# ISOLATION AND CHARACTERIZATION OF LIGHT-INSENSITIVE MUTANTS OF *NEUROSPORA CRASSA*

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## ABSTRACT

As part of a genetic analysis of blue light photoreception in Neurospora, three mutants were isolated that do not exhibit photosuppression of circadian conidiation, *i.e.*, they show periodic conidiation in constant light. The mutations have been given the designations *lis-1*, *lis-2* and *lis-3* ("light insensitive"). The three mutations segregate as single nuclear genes, are nonallelic and are recessive to wild type in heterokaryon tests. The linkage groups of the mutations are as follows: *lis-1*, I; *lis-2*, VI; and *lis-3*, V. The light-insensitive phenotype of the mutants is restricted to the photosuppression response; other responses such as photoinduced phase shifting of the conidiation rhythm and photoinduced carotenogenesis are not altered. The physiological or biochemical defects of the mutants have not been established, but they are not similar to previous reported cases (*i.e.*, *rib* and *poky*) in which a reduction in light sensitivity has been observed.

PHYSIOLOGICAL responses to blue light are known in a wide variety of organisms. Examples include phototaxis in Euglena (DIEHN 1969), phototropism of coleoptiles in Avena (SHROPSHIRE and WITHROW 1958), induction of carotenoid synthesis in Neurospora (ZALOKAR 1955) and entrainment of circadian pupal eclosion in Drosophila (FRANK and ZIMMERMANN 1969). For many of these responses a flavoprotein is thought to be the photoreceptor pigment (SCHMIDT 1980; SENGER and BRIGGS 1981). An exact identification of the photoreceptor has, however, not been accomplished.

The use of mutants with known biochemical defects has provided some general information about the Neurospora blue light photoreceptor(s). BRAIN, WOODWARD and BRIGGS (1977) found a correlation between a cytochrome *b* deficiency and a reduced sensitivity to light in the cytoplasmic mutant *poky*. PAIETTA and SARGENT (1981) observed a parallel effect in riboflavin mutants (*rib-1* and *rib-2*), in which a reduction in light sensitivity correlated with the presence of a flavin deficiency. These reductions in light sensitivity are apparently specific effects of the *rib* and *poky* mutations, since deficiencies induced in various other auxotrophs or respiratory mutants do not cause similar effects (BRIGGS 1980; PAIETTA 1982a). These findings suggest a role for a cytochrome *b*-

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flavin complex in photoreception. Further support for flavin involvement comes from the demonstration that riboflavin analogs can act as photoreceptors in the rib-2 mutant (PAIETTA and SARGENT 1983). Nitrate reducase, a potentially photoactive flavoprotein in Neurospora (KLEMM and NINNEMANN 1979), does not, however, appear to function generally as a photoreceptor since a number of photoresponses were found to be normal in nitrate reductase (nit) mutants (BELOZERSKAYA et al. 1982; PAIETTA and SARGENT 1982b).

Another approach to the study of photosensory transduction in Neurospora involves the isolation of mutants with an altered sensitivity to light. As a starting point for the genetic dissection of photoreception in Neurospora, the photosuppression of circadian conidiation (SARGENT, BRIGGS and WOODWARD 1966) was chosen for analysis. With this photosuppression response there is continuous conidiation in constant light, rather than a rhythm of conidial formation as in dark controls. A screening technique for mutants that would exhibit circadian conidiation in constant light was developed. A genetic, biochemical and physiological characterization of three such mutants which have been isolated is presented here. A preliminary account of this study has been given (PAIETTA and SARGENT 1982a).

## MATERIALS AND METHODS

*Strains:* The following strains were obtained from the Fungal Genetics Stock Center (FGSC) (Humboldt State University, Arcata, California): ad-3 (38701, FGSC no. 368); al-1 (80–96, FGSC no. 901); bd (41-4, FGSC no. 1859); chol-2 (47904, FGSC no. 164); cr-3 (R2509, FGSC no. 3449); ilv-1, inl (16117, 64001, FGSC no. 676); trp-1 (8, FGSC no. 2038); and trp-2 (S4266, FGSC no. 990).

