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REGULATION OF PYRIMIDINE METABOLISM IN PLANTS

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1. ABSTRACT

Pyrimidine nucleotides represent one of the most fundamental of cellular components. They are the building blocks for the direct synthesis of DNA and RNA that function in information storage and retrieval within the cell, but they also participate in the metabolism of a large number of other cellular components from sugar interconversion to cellular polysaccharides to glycoproteins and phospholipids. Thus, the metabolism of pyrimidine nucleotides and their intracellular pool sizes influence vast areas of normal cellular metabolism. The first pyrimidine, UMP, is synthesized by a *de novo* pathway that appears to be mechanistically invariant in all organisms. UMP is then further modified to form other pyrimidines. Breakdown of deoxyribo- and ribonucleic acids, the main sink for pyrimidine nucleotides, allows pyrimidines to be reutilized for resynthesis of these important cellular components. Pyrimidines are salvaged by converting the modified components into the free base, uracil for reutilization. Finally, pyrimidines are degraded into simple cellular metabolites permitting reutilization of nitrogen and carbon from pyrimidine ring systems into cellular metabolic pools. The regulation of pyrimidine metabolism is tightly controlled in plants. Additionally, plants produce toxic secondary metabolites derived from pyrimidines for use as defense compounds.

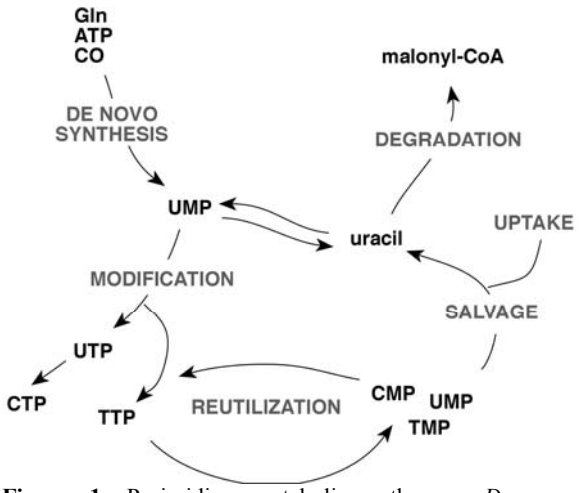

Figure 1. Pyrimidine metabolic pathways. *De novo* synthesis of UMP requires glutamine, ATP and CO_2. Modification of nucleotides converts UMP into the UTP, CTP, dCTP and TTP. The primary use of pyrimidines is in DNA and RNA synthesis. Degradation of nucleic acids converts pyrimidines to the monophosphate form which can be reutilized by conversion back to the pyrimidine triphosphates. Salvage of nucleotides requires the conversion of the nucleotides to either nucleosides or to the free base, uracil. Uptake from extracellular compartments also provides a source of salvageable nucleotides. Degradation of nucleotides results in the production of malonyl-CoA which can be completely metabolized via other pathways

2. INTRODUCTION

Within the cell there are two classes of nucleotides, purines and pyrimidines. Each class of nucleotides is synthesized by independent pathways. The pools of these molecules are tightly regulated and stringently maintained in proportion to one another in plants (1).

The rationale for studying the regulation of pyrimidine biosynthesis is two fold. First, pyrimidine biosynthesis must surely be one of the most ancient of all biochemical pathways; enzymatically, the *de novo* pathway is invariant among all life forms examined. Second, nucleotides are key components in a myriad of cellular reactions and they are absolutely essential for normal growth and development. Because of this key role in such a large number of major biosynthetic pathways, their study is of intrinsic, and potentially practical value, yet how this essential pathway is regulated has been largely ignored.

The overall metabolism of pyrimidines can be broken into several different phases: *de novo* synthesis, modification, reutilization, salvage, and degradation (Figure 1). Nucleotides can be synthesized *de novo* as well as from salvage of preformed bases or by recycling from nucleic acid turnover. It is obviously beneficial from an energetic standpoint for an organism to recycle nucleotides from RNA degradation. Pyrimidine nucleotides and/or nucleosides can be converted back to UMP or simply reused without any conversion other than phosphorylation. Plants have all the enzymes necessary for this recycling with two notable exceptions, cytosine deaminase and dCMP deaminase.

3. PYRIMIDINE METABOLIC PATHWAYS

The completion of the *Arabidopsis* genome project has allowed us to characterize the genes (Table 1) and pathways of pyrimidine metabolism as well as the genomic organization of the pathways (Figure 2). Based on this genomic analysis it appears that pyrimidine biosynthesis is quite similar to other eukaryotic organisms with few exceptions.

3.1. *De novo* pyrimidine biosynthesis

Pyrimidine metabolism is evolutionarily conserved in all species examined. The *de novo* biosynthetic pathway consists of six enzymatic steps. Carbamoyl phosphate synthase (CPSase) forms carbamoyl phosphate which is used in both pyrimidine and arginine biosynthesis. Aspartate is condensed with carbamoyl phosphate by the enzyme aspartate transcarbamoylase (ATCase) and is the first committed step in pyrimidine biosynthesis. The carbamoyl aspartate is cyclized by the enzyme dihydroorotase (DHOase) to yield the pyrimidine ring. Dihydroorotate is subsequently oxidized via the enzyme dihydroorotate dehydrogenase (DHODH) to yield orotate. The resulting orotate is used by the bi-functional enzyme UMP synthase (UMPS) along with phosphoribosyl pyrophosphate to form orotidine monophosphate (OMP). The OMP molecule is also decarboxylated by UMPs. The resulting uridine monophosphate (UMP) is the precursor to all other pyrimidine nucleotides within the plant cell.

There are some notable similarities and differences in the enzymes of the *de novo* pathway between plants and other organisms. In animals, the first three steps are carried out by a large multifunctional polypeptide termed CAD (CPSase-ATCase-DHOase). In plants these three steps are carried out by individually encoded proteins (2), as is found in prokaryotes (3). Like animals, in plants, UMP synthase is found as a single bi-functional polypeptide with discreet domains for each function. In prokaryotes these last two steps are found in individually encoded proteins (3).

3.1.1. CPSase

Two cDNAs have recently been cloned which encode the small and large subunit (carA and carB) of this enzyme (4, 5). Like other enzymes of the *de novo* pathway, CPSase is more similar to a prokaryotic enzyme than its eukaryotic homologues. The small subunit shows 46% identity to the *E. coli* enzyme and 51% identity to the *Synechocystis* enzyme. The large subunit shares 66% identity to the *Synechocystis* enzyme. The C-terminus of the *Arabidopsis* large subunit contains a unique domain not found in other CPSases that may be important in subunit interaction (5).

