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Thymine-Starvation Up-Regulates a Region of the *Arabidopsis thaliana* Chromosome III Containing Both the UMP Synthase Gene and a Proximal Gene Encoding a Novel F-Box Protein

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We have cloned the *Arabidopsis thaliana* uridine 5'-monophosphate synthase (UMP synthase) locus and characterized transcript levels of this gene as well as the transcript encoding a novel F-box protein found upstream of UMP synthase, *Skp1*-interacting partner 5 (*SKIP5*). The 3' end of the *SKIP5* gene is 405 bp from the 5' end of the UMP synthase gene. To determine whether these proximate genes are coordinately or independently regulated, we have used an RT-PCR method to quantitate the transcript levels of each gene relative to 18S ribosomal RNA. Previous work has demonstrated an up-regulation of the UMP synthase gene in tobacco callus in the presence of compounds such as 5-fluoro-orotate and aminopterin that induce thymine starvation. Here, we present results showing that both the UMP synthase and *SKIP5* genes are coordinately up-regulated in the presence of fluoroorotic acid.

Keywords: *Arabidopis thaliana*, F-Box protein, fluoroorotic acid, *Nicotiana plumbaginifolia*, pyrimidine metabolism, *SKIP5*, thymine, UMP synthase

Pyrimidine metabolism is fundamental in all life forms. Pyrimidines are required for DNA and RNA biosynthesis, for the interconversion of carbohydrates, for polysaccharide and glycoprotein biosynthesis, for glycolipid and phospholipid biosynthesis, and many other cellular processes. When pyrimidine nucleotides become limiting, growth ceases (Jones and Hahn, 1979; Sung and Jacques, 1980; Löffler et al., 1997; Santoso and Thornburg, 1998). Therefore, understanding nucleotide biosynthesis is essential to gain a complete understanding of primary cellular metabolism.

De novo pyrimidine biosynthesis in plants is catalyzed by 6 enzymatic steps. cDNAs encoding every step have been cloned from *Arabidopsis* (Minet et al., 1992; Nasr et al., 1994; Williamson et al., 1996; Zhou et al., 1997; Brandenburg et al., 1998). The final two steps of the pathway result in the conversion of orotic acid into UMP. In the first of these steps, the ribosyl-5'-phosphate group from phosphoribosyl pyrophosphate (PRPP) is transferred onto orotic acid to form orotidylate monophosphate (OMP) by the chloroplastic enzyme orotate phosphoribosyltransferase. OMP is subsequently decarboxylated to form UMP by OMP decarboxylase. In prokaryotes and lower eukaryotes (yeast), these two enzymes are separate and distinct. However, in higher eukaryotes, including plants, the genes for these two enzymes have been fused to form a single transcriptional unit that produces a single bifunctional protein, termed UMP synthase. The plant UMP synthase thus harbors both orotate phosphoribosyl transferase and OMP decarboxylase activity (Santoso and Thornburg, 1992; Santoso and Thornburg, 1998). UMP synthase is also the rate limiting step in pyrimidine biosynthesis in plants (Santoso and Thornburg, 1992).

While there is considerable information regarding the biochemistry of pyrimidine biosynthesis, regulation of expression of the pyrimidine biosynthetic genes has lagged far behind. UMP synthase is the best studied of the de novo pyrimidine biosynthetic genes in plants. It is transcriptionally-regulated by thymine levels within cells (Santoso and Thornburg, 1998). When thymine becomes limiting, cells respond by activation of a transcriptional program that results in the up-regulation of several genes in the de novo pyrimidine biosynthetic pathway, including UMP synthase. Recently, we have demonstrated that some fluoroorotic acid-selected *Nicotiana plumbaginifolia* cell lines have lost this thymine-regulated transcriptional program and are unable to respond to alteration in thymine levels (Santoso and Thornburg, 2000). In an effort to better understand the regulation of the UMP synthase, we have isolated the locus encoding UMP synthase and have characterized gene expression from this locus.

