Genetic Regulation of Cercosporin Production in *Cercospora kikuchii*

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ABSTRACT: The large and diverse *Cercospora* genus of plant pathogenic fungi includes many species that are causal agents of economically relevant leaf, stem, and seed blights of numerous crop plants. Several of these pathogens produce the red, photoactivated, phytotoxic polyketide toxin cercosporin. This mycotoxin is a crucial pathogenicity factor in the development of leaf and pod blights by the seed-borne soybean fungal pathogen *Cercospora kikuchii*. Although certain cultivars may be less susceptible to the leaf- and pod-infection phases of the fungus, there are no soybean cultivars with resistance to cercosporin. A newly isolated gene from *C. kikuchii*, known as LE6, is essential for cercosporin production and pathogenicity. Therefore, genetic manipulation of this gene may affect resistance to cercosporin. Transcription of LE6 is regulated by light. The expression of cercosporin also may be inhibited by certain growth media and other natural products. Modification of cultivar screens that target LE6 may greatly enhance the possibility of finding native resistance to this soybean pathogen. Soybean germplasm that produces strong LE6 downregulating or inhibiting compounds may enhance pathogen resistance. Thus knowledge of the genetic and physiological regulation of cercosporin should provide new technological strategies for biocontrol of mycotoxins and the development of soybean breeding lines that exhibit durable resistance to *C. kikuchii*.
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KEY WORDS: *Cercospora kikuchii*, cercosporin, fungal pathogens, *Glycine max*, molecular genetics, mycotoxin, regulation, seed quality, soybean, weather damage.

Cercosporin (4,9-dihydroxyperylene-3,10-quinone) is a low molecular-weight (MW 534), red, lipid-soluble, secondary metabolite that is produced by several species of the imperfect fungal genus *Cercospora* (1). The isolation and purification of cercosporin have been described and may be achieved with simple organic extraction methods (1). Qualitative and quantitative assays of the mycelial mass and growth medium of *Cercospora* species for the presence of cercosporin involve extraction of mycelial plugs with alkali solution (5N KOH) in which cercosporin changes from bright red to emerald green (2). Little is known about the biosynthesis of this mycotoxin. A metabolic study, conducted with ^{13}C-labeled acetate or malonate in a culture feeding technique, followed by mass spectroscopy and nuclear magnetic resonance (NMR) analysis, did suggest a chemical structure for a potential cercosporin intermediate (3). Results of this study also provided strong evidence that cercosporin was derived from the polyketide biosynthetic pathway.

A substantial body of research has focused on factors that regulate cercosporin accumulation during growth in laboratory culture. Although medium components, particularly carbon (C) and nitrogen (N) sources (C/N) and the C/N ratio, can strongly affect cercosporin accumulation in several species examined to date, nutrient and isolate interaction often is complex and affects species differently. Cercosporin accumulation also is regulated by temperature in some isolates. Several studies indicate that the primary regulatory cue for the induction of cercosporin production is blue light (4,5). In addition, an important and unique feature of this fungal toxin is that it is photoactivated by the same wavelengths that induce production. In the presence of molecular oxygen and light, activated cercosporin promotes the production of singlet oxygen and superoxide (1). These active oxygen species can damage several components of living organisms, most notably by compromising membrane integrity.

There is no evidence that this polyketide secondary metabolite is required for the growth of the fungus, but cercosporin is essential for fungal pathogenicity. Because of its unique mode of action, cercosporin is a nonhost-selective mycotoxin with almost universal biological activity. Only *Cercospora* species and a few other fungi have resistance to this toxin (1). Therefore, all living plant material is susceptible to cercosporin damage in the light. Cercosporin has been isolated from cercosporin-induced plant lesions. Infected plant tissue typically exhibits structural changes that increase membrane rigidity and electrolyte leakage. Thus lipid peroxidation is consistent with the mode of toxin action (1). The application of purified cercosporin to plant tissue in the light reproduces disease symptoms of fungus-inoculated tissue (1). Cercosporin-deficient mutants of the soybean pathogen *Cercospora kikuchii* have been isolated. These mutant strains do not produce leaf or pod lesions in the light when inoculated

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onto greenhouse-grown plants (6). A subtractive hybridization technique, followed by analysis of fungal mRNA accumulation in these mutants, led to the identification of a cDNA, defined as light-enhanced (LE6) (4). This gene has been shown to be a critical element in the genetic regulation of cercosporin synthesis. The product of this gene also may provide the fungus significant protection from injury by the toxin.

