Tryptophan Analog Resistance Mutations in *Chlamydomonas reinhardtii*

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ABSTRACT

Forty single gene mutations in *Chlamydomonas reinhardtii* were isolated based on resistance to the compound 5′-methyl anthranilic acid (5-MAA). In other organisms, 5-MAA is converted to 5′-methyltryptophan (5-MT) and 5-MT is a potent inhibitor of anthranilate synthase, which catalyzes the first committed step in tryptophan biosynthesis. The mutant strains fall into two phenotypic classes based on the rate of cell division in the absence of 5-MAA. Strains with class I mutations divide more slowly than wild-type cells. These 17 mutations map to seven loci, which are designated *MAA1* to *MAA7*. Strains with class II mutations have generation times indistinguishable from wild-type cells, and 7 of these 23 mutations map to loci defined by class I mutations. The remainder of the class II mutations map to 9 other loci, which are designated *MAA8–MAA16*. The *maa5–1* mutant strain excretes high levels of anthranilate and phenylalanine into the medium. In this strain, four enzymatic activities in the tryptophan biosynthetic pathway are increased at least twofold. These include the combined activities of anthranilate phosphoribosyl transferase, phosphoribosyl anthranilate isomerase, indoleglycerol phosphate synthetase and anthranilate synthase. The slow growth phenotypes of strains with class I mutations are not rescued by the addition of tryptophan, but the slow growth phenotype of the *maa6–1* mutant strain is partially rescued by the addition of indole. The *maa6–1* mutant strain excretes a fluorescent compound into the medium, and cell extracts have no combined anthranilate phosphoribosyl transferase, phosphoribosyl anthranilate isomerase and indoleglycerol phosphate synthetase activity. The *MAA6* locus is likely to encode a tryptophan biosynthetic enzyme. None of the other class I mutations affected these enzyme activities. Based on the phenotypes of double mutant strains, epistatic relationships among the class I mutations have been determined.

IN *Chlamydomonas reinhardtii*, strains that exhibit an amino acid auxotrophic phenotype are rare (LI, REDEI and GOWANS 1967). There are mutant strains that require the addition of the vitamins nicotinamide (7 loci), thiamine (7 loci), or *p*-aminobenzoic acid (2 loci) to the medium (EVERSOLE 1956; EBERSOLD 1962; EBERSOLD *et al.* 1962; HASTINGS *et al.* 1965; LEVINE and GOODENOUGH 1970). The only amino acid auxotrophic strains that have been described require arginine; these strains define six loci (EBERSOLD 1956; EVERSOLE 1956; HASTINGS *et al.* 1965; LOPPES 1969; LOPPES and HEINDRICKS 1986). The paucity of amino acid auxotrophic mutations is not unique to Chlamydomonas; this class of mutations is rare in photosynthetic organisms in general (MCCOURT and SOMERVILLE 1987).

The failure to find other amino acid auxotrophs could result from an inability to import these amino acids (KIRK and KIRK 1977). This cannot be true for all amino acids in *C. reinhardtii* because cells can grow on medium that contains either leucine (LOPPES 1969) or glutamine (SAGER and GRANICK 1953) as the sole nitrogen source. Additional evidence for the ability to import amino acids comes from the relief of inhibition by a number of compounds that interfere with amino acid biosynthesis by the addition of specific amino acids to the medium. Glyphosate inhibits 5′enolpyruvylshikimate 3-phosphate synthase (EPSP), which is the penultimate enzyme in the shikimic acid pathway (STEINRUCKEN and AMRHEIN 1980). Glyphosate inhibition is relieved by the addition of phenylalanine and tyrosine to the medium (GRESSHOFF 1979). The effect of methionine sulfoximine, which inhibits glutamine synthetase (CULLIMORE and SIMS 1980; YOUNG and RINGOLD 1983), can be negated similarly by the addition of methionine (NAKAMURA, LEPARD and MACDONALD 1981). However, not all inhibitors of amino acid biosynthetic enzymes can be blocked by addition of amino acids in Chlamydomonas. Addition of 100 mM valine, leucine or isoleucine does not relieve inhibition by sulfometuron-methyl (HARTNETT, NEWCOMB and HODSON 1987). Sulfometuron-methyl inhibits acetolactate synthase, which is the first enzyme in branched amino acid biosynthesis.

A second reason for the failure to find amino acid auxotrophic mutations is that the mutations have a pleiotropic effect on cells that can not be rescued by the addition of amino acids. In higher plants, amino

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acid auxotrophic mutations have been isolated in plant tissue culture cells, although fertile plants have not been regenerated (SIDOROV, MENCZEL and MALIGA 1981; ROTH and LARK 1982; GEBHARDT *et al.* 1983; HORSCH and KING 1983) or the phenotype is not transmitted as a single gene trait (NEGRUTIU *et al.* 1985). Auxotrophs that require valine, threonine, or isoleucine and tryptophan, tyrosine, or phenylalanine have been isolated in the alga *Scenedesmus obliquus,* but the lack of a sexual cycle has made it difficult to determine the genetic properties of these mutant strains (MATAGNE and VINCENZOTTO 1980).

Another potential reason for the failure to find amino acid auxotrophs is the existence of redundant genes. An auxotrophic phenotype may require multiple mutations or mutations in regulatory genes. Molecular evidence for multiple genes has been obtained for glutamine synthetase in a number of plants (TISCHER, DASSARMA and GOODMAN 1986; TINGEY *et al.* 1988), for EPSP of the shikimic acid pathway in Arabidopsis (KLEE, MUSKOPF, and GASSER 1987), and for the tryptophan synthase β-subunit, which is present in at least two copies in *Arabidopsis thaliana* (BERLYN, LAST and FINK 1989) and in maize (WRIGHT *et al.* 1990). The number of copies of these genes in green algae is not known.

In bacteria (CASTER 1967; ZURAWSKI *et al.* 1978) and in yeast (SINGH and SHERMAN 1974; BRETON and SURDIN-KERJAN 1977; CHATTOO *et al.* 1979; HSU, KOHLHAW and NIEDERBERGER 1982; BOEKE, LACROUTE and FINK 1984) it has been possible to select auxotrophic mutations directly through the use of analogs of various precursors. In many of these cases, failure to metabolize the analog confers resistance; that is, the loss of activity of enzymes in the biosynthetic pathway provides resistance. 5′-Methyl anthranilic acid (5-MAA) is an analog of anthranilic acid that is enzymatically converted to 5′-methyltryptophan (5-MT). In *Escherichia coli* (MOYED 1960), yeast (SCHURCH, MIOZZARI and HUTTER 1974), and the plant *Datura innoxia* (RANCH *et al.* 1983), both tryptophan and 5-MT act by negative feedback control to repress the activity of anthranilate synthase (AS), which catalyzes the first committed step in tryptophan biosynthesis (see Figure 1 for diagram of pathway). Although 5-MT represses tryptophan biosynthesis in these organisms, it cannot replace tryptophan in protein synthesis and cells starve for tryptophan. Mutations in genes of the tryptophan biosynthetic pathway are one means to obtain resistance to 5-MAA. Cells unable to convert 5-MAA to 5-MT are not inhibited. Survival of cells carrying this type of mutation requires the addition of limiting amounts of tryptophan. Alternatively, cells carrying a hypomorphic mutation in a step in tryptophan biosynthesis may survive because they still complete some tryptophan biosynthesis

