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Isolation and characterization of cDNA clones encoding ADP-glucose pyrophosphorylase (AGPase) large and small subunits from chickpea (*Cicer arietinum* L.)☆

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Abstract

Four cDNA clones encoding two large subunits and two small subunits of the starch regulatory enzyme ADP-glucose pyrophosphorylase (AGPase) were isolated from a chickpea (*Cicer arietinum* L.) stem cDNA library. DNA sequence and Southern blot analyses of these clones, designated *CagpL1*, *CagpL2* (large subunits) and *CagpS1* and *CagpS2* (small subunits), revealed that these isoforms represented different AGPase large and small subunits. RNA expression analysis indicated that *CagpL1* was expressed strongly in leaves with reduced expression in the stem. No detectable expression was observed in seeds and roots. *CagpL2* was expressed moderately in seeds followed by weak expression in leaves, stems and roots. Similar analysis showed that *CagpS1* and *CagpS2* displayed a spatial expression pattern similar to that observed for *CagpL2* with the exception that *CagpS1* showed a much higher expression in seeds than *CagpS2*. The spatial expression patterns of these different AGPase subunit sequences indicate that different AGPase isoforms are used to control starch biosynthesis in different organs during chickpea development.

Keywords: ADP-glucose pyrophosphorylase; Chickpea (*Cicer arietinum* L.)

1. Introduction

ADP-glucose pyrophosphorylase (AGPase) plays a central role in starch biosynthesis in both photosynthetic and nonphotosynthetic plant tissues. It catalyzes the first step of starch biosynthesis by generating the sugar nucleotide ADP-glucose and inorganic pyrophosphate (Pi) from glucose 1-phosphate and ATP. ADP-glucose functions as the glucosyl donor for α-glucan synthesis by starch synthase. The catalytic activity of AGPase is allosterically regulated by small effector molecules whose levels control the rate of starch biosynthesis (Preiss, 1982). Plant AGPases including those from maize (Plaxton and Preiss, 1987) and rice (Sikka et al., 2001) endosperm is activated by 3-PGA and inhibited by Pi. However, recent studies have shown that the allosteric regulatory property may not be a universal feature of all AGPases. The AGPases from the endosperm of barley (Kleczkowski et al., 1993), wheat (Duffus, 1992), and some maize lines (Hannah et al., 1995) have been reported to be relatively insensitive to 3-PGA/Pi regulation indicating some degree of heterogeneity in metabolic regulation among the plant enzymes.

Bacteria employ the same enzyme activity to synthesize glycogen. A single gene whose expressed product of subunit mass of 48 kDa forms a homotetrameric enzyme encodes the AGPase from *Escherichia coli*. The higher plant AGPase has a much more complex structure as it is composed of two large subunits and two small subunits encoded by different genes (Preiss et al., 1991). Depending on the plant species, the small subunits range from 50 to

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; AMP, adenosine 5′-monophosphate; PGA, 3-phosphoglycerate; Pi, inorganic phosphate; *CagpL1* and *CagpL2*, chickpea AGPase large subunits 1 and 2; *CagpS1* and *CagpS2*, chickpea AGPase small subunits 1 and 2.

☆ The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Database with the following Accession Nos: *CagpL1*, AF356002; *CagpL2*, AF356003; *CagpS1*, AF356004; *CagpS2*, AF356005.

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56 kDa, whereas the large subunits range from 51 to 60 kDa (Sowokinos and Preiss, 1982; Morell et al., 1987; Lin et al., 1988). Multiple cDNA clones encoding isoforms of AGPase have been isolated from several plants such as maize (Bhave et al., 1990; Giroux and Hannah, 1994), potato (Müller-Röber et al., 1990; Nakata et al., 1991; La Cognata et al., 1995), sugar beet (Müller-Röber et al., 1995), wheat (Olive et al., 1989; Villand et al., 1992b), barley (Villand et al., 1992a,b), *Arabidopsis* (Villand et al., 1993), sweet potato (Harn et al., 2000), and tomato (Park and Chung, 1998). Therefore, it is now generally recognized that higher plants have multiple AGPase genes, which are expressed in a developmental- and tissue-specific manner. Physiological stimuli such as carbohydrates and light signals have also been shown to control the expression of these genes (Sokolov et al., 1998).

