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5-Fluoroorotic acid-selected cell lines and regulation of UMP synthase
gene expression in tobacco cells

by

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CHAPTER 1. INTRODUCTION

UMP biosynthesis

Nucleotides are indispensable precursors for the synthesis of DNA and RNA. Without DNA biosynthesis, normal cells can not divide. RNA is required for translating the genetic information from DNA into the proteins. It is thus not surprising that inhibitors of nucleotide biosynthesis are very toxic to cells.

UMP de novo biosynthesis

Like purine nucleotides, pyrimidine nucleotides can be synthesized either by de novo from various metabolic intermediates or by salvage pathways from free nucleobases or nucleotides. A key intermediate for these synthesis of pyrimidines is uridine-5'-monophosphate (UMP) at which both the de novo pathway and the salvage pathway merge, and from which all pyrimidine nucleotides can be synthesized. The pathways of UMP biosynthesis are presented on Figure 1.1.

In contrast to the synthesis of inosine-5'-monophosphate (IMP) which is the precursor for all purine nucleotides, in the de novo biosynthesis of UMP the pyrimidine ring is constructed before incorporation of ribose-5'-phosphate. The ring formation is initiated by the synthesis of carbamoylphosphate from bicarbonate ion, glutamine and ATP. This reaction is catalyzed by carbamoylphosphate synthase (CPSase). This enzyme utilizes bicarbonate but is

\[^{1}\text{Abbreviations are listed in appendix A}\]
Figure 1.1. The pathway of UMP synthase biosynthesis. The *de novo* pathway initiates with $\text{H}_2\text{CO}_3$, glutamine and ATP. This is catalyzed by carbamoyl phosphate synthase (CPSase), aspartate carbamoyl transferase (ATCase), dihydroorotase (DHOase), dihydroorotate dehydrogenase (DHO DH), orotate phosphoribosyl transferase (OPRTase), and OMP decarboxylase (ODCase). In higher eukaryotes, these two enzymes are fused together into single polypeptide UMP synthase. The salvage pathway from uracil is catalyzed directly by uracil phosphoribosyl transferase (UPRTase). UMP synthase can also use 5-fluoroorotic acid (FOA) as substrate.
somewhat unusual in that it does not require the cofactor biotin which is usually required for enzymes that utilize CO₂. The carbamoylphosphate synthase was reported to be activated by phosphoribosyl pyrophosphate (PRPP) and inhibited by UDP, dUDP, UDP-glucose, CTP or dUTP (Mori and Tatibana, 1978). The carbamoyl group of carbamoylphosphate is then transferred to the amino group of aspartate to form carbamoylaspartate. This step was identified as the first committed step in UMP synthesis (Jones, 1980). By the action of dihydroorotase (DHOase), the pyrimidine ring is closed to form dihydroorotate.

Before reacting with PRPP, dihydroorotate is reduced to orotate by dihydroorotate dehydrogenase (DHO DH) in the presence of NAD⁺. The addition of a phosphoribosyl group from PRPP, converts orotate into orotidine-5'-monophosphate (OMP) by the catalysis of orotate phosphoribosyl transferase (OPRTase). Finally, CO₂ is released from OMP to give UMP by catalysis of OMP decarboxylase (ODCase). In higher eukaryotes, the last two steps of UMP de novo biosynthesis are catalyzed by a single polypeptide termed UMP Synthase (Jones, 1980; Walther et al., 1984).

In most prokaryotes, each of the six steps is catalyzed by separate enzymes. All of these are soluble except DHO DH which is reported to be membrane-bound (O'Donovan and Neuhard, 1970). In simple eukaryotes such as Neurospora and yeast, CPSase activity resides on the same soluble polypeptide as aspartate transcarbamoylase (ATCase), while the remaining enzymes are separate and soluble except DHO DH which is bound to the mitochondrial membrane (Miller, 1975). In animal cells, de novo biosynthesis of pyrimidine nucleotide is carried out by a multifunctional protein containing the first three enzyme activities (CPSase, ATCase and DHOase), DHO DH, and
UMP synthase having the last two steps activities (OPRTase and ODCase) (Jones, 1980). These multienzymic proteins were thought to be the products of a series of ancestral gene fusion events and both are cytosolic, whereas DHO DH is a mitochondrial, electron transport chain-linked enzyme.

The data available so far have indicated that the organization of the enzymes required for the de novo biosynthesis of UMP in plants may be different from those in other types of organisms. In plants, the first three enzymes (CPSase, ATCase and DHOase) are organized as in prokaryotes whereas the last two enzymes (OPRTase and ODCase) reside in a single polypeptide as in animals. This conclusion is based on several independent studies. According to O'Neal and Naylor (1976), there is only one CPSase in pea. Yet carbamoyl phosphate produced by catalysis of CPSase is required for both arginine and pyrimidine biosynthesis. Therefore it is inferred from these studies that CPSase and ATCase may not reside on the same polypeptide. In Chlorella grown in a synchronous culture, the ATCase and DHO DH activities did not always change in tandem, Dunn et al. (1977) suggested that these enzymatic activities are on separate polypeptides. The last two steps of the pathway were shown to be catalyzed by a single multienzymic polypeptide containing OPRTase and ODCase activities (Wather et al., 1984). The existence of this type of organization in plants has been confirmed by a report on Pisum sativum L. (Doremus, 1986). Recently, cDNA clones of UMP synthase were isolated from Arabidopsis thaliana (Minet et al., 1992) and N. tabacum (Maier et al., in press).
Salvage pathway of UMP biosynthesis

In addition to the de novo biosynthetic pathway, plants also have the ability to utilize preformed uracil in the synthesis of pyrimidine nucleotides. There are two different ways to convert uracil into UMP. One is direct conversion by uracil phosphoribosyl transferase (UPRTase) and the other requires two steps. First, ribose can be added to form uridine by the action of uridine phosphorylase and can subsequently be converted into UMP by uridine kinase. In mammalian cells, the substrate specificity of uridine phosphorylase is rather wide. In addition to uracil and uridine, uridine phosphorylase can also accept deoxyuridine as well as thymine as a substrate (Backley, 1989).

In Dictyostelium discoideum, the salvage pathway of UMP biosynthesis from uracil was thought to be less efficient than the de novo pathway (Kalpaxis et al., 1991). This was indicated by the fact that the growth of UMP synthase (umps) mutants of D. discoideum in the presence of sufficient uracil was lower than the wild type. In general, UMP biosynthesis from orotate is considerably more efficient than from uracil (Beckwith et al., 1962; Heidelberger, 1982).

UMP synthase

In higher eukaryotes, the last two steps of de novo UMP biosynthesis are catalyzed by a single multi-functional enzyme, UMP synthase. This enzyme has both OPRTase and ODCase activities.

The kinetic data of the UMP synthase in mammalian cells show that the orotate phosphoribosyl transferase activity of the enzyme determines the rate of overall enzymatic catalysis. This is indicated by the high $K_m$ and low $V_{max}$ of the OPRTase. The $K_m$ values of the OPRTase for orotate and PRPP are respectively 7 and 50 fold higher than that of the ODCase. The relative velocity of the
decarboxylase is about two fold higher than that of the transferase (Jones et al., 1978; Santoso and Thornburg, 1992), and the stoichiometric production of OMP can be detected only if the decarboxylation is completely inhibited (Traut and Jones, 1977). Indeed both in vitro and in vivo assays for the reaction demonstrated that OMP concentration is very low, 0.05 μM (Traut and Jones, 1977; Hitching, 1974). The lower than expected level of OMP in the medium implies that a channelling effect may be occurring (Traut and Jones, 1977). Thus, even though ODCase can utilize exogenously supplied OMP, the OMP produced by the OPRTase site is not readily released into the medium but is rather thought to be transferred to the ODCase site of the enzyme through a groove or channel without dissociation of the enzyme substrate complex.

Less is known about UMP biosynthesis in higher plants. Walther et al. (1984) isolated and purified OPRTase and ODCase from suspension cultures of tomato cells. Using a four-step purification, they purified the enzymes 1000 fold. The analysis of the purified enzymes by gel electrophoresis coupled with assays for enzymatic activity indicate that these two activities were not separable during purification. Therefore, they concluded that OPRTase and ODCase activities also reside on a single polypeptide, termed UMP synthase. The presence of this multifunctional enzyme was also identified in Euglena gracilis (Walther et al., 1981) and Phaseolus mungo (Ashihara, 1978). In addition, Walther et al. (1984) also demonstrated that the native form of the UMP synthase exists as a dimer with molecular weight about 100,000 kDa.

In pea (Pisium sativum) leaves, UMP synthase was found to be strictly confined in the plastid/chloroplast (Doremus and Jargendorf, 1985; Doremus, 1986). However, in cultured Vinca rosea cells and P. mungo seedlings the bulk
of OPRTase and ODCase are located in the cytosol (Kanamori et al., 1980; Ashihara, 1978). Biosynthetic studies performed with Cucurbita pepo callus and Catharanthus roseus suspension culture indicated that the expression of UMP synthase gene may be regulated during cell growth (Lin and Lovah, 1986; Sasamoto and Ashihara, 1988). This gene is expressed at high level when the cells are actively dividing.

Since the organization of orotate phosphoribosyl transferase and OMP decarboxylase in higher plants is similar to that in mammalian cells, it is possible that the catalytic mechanism of UMP synthase in the both plants and animals are similar as well. Therefore a channelling process of OMP produced by OPRTase to ODCase may also occur in higher plants.

**Mutagenesis and selection**

Tissue culture techniques have been utilized to produce several different mutants in plants (Maliga et al., 1982; Blonstein and King, 1985; Negrutiu et al., 1984), including a wide variety of drug-resistant, stress tolerant and auxotrophic mutants. Some of these mutants are presented in Appendix B. Several factors determining the production of mutants have been identified. They can be classified into three groups; plant species, culture system and methods of mutagenesis.

**Plant species**

There are many species of plants from which mutants have been isolated. For some purposes, a model species having specific characters is required. The desirable properties of the species usually are: fast growth in culture, maintained plant regeneration ability, ease of initiation or
establishment of the culture, low chromosome number, availability of haploid plants and existence of a genetic map (Maliga et al., 1982). The first three of these are the minimum requirements for utilizing tissue culture technique for mutant production. Whereas the remaining characters might provide some benefit in the application of the mutant for further study, such as for elucidation of biochemical processes or mutant gene isolation.

Plants of the genus *Nicotiana* have these advantageous criteria. A member of this genus which is widely used in tissue culture for many purposes, is *Nicotiana tabacum*. Tissue culture and regeneration of *N. tabacum* are considered as routine procedures. Many mutant lines of *N. tabacum* have been developed. Nitrate reductase-deficient lines were isolated from suspension cultures (Muller and Grafe, 1978). Mutants resistant to amino acids analogs (Negrutiu at al., 1984), temperature sensitive (Malmberg, 1979), and tolerant to high level of salt (Nabors et al., 1980) have also been reported. *N. tabacum* does have one drawback as a model plant system, its ploidy. It is now generally agreed that *N. tabacum* is a natural hybrid of *N. sylvertris* X *N. tomentosiformis*. Both *N. sylvertris* and *N. tomentosiformis* have chromosome number (2n) 24 (Akehurst, 1968; Goodspeed, 1954). *N. tabacum* has one complement from each of these species and is thus amphidiploid (2x = 4n = 48). Therefore for most loci, *N. tabacum* has two alleles, one from *N. sylvertris* and the other from *N. tomentosiformis*. This can be a problem in the selection of mutants, however the other characteristics of *N. tabacum* offset this problem and make *N. tabacum* a very useful plant species for diverse biological, biochemical and molecular genetics studies.
Another member of *Nicotiana* genus which is commonly employed for this purpose is *N. plumbaginifolia*. The advantages of *N. plumbaginifolia* over *N. tabacum* are its smaller chromosome number (2n = 20) and it is a real diploid plant. There have been some reports describing wide variety of biochemical mutants isolated from *N. plumbaginifolia* (Shabtai et al., 1992; Maliga, 1984).

**Culture systems**

The culture systems which are usually used for isolation of mutants are callus culture, cell suspension culture and protoplast culture. The rationale for choosing a system depends on the desired phenotype of mutant, malleability of the plant species and the simplicity of the tissue culture system.

*Callus culture.* For the selection of an inhibitor-resistant line, callus culture may be the simplest one. Callus is essentially cells growing together to form a mass. One small callus may consist of hundreds to thousands of cells. This system can be easily applied for either one-step or multistep selections (Gonzales and Widholm, 1985). Streptomycin-resistant lines of tobacco (Maliga et al., 1973) and maize resistant to the pathotoxin of *Helminthosporium maydis* have been isolated through callus culture (Maliga et al., 1973; Gengenbach and Green, 1975). Due to some problems, the application of callus culture to isolate mutants is limited only for practical purposes. This culture system is utilized primarily when cell suspension culture methods are not available for the plant species of interest. Mutants isolated from callus culture are less stable than those isolated from cell culture. The instability of the mutants occurs because the contact with inhibitor during selection is unequal among the cells in the
callus. In addition, the use of callus cultures compounds the difficulty when mutations need to be induced by mutagenesis.

**Cell suspension culture.** In contrast to the growth of calli on plates, plant cells can also be grown as a single cells or as very small clumps of cells in a fine suspension culture. The advantage of using cell suspension cultures is that the cells can be manipulated like microbes. The mutagenesis of cells in a suspension culture and selection for mutants can be performed more effectively because of the increased contact between cells and mutagen or inhibitor. Another advantage is that cells in suspension cultures are available in large amounts. This permits quantitative measurement of parameters involved in production of mutants from the suspension culture. Because plant cells are totipotent, they can regenerate whole new plants from single cells. Therefore selections performed on single cell can often give rise to complete mutant plants.

**Protoplast culture.** Protoplasts are cells which lack cell walls. They can be prepared by incubating growing tissues or cell suspension in a solution of wall-degrading enzymes. Because of their wall-less nature, protoplasts in a culture are more sensitive to their environment than either suspension cultures or callus cultures. In order to keep protoplasts viable and growing they therefore need to be handled very carefully. A culture medium with isotonic osmotic pressure is required for the first two weeks of culture to prevent autolysis of the protoplasts. In such medium however protolasts grow very slowly. These problems are a major concern in protoplast culture. On the other hand, cells with rigid cell wall in suspension culture are more resistant to environmental
changes. There is no need to worry about their viability or growth as long as standard culture procedures are followed. Therefore to isolate mutants responding to stressed conditions such as wounding or high salt, cell suspension culture systems are often preferred over protoplast culture or callus culture if the culture system has been established for the plant species of interest.

**Mutagenesis**

Plants have natural mechanisms to generate their own genetic variability. In cell cultures this variation is known as somaclonal variation in which novel genetic phenotypes frequently arise. Somaclonal variation has been useful for plant improvement (Scowcraft and Larkin, 1982). Plant cells can act as intact organisms and can regenerate into whole plants. Therefore any stable genetic alteration in those cells is likely to be phenotypically expressed in the regenerated plants. Shepard (1980) reported that new variants of potato were isolated from cell culture of the Russet Burbank variety. These new varieties had improved characteristics such as greater tuber uniformity and earlier onset of tuberization. Krisnamurthi (1974) reported that sugar cane varieties resistant to Fiji disease and Downy Mildew (*Sclerospora sacchari*) had been isolated from the parental lines sensitive to these diseases. Many factors are known to be involved in creating somaclonal variants (Larkin and Scowcroft, 1981).

