APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1991, p. 2671–2676
0099-2240/91/092671-06$02.00/0

Vol. 57, No. 9

Isolation of Light-Enhanced cDNAs of *Cercospora kikuchii*

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Received 8 May 1991/Accepted 2 July 1991

***Cercospora kikuchii* is a fungal pathogen of soybeans which produces a photosensitizing phytotoxic polyketide metabolite, cercosporin. Cercosporin synthesis in culture is modulated by several environmental factors. In addition to the light requirement for toxin action, cercosporin biosynthesis is also highly light regulated. As a first step towards identifying genes involved in cercosporin regulation and biosynthesis, we have used subtractive hybridization to isolate light-enhanced cDNA clones. Six distinct cDNA clones representing genes from a wild-type *C. kikuchii* strain for which transcript accumulation is positively regulated by light were isolated. To assess the relationship of these light-enhanced cDNAs to cercosporin biosynthesis, we compared corresponding steady-state RNA levels in the wild type and in three mutant strains altered in toxin biosynthesis. Two of the mutant *C. kikuchii* strains which fail to accumulate cercosporin in response to light also fail to exhibit light-enhanced accumulation of transcripts corresponding to all six light-enhanced cDNAs. Cercosporin accumulation in the third mutant strain, S2, is regulated by medium composition as well as light. S2 fails to accumulate cercosporin in complete medium, a medium which allows significant cercosporin accumulation by the wild-type strain. When cultured in complete medium, this mutant strain also fails to show the wild-type, light-enhanced accumulation of transcripts corresponding to five of the six light-enhanced cDNAs. Kinetic analysis demonstrated that transcript accumulation for two of the six light-enhanced cDNAs strongly paralleled cercosporin accumulation in light-grown wild-type culture.**

Polyketides are products of secondary metabolism in bacteria, fungi, and plants. This group of compounds includes important bacterial and fungal antibiotics, plant flavonoids, and fungal mycotoxins and phytotoxins (13). Many phytopathogenic fungi of the genus *Cercospora* produce a red polyketide toxin, cercosporin (7, 15). Cercosporin is a non-host-specific toxin which, in the presence of light, interacts with molecular oxygen to produce both superoxide radicals and singlet oxygen (9). These activated oxygen species cause peroxidation of cell membrane lipids, resulting in electrolytic leakage, a decrease in membrane fluidity, and cell death (8). *Cercospora* species incite disease in numerous economically important plants such as banana, sugar beet, coffee, tobacco, corn, peanut, and soybean. Several lines of evidence indicate that cercosporin plays an essential role in *Cercospora* pathogenicity: high light intensity is absolutely required for both disease development (4, 5) and toxin action (7), toxin can be isolated from naturally infected tissues (10, 23), application of the toxin alone can produce disease symptoms on host plants (1, 10), and non-toxin-producing mutants of *Cercospora kikuchii* fail to induce disease symptoms in soybean plants (23).

Environmental conditions, such as light intensity, temperature, and nutrient relationships, affect toxin production in culture (14, 16). While temperature and growth medium composition affect the quantity of toxin produced, light appears to be the dominant regulatory cue. Light-grown cultures of *C. beticola* and *C. rincinella* produce from 3- to 100-fold-higher levels of toxin than do dark-grown cultures (1, 10, 16). *C. kikuchii* cultures incubated in continuous white light accumulate approximately 50-fold-higher levels of toxin than those grown in continuous darkness (18, 23). Recent two-dimensional gel electrophoretic analyses of both extracted proteins and in vitro translation products (18) demonstrate that cultures grown in continuous light contain several proteins and translatable transcripts not present in cultures grown in continuous darkness. These data led us to clone light-enhanced cDNAs as the first step towards identifying genes involved in cercosporin biosynthesis.