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MATERIALS AND METHODS

Materials

The *Escherichia coli* strain, BL21:p33, expressing *Taq* polymerase (Engelke et al., 1990) was kindly supplied to us by Dr. Dan Voytas, Iowa State University. The Taq polymerase was purified by the method of Desai and Pfaffle (Desai and Pfaffle, 1995). α-[^{32}P]-dCTP was obtained from New England Nuclear (Boston, MA, USA). MMLV reverse transcriptase was purchased from Promega, (Madison, WI, USA) and used according to the manufacturer's instructions. Random hexamers were obtained from the Iowa State University DNA synthesis facility. All other materials were of the highest quality obtainable and were obtained from Fischer Scientific Co. (Pittsburgh, PA, USA) or from Sigma Chemical Co. (St. Louis, MO, USA).

Gene Isolation and Sequencing

To isolate the *Arabidopsis* UMP synthase gene, an *Arabidopsis thaliana* genomic library (Voytas et al., 1990) was screened with the *Arabidopsis* UMP synthase cDNA (Nasr et al., 1994). Positive plaques were taken through three rounds of screening. This yielded two positive clones called *1At-UMPS1* and *1At-UMPS2*. Both bacteriophage contained a 7 kb XbaI insert that hybridized with the UMP synthase cDNA. The hybridizing band was subcloned into the XbaI sites pBlueScript (SK+). The resulting clone, pRT357, contained the full-length UMP synthase gene. All sequencing was performed at the Iowa State University DNA facility using the Applied Biosystems Prism Dye-deoxy Cycle Sequencing Kit (Perkin-Elmer Corp., Norwalk, CT, USA). The reactions were run on an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer Corp., Norwalk, CT, USA). Sequencing was initiated from known vector sequences. On the basis of these runs, primers specific to the UMP synthase sequence were constructed. Both strands were sequenced in duplicate or triplicate.

Plants and Plant Growth

A. thaliana cv. Columbia plants were used for all studies. Aerial tissues (leaves, stems, flowers and siliques) were harvested from young, wild type plants grown in soil. Tissue was either harvested and used immediately or frozen at –70°C until use. Root tissue was obtained from plants grown in vitro as described (Ostin et al., 1998). For in vitro grown plants, approximately 25 surface sterilized (50% NaOCl, 10 min; 2 washes in sterile water) *Arabidopsis* seeds were used to inoculate 250 mL flasks containing 25 mL of MS medium + 2% glucose (Murashige and Skoog, 1962). The flasks were maintained with constant shaking at 100 rpm under continuous illumination for various times. After approximately 14 days of growth, the mass of plants was aseptically removed from the flask and briefly blotted to remove excess growth medium. The root tissue was excised and either used immediately for RNA isolation or frozen in aliquots at –70°C until use.

RNA Isolation

RNA was isolated using a modified guanidine thiocyanate method (Chomczynski and Sacchi, 1987). Frozen or freshly harvested tissue (100 - 300 mg) was ground in 1 mL of extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol, 0.3 M sodium acetate pH 4.8) and extracted with 0.5 mL of 0.1 M sodium acetate saturated phenol:chloroform (5:2) pH 4.8 by vigorous vortexing in a 1.5 mL microfuge tube followed by a 3 min centrifugation at 17,000*g*. The upper aqueous phase was extracted the same way once more followed by a final chloroform extraction. RNA was precipitated by adding an equal volume of 2-propanol to the aqueous phase, mixing by inversion and then room temperature incubation for 5 min followed by a 10 min centrifugation at 17,000*g*. The RNA pellet was washed with 70% ethanol and centrifuged for 1 min. The pellet was dried and resuspended in 90 µL water. Contaminating DNA was removed by incubation with 2 units of RNAse-free DNAse in the manufacturers buffer (Promega, Madison WI, USA) at 37°C for 45 min followed by a single phenol:chloroform extraction and 2 propanol precipitation as before. The final dried pellet of RNA was resuspended in 35 µL of RNAse free water. The RNA was quantitated by measuring absorbance at 260 nm on a Beckman DU-7400 spectrophotometer. RNA was stored at –70°C until use.

Reverse Transcription

Approximately 2.5 µg of RNA was incubated in a total volume of 15 µL with 2.5 µM random hexanucleotides and 2.5 mM each dNTP at 75°C for 5 min. then cooled to room temperature. Four µL of the manufacturers 5x MMLV buffer and 200 units of MMLV reverse transcriptase (Promega, Madison WI, USA) were added to each tube and mixed gently. The reverse transcription reaction was carried out at 37°C for 1 h and the reaction products were stored at –20°C until use.

