Isolation and Characterization of UMP Synthase Mutants from Haploid Cell Suspensions of *Nicotiana tabacum*

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ABSTRACT

Uridine 5'-monophosphate (UMP) synthase mutants of tobacco have been produced from haploid cell-suspension cultures of a transgenic *Nicotiana tabacum* line, Tr25. The mutants were induced by incubating the suspension-cultured cells with 1 mM N-nitrosomethylurea for either 5 or 12 hours. Twenty mutant calli were isolated on selection medium containing 20 milligrams per liter of 5-fluoro-orotic acid. Of those tested, most had reduced regeneration capacity. Characterization of UMP synthase activity in the isolated calli showed that UMP synthase activity varied from 8 to nearly 100% of the wild-type activity. The growth of the calli on the media containing different levels of 5-fluoro-orotic acid correlated with decreasing UMP synthase activity. Because the UMP synthase enzyme has two separate enzymic activities (orotate phosphoribosyltransferase and orotidine-5'-monophosphate decarboxylase), several mutants were further characterized to determine how the mutations affected each of the two enzymic activities. In each case, the enzymic activity affected was the orotate phosphoribosyl transferase and not the orotidine-5'-monophosphate decarboxylase. The wound-inducible phenotype of the Tr25 plants as measured by the activation of the *pin2-CAT* gene remained unchanged by introduction of the UMP synthase mutations.

The Solanaceae contain genes induced locally and systemically in response to wounding of leaf tissue that provide defense against insect attack. The induction mechanisms have been extensively studied using biochemical and molecular biological methods (4, 15–17, 24, 26, 31–34). However, one approach to understanding the activation process that has received little attention to date is a genetic approach. This is due to the difficulty in screening for mutants in the wound induction pathway. In other eukaryotic systems, techniques have been developed for the selection of, rather than screening for, mutants in complex pathways. Among the methods available to select for mutants is the selection against UMP synthase. This scheme has proven to be a useful tool for the selection of mutants in other systems, most notably *Saccharomyces cerevisiae* (2), *Candida albicans* (20), *Aspergillus flavus* (38), and *Dictyostelium discoideum* (12), but such a selection scheme has not previously been applied to plants. Our long-term goal is to develop such a selection scheme in plants for studying wound-inducible genes in the Solanaceae. In this paper we describe the first step in such a selection scheme, the production and characterization of mutants that have reduced levels of UMP synthase. These mutants will be used in the future for selection of mutants that are blocked in the wound-induction pathway. In addition, these mutants should also be useful to understand the biosynthesis of pyrimidines in plants.

UMP biosynthesis is a very highly conserved pathway in both prokaryotes and eukaryotes. In nearly all organisms, six enzymic activities make up the *de novo* biosynthesis of UMP. In prokaryotes, these enzymic activities are encoded by six separate enzymes; however, in higher eukaryotes, these six enzymic activities are encoded by only three genes (11). The last two steps of this pathway are encoded by a single gene that produces one polypeptide termed UMP synthase with two enzymic activities, OPRTase and OMPdeCase (see Eq. 1). In animals (21) and plants (1, 14, 37), both enzymic activities reside on a single UMP synthase polypeptide.

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\text{Orotic Acid} + \text{PRPP} \xrightarrow{\text{OPRTase}} \text{OMP} \xrightarrow{\text{OMPdeCase}} \text{UMP} \xrightarrow{\text{CO}_2} \text{Urea} \]

Mutants that are blocked in the *de novo* UMP biosynthetic pathway can be effectively selected with the antimetabolite, 5-FOA (2). Fluoroorotic acid mimics the UMP biosynthetic intermediate orotic acid in that 5-FOA serves as a substrate for UMP synthase and is converted into 5-fluoro-UMP. The 5-fluoro-UMP is then incorporated into RNA and can also be converted into other nucleotides. The result of the incorporation of 5-fluoro-UMP into nucleic acids is cell death. Although the exact mechanism that leads to cell death is not fully known, clearly those cells that contain a functional UMP synthase will die in the presence of 5-FOA. However, because plants and other organisms contain a salvage path-

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1 Abbreviations: OPRTase, orotidine phosphoribosyltransferase; OMP, orotidine-5'-monophosphate; OMPdeCase, OMP decarboxylase; CAT, choramphenicol acetyltransferase; 5-FOA, 5-fluoroorotic acid; NMU, nitrosomethylurea; NAA, naphthalene acetic acid; MS, Murashige-Skoog plant tissue culture medium; PRPP, 5-phosphoribosyl-1-pyrophosphate.

