Characterization of Mutants of *Cercospora nicotianae* Sensitive to the Toxin Cercosporin

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ABSTRACT

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Six mutants of *Cercospora nicotianae* that were isolated for sensitivity to the photoactivated toxin cercosporin were characterized for cercosporin sensitivity and production, presence of protective compounds, and pathogenicity. The mutants fell into two classes. Five of the mutants (CS2, CS6, CS7, CS8, and CS9) were inhibited when grown on medium amended with cercosporin concentrations as low as 0.1 μM. Cercosporin sensitivity of these mutants increased markedly with increasing light intensity from 3 to 40 μE m^{-2} s^{-1}. These mutants showed no general light sensitivity in the absence of cercosporin. These mutants were capable of synthesizing cercosporin even though their growth was inhibited when cercosporin was produced. These five mutants were protected against cercosporin toxicity by exogenously added ascorbate, cysteine, and reduced glutathione. The sixth mutant (CS10) was not inhibited by cercosporin concentrations below 10 μM and showed little change in cercosporin sensitivity under increasing light intensity. CS10 also synthesized cercosporin. However, growth of this mutant was unaffected under cercosporin-producing conditions. CS10 was protected against high concentrations of cercosporin by exogenously added cysteine and reduced glutathione, but not by ascorbate. None of the six mutants were altered in endogenous levels of β-carotene, ascorbate, cysteine, reduced glutathione, or total soluble or protein thiols. CS2, CS8, and CS10 were compared to wild-type for ability to produce lesions on tobacco. CS2 and CS8 produced significantly fewer lesions than did wild-type; CS10 was intermediate.

Additional keywords: active oxygen, fungus, perylenequinone, photosensitizer, singlet oxygen.

Species in several genera of plant pathogenic fungi, including *Cercospora*, *Alternaria*, *Cladosporium*, *Stemphylium*, and *Hypocrella*, produce light-activated perylenequinone toxins (5,13,22,27,28,32,33,37,39,40). Of these, the toxin studied the most is cercosporin, a toxin produced by *Cercospora* species. Cercosporin is a photosensitizing compound which in the presence of light transfers absorbed light energy to oxygen, producing the highly toxic, activated singlet form of oxygen (9,15,21). Singlet oxygen ($^1O_2$), in turn, damages cells by causing peroxidation of membrane lipids, leading to membrane breakdown and cell death (6,7). Studies demonstrating the dependence of symptom development on light intensity (3,4,16,34) and the lack of disease production by mutants deficient in toxin synthesis (35) suggest that cercosporin plays an important role in diseases caused by *Cercospora* species.

Singlet oxygen is almost universally toxic to living cells, and cercosporin has been demonstrated to have toxicity not only to plants and cultured plant cells, but to mice, human tumor cells, bacteria, and many fungi (reviewed in [8]). *Cercospora* species, by contrast, appear resistant to cercosporin as they produce and excrete up to millimolar concentrations of cercosporin in culture in the light, with no observable toxic effects. High-level resistance to a $^1O_2$-generating photosensitizer is uncommon, and thus this resistance is currently under investigation in our lab. Genes responsible for resistance may have potential usefulness in the development of *Cercospora*-resistant plants.

In previous studies we showed that resistance of fungi to cercosporin was correlated with their ability to transiently reduce and detoxify cercosporin in contact with hyphae (10,26,31). Using fluorescence microscopy with bandpass filters specific for fluorescence emission from cercosporin and reduced cercosporin, we demonstrated that cercosporin in contact with the hyphae of *Cercospora* species and *Alternaria alternata* (also cercosporin-resistant) was in a reduced (nontoxic) form. By contrast, two cercosporin-sensitive fungi, *Neurospora crassa* and *Aspergillus flavus*, were not able to reduce cercosporin. *Cercospora* cultures killed by heat, chloroform vapor, or exposure to UV light also did not reduce cercosporin, indicating that cercosporin reduction is an active function of resistant hyphae. We showed that reduced cercosporin is highly labile and immediately reoxidizes upon aeration or extraction away from a reducing agent (26). This property would allow for the spontaneous reoxidation of reduced cercosporin to yield the active form needed for plant infection, a hypothesis supported by the observation that crystals of active (nonreduced) cercosporin accumulate adjacent to hyphae in culture.

We recently isolated six mutants of *Cercospora nicotianae* Ellis & Everh. that are sensitive to cercosporin (24). These mutants were isolated from UV-mutagenized protoplasts and were screened for cercosporin sensitivity by replica plating onto cercosporin-containing medium. Growth of five of the mutants, CS2, CS6, CS7, CS8, and CS9, was completely inhibited by 10 μM cercosporin, and also was totally inhibited by five other $^1O_2$-generating photosensitizers. The sixth mutant (CS10) was partially inhibited by 10 μM cercosporin, but was unaltered in its response to the other photosensitizers. Fluorescence microscopy showed that the five completely sensitive mutants were unable to reduce cercosporin, while the partially sensitive CS10 was normal in cercosporin-reducing ability.

