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Genetics

A chimeric light-regulated amino acid transport system allows the isolation of blue light regulator (*blr*) mutants of *Neurospora crassa*

(signal transduction/photoregulated genes/carotenoids)

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ABSTRACT **We have developed a system for the isolation of *Neurospora crassa* mutants that shows altered responses to blue light. To this end we have used the light-regulated promoter of the albino-3 gene fused to the neutral amino acid permease gene *mtr*. The product of the *mtr* gene is required for the uptake of neutral aliphatic and aromatic amino acids, as well as toxic analogs such as *p*-fluorophenylalanine or 4-methyltryptophan. *mtr trp-2*-carrying cells were transformed with the *al-3* promoter–*mtr* wild-type gene (*al-3p–mtr*$^+$) to obtain a strain with a light-regulated tryptophan uptake. This strain is sensitive to *p*-fluorophenylalanine when grown under illumination and resistant when grown in the dark. UV mutagenesis of the *al-3p–mtr*$^+$-carrying strain allowed us to isolate two mutant strains, BLR-1 and BLR-2 (blue light regulator), that are light-resistant to *p*-fluorophenylalanine and have lost the ability to grow on tryptophan. These two strains have a pale-orange phenotype and show down-regulation of all the photoregulated genes tested (*al-3, al-1, con-8,* and *con-10*). Mutations in the BLR strains are not allelic with white collar 1 or white collar 2, regulatory genes that are also involved in the response to blue light.**

Light is essential for life on the earth. Different organisms, from plants to bacteria, use light not only as a source of energy for growth but also as a stimulus to regulate several metabolic and developmental processes. In the filamentous fungus *Neurospora crassa*, blue light induces a variety of responses, including carotenoid biosynthesis in mycelia and phase shifting and photosuppression of the circadian rhythm of conidiation (1, 2). During the sexual cycle, light induces photodifferentiation and positive phototropism of perithecial beaks (3). In recent years many of these responses have been studied, and several light-induced genes have been identified that are related to developmental programs of the fungus. Examples are the *con* genes (preferentially expressed during conidiation) (4, 5) and the *bli* genes (blue light inducible) of which the functions are still mostly unknown (6, 7). In *N. crassa* the synthesis of carotenoids is constitutive in conidia, whereas in mycelia it is induced by blue light (8). Three loci involved in carotenoid biosynthesis (*al-1, al-2,* and *al-3*) show increased expression after exposure to blue light (9–13). Mutations in these structural genes result in inability to synthesize carotenoids. Furthermore, two light-regulatory mutants defective in all blue light responses have been isolated and termed white collar (*wc*) because they produce white mycelia and orange conidia after exposure to light (8, 14). Whether any of these mutations corresponds to the blue light photoreceptor or to an element in the light signal transduction is still unknown. In addition, other mutants that show a reduction in light sensitivity have been described, such as the poky, rib, and lis mutants (15–18).

In this work we describe another system for the isolation of mutants altered in the response to blue light in *N. crassa*. For this purpose we constructed a strain with a light-regulated uptake of several amino acids. We used the light-regulated promoter of the albino-3 gene (13, 19) fused to the *mtr* gene (20, 21). The functional product of the *mtr*$^+$ gene is required for uptake of neutral aliphatic and aromatic amino acids as well as of toxic analogs such as *p*-fluorophenylalanine (FPA) and 4-methyltryptophan (22). *mtr trp-2* cells were transformed with the *al-3p–mtr*$^+$ gene fusion to obtain a strain able to produce the neutral amino acid permease only after light induction of the mycelium. This strain can grow on tryptophan only when it is light-stimulated; however, when cultured in a medium containing a toxic amino acid analog such as FPA, it grows only in the dark because light induces the production of the permease and the subsequent uptake of the drug inhibits cellular growth. UV mutagenesis of the *al-3p–mtr*$^+$ strain allowed us to isolate mutants that are incapable of responding to blue light.