Chromosomal rearrangements are considered to be a major mechanism that generates such somaclonal variation. Somatic crossing over, transposable elements, gene amplification and diminution are other factors which could participate in somaclonal variation.
For the production of defined biochemical mutants, the occurrence and intensity of the somaclonal variation occurs at too low a frequency to be useful. Therefore it is necessary to increase the frequency of mutation to produce these defined mutations. To increase the frequency of mutation, cells can be exposed to mutagens. The amount of mutagen and duration of exposure are dependent on the species of plant, the culture system and the nature of mutagen. Maliga et al. (1982) reported that mutagenesis of *N. plumbaginifolia* protoplasts with 0.1 mM N-ethyl-N-nitrosourea increased the frequency of streptomycin-resistant cells by 32-fold. Mutagenesis of protoplasts with UV irradiation also increase the frequency of nitrate reductase-deficient mutants by 20-fold (Negrutiu, 1983). In cell suspension cultures of *Petunia hybrida*, 5 mg/L N-methyl-N′-nitro-N-nitrosoguanidine increased the frequencies of 6-fluorotryptophan-resistant mutant and HgCl₂-resistant mutant by 15- and 68-fold respectively (Colijn, 1979). Therefore mutagens can significantly increase the rate of occurrence of defined biochemical mutations.

Many mutagens function by alkylating DNA at specific nucleotides. Nucleotides contain many functional groups that can react with alkylating agents. Some nucleotide functional groups are preferentially sensitive toward alkylation. These include: N-7 of guanine, O-6 of guanine, O-4 of thymine and N-7 of adenine. The mutagenic process occurs after alkylation, when DNA is replicated. Alkylation of nucleotides affects base-pairing, resulting in mismatched base pairs (Lewis, 1978). For example, instead of pairing with cytosine an alkylated guanine on DNA will pair with thymine. Therefore during replication, the mismatched base pairing generates base pair substitutions which result in mutations.
N-Nitroso-N-methylurea (NMU) is an alkylating agent whose activity is similar to that of other N-nitroso compounds. The mutagenic activity of NMU is initiated by its spontaneous decomposition to form a methyl-carbonium cation (Lawley, 1984). This ion then attacks nucleophilic sites on the nucleotides of DNA to produce alkylated nucleotides which result in mutations as described above. Generally, the mutagenic activity of N-nitroso compounds such as NMU are more potent than many other classes of alkylating agents such as alkylsulfonate, dialkylsulfonate, or alkylhalide (Lawley, 1976). The major difference of their mutagenic potencies is due to the difference of alkylating pattern (Guttenplan, 1984). The alkylation of DNA by the N-nitroso compounds might produce a high degree of alkylated oxygen. Since the oxygen sites of nucleotides are less nucleophilic than most the nucleotide nitrogens, their alkylation would be expected to proceed mainly through highly reactive electrophilic such as alkyldiazonium ion (Lawley, 1976).

In plants the application of NMU to induce mutation was shown quite effective. Compared to either ethyl-methylsulfonate (EMS) or gamma-rays from $^{60}$Co, NMU is the most effective (Chaturvedi and Singh, 1981). On dry seeds of mung bean, mutagenic activity of 0.03% NMU is equivalent to that of 0.3% EMS.

5-Fluoroorotic acid

The antimetabolite 5-fluoroorotic acid can enter the pathway of de novo UMP biosynthesis just as orotic acid does. It can thus act as the receptor for the phosphoribosyl group transferred from PRPP to form 5-fluoro UMP (FUMP). From FUMP, a variety of toxic pyrimidines fluorinated at the 5-position can be produced by various pathways.
The mechanism of toxicity of these compounds, has not yet been fully elucidated. These 5-fluoropyrimidine nucleotides are likely to be incorporated to nucleic acids. However it is not entirely clear whether the toxicity of these compounds is due to incorporation into DNA or into RNA. Another possible mechanism of toxicity is through conversion to 5-fluorodeoxy UMP (FdUMP). The FdUMP binds covalently to thymidilate synthase rendering this enzyme incapable of further catalysis, thereby leading to cell death (Zubay, 1988; Kalpaxis et al., 1991). In spite of the lack of information on the mechanism of toxicity, the net result is that cells which express an active UMP synthase will die in media containing a 5-fluoroorotic acid. The use of 5-fluoroorotic acid to kill cells therefore provides a very strong selection for mutant cells lacking UMP synthase. Because of the salvage pathways, mutants which do not express an active UMP synthase can be rescued by providing uracil in the selection media. This technique has been used to isolate UMP synthase mutants from yeast (Boeke et al., 1984), *D. discoideum* (Kalpaxis et al., 1991) and tobacco (Santoso and Thornburg, 1992). Uracil auxotrophic mutants have also been isolated in plants but by different selection scheme (Sindorov and Maliga, 1982). These mutants however, were not completely characterized. So, whether these were mutant in UMP synthase or other enzymes of UMP biosynthesis pathway is not known.

**Protein purification**

Enzymes are necessary for cells to perform various metabolic processes. Enzymes are extremely efficient catalysts with high substrate specificity. Under optimum conditions, for example, catalase can dismutate 40 million molecules of hydrogen peroxide per second. Carbonic anhydrase converts 1 million
molecules of CO$_2$ to HCO$_3^-$ per second (Fersht, 1985). Enzymes are highly specific both in binding substrates and in catalyzing their reactions. Trypsin, for instance, readily hydrolyzes polypeptides composing L-amino acids but not those with D-amino acids. (Voet and Voet, 1990).

Contributors to the catalytic action of an enzyme are mainly its structure and functional groups especially those present on the binding site and fit to its substrates. The stage of these two factors is effected by surrounding environment such as pH, ionic strength, temperature, and affecting molecules. Changes of cellular condition affecting an enzyme might occur in response to environmental changes. To study an enzyme and all the affecting factors requires a purified enzyme.

Considerations in purification of proteins

Purification of proteins is often a formidable task because a typical cell or tissue contains thousands of different substances many of which closely resemble each other in their properties. Furthermore, the proteins of interest may be unstable and present in a small amount. Therefore searching tissue sources that contain significant level of the protein of interest is reasonably important. Several different available tissue sources may be tested for the starting material.

Handling of the proteins is the next consideration. The stability of proteins is dependent on the environment. During the course of purification, proteins should be handled carefully. Cell-free enzymes usually retain their catalytic activities at low temperature, 0 to 4°C and pH ranging 6 to 8. Specific tissue sources often contain some proteases that can digest the proteins of
interest. To block the activity of these proteases, protease inhibitors and chelating agents are included in the extraction buffer. Finding a reliable assay that quantitatively can monitor the presence of the protein of interest in each step of purification is also required. Several approaches can be applied including, the use of a reaction that produces spectrophotometrically detectable products, radioactively-labeled substrates, and immunological methods using antibody specific for the protein of interest.

Purification of a protein from the other similar substances in cellular extract usually consists of a series of several different separation procedures. Selection of these procedures is based primarily on the physical properties of the proteins.

Precipitation

Solubility of a protein in a solution is largely dependent on the ionic compatibility of the solvent-protein interaction. Perturbation of this interaction may decrease the solubility and cause precipitation of the protein. Concentration of salt in the solution, polarity of the solvent, the pH, and the temperature are the factors affecting the solubility. In a given set of condition, a protein has its own specific solubility that may differ from the others. This characteristic is used as basis of the protein precipitation.

In general, the solubility of protein in aqueous solution decreases with increasing amounts of salt in the solution. This is because there is a competition between the salt and the proteins to interact with water molecules. This competition decreases the effective amount of water dissolving the proteins, resulting in precipitation or salting out of the protein. The salt that is most
frequently used to precipitate or fractionate proteins is (NH$_4$)$_2$SO$_4$. The advantages of using this precipitant are (1) it has high saturation molarity, 4.04 M; (2) It does not have a large heat of solution; (3) its saturated solution has relatively low density, 1.235 gram cm$^{-1}$; the concentrated solution limits or prevents most bacterial growth; and (5) in solution it protects most protein from denaturation (Englard and Seifter, 1990).

Similar effects also occur when water-miscible organic solvents such as methanol, ethanol, butanol, or acetone is added to a aqueous solution of proteins or other macromolecules. Polar functional groups of those solvents interact with water molecules thereby limiting the availability of water molecules to dissolve the proteins. The drawback of these organic solvent are they produce a significant heat of solution and have a tendency to denature the proteins (Englard and Seifter, 1990).

**Chromatography**

Chromatography is a technique of separation of components in a mixture solution based on their solubility in the moving solvent (mobile phase) and adsorption on the stationary phase. Different substances have varying degree of solubility on these two phases.

In size exclusion chromatography, gel filtration, the mixture is passed through a solid support with a range size of pores then eluted with a buffered-solvent. Molecules smaller than the pore will be retained longer on the solid support than those larger. Thus, gel filtration separating substances based on their molecular sizes where the large components will be eluted before the small components.
Ion-exchange chromatography is a type of adsorption chromatography in which the stationary phase has an electrochemical charge linked to its solid support, the ion exchanger. Different components bind to this exchanger with different degrees of tightness which are dependent upon their ionic properties. Elution can be performed by increasing the ionic strength or changing the pH of the eluent, thereby weaken the exchanger-components binding and displacing exchanger-bound components with an equivalent ion of the eluent.

In affinity chromatography, a specific molecule termed the ligand is covalently attached to the inert solid support. Affinity chromatography has been very useful to purify biological molecules such as proteins because the attached ligand has high and specific affinity to the molecule to be purified. The ligand could be competitive inhibitors, substrate analogs, product analogs, coenzymes, etc. After a mixture containing protein of interest is loaded into the column, the column is extensively washed to remove contaminant components. The protein to be purified will bind to the ligand and can be eluted by changing the ionic strength of the elution buffer or adding a competing molecule in the elution buffer.

Dyes, ligands having a rather wide specificity to proteins have been commonly used to purify several classes of proteins. Some of these have high affinity to proteins with nucleotide folds (Fultons, 1980). Blue A-agarose has been reported to purify orotate phosphoribosyl transferase and orotidylate decarboxylase from Baker's yeast with purification factor 202x and 5670x respectively (Reyes and Sandquist, 1978). Cibacron Blue 3GA was able to purify UMP synthase from cell suspension culture of tomato with purification factor 251x (Walther et al., 1984).
**Electrophoresis**

Electrophoresis is a technique of separation of macromolecules based on the movement of charged molecules in an electric field. Media through which the charged molecules separated are porous media such as gel (agar or polyacrylamide) or paper. The velocity, $v$ at which the molecules migrate in the electrical field is a function of the field strength, $E$ and expressed in the following equation

$$v = \frac{E \cdot q}{f}$$

where $q$ is charge and $f$ friction coefficient. In a given medium, the friction coefficient is proportional to the size of molecules being separated. The bigger the size of molecule is the larger its friction coefficient and in the gel it migrates slower.

To purify protein by electrophoresis techniques, polyacrylamide gels are commonly used. The gel is polymer of acrylamide and bisacrylamide. The pore size of the gel is controlled by total concentration of acrylamide. A 2.5% gel sieves molecules of $10^5$ to $10^6$ kDa molecular weight, 7% gel sieves molecules of $10^4$ to $10^5$ kDa, and 30% gel sieves molecules of $2 \times 10^2$ to $2 \times 10^3$ kDa (Robyt and White, 1990). For the polyacrylamide gel protein samples are denatured and uniformly charged by adding anionic detergent, SDS. Therefore in a certain field strength, the migration of proteins in the gel is dependent primarily upon the friction between gel and the proteins. Molecules smaller than then pore size of the gel migrate freely and faster on the gel than those bigger than the pore size.

**Gene expression in eukaryotes**

Of the thousands of genes in eukaryotic cells only some serve as house-keeping genes. These genes are constitutively expressed. Their protein products
are required most of the time in metabolic activities. Therefore these proteins are always present at relatively constant levels at any given time. Genes encoding enzymes that catalyze steps in central metabolic pathways such as the TCA cycle, are usually included in this category. Most of the cellular genes however are regulated; the level of their products is subject to induction or repression. The rate of the expression of inducible genes is usually responsive to environmental changes such as availability of nutrients, exposure to light, stresses, or metabolic requirement (Mason et al., 1992; Johnston, 1987; Ilag et. al, 1994; Berkel et al., 1994).

Regulation of expression of genes to synthesize their respective proteins involves a delicate balance of many processes. There are at least 4 potential points at which the synthesis of protein is regulated. These are regulations at the level of transcription, post-transcription, translation and post-translation (Johnston, 1987, Kimata and Theil, 1994; Reinbothe et al., 1993).

Transcription in eukaryotes

RNA polymerase is a enzyme complex playing key role in DNA-directed RNA synthesis. The binding of this enzyme to a specific site of DNA sequence to be expressed initiates the transcription. Besides, RNA polymerase also catalyzes the elongation process of RNA synthesis. To elongate the RNA, this enzyme moves along the DNA template to the 5'-end direction of the DNA and adds new ribonucleotides to the growing oligoribonucleotides at the 3'-end. When the RNA polymerase reaches the site of the DNA signaling termination of RNA synthesis, the newly synthesized RNA released from the enzyme complex and transcription is stopped.
In prokaryotes, there is only one RNA polymerase. In eukaryotes however, there are three different RNA polymerases which catalyze the synthesis of the three different subsets of RNA. RNA polymerase I catalyzes synthesis of ribosomal-RNAs, RNA polymerase II is responsible for the transcription of all protein-encoding messenger-RNAs, and RNA polymerase III involves in the synthesis of transfer-RNAs and 5S ribosomal-RNA. Moreover, prokaryotic RNA polymerase can bind directly to the promoter region of the DNA to initiate transcription. Whereas in eukaryotes, RNA polymerases have low binding affinity to DNA, They are not able to bind to the DNA without assisting factors (Manley et al., 1980; Weil et al., 1979). These factors are known as a complex of proteins termed general transcription factors. The binding of these factors to the promoter region directs the binding of the RNA polymerase to the DNA to be transcribed (Roberts and Green, 1993). Therefore the presence of general transcription factors is required for basal transcription by RNA polymerase. When RNA polymerase is bound to the promoter region, a preinitiation complex is formed for transcription. The formation of this preinitiation complex in the promoter region is an energy-requiring process (Bunick et al., 1982; Lin and Green, 1991). When preinitiation complex is formed, transcription proceeds simply by incorporating ribonucleotides.

Many reports indicate that formation of the preinitiation complex is one of the rate-limiting step in the transcription process. Therefore this step is the point of regulation in transcriptionally-regulated gene expression of eukaryotes.
Transcriptional activator proteins

Modification of preinitiation complex formation results in changes of transcriptionally regulated gene expression. These modifications in complex formation are regulated by transcriptional activator proteins. These proteins bind to the promoter and influence complex formation.

Most enzyme-encoding genes in eukaryotes possess sequences other than TATA sequence in their upstream region that are usually called upstream activation sequences (UAS). The TATA sequence is required for accurate initiation of transcription whereas the UAS regulates the rate at which a gene is transcribed. Deletion of UAS, normally eliminates responsiveness of a gene to transcription-activating signals.

The UAS is the binding site for transcriptional activator proteins which are capable of altering the rate of transcription of eukaryotic genes. The activator proteins carry two functions which are for DNA-binding and activation. These two functions could be carried by a single polypeptide that has two functional domains or two separate polypeptides that have ability to interact each other. In a single polypeptide activator, the DNA-binding domain of the activator protein interacts with the UAS of an inducible eukaryotic gene. This interaction is specified by structural motif of the DNA-binding domain and the sequence of the UAS. There are several well-studied motifs of the DNA-binding domain of activator proteins, zinc finger motif, helix turn helix, helix loop helix and basic-leucine zipper (Struhl, 1989; Murre et al., 1989).

When the activator proteins bind to the UAS, within proximity of the promoter region, the activation domain interacts with the general transcription factors and brings them to the site where the preinitiation complex is
assembled. This kind of interaction facilitates the formation of the preinitiation complex. Because formation of the preinitiation complex is the rate-limiting step in the transcription process, interaction of the activation domain with the general transcription factors overcomes the rate-limiting step and increases the overall rate of transcription. Unlike the DNA-binding domain, the activation domain generally has a less well defined structural motif. Nevertheless, some reports indicated that the amino acid composition of the activation domain characterizes the interaction with GTFs. Based on the amino acid composition, the activation domains of transcriptional activator protein are classified as acidic, glutamine-rich, proline-rich, and serine/threonine rich (Roberts and Green, 1993).