As an initial step toward using these mutants to isolate and identify genes involved in cercosporin resistance, we report here

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on the phenotypic and chemical characterization of the six CS mutants. First, we observed that these mutants are capable of synthesizing and excreting cercosporin, an observation which seems contradictory to their sensitivity to externally supplied cercosporin. We have thus characterized the production of cercosporin by the mutant isolates, the response of the mutants to differing levels of cercosporin either exogenously applied or synthesized during growth, and the effects of light on resistance. Second, we assayed the mutants for endogenous levels of carotenoids and low molecular weight reducing agents, both shown in previous studies to protect sensitive organisms against cercosporin toxicity (5,11,31). Finally, we assayed the mutants for their ability to infect tobacco (*Nicotiana tabacum* L.), their host plant.

MATERIALS AND METHODS

Fungal strains and culture conditions. Wild-type *Cercospora nicotianae* (ATCC 18366) and the cercosporin-sensitive (CS) mutants were routinely maintained on malt medium (23) at 28°C in the dark, conditions under which they do not produce cercosporin. Media used in experiments included potato-dextrose agar (PDA, Difco Laboratories, Detroit) and PCG, a peptone/casein/glucose medium (31). PDA was used for experiments requiring induction of cercosporin biosynthesis. All other experiments utilized PCG in order to suppress cercosporin biosynthesis. For all experiments, cultures were incubated in a lighted growth chamber at 25°C under continuous light provided by cool-white fluorescent bulbs. Light intensities ranged from 37 to 52 μE m^{-2} s^{-1}, with an average intensity of approximately 40 μE m^{-2} s^{-1}. Dark conditions were achieved by wrapping plates in foil. Low light treatments were produced by varying the distance of the plates from lights, or by screening plates with cheesecloth or metal screening. Light intensities were measured with a LI-COR quantum sensor (Li-Cor, Inc., Lincoln, NE) which measures photosynthetically active radiation between 400 and 700 nm. Cercosporin is activated by wavelengths between 400 and 620 nm (5).

Chemicals. Cercosporin was extracted and purified from mycelial cultures of *Cercospora kikuchii* as previously described (5) and stored as either crystals or a 2 mM acetone stock in the dark at –20°C. Reduced glutathione (GSH), sodium ascorbate, L-cysteine, 2,6-diclorophenol-indophenol (DCIP), 5,5′dithio-bis(2-nitrobenzoic acid), and *o*-phthalaldehyde were from Sigma Chemical Company (St. Louis).

Cercosporin sensitivity assays. Sensitivity to exogenously applied cercosporin was assayed in divided petri plates, with medium containing cercosporin on one side of the plate and control medium on the other. Cercosporin was added to autoclaved medium as an acetone stock and was used at a final concentration of 10 μM, unless otherwise indicated. Both cercosporin-containing and control medium were amended to a final acetone concentration of 0.5%. Mycelial plugs (6-mm diameter) cut from the margins of 1- to 2-week-old stock cultures were plated on the medium mycelium side down. Growth was measured as the increase in colony diameter (final colony diameter in millimeters minus 6 mm) and was measured at 4 days, unless otherwise indicated. In all assays, growth on cercosporin-containing medium was reported as a percentage of growth on control medium of colonies growing on the same plate.

For reducing agent protection assays, cysteine, ascorbate, and GSH were added to PCG medium after autoclaving at the concentrations indicated. Cercosporin sensitivity was assayed as described above.

Sensitivity to synthesized cercosporin was assayed by plating cultures on PDA and incubating them in the light, conditions which induce cercosporin synthesis. Cercosporin is red, and is easily visible on plates when synthesized and excreted into the medium. Increase in colony diameter was measured daily, both prior to and after the initiation of cercosporin accumulation in the medium.

Assays of cercosporin production. Cercosporin production by the CS mutants was assayed as described (23). Agar plugs (6-mm diameter) cut from the margins of the colonies were extracted in 5 N KOH for 4 h, and the absorbance of the solution measured at 480 nm. Cercosporin concentration was calculated using a molar extinction coefficient of 23,300 (38). Cultures were assayed when cercosporin accumulated in cultures of all strains, at 5 days under high light conditions and at 7 to 9 days under low light conditions.