MATERIALS AND METHODS

Strains of *N. crassa*. Strain 82-59 (*trp-2, mtr, cot-1, ylo-1, A*): The *mtr* mutation was generated by David Stadler at the University of Washington (23). The Mtr phenotype is nonrevertible by UV because the mutant gene contains a small deletion at nt 1536 in the open reading frame (21, 23). Strains 13-1, 22-6, 29-4, 29-5, and 29-6 were generated by transforming strain 82-59 with the light-regulated chimera pLRN.

Strains were maintained on slants of 1× Vogel's medium/2% sucrose, supplemented with the necessary amino acids (24).

Recombinant Plasmid. The *al-3* promoter fragment was obtained by PCR amplification from a plasmid containing the full-length promoter sequence (GenBank accession no. U20940) using the oligonucleotide 5′-CTGCAG(3)CTTTATGCACTCGG(16)-3′, which carries a *Pst* I site, and the oligonucleotide 5′-GTCGAC(1170)TTGGAAACCCG(1160)-3′, which carries a *Sal* I site, and then treated with the restriction enzymes *Pst* I and *Sal* I. The pN846 plasmid containing the *mtr* gene (20, 21) was cut at position −40 from the AUG/ATG initiator codon using the *Sal* I restriction enzyme and at the polylinker located upstream of the cloned *mtr* gene using *Pst* I. The resulting product was ligated to the PCR fragment containing the *al-3* gene promoter. The ligated DNA was transformed

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Abbreviation: FPA, *p*-fluorophenylalanine.
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into *Escherichia coli* strain DH-5α cells made competent with $CaCl_2$ (25). The new chimeric construct was named pLRN for plasmid light regulated neutral (transport system). The sequence of the *al-3p–mtr*$^+$ gene fusion was verified by the standard procedure of Sanger *et al.* (26).

Transformation of *Neurospora*. Spheroplasts of host strain 82-59 were prepared by using the methods described by Vollmer and Yanofsky (27) and were transformed with pLRN (28). For selection of transformants, bottom plates and top agar were made with Vogel's medium and FIGS (0.5% fructose/2% inositol/0.5% glucose/1% sorbose) supplemented with 0.01 mg of tryptophan and 0.1 mg of arginine per ml. The arginine served as competitor for the general amino acid permease to avoid the light-independent entrance of tryptophan or FPA through this pump. Cultures were incubated at 28°C in a light-sealed incubator. To regulate the light conditions, a Micronta programmable timer (Radio Shack no. 63-864) was used to achieve an illumination cycle of 4-hr light (15-W white fluorescent) and 4-hr dark. To obtain full responsiveness of the *al-3* promoter, the light exposure was always followed by a period of dark incubation; this procedure is necessary to obtain full photoinducibility of the *al-3* promoter (29, 30). Several homokaryotic colonies growing on tryptophan were isolated by successive platings. These were screened for the transformant that showed the best growth after light induction in our experimental conditions.

Screening the Transformants. To identify the transformants containing a light-regulated amino acid transport system, 60,000 conidia of each transformant were inoculated in liquid Vogel's medium supplemented with FPA at 0.015 mg/ml. Because the transformants require tryptophan, the medium was also supplemented with anthranilic acid at 0.05 mg/ml, a precursor of tryptophan that is transported into the cell via a different pathway from that controlled by *mtr* (31). Cultures were incubated at 28°C for 5 days, either with light–dark cycles or in constant darkness, after which time the mycelia were harvested by filtration, and the dry weight of the cell masses was determined. Transformants were also grown on tryptophan-supplemented liquid medium to verify that tryptophan transport was under light control; the best response was obtained with tryptophan at 0.001 mg/ml.