Polymerase Chain Reaction

For all analyses a 50 µL "Hot Start" PCR was used (Klebe et al., 1999). The reaction mixture contained 50 mM KCl, 10 mM Tris pH 9.0, 0.1% Triton X-100, 0.2 mM of each dNTP, 0.5 µM each primer, 1 µL Taq polymerase (Desai and Pfaffle, 1995), 2.5 mM $MgCl_2$ and 1 µL of the reverse transcription reaction. The PCR was performed in an MJ Research PTC-100 thermal cycler (MJ Research, Incline Village, NV, USA) with the following cycling parameters: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. The number of cycles varies with each experiment and is presented in the individual figure legends.

[^{32}P]-labeled nucleotide triphosphates are incorporated in the PCR reaction to quantitate the PCR product yields. Following PCR, the reaction products were separated on an 8% polyacrylamide gel. The radiolabeled PCR products within the gel were visualized and quantitated using a Molecular Dynamics PhosphorImager and associated ImageQuant software (Amersham Biosciences, Piscataway, NJ, USA).

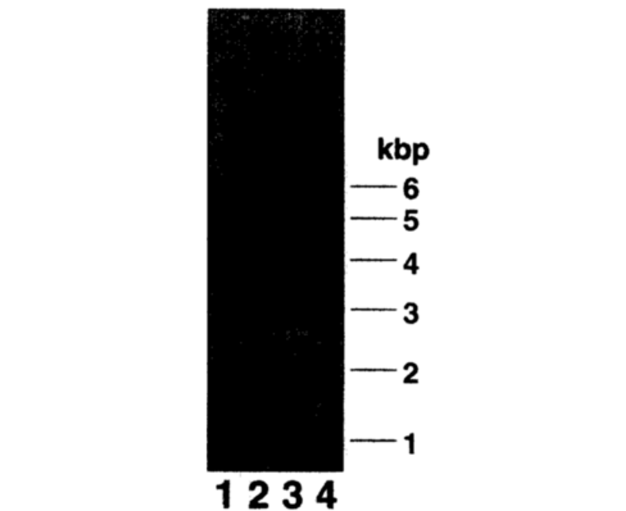

Figure 2. Genomic Southern. Ten µg of genomic DNA isolated from *A. thaliana* cv. Columbia plants was digested to completion with the following restriction endonucleases: Lane 1, EcoRI; lane 2, HindIII; lane 3, BamHI; and lane 4, XbaI. The digested DNA was run on a 1% agarose gel. Following electrophoresis, the DNA was blotted to a nylon membrane and hybridized with the radiolabeled *Arabidopsis thaliana* UMP synthase cDNA. A 1 kb ladder was used for molecular mass markers.

Oligonucleotides

Table I shows the various oligonucleotides used in this study. Non-modified oligonucleotides were synthesized at the Iowa State University Nucleic Acid Facility. Oligonucleotides modified at the 3' end were synthesized by Sigma/Genosys (St. Louis, MO, USA).

RESULTS

Cloning of the *Arabidopsis* UMP Synthase Locus

The recent completion of the sequence of the *A. thaliana* genome has confirmed our experimental characterization of the UMP synthase locus as well as filling some gaps in our knowledge. We originally cloned this region (Fig. 1) via traditional genomic library screening (Voytas et al., 1990) with the *Arabidopsis* UMP synthase cDNA (Nasr et al., 1994). This locus was found to be single copy by Southern blot analysis (Fig. 2). The full length UMP synthase gene as well as 2090 bp upstream and 448 bp downstream were isolated. The most interesting feature of this locus is the proximity of an open reading frame immediately upstream of the coding region of the UMP synthase gene. The proximity of this gene was verified in genomic DNA by PCR analysis (Fig. 3). This ORF was originally identified as EST clone T88668. Recently a routine BLAST search of the T88668 protein sequence has revealed that this gene codes for the F-box protein Skp1 Interacting partner 5 (SKIP5) isolated by Koncz and coworkers using SKP1 (ASK1) as bait (Farras et al., 2000). Due to the release of