Analysis of endogenous reducing agents. Endogenous reducing agents were assayed in mycelial extracts of 5- to 7-day-old shake cultures grown in liquid malt medium in the dark at 25°C. Extracts were prepared by grinding filtered mycelium in liquid nitrogen and extracting the mycelial powders with 0.1 M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA. Total thiol content was assayed using 5,5′dithio-bis(2-nitrobenzoic acid) by the methods of Grill et al. (18), and was assayed both from mycelial extracts (soluble thiols) and from mycelial acetone powders (protein thiols). Cysteine was measured by the acid ninhydrin technique (19). Mycelial GSH levels were assayed by reaction with *o*-phthalaldehyde using the method of Barak and Edgington (1). Ascorbate content was determined by a modification of the method of Guri (20). Mycelial extract (3.5 ml) was added to 0.5 ml DCIP solution (0.86 mM DCIP + 2.5 mM $NaHCO_3$, filtered and stored at 4°C in the dark). The absorbance was measured at 600 nm in a Beckman DU 650 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

β-Carotene production. Cultures of wild-type and CS mutants grown in shake culture in liquid malt medium for 3 days in the light were used for analysis of production of β-carotene, the sole carotenoid produced by *C. nicotianae* (11). The mycelia were

TABLE 1. Growth of cercosporin-sensitive mutants in the presence of exogenous cercosporin[a]

Isolate	Mean increase in colony diameter (mm) at 4 days in absence of cercosporin	Growth (% of control) (Cercosporin concentration [μM])			
		0.1	0.5	1.0	10
Wild-type	4.1 ± 0.3[b]	113[c]	124	116	89
CS2	2.3 ± 0.3	69*[d]	40*	0*	0*
CS6	2.1 ± 0.2	73	42*	5*	0*
CS7	2.2 ± 0.1	75*	45*	0*	0*
CS8	2.0 ± 0.2	67*	56*	11*	0*
CS9	2.0 ± 0.0	78*	61*	0*	0*
CS10	3.1 ± 0.4	93	88	79	36*

[a] Cultures grown on PCG medium amended with 0.5% acetone (control) or with cercosporin at concentrations indicated under continuous exposure to fluorescent light (40 μE m^{-2} s^{-1}).

[b] Means and their standard errors from three trials, each with three replicates.

[c] Numbers are means of growth on cercosporin-amended medium as a percentage of growth on control medium.

[d] * Indicates growth on cercosporin-containing medium significantly different from growth on control medium by Dunnett's *t* test ($P < 0.05$).

filtered and divided into two equal samples on the basis of fresh weight. One half was used for β-carotene extraction and the other half dried overnight for dry weight measurement. β-Carotene was extracted as previously described (11). The purity of the extract was assayed by determining the absorption spectrum between 350 and 550 nm. β-Carotene concentration was determined based on absorbance at 449 nm using the extinction coefficient for a 1% (wt/vol) solution in hexane ($E^{1\%} = 2,592$) (12).

Tobacco inoculation. Cultures of wild-type and the mutants CS2, CS8, and CS10 were induced to sporulate by culturing them on solid V8 juice medium containing 1 g of powdered, dried soybean leaves per liter (23). Cultures were incubated at 20°C for 7 days under continuous light. Conidia were harvested by adding 4 ml of sterile water to each plate and dislodging the conidia with an artist's brush. The conidial suspensions were adjusted to approximately 5×10^4 conidia per milliliter and then atomized onto leaves of 7- to 8-week-old tobacco plants, cultivar 'Burley 21'. Inoculated plants were incubated at 100% relative humidity for 4 days and then transferred to a greenhouse bench. Light intensity readings taken during the day (9 a.m. to 4 p.m.) varied greatly with the time of day and weather conditions. Readings ranged from 15 to 120 μE m^{-2} sec^{-1} for the high humidity incubation during the first 4 days and from 60 to 960 μE m^{-2} sec^{-1} during the rest of the incubation period. The number of lesions per leaf and size of lesions were determined at 7 and 14 days after inoculation. Each experiment involved four plants per treatment, and the experiment was performed four times.

Statistical analysis. Data were analyzed using the GLM procedure of SAS/STAT (30).

