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# Molecular Cloning and Characterization of the *pyrB1* and *pyrB2* Genes Encoding Aspartate Transcarbamoylase in Pea (*Pisum sativum* L.)[1]

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**We cloned cDNAs encoding two different pea (*Pisum sativum* L.) aspartate transcarbamoylases (ATCases) by complementation of an *Escherichia coli* Δ*pyrB* mutant. The two cDNAs, designated *pyrB1* and *pyrB2*, encode polypeptides of 386 and 385 amino acid residues, respectively, both of which exhibit typical chloroplast transit peptide sequences. Wheat germ ATCase antibody recognizes a 36.5-kD polypeptide in pea leaf and root tissues that is similar in size to other plant ATCase polypeptides and to the catalytic polypeptides of bacterial ATCases. Northern analyses indicate that the *pyrB1* and *pyrB2* transcripts are 1.6 kb in size and are differentially expressed in pea tissues. The small transcript size and data from biochemical studies indicate that plant ATCases are simple homotrimers of 36- to 37-kD catalytic subunits, rather than part of a multifunctional enzyme containing glutamine-dependent carbamoylphosphate synthetase and dihydroorotase activities, as is seen in other eukaryotes. In the pea ATCases, the carbamoylphosphate- and aspartate-binding domains are highly homologous to those of other prokaryotic and eukaryotic ATCases and critical active-site residues are completely conserved. The pea ATCases also exhibit a putative pyrimidine-binding site, consistent with the known allosteric regulation of plant ATCases by UMP in vitro.**

Pyrimidine nucleotides are activated precursors of DNA and RNA biosynthesis, and their derivatives function as intermediates in many biosynthetic pathways. The individual enzymic steps in de novo pyrimidine biosynthesis have been highly conserved in bacteria, fungi, and higher eukaryotes, including plants (Markoff and Radford, 1978; Jones, 1980; Ross, 1981). Despite this fact, prokaryotes and eukaryotes have adopted many different strategies for the regulation of pyrimidine and Arg biosynthesis, both of which utilize carbamoyl-P as an intermediate.

In prokaryotes such as *Escherichia coli*, a single Gln-dependent CPSase provides carbamoyl-P to both pathways and is metabolically regulated by antagonistic effectors of either pathway: UMP, the end product of the pyrimidine pathway, inhibits CPSase, whereas ornithine, a precursor of Arg biosynthesis, activates the enzyme. Another pyrimidine, CTP, also inhibits ATCase (EC 2.1.3.2), which carries out the first step committed to pyrimidine biosynthesis (Markoff and Radford, 1978). According to the bacterial model, when pyrimidines accumulate to some critical level, the activities of both CPSase and ATCase are inhibited. Decreased carbamoyl-P availability for Arg synthesis, resulting from CPSase inhibition, causes the accumulation of ornithine and reversal of UMP inhibition of CPSase (ATCase inhibition by CTP continues). Release of CPSase from inhibition provides carbamoyl-P for Arg biosynthesis until pyrimidine levels become limiting, releasing ATCase from CTP inhibition and diverting carbamoyl-P to the pyrimidine pathway.

In eukaryotes other than plants, two different CPSases commit separate pools of carbamoyl-P to the pyrimidine and Arg pathways. An Arg-specific, ammonia-utilizing CPSase (CPSase I, *N*-acetylglutamate cofactor) is localized in mitochondria (except in yeasts), along with OTCase, which utilizes carbamoyl-P in the synthesis of citrulline, an intermediate in Arg biosynthesis (Davis, 1986). A pyrimidine-regulated, Gln-dependent CPSase activity (CPSase II) resides as a superdomain on a multifunctional protein that also contains ATCase (nuclear localized yeast CA protein; Nagy et al., 1989) or both ATCase and DHOase activities (cytosolic CAD protein of higher eukaryotes; Davidson et al., 1990). In these organisms, the pyrimidine pathway is regulated by inhibition of CPSase, whereas ATCase is not regulated (Wild and Wales, 1990).

In plants, mechanisms by which activities of the pyrimidine and Arg pathways are coordinated are poorly understood. Biochemical studies to date have provided evidence for only a single Gln-dependent CPSase activity, although Maley et al. (1992) have recently provided evidence for the possible existence of both Arg- and pyrimidine-specific CPSases in alfalfa, based on sequence analyses of partial cDNA clones. Metabolic studies also suggest that the Arg and pyrimidine pathways share a common pool of carbamoyl-P (Lovatt and Cheng, 1984). Whether one or two CPSases occur in plants, cell fractionation studies have demonstrated that a Gln-dependent CPSase activity is localized in the chloroplast, along with OTCase (Arg pathway) and ATCase (pyrimidine pathway) activities (Shargool et al., 1978; Doremus and Jagendorf, 1985; Shibata et al., 1986); thus, allocation of carbamoyl-P to each pathway must be regulated.