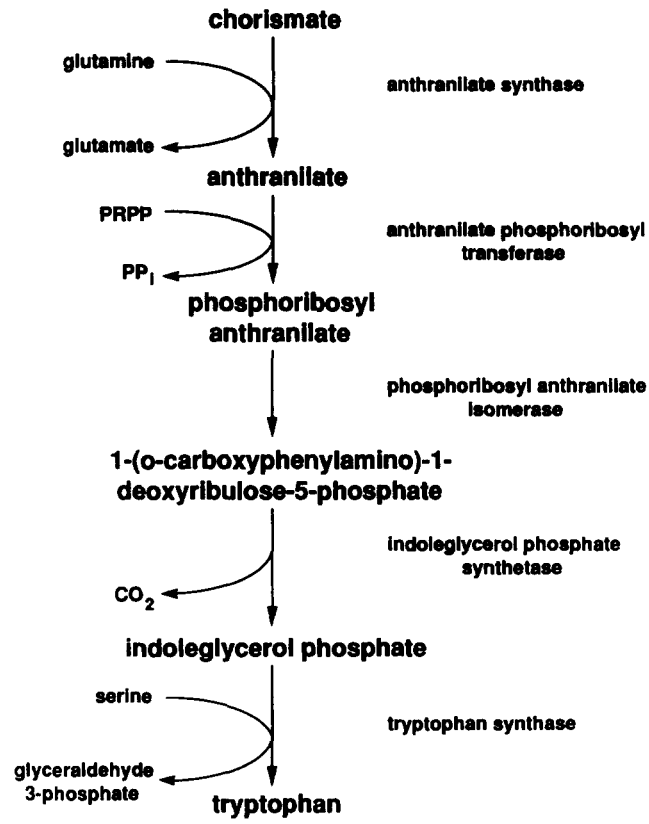

FIGURE 1.—The tryptophan biosynthetic pathway in bacteria and fungi (YANOFSKY and CRAWFORD 1987; BRAUS 1991). Anthranilate production is the first committed step in bacteria and fungi. Chorismate is also the precursor to tyrosine and phenylalanine biosynthesis. Anthranilate synthase is thought to be the control point in higher plants (SINGH and WIDHOLM 1974). 5-MAA is converted to 5-MT by the biosynthetic enzymes of the pathway. 5-MT represses anthranilate synthase, but is not incorporated into proteins. 5-FI is metabolized by the β-subunit of tryptophan synthase to 5-FT, which is toxic to cells.

but produce only sublethal amounts of 5-MT.

In *Arabidopsis thaliana,* LAST and FINK (1988) isolated rare tryptophan auxotrophic mutations by screening for plants resistant to 5-MAA. Extracts of plants with the *trp1–1* mutation lack anthranilate phosphoribosyl transferase activity (LAST and FINK 1988) and those from *trp2–1* mutants lack tryptophan synthase (LAST *et al.* 1991). These mutations suggest that a tryptophan biosynthetic pathway similar to the one in bacteria and fungi exists in photosynthetic organisms and that the mode of action for 5-MAA is similar.

We have selected mutations in *C. reinhardtii* that confer resistance to 5-MAA with the goal of identifying genes involved in tryptophan biosynthesis and in its regulation. In our selection, we obtained 41 mutant strains that fall into two phenotypic classes. One class grows more slowly than wild-type cells in the absence of 5-MAA, while the growth rate of the second class is indistinguishable from wild-type cells. In this paper, we primarily discuss the set of 17 mutations that grow

more slowly than wild-type cells; these mutations define seven loci. Seven additional mutations that have normal growth phenotypes map to three of these loci. The loci defined by mutations that result in slow growth were studied initially with the hypothesis that they were most likely to be tryptophan auxotrophs. We find that only the *MAA6* locus appears to encode a tryptophan biosynthetic enzyme. A possible pathway for the action of several of these loci in tryptophan biosynthesis is proposed.

MATERIALS AND METHODS

Media and culture conditions: Standard medium was SAGER and GRANICK (1954) Medium I as modified by HOLMES and DUTCHER (1989), and is referred to as rich medium. Other types of media are described in DUTCHER *et al.* (1991). Cultures grown in the presence of 5-MAA or 5-MT were exposed to low light intensities (8 μEinsteins/m^2/sec) to prevent the light-induced breakdown of the two compounds. Standard growth conditions were 21° and 17.5 μEinsteins/m^2/sec cool white fluorescent light. For examining heat or cold-sensitive growth defects, cells were grown at 32° or 16°. Filter-sterilized urea or acetamide in 95% ethanol were added as alternative nitrogen sources to autoclaved media.

Mutagenesis and mutant isolation: Mutagenesis was performed by ultraviolet irradiation of wild-type strain 137c *mt*$^-$ as described in DUTCHER, GIBBONS and INWOOD (1988). Mutagenized cells were plated onto medium containing 1.16 mM 5-MAA and 0.12 mM tryptophan. Resistant colonies were isolated after 2 weeks of growth at 21° and rescreened for the inhibitor resistance phenotype. Single colonies were backcrossed to the wild-type 137c *mt*$^+$ strain to determine if the resistance phenotype segregated two sensitive progeny to two resistant progeny. Each resistant isolate was obtained from a separate mutagenized population of cells to assure independence. The frequency of analog resistance mutations after mutagenesis was 2×10^{-5}.

Genetic analysis: 5-MAA resistant progeny from backcrosses of the mutant isolates to wild-type cells were used in mapping crosses. All dissections were performed as described by LEVINE and EBERSOLD (1960) and HARRIS (1989). The mutant strains used for linkage group assignments were obtained from the Chlamydomonas Genetics Center. The *pf6, pf10, pf14, pf15, pf19, pf24, pf25, pf27, pf28, pf29* mutant strains have flagellar phenotypes. These strains are nonmotile and accumulate as a pellet when resuspended in liquid medium. The *pf2* and *pf9* mutant strains have a slow swimming phenotype that is scored visually with the light microscope. The *fla10–15, fla9* and the *uni1* mutant strains were scored by a pellet-forming phenotype following incubation at 32°. The acetate-requiring phenotypes of *ac17, ac177* and *ac210* were determined by failure to grow on minimal medium. The *nic13* and *nic7* mutant alleles were scored by failure to grow on rich medium with 62 μM acetylpyridine. The *ery2, apm1* and *act2* mutant strains were scored by resistance to erythromycin, oryzalin and cycloheximide, respectively. The wild-type *NIT2* allele was scored on rich medium prepared with washed agar and sodium nitrate (2.8 mM) substituted for ammonium nitrate.

Mating-type was determined by mating with *mt*$^-$ and mt$^+$ tester strains and scored by the formation of pellicle or the microscopic observation of quadriflagellate cells. Quantitation of the ability of cells to mate was assayed microscopically by the appearance of quadriflagellate cells. Each mutant was grown in the presence of ammonium nitrate for 2–5 days. Cells were resuspended into M-N/5 medium in the presence of wild-type (137c) cells that were competent to mate. Cultures were examined after 30 min and 500 cells were counted.

Light microscopy analysis of the morphology of mutant strains: Cells grown in liquid rich medium without agitation were examined by phase contrast microscopy (Zeiss Axiophot) equipped with a 20× Neofluor objective and photographed with 400 ASA Tri-X pan film. Negatives were projected onto a screen and the cells traced. Areas within the traced cells were measured with a Keuffel and Esser compensating polar planimeter. For each mutant strain and growth condition 20–30 cells were measured and the average area calculated. Photographs of hemacytometer squares were similarly traced and the area measured by planimeter. This measurement was used to calibrate the magnification of the cells. One square centimeter measured by the planimeter was equal to 19.24 m^2. Cells were approaching stationary phase when they were photographed.

Analysis of growth defects and other phenotypes: To analyze the cosegregation of the slow growth phenotype and the 5-MAA resistance phenotype, the growth defect was monitored in several different ways. In most cases, the slow growth phenotype was determined by visual inspection of relative colony sizes in tetrads. The two smaller colonies in a tetrad were designated as slower growing, while the two larger colonies were designated as wild type in phenotype. Growth was also assessed by comparing mutant and wild-type density after growth on solid medium of a 5-μl drop of liquid culture. For a quantitative measure of differences in growth, colony diameters were measured using an ocular micrometer on a dissecting microscope that was calibrated with a stage micrometer. Twenty colonies of each mutant strain were measured and the average was calculated to obtain a measure of colony size for a given time period of growth. Finally, the growth rates of liquid cultures were determined by hemacytometer counts in combination with plating serial dilutions to obtain the number of viable cells. To analyze the effects of tryptophan or indole on the slow growth phenotype, cells were plated on solid media that contained urea (1 mM) or acetamide (10 mM) as a source of reduced nitrogen (GRESSHOFF 1981).

Resistance or sensitivity to other inhibitors was scored as a qualitative difference in growth on control medium versus inhibitor-containing medium. Phenotypes on 5-MT-containing media were scored at concentrations of 0.46, 1.05 or 1.51 mM 5-MT, which was added in 95% ethanol after autoclaving. Drug resistance phenotypes were assayed with the following concentrations of filter-sterilized inhibitors: anisomycin (60 μM), aminotriazole (12 μM), atrazine (3 μM), canavanine (2.8 mM), chlorsulfuron (22 or 44 μM), cycloheximide (64 μM), erythromycin (136 μM), 5-fluoroindole (7 μM), glyphosate (5.9–59 μM), methionine sulfoximine (2.8 mM), oryzalin (15 μM), spectinomycin (150 μM), streptomycin (86 μM), sulfometuron methyl (10 μM), triazole (5–30 mM) and tunicamycin (4 μM). They were added to autoclaved media.