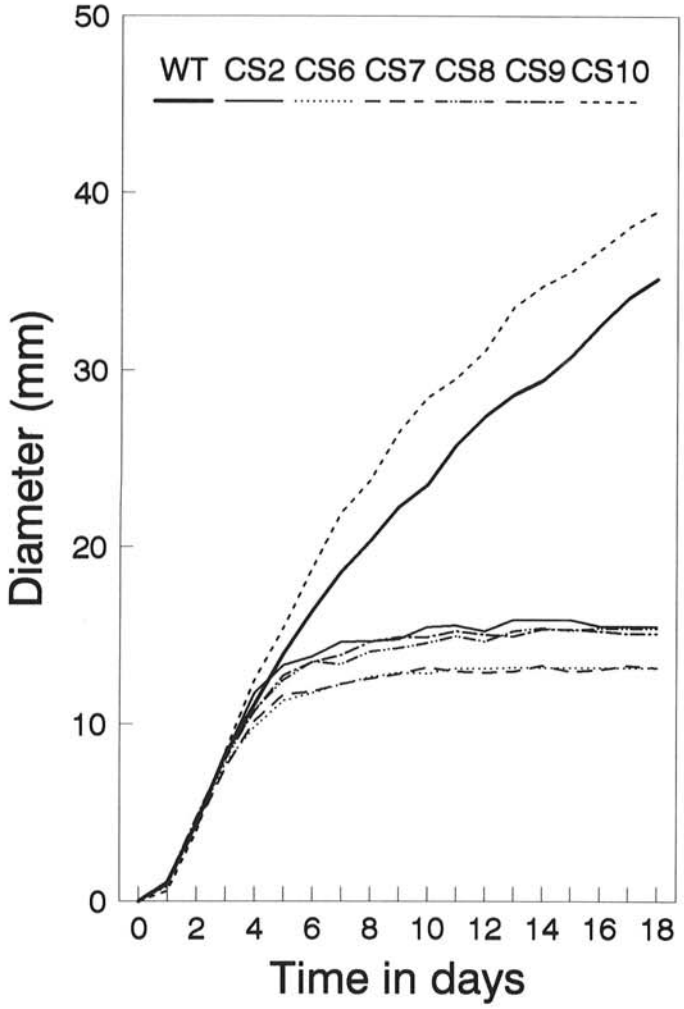

Fig. 1. Radial growth of colonies of wild-type and of the cercosporin-sensitive (CS) mutants grown on PDA under high light intensities (40 μE m^{-2} s^{-1}). Cercosporin production was visible at day 5. Values for colony diameter are reduced by 6 mm to account for the contribution of the original inoculum plug.

RESULTS

Growth of CS mutants on cercosporin-containing medium. The CS mutants were identified by screening for sensitivity to 10 μM cercosporin. As previously reported, when plugs cut from the edges of mycelial cultures were transferred to PCG medium containing 10 μM cercosporin, growth of five of the mutants was completely inhibited, whereas growth of mutant CS10 was partially inhibited (24, Table 1). At lower levels of cercosporin, CS10 was not significantly inhibited, but the other five mutants showed differential sensitivity (Table 1). CS2, CS7, and CS9 were still completely inhibited on 1 μM cercosporin, whereas CS6 and CS8 showed some limited growth. At 0.5 and 0.1 μM cercosporin all five mutants grew, but, with one exception, all were significantly inhibited even at 0.1 μM cercosporin. This level of sensitivity is comparable to that seen with plant cells (5).

As shown in Table 1, all of the CS mutants except CS10 grew significantly more slowly than wild-type when cultured on PCG medium without cercosporin. This slow growth was specific to PCG medium; on PDA the mutants grew at a rate comparable to wild-type until cercosporin was produced (Fig. 1).

Production of cercosporin by CS mutants and toxicity to mutants. The six CS mutants were induced to synthesize cercosporin by growing them on PDA medium in the light (Table 2). Cercosporin concentration was assayed when cercosporin accumulation was visible in all cultures, which occurred at 5 days under high light intensity (40 μE m^{-2} s^{-1}) and at 7 to 9 days under low light intensity (10 μE m^{-2} s^{-1}). Wild-type and CS mutants made less cercosporin under high light than under low light. Under both sets of light conditions the CS mutants made less cercosporin than did wild-type. However, even the lowest levels of cercosporin produced were higher than the 10 μM used in the toxicity assays.

Cultures of CS2, CS6, CS7, CS8, and CS9 growing on PDA under the high-light conditions used in the sensitivity assays (40 μE m^{-2} s^{-1}) grew at the same rate as wild-type for the first 4 to 5 days until cercosporin was produced, and then their growth leveled off (Fig. 1). There continued to be a slight increase in growth of these colonies up until 8 to 10 days. The growth rate of wild-type did not change when cercosporin was produced. Surprisingly, the growth rate of CS10 also was unaltered when cercosporin was produced.

TABLE 2. Cercosporin production by *Cercospora nicotianae* wild-type and cercosporin-sensitive mutants under low and high light[a]

	Mean cercosporin production (Light intensity [μE m^{-2} s^{-1}])			
	10		40	
Isolate	nmol/plug	μM[b]	nmol/plug	μM
Wild-type	107 ± 5[c]	757	37 ± 7	262
CS2	70 ± 6	495	2 ± 1	14
CS6	68 ± 20	481	14 ± 8	100
CS7	81 ± 3	573	13 ± 4	92
CS8	61 ± 18	431	7 ± 2	50
CS9	83 ± 4	587	7 ± 1	50
CS10	11 ± 2	78	6 ± 2	42

[a] Cultures grown on PDA. Cercosporin concentration determined by extracting cercosporin from agar plugs cut from colony margins in 5 N KOH. Cultures extracted at 5 days under high light and 7 to 9 days under low light. Absorbance of the cercosporin solution was measured at 480 nm.
[b] Molarity estimated based on a plug volume of 0.14 ml.
[c] Means and their standard errors from two trials.