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way, uracil phosphoribosyl transferase to convert uracil into UMP (28), such mutants can be rescued by the addition of uracil to the medium.

MATERIALS AND METHODS

Chemicals and Plant Material

OPRTase, OMPdeCase, CAT protein, orotic acid, OMP, UMP, uracil, NMU, and PRPP were purchased from Sigma. Media for plant tissue culture and plant hormones were obtained from Gibco Laboratories. 7-[^14]C]orotic acid (52.5 mCi/mmol), 7-[^14]C]orotidine monophosphate (39.6 mCi/mm), and [^14]C]loramphenicol (60 mCi/mm) were purchased from New England Nuclear. Other materials were of the highest purity available and were obtained locally.

The plants used in these studies were an inbred line of Nicotiana tabacum cv Xanthi, previously transformed with a wound-inducible proteinase inhibitor II-CAT (pin2-CAT) construct (31). These plants were previously described (16, 32).

Plant Tissue Culture Methods

Preparation of Haploid Plants

Haploid plants of the tobacco line Tr25 were produced from anther culture by the method of Sunderland (29). Flower buds with the corolla just emerging from the calyx were harvested from plants undergoing their first flowering. After surface sterilization using bleach solution containing 0.5% sodium hypochlorite, anthers were excised from the buds and placed on MS solid medium (22) supplemented with 0.5 mg/L NAA, 0.05 mg/L kinetin, and 30 g/L sucrose. The culture was maintained in an illuminated incubator set to an 18-h day at 26°C and an 8-h night at 18°C. After about 1 month, the germinated microspores reached 5 to 10 mm. The anther-derived plantlets were then transferred to a root-inducing medium (MS solid medium with 30 g/L sucrose but no hormones) and maintained under the same conditions as the previous cultures. The chromosome numbers of the plantlets were examined using the method of Collins (5).

Suspension Cultures

Callus was induced from the haploid plants by placing young leaf and stem pieces onto callus-inducing medium (MS solid medium containing 2 mg/L NAA, 0.5 mg/L benzylaminopurine, and 30 g/L sucrose). The resulting yellowish white, friable, fast-growing callus was then used to initiate cell-suspension cultures. These were begun by placing 1 to 2 g of the callus into a 250-ml Erlenmeyer flask containing 50 ml MS liquid medium supplemented with 0.2 mg/L 2,4-D, 0.1 mg/L kinetin, and 30 g/L sucrose. These cultures were incubated with constant shaking at 120 rpm with 16-h days at 26°C and 8-h nights at 18°C. The suspensions were subcultured every 2 to 3 weeks. Allowing clumped cells to sediment under the influence of gravity and pipetting the free cells to another flask for further growth produced fine cell suspensions.

Mutagenesis and Selection

The tobacco cell suspensions that were in the linear phase of their growth were resuspended at a density of 40 to 60 g of cells/L and exposed to medium containing NMU. After the exposure to NMU, the mutagen was washed away with three changes of fresh culture medium. After the cultures were concentrated to 300 g of cells/L, the cells were plated onto an enriched selection medium (medium A; Table I) modified from that described by Kao and Michayluk (13) containing 20 mg/L (114 μM) 5-FOA and 40 mg/L (367 μM) uracil.

Callus Growth

Unless otherwise stated, mutant calli were maintained on a maintenance medium (medium B; Table I) containing 20 mg/L (114 μM) 5-FOA and 40 mg/L (367 μM) uracil. Calli were subcultured every 2 to 3 weeks. Growth of these tobacco callus lines was monitored by periodically weighing the calli under sterile conditions (27). For each callus line, three to five replicates were preformed for the growth studies. The relative growth of calli and suspension-cultured cells was calculated using the following equation.

Relative growth (%) = \( \frac{W_n - W_0}{W_0} \times 100\% \) (2)

where \( W_n \) is the weight of the callus or the wet weight of the cells from 1 mL of suspension culture at the start of the experiment, \( W_n \) is the weight of the callus or the wet weight of the cells from 1 mL of suspension culture at the \( n \)th day in the culture.

Plant Regeneration

Calli of mutant lines having diameters of 5 to 20 mm were transferred to shoot-inducing medium (medium C; Table I). Shoots usually formed after 3 to 5 weeks of culture on this medium.

Enzyme Assays

UMP Synthase Assay

Plant tissues, either callus or leaf, was weighed, and an equal volume of homogenization buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, and 5 mM DTT was added. Homogenization of plant tissues was performed at 0°C in a small mortar and pestle. Following homogenization, the plant tissues were twice centrifuged at 10,000g for 5 min. Homogenates were stored at -20°C.