Dominance tests: Heterozygous diploid strains were selected as arginine prototrophs at 25° following mating between strains carrying the *arg2* and the *arg7* mutations (EBERSOLD 1967). One of the *arg*$^-$ parents carried a 5-MAA resistance mutation. Larger colonies that appeared within 4 days were isolated and restreaked twice for single colony isolates on medium that lacked arginine. For each diploid construction 16 colonies were selected and analyzed. Growth rates and phenotypes on media with inhibitors were determined for each set of diploid strains as well as for the

parental haploid strains. The wild-type haploid strain 137c and a diploid strain heterozygous for the *pf9–2* mutation were used as controls.

Enzyme assays: Cells for enzyme assays were grown on solid rich medium. Approximately 2 g of cells were harvested for each assay. They were suspended into liquid minimal medium with 1/5 the normal concentration of nitrogen (M-N/5), rinsed once in M-N/5 and resuspended in 2 ml breaking buffer (200 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 8 mM $MgCl_2$, 0.2 mM dithiothreitol and 60% glycerol). Cells were placed in liquid nitrogen and frozen overnight at −70°. Upon thawing, the suspension was centrifuged at 10,000 rpm in a Sorvall SS-34 rotor for 10 min. The pellet was resuspended in 1 ml of the breaking buffer and placed overnight at −70°. The suspension was sonicated on ice using 10 repetitions of 10-sec pulses with a microtip. Cell lysates were clarified by centrifugation at 10,000 rpm for 10 min in a SS-34 rotor. Protein concentrations of crude cell extracts were determined with a Bio-Rad Protein Assay reagent, which was quantitated by absorbance at 595 nm against bovine serum albumin standards run in the same assay.

Enzyme assays were performed essentially as described by LAST and FINK (1988). AS activity was monitored by mixing 0.1–25 mg protein to a 2-ml reaction mix containing glutamine, chorismic acid (barium salt), Tris-HCl and $MgCl_2$. Reactions were incubated from 10 to 30 min at room temperature (approximately 25°). Wild-type cell lysates produce reaction rates that are linearly dependent upon time and protein concentration for both reactions (data not shown). Reactions were stopped by the addition of 0.2 ml of 1 M HCl. Anthranilate was extracted from the reaction mix by vortexing with 2 ml of spectrophotometric grade ethyl acetate. After centrifuging to separate the layers, the ethyl acetate layer was removed and its fluorescence measured with a Perkin-Elmer fluorescence spectrophotometer (340 nm excitation/400 nm emission). Fluorescence levels were converted to nmoles anthranilate by extrapolation using a standard curve prepared from measured levels of fluorescence from known concentrations of anthranilate. Conversion of anthranilate to indole-3-glycerol phosphate, which is not fluorescent, by the combined anthranilate transferase/isomerase and indoleglycerol phosphate synthetase activities was measured using the method of LAST and FINK (1988) with a 1-ml reaction buffer containing Tris-HCl, pH 8.0, $MgCl_2$, anthranilate, and 5-phosphorylribose 1-pyrophosphate. Incubation of the reaction was at 30°. Reactions were stopped by addition of acid. Anthranilate was extracted with ethyl acetate and fluorescence of the extract was measured. In these reactions the rate of disappearance of anthranilate was measured.

Identification of anthranilate in medium: After approximately 1 month of growth on solid rich medium, agar devoid of cells was removed from wild-type and *maa5–1* conditioned plates. The agar sections were placed in 4 ml of distilled water in 13 × 100 mm screw cap test tubes and heated in a water bath (80°) until the agar dissolved. The tubes were cooled and 1.5 ml ethyl acetate added to each sample to extract anthranilate. After shaking the tubes and centrifuging the sample to separate the two phases, the ethyl acetate fractions were removed and the fluorescence emission spectrum of the samples from 200–700 nm was examined on a Aminco Bowman scanning spectrofluorometer (340 nm excitation). The fluorescence of a standard solution of anthranilate (7.3 μM) was used as a standard for comparison. To determine whether the browning of the anthranilate was an oxidative reaction, two solutions were prepared. The first was a 7.3 μM anthranilic acid solution in distilled water in a 16 × 150 mm screw cap test tube under room air and the second was a 7.3 μM anthranilic acid solution that was bubbled with N_2 for 30 min. The sample was placed in a 16 × 150 mm screw capped test tube under a N_2 atmosphere. After 6 weeks the optical density of the samples were measured on a Varian Model 635 spectrophotometer (420 nm, 1 cm path length).

For the determination of phenylalanine in the medium, cells were grown in standard medium lacking tryptophan for 2 weeks and then removed by centrifugation. The medium was concentrated by lyophilization and assayed using high performance liquid chromatography with the conditions of JONES and GILLIGAN (1983).

RESULTS

Wild-type *C. reinhardtii* is sensitive to 1.16 mM 5-MAA, which is an analog of the tryptophan precursor anthranilic acid. Following mutagenesis of strain 137c mt^- with ultraviolet irradiation (see MATERIALS AND METHODS), strains resistant to 5-MAA were selected in the presence of ammonium nitrate as the nitrogen source and limiting amounts of tryptophan. Forty-one independent 5-MAA-resistant mutant strains were recovered. Each mutant strain was crossed to a wild-type tester strain (137c mt^+). In 40 of the 41 mutant strains, the drug resistance phenotype results from a single gene mutation (Table 1). One mutant strain, M1, has at least two mutations that confer resistance to 5-MAA and these mutations segregate independently of one another. We have not characterized this strain further.

Class I 5-MAA resistance mutations: The remaining 40 mutations were grouped into two classes. 5-MAA-resistant mutant strains in the first class grow more slowly than wild-type cells under our standard conditions while members of the second class have growth rates indistinguishable from wild-type cells. The addition of 5-MAA does not rescue the slow growth phenotype; these strains are not dependent on the presence of 5-MAA. The slow growth phenotype cosegregates with the drug resistance phenotype for the 17 single gene class I mutations in crosses to wild-type strains (Table 1), which suggests that the two phenotypes are the result of the same single gene mutation.

The class I 5-MAA resistance mutations map to at least seven loci based on recombination mapping (Table 1). Because several of the loci are defined by dominant alleles (see below), we have relied primarily on recombination mapping to define the number of loci. Therefore, our estimates represent the minimum number of loci involved. For the loci that have multiple recessive alleles, we have performed complementation tests among the alleles (Table 3). The loci have been named *MAA* for *m*ethyl *a*nthranilic *a*cid. These seven loci map to six different linkage groups in *C. reinhardtii* (Figure 2 and DUTCHER *et al.* 1991). The *maa3* and *maa4* mutations are closely linked to the

