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Selection and Characterization of α-Methyltryptophan-Resistant Lines of *Lemna gibba* Showing a Rapid Rate of Indole-3-Acetic Acid Turnover[1]

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Turnover rate is an important aspect of the regulation of plant processes by plant growth substances. To study turnover of indole-3-acetic acid (IAA), two α-methyltryptophan-resistant lines (MTR1 and MTR2) of *Lemna gibba* were generated by nitrosomethyl urea treatment of an inbred line derived from *L. gibba* G-3. In this report we describe: (a) the development of a selection system using this near isogenic line of *L. gibba*; (b) techniques for chemical mutation of the lines and selection for α-methyltryptophan resistance; and (c) the partial characterization of the selected lines. MTR lines contained 3-fold higher levels of anthranilate synthase activity. The enzyme in the MTR lines required higher levels of tryptophan for feedback inhibition. MTR lines also contained 8-fold higher levels of tryptophan, 3-fold higher levels of free IAA, and similar levels of total IAA compared to the inbred line. Turnover rates in the inbred and selected lines were calculated, using the first-order rate equation, based on the decrease over time in isotopic enrichment of $^{13}C_6$-IAA introduced into *L. gibba* during a 1-h pulse period. Isotope enrichment in IAA was determined by using gas chromatography-mass spectrometry. Both MTR lines had an approximately 10-fold higher rate of IAA turnover than the parent inbred line.

IAA plays an important role in plant growth, development, and responses to the environment, yet after more than a century of study we still have large gaps in our knowledge of fundamental aspects of how the hormone acts and what controls its metabolism. One aspect of plant hormone metabolism that has attracted attention over the last 15 years is the rate of hormone turnover (Epstein et al., 1980; Nonhebel and Cooney, 1990). Hormonal homeostasis requires that increased rates of synthesis of IAA be offset by increasing rates of degradation, resulting in more rapid turnover. Studies of hormone turnover have been limited in number in part by lack of plant systems that overcome the difficulties of labeling internal pools. In many cases, IAA levels in plant tissues remain relatively constant, but changes in the rates of IAA biosynthesis and degradation are seen only as changes in turnover rate. To date we have information on turnover in only a few plants and no knowledge of how turnover might change relative to genetic, developmental, and environmental variation. Turnover can be expected to be an important metabolic parameter for hormone action, since rates of turnover must be rapid relative to the processes that are controlled by the particular hormone (see Cohen, 1983). A mechanism linking turnover and hormone action may involve the co-oxidation of plant fatty acids (Reineke, 1990).

Studies of IAA biosynthesis have shown the utility of using mutants to study IAA and Trp metabolism (Wright et al., 1991; Normanly et al., 1993). Unlike mutants used for studies of metabolic pathways, those most useful for understanding the function of IAA turnover would not be auxotrophs, but rather would be modified in metabolism so as to result in changes in turnover. We reasoned that mutants with constitutively high production of early precursors to indolic compounds might exhibit higher rates of IAA turnover than parent lines. Based on analogy to selections for microbial mutants, mutants in the key regulatory enzyme, anthranilate synthase, can be obtained by selection for plants that are able to grow on the Trp analogs 5-MT or α-MT (Widholm, 1972b; Kreps and Town, 1992), although this technique also selects for other types of mutations (Widholm, 1977b). The strategy for such selections is that mutations that result in unregulated activity of anthranilate synthase (not as sensitive to feedback regulation by Trp) will result in unregulated production of Trp precursors and Trp including the level of IAA precursors in common with Trp production (Fig. 1).

As with other higher plants, *Lemna* can be selected for specific changes in indolic metabolism (Last and Fink, 1988). Unlike most other plants, however, *Lemna* cultures allow rapid isotope labeling and pulse/chase experiments under sterile conditions (Slovin and Tobin, 1982; Baldi et al., 1991), making them well suited for turnover studies. In addition, problems related to wounding encountered with tissue sections can be avoided with the *Lemna* whole plant system. Likewise, the complexities of exogenous hormone

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Abbreviations: α-MT, α-methyl-DL-tryptophan; 5-MT, 5-methyl-DL-tryptophan; NMU, *N*-nitroso-*N*-methyl urea; SPE, solid phase extraction.

