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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.

☆ Accession numbers: FJ388884 *MtArgF*, FJ388885 *MtPyrB*, FJ388886 *MtCPSl*, FJ388887 *MtCPSs2*, FJ388888 *MtCPSs1*.

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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

an iCycler (Bio-Rad, Hercules, CA). Fluorescent signal indicative of PCR product accumulation was measured at 490 nm. Real time PCR conditions were as follows: 95 °C for 30 s, 60 °C for 15 s, and 72 °C elongation for 20 s (40 cycles). Four ten-fold serial dilutions of approximately 100 ng cDNA were used as template for Q-RTPCR to determine primer efficiency for each reaction (Souaze et al., 1996). A separate and independent verification of Q-RTPCR amplification efficiencies for each individual reaction was determined by methods described by Zhao and Fernald (2005). Melting curve analysis was performed for each primer pair to confirm amplification of a single product. Sizes of amplified products were verified by electrophoresis in a 1% agarose gel (w/v). The ratio of transcript that accumulated in treated plant tissue to that of control plant tissue was calculated using the equation described by Pfaffl (2001), and subsequently by the method described by Zhao and Fernald (2005). Actin mRNA was used as the reference to normalize results across treatments and plates. Sequences of Q-RTPCR primers are found in Supplementary Table 2.

2.6. Transfer hybridization analysis

CPSase small subunit and OCTase encoding gene transcripts were amplified by PCR and labeled with ^{32}P-dATP (Perkin Elmer, Boston, MA) using DECAprime II Random Priming DNA Labeling Kit (Ambion, Austin, TX). Northern transfer hybridization analysis and X-ray film (Kodak, Rochester, NY) exposure were conducted using standard methods (Sambrook et al., 1989).

2.7. Statistical analyses

Two technical Q-RTPCR replicates were averaged to give a single value for each treatment per experiment. The mean value obtained from three independent experiments representing 20 individual plants per experiment is reported. To accommodate the non-parametric nature of the data, all means were transformed by determining the square root of the arcsine. All statistical analyses including ANOVA and Pearson correlation were performed using the General Linear Model component of SAS (SAS Institute Inc, Cary, NC). Only correlations with r-values ≥ 0.6 and P-values ≤ 0.05 were considered biologically significant.

3. Results

3.1. Identification and sequence analysis of M. truncatula CPSase, ACTase and OCTase transcripts

To determine whether the genome of the model legume *M. truncatula* contains a CPSase multi-gene family, an EST sequence database (Tigr.org) was queried using an *M. sativa* CPSase small subunit gene nucleotide sequence (accession no. AF191301) to identify putative *M. truncatula* CPSase transcripts. One partial cDNA clone (NFO47E05FL) with sequence similarity to the alfalfa CPSase small subunit transcript was identified and used to construct primers for 5' RACE and RT-PCR reactions. Two transcripts were identified and characterized using a combination of RT-PCR and 5' RACE; these are termed *MtCPSs1* and *MtCPSs2*. Alignment of the duplicated *M. truncatula* CPSase small subunit amino acid sequences with those of the model dicot *A. thaliana* (accession no. AB018114), the model monocot *Oryza sativa* (accession no. NC_008395), and with *M. sativa*, *N. tabacum*, and *E. coli* provides evidence that the two *M. truncatula* putative small subunit sequences are highly conserved with regard to organisms that also possess type II CPSase (Table 1). In particular, the essential Cys-His-Glu catalytic triad of amino acids essential to glutamine hydrolysis remains intact in deduced *MtCPSs1* and *MtCPSs2* protein sequences, along with the synthetase and glutamine amido-transferase domains found at the amino- and carboxy-termini, respectively (Supplementary Fig. 1). The identification of two CPSase small subunit genes is consistent with transfer hybridization analysis (data not shown). Comparison of these transcripts with the partially annotated *M. truncatula* genome, in which two CPSase genes have been catalogued, demonstrated that both genes contain ten exons separated by nine introns (Fig. 1). Strikingly, the positions of the nine introns in both *MtCPSs1* and *MtCPSs2* genes are fully conserved with their respective locations in the CPSase small subunit gene architectures of *A. thaliana*, *O. sativa*, the Bryophyte *Physcomitrella patens* (accession no. DS544931), and California Poplar, *Populus trichocarpa* (accession no. AC208999.1). In contrast, the unicellular green alga *Chlamydomonas rheinhardtii* CPSase small subunit gene architecture is characterized by 7 exons and 8 introns (not shown), in which only the exon/intron boundaries between exons 3 and 4, and 7 and 8 are shared with the plants listed here. Hence, the remainder of the conserved exon/intron boundaries shared by land plants may have arisen in the lineage leading from the green algae to non-vascular land plants and persisted through the evolution of vascular plants.