TABLE 1

Genetic characterization of *maa*⁻ mutations

Locus and allele	Isolation name	Class[a]	2ˢ:2ʳ Segregation[b]	Mapping data: Marker	Linkage group	No. of tetrads[c]
maa1-1	M62	I	148	*nic15*	XII/XIII	239:0:1[d]
maa1-2	M41	I	188	*maa1-1*		22:0:0
				pf27		110:0:53
maa1-3	M11	I	33	*maa1-1*		11:0:0
				nic15		8:0:0
maa2-1	M45	I	150	*ac210*	III	3:0:10
				pf15		48:0:5
				nit2		17:0:11
				ac17		6:0:14
maa2-2	M29	I	31	*maa2-1*		33:0:0
				pf15		20:0:0
maa2-3	M39	I	39	*maa2-1*		79:0:0
maa2-4	M22	I	19	*maa2-1*		15:0:0
maa2-5	M54	I	28	*maa2-1*		21:0:0
maa2-6	M31	II	55	*maa2-1*		37:0:0
maa2-7	M51	II	63	*maa2-1*		31:0:0
maa3-1	M17	II	122	*pf19*	X	64:0:0
				nic13		72:0:13
				pf25		3:0:19
				pf24		64:0:1
				pf6		16:0:4
				ac17		8:5:4[e]
maa3-2	M36	II	14	*pf19*		21:0:0
maa3-3	M40	II	26	*pf19*		15:0:0
maa3-4	M32	I	40	*pf19*		20:0:0
maa3-5	M46	I	82	*pf19*		29:0:0
maa3-6	M28	I	47	*maa3-5*		13:0:0
maa3-7	M58	II	47	*pf19*		10:0:0
maa4-1	M38	I	301	*ac177*	XIV	15:0:7
				ery2		56:0:0
				pf27		12:19:2[e]
maa4-2	M43	I	100	*maa4-1*		44:0:0
				ac177		17:0:5
maa4-3	M47	I	50	*maa4-2*		10:0:0
				ac177		19:0:12
maa5-1	M15	I	430	*fla10-15*	XIX	32:0:3
				uni1		56:1:23
				pf10		10:0:19
				apm1		34:0:37
				pf27		23:19:38[e]
maa6-1	M19	I	346	*mt*	VI	144:0:1
				nic7		35:0:0
				pf14		9:2:29
maa7-1	M44	I	161	*maa2-1*	III	75:0:4
				maa2-3		83:0:6
maa7-2	M21	II	55	*maa7-1*		109:0:0
				maa2-1		160:0:11
maa8-1	M60	II	109	*mt*	VI	25:0:36
				pf14		78:0:18
				maa6-1		10:0:27
maa9-1	M23	II	71	*pf28*	XI	152:0:4
				pf2		76:0:3
				pf29[f]		309:0:19
				ery1		131:0:95
maa9-2	M55	II	51	*maa9-1*		89:0:0
maa10-1	M27	II	51	*fla9*	XIX	9:0:10
				uni1		119:0:20
maa10-2	M35	II	38	*maa10-1*		42:0:0
				uni1		15:0:4
maa10-3	M61	II	78	*maa10-1*		141:0:0
				uni1		10:0:1
				fla10		11:0:21
				pf10		4:0:14
				maa5-1		30:0:13
maa11-1	M57	II	91	*maa5-1*	XIX	367:0:0
				fla10-15		115:0:16
				pf10		40:0:50
				maa10-1		34:0:27
				maa10-2		16:0:11
				maa10-3		41:0:27
				apm1		47:0:27
maa12-1	M53	II	23	*mt*	VI	366:0:15
				pf14		19:1:77
				maa6		127:0:8
				maa8		44:0:56
maa13-1	M37	II	71	*maa7-1*	III	32:0:3
				maa7-2		35:0:4
				maa2-1		48:0:0
maa13-2	M56	II	47	*maa13-1*		49:0:0
				maa7-1		26:0:1
				maa7-2		32:0:3
				maa2-1		43:0:0
maa13-3	M52	II	28	*maa13-2*		24:0:0
				maa7-1		36:0:0
				maa7-2		30:0:1
				maa2-1		49:0:0
maa14-1	M33	II	63	ND	ND	
				pf2		43:38:7[e]
maa14-2	M49	II	74	*maa14-1*		43:0:0
maa15-1	M34	II	42	ND	ND	
maa16-1	M24	II	98	ND	ND	
				pf29		8:9:48[e]
maa16-2	M26	II	59	*maa16-1*		131:0:0

[a] Class I mutations show the slow growth phenotype described in the text. Class II mutations are indistinguishable from wild-type cells in the absence of 5-MAA under our normal conditions.

[b] The number of tetrads that gave two sensitive (s) and two resistant (r) meiotic progeny in crosses to wild-type and/or mapping strains. No other types of tetrads were observed. All class I resistant progeny exhibit the slow growth phenotype in crosses to wild-type cells; the two phenotype cosegregate. The isolate M1, which is not included in the table, did not segregate 2ˢ:2ʳ meiotic progeny.

[c] Types of tetrads, PD (parental ditype), NPD (nonparental ditype) and TT (tetratype).

[d] Data from DUTCHER *et al.* 1991.

[e] This is a cross of an unlinked centromere-linked marker to determine the distance of the *MAA* locus to the centromere.

[f] The *pf29* mutation was incorrectly mapped to linkage group XIX by RAMANIS and LUCK (1986). This mutation shows linkage to *pf28* (195:0:8), to *pf2* (85:0:1) and to *ery1* (40:0:28) (J. A. HOLMES, D. E. JOHNSON and S. K. DUTCHER, unpublished data).

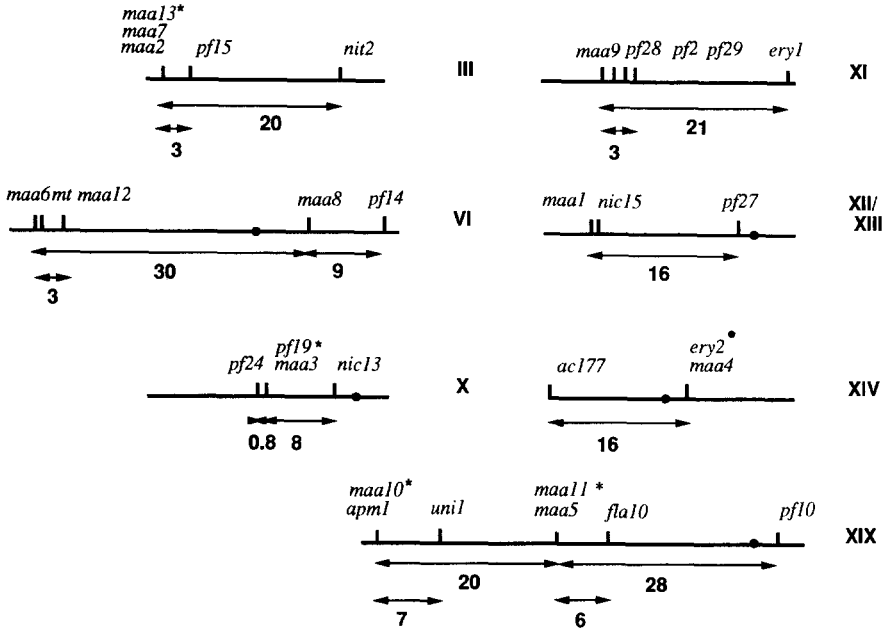

FIGURE 2.—Partial genetic map of *C. reinhardtii*. Shown is the position of each 5-MAA-resistance locus and the loci used in mapping them. *indicates the order of the loci is not known. The location of the centromere on linkage group XI was not determined. We have shown recently that linkage groups XII and XIII are not independent linkage groups; they represent a single linkage group that has been named linkage group XII/XIII (DUTCHER *et al.* 1991). The mapping data are from Table 1 and DUTCHER *et al.* 1991.

pf19 and *ery2* mutations on linkage group X and XIV, respectively (Table 1). Complementation was observed in stable heterozygous diploid strains with the linked markers.

Class II 5-MAA resistance mutations: Seven of the class II mutations map to three of the loci defined by class I mutations. The remaining 16 mutations map to at least nine loci. Six loci have been mapped (Table 1 and Figure 2). The map positions of the *MAA11*, *MAA15* and *MAA16* loci have not been determined, but each is unlinked to other *MAA* loci (data not shown). The *maa5* and *maa11* mutations have not been separated by recombinational analysis, but they complement one another for resistance to 5-MT (see below, Table 3). The *maa2* and the *maa13* mutations have not been separated by recombinational analysis but each *maa13* allele complements the *maa2–1* allele for the 5-MT phenotype (see below, Table 3). For the class II mutant strains, the complete pairwise matrix of heterozygous diploid strains (23 by 23 mutants) was examined (data not shown). All combinations were resistant to 5-MAA as would be expected from the dominance patterns obtained (see below, Table 3). In the remainder of the work, we have concentrated on the characterization of the loci defined by the slow growing class I mutant strains with the rationale that these mutations were more likely to define genes encoding enzymes involved in tryptophan biosynthesis.

Class I mutant phenotypes are not rescued by tryptophan: All class I mutant strains, by definition, produce smaller colonies than wild-type cells after comparable periods of growth. A quantitative measure of this phenotype at 21° is shown in Figure 3. Cells were plated onto solid medium without tryptophan, grown for 6 days and the diameter of colonies measured. From these data, it is clear that colony size is allele-specific and not locus-specific. A similar distribution, but shifted toward larger colony sizes, is observed after 10 days of growth (data not shown). The small colony phenotype on solid medium is correlated with an increase in cell doubling time in liquid cultures. At 21° the doubling times for three mutant strains *maa1–1*, *maa2–1* and *maa5–1* are 19.4, 17.0 and 23.5 hr, respectively, as compared to a 10.0-hr doubling time for wild-type cells.

Since 5-MAA-resistant strains in Arabidopsis are tryptophan auxotrophs (LAST and FINK 1988; LAST *et al.* 1991), we asked if the small colony/slow doubling time phenotype could be rescued by the addition of tryptophan to the medium. Addition of 0.12 mM (Figure 3) or 0.74 mM tryptophan does not restore a wild-type phenotype to any of the mutant strains. In contrast, addition of tryptophan at 0.24 mM is sufficient to relieve the inhibition of growth of wild-type cells by 1.16 mM 5-MAA.