UMP synthase was measured using a protocol slightly modified from that described by Walther et al. (36). The enzymic reaction occurred in a 25-ml Erlenmeyer flask plugged with a rubber septum. Reaction mixture (1 ml) contained 20 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 1 mM DTT, and 2 μM 7-[¹⁴]C]orotic acid. To this reaction mixture an extract of callus or whole plants containing 100 μg of total protein as determined by the method of Bradford (3) was added. A 1.5-ml microfuge tube holding a KOH-impregnated filter paper was placed into the flask to trap the [¹⁴]C]CO₂ generated.
Table I. Composition of Media for Selection of UMP Synthase Mutants

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<sup>a</sup> Medium A, Initial selection of mutants; medium B, use with wild-type feeder cultures; medium C, regeneration medium; medium D, root induction medium.  
<sup>b</sup> Salts are either Gamborg's B5 salts (6) or MS salts (20).  
<sup>c</sup> All 20 common amino acids were included in the media. Those not listed were included at a concentration of 0.1 mg/L.  
<sup>d</sup> mL/L. Fresh coconut water was deproteinized by heating to 80°C for 30 min and sterilized by filtration.

by the reaction. After capping the tubes, injection of 8 μL of 2.5 mM PRPP into the reaction mixture started the reaction. The tubes were incubated at 25°C for 15 min. The addition of 4 mL of 2 M perchloric acid stopped the reaction, and the KOH-impregnated filter paper was removed, dried, placed in scintillation fluid, and counted in a Packard model 1500 scintillation counter. For kinetic studies, the concentration of PRPP was varied as shown, and different concentrations of unlabeled orotic acid were added to achieve the indicated orotic acid levels.

**OMPdeCase Assay**

OMPdeCase was determined using a modification of the above procedure for UMP synthase. The reaction mixture was identical with the one for measuring UMP synthase except for the substrate used. For determination of OMPdeCase, 7-[14C]orotidine monophosphate was included at a concentration of 2 μM. Other conditions were as described above.

**Induction of the pin2-CAT gene**

To monitor the wound-inducible phenotype of the pin2-CAT transgene in these plants, we routinely tested the plants for wound-inducible CAT activity by the method of Gorman et al. (8) as described in ref 16. Alternatively, we monitored induction in the presence of high levels of sucrose or in the absence of auxin, both of which are established methods of inducing the wound-inducible pin2-CAT transgene (10, 16).

**RESULTS**

**Isolation of UMP Synthase Mutants**

Because we wanted to combine plants lacking UMP synthase with plants containing a well-characterized wound-
inducible gene, we chose as our starting material a line of transgenic tobacco, Tr25, that contains a pin2-CAT construct (31). These plants show high levels of wound-inducible CAT activity from the transgene and have been well characterized (32).

Preparation of Haploid Plants

Tobacco haploid plants were generated from anthers cultured on MS medium. The plants produced from anther culture were shown to be haploid by counting the chromosomes of root tip squashes. Analyses of these root tip squashes confirmed that the haploid numbers of 24 chromosomes were found in root-tip preparations from these plants.

To demonstrate that these haploid plants still maintained the wound-inducible phenotype, plants were assayed for expression of the CAT protein following either wounding or sucrose induction as earlier described (data not shown). There was not an observable difference between the expression of the pin2-CAT gene in whole plants as a result of ploidy.

Preparation of Cell-Suspension Cultures

Callus lines were prepared from the stem and leaf pieces of the haploid Tr25 plants. We also tested the ability of the pin2-CAT gene to be derepressed in callus in the absence of auxin by plating the haploid calli on auxin-deficient medium (16). We observed that the wound-inducible gene is strongly induced on media deficient in auxin and is not expressed on media containing auxin. Thus, the haploid plant materials responded like the diploid parental material both in whole plants and in callus.

From these fast-growing calli, we prepared a fine suspension culture with a doubling time of about 4 d. We measured the growth of these cells in MS liquid media with various auxin levels. The best growth for these cells was in MS liquid medium supplemented with 0.2 mg/L 2,4-D.

Effect of 5-FOA of Cell Growth

After preparing the suspension culture, we first determined whether 5-FOA was toxic to plant cells. We incubated the cultured cells in media with varying levels of 5-FOA for 10 d. The growth of the cultured cells was measured every 2 to 3 d by weighing the medium-free cells. Figure 1 shows that 5-FOA is indeed toxic to plant cells and dramatically inhibits their growth. From this figure we determined a 50% lethal dosage for fluoroorotic acid on haploid tobacco cells of about 50 μM.