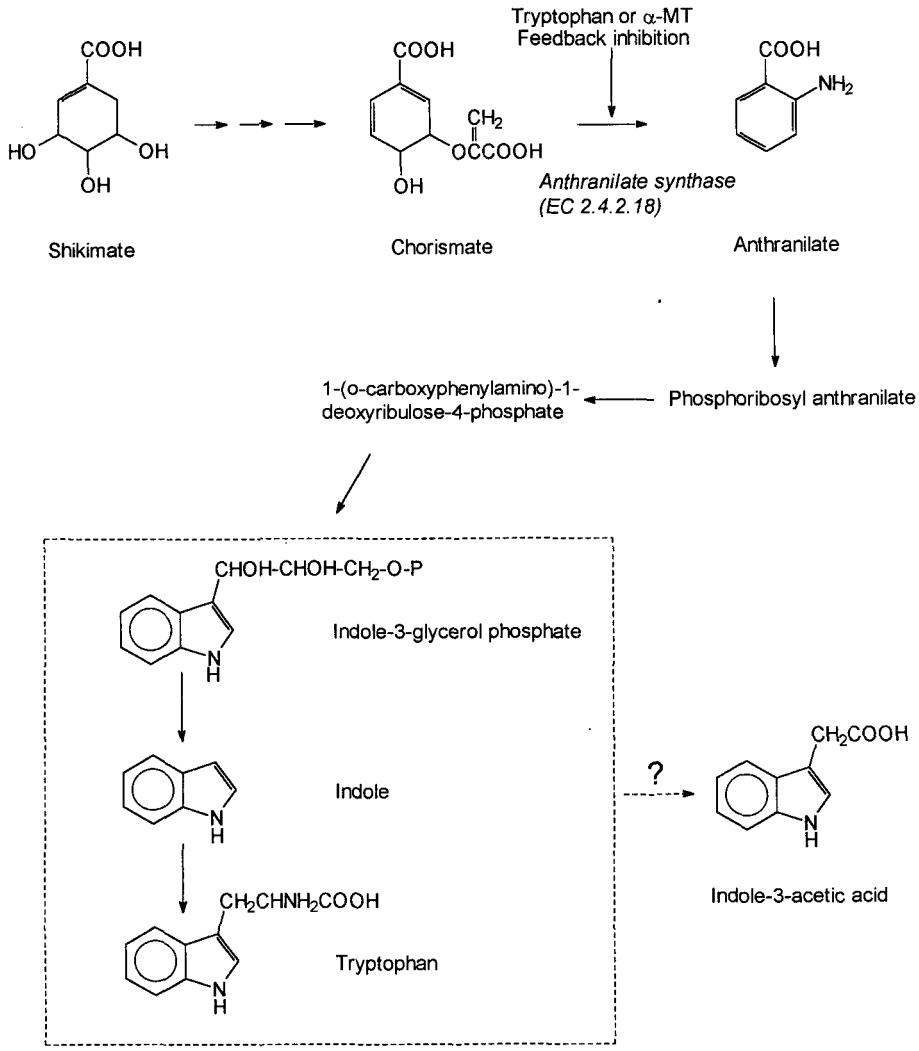

Figure 1. Like other aromatic compounds, IAA is a product of the shikimic acid pathway. The conversion of chorismate to anthranilate by anthranilate synthase is the first committed step to IAA and Trp. The boxed steps indicate potential branch points to IAA.

requirements encountered with cell cultures are avoided. In this report we describe: (a) the development of a selection system using near-isogenic lines derived from *Lemna gibba* G-3; (b) techniques for chemical mutation of the lines and selection of α-MT resistance; and (c) the partial characterization of the selected lines. The selected lines do not produce viable seeds; thus, it has not been possible to do genetic analysis. However, the selected lines have been maintained under nonselective conditions for 4 years through an estimated 400 vegetative generations. During this time all four independent lines have maintained their biochemical phenotype, demonstrating that the selected characteristic is highly stable.

MATERIALS AND METHODS

Plant Growth

A near-isogenic line of *Lemna gibba* G-3, 3F7–11, was produced by sequential selfings through seven generations. A single mother/daughter two-frond pair was grown under standard conditions; 50 mL of E medium (Cleland and Briggs, 1967) in a 125-mL flask kept in a growth chamber (model Q6521, Environmental Growth Chambers[2], Chagrin Falls, OH) at 26°C under continuous light, provided by a mixture of cool-white fluorescent and incandescent lamps at 25 μmol m^{-2} s^{-1} (Slovin and Cohen, 1988). When flowering occurred the cultures were placed on a rotary shaker and gently rotated at 75 rpm for 1 h three times per day to effect pollination. Seeds were collected from the bottom of the flask with an aseptic Pasteur pipette. At each generation, plant colonies derived from individual seeds were selected based on normal appear-

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

ance, uniform and rapid growth, and ability to flower and set viable seed (Slovin and Cohen, 1985). For growth rate determinations, cultures were started with two mother/daughter frond pairs at d 0 and grown under standard conditions. Growth was determined by counting frond number.

Obtaining the MTR Lines

Mutagenesis

An NMU stock solution consisted of 100 mM NMU (Sigma, No. N-1517) in 70% ethanol containing 1% acetic acid. Using aseptic conditions, approximately 250 fronds of 3F7–11 were floated for 15 min at 23°C on 10 mL of E medium in a 100 × 60 mm Petri dish containing 5% (v/v) of NMU stock, then washed three times with E medium. Fronds were then grown vegetatively on E medium as described above.

Selection

After 6 to 8 weeks of growth following NMU treatment, the number of fronds increased to approximately 6×10^6. At this point fronds with aberrant pigment levels were produced and this was used as an indicator of the efficacy of the NMU treatment. Some of the unusually pigmented fronds were clearly chimeras, and others appeared to be totally without chloroplast pigments (Slovin, 1990). Using approximately 250 fronds per dish, all the fronds were placed in 100 × 60 mm plastic Petri dishes containing E medium supplemented with 10 μM α-MT (Sigma, No. M-8377). Plants able to survive on the selection medium, as determined by their green color, were picked after 6 to 7 weeks, then grown on nonselective medium and retested for resistance to α-MT. Resistant lines were maintained on nonselective medium and checked for resistance prior to experimentation.

Anthranilate Synthase Activity

Anthranilate synthase activity was assayed by a modification of the method of Last and Fink (1988). All steps in the preparation of plant materials were done at 4°C. Plant extracts were prepared by grinding 1 to 2 g of plant material in a glass homogenizer containing 5 to 7 mL of grinding buffer (200 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 8 mM $MgCl_2$, 0.2 mM DTT, 60% glycerol, 0.08 μg/mL PMSF, and 200 mg of polyvinylpolypyrrolidone [Sigma, No. P-6755]). The suspension was clarified by centrifugation for 20 min at 27,000*g*. A 5-mL portion of the supernatant was loaded onto a Nap-25 column (Pharmacia), then eluted with column buffer (50 mM Tris-HCl, pH 7.5, 0.05 mM EDTA, 2 mM $MgCl_2$, 0.05 mM DTT, 5% glycerol). Enzyme activity in 1 mL fractions was measured as the chorismate-dependent production of anthranilate in a 2-mL reaction containing 1 mL of column buffer and 1 mL of reaction mixture (0.1 μmol of chorismic acid, 25 μmol of Tris-HCl, pH 8.0, 2 μmol of $MgCl_2$, 20 μmol of Gln) at 25°C for 30 min. Preliminary studies showed that chorismate in the reaction was saturating with respect to the enzyme from the inbred line under these conditions. The reaction was started by adding 0.1 mL of extract to the reaction mixture and terminated by adding 0.2 mL of 1 M HCl. The anthranilate product was extracted into 2 mL of ethyl acetate and the ethyl acetate phase was clarified by centrifugation at 1650*g* for 10 min at room temperature. Anthranilate was quantified using fluorescence spectrophotometry on a Perkin Elmer 650–10s fluorimeter (excitation 345 nm, emission 400 nm). Trp inhibition of anthranilate synthase activity was measured by addition of Trp to the 1 mL of column buffer used to dilute the Nap-25 column fractions. Kinetic studies by fluorescence monitoring of the total reaction mixture showed that the reaction was linear for up to 2 h.