Effect of light intensity on cercosporin toxicity to CS mutants. Growth of CS6, CS9, CS10, and wild-type on medium containing 10 μM cercosporin was measured in cultures incubated in the dark and at five different light intensities (3, 10, 20, 30 and 40 μE m^{-2} s^{-1}) (Fig. 2). In the dark (0 μE m^{-2} s^{-1}), cercosporin did not significantly inhibit growth of any of the strains (Dunnett's t test, $P < 0.05$). Continual exposure to light intensities as low as 3 μE m^{-2} s^{-1} did not significantly affect growth of wild-type, but was sufficient to inhibit growth of all three mutants, even CS10. For CS6 and CS9, cercosporin sensitivity was strongly influenced by light intensity. For these mutants there appeared to be a threshold of light between 10 and 20 μE m^{-2} s^{-1} at which cercosporin was totally inhibitory; below that threshold, growth increased dramatically with decreasing light intensity. By contrast, cercosporin toxicity to CS10 changed only slightly with increasing light intensity.

The pronounced cercosporin sensitivity shown by CS6 and CS9 with increasing light intensity was not due to general light sensitivity. Growth of CS6, CS9, CS10, and wild-type in the absence of cercosporin was measured in cultures incubated in the dark and at the highest light intensity used in these studies (40 μE m^{-2} s^{-1}). The wild-type strain grew better in the dark than in the light; growth in the light was 65% of growth in the dark. For CS6, CS9, and CS10, growth in the light was 76, 81, and 89%, respectively, of that in the dark.

Protection of CS mutants by reducing agents. We previously reported that reducing agents such as cysteine, ascorbate, and GSH can protect cercosporin-sensitive fungi against cercosporin toxicity (31). To determine if the CS mutants were similarly protected, cercosporin sensitivity assays were conducted on medium amended with 1 to 40 mM ascorbate, 1 to 10 mM cysteine, or 1 to 20 mM GSH; concentrations above these levels were toxic to *C. nicotianae*. Results are shown in Table 3. The response of wild-type to cercosporin was not affected by any of the reducing agents at the concentrations used. Mutants CS2, CS6, CS7, CS8, and CS9 were completely protected against cercosporin at ascorbate levels of 30 or 40 mM. By contrast, CS10 was not protected by ascorbate. However, high to complete levels of protection were obtained for all CS mutants, including CS10, with either 10 mM cysteine or GSH.

Endogenous levels of reducing agents. The dramatic protection of the CS mutants by reducing agents suggested that these mutants may be deficient in endogenous levels of these agents. However, extraction and quantification of ascorbate, cysteine, GSH, and total soluble or protein thiols from wild-type and mutant mycelium indicated that the CS mutants were not significantly different from wild-type in levels of any of these agents (Dunnett's t test, $P < 0.05$). Ascorbate levels in the CS mutants ranged from 1.5 to 2.8 μmol/g of dry weight as compared to wild-type which had 2.3 μmol/g of dry weight. Cysteine levels in the CS mutants ranged from 2.6 to 3.9 μmol/g of dry weight; wild-type had 2.1 μmol/g of dry weight. Reduced glutathione content ranged from 1.5 to 2.6 μmol/g of dry weight with wild-type at 2.0 μmol/g of dry weight. Total soluble and protein thiol content of wild-type was 1.8 and 3.1 μmol/g of dry weight, respectively, and for the mutants ranged from 1.5 to 2.5 and from 2.1 to 4.7 μmol/g of dry weight for soluble and protein thiols, respectively.

Carotenoid production. Carotenoids are potent quenchers of $^1O_2$ and have been shown to protect plant cells and cercosporin-sensitive fungi against cercosporin toxicity (5,11). None of the mutants, however, differed from wild-type in β-carotene accumulation. In three independent experiments, mean β-carotene content for wild-type was 15.9 ng/mg of dry weight of mycelium. Mean β-carotene content of the six mutants ranged from 11.5 to 21.1 ng/mg of dry weight, and none were significantly different from that of wild-type (Dunnett's t test, $P < 0.05$).