N. tabacum (Giermann et al., 2002) and the legumes *P. sativum* (Williamson and Slocum, 1994) and *Canavalia lineata* (Lee et al., 2001) have been reported to possess multi-gene families encoding the CPSase large subunit, ACTase, and OCTase, respectively. To determine whether *M. truncatula* also possesses multiple genes encoding each of these products, an *M. truncatula* EST sequence database (Tigr.org) was queried using *M. sativa* CPSase large subunit (Zhou et al., 2000), *P. sativum* ACTase (Williamson and Slocum, 1994), and *C. lineata* OCTase (Lee et al., 2001) nucleotide sequences. Three useful ESTs were identified: CPSase large subunit (NF101A05ST), ACTase (TC98462), and OCTase (TC95434). Based upon these sequences, primers were designed for RACE reactions (Supplementary Table 1). Transfer hybridization analysis (not shown) suggested the presence of two possible genes encoding OCTase in *M. truncatula*; however, an exhaustive search for multiple transcripts within the EST database yielded only a single sequence with the methods employed here (Fig. 2). Consistent with analyses of deduced N-terminal sequences of other described plant CPSase, ACTase, and OCTase polypeptides (Slocum, 2005; Zrenner et al., 2006), the deduced amino acid

Table 1
Putative amino acid sequence motifs necessary for enzymatic function identified using pairwise alignment against identified *E. coli* sequences.

Gene product	Sequence motif function	Amino acid sequence motif	References	Molecular weight	Length (amino acid)	Exon/intron architecture
MtCPSs1	Catalytic triad	C308, Q390, H392	Rubino et al. (1986)	47 kDa	432	10/9
MtCPSs2	Catalytic triad	C309, Q391, H393	Rubino et al. (1986)	47 kDa	431	10/9
MtCPSl	Carboxyphosphate formation	R157, R204, R243, E289, H317, N357, Q359, N375, R377	Post et al. (1990), Stapleton et al. (1996)	128 kDa	1165	4/3
MtCPSl	Carbamate formation	G807, L820, H868, H875, K1081	Post et al. (1990), Miles et al. (1993), Fresquet et al. (2000)			
MtPyrB	CP binding	S135, T136, R137, T138, R139, S152, K156,	Gouaux and Lipscomb (1988)	44 kDa	392	6/5
MtPyrB	Catalytic site	H205, P206, T207, Q208	Gouaux and Lipscomb (1988)			
MtArgF	CP binding	S117, M118, R119, T120, R121	Gouaux and Lipscomb (1988)	40 kDa	369	Undetermined
MtArgF	Catalytic site	H195, P196, C197, Q198	Gouaux and Lipscomb (1988)			
MtArgF	Ornithine Binding	F324, M325, H326, C327, L328, P329	Marshall and Cohen (1980), Itoh et al. (1988)			