*Media and general procedures:* Stock cultures were grown on Vogel's sucrose medium (DAVIS and DESERRES 1970) and stored on silica gel (PERKINS 1977). Standard formulas were used in preparing glucose-arginine (SARGENT, BRIGGS and WOODWARD 1966), sucrose-Tween 80 (HARDING 1974), complete, and acetate minimal (DAVIS and DESERRES 1970) media. Crosses were carried out according to standard procedures (DAVIS and DESERRES 1970) using either WESTERGAARD and MITCHELL'S (1947) or corn meal crossing media. Construction and analysis of forced heterokaryons were done according to the techniques outlined by DAVIS and DESERRES (1970). All biochemicals were of reagent grade.

*Mutagenesis:* Nitrous acid or ultraviolet light were used as mutagens according to standard procedures (DAVIS and DESERRES 1970). The treatments chosen resulted in about 50% survival of spores.

*Screening for mutants:* The phenotype screened for was the occurrence of circadian conidiation in constant light (cool white fluorescent, 400 lux). In all cases, the bd marker was present, so that the strains isolated were actually double mutants, i.e., containing the bd mutation plus a light sensitivity mutation. Two approaches were used to screen for such "blind" mutatants. In one approach, the combination of a colonial marker (cr-3), a growth inhibitor (sodium desoxycholate, 10 mg/liter; TATUM, BARRATT and CUTTER 1949) and a cool temperature (22°) was used to restrict the growth rate so that a number of colonies, i.e., ten to 15, could be scored on a single Petri plate (Figure 1). With this procedure mutagenized spores were plated and held in darkness for about 24 hr. The plates were then transferred into constant light, and any colonies that subsequently showed evidence of rhythmicity were retained for further study. Mutants isolated in this way were subsequently crossed to remove the cr-3 marker in order to obtain strains that could be more easily studied.

The second approach was to take mutagenized bd spores and plate them onto sucrose minimal medium containing 3% l-sorbose. Individual colonies were then picked and transferred into separate minigrowth tubes (15- × 150-mm test tubes kept horizontal and containing a thin film of medium). The tubes were kept in darkness for 24 hr and then transferred into constant light. As with the
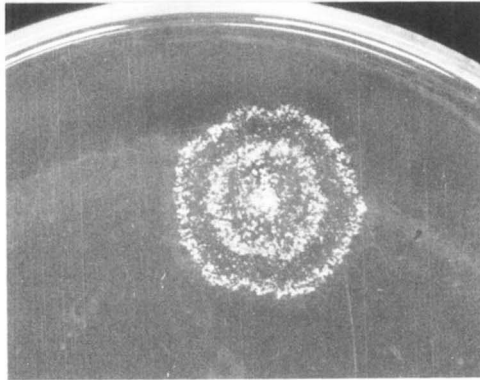
FIGURE 1.—Presumptive light-insensitive mutant growing in a Petri dish culture and kept in constant light (400 lux).

other technique, isolates that showed evidence of rhythmicity were kept for analysis. Mutants isolated by either technique were cycled through backcrosses and reisolations a minimum of three times before study.

*Photoresponse assays:* The procedures outlined in PAIETTA and SARGENT (1981) were used for the assays of photosuppression, phase shifting and carotenogenesis.

*Cytochrome extractions and respiration measurements:* The method of BERTRAND and PITTENGER (1969) was used to isolate and prepare mitochondrial cytochromes for spectrophotometric analysis. Air-oxidized *vs.* dithionite-reduced difference spectra of the cytochromes were measured with a Cary 14 spectrophotometer.

Oxygen consumption of mycelia was measured with a YSI model 53 oxygen monitor with a Clark type YSI 5331 probe. Temperature was maintained at $25° \pm 0.1°$ with a Polytemp circulator and a PSC KR-30 refrigeration unit. The general procedures outlined by EDWARDS, KWIECINSKI and HORTSMANN (1973) were followed.

<div style="text-align:center">RESULTS</div>

## Isolation of the mutants

Of approximately 30,000 colonies screened, a total of 35 presumptive light-insensitive mutants were isolated. Three single-gene mutants actually insensitive to light were obtained after characterization of the presumptive strains. The three mutants described here have been given the designation "light insensitive" (*lis*), and all exhibit periodic conidiation in constant white light (Figure 2). Note that the parental *bd* strain shows continuous conidiation under the same conditions. The conidial banding exhibited by the mutants in constant light (400 lux) is circadian in that the period lengths are: *bd lis-1*, 20.1 hr; *bd lis-2*, 21.4 hr; and *bd lis-3*, 20.6 hr. Additionally, these effects are not temperature sensitive since circadian conidiation can be observed in constant light within the range of temperatures that the responses can be accurately monitored (*i.e.*, 18–32°).