In this article, we describe the cloning and characterization of six light-enhanced *C. kikuchii* cDNAs. Corresponding poly(A)$^+$ RNA levels ranged from slightly to highly light enhanced. Steady-state levels of these messages in three mutants altered in toxin biosynthesis were also measured. Two mutants that failed to accumulate cercosporin in response to light also failed to accumulate enhanced levels of these transcripts in response to light. In a third, medium-regulated toxin mutant, the accumulation of RNAs corresponding to five of the six cDNA clones was strongly down-regulated in a medium which does not sustain cercosporin accumulation. Finally, we show that, in the wild-type strain, the transcript accumulation kinetics for two of these light-enhanced cDNAs closely parallels the cercosporin accumulation kinetics.

MATERIALS AND METHODS

Fungal cultures and cercosporin determinations. Cultures of *C. kikuchii* were grown in either continuous light or continuous darkness in either potato dextrose (PD) broth (Difco Laboratories) or a complete medium (CM) containing salts, yeast extract, and Casamino Acids (13). The cultures were grown in shake culture (200 rpm) until the early stationary phase unless otherwise specified. Five-milliliter aliquots of culture (mycelium plus medium) were blended for 30 s in a Waring blender with 5 ml of distilled water, and the resulting slurry was used to determine cercosporin concentration as previously described (14). Dry weights were determined after lyophilization.

RNA extraction and cDNA library construction. Mycelia were harvested from liquid cultures by vacuum filtration

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through Miracloth, frozen in liquid nitrogen, and lyophilized. Lyophilized tissue was refrozen in liquid nitrogen and ground to a powder in a mortar and pestle prechilled with liquid nitrogen. Total RNA was extracted essentially as previously described (25), and poly(A)$^+$ RNA was extracted from total RNA by oligo(dT) cellulose chromatography (17). Five micrograms of poly(A)$^+$ RNA extracted from light-grown cultures of the wild-type *C. kikuchii* strain PR was used to construct a cDNA library in lambda-ZAPII (Stratagene).

Differential screening of the cDNA library. Light-enhanced cDNA clones were isolated from the cDNA library constructed from light-grown *C. kikuchii* poly(A)$^+$ RNA by a subtractive hybridization technique. First-strand cDNA was synthesized from light-grown *C. kikuchii* poly(A)$^+$ RNA as described elsewhere (17) by using [α-^{32}P]dCTP. Dark-grown *C. kikuchii* poly(A)$^+$ RNA was biotinylated and hybridized to the first-strand light-enhanced cDNA, and hybrids and nonhybridized dark-grown *C. kikuchii* poly(A)$^+$ RNA were subtracted from the mixture with streptavidin (20). After two rounds of subtraction, the remaining light-enhanced cDNA was labeled with [α-^{32}P]dCTP by random hexamer priming (11) and was used to probe duplicate plaque lifts (17) containing the light-enhanced cDNA library. Hybridizing areas containing multiple plaques were subsequently plaque purified and reprobed. Individual hybridizing plaques were isolated, and the cloned DNA was converted into plasmid DNA by using a helper bacteriophage and an in vivo plasmid excision technique described by the vector manufacturer (Stratagene).

RNA hybridization analysis. To screen the differentially selected cDNAs, 2-μg samples of glyoxylated poly(A)$^+$ RNA were electrophoresed under denaturing conditions through a 1.2% agarose gel (17) and transferred to nitrocellulose. For slot blot analysis, either 10- or 20-μg samples were applied to nitrocellulose with a Schleicher & Schuell Minifold II. Hybridizations were performed in a 50% formamide buffer at 42°C with insert DNA labeled to a high specific activity with [α-^{32}P]dCTP by random hexamer labeling (11). After experimental hybridizations were performed, the probe was removed from the slot blots by washing in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 95°C. The blots were then hybridized with a *Neurospora crassa* probe consisting of rDNA (genes coding for rRNA) (19). These control hybridizations verified that all samples within a slot blot were equally loaded. To quantitate intensity of slot blot hybridization, the autoradiographs were analyzed by laser scanning densitometry.