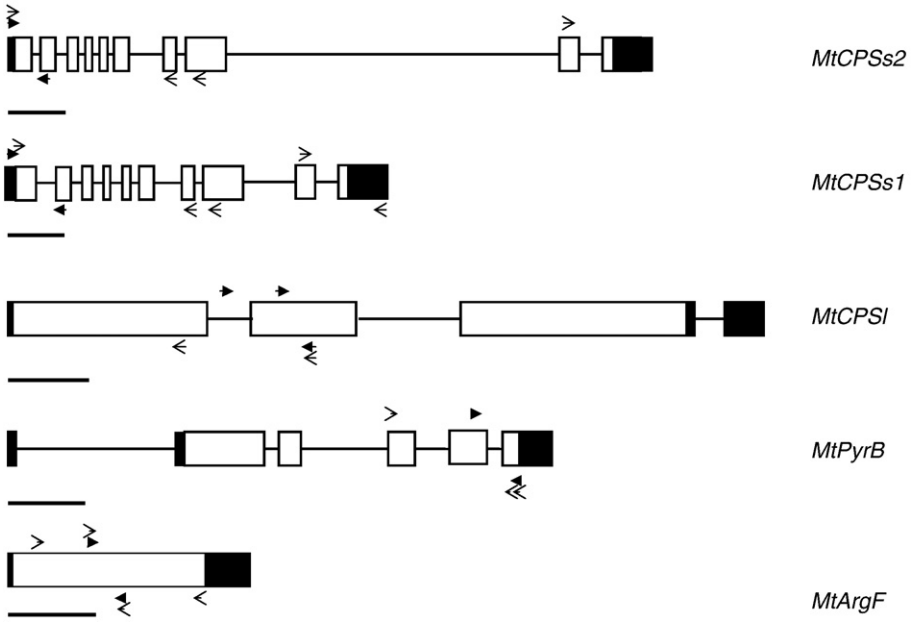

Fig. 1. Schematic representations of *M. truncatula* CPSase small subunits (*MtCPSs1* and *MtCPSs2*), large subunit (*MtCPSl*), ACTase (*MtPyrB*), and OCTase (*MtArgF*) genomic DNA architectures. Exons are represented by rectangles and introns are shown as adjoining lines. The 5'- and 3'-untranslated regions for each gene are shown as a black region with the first and last exons, respectively. Open arrows indicate the positions of primers used for RACE PCR; solid arrows, primers used in Q-RTPCR analysis.

sequences of all *M. truncatula* transcripts described here are predicted to localize to the plastid (ChloroP scores>0.05).

3.2. Influence of exogenous uridine and/or ornithine on CPSase and ACTase transcript accumulation

In order to determine whether *M. truncatula* CPSase and ACTase genes are transcriptionally responsive to feedback control by pyrimidine pathway end products, 4-day-old seedlings were incubated in the presence of 10 mM uridine for up to 24 h. Over this period, exogenous uridine reduced (defined as >50% decrease in mRNA expression relative to the untreated control) the amount of mRNA that accumulated for *MtCPSs1* (87%), *MtCPSs2* (88%), *MtCPSl* (95%), and *MtPyrB* (97%). These reductions were observed to be the greatest after 3.5 h of incubation (Supplementary Fig. 1A, B, C, D). Thus, a 3.5-h incubation period was used in the experiments reported below. Qualitative validation of Q-RTPCR data was demonstrated by using transfer hybridization to reveal steady state accumulation of CPSase small and large subunits against two independent fractionated total RNA samples from MS (control) and uridine treatments (Supplementary Fig. 3A).

The *de novo* arginine biosynthesis intermediate ornithine is able to reverse allosteric inhibition of CPSase by uridine in prokaryotes, eukaryotes, and plants. To determine whether ornithine could prevent or overcome the approximately 85% reduction in mRNA