Chickpea (*Cicer arietinum* L.) is an important pulse crop used for food and is a significant source of protein especially for the primarily vegetarian population. The major constituents of chickpea seeds are starch (47.2%), storage proteins (25.7%), lipids (6.0%) and ash (3.4%) (B.-K. Baik, personal communication). One approach to increase the productivity and the nutritional value of chickpea is to improve the rate of starch biosynthesis (Okita et al., 1993, 2001). However, the biochemical and molecular aspects of starch biosynthesis in chickpea have not been studied. As an initial step in efforts to increase starch production during chickpea development, we report here the isolation and transcriptional characterization of cDNA clones encoding the large and small subunits of AGPase from chickpea. The presence of multiple large and small subunit gene sequences which display distinct spatial expression patterns during chickpea growth and development indicates that different AGPase isoforms are used to control starch biosynthesis in different organs during chickpea development.

2. Results and discussion

2.1. Isolation of AGPase cDNA clones

Using the potato AGPase large subunit as a probe, we screened a stem cDNA library under high stringency conditions and isolated 15 positive phage clones. Phages were converted to pBluescript SKII by in vivo excision. Restriction mapping and sequencing analysis led to the identification of two different cDNAs encoding AGPase large subunits. Similar efforts using the AGPase small subunit of *Perilla frutescens* as a probe resulted in the isolation of 10 positive clones, which led to the identification of two different AGPase small subunit cDNAs. Multiple cDNAs for the AGPase large subunits have been isolated from potato (Müller-Röber et al., 1990; Nakata et al., 1991; La Cognata et al., 1995), tomato (Park and Chung, 1998), *Arabidopsis* (Villand et al., 1993; Park and Chung, 1998) and sweet potato (Harn et al., 2000; Lee et al., 2000). In contrast, only a single small subunit has been isolated so far in these plants except in potato where two near-identical small subunit cDNAs have been cloned. The presence of multiple large and small subunits suggests that chickpea expresses several different AGPase isoforms during growth development.

The complete nucleotide sequences of the two large subunit clones, *CagpL1* and *CagpL2*, and two small subunit clones, *CagpS1* and *CagpS2*, were determined and contained 1826, 1996, 1908 and 1722 bp, respectively. Three of the cDNA clones contained the complete coding sequences of 526 residues for *CagpL1*, 522 residues for *CagpL2* and 517 residues for *CagpS1*, respectively. In contrast, *CagpS2* was incomplete and lacked nucleotides coding for the *N*-terminal end of the polypeptide. Further efforts to obtain the complete coding sequence of *CagpS2* by PCR of the stem cDNA library resulted in the identification of 51 additional coding nucleotides. Alignment of *CagpS1* and *CagpS2* sequences indicated that the latter lacked coding information for the extreme N-terminus. Polyadenylation signals (AATAAA) were found in the 3′ untranslated regions at positions 1659–1664 for *CagpL1*, 1861–1866 for *CagpL2*, 1758–1763 for *CagpS1* and 1660–1665 for *CagpS2*.

2.2. Comparison of two isoforms of chickpea AGPase with known large and small subunit polypeptides

The deduced amino acid sequences of *CagpS1*, *CagpS2*, *CagpL1* and *CagpL2* are shown in Fig. 1A and B. CagpS1 and CagpS2 proteins shared higher homology (87.9%) as compared to the two large subunit sequences of CagpL1 and CagpL2, which were more divergent (59% sequence identity). This trend is not only evident in the putative plastid targeting leader sequences (see below) but also in the coding sequences for the mature polypeptides.

The AGPase subunits that are localized to the plastids are initially synthesized as large precursor polypeptides containing an *N*-terminal plastid targeting leader sequence (Choi et al., 2001). The *N*-terminal sequence for the mature small subunit from spinach leaf has been determined by Edman degradation to be VSDSQNSQ (Morell et al., 1987). A nearly identical sequence VSDS (K/Q)N(A/S)Q is also found in the primary sequences of both CagpS1 and CagpS2 (Fig. 1A), indicating that the preceding residues comprise the plastid targeting leader sequence. CagpS1 has a putative plastid targeting sequence of 67 residues, rich in hydroxylated and basic amino acids, a feature commonly observed in chloroplast targeting leader peptides as well as in other small subunit sequences from dicots such as potato (La Cognata et al., 1995), tomato (Park and Chung, 1998) and *Vicia faba* (Weber et al., 1995). The AGPase small subunits of monocot endosperm lack this motif (Villand et al., 1992b) and are likely located in the cytoplasm