RESULTS

Identification of cLEs. A cDNA library was constructed with the bacteriophage lambda vector lambda-ZAPII by using poly(A)$^+$ RNA isolated from light-grown, wild-type *C. kikuchii* PR. The library was maintained as a bacteriophage stock and infected into *Escherichia coli* XL1-blue cells for screening. To isolate light-regulated cDNAs from this library, a "subtracted" probe was made from light-grown *C. kikuchii* poly(A)$^+$ RNA as described in Materials and Methods and used to probe high-density plaque lifts. Single hybridizing plaques were isolated and converted into plasmids by using a helper bacteriophage. Inserts from these plasmids were then used to probe Northern (RNA) blots containing poly(A)$^+$ RNA from light- and dark-grown *C. kikuchii* cultures. Six light-enhanced cDNA clones (cLEs), each of which hybridized to a single distinct mRNA band on Northern blots, were identified (Fig. 1). The lengths of these transcripts ranged from 0.8 to 6.0 kb, while the lengths of the cDNA inserts ranged from 0.8 to 2.4 kb. Two of the cDNA clones are nearly full length, while the others range from 32 to 79% of the lengths of their respective mRNAs. No transcripts for cLE6 were detected in the dark-grown *C. kikuchii* poly(A)$^+$ RNA. mRNAs for the other light-enhanced sequences were detected at various levels in the dark-grown *C. kikuchii* poly(A)$^+$ RNA.

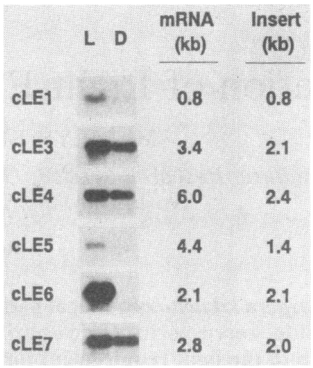

FIG. 1. Northern hybridization analysis of wild-type *C. kikuchii* PR. Two micrograms of poly(A)$^+$ RNA from light (L)- and dark (D)-grown PD broth cultures per lane was electrophoresed through a denaturing 1.2% agarose gel and transferred to nitrocellulose. Duplicate blots were probed with ^{32}P-labeled insert DNA from each of the cLEs (cLE1 and cLE3 to cLE7).

Expression of the light-enhanced cDNA clones in toxin mutants. To assess the correlation between cercosporin accumulation and expression of the cLEs, we examined accumulation of the corresponding mRNAs in three stable mutant derivatives of strain PR altered in cercosporin biosynthesis. *C. kikuchii* U2 and U4 were isolated after exposure of PR conidia to mutagenizing UV light (23). Both mutant strains appear to have lost the ability to produce cercosporin in response to light; when they are grown in continuous light, their toxin accumulation matches that of dark-grown wild-type PR. To determine whether this toxin-deficient phenotype correlated with an alteration in message accumulation for the light-enhanced sequences, slot blots containing total RNA isolated from both light- and dark-grown PR, U2, and U4 were probed with each of the labeled cLEs (Fig. 2), and the autoradiographs were analyzed by laser scanning densitometry. To directly compare the effect of light on the levels of transcript accumulation in the wild-type and mutant strains, the ratio of accumulation of each message in the light to its accumulation in the dark (L/D ratio) was determined (Table 1).

In the wild-type strain, PR, the light-enhanced sequences accumulated to between 2- and 20-fold-higher levels in the light than in the dark (Fig. 2 and Table 1). In contrast, the L/D ratios in the two mutant strains ranged from approximately 0.2 to 2. In U4, this was a result of a marked decrease in the accumulation of transcripts in the light relative to the wild type (Fig. 2). Interestingly, although U2 also failed to show light-enhanced message accumulation, this was due to an apparent increase (relative to dark-grown PR) in cLE transcript levels in dark-grown U2. The intense light enhancement of cLE6 transcript accumulation in PR (L/D = 20.7) was virtually eliminated in both U2 and U4 (L/D = 0.6 and 1.2, respectively). There is thus a strong correlation

TABLE 1. L/D ratios for cultures grown in PD broth

Clone	L/D ratio for strain[a]:		
	PR (wt)	U2	U4
cLE1	3.0	0.7	1.8
cLE3	2.2	0.9	0.2
cLE4	3.6	0.7	1.7
cLE5	1.9	0.9	0.7
cLE6	20.7	0.6	1.2
cLE7	2.6	1.4	2.1

[a] The slot blots in Fig. 2 were analyzed by scanning laser densitometry, and the L/D ratios were determined from the densitometry data. wt, wild type.

between light-induced cercosporin production and light-enhanced transcript accumulation. The wild-type fungus, PR, responds to light by synthesizing cercosporin and by increased accumulation of cLE transcripts. Mutant strains U2 and U4 fail to produce cercosporin in response to light and also fail to accumulate light-enhanced levels of cLE transcripts.