Mutant Selection by Filtration Enrichment. To isolate the mutants defective in light response, we used a filtration enrichment protocol (32). For mutagenesis, 10^7 conidia of strain 13-1, in 20 ml of distilled water, were exposed directly to UV light for 10 min with a 30-W UV bulb at a distance of 78.5 cm (20–50% lethality). After UV treatment, the cell suspension was cultured in medium containing tryptophan (0.01 mg/ml) and arginine (0.1 mg/ml) at 28°C in light–dark cycles, conditions that were conducive to the growth of cells with normal blue-light responses. Growing cells formed chains of mycelia that were easily filtered out of the medium. After filtration through sterile cheesecloth, most of the eluted culture should consist of poorly growing mutants and nonviable cells. Fifty milliliters of fresh medium/tryptophan/arginine was then added to the filtrate and incubated as described above. The cycle of growth and filtration continued for 3 days, varying the interval between filtrations from 6 to 12 hr. After that, little or no additional growth was recorded, and the cells were spun down and washed with sterilized double-distilled water. A suspension of 5×10^6 cells was plated on a medium containing FPA and anthranilic acid, each at 0.05 mg/ml.

Southern and Northern Hybridizations. Small preparations of chromosomal DNA from strains 82-59 and 13-1 were made as described by Morelli *et al.* (33). The genomic DNA was digested with *Pst* I and *Eco*RV, blotted as described in Maniatis *et al.* (34), and probed using a 1099-bp *Hin*cII fragment specific for the *mtr* gene labeled by random priming with [α-^{32}P]dATP (Amersham).

Northern blots of *Neurospora* mRNA were prepared as described in Baima *et al.* (11) using 10 μg of total RNA. Light induction was performed illuminating 18-hr dark-grown cultures for 20 min using a constant saturating light regime (10 W/m^2). The RNA was electrophoresed on agarose gels, transferred onto Hybond-N membranes, and hybridized with the following labeled oligonucleotides and restriction fragments: the *al-3*-specific oligonucleotide 5′-ACGGCCATGGTGACG-TGTTCCATTTCC-3′ (13), the *con-10*-specific oligonucleotide 5′-CCGGAAGCCTTGCCGCCCTTGCGACCG-3′ (5), the *con-8*-specific oligonucleotide 5′-GGGGTTGCTACCGG-TGTCATCCATGG-3′ (35), the 1099-bp *Hin*cII fragment of *mtr* (20), and the *Xba* I–*Cla* I fragment of *al-1* (9). Oligonucleotides were end-labeled by T4 polynucleotide kinase with [γ-^{32}P]dATP according to standard methods (34). Restriction fragments were labeled by random priming with [α-^{32}P]dATP (Amersham).

RESULTS

We fused the *al-3* promoter to the coding sequence of the wild-type *mtr* gene to obtain the plasmid pLRN (light regulated neutral) with the aim of creating a strain that can grow on suitable medium only when irradiated with blue light; UV-induced mutants of such a strain should enable us to identify mutations in genes responsible for light induction and regulation of the *al-3* promoter.

Three major transport systems for the uptake of amino acids exist in *N. crassa*: neutral (N), basic (B), and general (G). These are controlled by the expression of the three loci *mtr*, *pmb*, and *pmg* (36). Strains with mutations in the *mtr* locus are characterized by their resistance to 4-methyltryptophan and FPA. The *mtr* gene can be used as a selectable marker on FPA-containing medium. Furthermore, positive selection of *mtr* wild-type transformants can be accomplished by using a double-mutant host cell containing both *mtr* and an amino acid auxotroph such as *trp-2*. Tryptophan is transported both by the neutral (mtr) and general (pmg) amino acid transport systems. Its transport by the general system can be blocked by adding large amounts of arginine and glutamic acid as competitors (37). The Mtr phenotype can be rescued by transformation with mtr wild-type cDNA followed by plating on arginine/tryptophan medium (22).

The *al-3* promoter fragment cloned in the pLRN plasmid was 1168 bp in length (from position 3 to position 1170) (GenBank accession no. U20940). The elements controlling the photoinduceability of *al-3* gene are restricted to the region between nt 904 and nt 1075. This region contains at least three upstream control elements important for photoregulation, each of which is present in the pLRN construct (13). Thus, after transformation of the host strain 82-59 (*mtr trp-2*) with the pLRN chimeric plasmid, we expected to obtain a strain that could transport tryptophan in the light but that would have little or no tryptophan transport in the dark. In addition, this strain should be resistant to FPA in the dark and sensitive to the drug in the light.

