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Regulation of CPSase, ACTase, and OCTase genes in *Medicago truncatula*: Implications for carbamoylphosphate synthesis and allocation to pyrimidine and arginine *de novo* Biosynthesis ☆

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ABSTRACT

In most prokaryotes and many eukaryotes, synthesis of carbamoylphosphate (CP) by carbamoylphosphate synthetase (CPSase; E.C. 6.3.5.5) and its allocation to either pyrimidine or arginine biosynthesis are highly controlled processes. Regulation at the transcriptional level occurs at either CPSase genes or the downstream genes encoding aspartate carbamoyltransferase (E.C. 2.1.3.2) or ornithine carbamoyltransferase (E.C. 2.1.3.3). Given the importance of pyrimidine and arginine biosynthesis, our lack of basic knowledge regarding genetic regulation of these processes in plants is a striking omission. Transcripts encoding two CPSase small subunits (*MtCPSs1* and *MtCPSs2*), a single CPSase large subunit (*MtCPSl*), ACTase (*MtPyrB*), and OCTase (*MtArgF*) were characterized in the model legume *Medicago truncatula*. Quantitative real-time PCR data provided evidence (i) that the accumulation of all CPSase gene transcripts, as well as the *MtPyrB* transcript, was dramatically reduced following seedling incubation with uridine; (ii) exogenously supplied arginine down regulated only *MtArgF*; and (iii) mRNA levels of both CPSase small subunits, *MtPyrB*, and *MtArgF* were significantly increased after supplying plants with ornithine alone or in combination with uridine or arginine compared to plants treated with only uridine or arginine, respectively ($P \leq 0.05$). A proposed novel, yet simple regulatory scheme employed by *M. truncatula* more closely resembles a prokaryotic control strategy than those used by other eukaryotes.

1. Introduction

The *Arabidopsis thaliana* genome encodes single carbamoylphosphate synthetase (CPSase) small and large subunit genes (The *Arabidopsis* Genome Initiative, 2000). However, analyses of the genomes of two additional plant species provide intriguing evidence suggestive of CPSase multi-gene families. Zhou et al. (2000) isolated two genomic fragments encoding partial, but distinctively different, sequences from the tetraploid *Medicago sativa*, and Giermann et al. (2002) reported multiple signals from transfer-hybridization analysis performed using DNA isolated from *Nicotiana tabacum*, although only one cDNA could be isolated. Whether the contrasting results obtained for *N. tabacum* are due to limitations of the methods employed or to the presence of pseudogenes remains unresolved. Interestingly, genomic sequence data suggest a CPSase pseudogene resides in the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000). Taken together, these reports raise the possibility that some plants might in fact express functional multiple CPSase gene products. Such a finding would challenge the long-held paradigm that in plants the competing metabolic needs of the pyrimidine and arginine biosynthetic pathways for the common precursor CP are met by a single CPSase enzyme which is encoded by a single gene per subunit (Zrenner et al., 2006). The presence of multiple CPSase genes invokes possibilities for differential regulation with a special focus on pathway specific-genes that could be responsive to fluctuating levels of pyrimidines or arginine, respectively. In contrast, the presence of a single gene for each CPSase subunit requires that control at the level of transcription be coordinated in a manner that is responsive to the plant's needs for pyrimidine nucleotides and arginine.

Both pathway-specific and dually functional CPSase genes have been identified by their transcriptional and post-transcriptional response to pyrimidine and arginine pathway end products across widely ranging taxa. One example of dually functional CPSase-encoding genes is found in enterobacteria, such as *Escherichia coli* (Piette et al., 1984) and *Salmonella typhimurium* (Kilstrup et al., 1988). These species possess a single type II CPSase [L-glutamine dependent; carbon dioxide:L-glutamine amido ligase (ADP-forming, carbamate-

Abbreviations: ACTase, Aspartate carbamoyltransferase; CP, Carbamoylphosphate; CPSase, Carbamoylphosphate synthetase; OCTase, ornithine carbamoyltransferase.