Because we had no guidelines for selection of plant cells with 5-FOA and because fluoroorotic acid is a very toxic compound, we decided to use a concentration of fluoroorotic acid that was in the moderate killing range. Therefore, we chose a concentration of 20 μg/mL (114 μM) for the selections described below.

Mutagenesis and Selection

We initially investigated several concentrations of the mutagen, NMU, and various time periods for the mutagenesis of the tobacco suspension cultures. The treatment with 1.0 mM NMU proved the most satisfactory. Following treatment with NMU for either 5 or 12 h (conditions that resulted in 70–90% cell death), residual NMU was washed out of the cultures with three changes of fresh media. The remaining cells were plated on selection medium in the presence of 5-FOA.

Our first attempts to isolate mutants failed because of the very low density of surviving cells after mutagenesis. The inability of the cells to grow in culture at very low population densities may result from the excessive diffusion of metabolic intermediates into the medium, which results in their dilution within the cell to a level below that required for survival (9). To avoid this problem, we chose a very rich medium for the selection of these mutants (medium A; Table I).

This medium modified from that described by Kao and Michayluk (13) proved useful for growth of plant cells at very low densities. It contains many vitamins, all amino acids, several TCA cycle intermediates, two carbohydrate sources, casein hydrolysate, and liquid endosperm from coconuts. This selection medium also contains Gamborg's B5 salts (7) which are low in ammonium ions. When we used this medium for 5-FOA selection, several mutant calli were obtained.

During the first 6 weeks of selection, the mutant cells grew very slowly. Small calli of the mutants with diameters of approximately 1 mm formed after 5 to 7 weeks of selection. Because of this slow growth rate, the very small calli were removed from fluoroorotic acid selection after 7 weeks. To further enhance their growth, the mutant calli were co-plated with feeder calli to provide extra nutrients. The feeder calli were small pieces of the wild-type Tr25 calli that were placed between the mutant calli on the enriched medium without

Figure 1. Effect of 5-FOA on the growth of the haploid Tr25 cell suspension culture. Growth was measured on the 10th d following the addition of fluoroorotic acid. Cell growth was determined by centrifuging an aliquot of cells, pouring off the liquid medium, and weighing the remaining cells. The relative growth was calculated using Equation 2. Error bars on each point represent the se; n = 4.
fluoroorotic acid selection (medium B; Table I). These feeder calli and the enriched medium enabled the mutant calli to grow much faster. After the selected mutant calli had grown to 3 to 8 mm, they were again transferred back onto media containing 5-FOA. The feeder callus treatment did not decrease the ability of the mutants to grow on media containing 5-FOA.

**Characterization of UMP Synthase Mutants**

**Growth of the Mutants in the Presence and Absence of 5-FOA**

After the mutant callus lines had been established, we measured the relative growth rates of these callus lines and the parental haploid Tr25 callus line on media containing 20 mg/L (114 μM) 5-FOA and 40 mg/L (367 μM) uracil. These concentrations provided a 3.25-fold molar excess of uracil over fluoroorotic acid in these studies. The mutant calli grew at various rates under these conditions, but callus of the wild-type haploid Tr25 control did not grow on this media in the presence of 5-FOA (Fig. 2). The mutant lines have various growth rates on this medium.

In addition to the growth in the presence of 5-FOA plus uracil, we also tested the growth of the mutants in the absence of both fluoroorotic acid and uracil. Under these conditions, the cell lines should grow at a rate limited by their ability to synthesize UMP from the de novo UMP biosynthesis pathway. As shown in Figure 3, the mutants again grew at variable rates. However, in the absence of both uracil and 5-FOA, the wild-type haploid Tr25 callus was the most rapidly growing callus line. The growth rates of the mutant callus lines were approximately the inverse of the growth rates observed in the presence of fluoroorotic acid plus uracil. Therefore, these growth studies either in the presence of the fluoroorotic acid plus uracil, or in the absence of both, correlate with the presence of a defect in the UMP synthase gene in these mutant calli. This defect appears most pronounced in the mutants umps511 and umps121, and we might, therefore, expect that these cell lines would have lower levels of UMP synthase activity than the other mutants.

**UMP Synthase Activity**

To determine whether the observed pattern of growth reflected the UMP synthase enzymic activity of the mutants, we examined both the OMPdeCase and total UMP synthase activities (OPRTase plus OMPdeCase) of all the isolated mutant callus lines. The UMP synthase and OMPdeCase activities of mutant calli are presented in Table II. The relative activities of UMP synthase from these mutants ranged from 8 to 99% of the wild type. From the 20 mutants examined, five of them had low UMP synthase activity, seven had medium activity, and eight had high UMP synthase activity. Never did we ever find calli that were completely deficient in UMP synthase.