Because we failed to observe rescue of the slow growth phenotype by the addition of tryptophan with our standard medium, we tried several variations. Ammonium ions inhibit the transport of different amino acids into several green algae (NORTH and STEPHENS 1971, 1972; KIRK and KIRK 1977), including Chlamydomonas (LOPPES 1970; LOPPES and STRIJKERT 1972). In place of ammonium ions, we used 10 mM acetamide as a source of reduced nitrogen (GRESSHOFF 1981), which leads to slightly slower growth rates for both wild-type and mutant cells after 6 days (data not shown). Neither tryptophan at 0.74 mM nor arginine at 1.15 mM, in the presence of acetamide as the source of reduced nitrogen, restore

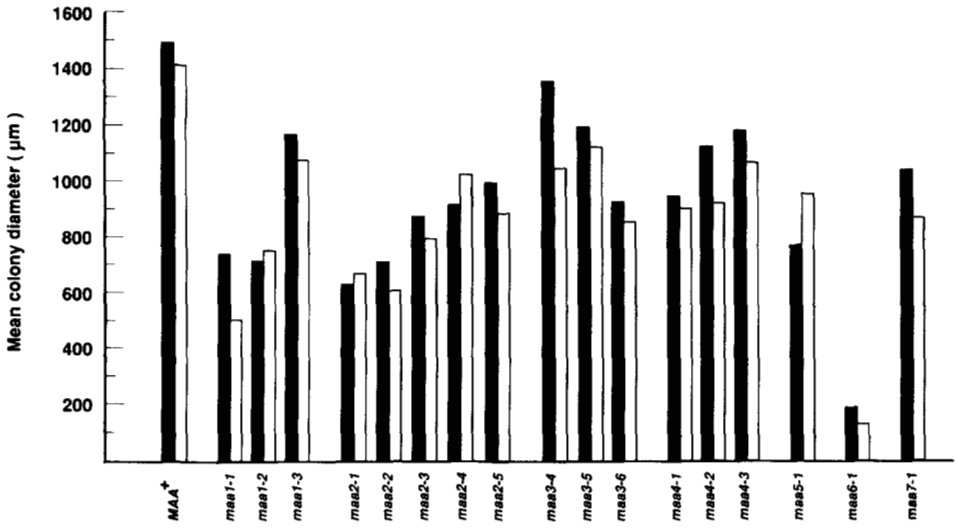

FIGURE 3.—Average colony diameter of class I mutations. Colonies were measured 6 days after individual cells were plated onto medium without tryptophan (solid bars) and with tryptophan (0.12 mM) (white bars). Cells were grown at 21° with 17.5 μEinstein/m^2/sec light intensity and with 1 mM urea as a nitrogen source. Twenty colonies were measured for each bar. Chi square tests showed no significant differences between the medium with tryptophan and without it.

a wild-type growth phenotype to any of the mutant strains as determined by the colony diameter after 10 days of growth (data not shown). As none of the *maa*$^-$ mutant strains are rescued by tryptophan, it is likely that either they do not define tryptophan biosynthetic genes or that tryptophan is not efficiently imported into these cells.

To ask if any of these mutant stains are starved for nitrogen in the presence of a reduced nitrogen supply, we examined their size by light microscopy (starved cells are smaller than nonstarved cells) and the ability of cells to mate (mating requires nitrogen starvation, SAGER and GRANICK 1954). Cultures were grown under conditions in which wild-type cells would be in logarithmic growth. The size of cells in mutant cultures were not statistically different from wild-type cultures and we could detect no increase in the frequency of mated cells by comparison to wild-type cells (data not shown). We conclude from these results that the mutant strains do not have general defects in nitrogen metabolism.

Rescue of the *maa6–1* mutant phenotype by indole: We tested whether two intermediates in the biosynthetic pathway could rescue the small colony phenotype of class I mutant strains with the rationale that these compounds may be imported more efficiently than tryptophan itself. We tested indole, which is the last intermediate in the tryptophan biosynthetic pathway and anthranilate, which is the first intermediate in the pathway. 0.043 mM indole negates the effects of 5-MAA on wild-type cells. The slow growth phenotype of one of the 17 class I mutant strains (*maa6–1*) is partially rescued by the addition of 0.043 mM indole. Higher concentrations of indole were not tested as they had deleterious effects on wild-type cells. Wild-type cells form colonies of the same size on indole and standard medium while *maa6–1* colonies are about 65% of wild-type colony size on indole medium and only about 12% of wild-type colony size on standard medium (n = 100 colonies). Anthranilate can rescue wild-type cells from inhibition by 5-MAA at concentrations of 0.073 mM, but it does not rescue the slow growth phenotype of any of the class I mutant strains. This results suggests that the *MAA6* locus may encode an enzyme in the biosynthetic pathway and that the other class I loci do not encode enzymes in this pathway prior to the last step.

TABLE 2

Comparison of mutant and wild-type growth rates at different temperatures

Strain	Ratio of mutant/wild-type doubling times		
	16°	21°	32°
Nonconditional mutant strains			
maa3-1	1.28	0.97	1.08
Cold-sensitive mutant strains			
maa1-1	0.21	0.24	0.67
maa1-2	0.29	0.34	1.14
maa6-1	0.20	0.22	0.67
maa7-1	0.04	0.43	1.19
Cold and temperature-sensitive mutant strain			
maa2-1	0.64	0.32	0.64

Conditional phenotypes of class I mutant strains: Although all class I mutant strains form small colonies at 21°, some of these mutant strains produce nearly wild-type sized colonies at 32° or 16° (data not shown). The conditional growth phenotypes are allele-specific and not locus-specific. To quantitate the effects of different temperatures, doubling times in liquid cultures were compared (Table 2). Strains with the mutations *maa1–1, maa1–2, maa6–1* and *maa7–1,* which grow more slowly at both 16° and 21°, show more wild-type-like patterns of growth at 32°. These mutations are cold-sensitive. Cold-sensitive mutations may define genes that encode proteins that have dif-

ficulty folding (GUTHRIE, NASHIMOTO and NOMURA 1969; STRAUSS and GUTHRIE 1991). The other conditional mutation is the *maa2–1* strain. This mutant strain exhibits its most severe phenotype at 21° and has a less severe phenotype at 16 or 32°. All strains with the conditional mutations retain the 5-MAA resistant phenotype at all three temperatures.

Effects of other inhibitors of amino acid biosynthesis: We tested a range of compounds (see MATERIALS AND METHODS) to ask if any mutant strain showed pleiotropic drug resistance (JAMES and LEFEBVRE 1989) or if any were hypersensitive to other amino acid analogs. We found that none of the class I mutations conferred resistance to any compound that does not affect tryptophan biosynthesis directly. The compounds tested included other amino acid analogs such as aminotriazole, glyphosate, chlorsulfuron, methionine sulfoximine and triazole. Four of the 17 class I mutations confer resistance to 5-MT, which is known to inhibit AS in other organisms. The *maa2–7* strain is hypersensitive to 5-MT (Table 3). In other organisms, 5-fluoroindole (5-FI) becomes toxic to cells only when it is converted to 5-fluorotryptophan (5-FT) by tryptophan synthase. Resistance to 5-FI may be conferred by the reduced activity of the β subunit of tryptophan synthase. Alleles at two class I loci show resistance to 5-FI. On the other hand, alleles at eight of the nine loci defined by class II mutations show resistance to 5-MT, alleles at two class II loci show resistance to 5-FI, and the *maa15* mutant strain is resistant to glyphosate (Table 3), which inhibits EPSP synthase (KLEE, MUSKOPF and GASSER 1987).

Anthranilate is excreted by *maa5–1* cells: After 3–4 weeks of growth of *maa5–1* cells in either solid or liquid medium, the medium is observed to turn a brown color, which progressively darkens with increasing time. This browning of the medium is never observed after growth of wild type or other *maa*$^-$ mutant strains. The brown producing phenotype cosegregates with the 5-MAA resistance phenotype of the *maa5–1* mutation in 207 tetrads.