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Abbreviations: ATCase, aspartate transcarbamoylase; CA, carbamoylphosphate synthetase/aspartate transcarbamoylase bifunctional protein; CAD, carbamoylphosphate synthetase/aspartate transcarbamoylase/dihydroorotase trifunctional protein; carbamoyl-P, carbamoylphosphate; CPSase, carbamoylphosphate synthetase; DHOase, dihydroorotase; OTCase, ornithine transcarbamoylase.

Early in vitro studies of plant CPSases and ATCases (reviewed by Lovatt and Cheng, 1984) demonstrated that both enzymes were inhibited by pyrimidines, UMP being most effective. Ornithine reversed UMP inhibition of CPSase activity, but not ATCase activity, suggesting that the pyrimidine and Arg pathways were regulated at the level of CPSase in a manner similar to that described for *E. coli*. The first comprehensive in vivo studies of pyrimidine biosynthesis in plants by Lovatt et al. (1979) confirmed that this pathway is feedback-inhibited at a step leading to the formation of carbamoylaspartate, but these investigators were unable to determine whether regulation occurred primarily at the level of CPSase or ATCase. In a later study, Lovatt and Cheng (1984) reported that pyrimidines inhibited ATCase, but not CPSase, activity in vitro. They also found that uridine blocked the incorporation of label from $NaH^{14}CO_3$ (via [$^{14}$C]carbamoyl-P), but not [$^{14}$C]carbamoylaspartate, into UMP and increased its incorporation into Arg in intact squash roots. Based on these findings and other types of evidence, these investigators concluded that ATCase, rather than CPSase, was the important site of regulation for the pyrimidine pathway in plants.

It is difficult to reconcile the very different results of these studies with regard to the metabolic regulation of the pyrimidine pathway at CPSase or ATCase or both. Furthermore, nothing is known regarding either the genetic organization of the pyrimidine pathway (separate genes, individual enzymes versus fused genes, multifunctional enzymes) or the molecular mechanisms by which the expression of CPSase and ATCase proteins might be regulated in plants. As a first step in this direction, we have cloned cDNAs encoding two different ATCases in pea (*Pisum sativum* L.) and have examined their expression in pea tissues. A preliminary report on the isolation and initial characterization of one of these genes was previously published (Williamson and Slocum, 1993).

## MATERIALS AND METHODS

### Bacterial Strains

*Escherichia coli* strain XL1-Blue [*recA1, endA1, gyrA96, thi, hsdR17*($r_k^-m_k^+$), *supE44, relA1, lac*$^-$, (*F', proAB, lacIQ, lacZΔM15, Tn10*)] was used to rescue recombinant pBluescript phagemids from the λZAPII vector (Stratagene).

*E. coli* strain TB-2 was derived from strain E63, a wild-type *E. coli* K-12 that carries a partial F plasmid, by phage Mu d1 (*lac* Ap$^r$) mutagenesis (Roof et al., 1982). Imprecise excision of the integrated Mu d1 prophage removed part of the *pyrBI/argI* region of the *E. coli* chromosome, although the exact nature of the deletion has not been determined. The resultant TB-2 strain lacks both ATCase and OTCase activities and is auxotrophic for both uracil and Arg.

### Isolation of Pea ATCase cDNA Clones by Functional Complementation of the Δ*pyrB* Mutation in *E. coli* Strain TB-2

A pea (*Pisum sativum* L.) leaf cDNA library was constructed in λZAPII as previously described (Williamson and Slocum, 1992). Recombinant, single-stranded pBluescript phagemid DNA was excised from the λZAPII vector in vivo and packaged in filamentous bacteriophage particles (Short et al., 1988). *E. coli* strain TB-2 was then infected with this bacteriophage preparation, representing the complete cDNA library, and plated onto TF medium (0.1 M Tris, pH 7.8, 0.37 M $NH_4Cl$, 2.5 mM $MgCl_2$, 27 mM KCl, 1 mM $Na_2HPO_4$, 0.35 mM $Na_2SO_4$, 31 μM $FeCl_3$, 30 μM $Na_2EDTA$, 2.2 μM $CaCl_2$, 0.3 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.3 μM $CoCl_2$, 0.4% Glc, and 4 μg/mL vitamin B1) containing 40 μg/mL of each amino acid (Arg supplied at 125 μg/mL) and 100 μg/mL ampicillin, but lacking uracil. Details of this phagemid library screening method are described elsewhere (Williamson and Slocum, 1994).

Colonies exhibiting pyrimidine prototrophy were visible after overnight incubation at 37°C in most cases, although some clones required 2 to 3 d to form normal-sized colonies. The putative ATCase clones were then further plated on TF medium containing ampicillin and amino acids, except for Arg, in order to screen for possible ampicillin-resistant non-TB-2 cell contaminants. Only those isolates that failed to grow on the minus-Arg medium were further characterized.