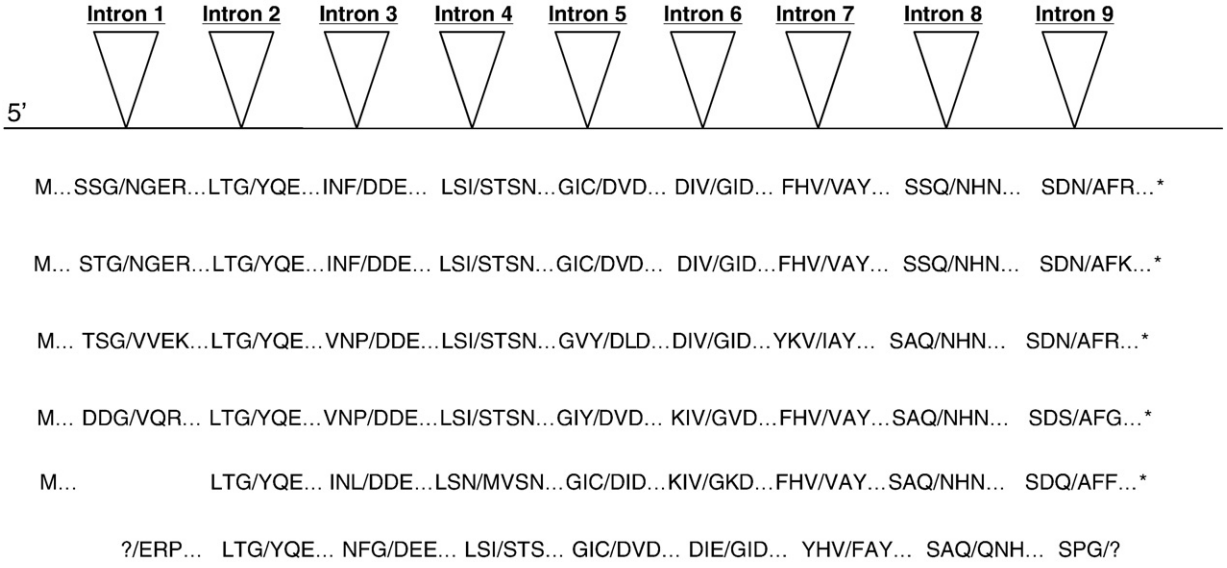

Fig. 2. The deduced amino acids encoded at the 3' end of the upstream and the 5' end of the downstream exons within CPSase small subunit genes from *M. truncatula* (*MtCPSs1*, *MtCPSs2*), *A. thaliana, O. sativa, Physcomitrilla patens* (a Bryophyte), and *Populus trichocarpa*. The solid line represents transcript exons; the triangles, intronic regions. There is no intron corresponding to intron 1 of other species in the *P. patens* gene. The sequence for *P. trichocarpa* is deduced from unannotated partial genomic sequence, and as such the spliced architecture is speculative.

accumulation by added uridine, or its metabolites, 4-day-old *M. truncatula* seedlings were incubated with ornithine alone or ornithine combined with uridine. Added ornithine generated an increase in accumulated mRNA levels relative to the untreated control for *MtCPSs1* (400%), *MtCPSs2* (205%), and *MtPyrB* (348%) (Fig. 3A, B, C). Supplying seedlings with both uridine and ornithine provided clear evidence that ornithine could prevent or overcome the inhibitory effect of uridine on transcription by increasing RNA levels of *MtCPSs1* (354%), *MtCPSs2* (263%) and *MtPyrB* (359%) (Fig. 3A, B, C). Plants treated with ornithine alone and ornithine in combination with uridine accumulated significantly greater *MtCPSs1*, *MtCPSs2*, and *MtPyrB* transcript levels than plants treated with uridine alone, respectively (Fig. 3A, B, C) ($P \leq 0.05$). In contrast, ornithine alone did not cause a dramatic increase in accumulated *MtCPSl* mRNA (Fig. 3E). Added ornithine did, however, significantly diminish the effect of added uridine ($P \leq 0.05$) such that *MtCPSl* transcript levels in plants treated with ornithine alone or ornithine plus uridine were not significantly different at the 5% level.

3.3. Influence of exogenously supplied arginine and/or ornithine on CPSase and OCTase transcript accumulation

Arginine has been demonstrated in both prokaryotes and eukaryotes to modulate CPSase gene expression. However, in *M. truncatula*, a 3.5-h incubation with added arginine (5 mM) reduced only the transcript level of *MtArgF*, the arginine pathway indicator gene encoding OCTase, below the level of the untreated control (Fig. 3A, B, D, E). In the presence of arginine, *MtArgF* transcript accumulation was reduced by 63%. Using the criterion that a decrease in transcript levels of >50% relative to the untreated control represents a down regulation of transcription, *MtCPSs1*, *MtCPSs2* and *MtCPSl* transcript levels were relatively insensitive to added arginine, suggesting that *MtArgF* alone is the point of transcriptional control for feedback by arginine.

To test whether the arginine biosynthesis intermediate ornithine could overcome or prevent the negative effect of arginine on *MtArgF* transcription, 4-day-old *M. truncatula* seedlings were incubated with