The activation domain positively affects the formation of preinitiation complex. Elimination or blocking of the interaction between this domain with GTFs will consequently decreases the rate of transcription. This effect was reported from the studies on regulation of well-studied GAL genes. Lin and Green (1991) reported that when the assembly of preinitiation complex is carried out without acidic activator of GAL4-AH, the assembly was inhibited at the TFIIB (a component of the general transcription factors) binding stage. This indicates that the acidic transcriptional activator protein may enhance the transcription by recruiting TFIIB to the promoter. The metabolite regulation of GAL4-GAL80 system of yeast also demonstrates that blocking the activation domain will inhibit the transcription. In this case when galactose is absent, the GAL80 protein binds to the activation domain of the GAL4 protein therefore preventing the GAL4 activation domain to interact with general transcription factors of the complex which in turn limits the synthesis of GAL RNAs. In the
presence of galactose, this inducer binds to the GAL80 protein, releasing GAL4 from the repressor GAL80 and enabling GAL4 activation domain to interact with general transcription factors to form preinitiation complex thereby transcription is activated (Johnston, 1987).
CHAPTER 2. MATERIALS AND METHODS

Materials

Haploid plants of *Nicotiana plumbaginifolia* which are in sterile culture were kindly provided by Dr. Laszlo Martón, Department of Biology, University of South Carolina. These plants were tested to be sensitive to the infection by tobacco mosaic virus. The fruits of summer squash (*Cucurbita pepo*) were obtained from a local market.

Plasmid pRT327 that contains the 1.2-kb clone of UMP synthase cDNA fragment isolated from *Nicotiana tabacum* (Maier et al., in press) has been used routinely as the template to prepare either [32P]-labeled DNA or RNA probes. The pRT327 plasmid was constructed by inserting the cDNA fragment of UMP synthase into EcoR I site of an expression vector pBluescript II SK+.

A polyclonal antibody was raised against UMP synthase in a white female rabbit of New Zealand, against UMP synthase protein isolated from squash fruit. This antiserum was previously described (Santoso and Thornburg, submitted).

PRPP, orotic acid, 5-fluoroorotic acid, uracil, NMU, nucleotide metabolites, dihydrofolate reductase inhibitors, Cibacron Blue 3GA and Sephadex DEAE A25 were bought from Sigma Chemical Co., St. Louis, MO. Sephadex G-150 (particle size 40-120 μm) was from Pharmacia, Piscataway, NJ. Media for plant tissue culture and plant hormones were purchased either from Sigma Chem. Co. or GIBCO Laboratories, Grand Island, NY. Cell-wall digestion enzymes, Cellulase of *Trichoderma reesei* was from Sigma Chem. Co. and Pectolyase Y-23 was from Seishin Pharmaceutical Co., Tokyo. Restriction enzymes were from Promega, Madison, WI. RNA transcription Kits were from Stratagene, LaJolla,
CA. Carmine was purchased from Fluka Chemical Corp., Ronkonkoma, NY. Radiochemicals, carboxyl-$[^{14}\text{C}]-\text{orotic acid with specific activity of 48.5 mCi/mmol}, 6-[^{14}\text{C}]-\text{orotic acid (51.3 mCi/mmol)}, [^{125}\text{I}]-r\text{ProteinA (9.0 mCi/mg)}, \alpha-[^{32}\text{P}]-\text{UTP (800 Ci/mmol)} and \alpha-[^{32}\text{P}]-\text{dCTP (3000 Ci/mmol)} were purchased from New England Nuclear, Boston, MA. Other materials were of the highest purity available obtained either locally or from Fisher Chemical Co., Pittsburgh, PA.

Methods

Cell and protoplast cultures

As described for \textit{Nicotiana tabacum} (Santoso and Thornburg, 1992), cell suspension culture of \textit{Nicotiana plumbaginifolia} was initiated by shaking a fast-growing and friable callus of the tobacco in a liquid MS media. The calli were induced from small slices of the plant tissues, stems and leaves, on the solid MS media supplementing with 2 mg/L \(	ext{\alpha-NAA or IAA, 0.2 mg/L BAP and 3\% sucrose (Maliga, 1984). Friable calli were usually developed after 3 to 4 subcultures with 3-weeks period of time for each subculture. The composition of liquid media for cell suspension cultures were similar to that for callus cultures except without agar. The suspension cultures were incubated in 16 h day and 8 h night period at 26^\circ\text{C} with constant shaking at 120 rpm. The suspension culture was subcultured every 2 to 3 weeks with 2 volumes of fresh media.

Protoplasts were isolated from leaves or calli with the protocols slightly modified from Dixon (1985) for unpeelable leave tissues. About 2 gram of tissue was incubated in 15 mL solution of digestion enzyme contained 0.4\% Cellulase, 0.1\% Pectolyase, 0.55 M Mannitol, 0.06\% MS salt and 6 mM MES. The pH of the solution was adjusted to 5.6. The digestions were performed at 25^\circ\text{C} with slow
shaking for about 4 hours. After digestion, the protoplast suspensions were filtered through 80-μm nylon mesh then spun at 200 X g for 3 minutes. The pellet was gently resuspended in 6 ml of 0.6 M sucrose solution and spun at 100 X g for 10 minutes. Protoplasts floating on the surface of the sucrose solution were transferred using Pasteur pipet into a fresh tube, washed with 10 mL of liquid culture medium and cultured at a density of about 1 X 10⁴/mL in the dark at 26°C. After 3 days in the culture, the cells were exposed to NMU to induce mutation. At that time, the cell walls are usually regenerated but the cell division has not started yet (Nagata and Takebe, 1971).

Selection for mutants

Cell suspension or protoplast cultures from the haploid plants were employed as the starting material for mutant selection. The methods for induction and selection of mutants were previously described (Santoso and Thornburg, 1992). Cell suspensions were incubated in the liquid media containing 1 mM NMU. After washing with the liquid media to remove NMU, the cells were resuspended with the media at density of about 10 times higher than the regular cell cultures. The concentrated cell suspension was then plated on the selection media, aspirated for about 30 minutes to remove excess liquid media. The FOA-selected calli were maintained in the MS solid culture media containing 50 mg/L uracil, 2 mg/L α-NAA, 0.2 mg/L BAP, 250 mg/L casein hydrolysate and 20 mL/L coconut water, with or without FOA. They were incubated at 26°C with a 16-hour day and 8-hour dark period. Subculture was done every 3 to 5 weeks.
Growth of calli

The growth of calli was determined with the method as described in Santoso and Thornburg (1992). Five to nine (approximately 0.3 gram) calli were initially inoculated on a 9 cm diameter petri dish containing 40 mL solid media.

Enzymatic assays

ATCase activity was determined spectrophotometrically by converting the cabamylaspartate into a conjugated compound as described in Gerhart and Pardee (1962). To increase the sensitivity of this assay, 2 μM orotate and 0.1 μM aza-UMP were added to the reaction mixture.

DHOase and DHO DH activities were also assayed with spectrophotometric techniques according to Beckwith et al. (1962) and Caroline (1969) respectively. To increase the sensitivity of both enzymatic assays, 2 μM orotate and 0.1 μM aza-UMP were added to the DHOase reaction mixture and 0.1 μM aza-UMP was added to the DHO DH reaction mixture.

UMP synthase was determined by two different techniques, CO₂ release assay and TLC assay. In the CO₂ release assay, [¹⁴C]-labeled orotic acid at the carboxyl group was used as primary substrate. The reaction was stopped after 5 to 10 minutes to insure that the initial velocity was being measured. The details of this protocol were described previously (Walther et al., 1984; Santoso and Thornburg, 1992).

In the TLC assay, the reaction condition was similar to that of CO₂ release assay except that a smaller volume of the reaction mixture (100 μL) containing ring-labeled orotic acid was utilized. After stopping the reaction by boiling for 3 minutes, the reaction mixtures were spotted and dried onto polyethylene-imine
cellulose TLC plates (Sigma Chem. Co.). The development was performed with 0.3 M sodium chloride and the autoradiography was done at -70°C for 3 days.

Thymidylate synthase activity was determined based on the light absorption at 340 nm of the 7,8-dihydrofolate produced in the reaction as described by Dunlap (1978).

**Purification of the UMP synthase**

In attempt to characterize molecularly UMP synthase mutants from the tobacco, we need to use UMP synthase antibody. For this purpose we purified UMP synthase protein from squash fruits, a plant tissue readily available that contains high levels of UMP synthase activity. This purified UMP synthase was then used to raise antiserum. All operations in the UMP synthase purification were carried out at 0 to 4°C.

**Enzyme extraction.** Typically, one kilogram of the fruits from fresh summer squash (*Cucurbita pepo* L.) were homogenized in a Waring blender using an equal volume of 2 X homogenization buffer (100 mM Tris, pH 7.6, 20 mM MgCl₂, 10 mM β-mercaptoethanol, 4 mM EDTA). The homogenate was centrifuged at 20,000 X g for 30 min to remove particulate material or filtered with six-layer of cheesecloth. The supernatant was subjected to ammonium sulfate fractionation to concentrate protein from the extract as described by England and Seifter (1990). Typically, 90 to 100% of the total UMP synthase activity was recovered in the 45 to 70% ammonium sulfate fraction with a one-to two-fold enrichment.

**Size exclusion chromatography.** The pellet from the 45 to 70% ammonium sulfate cut was dissolved in 10 to 20 mL of homogenization buffer. Afterward, it
was spun at 30,000 X g for 30 min to remove any particulate and the supernatant was applied directly to a Sephadex G-150 column (5 X 90 cm) pre-equilibrated with 1X homogenization buffer. The column was washed at a flow rate of 1.1 mL min⁻¹ with 1X homogenization buffer. Fractions of 10 mL each were collected and tested for their UMP synthase activities. The fractions containing the UMP synthase were pooled and used in the subsequent separation.

**Anion exchange chromatography.** The collected UMP synthase pool from the gel filtration was applied to a Sephadex DEAE A-25 column (2.5 X 12 cm) equilibrated in one-half concentration homogenization buffer without EDTA. The column was previously washed according to Robyt and White (1990). After the application of the protein sample, the DEAE column was eluted at a flow rate of 0.75 mL min⁻¹ with a linear gradient of 0 to 0.6 M NaCl in one-half concentration homogenization buffer without EDTA. The fractions containing the active UMP synthase were pooled, concentrated, and desalted using an Amicon protein concentrator fitted with a YM30 filter. The sample was further washed with one-half concentration homogenization buffer without EDTA to remove excess NaCl.

**Affinity chromatography.** The pooled enzyme from the ion-exchange chromatography was applied to a Cibacron Blue 3GA column (1.0 X 10 cm). After the application of the pooled sample, the column was washed extensively with about 40 to 50 volumes of a buffer containing 10 mM Tris pH 8.0 and 1 mM DTT and then eluted with 0.08 mM of OMP in the buffer. The loading, washing and elution of the sample were conducted at a flow rate of about 0.33 mL min⁻¹. The fractions containing UMP synthase were pooled and
concentrated with an MSI centrifugal ultrafiltration device (Micron Separations Inc., Westborough, MA, USA) with a 10 kDa molecular weight cut-off. To remove the substrate, OMP, for kinetic studies the concentrated sample was twice diluted with the buffer then reconcentrated. Residual OMP carried over to the enzyme assays was calculated to be less than 0.15 nM. Samples of UMP synthase were prepared for SDS-PAGE using the procedure of Laemmli (1970).

**Preparation of antiserum**

The band corresponding to UMP synthase was carefully excised from preparative 12% SDS PAGE gels, and stored at -70°C until use. To prepare antiserum, a portion of the gel, corresponding to approximately 25 μg of protein in the gel was ground in a mortar and pestle, then resuspended in 1 mL of 0.85% NaCl (Dunbar and Schwoebel, 1990). This saline solution was combined with 1 mL of Freund's complete adjuvant and homogenized until a stiff emulsion formed. This emulsion was injected at several locations into the flanks of a female New Zealand White rabbit. After 14 days and again after 31 days, this procedure was repeated except that incomplete adjuvant was used to make the emulsion. Blood samples were taken prior to injection on days zero, 14, 31. Western blot analysis of the antisera demonstrated that high titer anti-UMP synthase was produced after 31 days of immunization.

**Uptake of orotic acid**

Small pieces of FOA-resistant calli and wild type calli were inoculated onto culture media containing 5 μM [6-14C]orotic acid of 50 μM total orotic acid. At several different culture times, the calli were harvested, washed 3 X with liquid culture media, the excess liquid was removed with filter paper and the
calli were stored at -70°C until ready for the next step. The calli were homogenized and placed in vials, scintillation cocktail added and counted with Packard model 1500 liquid scintillation counter.

**Induction of UMP synthase by metabolites**

To induce UMP synthase, small pieces of calli were cultured in induction media (callus-maintaining media without casein or coconut water). The media for the induction were prepared by adding filter-sterilized inducer, FOA, thymine, or inhibitors of dihydrofolate reductase (DHFR), into the MS media to a final concentration as indicated in each experiment. For the induction experiments, uracil was not included. Small pieces of calli of 0.2 to 0.3 gram were inoculated onto the induction media with density about 1 to 1.2 gram calli per 10 mL media and incubated at 26°C with a 16-hour day and 8-hour dark period. After several days the calli were harvested, washed with half strength of MS liquid media, the excess liquid was removed with filter paper and the calli stored at -70°C until all samples were ready for assay.

**Western blotting**

Analysis of UMP synthase protein by Western blot was modified from the method described by Timmons and Dunbar (1990). Samples containing 50 to 100 µg protein were fractionated on 12% polyacrylamide gel electrophoresis. The fractionated proteins were then electrotransferred onto nitrocellulose membranes in the buffer containing 39 mM Glycine, 48 mM Tris, 0.037% SDS and 20% methanol (Sambrook et al., 1989). The protein transfer from 14 X 10 X 0.2 cm gel could be completed for about 4 h with 200-mA current. The membrane was then incubated at 25°C for 6 hours in prehybridization solution
containing 5% non-fat dry milk, 0.01% antifoam A (Sigma Chem. Co.) and 0.02% sodium azide in phosphate-buffered saline (PBS). After washed 2 X 15 minutes with PBS, the membranes were incubated in the hybridization solution which was the prehybridization solution plus 0.1 to 0.2% (v/v) the antiserum raised against UMP synthase purified from squash fruits. The incubation of the transferred proteins in the antiserum was performed overnight at 4°C. In the next day the membrane were washed 3 X with PBS at 25°C, 2 X for 20 minutes each and 1 X for 8 to 24 hours. After the washing, the membranes were incubated in the milk solution plus [125I]-Protein A at 25°C. The activity of the [125I]-Protein A was about 2 X 10^4 dpm per cm^2 membrane. Except in prehybridization and washing, all incubations used just enough solution to cover the membrane and performed with rocking shaker set at medium speed. After 6 hours in the [125I]-rProtein A solution, the membranes were washed 3 X 15 minutes with PBS, air-dried and then autoradiography for about 3 days at -70°C.

**Southern blot analysis**

The analysis of the genomic DNA was performed with the Southern blot method modified from Sambrook et al. (1989). The preparation of small scale DNA samples was performed as described by Bloksberg (1991) except for the amount of tissue. Callus of 200 mg was homogenized in 1.5 mL-tube with 0.6 mL of extraction buffer. Using this method, the average yield of the DNA was about 100 μg/gram callus.

Restriction endonuclease-digested DNA samples were separated on 1% agarose gel electrophoresis. The fractionated DNA fragments were transferred by capillary action on nylon membranes (GeneSreen plus, DuPont, Boston,
MA). Transfer and hybridization protocols were performed as described by the manufacturer. The DNA probe labeled with $^{32}$P was used in the hybridization. This probe was synthesized using the 1.2 kb DNA template of the tobacco UMP synthase cDNA in pRT327 and Multiprimer labeling system (Amersham Co., Arlington, IL).

**Northern blot analysis**

Total RNA was isolated from calli of *N. plumbaginifolia* with the method of Wadsworth et al. (1988) and quantified spectrophotometrically at 260 nm. The average yield was about 50 μg RNA/gram callus.