K_i was determined at saturating substrate concentration from a plot of enzyme activity obtained after the addition of Trp to the reaction at eight different concentrations (from 5–12.5 mM), as per Widholm (1972a).

Trp Analysis

[$^2H_5$]Trp (MSD Isotopes, St. Louis, MO, 98.5 atom%) was used as an internal standard for the GC-MS determination of Trp levels as described by Michalczuk et al. (1992). Briefly, 1 g of tissue (fresh weight) was ground in a liquid N_2 chilled mortar and pestle containing 65% isopropanol, 35% 0.2 M imidazole buffer at pH 7.0 for grinding and extraction. Approximately 0.84 kBq of [5-^{3}H]Trp (Amersham, 999 GBq/mmol) was added as a tracer and 1.5 μg of [$^2H_5$]Trp was added as the internal standard. After equilibration for 1 h, the sample was centrifuged to remove solids and the isopropanol was removed in vacuo. A 5-mL bed volume column of Dowex 50-X2,200–400 (J.T. Baker, No. 1922) in a 10-mL plastic syringe was used to purify the sample. The column was washed with 2 N HCl and 5 bed volumes of distilled water before use. The sample was applied at pH 7 and Trp was eluted with 20 mL of 2 N NH_4OH. The elute was evaporated to dryness and traces of water were removed by multiple azeotropic distillation with pure ethanol and dichloromethane. The dry residue was dissolved in 2 mL of absolute methanol plus 0.5 mL of acetic anhydride and incubated at 65°C for 1 h to form the *N*-acetylmethyl ester of Trp. The methanol was then removed in vacuo and the derivatized amino acid was dissolved in distilled water. A Fisher PrepSep C_{18} SPE column and a Waters (Milford, MA) NovaPak C_{18} HPLC column (on a Varian [Sunnyvale, CA] 5000 HPLC) were used for further purification before analysis by GC-MS (Hewlett-Packard 5890 series II GC with a Hewlett-Packard 5971A mass selective detector), as described by Bialek et al. (1992).

IAA Analysis

Free and total IAA were determined using [$^{13}C_6$]IAA as an internal standard for GC-MS analysis as described by Cohen et al. (1986).

Free IAA was purified from an aqueous acetone extract of 0.5 g of tissue (fresh weight) using diethyl ether for partitioning. Diethyl ether was shaken with 10 mM ascorbic acid (pH 7) prior to use. HPLC was carried out on a C_{18} 50 mm × 4.6 mm Phenomenex Ultracarb 30 5μ ODS column

using a Waters 550 pump, a Rheodyne (Cotati, CA) 7125 valve, and a Gilson (Middleton, WI) 203 fraction collector. The sample was eluted isocratically using a mobile phase of 25% methanol containing 1% (v/v) acetic acid. The IAA fraction was methylated using diazomethane and analyzed by GC-MS selected ion monitoring as described by Slovin and Cohen (1988).

Total IAA was determined essentially by the method of Chen et al. (1988). The extract was hydrolyzed with 7 N NaOH at 100°C for 3 h using oxygen-scrubbed N_2 (Bialek and Cohen, 1989). IAA was purified on a 3-mL C_{18} SPE column (J.T. Baker No. 7020–03) and further purified by HPLC prior to analysis by GC-MS.

Indole Analysis

The level of indole in *Lemna* was determined by GC-MS using a [$^2H_7$]indole internal standard (MSD Isotopes; 91 atom%) essentially as described by Normanly et al. (1993). Approximately 1.5 g fresh weight of tissue was used for each determination. The plant tissue was rinsed and fresh weight was determined before freezing in liquid N_2. The plant tissue was ground using a liquid-N_2-chilled mortar and pestle with about 200 mg of glass beads (100–130 μm size "Superbrite" beads, 3M, St. Paul, MN) and extracted using 5 mL of 35% 0.2 M imidazole buffer, pH 7.0, and 65% isopropanol. The internal standard was added (200 ng/g) and the extract was equilibrated in the dark at 4°C for 1 h. The sample was centrifuged for 10 min at 1300*g* to remove solids and the supernatant was reduced in volume using a rotary evaporator to remove isopropanol. The sample was diluted 10-fold with distilled water. The diluted sample was passed through a chilled and preconditioned amino SPE column (2.5 g size, Lida [Bensenville, IL]); the column was preconditioned with 5 mL of hexane, acetonitrile, water, 0.2 M imidazole buffer, and water, consecutively). The collected sample was then passed through a chilled and preconditioned C_{18} SPE column (the column was preconditioned with methanol and distilled water, 5 mL each, before use). Pentane (6 mL) was used to elute the sample from the C_{18} column. The pentane eluate was passed slowly through a cartridge-type Sample Drying Device (Whatman) containing 1.5 g of sodium sulfate. N_2 was used to reduce the volume of the pentane to about 1 mL, and the indole content was determined by GC-MS monitoring ions at m/z 117 and 123 (the molecular ions for indole and [$^2H_6$]indole, respectively). Because of the low enrichment of the internal standard, the major peak was for [$^2H_6$]indole rather than for [$^2H_7$]indole (see Bialek et al., 1992, for calculations). The correction factor ("R value"; Cohen et al., 1986) for indole measured at m/z 117 and the internal standard measured at m/z 123 was determined empirically to be 1.65.