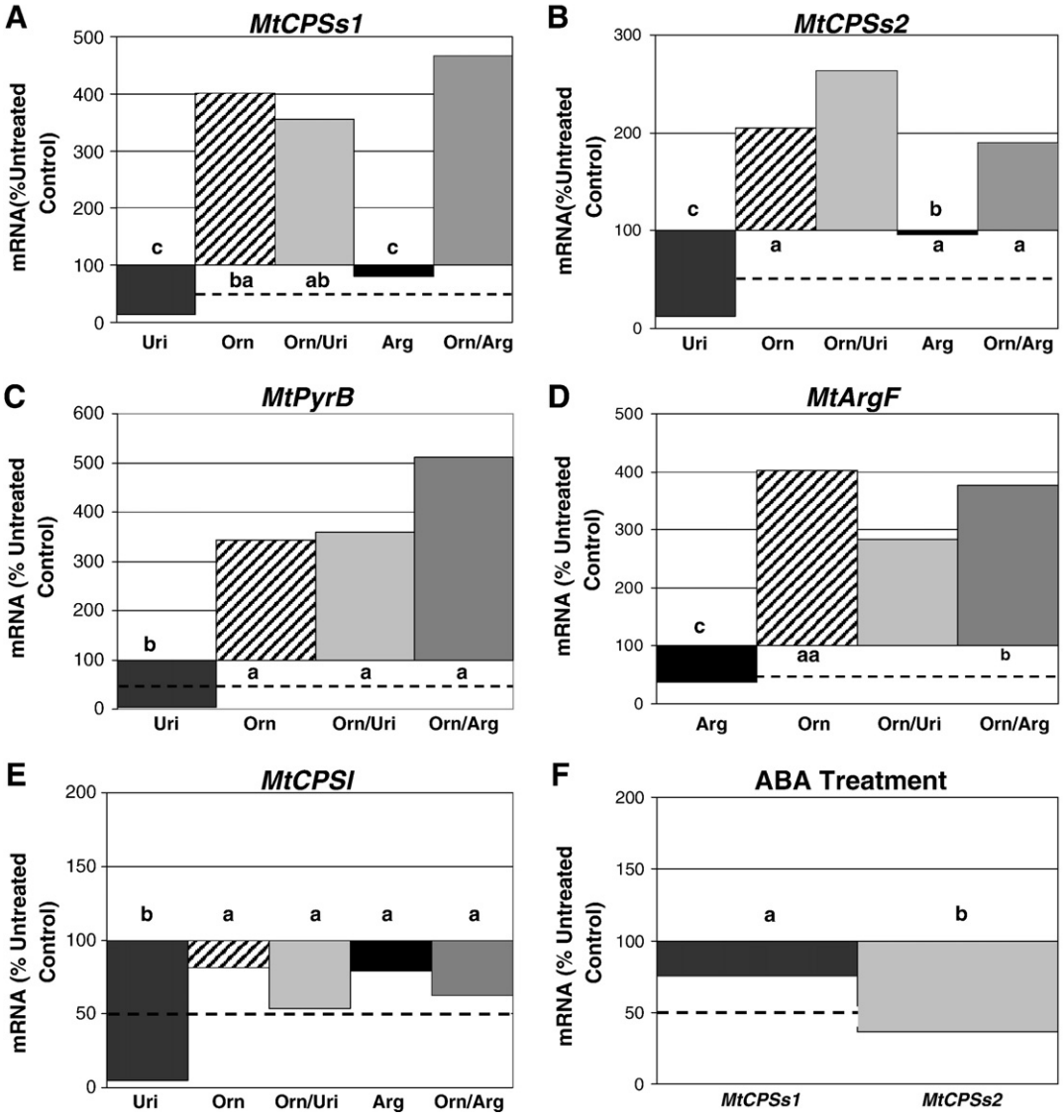

Fig. 3. Effects of arginine (5 mM), uridine (10 mM), ornithine (5 mM), ornithine plus arginine, and ornithine plus uridine on (A) *MtCPSs1*, (B) *MtCPSs2*, (C) *MtPyrB*, (D) *MtArgF*, (E) *MtCPSl*, and (F) ABA (100 μM) on *MtCPSs1* and *MtCPSs2* mRNA levels. Four-day-old plants were treated with each effector molecule and transcripts were measured after 3.5-h treatment using Q-RTPCR and analyzed using the method of Pfaffl (2001). Transcript levels were normalized to *Mt-actin* mRNA levels and are reported relative to mRNA level of each gene detected in the untreated control (set at 100%). Bars within a panel having different letters are significantly different by Fisher's protected LSD at $P \leq 0.05$. The dashed line indicates a 50% decrease in expression and used as the threshold to define down regulation.

ornithine alone or in combination with arginine. Exogenously supplied ornithine, either alone or with arginine, increased *MtArgF* transcript levels (400% and 377%, respectively) to amounts significantly greater ($P \leq 0.05$) than those that accumulated in the presence of arginine alone (Fig. 3D). *M. truncatula* plants treated with both ornithine and arginine accumulated transcripts of *MtCPSs1* (466%), *MtCPSs2* (189%), *MtPyrB* (511%), and *MtArgF* (377%) to levels greater than or equal to the amount of transcript that accumulated in shoot apices treated only with ornithine ($P \leq 0.05$) (Fig. 3A–D). In contrast, the transcript level of *MtCPSl* (38%) was insensitive to the addition of arginine and/or ornithine at the 5% confidence level (Fig. 3E).