Carbamoylphosphate Synthase (CPSase) is encoded within the nucleus, but the mature protein is targeted to and functions within the chloroplast. This enzyme

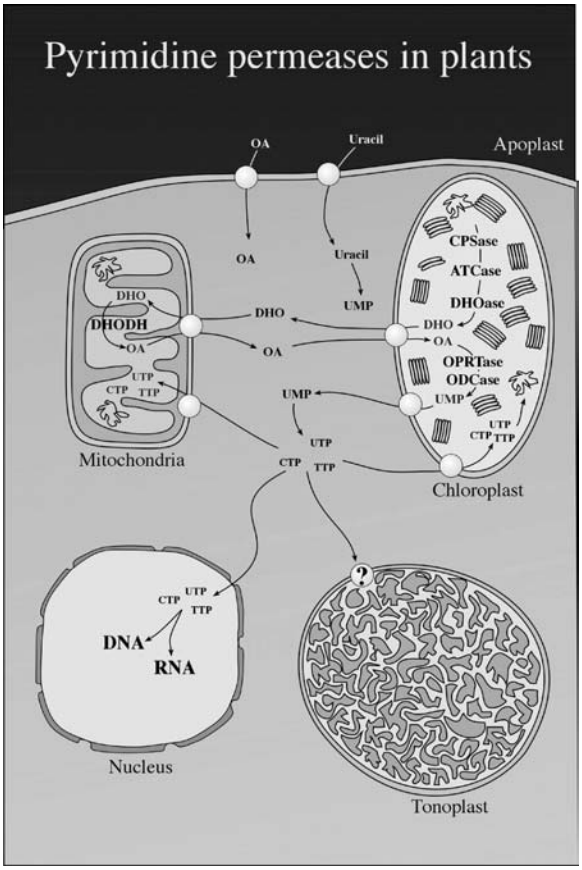

Figure 2. Cellular compartmentalization of pyrimidine metabolism. Pyrimidine permeases and transporters shuttle pyrimidine nucleotides through various cellular compartments as well as between cells. The bulk of *de novo* synthesis occurs within the plastid. Intermediates in the pathway are transported between organelles. Most of these pyrimidine permeases have not been fully characterized or identified

is responsible for the production of carbamoylphosphate for both pyrimidine and arginine biosynthesis. Ornithine stimulates incorporation of $NaHCO_3$ into both UMP and arginine. However, in the presence of exogeneously added uridine, incorporation of $NaHCO_3$ into UMP was reduced while incorporation into arginine was unaffected (6).

3.1.2. ATCase

Several cDNAs encoding ATCase (pyrB1, pyrB2, pyrB3) have been cloned from *Pisum sativum* (7, 8) along with a single cDNA from *Arabidopsis* (9). A motif within the ATCase protein has been identified as a putative pyrimidine-binding site, based upon its homology to the known allosteric pyrimidine-binding site of the *E. coli* regulatory ATCase subunit. This supports the argument that ATCase is the major regulated enzyme of pyrimidine biosynthesis. This binding site is also conserved in the *Arabidopsis* protein. The pea cDNAs contain N-terminal chloroplast targeting sequences that are in agreement with previous work reporting localization of ATCase activity to the chloroplast.

As in other organisms, this step is likely the major regulatory site. Indeed, studies in squash (*Cucurbita pepo*) tissue cultured cells have shown a 90% decrease in ATCase activity after the addition of UMP to the growth media (6). Further, addition of exogenous uridine to the growth media was able to inhibit incorporation of radiolabeled $NaHCO_3$ into UMP but had no effect on the incorporation into arginine. This also lends support to the claim that CPSase is not the major regulatory step in pyrimidine biosynthesis (6; 10).

3.1.3. DHOase

Like most other members of the pathway, DHOase is a nuclear encoded chloroplastic enzyme. The cDNA encoding this enzyme has been cloned from *Arabidopsis* and shares 54.5% identity to the *E. coli* enzyme (11). Expression of DHOase has been examined during wheat germination. It was found that DHOase drops 50% during the first 30 min of imbibition and remains very low until approx. 12 hours after imbibition at which point, activity again rises. Two days after germination the activity is four fold higher than in the dry seeds (12) and therefore, this enzyme must be developmentally regulated.

3.1.4. DHODH

To date the only dihydroorotate dehydrogenase homologue cloned and characterized from a higher plant was from *Arabidopsis* by complementation of a yeast auxotroph with plant cDNAs (13). In this case, the enzyme appears to be similar to its eukaryotic homologues. This protein also has N-terminal targeting sequences that direct the protein to the mitochondrial intermembrane space.

Dihydroorotate is oxidized by DHODH within the mitochondria with concomitant reduction of ubiquinone. Therefore, nucleotide biosynthesis is inextricably linked to respiration. Plants frequently undergo oxygen stress induced principally by flooding. In many parts of the globe, flooding is an annual or a semiannual occurrence. Pyrimidine biosynthesis would likely be affected by these events resulting in unknown consequences for the plant. The plant DHODH apparently differs significantly from the animal enzymes, because potent inhibitors of the animal enzymes do not significantly affect the plant enzymes (14).

There are several interesting features of the promoter region of this gene. It is relatively short (290 nt) and contains no TATA box. Furthermore the promoter is, most likely bi-functional, as an expressed superoxide dismutase gene is found immediately upstream and in opposite orientation to the DHODH gene.

3.1.5. UMPS

In plants, UMP synthase is a bifunctional protein catalyzing the final 2 steps of *de novo* pyrimidine biosynthesis. As in other higher eukaryotes, the plant UMP synthase has both orotate phosphoribosyl transferase (OPRTase) and orotidylate decarboxylase (ODCase) activities.