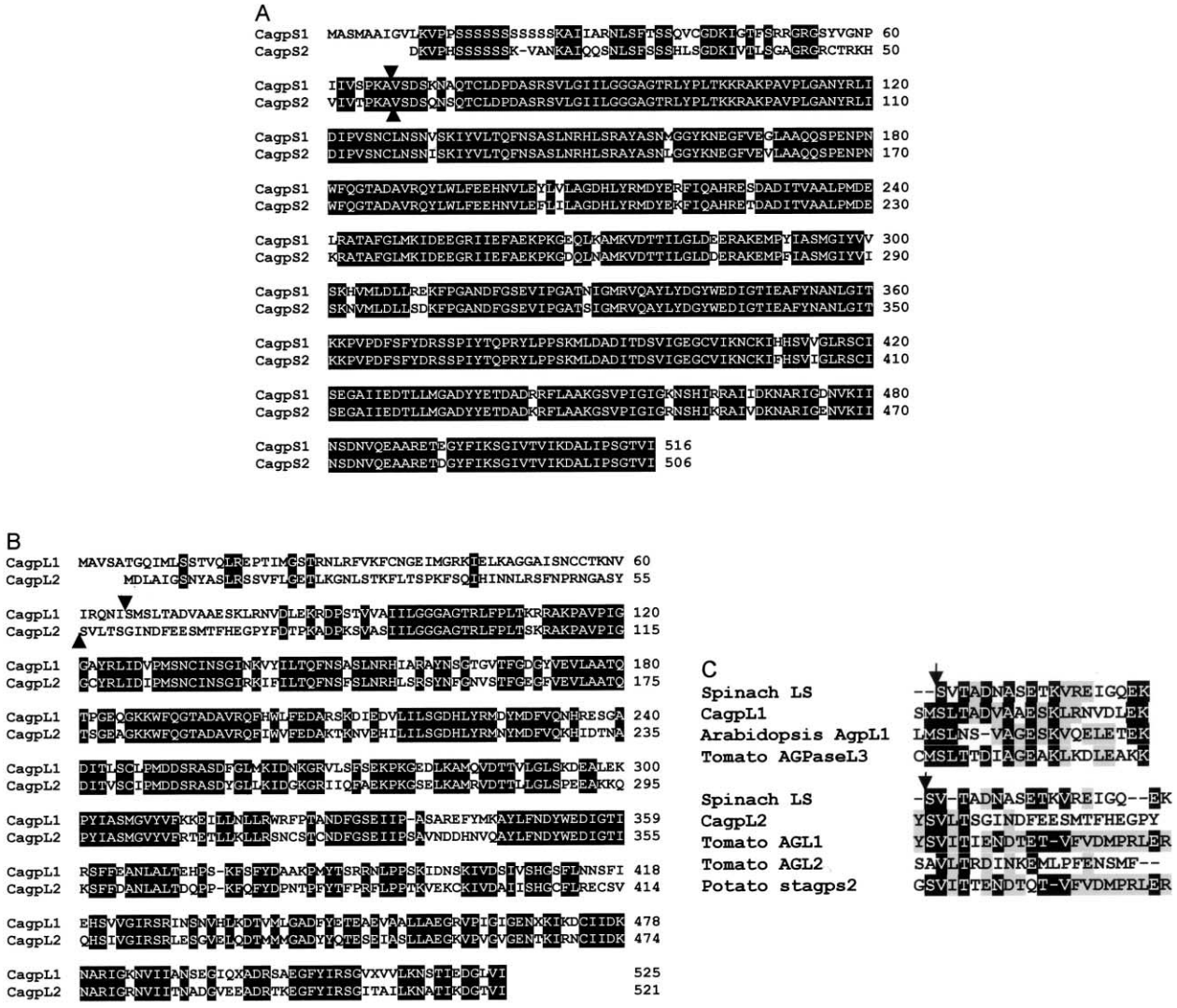

Fig. 1. Comparison of the deduced amino acid sequences of cDNA clones encoding chickpea (A) small subunits and (B) large subunits. (C) Comparison of the derived *N*-terminal sequences of *CagpL1* and *CagpL2* with mature *N*-terminal sequences from spinach leaf (Morell et al., 1987), tomato (Park and Chung, 1998) and potato (La Cognata et al., 1995) LS AGPases. Coding sequences for the *N*-terminal region as well as the 5′UTR (not shown) were used for the preparation of gene specific probes used in the northern and Southern blot analysis. Arrowhead indicates the putative proteolytic cleavage site. Gaps (denoted by dashes) are included to maximize homology.