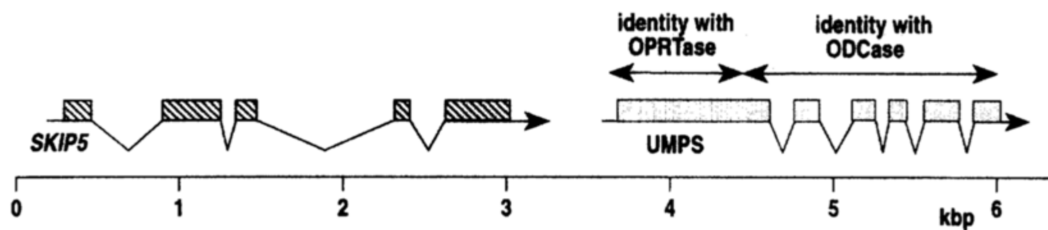

Figure 1. Structure of the UMP synthase genomic locus. A 5.2 kb stretch of DNA was sequenced and analyzed. The location of the *SKIP5* cDNA (hatched boxes) and the UMP synthase cDNA (open boxes) are indicated.

Figure 3. PCR amplification of the T88668/UMP synthase intergenic region. Lane 1 is HindIII cut bacteriophage lambda standards. Lane 2 is amplified from genomic DNA isolated from *A. thaliana* cv. Columbia plants. Lane 3 is from the lambda clone *ΦAt-UMPS1*. The oligonucleotides used for this amplification were T88668-oligo F (5'-GAGACAAAC-CCTTTGGATCAT-3') and UMPS-oligo 6 (5'-GTCTTATTTTT-GGTGTTTCA-3').

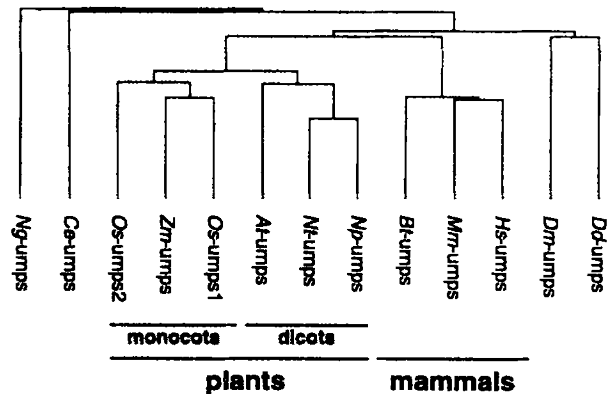

Figure 4. Phylogenetic analysis of the UMP synthases present in the GenBank. The amino acid sequences were compared using the Wisconsin GCG tool, "pile-up". The sequences used for this study were *Ng-UMPS*, *N. gruberii* (GenBank Accession # L08073); *Ce-UMPS*, *C. elegans* (Z29443); *Os-UMPS1*, *O. sativa UMPS1* (AF210323); *Os-UMPS2*, *O. sativa UMPS2* (AF210325); *Zm-UMPS1*, *Z. mays UMPS1* (AF277454); *At-UMPS*, *A. thaliana* (X71842); *Nt-UMPS*, *N. tabacum* (U22260); *Np-UMPS*, *N. plumbaginifolia* (AF277455); *Bt-UMPS*, *B. taurus* (X65125); *Mm-UMPS*, *M. musculus* (P13439); *Hs-UMPS*, *H. sapiens* (NM_000373); *Dm-UMPS*, *D. melanogaster* (L00968); *Dd-UMPS*, *D. discoideum* (X07560).

the *Arabidopsis* genome sequence, we have been able to verify that our clone was in fact correct and not a rearrangement during library construction, to place the gene on chromosome III at 92.5 cM and to verify our Southern analysis, that this gene is in fact single copy within the *Arabidopsis* genome.