The *Arabidopsis* UMPS gene has an interesting physical structure. The 5'-half of the gene encodes the OPRTase function while the 3'-half encodes the ODCase function. Interestingly, all five introns within the UMPs gene

Table 1. Genes of Pyrimidine Metabolism in Arabidopsis thaliana

Step	Enzyme	Gene Symbol	TAIR Locus
Biosynthesis			
1a	Carbamoyl phosphate synthase (ssu)	*carA*	AT3G27740
1b	Carbamoyl phosphate synthase (lsu)	*carB*	AT1G29900
2	Aspartate transcarbamoylase	*pyr2*	AT3G20330
3	Dihydroorotase	*pyr4*	AT4G22930
4	Dihydroorotate dehydrogenase	*pyrD*	AT5G23300
5/6	UMP synthase	*pyrE-F*	AT3G54470
Modification			
7	UMP/CMP kinase UMP/CMP kinase-like	*pyr6* [c]*PYR6-L*	AT5G26667 AT4G25280
8	Nucleotide diphosphokinase	*ndp1a* *ndp2* *ndp3*	AT5G63310 [a]NI [a]NI
9	CTP synthase	*ctps1* *ctps2* *ctps3* *ctps4* *ctps5*	AT4G02120 AT3G12670 AT1G30820 AT4G20320 AT2G34890
10a	Ribonucleotide reductase (lsu)	*rnr1*	AT2G21790
10b	Ribonucleotide reductase (ssu)	*rnr2*	AT3G23580
11	dUTPase	*dut1*	AT3G46940
12	Thymidylate synthase	*thy1* *thy2* *thy3*	AT2G16370 AT2G21550 AT4G34570
13	Thymidylate kinase	*tk*	AT5G59440
14	DCMP kinase	*dck*	[a]NI
Salvage			
15	Uracil phosphoribosyltransferase	*uprt1*	AT1G55810
		uprt2	AT3G27190
		uprt3	AT3G53900
		uprt4	AT4G26510
15a	UPRTase/UrK	*utk*	[d]AT3G27440
16	Uridine kinase	*urk1*	AT1G26190
		urk2	AT1G73980
		urk3	AT5G40870
17	5'-nucleotidase	*5nt*	[a]NI
18	Cytidine deaminase	*cyd1*	AT2G19570
		cyd2	AT4G29620
		cyd3	AT4G29630
		cyd4	AT4G29650
		cyd5	AT4G29640
		cyd6	AT4G29610
		cyd7	AT4G29600
		cyd8	AT4G29580
		cyd9	AT4G29570
19	Cytosine deaminase	*cod*	[b]NP
Degradation			
20	Dihydropyrimidine dehydrogenase	*dpyd*	[a]NI
21	Dihydropyrimidinase	*dpys*	AT5G12200
22	β-ureidopropionase	*βUP*	AT5G64370
22	β-alanine synthase	*βas*	[a]NI

[a]NI=Not yet identified, [b]NP=This enzyme is not present in plants, [c]encodes a uridine 5'-monophosphate (UMP)/cytidine 5'-monophosphate (CMP) kinase. This gene is not present in the Columbia genome sequence but has been identified in the Ler shotgun sequence. Note: cDNA does not hit in Col, hits Ler ATL8C35419 and ATL8C38565 ATL7C171423_1, [d]uridine kinase/uracil phosphoribosyl transferase-related, N-term half similar to uridine kinase GB:AAB50568 (*Mus musculus*) (Arch. Biochem. Biophys. 336 (1), 105-112 (1996)), C-term portion similar to uracil phosphoribosyl transferase GB:AAB60213 (*Toxoplasma gondii*); unlikely two separate genes

are located in the ODCase half and no introns are found in the OPRTase half. The *Nicotiana plumbaginifolia* gene contain introns in identical positions to that of the *Arabidopsis* gene; however there is an additional intron

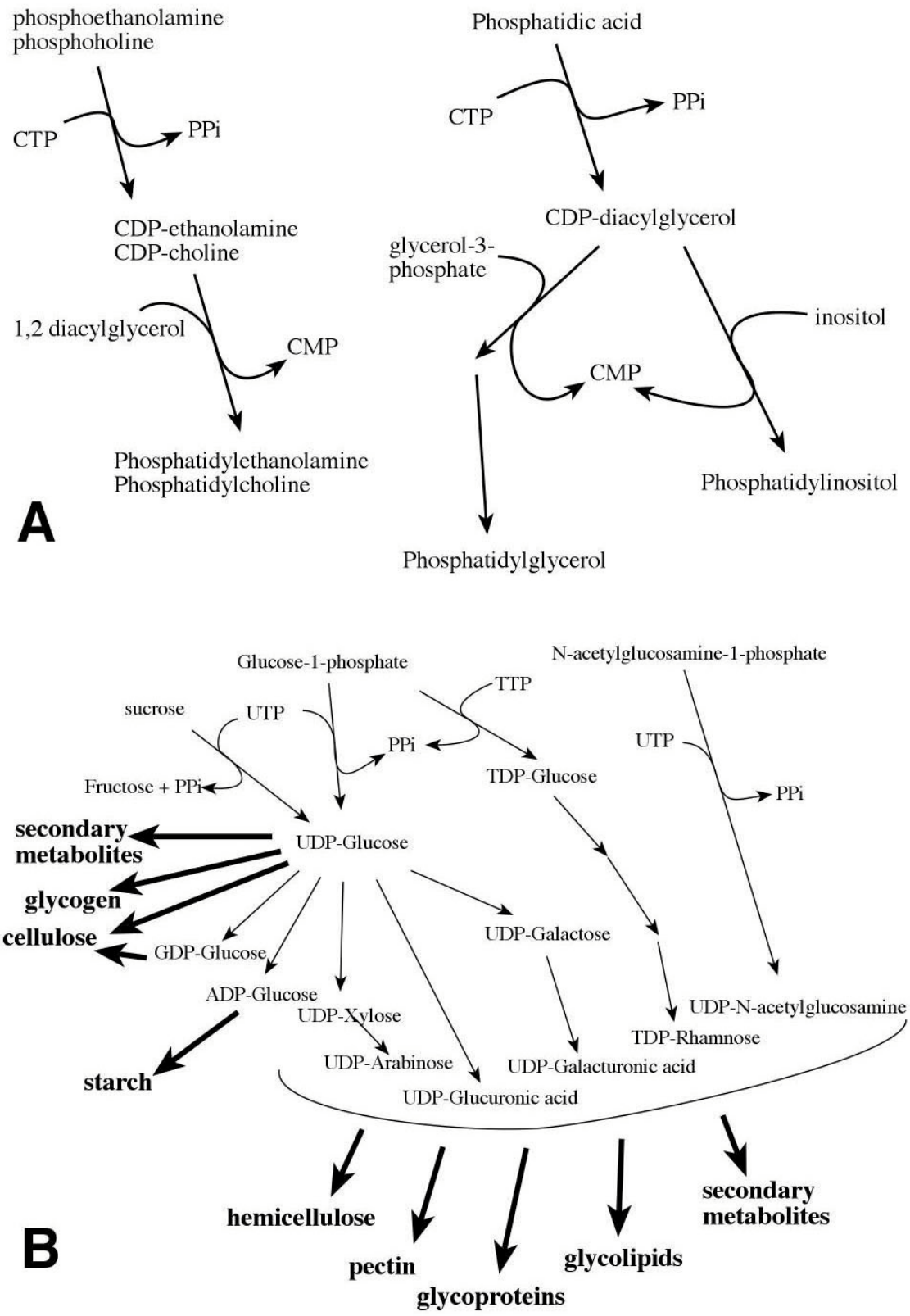

Figure 3. Pyrimidine cofactors affect broad areas of metabolism. A. Phospholipid metabolism requires the cytidine diphosphate activated forms of ethanolamine, choline, and diacyl glycerol to produce phosphatidylcholine, phosphatidylethanolamine, phosphatidyl glycerol, and phosphatidylinositol. B. A wide variety of carbohydrates require pyrimidine nucleotide cofactors in their biosynthesis.