### Restriction Mapping and Sequencing of ATCase cDNA Clones

Putative ATCase clones were inoculated into Terrific Broth (BRL) + 100 μg/mL ampicillin. Plasmid DNA was isolated from overnight cultures using the standard boiling miniprep protocol of Holmes and Quigley (1981). However, plasmid DNA prepared from these cells was generally of poor quality, as had been found in previous studies with this strain (M.E. Wales, personal communication). For this reason, isolated plasmid DNA was transformed into competent *E. coli* DH5α cells (BRL) and miniprepped a second time. cDNA inserts from the respective clones were characterized by restriction mapping, and several clones representing various classes of cDNAs were selected for sequencing.

Nested deletion subclones of clone pATC57 (*pyrB1* gene) were made as described by Slatko et al. (1991). Exonuclease III was used to create unidirectional deletions, followed by S1 nuclease blunt-end formation, fill-in with the Klenow fragment of DNA polymerase I, and ligation with T4 DNA ligase to create circularized plasmids. The plasmids were transformed into *E. coli* DH5α and miniprepped as described above. A series of plasmids 100 to 400 bp apart in size were then selected for sequencing. Additional sequence data were obtained in both directions from an internal *Pst*I site in pATC57 (Fig. 1). Several overlapping restriction fragments (Fig. 1) of the second clone (pATC22; *pyrB2* gene) were subcloned into pBluescript for sequencing. Both strands of each cDNA were completely sequenced using the Sequenase version 2.0 kit (United States Biochemical).

### Northern Blot Analysis

Large-scale purification of total RNA from 2-week-old green pea leaf tissues and fractionation of poly(A)$^+$ RNA and poly(A)$^-$ RNA were carried out according to Slocum et al. (1990). Isolation of total RNA from 3-d-old and 3-week-old pea seedlings was performed using the method of Chomczynski and Sacchi (1987). RNA samples were electropho-

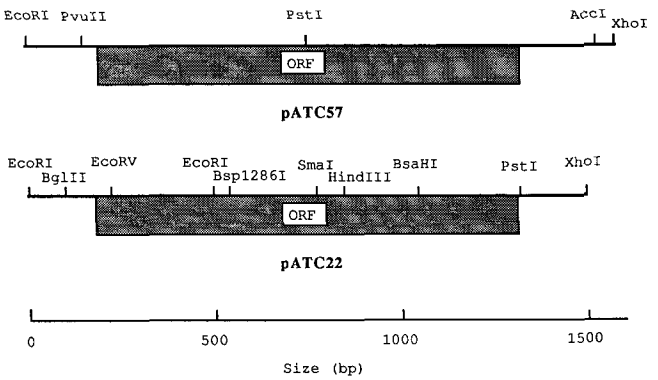


**Figure 1.** Restriction maps of cDNA clones pATC57 (*pyrB1*) and pATC22 (*pyrB2*).

resed in a 1% agarose-0.6 M formaldehyde gel and transferred to nylon membrane as described previously (Williamson and Slocum, 1992). A *pyrB1*-specific probe (390-bp *Bsp*1286I fragment of the pATC57 insert), a *pyrB2*-specific probe (300-bp *Eco*RI/*Hinc*II fragment of the pATC22 insert), and an 18S rRNA probe (1037-bp *Bam*HI/*Eco*RI fragment of a soybean 18S rRNA; Eckenrode et al., 1985) were labeled with [$\alpha$-$^{32}$P]-dCTP (New England Nuclear) to a specific activity of $2 \times 10^8$ to $8 \times 10^8$ dpm/$\mu$g using a random-primed DNA labeling kit (United States Biochemical). The probes were hybridized to the blots in hybridization buffer (5× SSPE [0.75 M NaCl, 0.05 M $NaH_2PO_4$, 6 mM EDTA, pH 7.4], 5× Denhardt's solution, 50% formamide, 0.5% SDS, 200 $\mu$g/mL of salmon sperm DNA) for 16 h at 42°C. Blots were washed at room temperature in 5× SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.4) + 0.5% SDS, then at 42°C in 1× SSC + 0.5% SDS and 0.2× SSC + 0.5% SDS, and exposed to film.

### Southern Blot Analysis

Genomic DNA was isolated from pea leaf tissues, cut with restriction enzymes, electrophoresed through an 0.8% agarose gel, and transferred to nylon membrane by capillary blotting as previously described (Williamson and Slocum, 1992). Blots were hybridized to $^{32}$P-labeled probes as described above.