We also examined message levels in a spontaneously occurring, medium-regulated toxin mutant, S2. When grown in parallel PD broth cultures in light and darkness, mutant strain S2 showed a wild-type pattern of light regulation of both cercosporin and transcript accumulation (data not shown). When cultured in continuous light in CM broth, however, S2 accumulated no detectable toxin. This failure to synthesize cercosporin is not directly related to growth; accumulation of fungal tissue is approximately equal in the two media under either light regimen. Total RNA from the wild-type strain PR and the mutant strain S2 grown in continuous light in both media was slot blotted and probed with each cLE sequence (Fig. 3) and quantitated by laser scanning densitometry. Steady-state transcript levels for the light-enhanced cDNAs were not conspicuously different among the three cercosporin-producing cultures (wild type

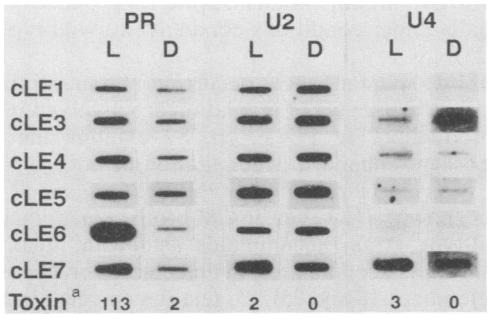

FIG. 2. Slot blot hybridization analysis of wild-type and mutant strains altered in cercosporin biosynthesis. Twenty micrograms of total RNA extracted from wild-type (PR) or mutant (U2 and U4) *C. kikuchii* strains grown in either continuous light (L) or continuous darkness (D) in PD broth was slot blotted onto nitrocellulose and probed with ^{32}P-labeled insert DNA from each of the cLEs. After hybridization and autoradiographic analysis with the cDNAs, the blots were stripped of signal. Hybridization with ^{32}P-labeled insert DNA from an *N. crassa* rDNA clone (19) verified that all slots contained equal amounts of total RNA (data not shown). Autoradiographs were analyzed by scanning laser densitometry, and the ratios of RNA accumulation in the light to RNA accumulation in the dark (L/D) are presented in Table 1. Toxin[a], cercosporin accumulation in nanomoles of toxin per milligram (dry weight) of fungus.

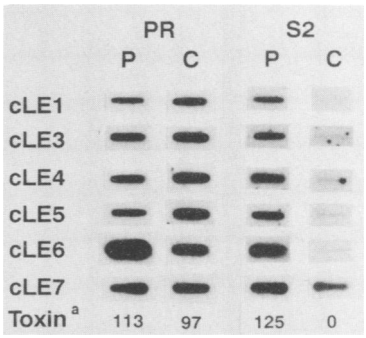

FIG. 3. Slot blot hybridization analysis of the wild type (PR) and a medium-regulated toxin mutant strain (S2). Twenty micrograms of total RNA extracted from PR or S2 grown under continuous light in either PD (P) or CM (C) broth was slot blotted onto nitrocellulose and probed with ^{32}P-labeled insert DNA from each of the cLEs. After hybridization and autoradiographic analysis with the cDNAs, the blots were stripped of signal. Hybridization with ^{32}P-labeled insert DNA from an *N. crassa* rDNA clone (19) verified that all slots contained equal amounts of total RNA (data not shown). Autoradiographs were analyzed by scanning laser densitometry, and the ratios of RNA accumulation in the PD broth culture to RNA accumulation in the CM culture (P/C) are presented in Table 2. Toxin[a], cercosporin accumulation is given in nanomoles of toxin per milligram (dry weight) of fungus.