within the 5' half of the coding region of the tobacco gene that is not found in the Arabidopsis gene. Like all of the other genes in the *de novo* pathway UMP synthase is single copy.

3.1.6. Intracellular Organization of the *de novo* Pathway

Almost the entire pathway of pyrimidine biosynthesis occurs within the chloroplast (Figure 3). There is one notable exception, DHODH, which is localized in the mitochondria. (15). The biosynthetic product of the first three *de novo* pathway enzymes, dihydroorotate, accumulates in the chloroplast. To produce UMP, dihydroorotate must be transported from the chloroplast and be taken up by the mitochondria where it is converted into orotic acid. Subsequently, orotic acid must be transported from the mitochondria and re-enter the chloroplast where it is converted into UMP by the enzyme UMP synthase.

In maize, the shuttling of nucleotides between the chloroplast and the mitochondria may be mediated by a protein with similarity to microbial pyrimidine/purine transport proteins. This protein is encoded by the *lpe1* gene (16). Indeed, normal plant development appears to be dependent upon such pyrimidine transport. Mutations in the *lpe1* gene in maize result in abnormal chloroplasts and altered leaf development. Several homologues have been identified in the *Arabidopsis* EST databases. Recently, a large family of nucleoside transporters was identified in Arabidopsis (15). One of these, *AtENT3* appears to localize to the plasma membrane and may be the functional equivalent of the yeast *fur4* gene, uracil permease. Because the control of pyrimidine biosynthesis in plants involves this unusual subcellular compartment shuttling, these proteins may be important and previously overlooked sites of control for nucleotide metabolism in plants.

3.2. Pyrimidine Salvage and Recycling

While *de novo* pyrimidine biosynthesis can generally provide sufficient levels of nucleotides for growth and development, this pathway is energetically expensive, directly requiring the hydrolysis of 2 ATPs and additionally requiring several others due to the involvement of PRPP. Therefore, cells have developed the strategy to repeatedly reuse the preformed nucleotides. In general, cells at rest can meet their metabolic requirements by salvage of these preformed nucleotides, perhaps supplemented to a small degree with newly synthesized nucleotides to balance nucleotide degradation. It is only when cells are undergoing rapid growth or development that large amounts of new nucleotides are required thereby necessitating a large flux through the *de novo* biosynthetic pathway.

The salvage of pyrimidines therefore permits the reutilization of the preformed nucleotides to meet normal metabolism. Both pyrimidine ribosides and pyrimidine free bases are salvaged in these processes. Pyrimidine nucleosides are principally taken up into plant cells via one of two routes, Wu and King (17), have identified 5-fluoro-2'-deoxyuridine FUdR resistant mutants of *Arabidopsis* that are deficient in uptake of FUdR from tissue culture media. This protein also transports thymidine and may be similar to thymidine transporters from yeast and other eukaryotes. Uracil transport was unaffected in these mutants. In addition, work in our lab has demonstrated that the pyrimidine free bases orotic acid, uracil and cytosine and each of their fluorinated analogs are all taken up into plant cells (18). The proteins responsible for this transport have not been identified, but are apparently different from the transporter identified by Wu and King.

3.2.1. Cytosine deaminase

It has been previously demonstrated that plants, like animals and unlike microorganisms, lack a cytosine deaminase activity (19). This lack of cytosine deaminase points to the inability of plants to salvage the cytosine free base. Indeed, because of this inability to salvage the free base, fluorinated cytosine cannot be metabolized in plants and animals and is therefore an effective antimicrobial compound. Such fluorinated compounds have been very effectively utilized in animal systems, but have been widely overlooked in plant systems, especially plant tissue cultures. Recently, a negative selection scheme utilizing a bacterial cytosine deaminase has been proposed (20). In this scheme the *codA* gene was introduced into the plastid genome of tobacco. A functional *codA* gene causes cell death in the presence of the normally non-toxic 5-fluorocytosine by conversion to the toxic 5-fluorouracil.

3.2.2. Cytidine deaminase

Arabidopsis contains a gene family of nine genes encoding cytidine deaminase. Six of these genes *CDA2* through *CDA7* are present as a tandem repeat on a 15.7 kb stretch of chromosome IV (21). Interestingly, all of the plant cytidine deaminases are more similar to the *E. coli* enzyme than they are to other eukaryotic enzymes. The bacterial enzymes are $\alpha 2$ dimers of 31 kDa subunits. The eukaryotic enzymes from humans, yeast, *Caenorhabditis*, and insects are tetramers of 15 kDa subunits. The plant enzyme is translated as a 32.5 kDa monomer that shows conserved identity with the *E. coli* enzyme throughout its length (22).

In addition, the genomes of plant mitochondria and chloroplasts undergo a process known as RNA editing. During this process, numerous genetically-encoded cytidine residues in organelle-encoded RNAs are deaminated to form uridine residues. Similarly, in some species, uridine residues are modified to form cytosine residues. In the chloroplasts of lower plants such as hornwarts, there are 509 C-to-U and 433 U-to-C conversions (23). In tobacco chloroplasts there are 30 specific C-to-U conversions. (24). The mechanisms that permit the selective conversion of only a few RNA residues from C-to-U appear to require specific *cis*-sequences within the RNA chain (25) accompanied with *trans*-acting proteins that recognize the *cis*-sequences (26). The biochemistry of the C-to-U conversions is identical to the cytidine deaminase chemistry and it is intriguing to speculate that one or more of the cytidine deaminases present in Arabidopsis may be involved in the RNA editing process. While this is still an open question, recent studies utilizing zinc chelators

[cytidine deaminase is a zinc-containing enzyme] suggests that the plant cytidine deaminases may ***not*** catalyze the deamination reactions of RNA editing (27).