MATERIALS AND METHODS

Cercospora kikuchii PR was isolated from soybeans in Puerto Rico and was provided by J.B. Sinclair (University of Illinois, Urbana, IL). Growth media and culture conditions were exactly as previously described (6,7). The cLE clones were isolated from a *C. kikuchii* PR (grown in the light) cDNA expression library by subtractive hybridization as described by Ehrenshaft and Upchurch (4). The insertional disruption of LE6 previously has been described by Callahan *et al.* (5,8). DNA sequences of the full-length 2.1-kb cDNA cLE6 and the 6.5-kb genomic clone gLE6 have been described previously (5,8). Total RNA from *C. kikuchii* was isolated by the procedures described by Ehrenshaft and Upchurch (4). Transcript accumulation of LE6 and four other light-enhanced *C. kikuchii* cDNAs was visualized by using standard RNA slot blot analysis methods (4).

Extraction of pigments from fungal mycelium. Lyophilized fungal mycelium was ground to a powder in liquid nitrogen in a chilled mortar. Approximately 100 mg mycelia powder was extracted (2–3×) with 0.5 mL ethyl acetate for 12 h at 4°C. The ethyl acetate volumes were pooled and taken to dryness under a stream of nitrogen. Thin-layer chromatographic (TLC) analysis was conducted with a system designed to resolve cercosporin from other naturally fluorescent ethyl acetate-extractable compounds (9). Pigments were visualized under long-wave ultraviolet (UV) light (366 nm). Standards of purified cercosporin were run on a separate plate prepared in the same manner, and the R_f values were compared to those compounds resolved from the fungal extracts.

Cercosporin assay. Aliquots (10 mL) of fungal liquid cultures (mycelium plus medium) were mixed in a Waring blender (Dynamics Corp. of America, New Hartford, CT) and treated with one volume 5N KOH, as described by Jenns *et al.* (2), and clarified by centrifugation. Cercosporin concentrations were determined spectrophotometrically by absorbance at a wavelength of 480 nm (A_m = 23,300 for cercosporin in base). Preweighed amounts of lyophilized mycelia samples were utilized for these assays.

Fungal sensitivity to cercosporin. The sensitivity of *C. kikuchii* PR and LE6-disruptant stains to cercosporin was tested by growth inhibition. Standard-sized fungal plugs (5 mm) were inoculated on divided petri plates. Half the potato dextrose (PD) medium in each plate was amended by addition of a cercosporin solution to give a final concentration of 10 µm, as outlined by Daub (1). Because the cercosporin stock was prepared in acetone, the other half of each plate was amended with an equal volume of acetone. Plates were maintained at 25°C under continuous fluorescent light (80 µeinsteins • m^{-2} • s^{-1}) or constant dark. Radial growth was measured three and four days after inoculation.

Fungal pathogenicity assays. The pathogenicity of *C. kikuchii* transformants *in planta* was determined by leaf inoculation of greenhouse-grown five-week-old soybean plants, cultivar Lee (6). Fungal inoculum was prepared by blending approximately 0.5 g fresh weight of washed, dark-grown mycelium of each strain in 20 mL sterile water for two 20-s pulses. Mycelium suspensions were then atomized on the undersides of the leaves until inoculum runoff was achieved. Plants were covered with plastic bags for an initial 48-h period in reduced light to maintain humidity for infection. Lesion formation was monitored over a 14-d period. Disease severity was quantified by rating the area of host tissue covered by lesions. Five plants were inoculated with each fungal strain tested. The entire experiment was replicated twice.