IAA Turnover

[^{3}H]IAA (Amersham, 851 GBq/mmol) and [$^{13}C_6$]IAA were used as tracer and marker, respectively, for measurement of IAA turnover in *Lemna*. *Lemna* fronds (1.5–2.0 g/dish) were incubated under sterile conditions for 1 h with 20 mL of E medium supplemented with 296 kBq of [^{3}H]IAA and 370 ng of [$^{13}C_6$]IAA in a plastic Petri dish (100 × 25 mm). After washing with sterile distilled water, fronds were incubated with 25 mL per dish of fresh E medium in a growth chamber for 1.5 to 2.0 h. Samples were harvested after incubation with labeled IAA (T_0), and 1.5 or 2.0 h after incubation with fresh E medium. Harvesting consisted of washing fronds with distilled water, blotting

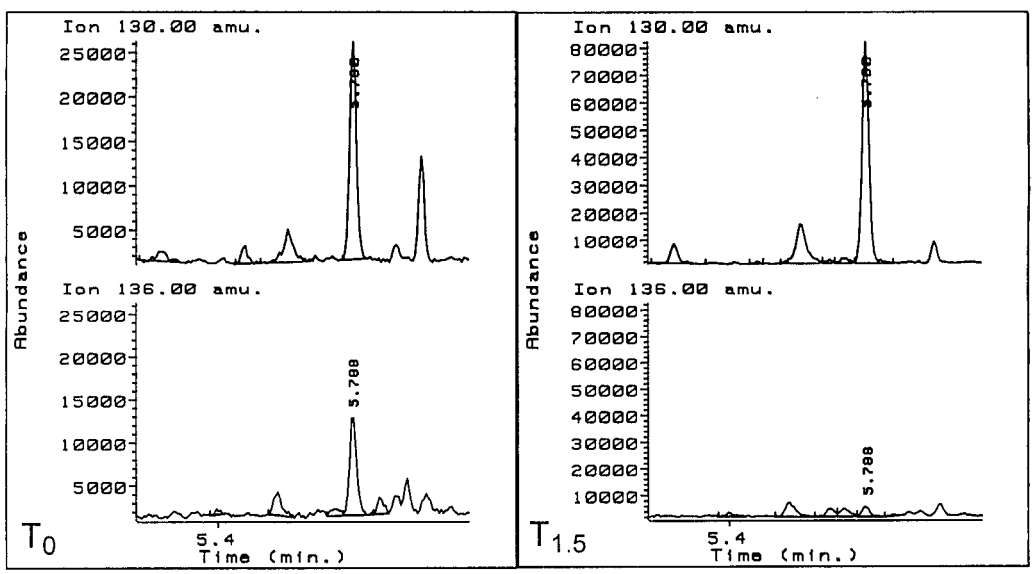

Figure 2. Selected ion chromatograms showing the isotope ratio change during a 1.5-h incubation of the MTR2 line of *L. gibba* on E medium after a 1-h labeling period with [$^{13}C_6$]IAA. Upper traces show the ion at m/z 130 characteristic of unlabeled IAA produced by the plants and lower traces are for m/z 136 from the supplied [$^{13}C_6$]IAA containing the six "heavy" carbons. Ion chromatograms on the left are at T_0 and those on the right were obtained following the 1.5-h incubation.

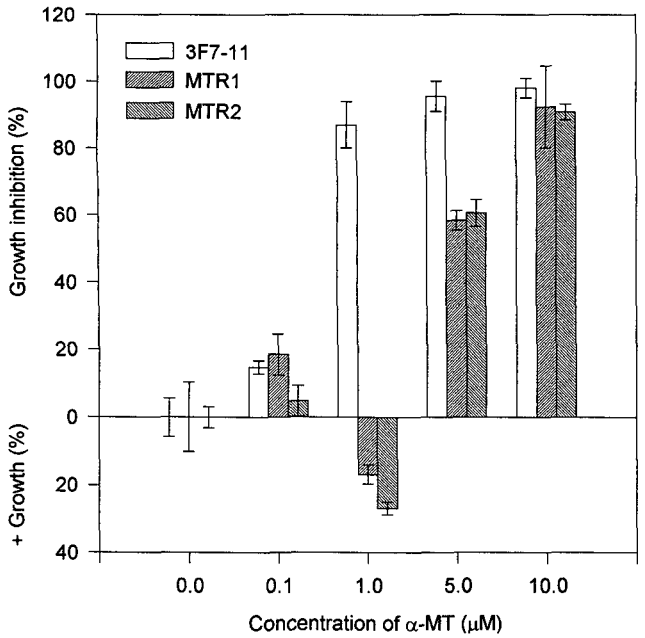

Figure 3. Percent inhibition of growth of various lines of *L. gibba* grown on E medium containing α-MT after 8 d. Data are mean values ± SE, $n = 4$. Control flasks containing two mother/daughter pairs (total of four fronds) were planted on E medium. After 8 d of growth control flasks contained 80 ± 4.5 fronds. Growth inhibition is calculated relative to this control growth. Although the growth of the MTR lines was inhibited at 8 d of culture at 10 μM α-MT, they remained viable for several additional weeks. During this period the inbred line dies, allowing for selection of resistant plants.

dry, weighing, and freezing in liquid N_2. Isotopic enrichment in the free IAA pool was determined by GC-MS (Fig. 2) essentially as described for free IAA quantification except that partitioning was with hexane (pH 7.0) and ethyl acetate (pH 2.5). The change of the ratio of m/z 136 to [m/z 130 + m/z 136], C_t, was used to determine the rate of IAA turnover. Based on the equation for a first-order reaction, turnover time ($t_{1/2}$) for a 50% reduction in enrichment was calculated as follows:

$$\log C_0/C_t = kt/2.303$$

$$t_{1/2} = \ln 2/k$$

where k is the first-order rate constant. As previously discussed (Zilversmit et al., 1943; Epstein et al., 1980; Nonhebel and Cooney, 1990), use of this equation assumes that the change in isotope ratios obeys first-order kinetics and that the labeled material is metabolized at a rate similar to that of the unlabeled material.