### SDS-PAGE Immunoblot Analysis of ATCase Protein

SDS-PAGE separations of total soluble proteins from pea tissues or *E. coli* TB-2 cultures, electroblotting onto nitrocellulose membrane, and immunodetection of ATCase protein with rabbit polyclonal antisera produced against purified wheat germ ATCase were carried out as described by Slocum et al. (1990). Primary antisera were used at 1:1000 dilutions.

### Enzyme Assay for ATCase

All extraction steps prior to assaying ATCase activities were carried out at 4°C. Pea tissues were homogenized at 0.5 g fresh weight tissue/mL of homogenization buffer (100 mM Hepes, 5 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF, pH 7.5). The homogenate was centrifuged at 20,000*g* for 20 min and the supernatant was dialyzed against several changes of 10 mM Hepes, 0.5 mM EDTA, pH 7.5 buffer. *E. coli* strain TB-2 was grown overnight in 50 mL of TF medium containing 50 $\mu$g/mL uracil; overnight cultures of TB-2 isolates harboring either pATC57 or pATC22 were grown in the same medium with or without uracil. Cells were pelleted at 10,000*g* for 20 min and resuspended in 2 mL of homogenization buffer containing 10% (v/v) glycerol. Lysozyme was added to 1 mg/mL, and the cells were further incubated for 10 min. The cells were then disrupted using a probe sonicator at 80% power output for 30 s (model 50, Heat Systems, Inc., Farmingdale, NY). The lysate was centrifuged at 16,000*g* for 10 min and the supernatant was used for ATCase assays.

ATCase activity was measured according to the method of Boyde and Rahmatullah (1980), based on the colorimetric detection of carbamoylaspartate with diacetylmonoxime. The reaction mixture contained 200 mM Hepes, 20 mM L-aspartate, 10 mM carbamoyl-P (dilithium salt), pH 8.5, and varying amounts of enzyme in a final volume of 0.4 mL. For nondialyzed samples, minus-carbamoyl-P reactions were run as blanks. The reaction was carried out in a 37°C bath for 20 min and stopped by addition of 0.4 mL of 10% perchloric acid. Half of the acidified assay mixture was combined with 0.9 mL of distilled $H_2O$ and 4.5 mL of chromogenic reagent (20 mM antipyrene, 5 mM ferric ammonium sulfate in 2.2 N $H_3PO_4$, 9 N $H_2SO_4$). Carbamoylaspartate formation was estimated from $A_{464}$ readings using a carbamoylaspartate standard curve, which was linear between 0 and 5 $\mu$mol.

### Protein Determination

Total soluble proteins in samples were estimated using the dye-binding method of Bradford (1976).

### Plant Materials

Pea (cv Wando) seeds were planted in washed vermiculite moistened with tap water and grown either in darkness or in illuminated growth chambers (16-h light/8-h dark photoperiod) at 22°C.

## RESULTS AND DISCUSSION

### Isolation and Characterization of ATCase cDNAs in Pea

We isolated the pea ATCase cDNA clones in the present study using a novel method of screening our λZAP expression library (Williamson and Slocum, 1994) for clones that would complement the Δ*pyrB* mutation in *E. coli* strain TB-2. Briefly, the entire cDNA library was excised in vivo from the λZAP vector, using helper phage VCSM13, and packaged into filamentous bacteriophage particles in the form of single-stranded pBluescript phagemid DNA. This phagemid library was then used to infect *E. coli* TB-2, in which double-stranded pBluescript phagemids would be stably propagated. Those colonies that grew on minus-uracil selection medium were assumed to contain pea homologs of the *pyrB* gene, whose expression resulted in the production of functional ATCase enzymes and pyrimidine biosynthesis in this bacterial host.

A large number of clones complementing the Δ*pyrB* mutant were isolated using the phagemid library screening method.

In one representative experiment, 61 complementing clones were isolated from a total of $1.6 \times 10^7$ cells, for a frequency of approximately 1 in 260,000. Fourteen independently isolated clones were characterized by restriction mapping and DNA sequencing. Ten of the clones belonged to one class of cDNAs, represented by pATC57 (Williamson and Slocum, 1993), but varied in the length of their 3′ untranslated sequences, suggesting the use of multiple polyadenylation sites in this gene (Dean et al., 1986). Three other clones, represented by pATC22, encoded a second ATCase gene whose sequence is moderately divergent from the first gene.

To the best of our knowledge this is the first instance in which more than one ATCase gene has been isolated from any organism; we refer to the two pea ATCase cDNAs as *pyrB1* and *pyrB2*, following the bacterial nomenclature, where the *pyrB* gene encodes the catalytic subunit of ATCase (Hoover et al., 1983). Restriction maps for the pATC57 and pATC22 cDNAs are shown in Figure 1.