To identify the compound responsible for the brown media, the ultraviolet absorbance profiles of liquid medium before and after the growth of *maa5–1* and wild-type cells were compared. Two new ultraviolet light-absorbing peaks were observed in the media of *maa5–1* cells but not from the medium of wild-type cells. These peaks indicate the presence of conjugated ring compounds. The *maa2–4* mutant strain, which does not have a brown producing phenotype, behaved similarly to wild-type cells (data not shown). Given the anthranilate accumulation phenotype of the *trp1–1* mutation in Arabidopsis (LAST and FINK 1988) and of the *blue fluorescent-1* and *orange pericarp* mutations in maize (SINGH and WIDHOLM 1975; WRIGHT and NEUFFER 1989), and the browning of medium that contains exogenously added anthranilate, we tested both liquid and solid media for the presence of anthranilate. By fluorescence spectroscopy, anthranilate emits a characteristic 400 nm fluorescence upon excitation by 340 nm light (BERLMAN 1971). Figure 4

TABLE 3

Cross-resistance phenotypes to analogs of intermediates in tryptophan biosynthesis and dominance of *maa*$^-$ mutations

	Resistance phenotype[a]				
	Haploid strains		Heterozygous diploid strains		
Locus and allele	5-MT	5-FI	5-MAA	5-MT	5-FI
No cross-resistance					
maa1-1	W	W	W	W	W
maa1-2, maa1-3	W	W	R	W	W
maa3-1, maa3-2, maa3-3, maa3-4, maa3-5, maa3-6, maa3-7[b]	W	W	W	W	W
maa4-1, maa4-2, maa4-3[b]	W	W	W	W	W
maa6-1	W	W	S	W	W
Cross-resistance to 5-MT and 5-FI					
maa2-1, maa2-2[b]	R	R	R	R[c]	W
maa2-3	R	R	R	W	W
maa13-1, maa13-2, maa13-3[b,d]	R	R	R	W	W
Cross-resistance to 5-MT					
maa5-1[d]	R	W	R	W	W
maa8-1	R	W	R	W	W
maa9-1, maa9-2[b]	R	W	R	W	W
maa10-1, maa10-2, maa10-3[b]	R	W	W	W	W
maa11-1	R	W	R	W	W
maa12-1	R	W	R	W	W
maa14-1, maa14-2[b]	R	W	R	W	W
maa15-1[e]	R	W	R	W	W
Cross-resistance to 5-FI					
maa2-4, maa2-6[b]	W	R	R	W	W
maa2-5	W	R	R	R[c]	W
maa2-7	S	R	R	W	W
maa7-1, maa7-2[b]	W	R	W	W	W
maa16-1, maa16-2[b]	W	R	R	W	R

[a] W indicates that the tested strain, like wild-type cells, fails to grow on 5-MT (1.05 mM) or on 5-FI (7 μM), R indicates that the tested strain is more resistant to 5-MT or 5-FI than wild-type cells, and S indicates that the tested strain is more sensitive than the wild-type cells. These hypersensitive cells fail to grow on 0.46 mM 5-MT. We measured the size of colonies on 5-MT for the four class I resistant mutant strains to ask if the slow growth phenotype was rescued by 5-MT. No rescue of the strains tested was observed (data not shown).

[b] All *maa*$^-$ alleles at this locus were tested in complementation tests with the first allele and fail to complement one another for resistance to 5-MAA, 5-FI and/or 5-MT. Only recessive phenotypes were used for complementation tests.

[c] This diploid strain shows an intermediate 5-MT resistance phenotype. The mutation is incompletely dominant.

[d] Diploid strains heterozygous for the *maa5-1* mutation and the *maa11-1* mutation were constructed. The diploid strain has wild-type growth properties and is sensitive to 5-MT. This last property suggests that these mutations are in different loci despite that observation that we have not recovered recombinants in 367 tetrads. Diploid strains heterozygous for the *maa2-3* mutation and each *maa13* allele were constructed. The diploid strains are sensitive to 5-MT. This property suggests these mutations are in different loci despite that observation that we have not recovered a recombination event between them in 140 tetrads.

[e] The *maa15-1* mutation is resistant to glyphosate and this resistance is dominant to the wild-type *MAA15* allele.

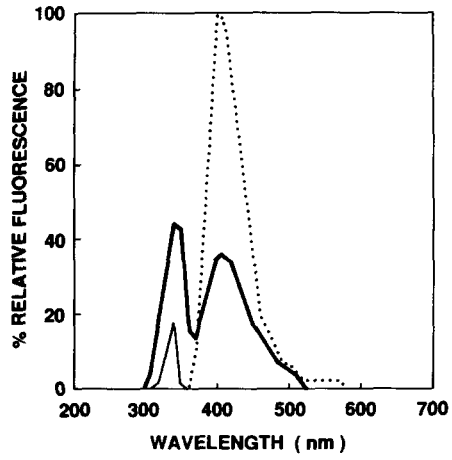

FIGURE 4.—Fluorescence of ethyl acetate extracts of medium after growth of *maa5–1* and wild-type cells were compared to that of an anthranilate standard. Fluorescence emission, after excitation at 340 nm, was measured over a range of wavelengths from 300 to 700 nm. The dotted line was an anthranilate standard. The heavy solid line and the light solid line indicate the profile of extracts of solid medium following growth of *maa5–1* and wild-type cells, respectively. The peaks at approximately 340 nm are from light scattering of the excitation beam by small amounts of agar in the extracts. The peak at 400 nm in the mutant extract corresponds to the peak fluorescence of anthranilate.

illustrates the fluorescence spectrum of anthranilate in ethyl acetate compared with ethyl acetate extracts of solid media that were used to grow wild-type cells or *maa5–1* cells. There is a peak in the spectrum of the medium from *maa5–1* cells that is coincident with the peak of the anthranilate standard. No peak is observed in medium from wild-type cells. No detectable browning of the medium is produced by the heterozygous diploid strain (*maa5–1/MAA5*$^+$) and excretion of anthranilate by the diploid strain is not detectable by spectroscopy.

We have analyzed the medium for the presence of amino acids after the growth of wild-type and mutant cells using high performance liquid chromatography for the assay. A 10-fold increase in the level of phenylalanine is observed in the medium of *maa5–1* cells compared to the medium from wild-type cells. The excretion of phenylalanine is not observed in heterozygous *maa5–1/MAA5* diploid cells. Thus, the *maa5* mutant strain appears to have a defect that affects the tryptophan pathway as well as least one other aromatic amino acid pathway.

Modified anthranilate is excreted by *maa6–1* cells: Unlike *maa5–1* cells that produce brown medium, medium containing *maa6–1* cells turns yellow after 4 weeks. Production of yellow medium is a single gene trait that cosegregates with the 5-MAA resistance phenotype of *maa6–1* in 178 tetrads from a cross to wild-type cells. This phenotype is recessive in heterozygous diploid strains. Because pure anthranilic acid turns brown under our conditions, we assume that this excreted product, which shows a new fluorescence peak at 400 nm (BERLMAN 1971), is a modified form of anthranilic acid. It may be a glycosylated anthranilate derivative like the ones observed in Arabidopsis (LAST and FINK 1988) and in maize (SINGH and WIDHOLM 1975). The synthetic orange pericarp phenotype in maize results from mutations in two genes, *orp1* and *orp2*. These plants accumulate a glycosylated anthranilate product. These mutations are linked to restriction fragment length polymorphisms identified by probes to the tryptophan synthase β genes (WRIGHT *et al.* 1990).

Enzymatic phenotypes of 5-MAA resistance mutations. In fungi and bacteria the first committed step in the tryptophan biosynthetic pathway is catalyzed by AS, which produces anthranilate from chorismate (YANOFSKY and CRAWFORD 1987; BRAUS, 1991). The fluorescence of anthranilate serves as the assay for the activity of AS. Conversion of anthranilate to indole-3-glycerol phosphate can be measured by monitoring the disappearance of the blue fluorescence at 400 nm in the presence of phosphoribosyl pyrophosphate. This serves as the assay for the combined activities of anthranilate phosphoribosyl transferase, phosphoribosyl anthranilate isomerase (transferase/isomerase or T/I) and indoleglycerol phosphate synthetase (IGPS). The enzymatic activities of AS and T/I/IGPS can be measured in crude extracts obtained from lysed Chlamydomonas cells (see MATERIALS AND METHODS).

Both enzyme activities were measured for mutant alleles at seven different loci using protein concentrations of 0.5 mg/reaction for the AS reaction and 0.1 mg/reaction for the T/I/IGPS reaction. Time points between 0 and 30 min were taken for each extract. Conversion of chorismate to anthranilate by AS occurs at comparable rates in assays of crude extracts from wild-type cells and from cells of six mutant strains (data not shown). Extracts from *maa5–1* cells show a 2.5-fold elevation in the specific activity of AS in four independent trials. The conversion of anthranilate to indole 3-glycerol phosphate by T/I/IGPS in the presence of phosphoribosyl pyrophosphate was similar to wild-type levels in four of the six mutant extracts tested. In extracts of the *maa5–1* mutant strain, however, there was increased activity. The specific activity was approximately twofold higher than in wild-type extracts. In extracts from the *maa6–1* mutant strain, there was no activity (Table 4). When *maa6–1* and wild-type extracts were mixed at equal protein concentrations, activity was observed (data not shown). We conclude that there is not an inhibitor in the *maa6–1* extracts that blocks the activity of wild-type extracts and the *MAA6* locus may encode one of these three enzymes.