3.2.3. UPRTase

Plants appear to have several different uracil phosphoribosyl transferase (UPRTase) genes that are responsible for salvage of free uracil. We have recently identified one gene from *Arabidopsis thaliana* which is most similar to the *Toxoplasma gondii* and yeast enzymes rather than the bacterial enzymes (28). However, the *Nicotiana tabacum* enzyme has greater identity with the bacterial enzymes than to eukaryotic enzymes. At least five UPRTase genes or putative genes are found in the Arabidopsis genome. None have been well characterized. In addition, Arabidopsis appears to have an unusual bi-functional UPRTase/uridine kinase. This bifunctional UPRTase/uridine kinase gene is also conserved in *C. elegans* (Kafer and Thornburg, unpublished). Both the *Arabidopsis thaliana landsberg erecta* cDNA and the *columbia* gene have a single nucleotide deletion that causes a frame shift mutation. For this transcript to encode a functional protein with both enzymatic activities the ribosome must shift to maintain the correct reading frame. It is unknown if this particular gene product is relevant in pyrimidine biosynthesis since both activities are also carried out by other individually encoded genes. However, several cDNAs from this potentially novel bi-functional gene have been isolated so the frame-shifted gene is transcriptionally expressed.

3.3. Pyrimidine Modification

Based upon current knowledge, it appears that formation of TMP and CMP is essentially identical to animal cell systems. Because fluorouracil induces thymine starvation in plant cells (29) as it does in animal cells, the biochemistry must be similar.

3.3.1. UMP/CMP kinase

An Arabidopsis cDNA encoding the UMP/CMP kinase has been cloned and the encoded protein shows 50% sequence homology to the mammalian enzyme (30). A conserved nucleotide-binding region (GGPGS/AGK) is located near the N-terminus. This motif is found in all eukaryotic monophosphokinases and anchors the gamma phosphate of the nucleotide (30; 31). Like other eukaryotic enzymes, UMP and CMP are equally acceptable as substrates for the plant protein (32).

3.3.2. NDP kinase

Nucleoside diphosphokinase catalyzes the conversion of the diphosphonucleosides to the triphosphonucleosides. The UTP/UDP ratio is kept similar to the ATP/ADP ratio through the action of nucleoside-5-diphosphate kinase. However, most of the work done on these kinases has centered on their involvement in signal transduction pathways. These important kinases have been shown to be autophosphorylating (33; 34), and involved in phytochrome mediated light perception (35, 34; 36), heat shock response (33) and the wound response (37). A family of three NDP kinases has been identified in *Arabidopsis thaliana*. The Arabidopsis type I enzyme is wound inducible (37), the type II enzymes are involved in phytochrome signaling (35), and the type III proteins are localized in the inner-membrane space of the mitochondria (38), where they interact with the adenine nucleotide transporter (39) and may participate in a variety of functions including the self-incompatibility response in *Brassica campestris* (40).

3.3.3. CTP synthase, NDP reductase, dUTPase

CTP synthases have not been studied in plants. Our searches have identified five genes putatively encoding CTP synthases from the Arabidopsis genome sequencing project (Table 1). Likewise, the NDP reductase subunits have not been studied. In a search for meristem specific mRNAs, Pri-Hadash and coworkers isolated a small cDNA from tomato that encoded a dUTPase (41). This mRNA was preferentially expressed in the growing meristems, but its expression dramatically declined in tissues further from the meristems. This enzyme is similarly expressed in root meristems (42) but is almost undetectable in mature root tissues.

3.3.4. Thymidylate synthase/Dihydrofolate reductase

Thymidylate synthase (TS) and Dihydrofolate reductase (DHFR) are responsible for the production of dTMP and regeneration of tetrahydrofolate, respectively. Like UMP synthase, the enzymes in both Arabidopsis and *Dacus carota* are bifunctional polypeptides. (43; 44) which apparently has arisen from a gene fusion. The N-terminus has identity with DHFR while the C-terminus has identity with TS.

The bifunctional nature of this enzyme is unusual. In bacteria and in most higher eukaryotes, including yeast, TS and DHFR proteins are separate enzymes encoded by distinct genes. Other bifunctional TS/DHFR enzymes occur in protozoans such as *Leishmania amazonesis* (45), *Plasmodium falciparum* (46), and *Paramecium tetraurelia* (47). Sequence analysis by Lazar and coworkers provides conflicting arguments on the two competing hypotheses for the origin of the plant and protozoan TS/DHFR enzymes (43). These enzymes have identical fusion sites between the functional domains. Based upon this, it is possible that the protozoan and plant enzymes have a common ancestor. Alternatively, these authors indicate that pairwise amino acid sequence analysis shows that these bifunctional enzymes are each more similar to the monofunctional enzymes than they are to each other. This raises the possibility that these enzymes may have arisen independently by convergent evolution. There are two copies of the gene in *Arabidopsis* that appear to have arisen by gene duplication (43).

3.4. Pyrimidine Catabolism

This pathway in plants has not been well characterized; however, it appears to function essentially as a reverse of the *de novo* biosynthetic pathway. Genes encoding dihydrouracil dehydrogenase (uracil reductase), dihydropyrimidine dehydrogenase, and β-ureidopropionase have all been identified in *Arabidopsis*. We conclude that pyrimidine degradation is in all likelihood identical to that in other organisms. In rat liver, the rate limiting step in this

pathway is β-ureidopropionase (48). It is unknown if this holds true for plants. The dihydropyrimidine dehydrogenase (amidohydrolases) from slime molds, plants and insects were recently found to be phylogenetically related to the *de novo* biosynthetic dihydroorotase (DHOase) enzymes (49), thereby confirming that the catabolic pathway is in many ways, the reverse of the biosynthethetic pathway.

Degradation of the pyrimidine ring proceeds via the "reductive" pathway after nucleoside phosphorolysis by uridine phosphorylase, which yields the free base and ribose-1-phosphate. The reductive enzymes utilize thymine or uracil and cytidine must therefore be converted to uridine by cytidine deaminase before degradation. The end product of pyrimidine ring degradation is β-alanine. The intracellular location of these enzymes in plants is not known for certain. In mammalian liver and *Euglena gracilis*, β-ureidopropionase was localized to the cytosol (48).