## Growth rates and nutritional effects

The growth rate of the mutants on different media is shown in Table 1. On the glucose-arginine (growth tube) medium, the *bd lis-1* strain exhibits a normal growth rate, whereas *bd lis-2* has a somewhat reduced growth rate, and *bd lis-*
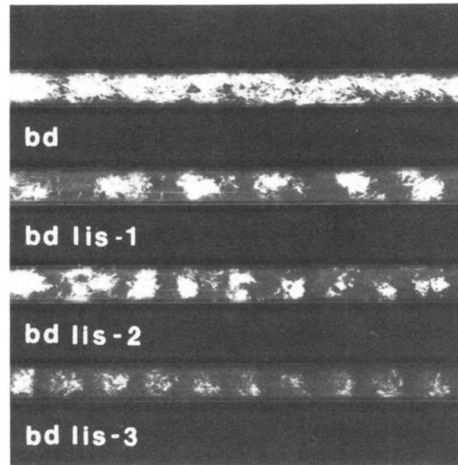
FIGURE 2.—Growth tube cultures of the *bd* and *bd lis* strains incubated in constant white light at 400 lux. Note that photosuppression of the circadian conidiation rhythm does not occur in the *bd lis* strains.

TABLE 1

*Effect of medium composition on growth rate*

| Strain | Growth rate (mm/day ± S.E.M.) | | |
| | Glucose-arginine | Complete | Acetate |
|---|---|---|---|
| *bd* | 38.3 ± 0.5 | 41.1 ± 0.6 | 33.7 ± 0.6 |
| *bd lis-1* | 42.3 ± 0.7 | 42.9 ± 0.6 | 31.2 ± 0.8 |
| *bd lis-2* | 22.6 ± 0.9 | 26.8 ± 0.8 | 13.8 ± 0.5 |
| *bd lis-3* | 13.6 ± 0.6 | 15.9 ± 0.9 | 3.7 ± 0.2 |

3 is very slow growing. Growth on different types of supplemented media (complete; riboflavin, flavin adenine dinucleotide or flavin mononucleotide supplementation) had little effect on the strains, and in no cases were normal growth rates restored for *bd lis-2* or *bd lis-3*. The slow growing mutants, *bd lis-2* and *bd lis-3*, do not appear to be leaky auxotrophs (flavin or otherwise) with defects that are nutritionally repairable. To the extent that photosuppression could be assayed, growth on complete or flavin-supplemented media appeared to have no effect on the sensitivity to light of the mutants. The only unusual effect noted that was that the *bd lis-3* strain grew poorly on acetate minimal medium, being 73% slower than on the glucose-arginine medium.

*Segregation of the mutant phenotypes*

The three *lis* mutations all segregate as single nuclear genes. For each cross between *bd lis*[+] and the *bd lis* strains, random spores were collected and the phenotypes scored (Table 2). One to one segregation is present in each case. Reciprocal crosses gave similar results, although the mutants function less effectively as the protoperithecial parent. The *lis-1* and *lis-3* mutations resulted

TABLE 2

*Segregation of the lis genes*

| Cross | Number of isolates | | % Germination |
| | *lis* | *lis*$^+$ | |
|---|---|---|---|
| *bd lis-1*$^+$ × *bd lis-1* | 229 | 241 | 85.5 |
| *bd lis-2*$^+$ × *bd lis-2* | 137 | 121 | 64.5 |
| *bd lis-3*$^+$ × *bd lis-3* | 182 | 170 | 78.2 |

from ultraviolet mutagenesis, whereas *lis-2* arose in an experiment employing nitrous acid.

## Mapping the mutations

The linkage of each mutant gene was determined by crosses with markers from each linkage group. Three-point crosses were then performed with appropriate double mutants. The three-point linkage data are shown in Table 3. *lis-1* is in linkage group I, and *lis-3* is in linkage group V. *lis-2* is apparently in linkage group VI, but the data must be regarded as preliminary due to problems with poor ascospore germination and scoring the mutant's phenotype in the presence of other mutations.