Dominance of the *maa*$^-$ mutations: Heterozygous diploid strains were constructed using complementing arginine auxotrophs. The phenotypes with respect to

TABLE 4

Enzymatic activities of extracts from class I *maa*⁻ mutant strains

Mutant extract	Transferase/isomerase/IGP synthetase	
	No. trials[a]	Units of activity[b]
Wild-type 137c	5	422 ± 55
maa1-1	5	389 ± 75
maa2-1	3	444 ± 158
maa3-1	3	456 ± 241
maa4-1	3	315 ± 152
maa5-1	3	897 ± 70
maa6-1	3	6 ± 6
maa7-1	4	421 ± 89

[a] At least two independent preparations were used.
[b] The units are picograms of anthranilate consumed/min/mg protein.

inhibition by 5-MAA, 5-MT and 5-FI were determined (Table 3) as well as the growth rate in the absence of these inhibitors. In all but one mutant strain, the slow growth phenotype is recessive to the wild-type growth phenotype. The generation time of the *maa7–1* heterozygous diploid strain is five times longer than the wild-type diploid strain at 21°, and two and one-half times longer than the homozygous *maa7–1* diploid strain. These results suggest that the mutant gene product is more deleterious in the presence of the wild-type product than in a homozygous diploid or in a haploid strain.

Among the class I mutations, alleles at three loci show a dominant 5-MAA phenotype. The *maa6–1* mutant strain is more sensitive to inhibition by 5-MAA in a heterozygous diploid strain than in the wild-type control strain. The 5-FI resistance phenotype of the *maa2* mutations, unlike the 5-MAA resistance phenotype, is recessive. Among the class II mutations, the alleles at eight of the nine loci are dominant to the wild-type allele for the 5-MAA resistance phenotype, only the *maa13* alleles are recessive.

Double mutant phenotypes: The epistatic relationship of mutations in the *MAA1* to *MAA7* loci were examined in double mutant combinations (Table 5). The *maa6–1* mutant strain is distinguishable from the other mutant strains by the production of yellow medium, its extremely slow growth phenotype, and the partial rescue of the slow growth phenotype by indole. In three of the six double mutant strains with the *maa6–1* allele, yellow medium is produced. In the *maa5–1 maa6–1* strain, brown medium is produced. We do not know if the yellow compound is present. In four of the six double mutant strains, the extremely slow growth phenotype is observed and in these double mutant strains, indole partially rescued the slow growth phenotype. The *maa6–1* allele is epistatic to alleles at least three loci. In the *maa3–1 maa6–1* and *maa7–1 maa6–1* double mutant strains, the slow growth phenotype is not observed and yellow medium is not produced.

The *maa5–1* mutant strain has two easily monitored phenotypes that distinguish it from the other class I *maa*⁻ mutant strains. It confers resistance to 5-MT and it produces brown medium due to the excretion of anthranilate into the medium. The *maa5–1* mutation is epistatic to mutations at the other six class I loci for the 5-MT resistance phenotype and is epistatic to mutations at five of the six loci for the brown medium phenotype. The exceptions to this pattern are combinations with mutations at the *MAA2* locus. The *maa2–1* and *maa2–4* mutations are epistatic to the *maa5–1* mutation; each double mutant strain fails to produce brown medium.

All *maa2* strains are resistant to 5-FI and the *maa2–1* mutant strain is resistant to 5-MT. The double mutant combinations with the *maa2* alleles are resistant to 5-FI. In double mutant combinations with the *maa2–1* allele where the second mutation is sensitive to 5-MT, the strains are resistant to 5-MT. The *maa2*⁻ alleles are epistatic to the alleles at other loci. The *maa3–1* strain, which is a class II mutation, is distinguishable from mutations at the other loci because it lacks a slow growth phenotype. In five of the six double mutant strains, the slow growth phenotype of the other mutant allele is observed. Interestingly, the extremely slow growth phenotype of the *maa6* strain was suppressed in both the *maa6–1 maa3–1* and *maa6–1 maa3–5* double mutant strains.

Some of the 54 class I double mutants examined grow slightly more slowly than either of the parental strains. Four *maa1 maa7* double mutant strains examined show a lethal or an extremely slow growing phenotype in sixteen independent crosses. The putative *maa1–1 maa7–1*, *maa1–3 maa7–1* and *maa1–3 maa7–2* double mutant spores divide mitotically only one or two times after the completion of meiosis at 21° or 32° with or without tryptophan (or indole) in the medium. The *maa1–1 maa7–2* double mutant strain has an extremely slow growing phenotype with a doubling time in liquid medium at 21° of 56 hr. Three lines of evidence suggest that the *maa1* and the *maa7* mutations are responsible for these phenotype rather than unknown mutations in the strains. In tetrads with three viable meiotic progeny, one of the spores was sensitive to 5-MAA; in tetrads with two viable meiotic progeny, both are sensitive to 5-MAA; and in tetrads with four viable meiotic progeny, all the meiotic progeny are resistant to 5-MAA. This pattern of viability is strongly suggestive that these two mutations together are responsible for the inviability. The inviability was not observed in over 30 other crosses that involve these four mutations. Mutations at the *MAA2*, *MAA13* and *MAA16* loci, which also have a 5-FI resistance phenotype like *maa7* mu-

TABLE 5

Epistasis tests of pairwise combinations of *maa*⁻ alleles at class I loci

maa⁻ mutations	Phenotypes[a]			
	Resistance to 5-MT	Slow growth	Brown or yellow medium production	Resistance to 5-FI
I. Single mutants				
maa1-1	W	S	–	W
maa2-1	R	S	–	R
maa2-4	R	S	–	R
maa3-1	W	W	–	W
maa3-5	W	S	–	W
maa4-1	W	S	–	W
maa5-1	R	S	B	W
maa6-1	W	S	Y	W
maa7-1	W	S	–	R
maa7-2	W	W	–	R
II. Double mutants				
maa1-1 maa2-1	R	S	–	R
maa3-1 maa2-1	R	S	–	R
maa3-5 maa2-1	R	S	–	R
maa4-1 maa2-1	R	S	–	R
maa5-1 maa2-1[b]	R	S	b	R
maa6-1 maa2-1	R	S	Y	R
maa7-1 maa2-1	R	S	–	R
maa3-1 maa2-4	W	S	–	R
maa5-1 maa2-4	R	S	–	R
maa1-1 maa3-1	W	S	–	W
maa4-1 maa3-1	W	S	–	W
maa5-1 maa3-1	R	S	B	W
maa6-1 maa3-1	W	W⁻	–	W
maa7-2 maa3-1	W	W	–	R
maa5-1 maa3-5	R	S	B	W
maa6-1 maa3-5	R	S⁺	–	W
maa1-1 maa4-1	W	S	–	W
maa5-1 maa4-1	R	S	B	W
maa6-1 maa4-1	W	S	Y	W
maa7-2 maa4-1	W	S	Y	R
maa1-1 maa5-1	R	S	B	W
maa6-1 maa5-1	R	S	B	W
maa7-2 maa5-1	R	S	B	R
maa1-1 maa6-1	W	S	Y	W
maa7-2 maa6-1	R	W⁻	–	R⁻

[a] 5-MT and 5-FI phenotypes: R indicates resistance phenotype; W indicates the wild-type sensitive phenotype; R⁻ indicates an intermediate resistance phenotype. Growth phenotypes: S indicates the slow growth phenotype of the most severely affected parent; S⁺ indicates the slow growth phenotype of the least severely affected parent; W indicates a wild-type growth phenotype; W⁻ indicates a partial rescue of the slow growth phenotype. Excretion into the medium phenotype: – indicates the lack of yellow or brown colored media; B indicates the production of brown medium; b indicates a reduction in the intensity of brown in the medium; Y indicates the production of yellow medium.

[b] Both strains are resistant to 5-MT.

tations, do not produce a lethal phenotype in combination with the *maa1-1* or *maa1-3* alleles. This phenotype appears to be a locus-specific as well as an allele-specific interaction.