3.4. Statistical evidence that both MtCPSs1 and MtCPSs2 gene expression is coordinated with that of MtPyrB and MtArgF

The fact that *M. truncatula* expresses two CPSase small subunit genes opens the intriguing possibility that one or both of these genes is pathway specific in its expression. Pearson correlation was employed to determine whether transcriptional regulation of one or both *M. truncatula* CPSase small subunits, or the large subunit, was correlated with either the pyrimidine pathway indicator gene encoding ACTase, *MtPyrB*, or the arginine pathway indicator gene encoding OCTase, *MtArgF*. Expression of *MtCPSs1* was strongly correlated at a statistically significant level with that of *MtPyrB* ($r = 0.82$, $P = 0.0002$) and correlated to a lesser degree with *MtArgF* ($r = 0.70$, $P = 0.0049$). Whereas *MtCPSs1* RNA levels correlated with those of *MtCPSs2* ($r = 0.71$, $P = 0.0005$), levels of *MtCPSs2* RNAs were not as strongly correlated with *MtPyrB* or *MtArgF* RNAs as *MtCPSs1* (Table 2). Transcript accumulation of the gene encoding the large subunit *MtCPSl* was only significantly related to that of *MtPyrB* ($r = 0.62$, $P = 0.0099$). Furthermore, analyses comparing the genes encoding ACTase and OCTase revealed that regulation of *MtPyrB* and *MtArgF* were not related at a statistically significant level ($r = 0.37$, $P = 0.2325$), thus establishing the apparent regulatory independence of these two genes.

It is likely that *MtCPSs1* and *MtCPSs2* are differentially regulated in response to other endogenous metabolic conditions. A single putative *cis*-acting element was identified approximately 130 nt upstream from *MtCPSs2* with substantial nucleotide sequence similarity to an abscisic acid (ABA)-responsive element (ABRE) (data not shown). A similar putative control sequence was not found upstream of *MtCPSs1*. To test the possibility that *MtCPSs2*, but not *MtCPSs1*, was regulated at the level of transcription by ABA, *M. truncatula* seedlings were incubated in the presence of 100 μM ABA. Added ABA reduced the transcript levels of *MtCPSs2* (64%) below that of the untreated control but had no effect on *MtCPSs1* (Fig. 3F). The responses of *MtCPSs1* and *MtCPSs2* to added ABA resulted in significantly different amounts of accumulated transcript ($P \leq 0.05$).

Table 2
Pearson's correlation coefficients (r) among *MtCPSs1*, *MtCPSs2*, *MtCPSl*, *MtPyrB* and *MtArgF* gene transcript accumulation as pairwise comparisons[a].

	MtCPSs2	MtPyrB	MtArgF	MtCPSl
MtCPSs1	$r = 0.70935$ ($P = 0.0005$)*	$r = 0.81634$ ($P = 0.0002$)*	$r = 0.69739$ ($P = 0.0049$)*	$r = 0.25203$ ($P = 0.2979$)
MtCPSs2		$r = 0.66198$ ($P = 0.0052$)*	$r = 0.54457$ ($P = 0.0512$)	$r = 0.39894$ ($P = 0.0814$)
MtPyrB			$r = 0.37296$ ($P = 0.2325$)	$r = 0.62350$ ($P = 0.0099$)*
MtArgF				$r = 0.02574$ ($P = 0.9274$)

[a] Correlations of effects from exogenous 10 mM uridine +/− 5 mM ornithine, 5 mM arginine +/− 5 mM ornithine, 5 mM ornithine, and 100 μM abscisic acid on transcript accumulation in 4-day-old *M. truncatula* seedling shoot apices. Data is expressed relative to the amount of transcript accumulated in the shoot apices of untreated control plants during 3.5-h incubation. *P*-values are given in parentheses. Statistically significant values are assigned asterisks.

4. Discussion

This work is the first to describe cloning and characterization of multiple functional CPSase small subunit genes and their encoded transcripts resident in a single plant genome. It appears that there exists a diversity of organizations within plant genomes with respect to CPSase, ACTase, and OCTase gene copy number, suggesting a diversity of control strategies by which CP is synthesized and allocated to competing downstream biosynthetic pathways.