## Complementation and dominance relationships of the mutations

The three *lis* mutants are recessive to their respective *lis*$^+$ alleles in forced heterokaryons (*trp-1* and *inl* as forcing markers), *i.e.*, essentially normal growth rates and sensitivity to light for photosuppression were observed for each pairwise combination (Table 4). Furthermore, each of the mutants complemented each other in forced heterokaryons (*trp-1* and *inl* as forcing markers) to give a normal growth rate and light sensitivity. Nuclear ratios were at approximately 1:1 for these determinations.

## Responses to light

*Photosuppression of circadian conidiation:* All of the *lis* mutants were isolated on the basis of exhibiting circadian conidiation in constant white light, which they clearly do (Figure 2). In constant light, the *bd lis-1* and *bd lis-3* mutants will generally show conidial banding for the entire length of the growth tube, whereas the *bd lis-2* mutant will usually conidiate rhythmically for 4–5 days and then damp out. The maximum intensity of white light under which circadian conidiation can be observed varies for each mutant and is as follows: *bd lis-1*, 700 lux; *bd lis-2*, 450 lux; and *bd lis-3*, 800 lux. The *bd* parental strain shows complete suppression of circadian conidiation at about 7 lux (SARGENT, BRIGGS and WOODWARD 1966), so that the mutants are about 60- to 100-fold less sensitive to light than *bd*.

*Entrainment of the conidiation rhythm:* The three mutants all show entrainment to a light/dark, 12-hr/12-hr, cycle. Entrainment is observed whether low intensity (50 lux) or high intensity (800 lux) white light is used. The period length for *bd* and all of the *bd lis* strains is 24 hr under these conditions. The *bd*

TABLE 3

*Three-point mapping data for the* lis *mutations*

| | | | | Parental combinations | Recombinations | | | Total; % germination; linkage group |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Zygote genotype and % recombination | | | | | Singles (Region I) | Singles (Region II) | Doubles | |
| + | lis-1 | | + | 118, 106 | 5, 11 | 21, 26 | 0, 0 | 282; 65.9; I |
| ad-3 | + 5.6 | 16.4 | al-1 | | | | | |
| + | lis-2 | | + | 84, 66 | 7, 12 | 22, 29 | 1, 2 | 223; 49.5; VI |
| chol-2 | + 11.2 | 25.5 | trp-2 | | | | | |
| + | + | | lis-3 | 115, 99 | 17, 12 | 2, 5 | 2, 0 | 252; 72.0; V |
| ilv-1 | inl 13.1 | 4.4 | + | | | | | |

TABLE 4

*Growth and light sensitivity of forced heterokaryons involving* lis *and* lis$^+$

| Heterokaryon genotype | Growth rate[a] (mm/day) | Photosuppression (at 100 lux) |
| --- | --- | --- |
| lis-1, inl; lis-1$^+$, trp-1 | 38.3 ± 0.2 | + |
| lis-1, trp-1; lis-1$^+$, inl | 35.8 ± 0.3 | + |
| lis-2, inl; lis-2$^+$, trp-1 | 36.1 ± 1.2 | + |
| lis-2, trp-1; lis-2$^+$, inl | 34.5 ± 0.3 | + |
| lis-3, inl; lis-3$^+$, trp-1 | 34.6 ± 0.5 | + |
| lis-3, trp-1; lis-3$^+$, inl | 35.4 ± 0.4 | + |

[a] Means of five to six determinations ± S.E.M.

*lis-3* mutant showed erratic banding patterns (primarily skipped bands) in some growth tubes, but in most cases (60%) entrainment clearly occurred.

*Phase shifting of the conidiation rhythm:* The data for this response in the *bd* and *bd lis* strains are shown in Table 5. All of the strains could be phase shifted by brief pulses of white light (15 or 240 sec; 800 lux). The *bd lis-1* and *bd lis-3* strains have response levels similar to that of *bd,* whereas *bd lis-2* was slightly less responsive. The sensitivity of *bd lis-2* to light was, however, relatively normal, unlike the case with the photosuppression response. The *bd* and *bd lis* strains also showed similar phase shifts with lower fluences, although the data are less reliable due to erratic phase shifts as has been observed in other cases (J. PAIETTA, unpublished data). Due to this technical problem, the possibility that some differences might exist between the strains when low fluences near the induction threshold are used cannot be excluded at present.