DISCUSSION

We have isolated a collection of mutations that confer resistance to 5-MAA. They define at least sixteen new loci in *C. reinhardtii* and map to at least seven different linkage groups. As genetic markers these mutations will provide new easily scored phenotypes for genetic mapping and manipulations (see DUTCHER *et al.* 1991). Three of the loci map to linkage group XIX, which is also known as the UNI linkage group (DUTCHER 1986; RAMANIS and LUCK 1986). These mutations provide the first clear examples of loci that map to linkage group XIX whose phenotypes do not affect flagella or other microtubule-based functions.

Tryptophan auxotrophs? We were interested in identifying genes involved in tryptophan biosynthesis by the isolation of mutant strains resistant to 5-MAA. Most of the mutant strains we isolated do not appear to be simple tryptophan auxotrophs. Loss of function mutations in the enzymes of the tryptophan biosyn-

thetic pathway are expected to display at least some of the following properties. First, the mutant phenotype may be rescued by the addition of tryptophan. None of the mutant strains is rescued by the addition of tryptophan (0.24 mM) at concentrations that relieve the growth inhibition of wild-type cells by 1.16 mM 5-MAA. This statement remains true when different nitrogen sources are used and when the intensity of the light source is varied from 0 to 85 μEinsteins/m^2/sec (data not shown). Tryptophan can enter cells in sufficient amounts to serve as the sole source of reduced nitrogen in wild-type cells (data not shown). However, the *maa6–1* strain is rescued by the addition of indole (0.043 mM), which is the immediate precursor to tryptophan. It is possible that the rescue by indole, but not by tryptophan results from a difference in permeability of the two compounds. Another possibility for the differential rescue is that indole is required for other biosynthetic pathways in addition to the biosynthesis of tryptophan. Slow growth could occur because insufficient quantities of indole are available to meet other metabolic requirements. In higher plants, indole is used in the production of the hormone, indole acetic acid (auxin). It is not known if Chlamydomonas makes and utilizes this compound.

Second, a 5-MAA-resistance phenotype due to mutations in genes encoding the structural enzymes in tryptophan biosynthesis should be recessive. Dominant loss of function mutations may be possible if the dosage of the products of these loci is important or if the mutant gene product assembles into multimeric complexes and disrupts function (Blair and Berg 1990). Third, we might expect that mutations in structural enzymes would decrease the intracellular pools of tryptophan and would make the mutant cells hypersensitive to the action of 5-MT. In most cases we observed wild-type levels or increased levels of resistance to this analog and not hypersensitivity to it. Fourth, we might expect to observe a decrease in the activity of one of the enzymes in the tryptophan biosynthetic pathway. The *maa6–1* mutation meets several of these criteria for a mutation in a gene involved in tryptophan biosynthesis. Extracts made from *maa6–1* mutant cells produce no activity in the combined assay for T/I/IGPS. We have been unsuccessful in attempts to determine which enzymatic activity is missing in *maa6–1* extracts using *in vitro* complementation of extracts from *trpC* and *trpD E. coli* mutant strains. Wild-type Chlamydomonas extracts do not complement these activities (data not shown). In addition, the *maa6–1* mutation like the *trp1–1* mutation in Arabidopsis (Last and Fink 1988) secretes a fluorescent compound, which may be an anthranilate derivative.

We conclude that most of the *maa*$^-$ mutations do not meet the expectations listed above for an auxotrophic mutation. Why did we isolate so many non-auxotrophic mutations? If the selection conditions provided insufficient amounts of tryptophan (or indole) to support the growth of auxotrophic mutations, this class of mutant strains would not have been recovered. Alternatively, tryptophan auxotrophs may be sparse if there are multiple genes for some of the tryptophan biosynthetic enzymes in Chlamydomonas. Loss of one copy may be sufficient to provide resistance to 5-MAA, but not create an auxotrophic phenotype. In Arabidopsis, multiple genes for the β-subunit of tryptophan synthase exist and they are differentially regulated by light intensity (Last *et al.* 1991). We have preliminary molecular data that suggest there are two genes for the β subunit of tryptophan synthase in Chlamydomonas. Also, the severity of the phenotypes of several of the class II mutations is altered by the intensity of light. These data are consistent with the possibility of light regulation of different members of the same gene family (G. Poortinga and S. K. Dutcher, work in progress).

The *MAA5* locus: The single mutant allele at the *MAA5* locus exhibits pleiotropic phenotypes, which are consistent with a mutation that produces excess anthranilate and perhaps its precursors. Excess anthranilate is toxic to Chlamydomonas. When added exogenously, 0.73 mM inhibits the growth of wild-type cells. Consequently, anthranilate might be expected to be toxic if it were overproduced internally. In *maa5–1* cells, slow growth could be a consequence of the overproduction of anthranilate and resistance to 5-MAA and to 5-MT could occur by a dilution of the inhibitor by the increased amounts of anthranilate present. Resistance to 5-FI would not be expected since 5-FT is incorporated into protein (Pratt and Ho 1975), while 5-MT is not. The recessive nature of the slow growth phenotype and the brown producing phenotype is consistent with this interpretation.

At least two possible steps that lead to tryptophan biosynthesis could be affected in the *maa5–1* mutant strain. There may be increased activity of a step or steps in the shikimic acid pathway. In other systems, the synthesis of the aromatic amino acids proceeds from the common pathway from shikimic acid to chorismic acid. At this point the pathway branches to produce phenylalanine and tyrosine, tryptophan, or the vitamin *p*-aminobenzoic acid (Braus 1991). A defect in this part of the pathway may result in higher levels of chorismate and thus higher levels of anthranilate. The excretion of phenylalanine is consistent with this localization of the lesion. The *maa5–1* mutant cells are not resistant to glyphosate, so we postulate that the lesion occurs after the step catalyzed by EPSP synthase (Klee, Muskopf and Gasser 1987). Therefore, the most likely step in the shikimic acid pathway to be affected is the conversion of EPSP to

chorismic acid by chorismate synthase, which could be overproduced or improperly regulated. On the other hand, the mutation may overproduce or improperly regulate anthranilate synthase. The dominance of the 5-MAA resistance phenotype would be consistent with a gain of function mutation in a regulatory or structural gene.

Epistatic relationships: Analysis of the phenotypes of double mutant strains can be used to suggest a possible order for the function of these genes in tryptophan biosynthesis and its regulation. In the best of circumstances, epistasis tests should be performed with null mutations. We do not know if any of our mutant alleles are null mutations and in fact several of the mutations are dominant. We have considered only recessive alleles at the class I loci in this analysis.

We believe that the phenotypes of the *maa6–1* mutant strain are consistent with a defect in one of three enzyme activities in the tryptophan biosynthesis pathway. We use this mutation as the starting point. The *maa3* and *maa7* mutations are epistatic to the *maa6* mutation while the *maa6* mutation is epistatic to the *maa1* and *maa4* mutations. The *maa4* mutation is epistatic to the *maa3* and *maa7* mutations. The *maa1 maa7* double mutant combination has a synthetic lethal phenotype. No relationship can be determined for the *maa1* and *maa4* mutations as they have no distinguishing phenotypes. As one possible pathway, we suggest that *MAA1* and *MAA4* may act upstream of the requirement for the *MAA6* gene and may positively regulate the tryptophan biosynthetic pathway. It is possible that the *MAA3* locus encodes a negative regulator and the pathway is turned up in the mutant strain. At present we find the *MAA7* locus to be the most puzzling in light of its 5-FI resistance phenotype, which argues for an alteration in the control of tryptophan synthase, specifically.

Starvation of *Saccharomyces cerevisiae* cells for any one of 10 different amino acids results in the increased expression of at least 30 amino acid biosynthetic enzymes. Two classes of genes have been identified that regulate this general control system. They are the *GCN* genes, which are positive regulators, and the *GCD* genes, which are negative regulators (HINNEBUSCH 1988; BRAUS 1991). Chlamydomonas might have both a general control system for amino acid biosynthesis as well as pathway specific control systems. It seems unlikely that any of our genes define *GCN*-like genes, but we may have identified at least one *GCD*-like gene. This statement is based on the observation that the *maa3* mutation partially suppresses a leaky arginine auxotroph (*arg2*) at 21°, which would be expected for a mutation in a negative regulator of general amino acid biosynthesis.

One class of mutations that we might have expected to isolate are mutations that block the transport of 5-MAA into the cell. If we make the assumption that indole is imported by the same transport mechanism as 5-MAA, then the ability of indole to rescue the slow growth phenotype of *maa6–1* double mutant strains argues that the *maa1, maa2, maa4* and *maa5* mutant strains are not defective in the import of 5-MAA. It will be interesting to determine if any of the class II mutant stains block the rescue of the *maa6–1* mutant phenotype by indole.

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