Arabidopsis UMP Synthase Protein

A multiple sequence alignment of the available UMP synthase protein sequences using the "pileup" program in the Wisconsin GCG package was performed. The sequences used are: *A. thaliana*, *Bos taurus*, *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Drosophila melanogaster*, *Homo sapiens*, *Mus musculus*, *Naegleria gruberii*, *N. plumbaginifolia*, *Nicotiana tabacum*, *Oryza sativa* UMP synthase1 & 2 and *Zea mays* UMP synthase1. This program grouped the proteins into 3 clades with 2 outliers (Fig. 4). The mammalian sequences and the plant sequences formed 2 individual clades with the Drosophila and Dictyostelium sequences forming the third. The plant clade was further branched into dicot and monocot sequences. The *C. elegans* and *N. gruberii* did not fall into any of the three clades formed by these sequences.

The bifunctional *Arabidopsis* UMP synthase is similar to other UMP synthases from higher eukaryotes. The N-terminal half of the gene (amino acids 1 - 220) encodes the orotate phosphoribosyl transferase function and shows 23% identity to the yeast *ura5* protein while the C-terminal half of the gene (amino acids 220 - 477) encodes the orotidine decarboxylase function and shows 49% identity to the yeast *ura3* protein.

When we examined the putative amino acid sequence of the *Arabidopsis* UMP synthase, we found at least 9 highly conserved residues (K27, R93, K94, K97, I104, E119, D120, R151, G425) that have been identified as catalytically important in crystallographic studies of prokaryotic orotate phosphoribosyl transferase and yeast orotidine decarboxylase (Smiley and Jones, 1992; Grubmeyer et al., 1993; Scapin et al., 1994; Ozturk et al., 1995; Scapin et al., 1995; Suchi, et al., 1997). These comparisons strongly suggest that the catalytic mechanism of the plant UMP synthases is identical to that of the yeast, Salmonella and Human UMP synthases.

Arabidopsis SKIP5

A routine blast search using the sequence of T88668 from our *Arabidopsis* UMP synthase clone has identified 2 overlapping EST clones (190E9T7 and 214F2T7) and a genomic BAC (T14E10). From these sequences we have determined a full length sequence of this transcript

as well as the gene structure. The full length transcript is 1001 nucleotides in length. The gene contains 4 introns of variable length (85 to 911 nucleotides). Comparison of the EST clones to the genomic sequence revealed that clone 190E9T7 contained an unspliced intron 4. To test whether this was a cloning artifact or a form of alternative splicing we performed a quantitative RT-PCR analysis using oligos that flank intron 4. We tested RNA isolated from root, leaf, stem, and flowers as well as thymine starved whole plants. In every case we observed a band that corresponded to unspliced transcript. Quantitation of this band relative to spliced transcript shows 5.4 ± 1.2% of all transcripts were unspliced. We conclude that this intron may be poorly spliced and the EST clone 190E9T7 simply reflects this.

The full length cDNA encodes a 274 amino acid protein of 30624 Da and a predicted pI of 8.59. A PROSITE analysis of the protein reveals 2 motifs. Amino Acids 12-29 are a predicted bipartite nuclear localization signal (Dingwall and Laskey, 1986), amino acids 32 - 79 encode a predicted F-box motif (Bai et al., 1996) and amino acids 154-175 encode a single imperfect leucine rich repeat. This suggests that T88668 may be a nuclear protein involved in transcriptional regulation of an as yet unknown factor.

An ortholog of this protein was identified in the tomato EST databases. This cDNA was obtained and sequenced (GenBank Accession # AY056053). The tomato homolog is 18 amino acids shorter at its N-terminus but has a 15 amino acid extension at its C-terminus. The central portions of the two proteins showed 68% amino acid identity. No orthologs are found in any vertebrate (human, mouse, rat, pig, cattle, or zebrafish), invertebrate (Drosophila or *C. elegans*), yeast or prokaryotic databases. Thus, this sequence may represent a novel plant specific F-box protein.

Koncz and coworkers recently isolated this clone in a yeast two-hybrid using *ASK1* (*SKIP1*) as a bait and have subsequently renamed it *SKIP5* (*SK*ip1 *I*nteracting *P*artner 5) (Farras et al., 2000). The fact that this protein interacts with *ASK1*, a member of the SCF complex, is further evidence that it may be involved in transcriptional regulation of unknown factor(s) via an SCF complex.

Expression of *SKIP5* and UMP Synthase mRNAs

To evaluate expression from this locus, we developed a semiquantitative RT-PCR assay to monitor the mRNA levels of the *SKIP5* and UMP synthase genes. This assay was developed using total RNA from 7-day old *Arabidopsis* plants grown in vitro as described in Experimental Procedures.