RESULTS AND DISCUSSION

Soybean, an oilseed of major importance to the world's economy, is susceptible to infection by *C. kikuchii* wherever the crop is grown. Infected soybean tissue develops disease lesions through stomatal penetration and colonization of the intracellular tissue spaces (10). This invasive action is followed by symptoms of stem, leaf, and/or pod blighting. Blighted pods often contain discolored seeds that have a characteristic "purple-stained" appearance. Seeds infected with *C. kikuchii* can have reduced seed density and weight, increased free fatty acid content, and reduced oil content (11). Purple stain often is related to lower seed quality. No more than 5% purple-stained seeds are permitted in lots of U.S. No. 1 standard yellow soybeans (3). Extensively stained seeds (≥50% of the seed coat) usually have significantly reduced germination and may produce stunted, low-vigor seedlings (3). Although the severity of the disease varies each year, depending on weather conditions, substantial crop losses have been attributed to the leaf phase of the disease in the southern United States (11). The search for enhanced or durable resistance to this pathogen is complicated by the results of previous studies, which have shown that cultivar susceptibility to foliar and seed infections have no strong relationship. Indeed, some cultivars show resistance to seed stain and susceptibility to leaf blight (10). To date, dietary health effects of cercosporin from purple-stained seeds or blighted foliage have not been investigated.

Present research supports the concept that cercosporin is a crucial pathogenicity factor for diseases caused by several *Cercospora* species. Experimental results are consistent with the conclusion that this mycotoxin enables the fungus to colonize and extract nutrients for growth and sporulation in infected plant tissue. Thus application of technologies that affect downregulation or inhibition of cercosporin production should be an effective disease-control strategy. The success of such a research strategy will depend on information. New knowledge on cercosporin biosynthesis and regulation at the molecular level is required to develop this concept. Acquir-

ing this database involves isolation and characterization of fungal genes involved in cercosporin biosynthesis, regulation, transport, and auto-resistance; cercosporin pathway intermediates; natural compounds that downregulate the transcription of cercosporin genes or inhibit gene products; and the nature of natural resistance to the toxin cercosporin in soybean germplasm. These principal elements of a toxin-control strategy should then suggest approaches or applications toward development of transgenic soybeans with resistance to cercosporin or *Cercospora* infection.

Progress has been made toward isolating genes involved in cercosporin metabolism through use of knowledge that cercosporin production is photoinduced. This means that light should regulate certain genes that encode at least one of the enzymes involved in cercosporin metabolism (4). In that regard, experiments were conducted with light- and dark-grown, wild-type, and cercosporin-deficient *C. kikuchii* mutants (Fig. 1). The *in vitro* translated mRNA products from these strains were subjected to two-dimensional protein gel electrophoresis and fluorographic analysis (12). Four translation products were found to be associated with either exposure to light and/or the appearance of cercosporin accumulation in culture (Table 1). After these observations, a subtractive hybridization approach was used to isolate light-enhanced transcripts from a cDNA library of light-grown *C. kikuchii* wild-type isolate PR (4). Six cDNA clones with light-enhanced transcript accumulation were isolated and characterized by Northern and slot blot mRNA analyses.

Transcript accumulation of one clone, LE6, was enhanced 20-fold by light compared to levels in the dark. In addition, growth studies revealed that cercosporin accumulation was nutrient-specific in a mutant (S2) derived from *C. kikuchii* PR. Results showed that cercosporin accumulated in this mutant at a rate similar to that of wild-type strains when grown on potato dextrose (PD) medium. However, cercosporin was not produced by the S2 mutant when grown on complete (CM) medium (Fig. 2). Transcript analysis showed that five of the six cDNA did not accumulate in the S2 mutant grown

TABLE 1
***In vitro* Translation Products from Light-Grown Cultures of *Cercospora kikuchii* Wild-Type PR and the Medium-Regulated Mutant S2 That Are Specific to Cercosporin Production**

M_r	pI[a]	Presence	Specificity
42.9	6.45	PR	LT[c]
55.4	5.96	PR, S2–CM[b]	LT, medium

[a]pI denotes isoelectric pH of proteins.
[b]CM denotes the complete medium on which the mutant S2 was grown.
[c]LT denotes continuous light.

on CM medium. Furthermore, transcript accumulation of LE6 and another cDNA clone, LE7, paralleled the kinetics of cercosporin production in wild-type PR.