Homokaryotic transformants were isolated on tryptophan/arginine medium and tested on FPA liquid medium for sensitivity to light. Fig. 1 shows the amount of growth of pLRN transformants 13-1, 22-4, 22-5, 22-6, 29-4, and 29-5 cultured in cycled light, and of wild-type strain 74a and the original *mtr* parental strain 82-59, which served as negative and positive controls. The results indicate that several transformants were partially (transformants 22-6, 29-4, and 29-5) or totally (transformant 13-1) dark-resistant and light-sensitive to FPA, as expected. Two of them (transformants 22-4 and 22-5) did not show any difference between light and dark growth, presumably because the ectopic integrated copies of pLRN were poorly expressed in a light-independent fashion.

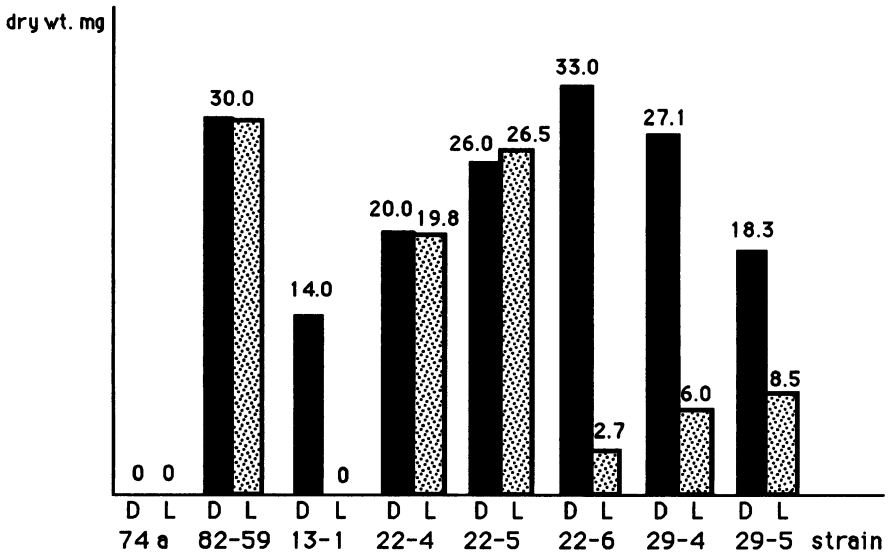

FIG. 1. Screening of the pRLN transformants. Histograms indicate dry weight (wt.) of pRLN transformant cultures obtained after growth in liquid medium containing FPA and anthranilic acid under light/dark regimes (L) or in the dark (D). Strains 74a and 82–59 were used as controls.

We tested the transformants on medium containing tryptophan at 0.05 mg/ml and arginine at 0.1 mg/ml to verify that tryptophan transport was controlled by light. Strain 13-1 showed the largest difference in growth between light and dark, and for this reason it was chosen for further study of the system.

Southern blots of genomic DNA from transformant 13-1 and strain 82-59 were probed with a *Hin*cII fragment specific for the *mtr* open reading frame. The *mtr* endogenous gene fragment is present in both lanes, and the 13-1 pattern is consistent with ectopic integration of three copies of the pLRN plasmid: in two of these events the *al-3p–mtr*$^+$ gene fusion is intact (size of the *Pst* I–*Eco*RV band is as expected) (Fig. 2). To examine light regulation of the *mtr* gene, a Northern blot of strain 13-1 RNA prepared from light- and dark-grown cultures was probed with the *mtr Hin*cII fragment. The 13-1 strain displayed a strong increase in mtr mRNA level after a 20-min exposure to blue light, as compared to the dark-grown culture (Fig. 3). In contrast, the parental strain 82-59 contained little or no detectable *mtr* message. Northern blots were normalized using a cDNA representing a nonphotoinducible gene of *N. crassa* as a probe (11).