4. REGULATION OF PYRIMIDINE METABOLISM

4.1. Control of Pyrimidine Metabolism in Plants

Plants undergo developmental processes that theoretically require high levels of pyrimidine nucleotides. This includes processes such as germination, pollen tube growth, flowering, and seed set. In spite of these numerous processes that require large amounts of nucleotides, nucleotide metabolism has not been particularly well studied in plants.

4.1.1. Germination

Expression of the enzymes of *de novo* pyrimidine and pyrimidine salvage have been examined in germinating seedlings. Following imbibition, dry seeds typically have high concentrations of free bases that appear within a few hours (50). These appear to arise by salvage from nucleosides stored within the seeds. Based upon incorporation of labeled precursors into RNA, salvage appears to be the preferred source during the first few hours following germination. After the first day, *de novo* synthesis becomes the predominant source of nucleotides (51).

Similarly, the *de novo* and salvage enzymes have been examined in dry seeds and most enzymes are present; however, following a brief decline in activity, the activity of the *de novo* enzymes dramatically increases within only a few hours following imbibition (12). In contrast, the salvage enzymes, UPRTase and Uridine kinase, were high in dry seeds and showed little change during the first 24 hours of germination. The fact that relative high levels of salvage enzymes are maintained following germination along with the induction of the *de novo* pathway results in maximum funneling of all nucleotides towards pyrimidine accumulation.

4.1.2. Tissue Cultured Cells

In some tissue cultures the relative activities of the salvage and *de novo* pathways change markedly depending upon the stage of the culture. The key salvage pathway enzymes UPRTase and Uridine kinase, increase just after transfer of the cultures. Enzymatic reactions of the *de novo* pathway, namely CPSase and UMP synthase, increased after the initial post transfer lag phase with a maximum velocity during the active cell division phase (52). The results are quite similar to the picture seen in germinating seeds where the initial phase is largely one of pyrimidine salvage which then quickly turns biosynthetic with salvage playing less of a role. The question of how the two pathways (*de novo* biosynthesis and salvage) are coordinately regulated still remains unanswered.

That the activities of CPSase and UMP synthase increased in *Vinca rosea* (Madagascar periwinkle) cultures during the cell division phase led Kanamori-Fukuda and coworkers to postulate that the *de novo* pathway may not be feedback regulated but may rather be controlled by enzyme synthesis (52). Likewise, these workers showed evidence that the salvage pathways were also controlled by the availability of the key salvage enzymes.

4.1.3. Studies with Metabolic Inhibitors

The pathways of arginine biosynthesis and pyrimidine biosynthesis share the carbamoylphosphate precursor pool and are tightly coordinately controlled. Metabolic inhibitors of each of these pathways should be useful in elucidating the regulatory mechanisms of each. The metabolite, 5-fluorouracil, is toxic to plant cells just as it is in animal cells (53, 54).

Phaseolotoxin is a pathogenesis factor from *Pseudomonas syringae* pv *phaseolicola*, a pathogen of beans. It inhibits the condensation of carbamoylphosphate with ornithine and is capable of reversing the toxic effects of fluorouracil (55). The result of treating cells with phaseolotoxin is the increase in intracellular ornithine that subsequently stimulates CPSase activity. The resulting carbamoylphosphate enters the *de novo* pyrimidine biosynthetic pathway thereby increasing the levels of UMP that overcomes the toxicity of fluorouracil.

In order to understand the regulation and expression of pyrimidine biosynthesis in plants, we have previously examined the effect of the metabolic inhibitor, 5-fluoroorotic acid, on UMP synthase expression in cell cultures of *Nicotiana plumbaginifolia* (29). UMP synthase is the rate limiting step of pyrimidine biosynthesis in plants (54). Addition of fluoroorotic acid causes an up-regulation of UMP synthase enzyme activity, resulting from transcriptional induction of the UMP synthase gene. Exogeneously added thymine reversed this up-regulation. Methotrexate and aminopterin, that affect thymine levels by inhibiting DHFR also up-regulate UMP synthase in both *N. plumbaginifolia* cells and *Arabidopsis* cells. (29).

Our lab has previously generated mutant *N. plumbaginifolia* cell lines which are capable of growing in the presence of 5-FOA. The most interesting of these mutant cell lines had constitutive high level expression of ATCase and UMP synthase. These cell lines have apparently lost the ability to regulate gene expression of members of the *de novo* pathway because addition of

thymine to the growth media does not repress expression of these two genes.

Thus, either thymine or a thymine metabolite is responsible for the transcriptional repression of UMP synthase in these tobacco cells. To explain this we have proposed the existence of a protein or proteins that respond to the intracellular levels of thymine and repress the transcription of UMP synthase and possibly other pyrimidine metabolic enzymes (29). To date this protein or cellular machinery remains unidentified.

4.1.4. Regulation of *de novo* Biosynthesis in Yeast and Plants are Different

Under conditions of pyrimidine starvation in *S. cerevisiae* the genes of the *de novo* biosynthetic pathway are transcriptionally up-regulated three to eight fold. (56) The yeast regulatory DNA binding protein *PPR1p* is in part responsible for regulation of this pathway by up-regulating transcription of DHODH, ODCase, DHOase and OPRTase. (57).

Under pyrimidine starvation conditions the dihydroorotate levels rise as ATCase expression is up-regulated under low UTP levels (58). When DHO levels rise to a critical concentration, the DNA bound *PPR1p* is converted to a transcriptionally active state by direct binding of DHO. (57). Thus, a specific and sensitive mechanism which senses pyrimidine levels controls *de novo* pyrimidine biosynthesis in yeast is mediated by *PPR1p*, the first eukaryotic regulatory gene to be characterized. There is no homologue of *PPR1p* found in Arabidopsis so plants must regulate pyrimidine biosynthesis in a different manner.

4.1.5. Regulation in Drosophila Shares some Identity with Plant Regulation

Because pyrimidines are co-factors in many other pathways, they may play an as yet undiscovered role in regulation of these pathways in plants. Examples of this regulation in other organisms are scattered throughout the literature. In *Drosophila*, the CAD complex (CPSase-ATCase-DHOase) is called *rudimentary* (*r*). Mutations in this gene cause developmental abnormalities and the eggs must be supplied with exogenous pyrimidine for survival. The wings of the surviving individuals are abnormal and show varying degrees of truncation and may also be wrinkled or blistered. (59) Homozygous females are sterile which can be partially alleviated by administering cytidine during development. These studies show that pyrimidine biosynthesis is required for normal ovarian and wing development. It is currently unknown what, if any, developmental requirements for pyrimidines are found in plants.