*Induction of carotenogenesis:* The data for this response are shown in Table 6. The mutant *lis* strains all showed similar response levels as compared with

TABLE 5

*Photoinduced phase shifting in the* bd, bd lis-1, bd lis-2 *and* bd lis-3 *strains*

| Strain | Phase shift advance (hr)[a] | |
| | 15 sec[b] | 240 sec[b] |
| --- | --- | --- |
| bd | 4.8 ± 0.2 | 5.9 ± 0.4 |
| bd lis-1 | 4.4 ± 0.3 | 5.6 ± 0.2 |
| bd lis-2 | 3.9 ± 0.4 | 4.8 ± 0.3 |
| bd lis-3 | 4.2 ± 0.4 | 5.2 ± 0.5 |

[a] Means of five replicates ± S.E.M.
[b] Irradiation time at 800 lux.

TABLE 6

*Photoinduced carotenoid synthesis in the* bd, bd lis-1, bd lis-2 *and* bd lis-3 *strains*

| Strain | Induced carotenoids[a] | |
| | 30 sec[b] | 480 sec[b] |
| --- | --- | --- |
| bd | 0.15 ± 0.03 | 0.23 ± 0.02 |
| bd lis-1 | 0.13 ± 0.02 | 0.21 ± 0.01 |
| bd lis-2 | 0.15 ± 0.01 | 0.24 ± 0.04 |
| bd lis-3 | 0.16 ± 0.02 | 0.25 ± 0.03 |

[a] Absorbance units per 100 mg dry weight; means of five replicates ± S.E.M.
[b] Irradiation time at 1000 lux.

the parental *bd* strain. The substantial effect on light sensitivity or photo-suppression in the mutants is not observed for this response.

*Cytochrome content and respiration rates*

Cytochrome spectra of isolated mitochondria for the *bd* and *bd lis* strains were obtained and compared (Figure 3). All strains show the presence of typical *a*-, *b*- and *c*-type cytochromes and are essentially normal.

Respiration, as measured by oxygen uptake, was also examined. The rates of respiration ($\mu$l $O_2$/hr/mg) were as follows: *bd*, 95.5; *bd lis-1*, 92.8; *bd lis-2*, 83.3; and *bd lis-3*, 54.4. Only *bd lis-3* showed a substantial difference from the control, and it is, as noted before, also the slowest growing among the group (Table 1). The respiration measured was completely cyanide sensitive. No cyanide (1 mM) insensitive or salicyl hydroxamic acid (0.78 mM) sensitive respiration was detected, indicating that alternate respiratory pathways are not operating in the mutants as is the case in the parental *bd* strain.

DISCUSSION

The three light-insensitive (*lis*) mutants described here are the first reported mutants of Neurospora to be isolated specifically on the basis of having a phenotype of reduced sensitivity to light. These mutants provide a start toward the genetic analysis of the transduction steps leading from the initial photo-
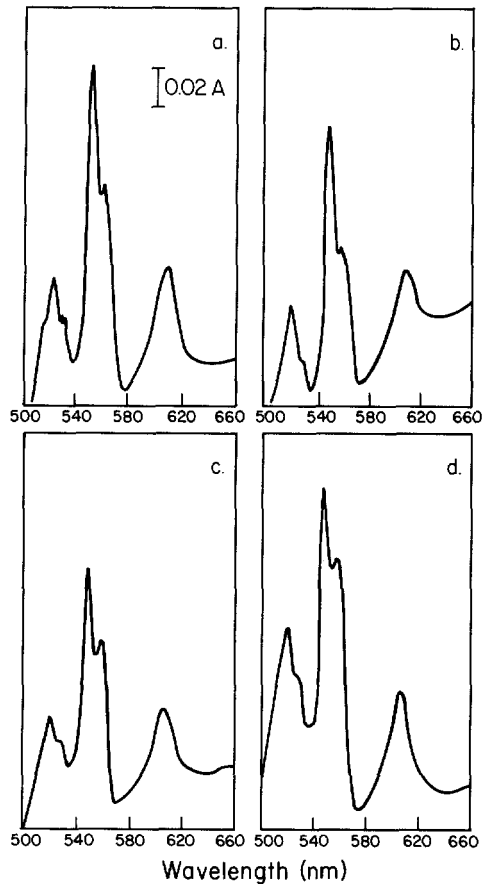
FIGURE 3.—Mitochondrial cytochrome spectra of the *bd* and *bd lis* strains: (a) *bd*, (b) *bd lis-1*, (c) *bd lis-2* and (d) *bd lis-3*. The mitochondrial suspensions contained 15 mg protein/ml.

chemical reactions to the observed physiological responses. The eventual goal is to obtain a number of such mutants that have defects for each of the different photoresponses in Neurospora and to determine the nature of the defects in the mutants.