grown in either PD or CM broth and S2 grown in PD broth). In the non-toxin-producing S2 (CM) culture, however, transcripts for five of the six cLEs were barely detectable. This alteration in transcript accumulation does not appear to be a response to medium composition intrinsic to *C. kikuchii*, since it is not seen in PR, the wild-type strain. To more clearly assess the effect of the medium on the transcript level, the ratio of the RNA level in PD broth to the RNA level in CM broth (P/C ratio) was determined (Table 2). In PR, the light-enhanced sequences generally accumulated to slightly lower levels in CM broth than in PD broth (P/C $<$ 1.0), while in S2, the P/C ratios ranged from 2.5 to 150. It appears, therefore, that in the toxin mutant strain S2, cercosporin accumulation and steady-state RNA levels for five of the six cLEs are strongly correlated.

Time-course analysis of toxin and transcript accumulation. To determine the relationship between toxin synthesis and expression of the light-enhanced cDNAs in *C. kikuchii* PR, we compared the kinetics of transcript and toxin accumulation. Samples were collected at 24-h intervals from a single large PD broth culture. Aliquots were used for toxin analysis (14), and the remaining culture was recovered and lyophilized for dry weight determination and RNA extraction.

TABLE 2. P/C ratios for cultures grown in continuous light

Clone	P/C ratio for strain[a]:	
	PR (wt)	S2
cLE1	0.5	12.1
cLE3	0.8	30.7
cLE4	0.5	6.5
cLE5	0.7	17.4
cLE6	2.0	150.5
cLE7	0.8	2.5

[a] The slot blots in Fig. 3 were analyzed by scanning laser densitometry, and the P/C ratios were determined from the densitometry data. wt, wild type.

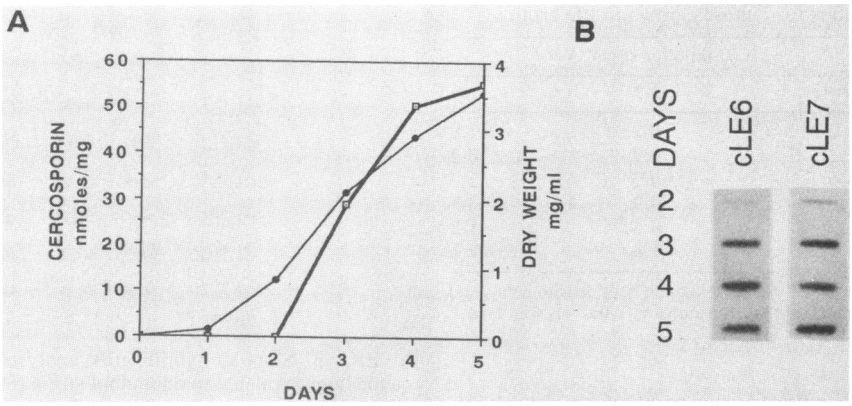

FIG. 4. Time-course analysis of dry weight, cercosporin accumulation, and steady-state levels of RNA corresponding to the light-enhanced cDNAs cLE6 and cLE7 in wild-type strain PR. (A) Aliquots from 50-ml samples collected at 24-h intervals from a single large PD broth culture were analyzed for toxin (⊡) and dry weight (●). (B) Slot blot hybridization analysis of total RNA extracted from the samples used in the experiments whose results are shown in panel A. Ten micrograms of total RNA was slot blotted onto nitrocellulose and probed with ^{32}P-labeled insert DNA from the cLEs. After hybridization and autoradiographic analysis with the cDNAs, the blots were stripped of signal. Hybridization with ^{32}P-labeled insert DNA from an *N. crassa* rDNA clone (19) verified that all slots contained equal amounts of total RNA (data not shown).

Transcript accumulation was assessed by slot blot analysis of total RNA from all samples except the day 1 sample, which yielded too little tissue for RNA extraction. Signal intensities were quantitated by laser scanning densitometry.

The onset of toxin accumulation occurred dramatically between days 2 and 3, lagging behind the onset of logarithmic growth by at least 24 h (Fig. 4A). Transcripts for both cLE6 and cLE7 exhibited accumulation kinetics identical to that of the toxin (Fig. 4B); i.e., each also increased dramatically between days 2 and 3. Between days 2 and 3, cercosporin levels increased from undetectable to 29 nmol/mg of fungal tissue, while steady-state RNA levels for cLE6 and cLE7 increased 16- and 4-fold, respectively. Transcripts for the other cLEs were already present at higher levels than those for cLE6 and cLE7 on day 2. They did not, however, show any marked change in steady-state level during the course of this experiment (data not shown).