The characteristics of pATC57 (*pyrB1*) have been previously reported (Williamson and Slocum, 1993; the molecular mass for this protein was incorrectly reported as 42,507 D; it is actually 42,622 D) and the deduced amino acid sequence for this ATCase is shown in Figure 2. pATC22 (*pyrB2*) contains a 1495-bp insert with an open reading frame that begins with the ATG codon at nucleotide 168 and ends with the stop codon TAA at nucleotide 1326. The 3′ untranslated region of this gene exhibits five tandem TTTTGC repeats 60 bp upstream from the polyadenylation site. The polyadenylation signal used in this gene was not obvious and appears to be highly divergent from the canonical AATAAA signal (Joshi, 1987). The pATC22 open reading frame consists of 385 amino acid residues with a predicted molecular mass of 43,750 D and an isoelectric point of 7.4, compared with an isoelectric point of 6.6 for the pATC57-encoded enzyme. The deduced sequence for this *pyrB2* ATCase is also shown in Figure 2.

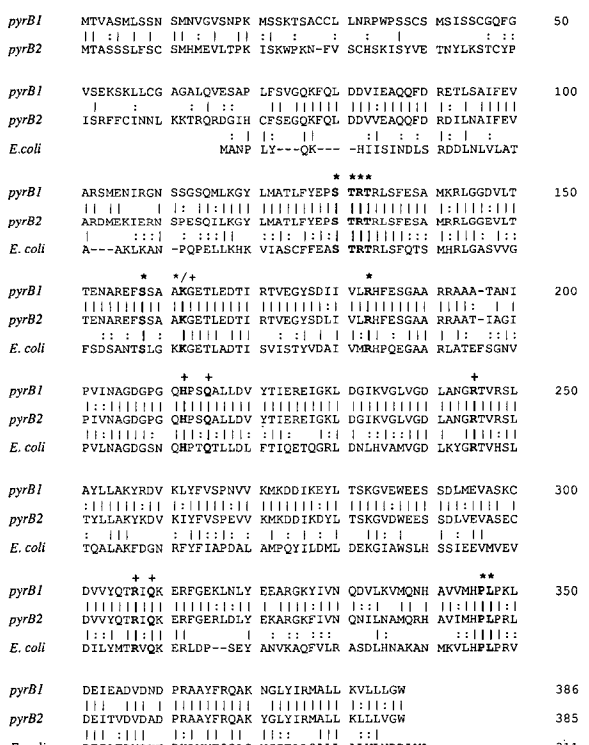


**Figure 2.** Comparison of the deduced amino acid sequences for the two pea ATCases (*pyrB1*, pATC57; *pyrB2*, pATC22) with that of the *E. coli* ATCase catalytic subunit (*pyr B*; Hoover et al., 1983). Gaps have been introduced to maximize sequence homologies. Residues identical to the pea *pyrB1* sequence are indicated by a bar and conserved residues are indicated by two dots (Dayhoff, 1978). Residues in boldface are involved in the binding of carbamoyl-P (*) and L-aspartate (+) to the *E. coli* ATCase catalytic subunit (Kantrowicz and Lipscomb, 1988).

## The *pyrB1* and *pyrB2* cDNAs Encode Chloroplast Precursor ATCases

Previous investigators have reported that ATCase activity is localized in chloroplasts (Doremus and Jagendorf, 1985; Shibata et al., 1986). The N-terminal sequences of the proteins encoded by the *pyrB1* and *pyrB2* cDNAs exhibit the general characteristics of chloroplast transit peptides (Keegstra et al., 1989). Although the cleavage sites for these precursors have not been determined, analysis of the *pyrB1* sequence (von Heijne, 1986) predicts $Leu^{64}$ as a likely cleavage site. This is supported indirectly by the observation that an N-terminal peptide sequence for purified wheat germ ATCase aligns to within two residues of this site (B. Ward and D. Gilchrist, personal communication). Cleavage at $Leu^{64}$ would generate a mature ATCase polypeptide consisting of 322 amino acid residues and a molecular mass of 36,089 D, which is close to the molecular mass of 36,500 D estimated for this protein on SDS-PAGE immunoblots of pea leaf tissues (data not shown). Analyses of the *pyrB2* precursor have not identified likely cleavage sites, but cleavage is assumed to occur at a similar position, since only a single 36,500-D protein band is seen on immunoblots of leaf proteins.

## Conservation of Structure in Plant and Bacterial ATCases

The amino acid sequences of the two pea ATCases are highly homologous (Fig. 2), differing from each other primarily at their N-terminal ends, within the presumed transit peptide sequences. The sequences for the mature ATCase proteins, beginning with $Leu^{64}$ of the *pyrB1* ATCase, are 85% identical, and 8% of the substitutions are conservative. The pea *pyrB1* sequence also exhibits 80% identity with an *Arabidopsis* ATCase (M. Minet, personal communication) and 83% identity with a partial-length sequence of the wheat germ ATCase (R.J. Yon, personal communication). In contrast, comparison of the amino acid sequence of the pea *pyrB1* ATCase with that of the catalytic polypeptide of *E. coli* ATCase (Fig. 2; Hoover et al., 1983) indicates that they are only 38% identical. However, the sequences that constitute the substrate-binding domains are highly conserved among all of the ATCases.