Pathogenicity. CS2, CS8, and CS10 were compared with wild-type for their ability to produce lesions on tobacco (Table 4). The wild-type isolate produced significantly more lesions per leaf than did CS2 and CS8. Lesion production by CS10 was intermediate and not significantly different from the other strains. Mean lesion size was greatest in leaves infected with wild-type, followed by CS10, CS8, and CS2, although there was no significant difference in the mean size of lesions produced by the four isolates. The lack of significance in lesion size differences was probably due in part to the wide range in lesion size caused by *C. nicotianae* infection. Lesion size caused by the wild-type strain ranged from lesions that were just visible (< 0.5 mm) up to 8 mm. Also, the three CS mutants all produced some expanded lesions.

DISCUSSION

The results of this study extend our preliminary characterization of two classes of mutants isolated for sensitivity to cercosporin (24). CS2, CS6, CS7, CS8, and CS9 were originally identified as mutants unable to grow on cercosporin-containing medium in the light and unable to reduce cercosporin. They also were shown to be totally inhibited by five other photosensitizers (methylene blue, toluidine blue, hematoporphyrin, rose bengal, and eosin Y) which are structurally different, but have the common property of producing $^1O_2$ when irradiated by light. Here we demonstrated that the mutants were capable of growing in the

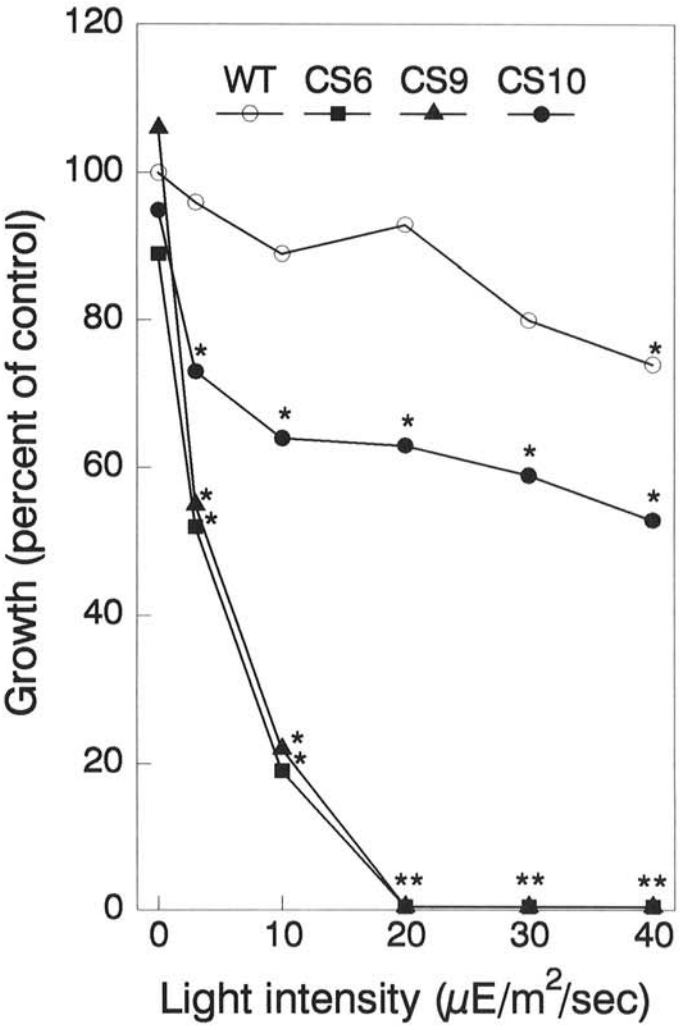

Fig. 2. Growth of wild-type, CS6, CS9, and CS10 on PCG medium containing 10 μM cercosporin as a percentage of growth on noncercosporin-containing medium, measured in the dark (0 μE m^{-2} s^{-1}) and at five different light intensities (3, 10, 20, 30, and 40 μE m^{-2} s^{-1}). * Indicates that growth on cercosporin-containing medium was significantly different from growth on control medium (Dunnett's t test, $P < 0.05$).

TABLE 3. Effect of ascorbate, cysteine, and reduced glutathione on sensitivity of *Cercospora nicotianae* wild-type and cercosporin-sensitive mutants to cercosporin

Reducing agent	Concentration of reducing agent (mM)	Growth (% of control)[a]						
		WT	CS2	CS6	CS7	CS8	CS9	CS10
None	0	87[b]	0*[c]	0*	0*	0*	0*	48*
Ascorbate	1	87	6*	3*	3*	5*	3*	47*
	5	77	17*	8*	9*	8*	7*	49*
	10	80	39*	34*	35*	28*	26*	50*
	20	95	67*	60*	50*	53*	53*	56*
	30	93	89	82*	90	88*	77*	58*
	40	105	97	97	100	94	95	68*
Cysteine	1	90	0*	0*	0*	0*	0*	50*
	5	89	17*	13*	14*	16*	0*	58*
	10	105	80	76	76*	71*	75	95
Glutathione	1	91	3*	0*	6*	0*	4*	44*
	5	105	53*	56*	60*	56*	67*	68*
	10	118	110	113	105	113	104	107
	20	96	91	100	111	91	88	92

[a] Mycelial plugs plated on medium containing reducing agents as indicated and either 10 μM cercosporin or 0.5% acetone (control). Cultures were incubated in the light and colony diameter measured at 4 days.
[b] Means of three replicates in each of three trials.
[c] * Indicates growth on cercosporin-containing medium is significantly different from growth on control medium (Dunnett's t test, $P < 0.05$).

