of cDNA clones for the small and large subunits of ADP-glucose pyrophosphorylase from barley tissues. Plant Mol. Biol. **19**, 381–389.

Villand P. and Kleczkowski L. A. (1994), Is there an alternative pathway for starch biosynthesis in cereal seeds? Z. Naturforsch. **49c**, 215–219.

Villand P., Olsen O.-A., Kilian A. and Kleczkowski L. A. (1992b), ADP-glucose pyrophosphorylase large subunit cDNA from barley endosperm. Plant Physiol. **100**, 1617–1618.

Villand P., Olsen O.-A. and Kleczkowski L. A. (1993), Molecular characterization of multiple cDNA clones of ADP-glucose pyrophosphorylase from *Arabidopsis thaliana*. Plant Mol. Biol. **23**, 1279–1284.

Wang S.-M., Chu B., Wue W.-L., Yu T.-S., Eimert K. and Chen J. (1997), *adg2–1* represents a missense mutation in the ADPG pyrophosphorylase large subunit gene of *Arabidopsis thaliana*. Plant J. **11**, 1121–1126.

Weber H., Heim U., Borisjuk L. and Wobus U. (1995), Cell-type specific, coordinate expression of two ADP-glucose pyrophosphorylase genes in relation to starch biosynthesis during seed development of *Vicia faba* L. Planta **195**, 352–361.