Alignment of the pea ATCase sequences with that of the *E. coli* catalytic polypeptide (Fig. 2) indicates that all 10 of the essential carbamoyl-P-binding residues and all 5 of the aspartate-binding residues (Kantrowitz and Lipscomb, 1988;

Wild and Wales, 1990) are conserved in the pea enzymes. These data suggest that the active sites of the plant and bacterial ATCases have been evolutionarily conserved. Conservation of pea ATCase residues corresponding to $Ser^{80}$ and $Lys^{84}$ of the *E. coli* enzyme, which are contributed to the active site by the adjacent catalytic chain in the homotrimer, further suggests that the quaternary structure of these enzymes has also been conserved. There is also a striking conservation of the C-terminal sequence spanning residues 340 to 385 of the pea ATCases and analogous C-terminal sequences of other prokaryotic and eukaryotic ATCases (data not shown). In the *E. coli* enzyme, residues 285 to 305 (helix 12) play a critical role in the folding and assembly of the catalytic subunits (Peterson and Schachman, 1991).

Unlike the *E. coli* ATCase, whose activity is regulated by allosteric mechanisms involving nucleotide binding to a separate regulatory subunit in the holoenzyme (Kantrowitz and Lipscomb, 1988), plant ATCases are simple homotrimers of catalytic subunits (Yon et al., 1982). Several studies have shown that UMP is an allosteric regulator of plant ATCases (Cole and Yon, 1984; Acaster et al., 1989), although the location of the pyrimidine-binding site on the catalytic polypeptide is unknown. Alignment of a highly conserved nucleotide-binding site on the *E. coli* regulatory polypeptide (*pyrI*) with the pea ATCase sequences (Fig. 3) has identified a putative pyrimidine-binding site in which 8 of 12 residues are identical or conserved substitutions, and 3 of the identical residues are directly implicated in NTP binding in the *E. coli* enzyme (Wild and Wales, 1990). This sequence is conserved in the *Arabidopsis* and wheat germ ATCases (data not shown) and within the ATCase domains of the yeast (*URA2*) and human (*CAD*) multifunctional proteins (Fig. 3). Interestingly, this site is not conserved in the *Bacillus subtilis* ATCase, which, like the plant enzyme, is a simple homotrimer of catalytic subunits but does not exhibit allosteric regulation by nucleotides (Brabson and Switzer, 1975). Similarly, the free catalytic subunits of the *E. coli* ATCase ($c_3$) do not display allosteric regulation (Gerhart and Schachman, 1965), although a sequence exhibiting weak conservation of the *pyrI* nucleotide-binding site is a also seen in the catalytic chain (Fig. 3).

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E. coli (pyrI)          VEAIKRGTVIDH
                        :: :: : | |
E. coli (pyrB)          LDNLHVAMVGDL
                          ::  :   |
B. subtilis (pyrB)      FKGLTVSIHGDI
                        |:::|  : : |
yeast (URA2)            VNGITVTFMGDL
                        |:::  : | |
human (CAD)             VNGMTITMVGDL
                        :::|| | | |
pea (pyrB1)             LDGIKVGLVGDL        (residues 228-239)
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**Figure 3.** A conserved nucleotide-binding domain of the *E. coli* ATCase regulatory subunit (*pyrI*; Schachman et al., 1984) and comparison with aligned sequences in catalytic polypeptides of *B. subtilis* (Lerner and Switzer, 1986), *E. coli* (Hoover et al., 1983), yeast (Nagy et al., 1989), human (Davidson et al., 1990), and pea ATCases after sequence alignments. The underlined residues between $Val^9$ and $Val^{17}$ of the *E. coli* regulatory subunit coordinate binding of the base and ribose moieties of both CTP and ATP; $Asp^{19}$ and $His^{20}$ residues (underlined) are involved in binding of the triphosphate moiety (Wales et al., 1993). Identical residues in other ATCases are indicated with a vertical bar, and conservative changes are indicated by two dots.

It should be possible to verify the location of this putative pyrimidine-binding domain in site-directed mutant pea ATCases that have lost the allosteric response. ATCase mutants with altered UMP inhibition characteristics would be useful in the kinetic modeling of this enzyme and in studies examining the role of ATCase in the regulation of pyrimidine biosynthesis in transgenic plants. The existence of a discrete nucleotide-binding site on the catalytic polypeptide of plant ATCases would suggest that this enzyme and its prokaryotic ancestors followed divergent evolutionary paths, perhaps reflecting fundamental differences in the regulation of pyrimidine synthesis in these organisms.