DISCUSSION

Several approaches are commonly used to study the regulation of secondary metabolite synthesis. The isolation and identification of pathway intermediates and enzymatic proteins has led to the identification of biosynthetic genes in both prokaryotic and eukaryotic microorganisms (2, 12, 13). Functional complementation of blocked mutants with genomic clones has also proved useful in some systems (3, 13, 21). These approaches, however, were not applicable to this study, as only a single intermediary product has been proposed for cercosporin and, as yet, no biosynthetic enzymes have been identified. We therefore chose to use a molecular approach to take advantage of the fact that light acts as a strong positive regulator of cercosporin biosynthesis. In addition, we have at our disposal mutants blocked in cercosporin production as well as a transformation system for wild-type and mutant strains of *C. kikuchii*. The transformation rate is low (approximately 0.4 to 7.0/μg of DNA, depending on the recipient strain), and it would be impractical to directly isolate genes by functional complementation.

We report the isolation of six cDNAs from a wild-type *C. kikuchii* strain for which corresponding mRNAs exhibit enhanced accumulation in the light. Cercosporin production in this fungus is strongly regulated by light, and these light-regulated cDNAs (cLEs) were isolated as the first step towards identifying genes involved in cercosporin metabolism. Accordingly, we further tested the correlation between expression of these cLEs and cercosporin production. Our analyses show that two kinds of mutant derivatives of *C. kikuchii* PR exhibit altered mRNA accumulation patterns for all six cLEs. Two UV-induced mutant strains, U2 and U4, have apparently lost the ability to produce cercosporin in response to light. Neither U2 nor U4 accumulates significant levels of cercosporin in either continuous light or darkness (Fig. 2 and references 18 and 23). In addition, these two strains fail to show wild-type, light-enhanced accumulation of RNAs corresponding to the cLEs (Fig. 2). Interestingly, these toxin mutants also exhibit an altered phenotype in a second developmental process which is positively regulated by light (6, 24). Mutant strains U2 and U4 produce no or very few conidia under conditions conducive to wild-type sporulation (23).

In a third, spontaneously occurring mutant strain, S2, it appears that two levels of regulation are involved in cercosporin production. In PD broth, S2 exhibits the normal, wild-type light-enhanced accumulation of both cercosporin and mRNAs corresponding to the light-enhanced cDNAs (data not shown). However, in CM broth, a medium in which the wild-type strain accumulates significant quantities of cercosporin, S2 accumulates no detectable cercosporin (Fig. 3 and references 18 and 23). To test the correlation between cercosporin production and cLE transcript accumulation, we measured transcript levels in light-grown cultures of PR and the medium-regulated toxin mutant S2 in both PD and CM broth. The three toxin-producing cultures in Fig. 3 (the wild-type fungus grown in either PD or CM broth and S2 grown in PD broth) accumulate cLE transcripts to comparable levels. In contrast, the non-toxin-producing S2 culture (grown in CM broth) shows a marked reduction in transcript levels for five of the six light-enhanced cDNAs (cLE1 and cLE3 through cLE6) and a slight reduction in the transcript level for the sixth (cLE7). This suppression of transcript accumulation in CM-grown S2 cultures is not a reflection of a response to the medium that is intrinsic to the fungus