For RNA blot analysis, total RNA samples were denatured and electrophoresed as described in Tirimanne and Colbert (1991). Samples containing 10 to 15 μg total RNA per lane were transferred onto nylon membrane (Gene Screen, Du Pont) using 10 X SSC solution which contains 1.5 M sodium chloride and 0.15 M sodium citrate for overnight. After briefly rinsing the membrane with 2 X SSC solution, the RNA samples were UV-crosslinked to the membrane. This step was done on 2 X SSC-wet filter paper for 60 seconds using a medium-wave UV illuminator (302 nm) from Spectroline. The membrane was then air-dried for 15 minutes followed with vacuum-baking at 80°C for 1 to 2 hours.

The membrane-fixed RNA samples were hybridized with 0.55 kb RNA probe. The $^{32}$P-labeled RNA probe was synthesized using the template of UMP synthase cDNA clone pRT327 from *N. tabacum*. The plasmids were linearized with *NsiI* and transcribed with T3 RNA polymerase (RNA transcription kit, Stratagene) and $^{32}$P-UTP, 800 Ci/mmol (New England Nuclear, Du Pont). The membrane was prehybridized and hybridized with a method slightly modified
from Barnes et al. (personal communication). Prehybridization was performed at 60°C for 4 hours in hybridization solution that contains 50% deionized formamide, 10% dextran sulfate, 1 M NaCl and 1% SDS. Fragmented salmon sperm DNA of 150 μg/mL and RNA probe of 3 X 10^5 cpm/mL were added into hybridization solution. After hybridization for overnight at 60°C, the membrane was washed with 2 X SSC twice at room temperature for 5 minutes each, 0.2 X SSC at 60°C for 15 minutes and 2 to 3 times with 0.1 X SSC at 62°C. Autoradiography was carried out at -70°C for 1 to 2 days. The radioactivity of hybridization was quantified by cutting the membrane corresponding to bands on developed film and counting with liquid scintillation counter (1900TR, Packard).

**Preparation of nuclei**

Nuclei were isolated from 10 to 14 day old calli using the method adopted from Watson and Thompson (1986). All operations were performed in RNAse-free conditions and at 4°C, otherwise indicated.

About 80 gram of callus was treated with 160 mL of ice-cold ether for 2 minutes. In a fume hood, the calli were washed twice with 160 mL ice-cold deionized H₂O per wash. To remove any trace of the ether, the calli were air dried on the 3MM paper until the smell of ether disappeared. This generally required about 10 to 15 minutes. The callus was homogenized in 160 mL of the ice-cold homogenization buffer using Osterizer blender at high speed chop for 3 bursts of 10 seconds per bursts with 1 min break in between blendings. The homogenization buffer contained 0.7 M hexylene glycol, 10 mM PIPES-KOH pH 7.0, 10 mM MgCl₂ and 10 mM 2-mercaptoethanol. The homogenate was filtered with 4 layers of sterile cheesecloth and through a 300 μm nylon mesh. While
stirring slowly, the 25% stock solution of Triton X-100 was slowly added to the filtrate to a final concentration of 0.5% and stirred for an additional 2 minutes. The filtrate was sieved with 80 μm and 20 μm nylon filters then spun at 1000 X g (2700 rpm on JA-17 rotor of Model J2-21 of Beckman centrifuge) for 10 min.

The pellet was gently resuspended with 10 mL of gradient buffer without percoll. The gradient buffers contained 0.5 M hexylene glycol, 10 mM PIPES-KOH, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.5% Triton X-100 and percoll varied from 0 to 80%. The suspension of nuclei was overlain onto a percoll step gradient of 30/50/80%(v/v/v) and spun at 1000 g for 30 min. The nuclei were collected from the 50/80 interface. The nuclei were checked for intact nature with microscope. This was performed by taking 10 μl of the nuclei suspension and mixing in a microtube with 10 μl of 1% carmine in 45% acetic acid. After 2 minutes they were counted in a counting chamber under microscope at 200 to 400 X magnification (Vlasak, 1981). If the nuclear preparation at the 50/80 interface contained 50% or more non-intact particles, the nuclei at this interface were resuspended in gradient buffer without percoll and recentrifuged as described above. In addition, the 30/50 interface was checked for the presence of nuclei. If significant proportion of nuclei were observed at this interface, the material at that interface was also resuspended in gradient buffer without percoll and recentrifuged as described above. The collected nuclei were washed once with gradient buffer containing 0% percoll and once with the storage buffer. The storage buffer contained 50 mM Tris.Cl pH 7.8, 5 mM MgCl₂, 10 mM 2-mercaptoethanol and 20% glycerol. The nuclei were resuspend at 10⁷ to 10⁸ per mL in storage buffer, aliquoted and stored at -70°C.
In vitro runoff transcription

The condition for the in vitro transcription assay was slightly modified from Kimpel et al. (1990). The reaction mixture for the transcription contained 25 mM Tris-Cl pH 7.8, 10 mM MgCl₂, 55 mM (NH₄)₂SO₄, 2.5 mM DTT, 0.5 mM (ATP, CTP, GTP), 12.5% Glycerol, 4 μL RNase-Block I (Strategene) block, 100 μCi [³²P]-UTP and 20 million nuclei in total volume of 400 μL.

The mixture was incubated at 30°C for 45 minutes. Add 40 μL (80 Unit) of RNase-free DNase I (Ambion Inc.), mixed then incubated at 30°C. After 10 minutes, 200 μL of the buffer containing 3% SDS, 0.5 M Tris pH 7.4, and 0.125 M EDTA was added along with. Also add 20 μg of proteinase K and the mixture was incubated at 37°C for an additional 30 min. Finally the mixture was extracted twice with 1 volume (650 μL) buffered (pH 7.4) - phenol:chloroform:isoamyl alcohol (25:24:1).

After adding 50 μg of tRNA, the RNA solution was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volume of ethanol. The precipitated RNA was kept at -20°C for overnight and spun for 20 minutes at about 10,000 X g. The pellet was washed briefly with 70% ethanol and air-dried. The pellet containing labeled RNA was dissolved in DEPC-treated deionized H₂O and counted to determine the amount of radioactivity.

RNA slot blot hybridization

UMP synthase antisense mRNA was transcribed in vitro using Stratagene's transcription kits and DNA template of the pRT327 plasmid DNA bearing the cDNA fragment of the tobacco UMP synthase. Binding of the RNA onto the GeneScreen membrane (Du Pont) was performed according to the manufacturer's instructions. The RNA samples from the in vitro transcriptions
were loaded into each well. After prehybridization for 4 hours at 58°C in 150 μL of the hybridization solution per cm² of the membrane, the membrane was hybridized overnight at 58°C with 2 to 8 x 10⁶ cpm of [³²P]-labeled RNA from the runoff transcription per mL hybridization solution. The hybridization solution also contained 10% Dextran Sulfate, 1% SDS, 200 μg/ml denatured-salmon sperm DNA and 1 M NaCl. The membrane was washed twice with 2 X SSC plus 0.1% SDS at room temperature for 5 minute each, once with 2 X SSC plus 0.1% SDS at 60°C for 15 minutes, once with 0.2 X SSC plus 0.1% SDS at 60°C for 15 minutes and once or twice with 0.1 X SSC plus 0.1% SDS at 60°C for 15 minutes. After each step of the washing, the background was monitored. Finally the wet membrane was wrapped with plastic and autoradiographed for 1 to 3 days.
CHAPTER 3. RESULTS

UMP synthase enzyme and its antibody

In most mature plant tissues, UMP synthase is expressed at very low levels. Because of this, we began a search of plant tissues to find a readily obtainable plant tissue which produced large amounts of UMP synthase and could serve as a facile source for enzyme purification. Various fruits, roots, tubers, seeds and leaves were obtained locally and were tested for UMP synthase activity. Table 3.1 shows that squash (Cucurbita pepo) fruits showed dramatically higher levels of UMP synthase activity than any other tissue tested. Therefore, we began the purification of this enzyme from squash fruit.

Purification of UMP synthase from squash fruits

UMP synthase was purified 2,080-fold from squash fruits with a 20.2% recovery of UMP synthase activity (Table 3.2). After homogenization of the squash fruit and clarification of the extract by centrifugation, the supernatant was fractionated by ammonium sulfate precipitation as described in Materials and Methods. The typical pattern of UMP synthase activity by this method is presented in Figure 3.1. The UMP synthase activity was recovered almost quantitatively in a 45 to 70% ammonium sulfate fraction resulting in a 1.7 fold purification. The number of 45 to 70% ammonium sulfate is higher than that required to precipitate tomato UMP synthase which is 35 to 50% (Walther et al., 1984). This is probably because the concentration of the proteins in the squash extract is lower than that in the tomato extract.
Table 3.1. UMP synthase activity in various common fruits and vegetables

<table>
<thead>
<tr>
<th>Species</th>
<th>Common Name</th>
<th>Tissue</th>
<th>UMP synthase activity unit/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brassica oleracea</td>
<td>Cabbage</td>
<td>leaf</td>
<td>1.12 ± 0.02</td>
</tr>
<tr>
<td>Brassica oleracea</td>
<td>Broccoli</td>
<td>head</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>Cucurbita pepo</td>
<td>Squash</td>
<td>fruit</td>
<td>5.73 ± 0.65</td>
</tr>
<tr>
<td>Daucus carota</td>
<td>Carrot</td>
<td>root</td>
<td>0.59 ± 0.41</td>
</tr>
<tr>
<td>Lactuca sativa</td>
<td>Lettuce</td>
<td>leaf</td>
<td>0.16 ± 0.00</td>
</tr>
<tr>
<td>Lysopersicon esculentum</td>
<td>Tomato</td>
<td>fruit</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Musa sp.</td>
<td>Banana</td>
<td>fruit</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>Green bean</td>
<td>pods</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>Pisium sativa</td>
<td>Pea</td>
<td>seed</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>Solanum tuberosum</td>
<td>Potato</td>
<td>tuber</td>
<td>0.44 ± 0.26</td>
</tr>
<tr>
<td>Zea maize</td>
<td>Maize</td>
<td>seed</td>
<td>0.01 ± 0.00</td>
</tr>
</tbody>
</table>

a various fruits and vegetables were obtained locally, homogenized, and tested for UMP synthase activity as described in materials and methods.

b 1 unit of activity = 1 nmol of CO₂ released per minute at 23°C.
Table 3.2. Purification of UMP synthase from fresh fruits of yellow squash.

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Total Protein (mg)</th>
<th>Total Activity (Unit)</th>
<th>Spec. Activity (Unit/mg)</th>
<th>Purification (X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>1,541.0</td>
<td>1,803</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>919.0</td>
<td>1,792</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sephadex G150</td>
<td>222.0</td>
<td>837</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>DEAE A25</td>
<td>56.4</td>
<td>756</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Cibacron Blue 3GA</td>
<td>0.2</td>
<td>365</td>
<td>2,433</td>
<td>2,080</td>
</tr>
</tbody>
</table>
Figure 3.1. Fractionated of squash UMP synthase activity by ammonium sulfate precipitation. The activity was measured after the salt was removed from fractionates by dialysis twice in extraction buffer for 3 hours in each dialysis. The stripped bars indicates the protein profile. The solid bars are the UMP synthase activity in corresponding fractions.
The ammonium sulfate precipitated UMP synthase fraction was chromatographed through a Sephadex G150 column. Fractions of 10 ml were collected and tested for their protein content and UMP synthase activity. The patterns of both the protein and the enzyme activity of those fractions are presented in Figure 3.2. The elution pattern of the squash proteins was very broad. Proteins were detected from the fraction numbers 16 to the fraction number more than 80. The elution pattern of the UMP synthase activity was narrower. The enzyme was eluted as a band of the activity whose peak corresponded to a molecular mass of 110 kDa.

Anion exchange was utilized as a second chromatographic step for enzyme purification. The pooled and concentrated fractions from the Sephadex G150 column were applied to a Sephadex DEAE A25 column. Elution was with a linear gradient from 0 to 0.6 M NaCl. The UMP synthase eluted as a single peak at a salt concentration of 0.14 M NaCl (Figure 3.3.). The purification steps through the DEAE chromatography resulted in removal of 96% of the protein with a 42% recovery of starting UMP synthase activity. The best results with this purification step were obtained when the Sephadex DEAE A25 was used only once, because washing, even in high salt buffer (2 M NaCl) does not completely remove bound protein and other colored materials from the column, and further ion exchange capacity is lost with subsequent runs with the same batch of Sephadex DEAE A25. One way to recover the binding capacity of the DEAE column is by washing the used column with acid-base treatment as described by Robyt and White (1990).
Figure 3.2. Elution pattern of UMP synthase on Sephadex G150. The column was equilibrated as described in Materials and Methods. Fractions of 10 ml were collected. Protein levels across the column are presented as opened squares and the levels of UMP synthase activity are represented by solid squares. The hatched bar represents those fractions pooled for further purification. The inset shows the determination of the molecular mass of the UMP synthase eluted from the Sephadex G150 column. Molecular weight markers used were: Thyroglobulin (670 kDa); Bovine gamma globulin (158 kDa); Phosphorylase B (97.4 kDa); and Bovine Serum Albumin (67 kDa).
Figure 3.3. Elution profile of UMP synthase on anion exchange column of DEAE A25. The sample separated was from the gel filtration pool. The elution was performed using linear gradient of NaCl as described in Materials and methods. The open circles represents protein concentration profile. The solid circles represent the UMP synthase activity.
The most productive purification step was the affinity chromatographic step using Cibacron Blue. This affinity chromatographic step binds proteins possessing a nucleotide binding pocket. As shown in Figure 3.4, the UMP synthase bound and was specifically removed from the column by elution with 0.08 mM OMP. SDS-PAGE analysis revealed a single band at 50 kDa, indicating that squash UMP synthase enzyme exists as dimer of 50 kDa subunits.

The protein profiles at each step of the purification are shown on the SDS-PAGE in Figure 3.5. After the Cibacron Blue affinity column the 50 kDa UMP synthase enzyme represents < 0.01% of total starting protein with 20.2% recovery. The final UMP synthase preparation had a specific activity of 2433 nmol min\(^{-1}\) mg protein\(^{-1}\). This enzyme was utilized for the characterization studies presented below. However, to raise antiserum against the UMP synthase, the enzyme was further purified using preparative SDS-PAGE which provided the protein in electrophoretically pure form.

**Properties of the UMP synthase**

When stored at 4\(^\circ\)C, UMP synthase has a half-life of approximately 1.8 days. At -20\(^\circ\)C, the half-life is approximately 1 week and the enzyme is relatively stable at -70\(^\circ\)C. After about 3 weeks in -70\(^\circ\)C, the activity retained was 60%. When stored at -20\(^\circ\)C, addition of 20% glycerol to the storage buffer can increase the half-life from about 3 days to 19 days. However, 20% glycerol reduces enzyme activity by 60% and must be removed prior to assay.
Figure 3.4. Elution profile of *C. pepo* UMP synthase from Cibacron Blue dye-ligand column. The column was washed as described in Materials and Methods until the protein background was reduced below 0.005 mg/ml then eluted with buffer containing 0.08 mM OMP. Protein levels across the column are presented as open squares and the levels of UMP synthase activity are represented by solid squares.
Figure 3.5. SDS-PAGE of C. pepo UMP synthase at different stages of purification. Molecular mass markers are shown in lane 1. They are: Phosphorylase B, 97.4 kDa; Bovine Serum Albumin, 66.2 kDa; Ovalbumin, 45 kDa; Bovine Carbonic Anhydrase, 31 kDa; Soybean Trypsin Inhibitor, 21.5 kDa; Lysozyme, 14.4 kDa. Lane 2 contains proteins from the crude extract. Lane 3 contains proteins from ammonium sulfate precipitation. Lane 4 contains the pooled fractions from Sephadex G150. Lane 5 contains the pooled fractions from DEAE A25. Lane 6 contains the pooled fractions from Cibacron Blue. The arrow indicates the UMP synthase band at 50 kDa.
**pH optima.** Because UMP synthase has two different enzymatic activities, the pH optimum was determined for each activity. The ODCase activity was measured directly using OMP as a substrate, while the OPRTase activity was measured indirectly by measuring the overall activity of UMP synthase with orotic acid as a substrate. Because the rate limiting step of plant UMP synthase is the OPRTase step (Kapoor and Waygood, 1965; Santoso and Thornburg, 1992), the overall UMP synthase activity actually measures OPRTase. The pH profile for the ODCase was very broad (Figure 3.6). The optimal pH for ODCase activity was 8.1. High levels of ODCase activity, however, occurred between 7.5 and 8.6. The OPRTase activity showed a much sharper pH optimum at 7.5. Both enzymes were slightly more active in Tris than in HEPES buffers. These values are somewhat lower than the pH optimum for UMP synthase from *Euglena gracilis* (Walther et al., 1984).