It is not clear why the mutants with high levels of UMP synthase activity such as umps122, umps124, and umps516 survived on fluoroorotic acid containing media. Perhaps our selection conditions permitted the escape of some clones. Alternatively, some of these cell lines might have been mutated so that they could not import 5-fluoroorotic acid for use in the de novo UMP biosynthesis pathway. This set of mutants clearly requires further characterization.
Table II. Enzymic Activities of Total UMP Synthase and OMPdeCase of the umps Mutants

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<th>Plant Line</th>
<th>Plant Specific Activity</th>
<th>OMPdeCase Specific Activity</th>
<th>Relative Activity</th>
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<td></td>
<td>UMP synthase</td>
<td>OMPdeCase</td>
<td>UMP synthase</td>
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<td>353 ± 16</td>
<td>76</td>
</tr>
<tr>
<td>umps127</td>
<td>53 ± 12</td>
<td>273 ± 27</td>
<td>56</td>
</tr>
</tbody>
</table>

* One unit, nanomoles of CO₂ released per min at 25°C, pH 8.0.  
* Assay was performed on fast-growing calli after 10 to 20 d of culture on maintenance media.  
* Not determined.

After we had determined UMP synthase activity for these mutants, we chose two of the mutants to determine whether the reduced enzymic activity was maintained stably without selection. We grew both umps121 and umps511 (the mutant lines with the lowest UMP synthase activity) on media lacking 5-FOA but containing uracil. After 2 weeks of growth without fluoroorotic acid, UMP synthase (Fig. 4) remained unchanged from the level found in the presence of fluoroorotic acid. Thus, these UMP synthase mutants were stably maintained alterations even without 5-FOA selection.

The OMPdeCase activities of all the mutants are also presented in Table II. Except for only two lines, umps57 and umps127, the OMPdeCase activity of the mutants did not differ significantly from the wild-type haploid Tr25 control. Some of these mutant lines such as umps52, umps53, and umps55 even showed elevated levels of OMPdeCase activity. The lack of alteration of the OMPdeCase activity was not entirely unexpected because the transferase activity of the UMP synthase enzyme and not the decarboxylase is rate limiting (see below).

Kinetic Analysis of the UMP Synthase Enzyme from Selected Mutant Calli

To verify further that the phosphotransferase and not the decarboxylase activity was the affected enzymic activity, we performed Lineweaver-Burk kinetic analysis on three of the mutants (umps511, umps121, and umps52). We chose mutant lines umps121 and umps511 because they had the lowest UMP synthase activity at 8 and 16% relative to the haploid Tr25 levels, and umps52 was the only mutant line capable of regenerating plants (see below).

UMP synthase catalyzed the last two steps of de novo UMP biosynthesis (see Eq. 1). The assay for UMP synthase uses a coupled enzyme assay that measures both the OPRTase and the OMPdeCase activities. Because UMP synthase channels OMP from the transferase site to the decarboxylase site (11), it is difficult to measure the phosphotransferase without also measuring the decarboxylase. However, one can perform kinetic analysis on such a coupled enzyme assay if the first step is rate limiting and the later steps are kinetically fast.

For plant UMP synthase, it is not known which of these enzymic steps is rate limiting. Therefore, we determined the rate-limiting step of our tobacco UMP synthase. We independently added known amounts of purified *Escherichia coli* OPRTase and OMPdeCase to the plant enzyme and compared the effect on the overall rate of the reaction. As shown in Table III, the addition of a 28-fold excess (330 milliunits) of the OMPdeCase into the reaction increased UMP synthase activity only slightly. However, the addition of the same amount (330 milliunits) of *E. coli* OPRTase increased the overall UMP synthase activity more than 20-fold. This result agrees with similar results from mammalian systems (35). Thus, like the mammalian enzyme, the rate-limiting step in the tobacco UMP synthase is the OPRTase, and standard kinetic procedures used to measure the UMP synthase are, in fact, measuring the rate-limiting step, OPRTase.