(Table 2); instead, it appears to be an indicator of the failure of S2 to be light responsive when grown in CM. Unlike sporulation of U2 and U4, sporulation of S2 is indistinguishable from wild-type conidiation (23). *C. kikuchii* sporulation medium, however, is more comparable to PD broth than to CM broth, as it contains plant extracts in the form of V8 juice and ground soybean leaves (24). The mutant phenotypes of U2 and U4 are slightly leaky; both strains accumulate small amounts of cercosporin (equal to dark-grown wild-type levels) when cultured under continuous light in either PD broth or CM broth (Fig. 2 and references 18 and 23). The mutant phenotype of S2 is, in contrast, more tightly regulated. Even though the toxin assay (14) used in this study can detect as little as 0.5 nmol of cercosporin, we could not detect cercosporin in S2 CM cultures grown under continuous light. In parallel with this apparently complete shutdown of cercosporin synthesis, transcript accumulation for the light-enhanced cDNAs is more radically suppressed in S2 than in either U2 or U4. If any of the cLEs are authentic cercosporin-related sequences, we would expect this kind of relationship, i.e., a more complete blockage of cercosporin synthesis would be characterized by a more extensive decrease in transcript accumulation for genes involved in its induction or biosynthesis.

Kinetic analysis of the wild-type strain also demonstrated a strong correlation between initiation of cercosporin accumulation and onset of transcript accumulation for two of the light-enhanced cDNAs, cLE6 and cLE7. Transcripts for the other four light-enhanced cDNAs did not show accumulation kinetics paralleling cercosporin accumulation. This does not, however, eliminate the possibility that they are involved in some other aspect of cercosporin metabolism. It is possible that the gene products of cLE1 and cLE3 through cLE5 perform a function necessary for, but auxiliary to, actual cercosporin biosynthesis, such as constituting part of the light-signal transduction pathway or participating in the ability of *C. kikuchii* to be resistant to its own toxin.

Of the six cLEs, cLE6 exhibits the most striking relationship to cercosporin production. Its expression is the most light enhanced, and accumulation of cLE6 transcripts was the most severely affected in the toxin mutants. Transcript levels for this cDNA clone were drastically reduced in non-toxin-producing S2 culture. In PR, accumulation of cLE6 transcripts in the light was increased by approximately 20-fold over transcript accumulation in the dark. In U2 and U4, this light enhancement of cLE6 transcript accumulation was not detected. In toxin mutant U2, cLE6 failed to show light enhancement, interestingly because of an apparent increase in the transcript level in dark-grown culture (Fig. 2). It is possible that light plays both a positive and a negative regulatory role in induction of cercosporin biosynthesis. This possibility is supported by the observation that protein extracts and in vitro translation products from dark-grown cultures contain proteins not detectable in comparable samples from light-grown cultures (18).

The results presented in this article demonstrate the feasibility of using the subtraction of dark-grown *C. kikuchii* RNA sequences from light-grown *C. kikuchii* RNA sequences to isolate cDNAs which are regulated, to various degrees, by light and show a persuasive correlation to cercosporin biosynthesis. We have recently constructed a cosmid library of wild-type strain PR genomic DNA with a *C. kikuchii* transformation vector (22) and used it to isolate genomic analogs of both cLE6 and cLE7. We are currently transforming these genomic clones into mutant strains to determine whether they can functionally complement toxin-altered phenotypes. We are also constructing disrupted versions of light-enhanced genes for transformation into the wild-type fungus to experimentally test their role (if there is any) in cercosporin biosynthesis or other related processes such as light-signal transduction or self-resistance to cercosporin. In this study, we have presented several lines of correlational evidence suggesting that the six cLEs play a role in cercosporin metabolism. The expression of one of these sequences is highly correlated with cercosporin accumulation. Our original and primary goal remains the isolation of cercosporin-biosynthetic genes. The correlational evidence presented in this article supports further characterization of the light-enhanced sequences. Even if they are not involved in some aspect of cercosporin synthesis, these sequences clearly represent a class of genes that respond to light, an important signal in development, sporulation, and pigment production in numerous species of fungi.

ACKNOWLEDGMENTS

We thank J. D. Williamson for many helpful discussions and critical evaluation of the manuscript and K. Everett for his advice and assistance with laser scanning densitometry. We also gratefully acknowledge the photographic expertise of Marvin Williams.