**Temperature optima.** Because plants are subjected to changing environmental conditions, we also determined the optimum temperature for both enzymatic activities of UMP synthase. Figure 3.7 shows that the optimum activity for each of the enzyme activities is about 30 to 32°C. At temperatures higher than 35°C, the activity decreased sharply. This optimum temperature is probably indicative of optimal plant growth temperature, summer. As plants grow, their need of nucleotides increases.
Figure 3.6. Effect of pH on the activity of UMP synthase. The buffers used were HEPES (closed circles and squares), and Tris (open circles and squares). The circles represent the data obtained using 7-[\textsuperscript{14}C]-orotic acid as a substrate, and the squares represent data using 7-[\textsuperscript{14}C]-OMP as a substrate.
Figure 3.7. Effect of temperature on the activity of UMP synthase. The circles represent the data obtained using 7-[\textsuperscript{14}C]-orotic acid as a substrate, and the squares represent data using 7-[\textsuperscript{14}C]-OMP as a substrate.
**Substrate kinetics.** The $K_m$ and $V_{max}$ values of each substrate, orotic acid, PRPP and OMP were determined by least squares analysis of Eadie-Hofstee plots (Table 3.3). The relationship between their initial velocities and the substrate concentration is presented on Figure 3.8. The $K_m$ values for the squash UMP synthase are similar to those from the *N. tabacum* enzyme (Santoso and Thornburg, 1992) and are lower than those of the *Euglena* enzyme (Walther et al., 1981). The $V_{max}$'s of the squash enzyme are similar to those of the tobacco callus UMP synthase and are approximately 10 to 20 fold lower than the values reported from the *Euglena* enzyme.

**Enzyme inhibitors.** All nucleotides tested (UMP, TMP, CMP, OMP, UDP, UTP, AMP, GMP, IMP, and XMP) slightly decreased both OPRTase and ODCase activity to various degrees as compared to the control (Table 3.4.). In general, the OPRTase activity was more strongly affected than was the ODCase activity. Interestingly, at high concentrations tested, the purines effectively inhibited the OPRTase activity. The regulation of UMP synthase by purines at high concentrations has been previously observed for both mammalian (Jones et al., 1978) and *Euglena* UMP synthases (Walther et al., 1981).

In mammalian cells, CMP and to a lesser extent TMP are competitive inhibitors of the ODCase activity (Jones et al., 1978). This does not appear to be the case with the squash UMP synthase, where these nucleotides do not effectively inhibit the ODCase activity. At the higher concentration tested, these nucleotides showed moderate inhibitory activity against both enzyme
Figure 3.8. Kinetic analysis of *C. pepo* UMP synthase. Plots A, B, and C show the effects of substrate concentrations on UMP synthase activity. When orotic acid (OA) was the variable substrate (Plot A), PRPP was held constant at 0.2 mM, and when PRPP was the variable substrate (Plot B), OA was held constant at 0.1 mM. When OMP was the variable substrate (Plot C), OA and PRPP were omitted from the reaction. The insets show Lineweaver-Burk plots of the presented data from which $K_m$ and $V_{max}$ values were derived.
Table 3.3. The $K_m$ and $V_{max}$ values of substrates for OPRTase and ODCase of squash UMP synthase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orotic acid</td>
<td>3.0 μM</td>
<td>0.34 μM/min</td>
</tr>
<tr>
<td>PRPP</td>
<td>3.1 μM</td>
<td>0.85 μM/min</td>
</tr>
<tr>
<td>OMP</td>
<td>2.6 μM</td>
<td>0.58 μM/min</td>
</tr>
</tbody>
</table>
Table 3.4. Enzymatic activity of OPRTase and ODCase of squash UMP synthase in the presence of pyrimidines, purines analogs.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>mM</th>
<th>OPRTase (%)$^a$</th>
<th>ODCase (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>---</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>UMP</td>
<td>0.005</td>
<td>85 ± 7</td>
<td>92 ± 12</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>79 ± 26</td>
<td>96 ± 7.8</td>
</tr>
<tr>
<td>TMP</td>
<td>0.005</td>
<td>88 ± 14</td>
<td>104 ± 12</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>55 ± 14</td>
<td>88 ± 11</td>
</tr>
<tr>
<td>CMP</td>
<td>0.005</td>
<td>85 ± 19</td>
<td>86 ± 19</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>67 ± 16</td>
<td>86 ± 18</td>
</tr>
<tr>
<td>OMP</td>
<td>0.005</td>
<td>14 ± 4</td>
<td>nd$^c$</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2 ± 2</td>
<td>nd</td>
</tr>
<tr>
<td>UDP</td>
<td>0.005</td>
<td>84 ± 3</td>
<td>100 ± 3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>55 ± 2</td>
<td>85 ± 6</td>
</tr>
<tr>
<td>UTP</td>
<td>0.005</td>
<td>77 ± 3</td>
<td>82 ± 2</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>68 ± 5</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Orotic Acid</td>
<td>0.005</td>
<td>nd</td>
<td>82 ± 3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>nd</td>
<td>79 ± 2</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.005</td>
<td>95 ± 12</td>
<td>75 ± 5</td>
</tr>
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<td>0.1</td>
<td>85 ± 13</td>
<td>84 ± 14</td>
</tr>
<tr>
<td>aza-UMP</td>
<td>0.005</td>
<td>9 ± 5</td>
<td>11 ± 4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3 ± 3</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>FOA</td>
<td>0.005</td>
<td>36 ± 7</td>
<td>92 ± 12</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>14 ± 4</td>
<td>78 ± 7</td>
</tr>
<tr>
<td>AMP</td>
<td>0.005</td>
<td>91 ± 12</td>
<td>108 ± 16</td>
</tr>
<tr>
<td></td>
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<td>70 ± 17</td>
<td>95 ± 14</td>
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<td>GMP</td>
<td>0.005</td>
<td>76 ± 9</td>
<td>94 ± 13</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>46 ± 12</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>IMP</td>
<td>0.005</td>
<td>75 ± 15</td>
<td>99 ± 12</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>52 ± 13</td>
<td>79 ± 7</td>
</tr>
<tr>
<td>XMP</td>
<td>0.005</td>
<td>63 ± 16</td>
<td>92 ± 12</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>21 ± 8</td>
<td>30 ± 2</td>
</tr>
</tbody>
</table>

$^a$OPRTase is measured as total UMPSase activity using [carboxyl-$^{14}$C]-orotic acid.

$^b$ODCase is measured using [$^{14}$C]-OMP.

$^c$nd = not determined
activities, however, this inhibition was most pronounced against the OPRTase activity rather than the ODCase activity.

We also tested the known metabolic inhibitors FOA and 6-aza-UMP. The FOA affected primarily the OPRTase activity and not the ODCase activity. In contrast, aza-UMP was quite inhibitory to both OPRTase and ODCase. However, because the OPRTase assay is dependent upon ODCase activity, care must be taken in interpretation of the OPRTase data when compounds inhibit both enzyme activities.

**Preparation and use of UMP synthase antiserum**

To monitor the expression of UMP synthase in plant tissues, we have prepared antiserum against the purified UMP synthase. Starting with the Cibacron Blue enzyme preparation, a preparative 12\% SDS PAGE was run and the major protein (UMP synthase) was excised from the gel. Careful analysis of the excised protein band from the preparative gel showed the presence of only a single band on subsequent analytical SDS PAGE gels. The acrylamide band containing the protein was emulsified with Freund's Adjuvants as described in Materials and Methods and injected into New Zealand White rabbits.

After several weeks of immunization, the antiserum produced was examined for its ability to detect UMP synthase by Western blot analysis. The extracts from tobacco calli having various UMP synthase activities were used for this analysis. As shown in Figure 3.9, the intensities of bands on this blot were proportional to the levels of UMP synthase activities. The higher the activity of UMP synthase, the more intense the band. This figure also indicates that the
Figure 3.9. Western blot for UMP synthase of the tobacco calli. Crude extracts of calli from mutants and wild type lines were fractionated on 12% SDS-PAGE. Each lane contained 100 µg protein. After transfer onto nitrocellulose membrane, the membrane was hybridized with the antiserum as described in Materials and Methods. Lane 1 is from the wild type; lane 2, 3, and 5 are from the mutants which were selected against 5-fluoroorotic acid and had UMP synthase activity of 21%, 50% and 83% relative to the wild type respectively.
antiserum recognizes a single polypeptide of molecular mass, 50 kDa. Moreover, there is no cross reactivity with other bands in this preparation. Thus, we conclude that the antiserum is specific in detecting UMP synthase and the level of UMP synthase can be approximated in these western blot assays.

To examine the utility of the anti-C. pepo UMP synthase antiserum, western blot analysis was conducted against a variety of different plant species (Figure 3.10). In all cases tested, this analysis shows crossreactive proteins having monomer sizes similar to that of the squash enzyme (Lane 1). These 50 kDa crossreactive proteins were present in most species tested and were visible in crude protein extracts. In carrot root and potato tubers (Lanes 8 and 9), however, the UMP synthase could only be observed if the crude extract was carried through the first step (ammonium sulfate precipitation) of our purification protocol. These differences in the crossreactivity may reflect the quantitative levels of UMP synthase present in the tissues tested, however, this variability could also result from differing avidity of the antiserum against more distantly related UMP synthase protein molecules.

In some cases immunoreactive bands smaller than 50 kDa monomer subunit appeared. In the case of squash (Lane 1), a 38 kDa band appears. This is thought to be a breakdown product of the UMP synthase because this band accumulates with storage of the squash UMP synthase preparations during which time, activity declines. There are also several immunoreactive bands that are larger than the 50 kDa band observed with squash. Although larger bands are found in all preparations, these bands are most evident in tobacco (N. plumbaginifolia) callus, maize (Zea mays) kernels, cabbage (Brassica oleracea) leaf, and broccoli (Brassica oleracea) heads (Lanes 3, 4, 6, and 7).
Figure 3.10. Western blot analysis of various plant species. Each lane contains 90 µg of protein extracted from: Squash fruit (C. pepo, Lane 1); tobacco callus (N. tabacum, Lane 2; N. plumbaginifolia, Lane 3); maize kernels (Z. mays, Lane 4); bean pods (P. vulgaris, Lane 5); cabbage leaf (B. oleracea, Lane 6); broccoli heads (B. oleracea, Lane 7); carrot root (D. carota, Lane 8); potato tuber (S. tuberosum, Lane 9). Each lane was loaded with either the sample as crude extract (Lanes 1 to 7) or after dialysis of a 45 to 70% ammonium sulfate fraction (Lanes 8 and 9). Blots were transferred and probed with anti-UMP synthase antiserum as described in Materials and Methods.
Whether these bands represent alternative forms of UMP synthase will require additional work.

**FOA-resistant cell lines**

**Plant tissue culture and selection**

Cell suspensions were developed after 3 weeks in liquid culture as described in Material and Methods. To separated single cells from calli and clumped cells, the suspension was allowed to sit without shaking for 1 to 2 minutes to let the calli settle. The fine cell suspension cultures were then transferred into fresh media by pipeting carefully without disturbing the precipitated, clumped cells. The cell suspension was subcultured every 2 to 3 weeks. Visible clumped cells often developed in the suspension after 2 to 3 subcultures, so to produce fine cell suspension cultures from clumped cells, the above procedure was repeated at each subculture.

The simple protocol to isolate protoplasts from the leaf tissues typically yielded 1 to 2 x 10^6 protoplasts per gram tissue. This result is about 5 to 10 times lower than if the lower epidermis of the leave tissue is peeled before enzymatic digestion (Takabe and Nagata, 1984). The removal of lower epidermis of the tissue ensures better access to the cell walls of the cell wall digesting enzymes. This simpler method was chosen because the peeling of the lower epidermis is not easy. To compensate for the decrease in protoplast yield with this simple method, the amount of starting leaf tissue was increased.

When using either the cell suspensions or protoplasts, mutations were induced with the alkylating agent, N-nitroso-N-methylurea (NMU). After washing with liquid MS media to remove the mutagen, the cells were plated on selection media containing 0.15 to 1.61 mM FOA plus 50 mg/L uracil (see
Materials and Methods for recipe of selection media). By estimation of the numbers of cells plated and the numbers of recovered cell lines, we calculated that this selection procedure yielded a selection rate of $0.4 \times 10^{-4}$ selectants per total cells. After 4 to 6 weeks, small calli grew on the selection media. These were subsequently transferred onto the fresh media.

**FOA-resistance, growth**

When FOA-resistant lines were isolated, they were first tested to ensure the resistant lines were really resistant to FOA. Therefore, quantitatively the growths in the presence of FOA plus uracil of these selected lines were measured. This was done by plating small pieces of calli on solid media containing 0.15 mM FOA plus 50 mg/L uracil followed by weighing the calli periodically at several different times. The calculation of the relative growth was described previously (Santoso and Thornburg, 1992). Result in Figure 3.11 demonstrated that the isolated lines tested are resistant to FOA. This resistance is indicated by their ability to grow in the presence of FOA plus uracil. This figure also shows that FOA resistance among the callus lines varied. In some cases, the cell lines were capable of growing quite well in the presence of FOA plus uracil whereas other cell lines grew only slightly better than the wild type callus line.

**UMP synthase activity of FOA resistant cell lines**

The next step in characterization of these cell lines was to determine whether the variation in the ability to grow in the presence of FOA plus uracil reflected the level of UMP synthase activity. As described in the Chapter 1 of this thesis, FOA is an antimetabolite that mimics the substrate, orotic acid. This
Figure 3.11. Growth of FOA selected calli of \textit{N. plumbaginifolia} on the maintenance media containing 20 mg/L FOA and 50 mg/L uracil. The growth was monitored by weighing the calli in sterile hood. The line with opened circle represent the of the hNp28, the closed circle is hNp28-umps2x4, the opened square is hNp28-umps2x27, and the closed square is hNp28-umps820.
metabolite is converted to a toxic compound FUMP by the action of UMP synthase. FUMP is toxic to cells because it can either be incorporated into RNA or it may be converted to FdUMP which is a suicide inhibitor of thymidylate synthase. Therefore, any cell having an active UMP synthase should die in the presence of FOA. However these cells can be rescued by adding uracil to the selection media. To provide the cells with pyrimidines, the uracil is converted to UMP by the enzymes of the salvage pathway. Cells resistant to FOA are expected to be impaired in UMP synthase expression. Cell lines lacking enzymes for UMP de novo biosynthesis can be rescued by providing uracil into the media to supply the need of UMP.