Mutants in *rudimentary* show truncated wings and undeveloped ovaries. Therefore, temporal and spatial expression of this gene is necessary for normal fly development. (60). Two mutants have been identified that show this phenotype. One maps to a gene now named *enhancer of rudimantary e(r)*^{p1}. This gene has been cloned and codes for a conserved protein also found in plants and animals (61). In Drosophila this mutation enhances wing truncation in the *r* mutant phenotype. It is currently unknown how this interaction occurs but does not appear to be regulating *r* gene expression at the transcriptional level.

The *Arabidopsis* enhancer of rudimentary homologue encodes a 109 amino acid protein. A protein database search reveals that homologues are also found in soybean and rice (62). Threonine 24 of the *Arabidopsis* protein is phosphorylated by casein kinase II *in vitro* but lacks the second phosphorylation site found on the *er* proteins of other organisms (61). The *Arabidopsis* amino acid sequence is 41% identical to the *Drosophila* sequence (61). No experimental data on the function of this gene in any plant has been reported.

5. PARTICIPATION IN DOWNSTREAM METABOLISM

Pyrimidine nucleotides are involved in broad areas of cellular metabolism. This involvement is primarily as the activated nucleotide intermediates of other compounds. The synthesis of phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl inositol, and phosphatidyl glycerol all high energy CDP-intermediates (Figure 4A). These high-energy intermediates then are modified to produce the phospholipids.

Overexpression of the CTP synthetase in *S. cerevisiae* led to a two fold increase in the utilization of the CDP-choline pathway and a reduction in phosphatidylserine synthetase activity (63). Neither of these results were due to a transcriptional upregulation of the biosynthetic genes, however. CTP was shown to be the limiting reagent in the phosphocholine cytidylyltransferase reaction and CTP had an allosteric inhibitory effect on the phosphatidylserine synthetase reaction. Similar experiments in plants have not been reported.

Sugar metabolism is also intimately linked to pyrimidine nucleotides. The side diversity of sugars within the cell arise from the interconversion of sugars by specific epimerases. Without exception, these enzymes require nucleotide intermediates as substrates. For the synthesis of most sugars, the starting substrate is UDP-glucose. A variety of enzymes synthesize UDP-xylose, UDP-arabinose, UDP-glucuronic acid, UDP-galactose, UDP-galacturonic acid, UDP-rhamnose and UDP-sulfoquinovose from UDP-glucose. These components then are incorporated into a large number of biochemicals from cell wall components (cellulose, hemicellulose, pectin) to glycoproteins to glycolipids to sulfolipids. Other carbohydrate components of these compounds also utilize pyrimidine nucleotide intermediates. UDP-N-acetylgalactosamine is synthesized from N-acetylgalactosamine-1-phosphate.

Mannose, which occurs in glycoproteins and fucose which occurs in plant cell walls (64) as well as plant glycoproteins (65) utilize the purine nucleotide GTP for activation (66). Interestingly, the enzymes of starch biosynthesis and at least isozyme of cellulose synthase utilize sugar nucleotides other than UDP-glucose. Starch synthases utilize ADP-glucose as a substrate (67, 68). ADP-glucose is synthesized from Glucose-1-phosphate by the action of ADP-glucose pyrophosphorylase (69).

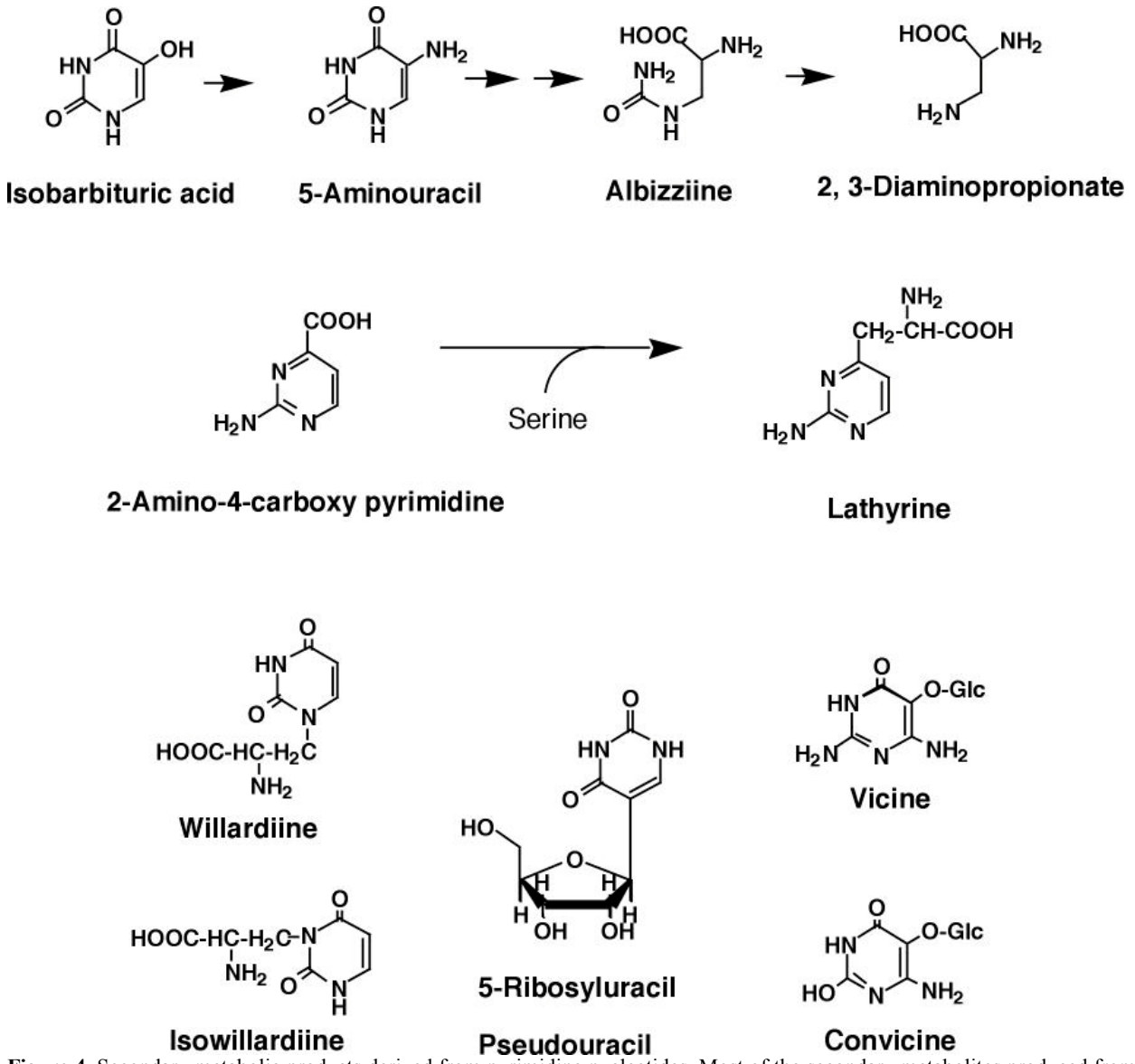

Figure 4. Secondary metabolic products derived from pyrimidine nucleotides. Most of the secondary metabolites produced from pyrimidines are thought to be defensive compounds