(Denyer et al., 1996; Thorbjornsen et al., 1996; Sikka et al., 2001). Although the *N*-terminal region of CagpS2 is incomplete, it shares considerable sequence homology with the plastid targeting leader of CagpS1. In addition, it has an amino acid composition consistent with a plastid targeting signal in being rich in hydroxylated amino acids (35%) and hydrophobic amino acids (31%), and having more positively charged amino acids (9%) than negatively charged amino acids (1%). The putative plastid targeting signal of CagpS1 and CagpS2 were also predicted by the TargetP (Emanuelsson et al., 1999) and PSORT (Nakai and Horton, 1999) algorithms.

The N-terminal sequences for the mature large subunits from spinach leaf has been determined by Edman degradation to be SVTADNASETKVREIGQEK (Morell et al., 1987). Comparison of the CagpL1 primary sequence to that of the mature spinach large subunit showed significant alignment with the sequence SMSLTADVAAESKLRNVDLEK beginning with serine at position 66 (Fig. 1C, upper panel). Similar analysis of CagpL2 also led to the identification of a sequence SVLTSGINDFEESMTFHEGPY, which showed significant homology beginning with serine at position 56 (Fig. 1C, lower panel). The N-terminal extension of 65 and 55 amino acids for CagpL1 and CagpL2, respectively, fit the pattern of an expected plastid targeting peptide. For instance, the putative N-terminal plastid targeting signals of CagpL1 and CagpL2 are also enriched with hydroxylated (41 and 46%, respectively) and hydrophobic (36 and 39%, respectively) amino acids, and have a higher percentage of positively charged amino acids (12 and 14%, respectively) than negatively charged amino acids (2 and 3%,

respectively). Overall, these features indicate that the N-terminal regions of CagpL1 and CagpL2 are plastid targeting leader signals.

2.3. Southern blot analysis of CagpL1, CagpL2, CagpS1 and CagpS2

Southern blot experiments were performed using chickpea genomic DNA digested with *Eco*RI, *Eco*RV, *Bam*HI and *Hin*dIII (Fig. 2). Only a few hybridizing fragments were evident when *CagpS1*, *CagpS2* and *CagpL1* were used as probes, suggesting that the genes for *CagpS1*, *CagpS2* are *CagpL1* are present in low copy number in the chickpea genome. In contrast, multiple reacting bands were detected with the *CagpL2* specific probe, suggesting the existence of other isoforms closely related to *CagpL2*. This suggestion is supported by the relatedness of the various AGPase sequences.

AGPase sequences are divided into two main branches, corresponding to the large and small subunit types. Large subunit sequences are divided into four subgroups, i.e. stem and root (including seeds), leaf, and monocot endosperm isoforms. *CagpL1* and *CagpL2* are closely aligned with the leaf and stem isoforms, respectively, observed in other plants. Closely related to the stem isoforms are large subunit sequences that are preferentially expressed in root tissue. The presence of multiple hybridizable bands on the Southern blot of *CagpL2* (Fig. 2) suggests that some of these bands may be contributed by a putative root specific *CagpL* gene. The phylogenic tree shows an interesting relationship in which stem and root forms are also expressed in fruit.

In contrast to the much broader sequence diversity exhibited by the large subunits, the small subunit sequences are much more conserved. They can be divided into two major sub-branches consisting of the cereal monocot endosperm and a second much larger group consisting of all non-endosperm SS (Fig. 3B). Overall, the distinct hybridization patterns obtained with the various chickpea AGPase probes indicate that the AGPase subunits are encoded by two multigene families.