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phosphorylating), E.C. 6.3.5.5] encoded by the *carAB* operon, that generates CP for both pyrimidine and arginine biosynthesis (Crabeel et al., 1980; Pierard and Wiame, 1964), and is transcriptionally regulated by both uracil and arginine (Bouvier et al., 1984; Piette et al., 1984). Furthermore, *E. coli* CPSase is allosterically inhibited by the pyrimidine pathway end product uridine 5'-monophosphate (UMP) and this inhibition is relieved by addition of ornithine (Anderson and Meister, 1966; Robin et al., 1989). In contrast, gram-positive species of the family *Bacillaceae* possess duplicated CPSase genes that are differentially regulated by pyrimidines and arginine, respectively (Paulus and Switzer, 1979). Eukaryotic fungal species *Neurospora crassa* and *Saccharomyces cerevisiae* also possess glutamine-dependent, arginine pathway-specific (CPS-A) and pyrimidine pathway-specific (CPS-P) type II CPSase genes, which are repressed by arginine and uracil, respectively (Cybis and Davis, 1975; Davis et al., 1981; Pierard et al., 1979).

Regulation of the genes and encoded enzymes immediately downstream of CPSase also plays an important role in CP allocation to pyrimidine and arginine biosynthesis in both prokaryotes and eukaryotes. In *E. coli,* aspartate carbamoyltransferase (ACTase; E.C. 2.1.3.2) is encoded by the *pyrBI* operon and is regulated by post-transcriptional attenuation and translational control (Clemmesen et al., 1985; Roland et al., 1985; Turnbough et al., 1983). *E. coli* ornithine carbamoyltransferase (OCTase; E.C. 2.1.3.3) is encoded by the *argF* gene and is both transcriptionally (Williams and Rogers, 1987) and post-transcriptionally (Faanes and Rogers, 1972) regulated by arginine. In eukaryotic species such as the yeast *S. cerevisiae*, CPS-P and ACTase genes are both encoded by the *ura2* gene cluster and are subject to both transcriptional (Potier et al., 1990) and enzymatic regulation (Serre et al., 2004) by UTP.

In plants, CPSase catalytic activity was first described for the legume *Pisum sativum* and found to be glutamine-dependent, strongly inhibited by UMP and stimulated by ornithine, like bacterial type II CPSase (E.C. 6.3.5.5) (O'Neal and Naylor, 1969). Subsequently, *in vitro* studies have demonstrated feedback control of CPSase (O'Neal and Naylor, 1976) or ACTase, depending on the plant species (Lovatt and Cheng, 1984; Ong and Jackson, 1972). The possibility that some plant species express multiple functional CPSase gene products still requires confirmation; if duplicated genes are expressed, it becomes important to understand whether CPSase gene family members are regulated in a pyrimidine pathway- or arginine pathway-specific manner, respectively, or they are redundant in their role of supplying CP to these competing pathways. Despite being a key aspect of basal nitrogen metabolism in plants, there are no reports describing transcriptional regulation of plant CPSase genes. Given the diversity of prokaryotic and eukaryotic species in which transcriptional regulation of CPSase genes by pyrimidine pathway end products has been described, it is striking that plants have been omitted from such investigations for more than 20 years (Piette et al., 1984).

The model legume *Medicago truncatula*, a congener to the agriculturally important legume crop *M. sativa* (the first plant species to have a CPSase gene sequenced Zhou et al., 2000), was used to address these fundamental questions. The *M. truncatula* genome includes a CPSase gene family comprising two genes encoding the small subunit of the CPSase holoenzyme and one gene encoding the large subunit. Quantitative real-time PCR was used to assess the effects of exogenous uridine, arginine and/or ornithine on CPSase, ACTase, and OCTase transcript accumulation in the shoot apices of 4-day-old *M. truncatula* seedlings.