The structure and function of LE6 have been partially characterized (4,5,8). Genomic Southern analysis showed that LE6 existed as a single-copy gene in *C. kikuchii* and also was present in the genomes of other phytopathogenic *Cercospora* species, such as *Cercospora beticola* (sugarbeet), *Cercospora nicotianae* (tobacco), and *Cercospora zeae-maydis* (corn). DNA sequence analysis of LE6 detected an open reading frame (ORF) of 1,818 base pairs (b.p.) that could be translated into a 606 amino acid hydrophobic protein (Fig. 3). This gene product had a mass of 65.4 kDa with an isoelectric pH of proteins (pI) of 5.08. Further analysis suggested that this hydrophobic protein may contain a region with significant amino acid sequence homology to both prokaryotic and yeast proteins involved in antibiotic resistance.

Recent experiments have resulted in the functional disruption of LE6. A plasmid that contained an altered copy of LE6 (5,8) was genetically transformed into the toxigenic, wild-type *C. kikuchii* strain PR. Several *C. kikuchii* transformants were recovered in which a single copy of the altered LE6 gene had replaced the native copy by homologous recombination. Functional assays of LE6-disruptant *C. kikuchii* transformants have given informative and consistent results. LE6-

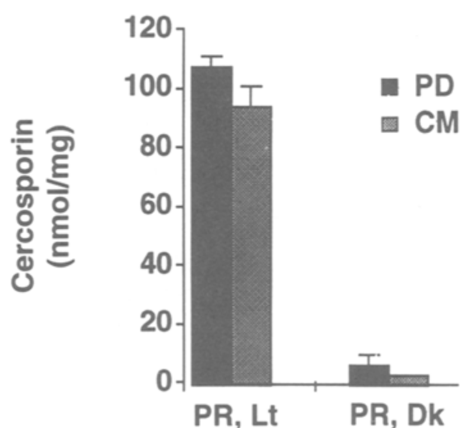

FIG. 1. Cercosporin accumulation (nmol/mg dry mass) in potato dextrose (PD) and complete (CM) media. Media were inoculated with *Cercospora kikuchii* PR and incubated for seven days in liquid shake culture at 23°C in continuous light (LT) or continuous dark (DK).

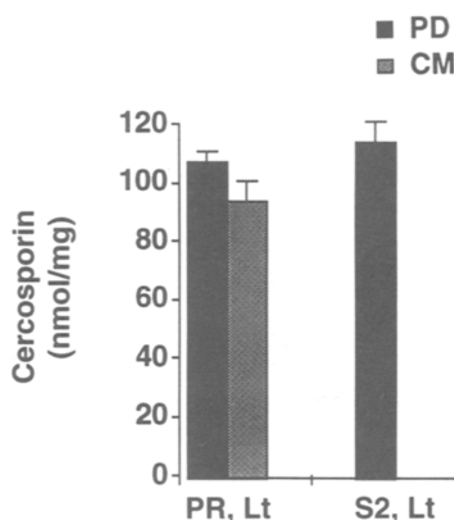

FIG. 2. Cercosporin accumulation in *C. kikuchii* wild-type PR and an S2 mutant. Strains were grown for seven days in liquid shake culture with PD and CM media under continuous light at 23°C. See Figure 1 for abbreviations.