2.4. RNA expression of CagpL1, CagpL2, CagpS1 and CagpS2

The spatial expression profiles of *CagpL1*, *CagpL2*, *CagpS1* and *CagpS2* were investigated primarily by northern blot analysis using total RNA isolated from developing seeds, leaf, stem and roots (Fig. 4 upper panel). To minimize the cross-reaction among the AGPase transcripts, we synthesized gene specific probes encompassing the 5′ regions for both small (Fig. 1A) and large subunit sequences (Fig. 1B). *CagpL1* was expressed strongly in leaves with weak expression in the stem. No detectable expression of RNAs was observed in seeds and roots. Contrary to the *CagpL1* expression pattern, *CagpL2* was expressed moderately in seeds followed by weak expression in leaves, stem and roots. Similar analysis showed that *CagpS1* was expressed highly in seeds followed by weak expression in leaves and very weak expression in stem and roots. *CagpS2* showed weak to moderate expression in seeds followed by very weak expression in leaves, stem and roots. Since Northern blot analysis showed a similar expression pattern between *CagpS1* and *CagpS2* in different tissues of chickpea plant, we further analyzed their expression pattern by RT–PCR (Fig. 4 lower panel). To semi-quantify the RNA expression level of *CagpS1* and *CagpS2* transcripts, unique DNA sequences at 3′UTR were synthesized (210 and 127 bp fragments for *CagpS1* and *CagpS2*, respectively). The amplified sequences of 210 and 127 bp for *CagpS1* and *CagpS2*, respectively, were verified by DNA sequencing (data not shown). The expression pattern showed that *CagpS1* was expressed

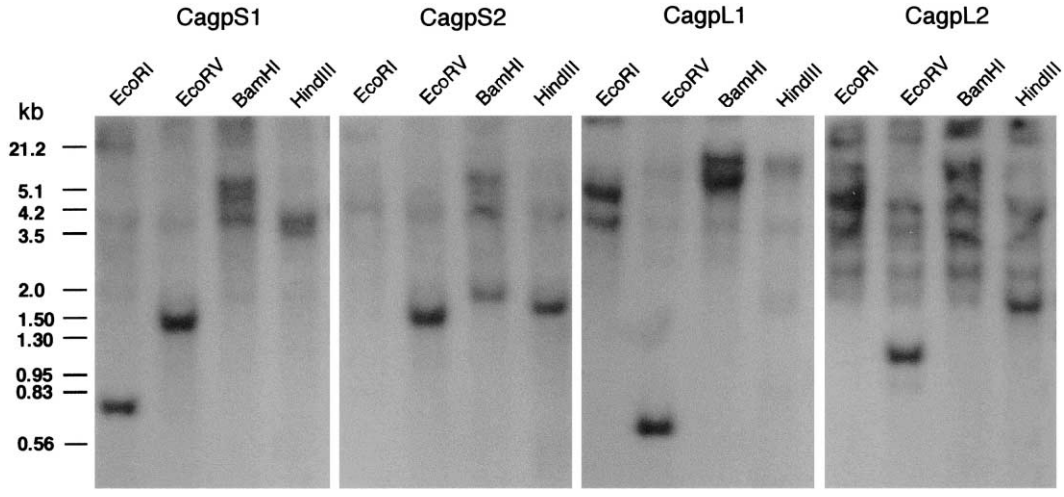

Fig. 2. Genomic DNA gel blot analysis. Genomic DNA (10 μg/lane) was digested with *Eco*RI, *Eco*RV, *Bam*HI and *Hind*III, separated on 0.8% agarose gel, transferred to Hybond N^+ nylon membrane and hybridized with gene specific probes (see Section 3.2).