4.1. CP synthesis and allocation to pyrimidine nucleotide and arginine biosynthesis in M. truncatula is transcriptionally regulated by both ornithine and pathway end products

The Q-RTPCR data reported herein were analyzed using the method described by Pfaffl (2001) and subsequently reanalyzed with the method of Zhao and Fernald (2005) for comparison. By either method, the most of the results and their apparent biological significance were the same (compare Fig. 3 to Supplementary Fig. 4). However, *MtArgF* transcript levels were not reduced below that of the untreated control after treatment with arginine when the data were analyzed according to Zhao and Fernald (2005) (Supplementary Fig. 4D), a result in conflict with that found using the method of Pfaffl (Fig. 3D). An independent transfer hybridization experiment was performed to determine whether steady state OCTase transcription was affected by exogenous arginine. These results presented in Supplementary Fig. 3B provide clear evidence that *MtArgF* expression is down regulated in the presence of added arginine, which is in agreement with the data analyzed by the Pfaffl method (2001). Hence, we included the data analyzed by the method of Zhao and Fernald (2005) as supplementary information. To our knowledge, this paper is among the very first to compare results of Q-RTPCR analysis using independent analytical methods [Pfaffl (2001) and Zhao and Fernald (2005)] and to employ Pearson's correlation coefficient in a statistical treatment of Q-RTPCR data.

The data presented here provide strong evidence of end product regulation of the *M. truncatula* CPSase gene family members by uridine, an output of the *de novo* pyrimidine biosynthetic pathway. This result provides the first demonstration in plants that pyrimidine pathway end products regulate plant CPSase genes. *MtPyrB* mRNA levels were also down regulated under these same conditions, further supporting the regulatory relationship between *M. truncatula* CPSases and the pyrimidine pathway. In this regard, the *M. truncatula* CPSase gene family is regulated in a pyrimidine pathway-specific manner using a two-pronged approach that coordinates CP synthesis by CPSase with CP utilization by ACTase, the first enzyme committed to pyrimidine biosynthesis. Furthermore, statistical analyses of quantitative expression data supported the hypothesis that duplicated CPSase small subunit genes are not differentially regulated in a pathway specific manner; rather, they are both regulated by pyrimidine feedback and insensitive to arginine. Thus, these genes are functionally redundant with respect to treatment conditions used in this investigation designed to probe CP allocation to pyrimidine and arginine biosynthesis.

In addition, these results provide the first evidence in any species that inhibition of CPSase and ACTase transcription by pathway end products can be overcome by ornithine. Up regulation of *MtPyrB* expression by ornithine was unanticipated, and provides additional evidence for coordination of CP synthesis and allocation to the pyrimidine pathway at the level of transcript accumulation. The observation that *MtArgF* transcript levels are also stimulated by the addition of ornithine is consistent with previous data demonstrating that ornithine stimulates synthesis of both UMP and arginine at the enzymatic level in plants (Lovatt and Cheng, 1984), and the coordinated up regulation of both pyrimidine and arginine biosynthesis in animal cells. (Tremblay et al., 1977). Ammonia feeding has also been shown to

stimulate production of urea cycle and pyrimidine pathway products in some mammals (Fico et al., 1984), demonstrating that these species are able to detoxify ammonia via CP incorporation into urea and orotic acid. Ammonia and arginine accumulation is also an index of stress in plants (Barker, 1999; Lovatt, 1990). Whether genes involved in pyrimidine and arginine biosynthesis in legumes such as *M. truncatula* are up regulated in the presence of ornithine to prevent ammonia toxicity awaits confirmation. This report also offers the first demonstration that inhibition of plant *MtArgF* transcription by arginine is reversible by exogenous ornithine. Taken together, the data provide additional evidence that this is the site of transcriptional inhibition in arginine biosynthesis; that up regulation of the two CPSase small subunit genes and *MtArgF* by ornithine, even in the presence of added arginine, is a putative strategy to meet the needs of the arginine pathway for CP; and that the pyrimidine biosynthetic pathway is also up regulated by ornithine.

4.2. Duplicated CPSase small subunit genes may be differentially regulated

Analysis of genomic DNA sequence upstream from both *M. truncatula* CPSase small subunit genes revealed putative ABA responsive element (ABRE) immediately adjacent to *MtCPSs2*, but not to *MtCPSs1*. Consistent with this observation, exogenously supplied ABA down regulated *MtCPSs2*, but not *MtCPSs1*. Whereas ABREs are generally responsible for up regulation of ABA-responsive genes, the ability of ABA to down regulate genes involved in cell division (Yazaki et al., 2004) and nitrogen assimilation (Li et al., 2006) has been reported previously. A plant CPSase small subunit gene that is regulated by ABA logically might play a role in responses to abiotic stress, defense responses, or in spatial and temporal control of development. Interestingly, data presented here suggest that the basal level of large subunit expression is adequate to accommodate the significant up regulation of both CPSase small subunit genes under the conditions tested.