The results of the UMP synthase assays as presented on Table 3.5 does not really satisfy the expectation of the mechanism of FOA toxicity. Not all FOA-resistant lines tested have low UMP synthase activity. Some of them even show elevated levels of the activity of that enzyme, several times higher than the wild type activity. Examples of those having elevated levels of UMP synthase activity are hNp28-umps820, hNp28-umps822, hNp28-umps2x26, hNp28-umps2x27, and hNp28-umps3x9. These results are derived from the CO₂ release assay. To verify these results we used a second method of assay which utilized a thin layer chromatography method to detect UMP, the other product of the UMP synthase activity beside CO₂. The results of TLC assay strongly supported the CO₂ release assay. (Figure 3.12). The extracts from high-UMP synthase calli (lanes 3 and 4) produced more [¹⁴C]-labeled UMP than that from low-UMP synthase calli (lane 1). These results indicate that the ability of N. plumbaginifolia calli to grow in media with FOA plus uracil did not correlated very well to their UMP synthase activity.
Table 3.5. UMPSase activity of selected FOA-resistant calli of tobacco assayed by CO₂ release method.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>mUnit/mg</th>
<th>% to wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>NtTr25 (wt)</td>
<td>94 ± 1</td>
<td>100</td>
</tr>
<tr>
<td>NtTr25-umps121</td>
<td>15 ± 5</td>
<td>16</td>
</tr>
<tr>
<td>NtTr25-umps127</td>
<td>53 ± 12</td>
<td>56</td>
</tr>
<tr>
<td>NtTr25-umps516</td>
<td>93 ± 17</td>
<td>99</td>
</tr>
<tr>
<td>hNp28 (wt)</td>
<td>393 ± 98</td>
<td>100</td>
</tr>
<tr>
<td>hNp28-umps1x15</td>
<td>476 ± 49</td>
<td>121</td>
</tr>
<tr>
<td>hNp28-umps1x17</td>
<td>51 ± 14</td>
<td>13</td>
</tr>
<tr>
<td>hNp28-umps2x4</td>
<td>163 ± 28</td>
<td>41</td>
</tr>
<tr>
<td>hNp28-umps2x26</td>
<td>992 ± 65</td>
<td>252</td>
</tr>
<tr>
<td>hNp28-umps2x27</td>
<td>1519 ± 54</td>
<td>387</td>
</tr>
<tr>
<td>hNp28-umps3x9</td>
<td>1104 ± 68</td>
<td>281</td>
</tr>
<tr>
<td>hNp28-umps82</td>
<td>740 ± 99</td>
<td>188</td>
</tr>
<tr>
<td>hNp28-umps820</td>
<td>811 ± 56</td>
<td>206</td>
</tr>
<tr>
<td>hNp28-umps822</td>
<td>970 ± 57</td>
<td>247</td>
</tr>
</tbody>
</table>

* A total of 32 FOA-resistant cell lines of *N. tabacum* was obtained from 3 rounds of selection on FOA. The level of UMP synthase in these cell lines varied from 8% to 99% of wild type activity.

* A total of 92 FOA-resistant cell lines of *N. plumbaginifolia* was obtained from 8 rounds of selection on FOA. The level of UMP synthase in these cell lines varied from 13% to 387% of wild type activity.
Figure 3.12. Activity of UMP synthase in various callus lines of tobacco. The activity of the callus lines (NtTr25umps121, lane 1; Nttr25, lane 2; hNp28-hNp28-umps822, lane 3; hNp28-umps820, lane 4; hNp28, lane 5; orotic acid, lane 6) were determined using TLC assay as described in Materials and Methods.
The other enzymes of UMP biosynthesis

In many cases, the expression of genes encoding enzymes which are involved in regulation of a metabolic pathway is coordinatively regulated by a protein regulator. Receiving a molecular signal, this protein acts on the promoter of target genes to regulate the synthesis of the regulatory enzymes of the metabolic pathway to either activate or inhibit the pathway, depending on the type of the signal received. One way to investigate if a change in UMP synthase enzyme is due local lesion or lesion at regulatory site of the pathway, is by checking all enzymes required for UMP de novo biosynthesis.

In this project some FOA-resistant calli of tobacco that have different UMP synthase activities were tested for this purpose. Along with UMP synthase activity, the activities of ATCase, DHOase, and DHO DH of those cell lines were determined. The results are presented in Table 3.6. In *N. tabacum*, the low-UMP synthase callus (NtTr25-umps121) has also significantly low ATCase and DHOase activities but DHO DH was unchanged. In *N. plumbaginifolia*, the low-UMP synthase callus lines have relatively unchanged ATCase, DHOase, and DHO DH. Like the low-UMP synthase callus of *N. tabacum*, the high-UMP synthase calli of *N. plumbaginifolia* have about the same change in pattern of their other enzymes for UMP biosynthesis, high ATCase and unchanged DHOase and DHO DH.

These observations indicate two things. First, ATCase and UMP synthase are likely to be coordinatively regulated for the synthesis of UMP. Increasing activities of these enzymes will increase the UMP biosynthesis (see
Table 3.6. The activities of the enzymes required for UMP \textit{de novo} biosynthesis in FOA-selected calli. The activities are in Unit/mg.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>ATCase</th>
<th>DHOse</th>
<th>DHO DH</th>
<th>UMPSase</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{NtTr25}</td>
<td>8.5 ± 1.8</td>
<td>11.8 ± 1.9</td>
<td>117 ± 17</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>\textit{NtTr25-umps} 121</td>
<td>3.9 ± 1.5</td>
<td>4.3 ± 0.6</td>
<td>101 ± 14</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>\textit{hNp28}</td>
<td>4.3 ± 0.6</td>
<td>12.3 ± 0.5</td>
<td>124 ± 4</td>
<td>0.39 ± 0.10</td>
</tr>
<tr>
<td>\textit{hNp28-umps} 1X17</td>
<td>4.7 ± 1.2</td>
<td>12.6 ± 1.0</td>
<td>76 ± 7</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>\textit{hNp28-umps} 2X4</td>
<td>6.3 ± 0.4</td>
<td>11.4 ± 1.6</td>
<td>127 ± 5</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>\textit{hNp28-umps} 82</td>
<td>7.5 ± 2.1</td>
<td>7.3 ± 1.6</td>
<td>95 ± 20</td>
<td>0.74 ± 0.07</td>
</tr>
<tr>
<td>\textit{hNp28-umps} 820</td>
<td>8.6 ± 0.9</td>
<td>12.8 ± 2.2</td>
<td>126 ± 19</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>\textit{hNp28-umps} 822</td>
<td>12.2 ± 1.6</td>
<td>10.9 ± 1.4</td>
<td>146 ± 9</td>
<td>0.97 ± 0.04</td>
</tr>
</tbody>
</table>
also Figure 3.12). In this regard DHOase and DHO DH are not regulated. This fact is also supported by those data showing that the activities of DHOase and DHO DH are significantly higher than those of ATCase and UMP synthase. Second, selection by FOA, results in at least three classes of cell lines. One class appears to be UMP synthase defective and two additional classes appear to be regulatory in nature. The first two of these will be briefly discussed and the third will be examined at length.

**UMP synthase - defective cell lines**

Some cell lines such as hNp28-umps1x17 and hNp28-umps2x4 have distinctive characteristics in the enzymes of UMP *de novo* pathway. From the four enzymes tested, these cell lines have only one of the enzymes altered. Their UMP synthase activities decreased to about 13% and 30% (of the wild type UMP synthase) for hNp28-umps1x17 and hNp28-umps2x4 respectively (see Figure 3.13). While the other enzymes for the *de novo* UMP biosynthesis are relatively unchanged.

The molecular phenotype of these FOA-resistant calli is predictable. They are able to grow in the presence of FOA plus uracil because they have low UMP synthase activity. Their UMP synthases are not able to convert FOA to toxic compound FUMP nor FdUMP. Therefore they stay alive and grow in the selection media that contains FOA and uracil.

**rpy1 cell lines**

The tobacco callus of *NtTr25-umps121* is different from the UMP synthase-defective cell lines. In addition to having low UMP synthase activity
Figure 3.13  The activity of enzymes required for *de novo* UMP biosynthesis in the UMP synthase-defective cell line and the wild type calli. The dotted-bars are those hNp28-umps1x17 cell line, the solid-bars represent hNp28-umps2x4 cell line, and the stripped-bars are of the corresponding wild type line. The quantitated levels of wild type enzymatic activities are presented in Table 3.6.
Enzymatic Activity (% of wild-type)
which is about 28% of the wild type, this cell line also has low enzymatic activity in both ATCase and DHOase. In this cell line the enzymatic activities decreased to about 35% (of the wild type) for ATCase and DHOase respectively (see Figure 3.14).

Like the UMP synthase-defective calli, the FOA resistance of this cell line is also predictable. It is resistant to FOA because of low UMP synthase activity. Even though it has low UMP synthase activity, we don’t automatically classify this cell line as a UMP synthase lesion. The reason is because the NtTr25-umps121 also has reduced activities in its ATCase and DHOase.

Regulation of a metabolic pathway usually occurs at more than one step of a pathway. The most efficient way for eukaryotes to regulate multiple steps is by using a single centralized regulator. This regulator is usually a protein. Since all three enzymes of the NtTr25-umps121 pathway are down regulated, we anticipate the lesion found in NtTr25-umps121 is a regulatory lesion that represses the UMP de novo biosynthesis. We have named this regulatory lesion as a regulation of pyrimidine biosynthesis (rpy1). This assumption is also based on reports observing regulatory lesions for other metabolic pathways that occurred in plants.

rpy2 cell lines

In N. plumbaginifolia, about 14% of all of the FOA-selected cell lines that were tested show elevated levels of UMP synthase activity. In N. tabacum however, none of the FOA-selected cell lines tested have UMP synthase activity significantly higher than the wild type enzyme (Santoso and Thornburg, 1992).

It was unexpected that callus lines should have elevated level of UMP synthase activity such as hNp28-umps820 and hNp28-umps2x27. Their
Figure 3.14  The activity of enzymes required for \textit{de novo} UMP biosynthesis in the \textit{rpy1} mutant and the wild type calli. The solid bars are those \textit{NtTr-umps121 (rpy1)} cell line, and the stripped-bars are of the corresponding wild type line. The quantitated levels of wild type enzymatic activities are presented in Table 3.6.
resistance to FOA leads our assumption that they do not follow the principle of the FOA toxicity. We named these cell lines as a regulation of pyrimidine biosynthesis \((rpy2)\). The characterization of this class of selected cells became a major objective to understand the mechanism of resistance of cell lines to FOA.

Resistance to toxic compounds in cultured cells has been widely studied in animal cells. At least four distinct mechanisms can result in resistance: altered transport of the toxic compound into the cells, detoxification of the toxic compound, altered affinity of the enzyme for the substrate or specific overproduction of the target enzyme (Schwitzer, et al., 1990; Volkenandt, et al., 1993). At first glance, the last mechanism (overproduction of the target enzyme) appeared to be responsible for the mechanism of resistance, however, we independently evaluated each of these possibilities.

**Orotic acid uptake**

To evaluate whether the \(rpy2\) calli were affected in their ability to take up substrate for UMP synthase, the wild-type and the \(rpy2\) calli were plated onto media containing 6-[\(^{14}\)C]-orotic acid. The level of radioactivity in both types of calli was determined with the culture times within a period of 84 hours. There was an initial uptake of approximately 7 pmoles of orotic acid within the first hour then a lower rate of approximately 1 pmoles per hour of orotic acid taken up (Figure 3.15.). This rate of uptake did not differ significantly between the
Figure 3.15. The rate of $^{14}$C-labelled orotate import from culture media into the *N. plumbaginifolia* cells. The opened-circle line is indicating the rate in hNp28 (the wild type), the closed-circle curve is for hNp28-umps820, the opened-square curve is hNp28-umps82, and the closed-square curve is for hNp28-umps83.
wild type and the FOA-resistant calli. From these results we concluded that the 
\textit{rpy2} calli are unaffected in their ability to take up \textsuperscript{14}C-labeled orotic acid.

\textbf{Affinity to bind FOA}

To evaluate whether there is altered specificity of the UMP synthase enzyme for FOA, we partially purified the UMP synthase enzyme (Santoso and Thornburg, submitted) from both the wild type and the \textit{hNp28-umps820} calli. We then used this partially purified enzyme to determine inhibition constants of these enzymes for FOA. As can be seen in Table 3.7, the inhibition constants of the wild type enzyme and the \textit{hNp28-umps820} enzyme do not differ significantly. Thus, the mechanism of resistance to FOA cannot be attributed to altered specificity of the enzyme for the toxic compound.

\textbf{Is FOA detoxified in the cells?}

Detoxification of FOA could occur by one of several mechanisms. First, replacement of the fluorine atom with a hydrogen would yield orotic acid, but enzymes catalyzing dehalogenation are extremely rare (Mohn and Tiedje, 1992) and to our knowledge are unknown in plants. Second, destruction of the pyrimidine ring might yield non-toxic products. Degradation of the pyrimidine ring occurs via 5 different pathways (Wasternack, 1978), however, all but one of these pathways utilizes uracil (or thymine) as the initial substrate and thus, would be unlikely to lead to the detoxification of FOA. The reductive pathway is the major pathway of pyrimidine degradation. In a few microorganisms such
Table 3.7. Kinetics parameters of the *rpy2* and the wild type calli. The substrate was orotic acid and the inhibitor is FOA, $n = 4$.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>wild type</th>
<th><em>rpy2</em> cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>$0.68 \pm 0.14$</td>
<td>$1.18 \pm 0.18$</td>
</tr>
<tr>
<td>$K_m$</td>
<td>$1.22 \pm 0.28$</td>
<td>$1.24 \pm 0.30$</td>
</tr>
<tr>
<td>$K_i$</td>
<td>$1.15 \pm 0.18$</td>
<td>$1.12 \pm 0.13$</td>
</tr>
</tbody>
</table>
as *Clostridium oroticum*, there is an additional reductive pathway starting from orotic acid. This pathway, which is found in organisms that overproduce orotic acid, is essentially the reverse of the biosynthesis pathway. However, this pathway is unknown from plants. Finally direct decarboxylation of orotic acid to uracil might also occur. This pathway is well known in many bacteria, but again, unknown from plants. Thus, none of these pathways seemed likely to occur in our cell lines.

We therefore considered the possibility that a hitherto for undescribed decarboxylase might be responsible for the direct conversion of orotic acid into uracil. To evaluate this possibility, we utilized a thin layer chromatography assay to monitor whether orotic acid was converted directly into uracil. This assay is shown in Figure 3.12. The positions of migration of orotic acid, OMP, UMP and uracil are indicated at the right of the figure. In none of these cases is any significant amount of [\(^{14}C\)]-labeled orotic acid converted directly into uracil. In each case, the major products are OMP and UMP. Lanes 1 and 2 show extracts from FOA-selected callus and the wild type of *N. tabacum*. Quantification of these results demonstrate that extracts of the wild-type *N. tabacum* containing 100 \(\mu\)g of protein convert 62% of the orotic acid into UMP, not free uracil. The mutant *NtTr25-umps121*, however converts only 6% of the orotic acid into UMP, confirming our earlier results that these *N. tabacum* mutants had reduced levels of UMP synthase (Santoso and Thornburg, 1993).

In contrast to the *N. tabacum* callus lines, lanes 3, 4, and 5 (Figure 3.12) show extracts of wild type and mutant *N. plumbaginifolia* calli. Similarly to the wild-type of *N. tabacum*, the wild type of *N. plumbaginifolia* can convert
approximately 75% of the orotic acid into UMP, not free uracil. However, the $Np28-umps820$ and $Np28-umps822$ calli convert >90% of the orotic acid into UMP again with no conversion into free uracil. Thus, degradation of the orotic acid into uracil seems unlikely. These results utilizing a different enzyme assay from the decarboxylation assay used in Table 3.5, independently confirm that these callus lines show elevated levels of UMP synthase enzymatic activity.

**Up regulation of UMP biosynthesis pathway**

One way to overcome the toxic FOA is that cells dilute the effect of FOA by increasing the cellular level of the competing substrate orotic acid. Inside the cell, orotic acid can be increased by up regulating the pathway of orotic acid synthesis. To investigate the occurrence of this possibility, all the enzymes required for the orotic acid biosynthesis on the FOA-resistant cell lines were also tested.