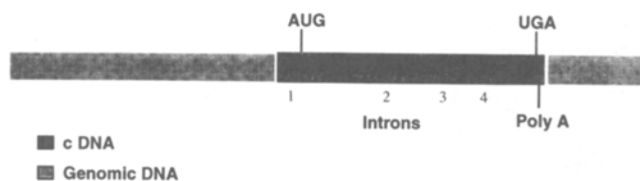

FIG. 3. Genomic map of LE6 cDNA. Structural organization of the 6.5 kb *EcoR1* genomic subclone restriction fragment containing the 2.1 kb cDNA LE6-hybridizing region. AUG and UGA, start and termination, codons of mRNA translation, respectively.

disruptive strains, grown in liquid medium in the light, produced ≤5% of wild-type levels of cercosporin. TLC of organic-soluble pigments extracted from the mycelial mass of disruptant and wild-type strains revealed an abundance of three orange-fluorescent compounds in the wild-type strains (Table 2). These compounds were dramatically reduced in the disruptants (11). Unexpectedly, fungal growth of LE6-disruptant strains in the light on PD medium that contained 10 μm cercosporin was significantly inhibited compared to the wild-type strain. Five-week-old soybean plants inoculated with these strains revealed that LE6 disruptants produced only a few nonspreading, pinpoint leaf lesions, in contrast to the large, advancing lesions observed in wild-type strains. Finally, transcript accumulation for the other five LE genes was determined in the LE6-disruptant strain grown in the light. This examination showed that accumulation of one transcript was dramatically reduced and that two others were slightly reduced (9). These results are consistent with the idea that LE6 may function in concert with and/or regulate transcription of up to three other LE genes. This hypothesis does not preclude involvement of the LE6 product in the resistance mechanism of *C. kikuchii* to cercosporin (auto-resistance). Efforts are being made to determine how mutations in LE6 alter pigment (possible intermediates in the cercosporin synthetic pathway) accumulation and how LE6 may regulate the transcript accumulation of the other LE genes.

These findings strongly suggest that LE6 is essential for fungal pathogenicity and plays a central role in toxin excretion. As mentioned previously, LE6 transcription and cercosporin production are strongly regulated by environmental signals, including light (4), by plant extracts and by nitrogen source in the S2 mutant and wild-type strain PR (13). Together, these findings suggest that LE6 is an appropriate target for biochemical manipulation to achieve downregulation of cercosporin production and also increased auto-sensitivity to cercosporin. Control of LE6 in *C. kikuchii* should diminish phytopathogenicity and decrease pathogen fitness. For these reasons, we constructed an in-frame GUS-reporter translational fusion of the putative "promoter region" of LE6 in a wild-type strain of *C. kikuchii*. The LE6 promoter has been characterized by deletion mapping to locate promoter regions involved in environmental regulation, such as light-enhancing elements (8).

TABLE 2
Relative Accumulation of Pigments Extracted from *Cercospora kikuchii* Cultures That Were Separated by Thin-Layer Chromatography and Visualized Under Long-Wave (366 nm) Ultraviolet Illumination

Pigment color	R_f	PR, LT	PR, DK[b]	LE6
Orange-yellow	0.81	++++	+	–
Cercosporin (red)	0.69	+++++++	++	+
Blue	0.64	–	+++++	–
Orange-yellow	0.59	+++++	–	+
Orange-yellow	0.51	++++	–	+

[a]LT denotes continuous light. [b]DK denotes continuous dark.

In conclusion, the translational fusion strain will be used to rapidly assay a variety of natural products (including soybean seed extracts) for compounds that may regulate LE6 expression. Compounds that downregulate LE6 transcription should eliminate cercosporin production and auto-resistance to the toxin. Compounds also may be identified that inhibit cercosporin production by affecting activity of the LE6 gene product. This targeted molecular approach may have advantages over traditional resistance screening in the field, where detection of cultivar resistance is influenced by variations in disease pressure. In that regard, the incidence of purple stain often seems unrelated to disease development on foliage (10). This new approach requires less labor and could be conducted in controlled environments. Hence, application of molecular genetics in a practical biocontrol method should significantly enhance research efforts to manage pathogenic fungi and to reduce the occurrence of mycotoxins in food products.

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