The phenotypes of the *bd lis* mutants indicate that the photoresponses studied here are independent of each other for at least some functions or components. The *bd lis* mutants all show a reduced sensitivity to light for photosuppression but are normal (or nearly so) for phase shifting, entrainment and the induction of carotenogenesis. At this point, these results are consistent with either a single photoreceptor coupled to different transduction pathways or the existence of multiple photoreceptors with their own pathways. In this regard, a determination of the effect of the Neurospora white collar (*wc*) mutation on rhythm-related photoresponses should be of value since it is apparently defective for the photoinduction of carotenogenesis (HARDING and TURNER 1981).

It is also of interest that the *lis* mutants show different thresholds for the

photosuppression of circadian conidiation. The different thresholds could be a result of a number of possibilities, including a decreased concentration of the photoreceptor, an alteration in photoreceptor function or an effect in the transduction pathway beyond the photoreceptor. The fact that the mutations are nonallelic indicates that at least three different components may be present in the photosuppression transduction pathway. Whether or not the effects are direct or indirect cannot be stated at this time. The pleiotropic effects of the *lis-3* mutation, however, suggest that an indirect effect may be responsible for its reduction in light sensitivity.

The phenotype of the *lis* mutants in constant light raises a question as to the nature of the photosuppression response. With the band (*bd*) strain, constant light appears to result in arrhythmicity in that spore formation is continuous. Is the circadian clock stopped by the constant light or is the clock running and only the assayed response being affected? Since the *lis* mutants do show rhythmicity in constant light, the latter possibility seems likely, *i.e.*, constant light affects the conidiation process itself. Photosuppression of circadian conidiation may actually involve photoinduction of conidiation and the *lis* mutants may be altered in the induction process. For comparison, in Gonyaulax circadian rhythmicity can be detected in constant light by monitoring cell division, but the rhythm of mechanically stimulated bioluminescence is suppressed due to a direct effect of light on the response (SWEENEY 1979).

Preliminary efforts were made to determine the nature of the defects in the *lis* mutants that are responsible for the reduced sensitivity to light. Previous experiments with Neurospora have shown that a flavin deficiency in *rib* mutants (PAIETTA and SARGENT 1981) or a cytochrome *b* deficiency in the *poky* mutant (BRIGGS 1980) can cause a decrease in light sensitivity for photosuppression. The *bd lis* mutants, however, do not seem to have flavin or *poky*-like respiratory deficiencies. A generalized flavin deficiency, as with the *rib* mutants, is not present in the *bd lis* strains since riboflavin, flavin adenine dinucleotide or flavin mononucleotide supplements do not substantially affect growth rates or photosensitivity. The *bd lis* mutants are not similar to *poky*, since they do not have a cytochrome *b* deficiency or similar defects in respiratory function. In no case were alternate respiratory pathways induced and operating which can occur (SLAYMAN 1977) when respiratory defects are present. The *bd lis-3* strain does, however, have a low respiration rate and shows poor growth on acetate which suggests some sort of defect in energy-related metabolism. A respiratory defect alone will not, however, necessarily result in a change in light sensitivity, since the mutant rsp-2, which is blocked in the normal respiratory pathway but has normal levels of cytochromes, has a normal sensitivity to light for photosuppression (BRIGGS 1980).

The *lis* gene products have, therefore, not yet been identified. At present, almost nothing is known regarding the molecular components in the transduction pathways. The only exception is the starting point, where the photoreceptor is probably a flavoprotein. Since there is some evidence that blue light-induced absorbance changes may be involved in the initial stages of these transduction pathways (MUNOZ and BUTLER 1975), it would be of interest to determine if the

*lis* mutants are altered in these light-induced absorbance changes. The search for other components that may couple the photoreceptor to cellular systems may also be aided by the use of an evolutionary model (PAIETTA 1982b).

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