RESULTS

Selection of MTR Lines

Plants treated with mutagen for longer than 15 min produced daughter fronds with highly aberrant morphological phenotypes. After mutagenized plants had been allowed to grow and reproduce vegetatively for 6 to 8 weeks, cells that had mutations affecting choloroplast pigmentation should have produced sufficient tissue to be clearly visible. Such pigment variants were observed and at this point selection was applied. Four lines (MTR1 to MTR4) were obtained as being resistant to α-MT. The two lines with the highest levels of resistance, MTR1 and MTR2, were used for the experiments described here. The two lines with lower resistance have not been as completely characterized.

Growth on α-MT or 5-MT

The MTR lines were demonstrated to be resistant to Trp analogs by measuring the growth rate (as increase in number of fronds) of plants grown on E medium containing from 0.1 to 10 μM α-MT (Fig. 3). Growth of 3F7–11, the inbred parent line, was inhibited over 95% by 5 μM αMT, whereas MTR lines maintained a growth rate around 40% of that on E medium alone (Fig. 3). The MTR lines also showed cross-resistance to 5-MT. The inbred line, 3F7–11, showed over 95% growth inhibition by 1 μM 5-MT, but the MTR lines actually grew better on 1 μM 5-MT than on E medium alone (data not shown)

Trp Reversed the Inhibition of α-MT

The growth of various lines of *L. gibba* was inhibited by both α-MT and 5-MT. If the mechanism of inhibition in *Lemna* is similar to that in other plants and microorganisms, then exogenous L-Trp should reverse the inhibition of growth caused by α-MT. Consistent with this expectation, 100 μM L-Trp added to the medium fully reversed the growth inhibition caused by 10 μM α-MT (Fig. 4). In fact,

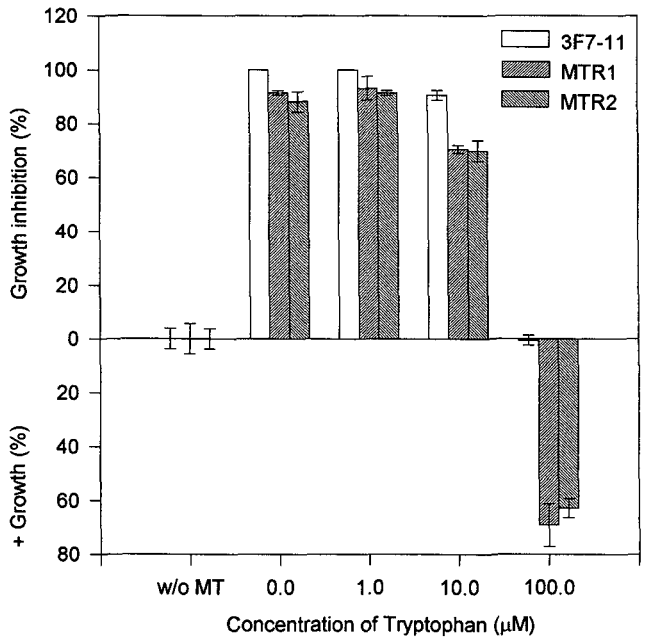

Figure 4. Percent inhibition of growth of various lines of *L. gibba* grown on the selective medium (E medium containing 10 μM α-MT) with 0 to 100 μM Trp after 8 d of treatment. Data are mean values ± SE, $n = 4$. The control flasks without α-MT contained 90.5 ± 3.5 fronds after the 8 d of growth.

100 μM L-Trp promoted significant growth of the MTR lines. A higher concentration (1 mM) of L-Trp did not further increase growth (data not shown).

Anthranilate Synthase Activity

The anthranilate synthase activity measured in extracts of MTR lines was higher than that of the inbred line by around 2- to 3-fold (Table I). Other MTR lines, which showed lower resistance to α-MT than MTR1 and MTR2, also showed higher anthranilate synthase activity than wild type, but this increased activity was less than that seen in MTR1 and MTR2 (data not shown). The anthranilate synthase enzyme from the inbred line was over 50% inhibited by 7.5 mM Trp. The enzyme from the MTR lines was less sensitive to Trp inhibition (only around 10% inhibition at 7.5 mM Trp). For the MTR lines, 12.5 mM Trp was required for approximately 50% inhibition of anthranilate synthase activity (Table I).

Levels of Indolic Compounds in Parent and MTR Lines

Quantitative analysis of Trp showed that in the inbred line 3F7–11, the amount of Trp was 1.44 μg/g fresh weight and in MTR lines (MTR1 and MTR2) the amounts of Trp were 8 to 12 times higher than that in the inbred line (Table II). Total IAA levels in the inbred line and the two MTR lines were not notably different; however, the free IAA levels in the two MTR lines were 3 to 4 times higher than that of the inbred line (Table II). No free indole could be detected in the *L. gibba* inbred line or in the MTR lines. Our estimated lower limit of detection was somewhat less than 0.1 ng/g fresh weight of indole, and thus the levels of free indole in *Lemna* appear to be lower than the levels reported for *Arabidopsis* and maize, the only other plant species for which reliable data on indole levels are available (Normanly et al., 1993; Rekoslavskaya and Bandurski, 1994).

IAA Turnover in *Lemna* Lines

The change in isotope enrichment in IAA obeys first-order kinetics over the first 4 h of incubation (Fig. 5). The whole-plant turnover time calculated for the parent inbred line 3F7–11 was about 10 h (Table III), a value similar to that reported by Nonhebel and Cooney (1990) for IAA turnover in young leaves of tomato. The MTR lines, however, showed a much more rapid turnover rate than the parent lines. In fact, a $t_{1/2}$ of about 1 h is the fastest rate of IAA turnover reported so far in any plant or plant tissue (Epstein et al., 1980; Nonhebel and Cooney, 1990). For both MTR lines, the IAA turnover was faster than the inbred line by almost 10-fold (Table III).