### Expression of Pea ATCase in *E. coli* Strain TB-2

No ATCase activity was detectable in lysates of *E. coli* strain TB-2 (Table I). In TB-2 isolates harboring pATC57, *pyrB1* expression resulted in ATCase activities that were approximately 10-fold higher than those seen in pea leaf or root tissues (Table I). In TB-2/pATC57 lysates, a number of proteins that were not present in TB-2 lysates were specifically immunostained with a wheat ATCase antibody (data not shown). These proteins ranged in size between 41.5 and 38.5 kD, including a major band at 40.5 kD. The proteins may be proteolytic degradation products of the predicted 42.6-kD precursor ATCase encoded by pATC57 or, alternatively, they might represent the use of internal Met residues to initiate translation. The presumed transit peptide sequence contains four such Met residues, initiation at which would produce truncated precursors of 42.1, 41.5, 40.6, and 38.4 kD. The two smaller bands (30 and 14 kD) are almost certainly proteolytic degradation products.

Purification of ATCase proteins from TB-2/pATC57 lysates by *N*-(phosphonacetyl)-L-aspartate-Sepharose affinity chromatography (data not shown) suggests that only the minor 41.5- and 38.5-kD bands are enzymically active, by virtue of their ability to bind the immobilized transition-state analog inhibitor. It appears that the partial transit peptide sequences associated with these two polypeptides do not interfere with the normal folding and assembly required for ATCase function in this bacterial host, whereas the other ATCase proteins are either nonfunctional or insoluble.

The *pyrB2* gene in TB-2/pATC27 (identical to pATC22 but three amino acid residues short at the N terminus) is expressed as a 48.5-kD β-galactosidase fusion protein (data not shown). We do not know whether the fusion protein itself, or a 32-kD polypeptide that is also immunostained in these lysates, is enzymically active.

### Patterns of ATCase Expression in Pea Tissues

The hybridization patterns on genomic blots probed with pATC57 and pATC22 inserts (Fig. 4) clearly support the existence of two different ATCase genes in pea, although weak cross-hybridization by the probes is indicative of the high degree of sequence conservation between the *pyrB1* and *pyrB2* genes. Recently, we have obtained evidence for a third pea ATCase gene (*pyrB3*; our unpublished data); thus, these genes may constitute a small gene family in this plant.

Northern analysis of poly(A)$^+$ RNA probed with full-length

**Table I.** *Activities of mature ATCase in pea leaf and root tissues and precursor ATCase overexpressed in E. coli TB-2 strain harboring the plasmid pATC57*

Values are means ± SE based on three replicates. N.D., Not detected. CarAsp, Carbamoylaspartate.

| | ATCase Specific Activity |
|---|---|
| | *μmol CarAsp $h^{-1}$ $mg^{-1}$ protein* |
| Pea leaf | 0.95 ± 0.08 |
| Pea root | 0.82 ± 0.09 |
| *E. coli* TB-2 | N.D. |
| *E. coli* TB-2/pATC57 | 8.74 ± 0.47 |

*pyrB1* or *pyrB2* cDNA probes showed a single low-abundance 1.6-kb transcript (Fig. 5). To examine the expression of the individual *pyrB1* and *pyrB2* genes in pea tissues, we used 5′ end fragments of the two clones, encoding the nonhomologous transit peptide sequences, as gene-specific probes. As is seen in Figure 5, steady-state levels of the *pyrB1* transcript were very low in leaf and root tissues from greened and etiolated seedlings of different ages, whereas levels of the *pyrB2* transcript were barely detectable. We are currently investigating possible tissue-specific differences in, and developmental regulation of, expression of these pea ATCase genes using a more sensitive RNase protection assay (Gilman, 1993).

Extremely low ATCase protein levels seen on SDS-PAGE immunoblots of leaf and root tissue proteins (data not shown) and low enzyme activities (Table I) reflect the low levels of ATCase mRNAs present in these tissues. We estimate that this enzyme constitutes <0.001% of the total soluble protein in pea tissues, based on studies of the purified protein (our unpublished data). At present we do not know the mechanisms by which expression of these genes are regulated in