Because the synthesis of important cellular metabolites such as cell wall polysaccharides, glycoproteins, glycolipids and sulfolipids all require pyrimidine nucleotide to participate in their production, an unexplored regulatory link exists in which pyrimidine nucleotide levels could affect large areas of cellular biochemistry.

Recently, Loef *et al.* have shown that potato tuber disks incubated with orotate show an increase in starch biosynthesis (70). Orotate and uridine stimulated an increase in UTP, UDP and UDP-glucose levels. Feeding with orotate led to an increase in glucose-1-phosphate levels by stimulating sucrose degradation two fold. These authors argue that uridine nucleotide levels are specifically stimulating sucrose degradation via sucrose synthase and concomitant production of hexose phosphate via the action of uridinedisphosphoglucose pyrophosphorylase. The net rate of starch biosynthesis increased 30% due to the increase in hexose phosphates which are precursors of starch biosynthesis (70).

6. PYRIMIDINES AS SECONDARY METABOLITES

Plants produce an incredibly rich diversity of biochemical compounds. Of the more than 100,000 secondary metabolites that have been identified by organic chemists, 80% of these are produced uniquely by plants. Most of these compounds serve a defensive function to combat bacteria, fungi, insects, or even other plants. Chief among these secondary metabolites are the terpenes and phenolic compounds; however, secondary metabolites derived from pyrimidines have also been described (see Figure 4).

Plants of the Mimosa family synthesize 5-aminouracil, a unique defensive compound (71). This

compound blocks the mitotic cycle and inhibits incorporation of guanosine into nucleic acids (72). This function makes it uniquely useful as a cell cycle inhibitor (73). This compound is presumably synthesized from uracil by way of isobarbituric acid (74). While this compound is a severe metabolic inhibitor in animal cells, plants can detoxify 5-aminouracil. The detoxification products are also known to accumulate in plant cells, and these detoxification products may also have defensive properties. As shown above, the normal pathway for degradation of uracil is ring cleavage between N3 and C4 by dihydropyrimidinease. The action of this enzyme on 5-aminouracil by ring cleavage results in the production of albizziine, another a non-protein amino acid. In plants that produce these pyrimidine-derived secondary metabolites, the *de novo* pyrimidine biosynthetic pathway is significantly up-regulated. Specifically, the relative activities of CPSase and ATCase were 4 to 6 times more active than non-metabolite producing plants (74). In addition, 2, 3-diaminopropionic acid, a product of 5-aminouracil catabolism also accumulates in these plants (74).

Other uracil-derived secondary metabolites include lathyrine which is produced from 2-amino-4-carboxypyrimidine by addition of an alanine side chain donated by serine (75). Uracil is the source of the 2-amino-4-carboxypyrimidine. Although this compound is known to accumulate in plants (76), the biosynthetic route remains unclear (77). Additional metabolic modifications of lathyrine that produce yielding γ-glutamyl-lathyrine (78) or *cis*-5-hydroxy-L-pipecolic acid (79) are also known. Lathyrine synthase is an unusual biotin-stimulated pyridoxal phosphate containing enzyme that catalyzes the simultaneous decarboxylation of 2-amino-4-carboxypyrimidine and condensation of this product with the alanyl side chain of serine (80). Lathyrine has antimicrobial activity, but surprisingly acts as a cytokinin in some but not all plant cells. Lathyrine stimulated soybean cells to divide and maintain chlorophyll in radish leaf discs (81).

Willardiine and isowillardiine are non-protein amino acids that function in a neuroexcitory pathway. They are amino acids that contain uracil in the side chain. These unusual amino acids appear to be synthesized from O-acetyl serine coupled with either the N1 or N3 of the pyrimidine ring (82). Enzymes capable of synthesizing willardiine and isowillardiine have been isolated from pea etioplasts (83, 84, 85, 86). Separate enzymes are involved in the synthesis of these two compounds.

Vicine and convicine accumulate in the stems and roots of *Lupinus hispanicus* (Spanish Lupins), *Vicia faba* (Faba beans), *V. sativa* (common vetch) and related species during seed and seedling development. In the guts of herbivores, vicine and convicine are hydrolyzed by β-glucanases into divicine and isouramil, respectively (87). These compounds are toxic to avian (88) as well as mammalian herbivores (89). The mechanism of action appears to be the inhibition of glucose-6-phosphate dehydrogenase (90) which leads to reduced growth.

Pseudouracil is another pyrimidine derivative that accumulates in plant tissues (91). Feeding experiments indicate that its synthesis proceeds from orotic acid through uracil and UTP. Pseudouracil is most commonly found in tRNAs, so presumably the accumulation arises as a result of RNA turnover.

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Abbreviations: ATCase–aspartate transcarbamoylase, DHOase–dihydroorotase, DHODH–dihydroorotate dehydrogenase, UMPS–UMP synthase, *car*–carbamoyl phosphate synthase, *pyr2*–aspartate transcarbamoylase, *pyr4*-dihydroorotase, *pyrD*-dihydroorotate dehydrogenase, *pyrE-F*-UMP synthase, *pyr6*-UMP/CMP kinase, *ndp*-nucleotide diphosphokinase, *ctps*-ctp synthase, *rnr*-ribonucleotide reductase, *dut*-dUTPase, *thy*-thymidylate synthase, *tk*-thymidylate kinase, *dck*-dCMP kinase, *uprt*-uracil phosphoribosyltransferase, *urk*-uridine kinase, *5nt*-5' nucleotidase, *cyd*-cytidine deaminase, *cod*-cytosine deaminase, *dpyd*-dihydropyrimidine dehydrogenase, *dpys*-dihydropyrimidinase, *βUP*-β-ureidopropionase, *βas*-β-alanine synthase

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