Table I. *Anthranilate synthase activity in inbred and MTR lines*

Lemna Line	Enzyme Activity	Ki_{50}[a]
	nmol anthranilate/g fresh weight ± SE	*mM*
3F7–11	6.98 ± 0.55	7.5
MTR-1	23.08 ± 1.48	12.5
MTR-2	20.54 ± 0.88	>10.0

[a] Concentration of Trp required to give 50% inhibition of anthranilate synthase activity.

Table II. *Free Trp, free IAA (without hydrolysis), and total IAA (free plus ester and amide IAA released by hydrolysis in 7 N NaOH for 3 h at 100°C) levels in the inbred line (3F7–11) and α-MT-resistant lines (MTR-1 and MTR-2).*

MTR lines showed about a 10-fold increase in Trp and a 3-fold increase in free IAA compared to the inbred line 3F7–11; however, total IAA was similar in all lines. Plants were grown for 3 weeks on nonselective medium and data are the mean ± SE of three determinations.

Lemna Line	Free Trp	Free IAA	Total IAA
	ng/g fresh weight ± SE		
3F7–11	1,440 ± 130	3.38 ± 0.68	349 ± 15
MTR1	12,590 ± 2160	15.59 ± 3.27	307 ± 60
MTR2	16,720 ± 2460	12.40 ± 1.00	317 ± 26

DISCUSSION

Anthranilate Synthase

Anthranilate synthase is a key enzyme in indole metabolism (Fig. 1). Anthranilate synthase not only catalyzes the synthesis of anthranilate but this reaction is also a regulated branchpoint step in Trp biosynthesis (Hütter and Niederberger, 1986; Niyogi and Fink, 1992). Trp analogs are toxic to microbial and plant cells because they generally inhibit anthranilate synthase activity by acting as false feedback inhibitors in place of Trp (Moyed, 1960; Widholm 1972c). In some organisms these compounds can also be incorporated into protein and result in nonfunctional en-

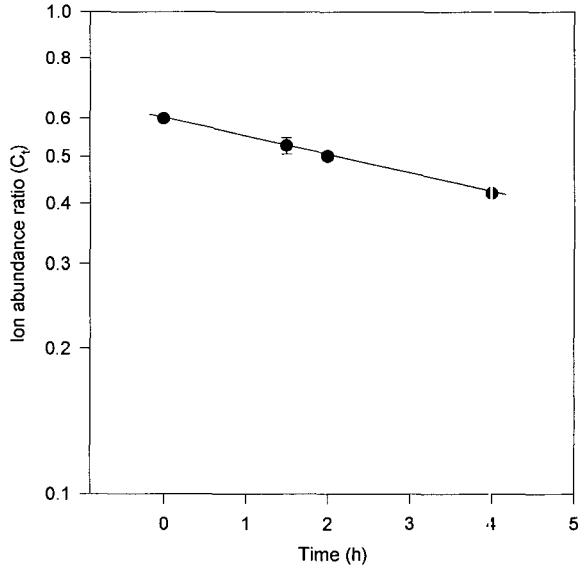

Figure 5. Semilogarithmic plot of isotope ratios in IAA at different times of incubation of the *L. gibba* inbred line 3F7–11 showing that the change in ratio is consistent with that expected for a first-order reaction. Data were normalized to the highest ratio obtained for T_0 (0.6). The SE for data at 1.5 h is indicated by error bars, and data for 2 and 4 h points are from single determinations.

Table III. *Turnover time ($t_{1/2}$) of IAA for inbred and MTR lines*

Lemna Line	$t_{1/2}$
	$h \pm SE$
3F7–11	9.6 ± 0.6
MTR-1	0.81 ± 0.06
MTR-2	0.89 ± 0.37

zymes that can lead to growth inhibition (Stäheli et al., 1981); however, in plants it has been suggested that little or no incorporation into protein occurs (Sasse et al., 1983). Resistance to Trp analogs obtained through an alteration in the activity of one or more enzymes in the Trp biosynthetic pathway would be expected to yield increased production of indolic precursors to IAA biosynthesis that are in common with the pathway to Trp. Thus, such selected lines have potential applications for a variety of studies aimed at understanding the pathway, regulation, and control of IAA production.

The increase in the levels of Trp seen in the MTR lines (Table II) is one of the expected results of high anthranilate synthase activity and reduced sensitivity to feedback inhibition (Widholm, 1977a). Unlike maize and *Arabidopsis*, where free indole is present in low but measurable quantities, indole levels in both inbred parent and MTR lines in *Lemna* were below our level of detection (less than 0.1 ng/g fresh weight). Therefore, it was not possible to determine the effect of high anthranilate synthase activity on the level of indole. It is not surprising to see low levels of indole, since most indole produced might be expected to remain with the Trp synthase complex and not accumulate in significant quantities (Brzović and Dunn, 1992). The reason for the higher levels of indole found in the other plant species is unknown. In *Lemna*, the non-Trp pathway for IAA biosynthesis is probably the predominant pathway during vegetative growth based on the low level of Trp conversion to IAA (Baldi et al., 1991), and, where the non-Trp pathway is active, anthranilate synthase may have a more pronounced effect on IAA biosynthesis than would be seen in plants where the Trp degradation pathway predominated.

In the inbred line of *Lemna*, anthranilate synthase activity per milligram of protein was higher than that reported for cultured carrot cells (Widholm, 1972a) but similar on a fresh weight basis. A much higher Trp concentration was necessary for the feedback inhibition of *Lemna* anthranilate synthase compared to that reported for anthranilate synthase enzymes from several plant and bacterial sources. For example, the Trp concentrations giving a 50% inhibition for anthranilate synthase activity in carrot cell lines and *Lemna* were 50 μM and 7 mM, respectively (Widholm, 1972b).