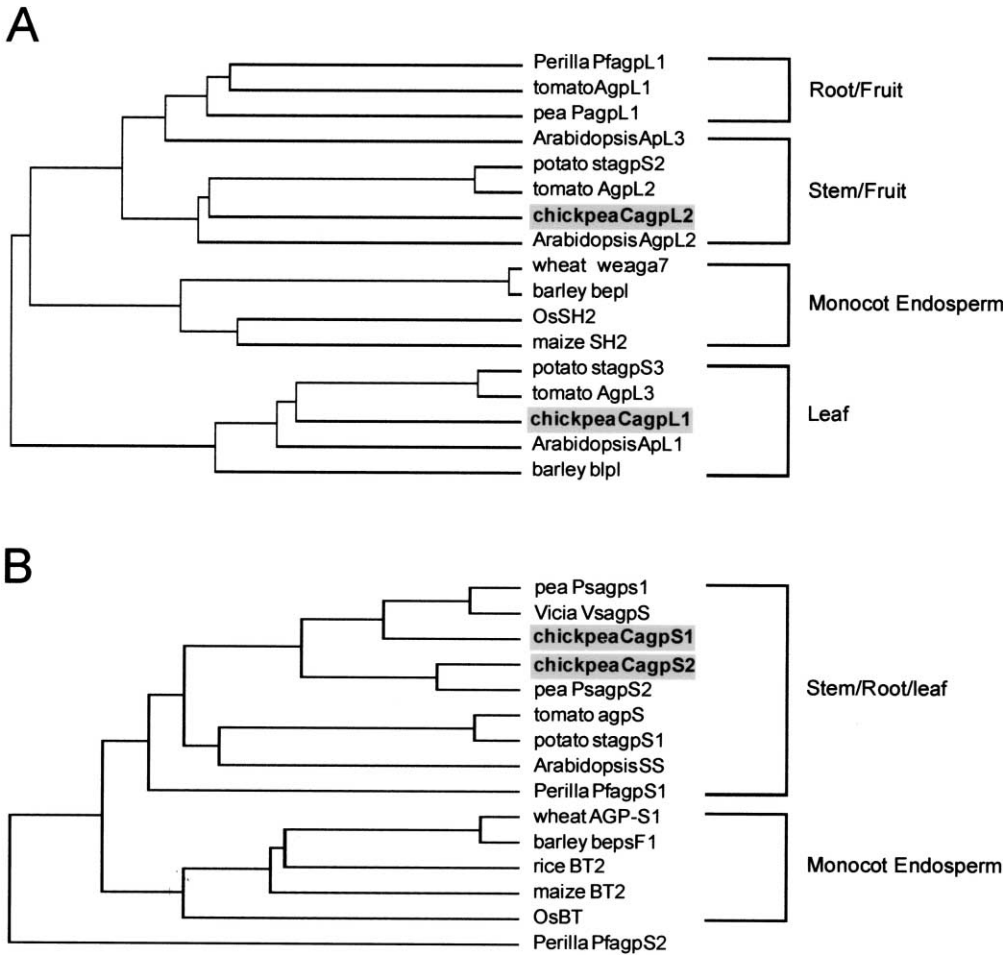

Fig. 3. A dendogram for AGPase (A) large and (B) small subunit proteins from diverse sources was generated by Vector NTI 6.0 software program. Amino sequences are from *Arabidopsis* (Villand et al., 1993), barley (Villand et al., 1992b), maize SH2 (Bhave et al., 1990), maize BT2 (Bae et al., 1990), Perilla (Choi et al., 2001), pea (Burgess et al., 1997), rice BT2 (Anderson et al., 1989), rice large (OsSH2) and small (OsBT) subunit (Sikka et al., 2001), *Vicia fava* (Weber et al., 1995), wheat (Olive et al., 1989), tomato (Park and Chung, 1998), potato (La Cognata et al., 1995), and chickpea (this work).

highly in seeds followed by weak expression in leaves and very weak expression in stem and roots. *CagpS2* also showed moderate expression in seeds followed by very weak expression in leaves, stem and roots (Fig. 4 lower panel). Unlike the large subunit isoforms, which showed completely different expression patterns in various tissues, both *CagpS1* and *CagpS2* small subunit transcripts showed identical spatial expression patterns. The two small subunit genes did differ in gene expression strength and *CagpS1* showed a higher steady state RNA level than *CagpS2* in the tissues examined (Fig. 4 lower panel).

The primary sequences of chickpea *CagpS1* and *CagpS2* are closely related to the pea *PsagpS1* and *Psagps2* (Burgess et al., 1997), respectively (Fig. 3B). Consistent with their sequence homology, the spatial expression patterns for the chickpea and pea isoforms are similar. For instance, *CagpS1* is expressed strongly in seeds followed by lower expression levels in leaves, stem and root. *PsagpS1* is also expressed strongly in seeds followed by weaker expression in leaves, stem and roots. Both *CagpS2* and *PsagpS2* showed the highest expression in seeds followed by weak expression in leaves, stem and root. Chickpea *CagpL1* shares significant homology with the tomato leaf *AgpL3* and other leaf isoforms found in other plants (Fig. 3A). Consistent with this sequence homology to other leaf AGPase large subunit sequences, chickpea *CagpL1* is expressed strongly in leaves with reduced expression in stem. This gene expression pattern is identical to that displayed by the tomato *AGPase L3* which also is expressed strongly in leaves (Park and Chung, 1998). Similarly *CagpL2* shares high homology with stem/root form of tomato *AGPaseL2* that are grouped under the seed/tuber isoforms (Fig. 3A). *CagpL2* is expressed moderately in seeds followed by weak expression in leaves, stem and roots. A similar, if not identical, spatial expression pattern is observed for the tomato *AGPaseL1* which is also expressed strongly in seeds followed by stem and roots but not in leaves (Park and Chung, 1998).