REFERENCES

1. **Balis, C., and M. G. Payne.** 1971. Triglycerides and cercosporin from *Cercospora beticola*: fungal growth and cercosporin production. Phytopathology **61**:1477–1484.
2. **Beck, J., S. Ripka, A. Siegner, E. Schiltz, and E. Schweizer.** 1990. The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. Eur. J. Biochem. **192**:487–498.
3. **Bibb, M. J., S. Biro, H. Motamedi, J. F. Collins, and C. R. Hutchinson.** 1989. Analysis of the nucleotide sequence of the *Streptomyces glaucescens* tcmI genes provides key information about the enzymology of polyketide antibiotic biosynthesis. EMBO J. **8**:2727–2736.
4. **Calpouzos, L.** 1966. Action of oil in the control of plant disease. Annu. Rev. Phytopathol. **4**:369–390.
5. **Calpouzos, L., and G. F. Stalknecht.** 1967. Symptoms of *Cercospora* leaf spot of sugar beets influenced by light intensity. Phytopathology **57**:799–800.
6. **Chen, M. D., S. D. Lyda, and R. S. Halliwell.** 1979. Environmental factors influencing growth and sporulation of *Cercospora kikuchii*. Mycologia **71**:1150–1157.
7. **Daub, M. E.** 1982. Cercosporin, a photosensitizing toxin from *Cercospora* species. Phytopathology **72**:370–374.
8. **Daub, M. E.** 1987. The fungal photosensitizer cercosporin and its role in plant disease. ACS Symp. Ser. **339**:271–280.
9. **Daub, M. E., and R. P. Hangarter.** 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. Plant Physiol. **73**:855–857.
10. **Fajola, A. O.** 1978. Cercosporin, a phytotoxin from *Cercospora* spp. Physiol. Plant Pathol. **13**:157–164.
11. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
12. **Hohn, T. M., and P. D. Beremand.** 1989. Isolation and nucleotide sequence of a sesquiterpene cyclase gene from the trichothecene-producing fungus *Fusarium sporotrichiochoides*. Gene **79**:131–138.
13. **Hopwood, D. A., and D. H. Sherman.** 1990. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annu. Rev. Genet. **24**:37–66.
14. **Jenns, A. E., M. E. Daub, and R. G. Upchurch.** 1989. Regulation of cercosporin accumulation in culture by medium and temperature manipulation. Phytopathology **79**:213–219.
15. **Lynch, F. J., and M. J. Geoghegan.** 1977. Production of cercosporin by *Cercospora* species. Trans. Br. Mycol. Soc. **69**:496–498.

16. **Lynch, F. J., and M. J. Geoghegan.** 1979. Regulation of growth and cercosporin photoinduction in *Cercospora beticola*. Trans. Br. Mycol. Soc. **73**:311–327.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Rollins, J. A., M. Ehrenshaft, and R. G. Upchurch.** Unpublished data.
19. **Russell, P. J., S. Wagner, K. D. Rodland, R. L. Feinbaum, J. P. Russell, M. S. Bret-Harte, S. J. Free, and R. L. Metzenberg.** 1984. Organization of the ribosomal ribonucleic acid genes in various wild-type strains and wild-collected strains of *Neurospora*. Mol. Gen. Genet. **196**:275–282.
20. **Sive, H. L., and T. St. John.** 1988. A simple subtractive hybridization technique employing photoactivatable biotin and phenol extraction. Nucleic Acids Res. **16**:10937.
21. **Smith, D. J., M. K. R. Burnham, J. H. Bull, J. E. Hodgson, J. M. Ward, P. Browne, J. Brown, B. Barton, A. J. Earl, and G. Turner.** 1990. β-Lactam antibiotics biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes. EMBO J. **9**:741–747.
22. **Upchurch, R. G., M. Ehrenshaft, D. C. Walker, and L. E. Saunders.** Appl. Environ. Microbiol., in press.
23. **Upchurch, R. G., D. C. Walker, J. A. Rollins, M. Ehrenshaft, and M. E. Daub.** Appl. Environ. Microbiol., in press.
24. **Vathakos, M. G., and H. J. Walters.** 1979. Production of conidia by *Cercospora kikuchii* in culture. Phytopathology **69**:832–833.
25. **Williamson, J. D., G. Galili, B. Larkins, and S. B. Gelvin.** 1988. The synthesis of a 19 kilodalton zein protein in transgenic petunia plants. Plant Physiol. **88**:1002–1007.