For RT-PCR methods to provide useful information, the measurements must be taken during the linear phase of the PCR process. Starting with the oligonucleotides specific for UMP synthase, we examined the accumulation of the UMP synthase PCR products from reverse transcribed leaf RNA. The UMP synthase PCR product began to accumulate after 18 cycles of amplification and the accumulation was linear through cycle 28 (data not shown).

A second concern is the use of an internal standard. Ideally, the internal standard should be invariant in all tissues and under all treatments. Because almost 80% of total RNA is rRNA, the level of the rRNA remains constant between samples and has a nearly constant steady state level. We have, therefore, chosen to compare all reactions to the 18S rRNA. This RNA species, however, is so abundant that it is difficult to find conditions that allow both the standard 18S and gene of interest to be simultaneously evaluated. To solve this problem, we are using modified competitive oligonucleotides (MCO).

These modified 18S rRNA oligonucleotides are identical in sequence with the non-modified 18S rRNA oligonucleotides (see Table 1) with the exception that the 3' terminal residue is modified so that cDNA can-

Table 1. Oligonucleotides used in the RT-PCR assays.

Identity	Sequence	n	Tm[a]
UMPS-1	5'-GATCCTGAGAGATGGCTGAG-3'	20	62°C
UMPS-2	5'-CAACGAGACATGAGTCTTAAAA-3'	22	60°C
T88668-1	5'-GAGACAAACCCTTTGGATCAT-3'	21	60°C
T88668-2	5'-GAAGATGGATCATTTCCCATC-3'	21	60°C
18S-1	5'-AACTTACCAGGTCCAGACATA-3'	21	60°C
18S-2	5'-TAGGAGCGACGGGCGGTG-3'	18	62°C
MCO-18S-1	5'-AACTTACCAGGTCCAGACATA-][b]	21	60°C
MCO-18S-2	5'-TAGGAGCGACGGGCGGTG-][b]	18	62°C

[a]Tm = 2(A + T) + 4(G + C)°C.

[b]3' end of the MCO oligonucleotides is not free. The oligonucleotides are modified at the 3' end and cannot elongate DNA.

not be extended from these oligonucleotides. These oligonucleotides can compete for binding to the 18S ribosomal RNA, however the 3' modifications block their ability to serve as primers in the polymerase chain reaction.

Using a mixture of the normal 18S oligonucleotides and the modified 18S oligonucleotides, we can reduce 18S PCR product to levels that are similar to that of the UMP synthase PCR products. Increasing the ratio of the modified to normal 18S oligonucleotides significantly reduces the accumulation of the 18S product. Over a range of ratios from 0.05 to 0.15 normal to modified 18S oligonucleotides, the levels of 18S rRNA to UMP synthase PCR products were roughly equivalent. Therefore, all further studies were performed at or near a ratio of 0.05 normal to modified oligonucleotides.

We next determined that all PCR products (UMPS, SKIP5 and 18S rRNA) could be measured in the linear PCR range as a function of cycle number. In this analysis, the ratio of normal to modified 18S rRNA oligonucleotides was evaluated at both 0.05 and 0.10. In both cases, the accumulation of all PCR products was linear from cycles 20 to 28. All additional analyses were performed at cycle 26.

Fluoroorotic Acid Induction

We initially evaluated whether the RT-PCR assay was capable of detecting changes in the UMP synthase mRNA levels. In our previous work with tobacco we have shown that UMP synthase levels are transcriptionally up-regulated by thymine starvation (Santoso and Thornburg, 1998). Therefore, we grew *Arabidopsis* plants in tissue culture flasks (Ostin, et al., 1998) and induced the plants with the addition of 20 μg/mL flurororotic acid (Santoso and Thornburg, 1998). Figure 5 shows the RT-PCR profiles of a representative experiment for uninduced and FOA-induced plants. The bands from these gels were quantitated by PhosphorImager and these data are presented in Table 2. In these studies, the UMP synthase mRNA was up-regulated greater than 3-fold. This induction of the UMP synthase mRNA is in-line with the previously published levels for the induction of this gene (Santoso and Thornburg, 1998). Thymine starvation, induced by the addition of FOA, also caused the up-regulation of the *SKIP5* mRNA. The *SKIP5* mRNA was induced greater than 10-fold. Because pool sizes of pyrimidines are large within cells, the induction requires several days to achieve maximal effect (Santoso and Thornburg, 2000). For both genes, maximal induction was observed

