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Targeted Gene Disruption of Carotenoid Biosynthesis in *Cercospora nicotianae* Reveals No Role for Carotenoids in Photosensitizer Resistance

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Many phytopathogenic *Cercospora* species synthesize the photoactivated phytotoxin cercosporin which is correlated with the ability of these fungi to cause plant disease. Cercosporin is a photosensitizer which upon illumination transfers energy to molecular oxygen, generating the universally toxic, activated oxygen species, singlet oxygen. Like other photosensitizers, cercosporin exhibits extensive toxicity. *Cercospora* spp., however, not only synthesize but are resistant to high concentrations of cercosporin. Because carotenoids are known to be potent quenchers of singlet oxygen, we tested the possible role of carotenoids in resistance of *Cercospora nicotianae* to autotoxicity. Targeted gene disruption was used to create carotenoid-minus derivatives of a wild-type and two cercosporin-sensitive *C. nicotianae* strains. These carotenoid-minus disruption mutants were no more sensitive to either cercosporin or five other photosensitizers than the parent strains from which they were derived. Pathogenicity tests of one of the carotenoid-minus disruption mutants indicated that it was also unaltered in its ability to infect plants. From these data, we conclude that carotenoids are not involved in cercosporin resistance nor are they required for plant infection. These results suggest that *Cercospora* have a distinct and highly effective mechanism for photosensitizer resistance which has the potential for widespread applicability in other organisms.

Cercospora species synthesize a photoactivated, nonhost specific polyketide toxin, cercosporin (Daub 1982a). When activated by light, cercosporin transfers energy to molecular oxygen, generating the energetically activated singlet form of oxygen (Dobrowolski and Foote 1983; Daub and Hangarter 1983). In turn, singlet oxygen causes membrane lipid peroxidation, leading to changes in structure and permeability, leakage of nutrients and cell death (Daub 1982b; Macri and Vianello 1979). Studies demonstrating reduced pathogenicity of cercosporin-deficient mutants (Upchurch *et al.* 1991b) and the importance of light in several *Cercospora*/host interactions (for review see Daub 1987b) provide compelling evidence that cercosporin plays an important role in disease development. The production of similar toxins by plant-pathogenic fungi in the genera *Alternaria*, *Cladosporium*, *Hypocrella*, and *Stemphylium* (Davis and Stack 1991; Robeson and Jalal 1992; Robeson *et al.* 1984; Stack *et al.* 1986; Weiss *et al.* 1987; Zhenjum and Lown 1990) suggest that photoactivated toxins may be important pathogenicity factors in several plant-fungal interactions.

Singlet oxygen has almost universal toxicity to cells, reacting destructively with critical components such as lipids, proteins, and DNA (Spikes 1989). It is not surprising, therefore, that cercosporin is highly toxic (at micromolar concentrations) to nearly all organisms tested, including plants, bacteria, most fungi, mice, and cultured human tumor cells (Daub and Ehrenshaft 1993). Despite its general and high toxicity, *Cercospora* species can accumulate large concentrations of cercosporin in culture, sometimes up to 1 mM, without adverse effects (Rollins *et al.* 1993). We have been investigating the biochemical basis for cercosporin resistance in *Cercospora* species. Because carotenoids are the primary defense against singlet oxygen in biological systems (Krinsky 1979, 1989, 1993) we examined their possible role in resistance of *Cercospora* species to autotoxicity.

Carotenoids are produced by both phototrophic and autotrophic prokaryotes, filamentous fungi, and plants (Goodwin 1980; Rau 1976). In plants, blocking carotenoid synthesis is lethal (Krinsky 1979; Young 1991a, 1991b). Without carotenoids, chlorophyll and the membranes containing the photosynthetic apparatus are irreversibly damaged by interaction with singlet oxygen which is an unavoidable byproduct of photosynthesis. Studies with carotenoid-minus derivatives of normally carotenoid-containing fungi and nonphotosynthetic bacteria indicate these pigments also serve as photoprotectants in nonphotosynthetic organisms (Ruddat and Garber 1993; Will *et al.* 1988). Carotenoids also function as light-harvesting pigments and photoprotectants in animals (Krinsky 1993). Carotenoids are the most potent quenchers of singlet oxygen known in biological systems (Krinsky 1979, 1993), and are the major mechanism of resistance so far identified against a diversity of light-activated, singlet oxygen producing toxins (Krinsky 1989).

We recently isolated the *C. nicotianae* gene encoding phytoene dehydrogenase, an enzyme in the carotenoid biosyn-

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thetic pathway (Ehrenshaft and Daub 1994). Here we report the creation of carotenoid-minus mutants of *C. nicotianae* using targeted gene disruption of the phytoene dehydrogenase gene. These disruption mutants were examined for alterations in sensitivity to cercosporin and other photosensitizers and for changes in their ability to infect *Nicotiana tabacum*, their host plant.