All results obtained with the 13-1 transformant were consistent with the expected phenotype, except that it showed slow growth in the dark in the presence of tryptophan. Several concentrations of tryptophan (0.05, 0.01, and 0.001 mg/ml) were tested to achieve the conditions in which strain 13-1 would give the best response in both dark and light. Optimal conditions were found using tryptophan at 0.001 mg/ml and arginine at 0.1 mg/ml in growth medium. Under these conditions, strain 13-1 is unable to grow in the dark but grows normally under cycled light. Control strains 74a and 82-59 were not affected by these growth conditions (data not shown).

Once conditions for the selection were established, UV mutagenesis was done on strain 13-1 to obtain mutants that had lost a functional light-regulated neutral transport system. Mutants were enriched by filtering through cheesecloth to remove germinating conidia after several hours of incubation. This step was repeated several times. The filtrate was then plated on medium containing FPA under light/dark cycles to isolate colonies resistant to the drug.

We obtained 50 independent FPA-resistant colonies grown in light. Three groups of phenotypes were observed among these strains: one group was similar to the parental strain, a second showed intermediate carotenoid accumulation, and the third had very pale-orange mycelia. Total RNA was extracted from the six isolated strains that showed pale-orange phenotype. Northern analysis was done by probing first with an *al-3*-specific probe and then with an *mtr*-specific probe. Two isolates, BLR-1 and BLR-2 (blue light regulator), showed a decrease both in *al-3* and in light-induced *mtr* expression after 20 min of light induction, as compared with the 13-1 parental strain. The Northern blot was then probed with specific probes for *al-1*, a light-regulated *Neurospora* gene involved in caro-

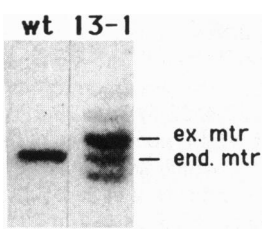

FIG. 2. Southern blot analysis of transformant 13-1. Genomic DNA from the transformant and the wild-type (wt) strain was digested with *Eco*RV and *Pst* I. The blot was probed with a 1099-bp DNA fragment specific for the *mtr* open reading frame. ex., Exogenous; end., endogenous.

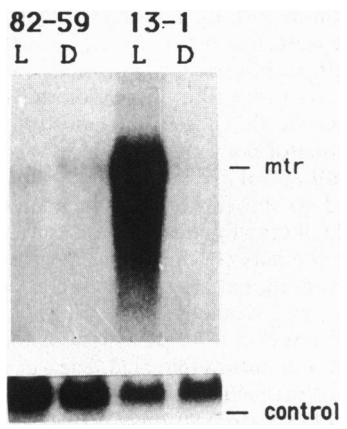

FIG. 3. Northern blot analysis of transformant 13-1. Ten micrograms of total RNA extracted from light-induced (L) and uninduced (D) cultures of transformant 13-1 and parental strain 82-59 was blotted and probed as for Fig. 2. The blot was normalized by reprobing with a nonphotoinducible *N. crassa* cDNA (IF2) (11).

tenogenesis, for *con-8*, and for *con-10*, other light-regulated genes preferentially expressed during conidiation. The BLR-1 and BLR-2 strains showed reduced expression of all the photoregulated genes probed except *al-1*, the mRNA level of which is only slightly reduced in BLR-2 (Fig. 4). The isolated strains were also cultured on positive selection medium to test for the loss of neutral amino acid transport. The two BLR strains were unable to grow.

The two BLR strains isolated with the *al-3p–mtr*$^+$ system are of special interest because, like *wc-1* and *wc-2*, their mutations affect the expression not only of the *al-3* gene but also of other light-regulated genes, indicating that one or more light-regulatory functions are impaired. Crosses with *wc-1* and *wc-2* mutants were done to determine whether the mutations in the BLR-1 and BLR-2 strains affect the function of at least one more locus besides *wc-1* and *wc-2*. In all crosses, ≈25% of progeny were phenotypically wild type, and no unexpected phenotypes were found in the progeny, indicating that the two mutations were not allelic with the white collar genes. However, owing to the *ylo-1* background of these two strains, further genetic analysis failed to be done. In fact, it was impossible to distinguish between a BLR *ylo*$^+$ strain and a wild-type strain.