Figure 3.16 compares the enzyme activities for several of the pyrimidine biosynthetic enzymes in wild type and the $Np28-umps820$ callus lines. These data are normalized to wild type activity. As can be seen, both UMP synthase and ATCase activities are up-regulated in the $Np28-umps820$ calli relative to the wild type, h$Np28$, calli. Further, this up-regulation for each of these enzymes is to approximately the same level. The level of DHOase and DHODH are not significantly different from wild type. Thus, we conclude that selection of these calli on FOA results in the up-regulation of multiple, but not all steps in the pyrimidine biosynthetic pathway. These results are indicative of a lesion which resembles known regulatory in nature that coordinately affect multiple steps in a single pathway. Therefore, we concluded that this group of calli has a lesion which we have termed *rpy2* for regulation of pyrimidine biosynthesis.
Figure 3.16. The activity of enzymes required for de novo UMP biosynthesis in the \textit{rpy2} mutant and the wild type calli. The solid bars are those hNp28-\textit{umps}820 (\textit{rpy2}) line, and the stripped-bars are of the corresponding wild type line. The quantitated levels of wild type enzymatic activities are presented in Table 3.6.
Enzymatic Activity
(% of wild-type)

ATCase
DHOase
DHO DH
UMPSase
Correlation between UMP synthase activity and the amount of the protein

Initially, to determine whether the high level of UMP synthase activity was caused by increasing amount of the UMP synthase protein, we used Western blot analysis of protein present in the wild type and the \( rpy2 \) callus lines using the specific UMP synthase antiserum. The antiserum was raised against summer squash, \textit{Cucurbita pepo}, UMP synthase. Because the culture condition of the FOA-selected and the wild type calli are different therefore before assaying their UMP synthase activities, they were preconditioned by culturing the calli on the same media, without or with FOA for 10 to 14 days. For these experiments uracil was not included in the media.

Figure 3.17, Panel A shows the level of UMP synthase enzyme activity present in the wild type and the \( rpy2 \) callus lines in the absence and presence of FOA. The level of UMP synthase enzyme activity in the \( rpy2 \) callus line is elevated relative to the level of enzyme activity present in the wild type \( hNp2S \) callus line (compare lanes 1 and 3). This figure also demonstrates that UMP synthase enzyme activity is elevated in wild type callus in the presence of FOA. However, FOA has no effect on the level of enzyme activity in the \( rpy2 \) callus line.

The western blot analysis (Panel B) demonstrates that the level of UMP synthase protein present in the wild type and \( rpy2 \) callus lines correlates with the level of the UMP synthase enzyme activity. Because the amount of UMP synthase protein changes in the wild type calli in response to FOA and these changes correlate with enzyme activity, we concluded that this increase in the
Figure 3.17. The levels of UMP synthase from the tobacco wild type and the hNp28-umps820 mutant calli. Panel A is UMP synthase activity of the calli assayed with the CO$_2$ release method. Panel B is the Western blot analysis UMP synthase protein from the calli. In both panels, lane 1 is protein from the wild type callus precultured in media without FOA for 13 days, lane 2 is from the wildtype precultured in the media with the FOA, lane 3 is from hNp28-umps820 mutant without FOA, and lane 4 is hNp28-umps 820 precultured with FOA.
activity of UMP synthase in response to FOA is not regulated post-translationally.

**Gene amplification**

To examine whether the increased level of UMP synthase in the *rpy2* calli showed signs of gene amplification we compared the wild type and mutant lines by southern blot analysis. The probe used for these studies was a *N. tabacum* UMP synthase cDNA. This cDNA recognizes a single 9.2 kb band in *EcoRI* digests of *N. plumbaginifolia* DNA and two bands of 23.6 and 11.2 kb in *BamHI* digests (Maier, et al., in press). Hybridization analysis demonstrated that there was no significant difference between the wild-type and mutant callus lines (Figure 3.18). This experiment indicated that gene amplification as occurred in cells tolerant to methotrexate, was not responsible for the increase in UMP synthase levels in these UMP synthase-overexpressing callus lines.

**Regulation of UMP synthase of the *rpy2* cell line**

Figure 3.18 indicates that the increase in UMP synthase protein of *rpy2* cell line was not due to amplification of UMP synthase gene. From this one can infer that the increase on the UMP synthase comes from the regulation of the UMP synthase gene expression.

To examine if this is what happening in the *rpy2* cell line, we performed northern blot analysis of the transcripts isolated from the mutant and compared it to the wild type. These studies are presented in Figure 3.19. Panel A shows again that the level of UMP synthase mRNA is higher in the *rpy2* callus line relative to the wild type line (Compare Lanes 1 and 3). Quantification of these
Figure 3.18. Southern blot analysis of the genomic DNA of tobacco. Probe used in this analysis is $^{32}$P-labeled DNA synthesized using UMP synthase cDNA from tobacco. The detail hybridization is described in Materials and Methods. Lane 1 is the DNA isolated from the wild type callus, lane 2 and 3 are from the tobacco mutant calli having elevated level of UMP synthase, hNp28-umps820 and hNp28-umps822 respectively.
Figure 3.19. The Northern blot analysis of transcripts isolated from the wild type and the hNp28-umps820 mutant calli. Panel A, lanes 1 to 4 are after hybridization, lanes 5 to 8 ethidium bromide gel RNA before hybridization. Panel B is radioactivity of lanes 1 to 4 of panel A. Panel A lane 1 (A1), A5, B1 are from the wild type callus without FOA preculture. A2, A6, B2 are from the wild type callus with FOA preculture. A3, A7, B3 are from hNp28-umps820 mutant callus without FOA preculture. A4, A8, B4 are from hNp28-umps820 mutant callus with FOA preculture.
bands by cutting the bands and liquid scintillation counting (Panel B) reveals that there is almost 3 times the level of UMP synthase mRNA present in the \textit{rpy2} callus line as there is in the wild type callus line. Again FOA causes an increase in the level of UMP synthase mRNA present in the wild type callus line (compare lanes 1 and 2).

**UMP synthase mRNA transcription**

Another point where a gene expression may be regulated is at the level of mRNA transcription. The northern blot analysis alone does not determine whether the observed increase in expression of UMP synthase is regulated at the level of mRNA transcription, mRNA stability, or even by mRNA processing. Therefore runoff transcription experiments were conducted on FOA-induced wild type, uninduced wild type, and \textit{rpy2} calli to examine the mode of regulation.

RNA slot blot analysis of the RNAs transcribed from isolated nuclei of FOA-induced and uninduced wild type calli suggested that the regulation UMP synthase gene expression occurs at the transcriptional level (Figure 3.20). Similar to the results of northern blot analysis, RNA slot blot analysis of wild type callus detected more UMP synthase RNA in FOA-induced nuclei than that in uninduced nuclei. A similar pattern of banding was also found in the experiment using \textit{rpy2} nuclei versus wild type nuclei. In that experiment, the \textit{rpy2} nuclei showed increased hybridization over the wild type nuclei.

Quantification of the bands on the Figure 3.20 and those on some other replications of this experiment are presented in Table 3.8. The numbers in first two columns are the UMP synthase activity of the calli. The UMP synthase
Figure 3.20. RNA slot blot analysis of the RNAs synthesized from the runoff transcription with the nuclei isolated from the uninduced-wildtype callus (lane 1 and 3) and from the FOA-induced wild type callus (lane 2 and 4). In row TP-05, the membrane-bound RNA (nonlabelled RNA) was transcribed with ratio of the DNA template to the RNA polymerase of 0.5 µg/Unit. In row TP-20, the ratio was 2.0 µg/Unit. The bands on lanes 3 and 4 were repetition of those on lanes 1 and 2 respectively except they were hybridized with a double amount of the $^{32}$P-labeled runoff transcripts. Panel B is ethidium bromide-stained RNA on the membrane before hybrization.
Table 3.8. The levels of UMP synthase mRNA transcribed from FOA-induced and uninduced hNp28 nuclei.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>UMPSase Activity</th>
<th>Hybridization #</th>
<th>Radioactivity</th>
<th>DNA/Polymerase Ratio(μg/U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninduced mUnit</td>
<td>Induced mUnit</td>
<td>Uninduced cpm</td>
<td>Induced cpm</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>330 100</td>
<td>714 216</td>
<td>43 100</td>
<td>101 234</td>
</tr>
<tr>
<td>2</td>
<td>208 100</td>
<td>625 251</td>
<td>371 100</td>
<td>960 259</td>
</tr>
<tr>
<td>3</td>
<td>270 100</td>
<td>672 248</td>
<td>47 100</td>
<td>93 198</td>
</tr>
</tbody>
</table>

^X±SD = 238±19; ^X±SD = 237±59
activities were determined before the isolation of nuclei. The values of UMP synthase activities and the radioactivities were normalized to that of the uninduced activities. The average value of the radioactivities is very similar to that of the UMP synthase activities. These results indicate that the observed increase in expression of UMP synthase is regulated at the level of mRNA transcription.

Regulation of UMP synthase gene expression

Kinetics induction of UMP synthase by FOA

Because previous experiments had demonstrated that UMP synthase was induced in cell lines grown in the presence of FOA, we decided to examine more closely this process of induction. Therefore an FOA induction experiment was performed. In a previous study we demonstrated that killing of the tobacco cell by FOA follows a hyperbolic curve. By increasing the concentration of FOA in the medium, the relative growth of the cells decreases. Because of this effect we want first to determine the optimum concentration of FOA for the UMP synthase induction purpose. We measured the UMP synthase activity of the wild type callus after culture for 13 days at various levels of FOA. The result presented on Figure 3.21 shows that the optimum concentration of FOA is about 0.12 mM. Using this concentration we determined the kinetics of induction of UMP synthase by FOA. Uracil was not included in this experiment.

Figure 3.22 indicates that after about 7 days in the FOA-containing media, as the time of the culture increased, the UMP synthase activity increased drastically until reaching the peak at about 11 days. After that time if the callus
Figure 3.21. The growth and UMP synthase activity of the wild type callus after being cultured for 13 days in the presence of various concentrations of FOA. The open circle points represent the UMP synthase activity. The solid circles indicate the growth of the callus. UMP synthase activity was determined with CO$_2$ release assay and the relative growth was measured as described by Santoso and Thornburg (1992).
Figure 3.22. Regulation of tobacco UMP synthase by FOA and thymine. Panel A indicates UMP synthase activity of the wild type callus after being cultured for several days in the MS medium without any additional supplement (solid squares), with 0.12mM of FOA (solid circles), and when 0.2 mM thymine was added to the FOA-containing medium. Panel B is Western blot analysis of the callus extracts using UMP synthase antibody rose against squash UMP synthase. Lane 1 is from the callus of 0 day, lane 2 is from the callus of 6 days in FOA, lane 3 is from the callus of 9 days in FOA, lane 4 is from the callus of 13 day (open circle), and lane 5 is from the callus of 13 days in FOA (closed circle).
is kept longer on that media, its UMP synthase start to slowly decrease. In the absence of FOA, however, the levels of UMP synthase in the callus did not change significantly. At the time that the induction is high, 9 days, addition of thymine to the culture dramatically decreases the UMP synthase activity (Panel A). Western blot analysis of the extract prepared from the cultured calli (shown Pin Panel B) demonstrates that the change of UMP synthase activity clearly corresponds to the level of the UMP synthase protein. This results suggested that FOA induces the expression of UMP synthase gene. Furthermore, the induction very likely to be mediated through the cellular level of thymine.

This result suggested that FOA induces the synthesis of UMP synthase. This induction seems to be mediated by the level of thymine metabolite. In the presence of FOA, thymidylate synthase is inhibited (Zubay, 1988; Kalpaxis et al., 1991). This inhibition drops the cellular level of thymine because the cellular pool of thymine is used up by cells to maintain their integrity and to grow. The low level of thymine signals cells to make more thymine by synthesizing more key enzymes for the biosynthesis of thymine or the precursor.

**Induction of UMP synthase by DHFR inhibitor**

To independtly evaluate whether thymine depletion was responsible for UMP synthase up-regulation, we investigated the effect of the culture containing other DHFR inhibitors on the UMP synthase activity. DHFR inhibitors are known to block the synthesis of thymine nucleotides by limiting the regeneration of dihydrotetrahydrofolate. This compound which is a one-carbon donor, is required for synthesis of dTMP from dUMP. Panel A of Figure 3.23 shows that both aminopterin (AMT) and methotrexate (MTX) induced the UMP synthase activity of the wild type callus.
Figure 3.23. The effect of DHFR's cultures on the UMP synthase activity of the tobacco calli. Numbers inside the bars represent preculture time in days.
Panel A: The open bars represent UMP synthase activity in the absence of inhibitors (0 day). Hatched bars represent UMP synthase activity in the presence of 0.2 mM aminopterin. Stippled bars represent UMP synthase activity in the presence of 0.2 mM methotrexate at the indicated times. Panel B: open bars represent UMP synthase activity of wild type hNp28 calli in the presence of 0.2 mM methotrexate at the indicated times. Hatched bars represent UMP synthase activity of the hNp28-umps820 (rpy2) cell line in the presence of 0.2 mM methotrexate at the indicated times.
When the experiment was repeated with inclusion of the \textit{rpy2} callus, again, the DHFR inhibitor induced the UMP synthase of wild type callus but not the \textit{rpy2} callus (Panel B of Figure 3.23). In the \textit{rpy2} the inhibitor even reduces the expression of UMP synthase. This is thought to be due the very toxic nature of the inhibitor on the somewhat unhealthy \textit{rpy2} callus. The DHFR inhibitor is very toxic to cells. It inhibits not only the synthesis of TMP but also the synthesis of purine nucleotides.

**Repression of UMP synthase by thymine**

To confirm that thymine decreases the UMP synthase in the wild type as shown on Figure 3.22, the wild type callus was cultured on the media with thymine as the only supplement. Because the \textit{rpy2} callus has been shown to behave differently from the wild type in this regard we also included the \textit{rpy2} callus in this experiment for the control purpose.

As presented on Figure 3.24, adding thymine alone to the culture also decreases UMP synthase activity of the wild type callus but not that of the \textit{rpy2} callus. Even though the effect thymine on the wild type was not as strong as when adding thymine at the point where UMP synthase of the wild type is highly induced (see Figure 3.20), comparison to its effect on the \textit{rpy2} callus strongly suggests that thymine indeed represses the UMP synthase of the wild type callus.
Figure 3.24. The effect of thymine culture on the UMP synthase activity of the wild type and hNp28-umps820 mutant calli. After being cultured for 3 days, their UMP synthase activities were assayed. The open bars are from calli precultured without thymine. The stripped bars are the preculture with 0.2 mM thymine. The solid bars are with 20 mM thymine.
CHAPTER 4. DISCUSSION

UMP synthase purification

We have evaluated a variety of plant tissues for their expression of UMP synthase. Squash fruit was found to have very high levels of UMP synthase activity therefore we chose this tissue for isolation of UMP synthase protein. We purified UMP synthase more than 2000 fold from crude extract of squash fruit. Among the purification steps employed in this study, the affinity chromatography step using Cibacron Blue column gave the highest level of purification. When the protein preparation that was eluted from the Cibacron Blue column by 0.08 mM OMP was electrophoresed on SDS polyacrylamide gels one 50 kDa band was apparent. To prepare antiserum, this band was extracted from preparative SDS polyacrylamide gel electrophoresis. The antiserum was able to specifically immunoprecipitate UMP synthase activity in a dose dependent manner. Use of this antiserum indicated the presence of UMP synthase in a variety of plant species.

The C. pepo UMP synthase is typical of UMP synthases from other higher eukaryotes. The monomer molecular mass of the UMP synthase is 50 kDa, and the native protein migrates on Sephadex G-150 as a 110 kDa peak. Therefore it is likely that the native protein exists as a dimer of 50 kDa subunits. This is similar in size to the previously purified mammalian (Jones, 1980) and tomato (Walther et al., 1984) UMP synthases.