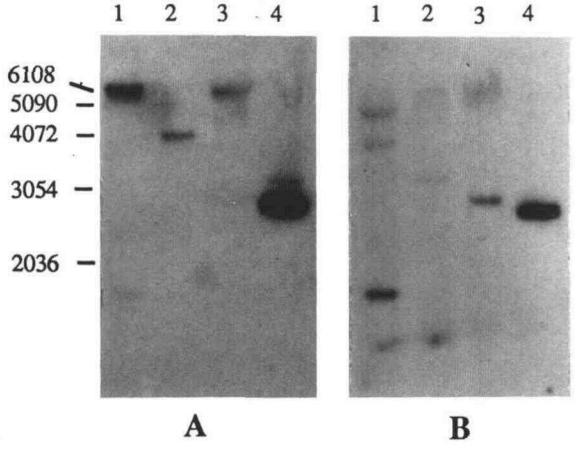


**Figure 4.** Southern blot analysis of pea leaf genomic DNA hybridized with a $^{32}$P-labeled 1593-bp *Eco*RI/*Xho*I fragment of clone pATC57 (A) or a 1268-bp *Eco*RV/*Xho*I fragment of clone pATC22 (B). DNA (8.8 μg/lane) was digested with *Bam*HI (lane 1), *Kpn*I (lane 2), or *Sac*I (lane 3), which do not cut at any sites within either clone (see Fig. 2). Lane 4 contains 190 pg of *Xho*I-linearized pATC57 or pATC22 DNA. DNA size markers (kb) are indicated.

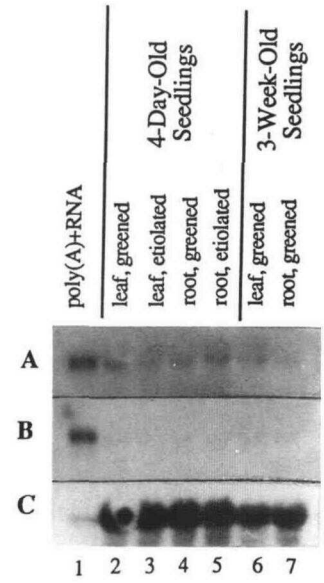


**Figure 5.** Northern blot analysis of ATCase mRNA levels in greened (lanes 2, 4, 6, and 7) and etiolated (lanes 3 and 5) pea tissues in 4-d-old seedlings (lanes 2–5) and 3-week-old seedlings (lanes 6 and 7). Lane 1, Pea leaf poly(A)$^+$ RNA (2.5 μg). Lanes 2 to 7, Total RNA (20 μg). The blots were probed with $^{32}$P-labeled gene-specific probes for *pyrB1* (A; 2-d exposure), *pyrB2* (B; 5-d exposure), and the 18S rRNA internal control (C; 6-h exposure).

pea, whether the polypeptides encoded by these genes are capable of interacting with each other to form ATCase isozymes consisting of homotrimers and heterotrimers with different kinetic or regulatory properties, or the possible functions of such isozymes in regulating pyrimidine biosynthesis. These studies are in progress.

## Structural Organization of Genes of the Pyrimidine Pathway in Plants

The structural organization of genes encoding the first three enzymes of the pyrimidine pathways of prokaryotes and eukaryotes has been summarized by Wild and Wales (1990). In bacteria, the *pyrB* and *pyrI* genes encoding the catalytic and regulatory subunits of ATCase are adjacent in a bicistronic operon, whereas the *carA* and *carB* genes encoding the Gln-dependent CPSase activity occur in another bicistronic operon. The *pyrC* gene for DHOase is unlinked to the other genes. In lower eukaryotes, such as *Saccharomyces cerevisiae*, the *carA/B* and *pyrB* genes are fused and the CPSase and ATCase activities reside as superdomains on a single bifunctional CA protein encoded by the *URA2* locus; DHOase is encoded by a separate *URA4* gene, although a nonfunctional DHOase-like domain is located between the CPSase and ATCase domains of the CA protein (Souciet et al., 1989). In higher eukaryotes, a functional DHOase domain is positioned between the CPSase and ATCase domains of the multifunctional CAD protein.

In plants, the activities of all the enzymes of the pyrimidine pathway are readily dissociable (Doremus, 1986) except for those of orotate phosphoribosyltransferase and orotidylate

decarboxylase, which reside on the single bifunctional protein UMP synthase (Walther et al., 1984). The separate CPSase, DHOase, and ATCase activities in plants might result from proteolytic processing of a larger multifunctional protein. Indeed, this is the case in *Dictyostelium discoideum*, where these enzyme activities can be purified independently as the result of proteolytic processing of a multifunctional enzyme encoded by a single *CAD* gene (Wales et al., 1989). However, the isolation of several independent ATCase genes, each of which exhibits a 1.6-kb transcript large enough to encode only the chloroplast precursor ATCase protein, suggests that the plant ATCases are encoded by independent genes and that this enzyme is not associated with a larger multifunctional enzyme complex. Recently, Minet et al. (1992) reported that an *Arabidopsis* cDNA encoding dihydroortate dehydrogenase had been isolated by complementation in a yeast *URA1* mutant. A similar approach was also used to isolate *Arabidopsis* cDNAs for ATCase and UMP synthase (M. Minet, personal communication). These clones will permit further characterization of the genetic organization of the pyrimidine pathway in plants, which, at this point, appears to resemble more closely that of bacteria than other eukaryotes.

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