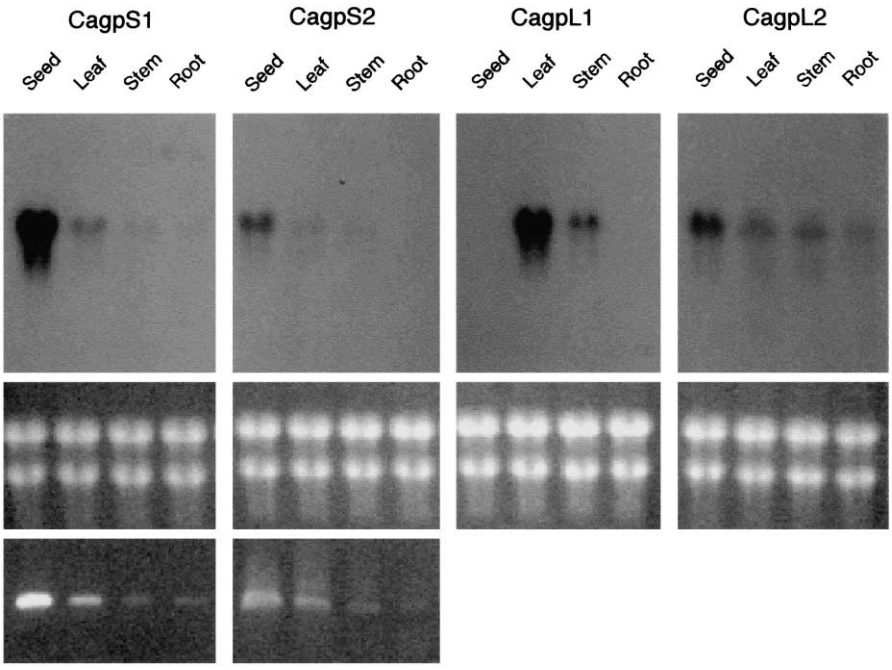

Fig. 4. Upper panel shows the northern blot analysis of *CagpS1*, *CagpS2*, *CagpL1* and *CagpL2* RNA expression in various tissues of chickpea plants. Total RNA (30 μg/lane) was separated on 1% formaldehyde gel, transferred to Hybond N^+ nylon membrane and hybridized with gene-specific probes (see Section 3.3). Middle panel shows EtBr-stained total RNA profile. Lower panel shows semi-quantitative RT–PCR analysis of *CagpS1* and *CagpS2* transcript levels.

Both the large and small subunit are essential for normal AGPase enzyme function (Iglesias et al., 1993). In most plants, there are multiple regulatory LSs and a single or two highly conserved catalytic SSs. The presence of multiple LS genes, which display significant sequence diversity, suggests that the coded AGPases may have different allosteric regulatory and catalytic properties. For instance, when considering the expression patterns for chickpea AGPase isoforms, *CagpS1* may form a heterotetramic structure with *CagpL2* and account for the predominant enzyme activity during starch synthesis in developing seeds. The availability of these different chickpea large and small subunit gene sequences will enable us to determine whether such tissue-specific enzymes have different properties.