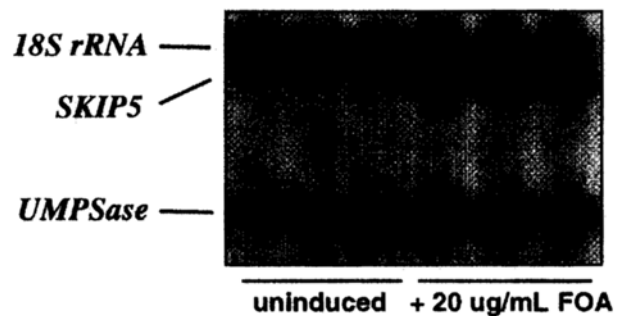

Figure 5. RT-PCR analysis of UMP synthase and *SKIP5* genes from normal or thymine starved (20 μg/mL, 3 days) plants. Replicates show the reproducibility of the assay.

Table 2. Expression of *SKIP5* and UMP synthase in response to FOA.

FOA concentration	days	*SKIP5*			UMP synthase		
		SKIP5/18S	Average[a]	ratio[b]	UMPS/18S	Average[a]	ratio[b]
None	7	0.12 0.11 0.12	0.12 ± 0.00	1.00	0.42 0.44 0.45	0.44 ± 0.02	1.00
20 μg/mL	3	0.41 0.77 0.68	0.62 ± 0.19	5.16	0.84 1.07 0.89	0.93 ± 0.12	2.11
20 μg/mL	5	1.68 0.93 1.23	1.28 ± 0.38	10.66	1.87 1.25 1.54	1.56 ± 0.31	3.55
20 μg/mL	7	0.56 0.53 0.53	0.54 ± 0.02	4.50	1.09 0.94 1.03	1.02 ± 0.08	2.32

[a]Average +/– standard deviation.
[b]Levels of mRNAs were normalized to those level found in plants incubated in the absence of FOA.

on day 5 and by day 7 the mRNA levels had begun to decline, implying a coordinated induction of both genes. A similar up-regulation of the UMP synthase and *SKIP5* mRNAs were observed when plants were thymine starved by the addition of 25 µg/mL aminopterin for 7 days (data not shown).

Tissue Specific Expression

Since we had verified that the RT-PCR assay successfully monitored the levels of UMP synthase in whole plants following the addition of fluoroorotic acid, we next examined the level of expression in various plant organs. Table 3 shows the results of a representative experiment. The highest levels of UMP synthase mRNA were observed in leaves and flowers. Stems and siliques were significantly lower and roots showed very low levels of UMP synthase mRNA. The tissue-specific pattern of expression for the *SKIP5* mRNA was similar. The highest levels were observed in leaves and flowers with stems and siliques significantly lower and roots showing the poorest levels of expression.

DISCUSSION

UMP synthase is the rate limiting and final step of pyrimidine biosynthesis in plants (Santoso and Thornburg, 1992). It is transcriptionally up-regulated in response to thymine starvation (Santoso and Thornburg, 1998). The UMP synthase locus in *A. thaliana* harbors two genes in close proximity, UMP synthase and *SKIP5*. To evaluate the expression of these two genes, we have developed a semi-quantitative RT-PCR assay using attenuated 18S rRNA as a standard. Because 18S rRNA comprises 80% of total cellular RNA, we designed modified competitive oligonucleotides (MCOs) to attenuate the signal from the 18S rRNA. These MCOs are identical in sequence to the unmodified primer and will compete for the binding site but have a 3' C3 linker. Therefore these MCOs cannot initiate chain elongation. Using the native 18S rRNA oligonucleotides plus the 18S rRNA MCOs together with the gene specific oligonucleotides in ratios that permit the linear amplification of each of the PCR products from reverse transcribed RNA has permitted us to develop a single pot semiquantitative RT-PCR assay to evaluate gene expression.