With these results in mind, we then undertook kinetic studies of both the OPRTase and the OMPdeCase enzymic activities for several mutants and the wild-type UMP synthase. Kinetic parameters, *Kₘ* and *Vₘₐₓ*, of the mutants and the wild type were determined by standard methods using double-reciprocal Lineweaver-Burk plots (Fig. 5). All three

Figure 4. UMP synthase activity in the presence and absence of 5-FOA. Wild-type and mutant calli were grown for 2 weeks in the presence (solid area) and absence (hatched area) of 5-FOA; after the incubation, calli were homogenized as described in "Materials and Methods," and 100 μg of total protein was assayed for UMP synthase activity. These results are the average of three repetitions for each callus. SD of each treatment is given by the error bars.
Table III. Effect of adding E. coli OMPdeCase and OPRTase to Tobacco UMP Synthase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Overall Enzymic Activity</th>
<th>Relative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMP synthase alone (no addition)</td>
<td>9.4 ± 0.1 milliunits</td>
<td>100%</td>
</tr>
<tr>
<td>UMP synthase plus OMPdeCase</td>
<td>12.3 ± 3.8 milliunits</td>
<td>132%</td>
</tr>
<tr>
<td>UMP synthase plus OPRTase</td>
<td>216.6 ± 47.2 milliunits</td>
<td>2304%</td>
</tr>
</tbody>
</table>

* A 28-fold molar enzymic excess of each E. coli enzyme (=330 milliunits) was added over the amount of total tobacco UMP synthase activity. 

Table IV when transferred to media containing 5-FOA. Those mutants showed a two- to threefold increase in K_m and a similar decrease in V_max for both orotic acid and for PRPP. However, the K_m and V_max for OMP (decarboxylase) were unchanged in any of these mutants. Thus, for each of these mutants, the phosphotransferase was the more affected enzyme activity. These results are similar to the enzymatic activities found in the flux experiments (Table II), although the V_max of the mutant enzymes showed an expected smaller difference from wild type than the total enzymic activity found in the flux experiments.

Growth Studies of the umps Mutants

To determine the relationship between UMP synthase activity and the growth rate of the mutant callus we plated mutant calli on media containing different levels of 5-FOA plus uracil. These experiments were performed with a subset of mutant calli varying in UMP synthase activity from 8 to 90% of wild type.

As shown in Figure 6, the growth of the UMP synthase mutants on media containing 5-FOA plus uracil was dependent on the UMP synthase activity expressed by these mutants. Those mutants having lower UMP synthase activities grew better than mutants with moderate to high enzyme activities. Always, as 5-FOA in the media increased, the growth rates of the mutants declined.

Plant Regeneration

It is clearly of interest to determine whether these mutant cell lines can be regenerated into whole plants. The data in Table IV show that most of our umps mutants had reduced capacity to regenerate whole plants. We plated 15 mutant callus lines on embryo/shoot-inducing medium (medium C; Table IV), however, only two lines, umps52 and umps121, were capable of regenerating a significant number of shoots. Typically, shoots formed after 3 to 6 weeks of culture on the shoot-inducing medium. Of these two lines that produced shoots, only the shoots of umps52 could develop into true plants. The shoots of line umps121 did not grow beyond 0.6 cm tall at which point all shoots died. These shoots died when transferred onto fresh shoot-inducing medium or root-inducing medium or simple MS medium without hormones all supplemented 40 mg/L uracil.

The formation of roots on the umps52 shoots occurred after the shoots had been cultured on root-inducing medium (medium D; Table I) for 2 weeks. We have maintained the umps52 mutant plant line in vitro by nodal propagation on MS medium without hormones. In addition, we have tried to transfer this mutant to soil. Five mutant plants (4–6 cm tall) with well-developed root systems were transferred to soil and maintained in a high-humidity chamber. The plants were

![Figure 5. Kinetic analysis of UMP synthase from wild-type Tr25 and umps mutant calli. Plots A, B, and C are Lineweaver-Burk double-reciprocal plots of the rate of reaction versus each of the substrates for UMP synthase. The initial velocity of the UMP synthase enzyme is linear for the first 15 min (D. Santoso, unpublished observation). The units of the abscissa are μM·min⁻¹ of CO₂ evolved. In A, the variable substrate is PRPP; in B, orotic acid (OA); and in C, orotidyl monophosphate. ■, umps52; ○, umps121; ●, umps511; □, haploid Tr25.](image)
Figure 6. Correlation between callus growth rate and the relative level of UMP synthase enzymatic activity. ■, Growth of each of the mutant calli on maintenance medium (medium B, Table I) containing 10 mg/L (57 μM) 5-FOA plus 40 mg/L (367 μM) uracil; □, growth of each of the mutant calli on maintenance medium (medium B; Table I) containing 20 mg/L (114 μM) 5-FOA plus 40 mg/L (367 μM) uracil; ○, growth of each of the mutant calli on maintenance medium (medium B; Table I) containing 40 mg/L (228 μM) 5-FOA plus 40 mg/L (367 μM) uracil. Growth was determined after 14 d in culture. The calli used in this experiment were umpS511, 8% of wild-type UMP synthase activity; umpS121, 16%; umpS57, 24%; umpS58, 36%; umpS54, 47%; umpS56, 66%; umpS516, 99%; and the wild-type haploid Tr25. Each data point is the average of three experimental replicates.