IAA Metabolism

Several Trp analog-resistant lines have been found in higher plants including the *amt-1 Arabidopsis* mutant (Kreps and Town, 1992), the R cell line in tobacco (Widholm, 1972b), and resistant cell lines in carrot (Widholm, 1972a, 1972b, 1972c). Among the resistant plant lines isolated, rice, *Datura*, and the *amt-1* line of *Arabidopsis* as well as MTR *Lemna* have shown resistance at the whole-plant level (Ranch et al., 1983; Lee and Kameya, 1991; Kreps and Town, 1992). Where examined, these Trp analog-resistant lines share characteristics such as elevated Trp levels, high anthranilate synthase activity, altered feedback inhibition of anthranilate synthase by Trp, and cross-resistance to 5-MT. The absolute levels of such changes appear to be quite different in the various species examined.

Although no mutants clearly blocked in auxin biosynthesis have been described (King, 1988; Reid, 1993), other classes of putative IAA mutants have been found in several plants. Examples include the jsR1 mutant in *Lemna* (Slovin and Cohen, 1988), a series of *trp* mutants in *Arabidopsis* (see Normanly et al., 1993), and the *orp* mutants of maize (Wright et al., 1991). Mutants blocked at the later stages of Trp biosynthesis show high levels of conjugated IAA (Wright et al., 1991; Normanly et al., 1993), indicating that these mutations may affect the rate of IAA biosynthesis, most probably by increasing indolic precursor pools. These results suggested to us that plants with increased rates of precursor biosynthesis at common steps of IAA and/or Trp biosynthesis may be useful for studies aimed at establishing the mechanisms by which plants regulate IAA metabolism. Such plants were obtained by selecting plants that are resistant to Trp analogs such as α-MT and 5-MT, since anthranilate synthase catalyzes the rate-limiting committed step in the indolic pathway. We have now shown that such selected lines are characterized by a high rate of flux through the IAA pool as measured by the apparent rate of turnover. An important difference between the MTR lines and mutants blocked in later steps of Trp biosynthesis (Wright et al., 1991; Normanly et al., 1993) is that the MTR lines contain somewhat increased levels of free IAA but normal levels of total IAA. The reverse is true of the Trp auxotrophic maize and *Arabidopsis* (Wright et al., 1991; Normanly et al., 1993). Thus, the MTR lines are important new tools for understanding the regulatory aspects of IAA biosynthesis in higher plants.

IAA Turnover

Relatively little is known about the turnover rates of plant hormones and almost nothing is known about the physiological aspects of turnover. It is implicit that a signal molecule must undergo turnover at a rate more rapid than that of the controlled process (see Cohen, 1983). If this was not true, then once the "message molecule" was made the signal would remain in the "on" state longer than necessary for the response to be completed and control would be lost. In addition, if the currently accepted concepts about hormone/receptors are not fully correct, or are only one component of the complex hormone response process, then turnover can be more critically important than previously thought. For example, IAA degradation in maize is accomplished by the concomitant oxidation of a fatty acid (Reinecke, 1990). If this activity results either in a change in lipid properties in the plant or the production of prostaglandin-like molecules (as, for example, jasmonic acid), then turnover could affect many plant processes. Similarly, in some plants such as bean (Bialek and Cohen, 1986), IAA is

found as a covalent modifier of peptides and proteins, and in such situations the rate of IAA production could affect the level of protein modification. Such considerations have remained untested, at least in part due to the lack of effective means to study changes in turnover. The fact that all of the four lines isolated as α-MT resistant were poorly fertile and set sterile seed when selfed suggests that sexual reproduction and/or embryogenesis are particularly sensitive processes in relation to auxin regulation or turnover, or high Trp levels, although similarly selected lines of other plant species are sometimes fertile (Ranch et al., 1983; Lee and Kameya, 1991; Kreps and Town, 1992). Our current results demonstrate that relatively large changes in the rates of IAA turnover can be obtained in selected lines (Table III) and that such plants show apparently normal vegetative morphology and growth.

Using *Lemna* for measuring IAA turnover, and with the selected lines now available, it should be possible to do detailed studies on the determinants of turnover in plants. For example, it should now be possible to determine what developmental and environmental events are accompanied by changes in IAA turnover. Such studies will be particularly enlightening for at least two reasons. First, classical studies of hormone levels could not detect situations in which IAA levels remain unchanged but turnover rates are affected. Second, changes in turnover could result from increased rates of synthesis balanced by an increase in the rates of degradation or, conversely, an enhanced rate of degradation could be balanced by increased biosynthesis. MTR lines should show effects similar to those shown by the parent line in the second case, but might be expected to show an enhanced change in the first case if precursor pools are, as expected, higher in the MTR lines.

We have described a plant line with an altered rate of turnover of a phytohormone. The characterization of this change in IAA turnover and the demonstration of the utility of using *Lemna* for such studies now makes it possible to ask meaningful questions about what affects turnover, and what the consequences of such changes are for the plant.