TABLE 4. Infection of tobacco by wild-type and cercosporin-sensitive mutants of *Cercospora nicotianae*

Isolate	Mean lesion number	Mean lesion diameter (mm)
Wild-type	144 A[a]	2.6 A
CS10	82 AB	2.5 A
CS8	48 B	1.9 A
CS2	12 B	1.7 A

[a] Numbers followed by the same letter are not significantly different (LSD test, $P < 0.05$).

presence of cercosporin, but that their sensitivity was dependent both on the concentration of cercosporin and on light intensity. The mutants were shown to be sensitive to cercosporin concentrations as low as 0.1 μM, a level of sensitivity equivalent to that of plant cells (5). In addition, their response to cercosporin was dramatically affected by changes in light intensity, although they showed no general light sensitivity in the absence of cercosporin. These mutants were all protected against cercosporin toxicity by ascorbate, a reducing agent shown previously to protect cercosporin-sensitive fungi (*Aspergillus flavus*, *Neurospora crassa*, *Penicillium* species) against cercosporin (31). We also have demonstrated that these mutants were capable of synthesizing cercosporin even though they remained sensitive to it.

Our results demonstrated that CS10 was distinctly different from the other mutants. CS10 was originally identified as a partially cercosporin-sensitive mutant, which was unaltered in cercosporin-reducing ability and in resistance to other $^1O_2$-generating photosensitizers. Here we have shown that, unlike the other mutants, growth of CS10 was not significantly inhibited when the cercosporin concentration was lowered below 10 μM. Also, CS10 showed little change in cercosporin sensitivity over a wide range of light intensities. At the lowest light intensity tested (3 μE m^{-2} s^{-1}) growth of CS10 on cercosporin was significantly different from growth in the dark, but decreases in growth in response to cercosporin at light intensities higher than that paralleled that of wild-type. CS10 also differed from the other mutants in its lack of response to protection by ascorbate, which may be related to the fact that CS10 is not altered in cercosporin-reducing activity (24). Most interestingly, the cercosporin sensitivity of CS10 was not expressed against endogenously synthesized cercosporin even though the cercosporin is excreted into the medium.

The only similarity found between CS10 and the other mutants was that both can be protected by the addition of cysteine and GSH. These agents were tested since they are effective reducing agents known to protect cells against oxidative damage, and they were previously shown to protect cercosporin-sensitive fungi against cercosporin (31). The fact that all mutants, including CS10, were protected by cysteine and GSH may indicate that the protective effect of these compounds was not due to their ability to act as reducing agents but to their ability to quench $^1O_2$ or to react with quinones. GSH and cysteine are both thiols, and thiols are known to be effective quenchers of $^1O_2$ (14,29). Thiols such as glutathione and cysteine also are known to react directly with quinones by enzyme-catalyzed reactions (36). Thus, these agents may be protecting by detoxifying cercosporin through the formation of cercosporin-thiol conjugates.

Our observation that the CS mutants were capable of synthesizing cercosporin was a surprise and appeared to be in conflict with their inability to grow on cercosporin-containing medium, since *Cercospora* species excrete the cercosporin they synthesize into the medium. Although the mutants synthesized less cercosporin than wild-type, we found that the concentrations of cercosporin produced by these mutants are higher than the concentrations used in our toxicity assays. The effective concentration of cercosporin may be equivalent to that used in our toxicity assays, however, since the solubility limit of cercosporin in the medium is approximately 10 μM, the rest being present as crystals in the medium.

Although direct comparisons are difficult because of differences in cercosporin concentration and stage of growth, CS2, CS6, CS7, CS8, and CS9 appear to be equally sensitive whether grown under conditions where they synthesize their own cercosporin or grown on externally supplied cercosporin under conditions that suppress endogenous synthesis. When induced to synthesize cercosporin, growth of the mutants leveled off sharply, but did not totally stop. Cercosporin accumulated in the culture approximately 2 mm behind the hyphal tips, which may have allowed for a small amount of continued growth. Also, actively growing cultures were more resistant to cercosporin than new colonies initiated from freshly cut plugs. We conducted experiments in which we plated mutants on cercosporin-containing medium, incubated them in the dark for various periods of time, and then transferred the plates to light. Growing colonies of CS2, CS6, CS7, CS8, and CS9 were all more resistant to cercosporin, growing 23 to 28% of control rather than the 0% seen with colonies from freshly cut plugs (data not shown).