Table IV. Regenerative Capacity of the umpS Mutants

<table>
<thead>
<tr>
<th>Mutant Line</th>
<th>Shoots</th>
<th>Roots-Plantlets</th>
</tr>
</thead>
<tbody>
<tr>
<td>haploid Tr25</td>
<td>++++*</td>
<td>+++</td>
</tr>
<tr>
<td>umpS52</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>umpS54</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>umpS56</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>umpS57</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>umpS58</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>umpS511</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>umpS512</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>umpS516</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>umpS121</td>
<td>+++*</td>
<td>–</td>
</tr>
<tr>
<td>umpS124</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>umpS127</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

*+++ , High; ++, moderate; +, low; –, none.

Figure 7. Activation of the pin2-CAT gene in various mutant callus lines. Various callus lines (umpS511, lanes 1 and 2; umpS121, lanes 3 and 4; umpS52, lanes 5 and 6, wild-type Tr25, lanes 7 and 8) were plated on MS solid medium in the presence (lanes 1, 3, 5, and 7) or absence (lanes 2, 4, 6, and 8) of 2.0 mg/L α-NAA. After 7 d of incubation, the calli were homogenized as described in “Materials and Methods.” After quantitation of protein, 100 μg of protein from each homogenate was assayed for CAT activity. Lanes 9 and 10 are a negative and positive controls containing, respectively, no added protein and 10 μg of purified E. coli CAT enzyme.

Watered every other day with a solution of 100 mg/L of uracil in water. Nevertheless, all five plants died within 4 weeks of transfer to soil. We are currently investigating grafting methods as a way to bring these plants to flower and reproduce.

CAT activity

One of the initial goals of this study was to determine whether the creation of umpS mutants would affect the wound-inducible phenotype; therefore, we examined the expression of the pin2-CAT gene in these callus lines in the absence of auxin (16). CAT assays were performed on calli of three mutant lines, umpS511, umpS121, and umpS52. These assays demonstrated that all three mutants still expressed the CAT gene, and like the parental type, haploid Tr25, the pin2-CAT gene was derepressed in the absence of auxin (Fig. 7). Therefore, the introduction of the mutations affecting the UMP synthase gene did not affect the regulation of the wound-inducible pin2-CAT gene in any of these mutants. We also tested the ability of the wound-inducible gene expression to function in the small regenerated plants from mutant umpS52. This plant showed fully wound- or sucrose-inducible levels of CAT activity in response to sucrose induction, indicating that the pin2-CAT gene was unaffected by the introduction of the umpS mutations.

DISCUSSION

We have selected many tobacco callus lines mutant in UMP synthase. Mutants were selected in the presence of 5-FOA following NMU mutagenesis on a highly enriched medium. Most, but not all, of the selected mutants had reduced activities of UMP synthase. Never did we find any mutant that was completely deficient in the expression of UMP synthase. Our best mutant lines, umpS511 and umpS121, showed 8 and 16% of the wild-type UMP synthase activity. This level of enzymatic activity is similar to the tryptophan
mutants of Arabidopsis thaliana (19), which show reduced expression (18% of the wild type) of tryptophan synthase β-subunit (18). The reduced levels of UMP synthase in these mutants were stably maintained even without the fluoroorotic acid-selective pressure. Most of the mutants had reduced capacity to regenerate into whole plants. However, one mutant, umps52, could regenerate whole plants.

The subunit of UMP synthase that harbored the genetic lesion was determined for several umps mutants. To conduct these kinetic analyses, we first determined that, like the mammalian enzyme, the OPRTase is the rate-limiting step of the tobacco UMP synthase. Our kinetic analysis of the UMP synthase activity for each of three independent mutant lines showed that the $K_m$ and $V_{\text{max}}$ of the OMPdeCase were unchanged, but the $K_m$ and $V_{\text{max}}$ values for both of the substrates of the OPRTase were altered. It is interesting that each of these independently derived mutants have the same lesion, OPRTase, and not OMPdeCase. Apparently, the fluoroorotic acid selection scheme favors selection of mutants in the OPRTase rate-limiting step. This is the first application of this selection scheme in plants.

The reduction in ability to regenerate plants is typical for selection of mutant plants and has also been observed in mutants from other species (23, 30). This problem may be associated with many undesirable secondary mutations caused by mutagenesis (23). However, another alternative is that the production of shoots is likely to require large amounts of UMP for the rapid growth rates that occur during shooting. It may be that most of these umps mutants are unable to synthesize or take up from the medium sufficient UMP for this process to occur.