The results of these studies confirm in *Arabidopsis*, our earlier observations on the induction of UMP synthase during nucleotide starvation in tobacco. These results also extend these observations by demonstrating that the F-box protein, *SKIP5*, is similarly regulated by thymine starvation. The FOA-induced patterns of expression for both *SKIP5* and UMP synthase implies coordinated regulation of these genes. However, a comparison of the 5' flanking regions of these genes failed to identify any sequences sharing significant identity between these two genes. Alternatively, FOA-induction of UMP synthase appears to result from derepression of that gene (Santoso and Thornburg, 2000). Therefore, it may be possible that a coordinated silenc-

Table 3. issue specific expression of *SKIP5* and UMP synthase

	SKIP5			UMP synthase		
Tissue	*SKIP5* / 18S	Average[a]	ratio[b]	UMPS / 18S	Average[a]	ratio[b]
	1.04			0.32	0.29 ± 0.03	
Leaf	1.08	1.06 ± 0.02	1.00	0.27		1.00
	1.08			0.28		
	0.54			0.24		
Stem	0.51	0.54 ± 0.04	0.51	0.25	0.25 ± 0.01	0.87
	0.58			0.26		
	0.04			0.04		
Root	0.03	0.03 ± 0.00	0.03	0.04	0.04 ± 0.00	0.13
	0.04			0.04		
	1.04			0.52		
Flower	1.07	0.83 ± 0.38	0.78	0.52	0.43 ± 0.15	1.50
	0.40			0.26		
	0.44			0.20		
Silique	0.41	0.41 ± 0.02	0.39	0.18	0.19 ± 0.01	0.65
	0.39			0.18		

[a]Average +/− standard deviation.
[b]Levels of mRNAs were normalized to the level of mRNA found in leaves.

ing mechanism is active at this locus to maintain low levels of expression of both mRNAs. Then as a result of thymine starvation a single event could result in the derepression of both genes.

F-box proteins are an increasingly large family of proteins (Xiao and Jang, 2000) that function in the transcriptional regulation of a wide variety of developmental and biosynthetic pathways. In plants, F-box proteins have been shown to function in jasmonate defense responses (Xie et al., 1998), auxin response (Ruegger et al., 1998), floral organ development (Samach et al., 1999) and circadian oscillations (Nelson et al., 2000; Somers et al., 2000).

F-box proteins have two distinct functions. First, they interact with target proteins that are frequently transcriptional repressor molecules. A great diversity of sequences associated with F-box proteins, WD40 repeats, Ankyrin repeats, Kelch repeats, and leucine rich repeats, results in a large number of molecules that can be recognized by the F-box proteins. Second, the F-box motif links the F-box protein to an SCF (Skp1-cullin-F-box protein) complex. These SCF complexes comprise a new class of ubiquitin E3 ligases that ubiquitinate proteins recruited to the F-box protein via specific protein-protein interactions of the diverse repeat sequences. The result is the targeted degradation of specifically recruited proteins (transcriptional regulators) at the 26S proteosome (del Pozo and Estelle, 1999, 2000).

Skip5 is an F-box protein that interacts with the *Arabidopsis Skp1* homolog (*Ask1*). Its expression is up-regulated in response to nucleotide starvation; however, the target of the *Skip5* protein is unknown. Studies to ascertain the function of *Skip5* are in progress.

FOOTNOTES

1) This work was sponsored by funds from the U.S. Department of Agriculture (91-37301-6208), from the Carver Trust, the Hatch Act and State of Iowa Funds.

2) Journal Paper No. J-19127 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project No. 3202.

4) Abbreviations: C_f-final concentration; MMLV- Maloney Murine Leukemia Virus; MCO, modified competitive oligonucleotides;

5) Keywords: UMP synthase, *N. plumbaginifolia*, fluoroorotic acid, thymine, pyrimidine metabolism, F-Box protein,

6) GenBank Accessions for sequences reported in these studies are: *A. thaliana* UMP synthase genomic locus, AF276887; *N. plumbaginifolia* UMP synthase gene, AF277455; *A. thaliana* SKIP5 cDNAs, AF276888, AF347971, and AF347972; *Lycopersicon esculentum* SKIP5 cDNA, AY056053.

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