UMP synthase catalyzes the last two steps of pyrimidine biosynthesis, the transfer of ribosylphosphate to orotic acid to form OMP and the decarboxylation of OMP to form UMP. Each of these activities of the squash UMP synthase was analyzed for the enzymatic stability, pHs, temperature optima, substrate
kinetics, and feedback inhibition properties. As has been found with other UMP synthases (Jones et al., 1978; Walther et al., 1984) the squash enzyme is labile and is not stable in storage. This storage apparently results in the proteolytic cleavage of the UMP synthase enzyme because a 38 kDa immunoreactive protein band accumulates with the loss of enzyme activity during storage.

Inhibition of the UMP synthase was examined by a wide variety of nucleotides. In general, all nucleotides tested showed inhibitory activity at high concentrations, but at lower concentrations, the nucleotides are not potent inhibitors of UMP synthase. The overall regulation of the *C. pepo* UMP synthase with respect to those pyrimidines tested appears to be similar to that observed in *S. cerevisiae*, where feedback regulation does not occur at either the OPRTase or ODCase steps, but at the earlier steps in the *de novo* biosynthetic pathway (Lacroute, 1968). We are currently investigating the regulation of this pathway in higher plants.

A Western blot analysis indicates that the anti-UMP synthase crossreacts with UMP synthases from a wide variety of plant species. These include species from the Brassicaceae, Cucurbitaceae, Graminaceae, Leguminosae, Solanaceae, and Umbelliferae families. In all cases, the denatured proteins show molecular masses of about 50 kDa. This size seems to be typical of UMP synthases isolated from plant (Santoso and Thornburg, 1992; Walther et al., 1984), microbial (Walther et al., 1981) and animal origin (Jones et al., 1978). Because this antiserum is immunoreactive with several species of tobacco, we were using this antiserum to characterize a variety of FOA-resistant callus lines which we have isolated from tobacco.
FOA-selected cell lines

Over the past five years, we have produced a large number of callus lines which have been selected for their ability to grow in the presence of FOA plus uracil. Biochemical characterization of these lines has demonstrated that there are several distinctly different phenotypic classes represented among our FOA resistant isolates. Some of these classes appear to affect only the UMP synthase enzymatic activity, while others appear to be much more complex. However, a common thread among the majority of the FOA resistant calli isolated to date is reduced level of expression of UMP synthase activity. One unusual phenotypic class of FOA resistant calli, however, which represents 14% of our *N. plumbaginifolia* isolates has elevated rather than reduced levels of UMP synthase.

To better comprehend the mechanisms of toxicity of FOA to plant cells, and to better understand the types of lesions which selection of FOA provides, we decided to fully characterize the *rpy2* phenotypic class of FOA-resistant calli. In this study, we mostly used the *hNp28-umps820* cell line for the *rpy2*.

Initially, it was unclear whether overexpression of UMP synthase was the root cause of the resistant phenotype or whether the resistant phenotype resulted in overexpression of UMP synthase. Hence, we began characterization of this class of FOA resistant calli. First we determined that the wild type and the resistant calli did not differ in either the uptake of labeled orotic acid, or in the recognition of the substrate by partially purified UMP synthase. Due to the nature of the biochemistry involved in pyrimidine metabolism, detoxification mechanisms for FOA seemed unlikely.
Because none of these mechanisms seemed responsible for FOA resistance, we decided to examine the expression of UMP synthase in more detail. Western and northern blot analysis of the wild type calli demonstrated that both the UMP synthase protein and the UMP synthase mRNA are expressed at low levels. However, in the rpy2 calli the UMP synthase protein and the UMP synthase mRNA are constitutively expressed at high levels proportional to the enzymatic activity.

When we tested for UMP synthase from wild type calli grown in the presence and absence of FOA for 12 days, the level of UMP synthase activity, protein and mRNA increased to near the levels in the rpy2 calli. These data imply that the addition of FOA was altering the regulation of UMP synthase. In contrast, FOA had no effect on the expression of UMP synthase from the rpy2 callus lines indicating that these calli were already expressing UMP synthase at maximum levels. Additional evidence that this lesion is a regulatory lesion comes from the fact that several, but not all enzymes are coordinately regulated.

There have been two mechanisms of toxicity proposed for FOA, destabilization of RNA and thymine starvation. It is likely that both mechanisms contribute to the toxic effects of FOA. However, the second mechanism (thymine starvation) appears to be the mechanism which the wild type cells respond to in our studies. Considering our experiments in which the levels of UMP synthase are induced in the presence of FOA, it appears that this represents the normal cellular response to thymine starvation.

Northern blot analysis demonstrated that the regulation of the synthesis of UMP synthase occurred at the level of mRNA. This observation led us to investigate whether the induced level of UMP synthase mRNA is regulated at
the level of transcription or mRNA stability. Our runoff transcription experiments using FOA-induced and uninduced nuclei isolated from the wild type callus, suggested that regulation of UMP synthase gene expression by FOA or thymine occurs at the level of its transcription.

Based on the experimental evidence contained herein, we have proposed the existence of a regulatory protein which we term RPY2 that is involved in the regulation of several steps in the pyrimidine biosynthetic pathway. We recognize that there are at least two independent functions which could be affected to give rise to the \emph{rpy2} phenotype.

First, the detection of intracellular concentrations of thymine in \emph{rpy2} cells could be affected. Either the regulatory protein of \emph{rpy2} cells has been altered so that it is unable to interact with thymine or \emph{rpy2} cells have been altered causing reduction in the synthesis of the regulatory protein. Several examples of this type of regulation are known in \emph{GAL} genes of \emph{S. cerevisiae} (Johnston, 1987), photosynthetic reaction center and light-harvesting genes of \emph{Rhodobacter capsulatus} (Sganga and Bauer, 1992), $\alpha$-prolamin genes of Maize (Yunes et al., 1994), anthocyanin pigmentation of plant species (Quattrocchio et al., 1993). Perhaps, other functions such as transmission of information to a promoter which involves protein-protein interactions and protein-DNA interactions have been affected.

Post-transcriptional regulations could involve in gene expression as reported in some other systems such as, light regulation of rubisco gene expression in \emph{Euglena} (Keller et. al, 1991), sucrose synthase in anaerobic seedling of maize (Taliercio et al., 1989), an insulin like growth factor in diabetic rat liver (Ooi et al., 1992). Nevertheless our runoff transcription experiment
suggested that the increased UMP synthase either in the *rpy2* callus or in the FOA-induced callus of the wild type is not due to post-transcriptional regulation. From these analyses it appears that the mechanism of regulation is at the level of the gene transcription.

We further conclude that the primary mechanism of toxicity in *N. plumbaginifolia* for FOA is through thymine starvation. Destabilization of mRNA is not the primary mechanism of the FOA toxicity. UMP synthase converts FOA into FUMP. By the following step, FUMP is reduced to FdUMP which is a suicide inhibitor of thymidylate synthase. When thymidylate synthase is inhibited, the availability of thymine for cells to grow is limited.

Figure 3.16 indicates that the activities of ATCase and UMP synthase of the *rpy2* are about twice as much as those of the wild type. The increase of these key enzymes may up regulate the flow of the metabolites in the UMP de novo biosynthesis. Such an up regulation of the pathway could lead to survival in the presence of FOA plus uracil by producing higher levels of cellular orotic acid. More orotic acid decreases the competition of FOA for the UMP synthase enzyme. Thus more orotic acid would be converted to UMP while less FOA was converted into FUMP by UMP synthase. Subsequently, the toxic effects of FUMP and FdUMP would be diluted.

We have observed that UMP synthase is normally expressed at very low levels in wild type *hNp28* plant cells, however, the addition of FOA induces UMP synthase. Several of our selected callus lines do not show this inducible mode of UMP synthase expression. These lines constitutively express UMP synthase at high levels, and UMP synthase is not induced when we add FOA to the media.
The cellular response to thymine starvation is to up-regulate several members of the \textit{de novo} pyrimidine biosynthetic pathway. Those members which are up regulated include ATCase (the first committed step of pyrimidine biosynthesis), UMP synthase (the rate limiting step) and Thymidylate synthase (a crucial step for thymidine biosynthesis). Other enzymes may also be up regulated, but DHOase and DHODH are not. When high levels of thymine are provided to the cells following the induction of the pathway, the level of the induced enzymes returns to low levels. Thus, these cells have some mechanism to detect the level of thymine in the cell and to communicate that information to the promoter of the UMP synthase gene and probably to the promoters of other genes in this pathway. Because multiple enzymatic steps are coordinately regulated, we propose the existence of at least one regulatory protein which is involved in this regulatory process. We propose that proteins must be capable of detecting the level of thymine (perhaps by binding thymine or a thymine metabolite [TMP, TDP, or TTP]). Subsequently they modify the expression of ATCase, UMP synthase, Thymidylate synthase, and perhaps other members of this pathway. Runoff transcription analysis indicates that this regulation takes place at the transcription level. It is unclear whether this process is mediated by a single factor as in the case of vertebrate steroid receptors (Gronemeyer, 1991; Kumar et al., 1987) or whether multiple factors are involved as in the case of the \textit{GAL} genes of \textit{S. cerevisiae} regulation (Johnson, 1989). It is clear that the \textit{rpy2} lesion is blocked in this process but it is not clear whether the \textit{rpy2} lesion is blocked at the ability to detect intracellular thymine or whether this lesion is blocked downstream of this point. Perhaps it is blocked at protein-protein interaction or a protein-DNA interaction.
Our investigation on the UMP synthase of *N. plumbaginifolia* suggested that FOA through thymine limitation induces the UMP synthase synthesis. This observation is supported by other experiments with similar purposes. DHFR inhibitors which limit the synthesis of TMP by inhibiting regeneration of dihydrotetrahydrofolate also induce the UMP synthase. Nevertheless, when thymine is included in the culture media UMP synthase is suppressed. This phenomenon is found in the wild type callus, but not in the *rpy2* callus.

Thus FOA and thymine regulation of UMP synthase synthesis leads to an interesting hypothesis that is summarized on Figure 4.1. It can be explained as follows; In the culture without FOA, orotic acid by a series of enzymatic reaction is converted to thymine metabolites. One of these metabolites, at high levels may inhibit the transcription of UMP synthase gene by interacting with regulatory protein, RPY2. This interaction produces a complex that somehow inhibits the transcription. In the presence of FOA, thymidylate synthase is irreversibly inhibited by FdUMP. The inhibition of thymidylate synthase decreases the thymine metabolites synthesized in this pathway. Less thymine decreases the formation of the inhibitory complex and finally relieves the blocking of the UMP synthase synthesis. This is why in the presence of FOA, UMP synthase is higher than in the absence of FOA. If we add thymine in the culture, again the inhibitory complex is formed and inhibits the gene expression, thus we observe a decrease UMP synthase expression.

Figures 3.17, 3.22, 3.23, and 3.24 all indicate that unlike the wild type, the *rpy2* calli is insensitive to FOA, thymine or DHFR inibitor, its UMP synthase level is not affected by the presence of those metabolites. In both the absence or in the presence of these metabolites, the UMP synthase level of the *rpy2* callus
Figure 4.1. Proposed mechanism of the regulation of UMP synthase gene expression in the wild type through RPY2 regulatory protein. In the absence of FOA, RPY2 protein interacts with a thymine metabolite to form a complex that somehow slow down the expression of the UMP synthase gene by inhibiting the transcription. In the presence of FOA, the level of the thymine metabolite decreases which limits the amount of the inhibitory complex. The inhibition by the complex is relieved, synthesis of UMP synthase goes up.
remains, high. This implies that UMP synthase gene of the \textit{rpy2} callus is constitutively expressed. It is likely that this change occurs at the regulatory protein RPY2, not the UMP synthase gene itself. One of the direct reason leading to this assumption is the data showing that both the UMP synthase and the ATCase of \textit{rpy2} callus are high (see Figure 3.16).

Regardless of the presence of FOA, thymine, or DHFR inhibitor, the UMP synthase of the \textit{rpy2} callus remains, high. This is because the \textit{rpy2} callus was somehow altered such that it does not produce the RPY2 protein, or produces the RPY2 protein but a defect causes inability to interact with the thymine metabolite. Thus in the \textit{rpy2} callus, the inhibitory complex is lower than in the normal wild type therefore the UMP synthase is synthesized constitutively at high rates.
CHAPTER 5. CONCLUSIONS

FOA-selection of NMU-induced mutation of tobacco cells produced at least three classes of cell lines. Cell line of rpy1 having reduced levels of ATCase, DHOase and UMP synthase. Cell line of rpy2 having elevated levels of both ATCase and UMP synthase, and UMP synthase lesion having only reduced UMP synthase. Further characterization of high-UMP synthase cell lines suggested that the rpy2 cells has been mutated in a regulatory protein that seemed no longer responsive to the cellular level of a thymine metabolite.

The mechanism of FOA toxicity in N. plumbaginifolia is primarily through inhibition of thymidylate synthase by FdUMP. This inhibition limits the level of thymine nucleotides.

The UMP biosynthetic pathway in N. plumbaginifolia is coregulated at ATCase and UMP synthase by regulatory protein RPY2 that responsive to the cellular level of thymine. It is up-regulated at a low level of thymine and inhibited by a high level of thymine.

Antibody raised against UMP synthase purified from squash has reactivity to wide variety of plant species. In all cases UMP synthase of plant sources tested have similar size, 50 kDa.
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APPENDIX A: ABBREVIATIONS

AMP = adenosine-5'-monophosphate
AMT = aminopterin
ATCase = aspartate transcarbamoylase
BAP = benzyl-amino purine
CMP = cytidine-5'-monophosphate
CPSase = carbamoylphosphate synthase
DEAE = diethylaminoethyl
DHFR = dihydrofolate reductase
DHOase = dihydroorotase
DHO DH = dihydroorotate dehydrogenase
DNA = deoxyribonucleic acid
EMS = ethyl-methyl sulfonate
FdUMP = 5-fluoro deoxyuridine-5'-monophosphate
FOA = 5-fluoroorotic acid
GMP = guanosine-5'-monophosphate
IMP = inosine-5'-monophosphate
MNNG = N-methyl-N'-nitro-N-nitroso guanidine
MS = Murasige and Skoog
MTX = methotrexate
NAA = naphthalene acetic acid
NMU = N-nitroso-N-methylurea
ODCase = orotidine-5'-monophosphate decarboxylase
OMP = orotidine-5’-monophosphate
OPRTase = orotate phosphoribosyl transferase
PAGE = polyacrylamide gel electrophoresis
PRPP = phosphoribosyl pyrophosphate
RNA = ribonucleic acid
SDS = sodium dodecyl sulphate
TLC = thin layer chromatography
TMP = thymidine-5’-monophosphate
UDP = uridine-5’-diphosphate
UMP = uridine-5’-monophosphate
UPRTase = uracil phosphoribosyl transferase
UTP = uridine-5’-triphosphate
XMP = xanthosine-5’-monophosphate
### APPENDIX B: PLANT MUTANTS

Some previously isolated plant mutants using tissue culture techniques

<table>
<thead>
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<th>Phenotypes *</th>
<th>System, species</th>
<th>Mutagen</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
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<td>Cells,</td>
<td>NEU</td>
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<td>-</td>
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<td>Cells, <em>O. sativa</em></td>
<td>-</td>
<td>Scaeffer (1981)</td>
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<td>Leucine</td>
<td>Protoplast, <em>N. plumbaginifolia</em></td>
<td>UV light</td>
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* * = resistant, * = sensitive, a = auxotroph
APPENDIX C: COMPOSITION OF BASAL MEDIA

The composition of the two major basal media employed in the plant tissue cultures

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<td>CuSO₄.5H₂O</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>0.75</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>27.8</td>
<td>-</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>37.3</td>
<td>-</td>
</tr>
<tr>
<td>EDTA Na Ferric salt</td>
<td>-</td>
<td>43.0</td>
</tr>
<tr>
<td><strong>Vitamins:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine.HCl</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Pyridoxine.HCl</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30,000</td>
<td>20,000</td>
</tr>
</tbody>
</table>
APPENDIX D: THE MAP OF pRT 327

pRT 327
3.2 kb

pBluescript II SK (+) → EcoRI

UMPS cDNA → pRT 327

NsiI

pRT 327