4.3. A model for CP synthesis and allocation in M. truncatula

Over the past quarter century, the regulation of genes involved in the synthesis and allocation of CP to accommodate the cell's need for pyrimidine and arginine has been described across a wide spectrum of taxa, with the notable exception of plants. The current paradigm for CP synthesis and allocation in plants posits a single CPSase enzyme, allosterically regulated by pyrimidine nucleotides, that generates CP for both pyrimidine and arginine biosynthesis and that both these pathways occur exclusively in the plastid (Slocum, 2005). Our studies addressing nitrogen flux in *M. truncatula* offer the first comprehensive model for genetic regulation of CP synthesis and allocation in a plant species (Fig. 4). Specifically, *M. truncatula* possesses a CPSase small subunit gene family comprised of two members, each similar in function with respect to feedback control by pyrimidine pathway end products and up regulation by the arginine pathway intermediate ornithine. Moreover, the *M. truncatula* ACTase gene is similarly regulated at the level of transcript accumulation by uridine and ornithine. The capacity of added ornithine to prevent or overcome the repression of *MtPyrB* and *MtArgF* transcription imposed by respective pathway end products implies that the *de novo* biosynthesis of pyrimidine nucleotides and arginine is coordinated in mitotically active tissues such as the shoot apex. Taken together, the results of this research describe a simple but unique regulatory model for CP synthesis and allocation in *M. truncatula* that accommodates the competing needs of the pyrimidine and arginine *de novo* biosynthetic pathways via a single pool of CP, in a manner more similar to prokaryotes than other eukaryotes.

With the expanding database of plant genomic sequences, it is likely that even more diverse strategies for regulation of genes encoding enzymes involved in CP synthesis and allocation will be revealed. It is expected that *A. thaliana*, which encodes a single CPSase small and large subunit gene, utilizes a strategy similar to *M. truncatula* to regulate dually functional CPSase genes. On the other hand, plants possessing CPSase, ACTase or OCTase gene families may employ different regulatory schemes. Hybridization events between closely related plant species that give rise to allopolyploids (as in the case of *M. sativa*) is one mechanism by which derived species are able to adapt new roles for functionally redundant genes. Gene duplication events and mutations within regulatory regions leading to new functionality (such as those that resulted in duplicated *M. truncatula* CPSase small subunit genes) are other mechanisms for evolution of gene function. Given the fact that multiple gene families have already been described for several plant species for which CPSase, ACTase, and OCTase gene sequences exist, it is likely that additional novel strategies for regulation of these genes exist in other plant species. Given the critical role that pyrimidine nucleotides play in nucleic acid and polysaccharide synthesis and the pivotal role that arginine has in protein synthesis and in the plant's responses to abiotic stress, additional research to determine the range of strategies utilized by a greater diversity of plants for the synthesis and allocation of CP might provide information of significant value to crop improvement.

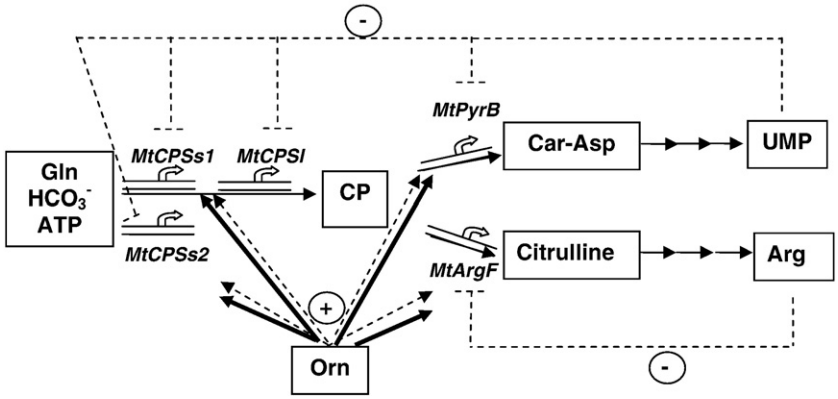

Fig. 4. Model illustrating transcriptional regulation of pathways for the *de novo* biosynthesis of pyrimidine nucleotides and arginine. Italicized text above or below double lines with bent arrows represents genes encoding pathway enzymes; solid arrows, enzymatic steps; boxed text, pathway intermediates and end products; broken lines, the regulatory effect of pathway end-products, or their metabolites, and ornithine on gene transcription (circles containing "+" or "−" indicate up and down regulation, respectively). Bold arrows emanating from the box labeled ornithine indicate the ability of ornithine to prevent or overcome inhibition of transcription by added uridine or arginine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2010.04.007.

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