CS10 was distinctly different. When grown under conditions

which induce cercosporin biosynthesis (PDA in the light), CS10 continued to grow at the same rate after induction of synthesis as before. The amount of cercosporin produced by CS10 under these conditions (approximately 42 μM) was sufficient to inhibit CS10 when added exogenously. The apparent resistance was not a medium effect; use of PDA instead of PCG in the toxicity assays did not change the sensitivity of CS10 to cercosporin added to the medium. The resistance also did not appear to be due to the stage of culture growth, since there was no major increase in CS10 resistance in the "dark to light" transfer experiments described above. Wild-type cultures express resistance whether or not they are grown under conditions that induce cercosporin synthesis, but it is possible that resistance genes also are up-regulated when toxin synthesis is initiated. If so, the CS10 phenotype could be explained by a mutation that partially inhibits accumulation of the resistance gene product resulting in levels not adequate for resistance. Up-regulation of the gene during synthesis, however, may allow for expression sufficient for resistance. Definitive testing of this hypothesis awaits isolation and characterization of the CS10 gene.

Initial evidence for the involvement of cercosporin in disease comes from early studies of several *Cercospora*-host interactions which document the importance of light in the development of normal symptom expression (3,4,16,34). On banana, this effect was so striking that it led in the 1940's to recommendations that bananas be grown under shade to reduce severity of Sigatoka disease (34). The inoculation studies reported here appear to confirm previous reports that cercosporin is involved in the development of disease by *Cercospora* species. CS2 and CS8 produced significantly fewer lesions on tobacco than did wild-type, and CS10 was intermediate. Mean lesion sizes produced by CS2 and CS8 also were less than wild-type, although the differences were not significant, due in part to the wide range in lesion size which normally occurs with this disease. Also, all three mutants produced some expanded lesions. We had originally expected to see greater differences in lesion size than in lesion number as studies with Sigatoka disease have demonstrated that light is not required for spore germination or penetration, but is required for development of lesions once the fungus has penetrated the leaf (3). We believe that the apparent lack of lesions may actually reflect the lack of expansion of infection sites into lesions that were visible. We did not attempt to detect lesions microscopically; such a study would have answered this question.

Disease development by CS2 and CS8 on tobacco in this study was greater than that shown on soybean by mutants of *Cercospora kikuchii* deficient in cercosporin biosynthesis (35). Differences may be due to the different hosts involved. However, the most likely explanation is that the ability of the CS mutants to synthesize cercosporin and grow in its presence under low light conditions may have allowed for the development of lesions. Light intensities in the greenhouse peak at levels 10- to 20-fold higher than those used in the toxicity assays, but the amount of light which penetrates into the leaf would be far less.

In spite of the protective effect of ascorbate, cysteine, and GSH, the mutants were unaltered in endogenous levels of these agents and in endogenous levels of total soluble or protein thiols. The mutants also were unaltered in levels of β-carotene. Carotenoids are the most potent quenchers of $^1O_2$ which exist in biological systems (2,25) and have been shown to protect plant cells and sensitive fungi against cercosporin toxicity (5,11). Recent studies (17) utilizing targeted gene disruption to produce carotenoid-deficient mutants of *C. nicotianae*, however, report no effect of carotenoids on *Cercospora* resistance to cercosporin, an observation consistent with the results of this study.

In summary, we have isolated and characterized two classes of mutants of *C. nicotianae* sensitive to the toxin cercosporin. One class consisted of five mutants which had levels of sensitivity equivalent to plant cells and whose sensitivity was dramatically affected by changes in light intensity. These mutants were unable to reduce cercosporin, could be protected by the addition of ascorbate, and were equally sensitive to cercosporin synthesized endogenously or added exogenously. The second class consisted of a single mutant which was only partially sensitive to cercosporin and whose cercosporin sensitivity did not change significantly under different intensities of light. This mutant was unaltered in cercosporin-reducing ability, could not be protected by the addition of ascorbate, and appeared to be resistant to cercosporin synthesized endogenously and excreted. We have further shown that all of the mutants were unaltered in endogenous levels of reducing agents and carotenoids commonly shown to protect cells against $^1O_2$. This observation increases the probability that resistance to cercosporin is due to specific gene products and not to the accumulation of low molecular weight compounds. Our current efforts are therefore directed at identifying genes involved in cercosporin resistance by complementation of the CS8 and CS10 mutants with a genomic library from the wild-type strain.

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