Finally, we determined that the introduction of the umps lesions in the transgenic Tr25 plant tissues had no effect on the ability of the pin2-CAT gene to be derepressed by the absence of auxin. Furthermore, the regenerated plant, umps52, showed good induction of the pin2-CAT gene in whole plants.

Because growth on fluoroorotic acid plus uracil is a very powerful selection method for the absence of functional UMP synthase, it should be possible to select for mutants in complex pathways. We have chosen to apply this selection scheme to the wound-induction pathway. By expressing UMP synthase under control of a wound-inducible promoter and selecting against the expression of UMP synthase, such mutants should grow and the wild-type cells should die. We are currently transforming these umps mutants with DNA constructions that will express a D. discoideum umps gene under control of the wound-inducible pin2 promoter (31). The ability to induce this promoter by a variety of biochemical methods (6, 10, 16, 24, 25) should permit us to select for such second site mutations in these umps cell lines.

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LITERATURE CITED

21. McClard RW, Black MB, Livingstone LR, Jones ME (1980) Isolation and initial characterization of the single polypeptide that synthesizes uridine 5’-monophosphate in Ehrlich ascites
26. Sanchez-Serrano
25. Penia-Cortes
22. Murashige
27. Sidorov
29. 23. Negrutiu I, Dirks R, Pearce
24. H, j, VA, N
uridine-5’-monophosphate synthase. Biochemistry of
carcinoma: purification by tandem affinity chromatography
of uridine-5’-monophosphate synthase. Biochemistry 19:
4699–4706
22. Murashige T, Skoog F (1962) A revised medium for rapid
growth and bio assays with tobacco tissue cultures. Physiol
Plant 15: 473–497
nitrile reductase-deficient mutants from protoplast culture
of Nicotiana plumbaginifolia (Viviani). Theor Appl Genet 66:
341–347
from tomato leaves induces wound-inducible proteinase in-
25. Peña-Cortes H, Sanchez-Serrano JJ, Mertens R, Willmitzer L,
Prat S (1989) Abscisic acid is involved in the wound-induced
expression of the proteinase inhibitor II gene in potato and
tomato. Proc Natl Acad Sci USA 86: 9851–9855
Identification of potato nuclear proteins binding to the distal
promoter region of the proteinase inhibitor II gene. Proc Natl
Acad Sci USA 87: 7205–7209
27. Shoemaker RC, Couche LJ, Galbraith DW (1986) Character-
ization of somatic embryogenesis and plant regeneration in
of auxotrophic and chlorophyll-deficient lines isolated in hap-
loidy Nicotiana plumbaginifolia protoplast cultures. Mol Gen
Genet 186: 328–332
Vasil, ed, Cell Culture and Somatic Cell Genetics of Plants.
Vol 1: Laboratory Procedures and Their Application. Academic
Press, Orlando, FL, pp 283–292
30. Sung ZR, Smith R, Horowitz J (1979) Quantitative studies of
embryogenesis in normal and 5-methoxytryptophan resistant
cell lines of wild carrot. Planta 147: 236–240
31. Thornburg RW, An G, Cleveland TE, Johnson R, Ryan CA
(1987) Wound-inducible expression of a potato inhibitor II-
chloramphenicol acetyltransferase gene fusion in transgenic
tobacco plants. Proc Natl Acad Sci USA 84: 744–748
32. Thornburg RW, Kernan A, Molin L (1990) CAT protein is
expressed in transgenic tobacco in field tests following attack
33. Thornburg RW, Li X (1991) Wounding of the foliage of Nicot-
tiana tabacum causes a decline in the levels of endogenous
foliar IAA. Plant Physiol 96: 802–805
34. Thornburg RW, Park S, Li X (1992) Hormonal regulation of
wound-inducible proteinase inhibitor II genes. In DPS Verma,
ed, Control of Plant Gene Expression. CRC Press, Boca Raton,
FL (in press)
35. Traut TW, Jones ME (1977) Inhibitors of orotate phosphorbo-
syl-transferase and orotidine-5’-phosphate decarboxylase
from mouse Ehrlich ascites cells: a procedure for analyzing
the inhibition of a multi-enzyme complex. Biochem Pharmacol
26: 2281–2291
bisyltransferase and orotidine-5’-phosphate decarboxylase of
Euglena gracilis: purification and properties. Biochem Physiol
Pflanz 176: 116–128
a single polypeptide catalyzes the two step conversion of orotate
to UMP in cells from a tomato suspension culture. J Plant
Physiol 116: 301–311
transformation system for aflatoxin-producing fungus, Asper-