DISCUSSION

A *N. crassa* strain called 13-1 (*al-3p–mtr*$^+$ *trp-2*) was obtained, expressing mtr mRNA under the control of the *al-3* promoter. Because the uptake of tryptophan in this strain is controlled by the light-regulated *mtr* transport system, 13-1 can grow on minimal medium supplemented with a low amount of tryptophan only in the light and is also light sensitive to the poisonous analog FPA. This strain reacts macroscopically to light, and its behavior can be used to screen for mutants that show altered responses to blue light. Under light/dark cycles, strain 13-1 grew as expected. UV mutagenesis of strain 13-1 provided mutants that had become FPA resistant even when grown in the light. This protocol of mutagenesis produced several independent derivatives resistant to FPA. However, none of them showed the expected white-collar phenotype.

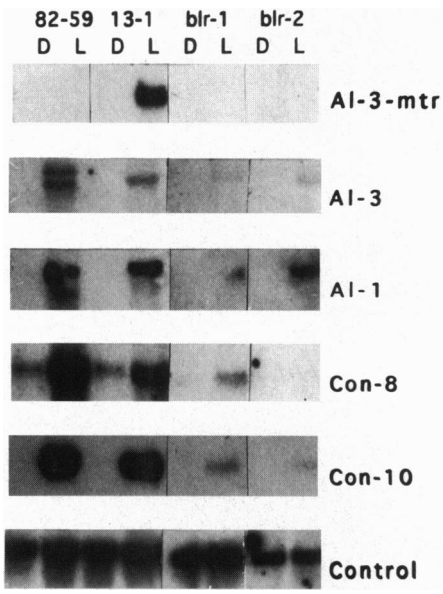

FIG. 4. Northern blot of mutant strains BLR-1 and BLR-2. Ten micrograms of total RNA extracted from light induced (L) and uninduced (D) cultures of BLR-1 and BLR-2, parental strain 13-1, and strain 82-59 was blotted and probed with DNA fragments specific for *mtr* and *al-1* open reading frames, and with *al-3*-, *con-8*-, and *con-10*-specific oligonucleotides, as described. The upper band in strain 82-59 mRNA hybridized with the *al-3*-specific probe represents an *al-3*-specific transcript expressed during conidiation (38). The double band observed with *al-1* could have a similar explanation, because it has been observed that strain 82-59 grows and develops faster than strain 13-1 in liquid cultures.

Several FPA-resistant colonies with normal carotenoid accumulation were obtained and discarded. These colonies could represent bradytrophs resistant to FPA (31) or mutations at other loci, unrelated to MTR-controlled uptake. Northern blot analysis revealed that the FPA-resistant strains BLR-1 and BLR-2 contain mutations important in light regulatory functions, as shown by the general reduction of light-regulated expression of several genes. These two strains are unable to grow on minimal medium/tryptophan in the light and grow normally when cultured in the presence of FPA. Furthermore, they are only lightly pigmented, indicating that carotenoid biosynthesis is reduced as a consequence of reduced expression of the albino genes.

In our experiment, no mutation was generated that fully abolished the expression of essential genes involved in the light signal-transduction pathway. The two mutants obtained respond very poorly to light, but they are not completely blind. The fact that they are leaky could indicate that the mutations lie in essential genes. On the other hand, the mutations could affect genes with a combinatorial role in the positive activation of gene expression; a null in such a gene could still show some regulation due to the action of other genes with overlapping functions but with reduced expression.

It is now possible to clone the wild-type alleles of the gene(s) that are mutated in the BLR-1 and BLR-2 strains by complementation with a genomic *N. crassa* library to rescue the parental growth phenotype on tryptophan/arginine medium.

The approach used here should readily allow isolation of other genes involved in the light signal-transduction pathway of *N. crassa*.

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