3. Experimental

3.1. Isolation and sequencing of cDNA clones

A cDNA library was constructed in λ Uni-ZAP XR vector (Stratagene) using poly $(A)^+$ RNAs isolated from stem tissues of greenhouse grown chickpea (*Cicer arietinum* L). Approximately 3×10^5 recombinant phages were screened by using either a ^{32}P-labeled potato large subunit cDNA (Nakata et al., 1991) or Perilla small subunit cDNA (Choi et al., 2001) as a probe. Probes were prepared using a random priming labeling method in the presence of α-[^{32}P]-dCTP and then purified through a Bio-gel P-60 spin column. The filters were pre-hybridized for 2 h followed by 16 h of hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA at 65 °C. Filters were washed twice in 2X SSC, 0.1% SDS at room temperature and once with 1X SSC, 0.1% SDS at 65 °C. In vivo excision of phage DNA was performed according to the manufacturer's protocol (Stratagene). cDNA inserts were sequenced by dye terminator sequencing mixtures using T3, T7 and sequence-specific primers.

3.2. Genomic DNA gel blot analysis

Genomic DNA was isolated from chickpea leaves as described by Shure et al. (1983). Ten micrograms of genomic DNA were digested with *Eco*RI, *Eco*RV, *Bam*HI and *Hin*dIII respectively, separated on 0.8% agarose gels, and then blotted onto Hybond N^+ nylon membrane (Amersham) in blotting buffer (10X SSC). To differentiate each cDNA, unique DNA sequences at the 5′ UTR and *N*-terminal region of each isoform were used as probes. Templates for probes were synthesized using PCR according to the following conditions: 1 min at 95 °C, 1 min at 53 °C and 1 min at 72 °C (30 cycles). Each pair of primers was synthesized on the basis of 5′ cDNA sequence as follows: 5′GCCACCGGTCAGA TAATGC3′ and 5′GCACGTGCAATATGCCTTG3′ for *CagpL1*, 5′GCTTCTCGTGAGGTCACCAG3′ and 5′C

CTAATGCCACTGTTGATGC3′ for *CagpL2*, 5′GC ATCAATGGCTGCGATCG3′ and 5′GGACTTTGC TGTGCAGCAAG3′ for *CagpS1*, and 5′CACACCTCT CAGGCGACAAG3′ and 5′CAGGACTCTGCTGAG CAG3′ for *CagpS2*. Fragments 427, 504, 523 and 415 bp, were obtained for *CagpL1*, *CagpL2*, *CagpS1* and *CagpS2*, respectively, which were used as probes after being labeled with α-[^{32}P]-dCTP. Filters were hybridized for 16 h at 42 °C in 5X SSC, 2X Denhardt's solution, 50% formamide, 10% Dextran sulphate, 0.25% SDS, 0.05% sodium pyrophosphate and 10 μg/ml denatured salmon sperm DNA. Filters were washed under high stringency conditions, i.e. twice in 2X SSC, 0.1% SDS at room temperature and twice in 0.1X SSC, 0.1% SDS at 65 °C. Filters were exposed for 3 days at −80 °C.

3.3. RNA gel blot analysis

Total RNA was isolated from developing seeds, leaf, stem and roots of chickpea as described by Pawlowski et al. (1994). Thirty micrograms of total RNA was resolved on 1% formaldehyde gels. After electrophoresis, the gel was transferred to a Hybond N^+ nylon membrane (Amersham) in blotting buffer (20X SSC). The same gene specific probes as described for genomic DNA blot analysis were used. Hybridization and washing were also performed as described above.

3.4. Reverse transcriptase–polymerase chain reaction (RT–PCR)

One microgram of total RNA was used for RT–PCR to quantify the RNA expression level of *CagpS1* and *CagpS2* transcripts. To differentiate *CagpS1* and *CagpS2* cDNA clones, unique DNA sequences at 3′ UTR were synthesized using Tth DNA polymerase (MasterAmp™, Epicentre) according to the following cycles: 5 min at 47 °C, 20 min at 60 °C and 5 min at 94 °C (1 cycle) followed by 1 min at 94 °C, 1 min at 47 °C, 1.4 min at 72 °C (20 cycles for *CagpS1* and 40 cycles for *CagpS2*). Each pair of primers were synthesized in the 3′ UTR region using the following primers: 5′ CGGTTTCCTCTTGCTGGT 3′ and 5′ CATATTCCATCCCTTTGATGAATTGC 3′ for *CagpS1* and 5′ CATATTTTGTATCCTAATGTGA GAGAGGC 3′ and 5′ GAGAGTCCTCTGTTCTGC-TATTATTTAC 3′ for *CagpS2*. Amplified fragments of 210 bp and 127 bp were obtained for *CagpS1* and *CagpS2*, respectively.

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