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LITERATURE CITED

Baldi BG, Maher BR, Slovin JP, Cohen JD (1991) Stable isotope labeling *in vivo* of D-and L-tryptophan pools in *Lemna gibba* and the low incorporation of label into indole-3-acetic acid. Plant Physiol **95:** 1203–1208

Bialek K, Cohen JD (1989) Free and conjugated indole-3-acetic acid in developing bean seeds. Plant Physiol **91:** 775–779

Bialek K, Michalczuk L, Cohen JD (1992) Auxin biosynthesis during seed germination in *Phaseolus vulgaris*. Plant Physiol **100:** 509–517

Brzović PS, Dunn MF (1992) Tryptophan synthase: catalysis, substrate channeling, and allosteric regulation. *In* BK Singh, HE Flores, JC Shannon, eds, Biosynthesis and Molecular Regulation of Amino Acids in Plants. American Society of Plant Physiologists, Rockville, MD, pp 37–51

Chen KH, Miller AN, Patterson GW, Cohen JD (1988) A rapid and simple procedure for purification of indole-3-acetic acid prior to GC-SIM-MS analysis. Plant Physiol **86:** 822–825

Cleland CR, Briggs WR (1967) Flowering responses of the plant *Lemna gibba* G-3. Plant Physiol **42:** 1553–1561

Cohen JD (1983) Metabolism of indole-3-acetic acid. What's New in Plant Physiology **14:** 41–44

Cohen JD, Baldi BG, Slovin JP (1986) $^{13}C_6$-[benzene ring]-Indole-3-acetic acid: a new internal standard for quantitative mass spectral analysis of indole-3-acetic acid in plants. Plant Physiol **80:** 14–19

Epstein E, Cohen JD, Bandurski RS (1980) Concentration and metabolic turnover of indoles in germinating kernels of *Zea mays* L. Plant Physiol **65:** 415–421

Hütter R, Niederberger P (1986) Tryptophan biosynthetic genes in eukaryotic microorganisms. Ann Rev Microbiol **40:** 55–77

King PJ (1988) Plant hormone mutants. Trends Genet **4:** 157–162

Kreps JA, Town CD (1992) Isolation and characterization of a mutant *Arabidopsis thaliana* resistant to α-methyltryptophan. Plant Physiol **99:** 269–275

Last RL, Fink GR (1988) Tryptophan-requiring mutants of the plant *Arabidopsis thaliana*. Science **240:** 305–309

Lee HY, Kameya T (1991) Selection and characterization of a rice mutant resistant to 5-methyltryptophan. Theor Appl Genet **82:** 405–408

Michalczuk L, Ribnicky DM, Cooke TJ, Cohen JD (1992) Regulation of indole-3-acetic acid biosynthetic pathways in carrot cell cultures. Plant Physiol **100:** 1346–1353

Moyed HS (1960) False feedback inhibition of tryptophan biosynthesis by 5-methyltryptophan. J Biol Chem **235:** 1098–1102

Niyogi KK, Fink GR (1992) Two anthranilate synthase genes in *Arabidopsis:* defense-related regulation of the tryptophan pathway. Plant Cell **4:** 721–733

Nonhebel HM, Cooney TP (1990) Measurement of the *in vivo* rate of indole-3-acetic acid turnover. *In* RP Pharis, SB Rood, eds, Plant Growth Substances 1988. Springer-Verlag, Berlin, pp 333–340

Normanly J, Cohen JD, Fink GR (1993) *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. Proc Natl Acad Sci USA **90:** 10355–10359

Ranch JP, Rick S, Brotherton JE, Widholm JM (1983) Expression of 5-methyltryptophan resistance in plants regenerated from resistant cell lines of *Datura innoxia*. Plant Physiol **71:** 136–140

Reid JB (1993) Plant hormone mutants. J Plant Growth Regul **12:** 207–226

Reinecke D (1990) The oxindole-3-acetic acid pathway in *Zea mays*. *In* RP Pharis, SB Rood, eds, Plant Growth Substances 1988. Springer-Verlag, Berlin, pp 367–373

Rekoslavskaya NI, Bandurski RS (1994) Indole as a precursor of indole-3-acetic acid in *Zea mays*. Phytochemistry **35:** 905–909

Sasse F, Buchholz M, Berlin J (1983) Site of action of growth inhibitory tryptophan analogues in *Cartharanthus roseus* cell suspension cultures. Z Naturforsch **38c:** 910–915

Slovin JP (1990) Chlorophyll-less mutant of *Lemna gibba* G-3 (abstract No. 522). Plant Physiol **93:** S-90

Slovin JP, Cohen JD (1985) Generation of variant lines of *Lemna gibba* G-3 via tissue culture, for use in selecting auxin metabolism mutants (abstract No. 53). Plant Physiol **77:** S-11

Slovin JP, Cohen JD (1988) Levels of indole-3-acetic acid in *Lemna gibba* G-3 and in a large *Lemna* mutant regenerated from tissue culture. Plant Physiol **86:** 522–526

Slovin JP, Tobin EM (1982) Synthesis and turnover of the light-harvesting chlorophyll a/b-protein in *Lemna gibba* grown with intermittent red light: possible translational control. Planta **154:** 465–472

Stäheli P, Kradolfer P, Neiderberger P, Hütter R (1981) Inhibition of yeast t-RNA^{trp} aminoacylation by 4-methyltryptophan. Arch Microbiol **129:** 146–149

Widholm JM (1972a) Anthranilate synthetase from 5-methyltryp-

tophan-susceptible and resistant cultured *Daucus carota* cells. Biochim Biophs Acta **279:** 48–57

Widholm JM (1972b) Cultured *Nicotiana tabacum* cells with an altered anthranilate synthetase which is less sensitive to feedback inhibition. Biochim Biophys Acta **261:** 52–58

Widholm JM (1972c) Tryptophan biosynthesis in *Nicotiana tabacum* and *Daucus carota* cell cultures: site of action of inhibitory tryptophan analogs. Biochim Biophys Acta **261:** 44–51

Widholm JM (1977a) Relation between auxin autotrophy and tryptophan accumulation in cultured plant cells. Planta **134:** 103–108

Widholm JM (1977b) Selection and characterization of amino acid analog resistant plant cell cultures. Crop Sci **17:** 597–600

Wright AD, Sampson MB, Neuffer MG, Michaliczuk L, Slovin JP, Cohen JD (1991) Indole-3-acetic acid biosynthesis in the mutant maize *orange pericarp*, a tryptophan auxotroph. Science **254:** 998–1000

Zilversmit DB, Enterman C, Fishler MC (1943) On the calculation of "turnover rate" from experiments involving the use of labeling agents. J Gen Physiol **26:** 325–331