2. Materials and methods

2.1. Plant material

Seeds of *M. truncatula* (cv. Jemalong A17) were scarified in concentrated sulfuric acid for 15 min, rinsed three times by soaking in sterile water for 5 min for each rinse, surface-sterilized with a 25% commercial sodium hypochlorite bleach solution, and rinsed three times with sterile water over a 5-min period. Imbibed seeds were transferred to Petri plates containing modified Murashige and Skoog (MS) agar (0.8%, w/v) medium without nitrogen (supplemented with KCl to replace KNO_3) for 48 h. Approximately 20 seedlings were then transferred to individual flasks containing complete MS liquid medium alone (control) or supplemented with one or more of the following effector molecules: 5 mM ornithine, 5 mM arginine, 10 mM uridine, or 100 μM racemic (+/−)-*cis*,*trans*-abscisic acid (all adjusted to a final pH of 5.8). Seedlings were incubated with effector molecules on shaking platforms in growth chambers at 20 °C under constant light for 3.5 h. At the end of the treatment period, approximately 20 shoot apices (terminal 1 mm of the epicotyl with leaves removed) per treatment were dissected and pooled. Dissected shoot apices were flash frozen in liquid nitrogen and stored in a freezer at −80 °C until further use.

2.2. Total RNA isolation

Total cellular RNA was extracted from the excised shoot apices using Trizol Reagent (Invitrogen, Carlsbad, CA). For RACE reactions necessary for molecular cloning, RNAs were isolated using Trizol Reagent and pooled from root and shoot apices, leaves, and flowers. Ten μg of total cellular RNA was treated with 2U RQ1 RNase-free DNase (Promega Corp. Madison, WI) according to the manufacturer's specifications. The RNA was further purified using a standard phenol/chloroform extraction (Sambrook et al., 1989). Spectroscopic analysis to quantify RNA amounts was performed using a Nanodrop ND1000 UV Spectrophotometer (Nanodrop Technologies, Wilmington, DE) at 260 and 280 nm. Electrophoretic fractionation of RNA was performed on denaturing formaldehyde gels containing 2% (w/v) agarose to determine the integrity of the RNA preparation.

2.3. Reverse transcriptase reactions

One μg of total cellular RNA was used as template for a first-strand cDNA synthesis reaction using 200 U MMLV reverse transcriptase (Promega Corp.), 5× MMLV RT buffer (Promega Corp.), 0.5 μg oligo dT primer (18 mer), 10 μM dNTPs, and 40 U RNasin. Reactions were allowed to proceed for 2 h at 42 °C after which time the reactions were heat-denatured at 70 °C for 10 min.

5' and 3' RACE was performed using the First Choice® RLM RACE kit (Ambion Inc., Austin, TX) using 1 mM of each primer as described in Supplementary Table 1. PCR products were purified using the QIAQuick PCR Purification Kit (Qiagen, Valencia, CA) and cloned into the pGEM T-easy cloning vector (Promega, Madison, WI). Sequencing reactions were carried out using an ABI 3730xl (Applied Biosystems Inc., Foster City, CA) at the University of California-Riverside Institute for Integrated Genome Biology.

2.4. Sequence analysis

Alignment of nucleotide and amino acid sequences were performed using BLAST (Altschul et al., 1990). Predicted molecular weights for *MtCPSs1*, *MtCPSs2*, *MtCPSl*, *MtPyrB*, and *MtArgF* gene products were determined using ExPASy Compute pI/Mw (http://ca.expasy.org/tools/pi_tool.html). Putative N-terminus protein targeting signals were identified using TargetP software (http://www.cbs.dtu.dk/services/TargetP/).

2.5. Quantitative real time PCR (Q-RTPCR)

Reverse transcriptase reactions were performed as described above. One μl aliquots of first-strand cDNA samples were subjected to Q-RTPCR using the iQ SYBR Green Supermix kit in conjunction with