RESULTS

Targeted disruption of the phytoene dehydrogenase gene.
The entire *C. nicotianae* phytoene dehydrogenase protein coding region plus flanking DNA is contained on a 3.1-kb *Nco*I fragment cloned in pGEM5Zf(+). To construct a disrupted version, this clone was digested at the unique *Sma*I site and ligated to a *Pvu*II fragment from pUCH1 (Turgeon et al. 1987) containing promoter 1 from *Cochliobolus heterostrophus* and the hygromycin B phosphotransferase gene from *E. coli*. *C. nicotianae* wild-type strain ATCC 18366 was transformed with the disruption construct, and hygromycin-resistant transformants were screened for production of β-carotene, the sole carotenoid produced by *C. nicotianae* (Daub and Payne 1989). Of 50 transformants examined, five independently isolated disruption mutants, designated DH1, DH9, DH25, DH37, and DH48, produced no detectable β-carotene (Fig. 1). In contrast, the wild-type and transformant VH1, a hygromycin-resistant, carotenoid-producing strain transformed with pUCH1 alone (Table 1) accumulated approximately 24 ng of β-carotene per milligram fungal dry weight.

The disruption construct was also used to transform two *C. nicotianae* mutant strains sensitive to cercosporin. These cercosporin-sensitive (CS) mutants were previously identified by screening UV mutagenized protoplasts for growth inhibition on cercosporin-containing medium (Jenns et al. 1995) (Table 1). The growth of one mutant, CS10, is partially inhibited by 10 μM cercosporin, while mutant CS8 does not grow at all at this concentration. Twenty hygromycin-resistant CS10 transformants were screened and two carotenoid-minus disruption mutants (CS10.15 and CS10.20) were identified. Out of 66 transformants screened, one CS8 carotenoid-minus disruption mutant (CS8B.19) was identified.

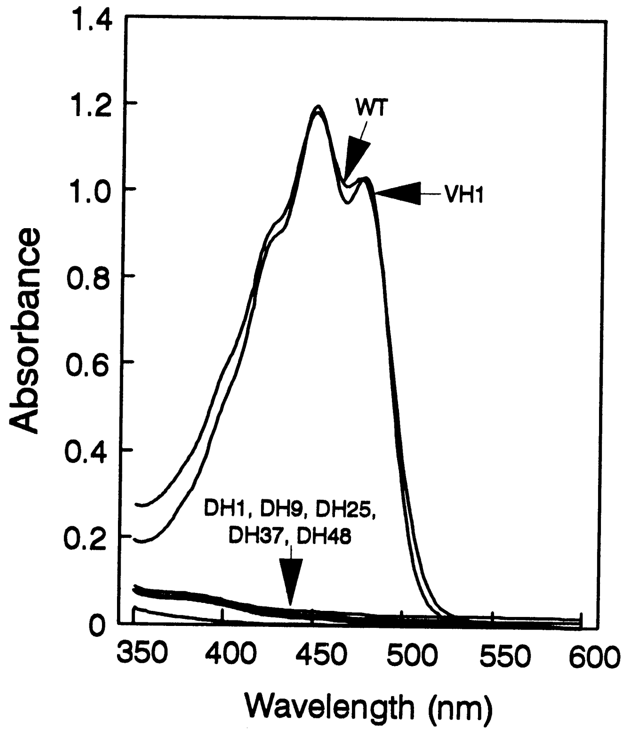

Fig. 1. Spectrophotometric analysis of β-carotene. Extracts were made from 200-mg samples of lyophilized tissue from strains of *Cercospora nicotianae* grown in continuous light. WT, wild-type; VH1, transformed with pUCH1; DH1, DH9, DH25, DH37, and DH48 are carotenoid-minus disruption mutants derived from the wild-type strain.

Southern hybridization analysis.
To confirm that the endogenous phytoene dehydrogenase gene was replaced by our disrupted version, Southern hybridization analysis was performed. *Hin*dIII-digested total genomic DNA was probed with a fragment of the phytoene dehydrogenase gene spanning the *Sma*I site into which the hygromycin-resistance cassette was inserted (Fig. 2). Both the wild-type strain and the vector-transformed control (VH1) had a 4.8-kb *Hin*dIII fragment which hybridized to the probe (Fig. 2A and B). The disrupted, carotenoid-minus mutants, however, all had 1.4- and 6.6-kb *Hin*dIII fragments hybridizing to the probe (Fig. 2A and C), indicating that the disrupted phytoene dehydrogenase gene had replaced the endogenous one. The same analysis was also performed with CS8, CS10, and their carotenoid-minus disruption mutants. As above, each carotenoid-producing strain had a single 4.8-kb *Hin*dIII fragment which hybridized to the probe, while the disruption mutants all had 1.4- and 6.6-kb *Hin*dIII hybridizing fragments (data not shown).

Table 1. *Cercospora nicotianae* strains used in this study

Strain	Characteristics	β-Carotene production	Cercosporin[a] resistance	Reference
WT	Wild-type	yes	105	ATCC18366
VH1	Vector transformed	yes	100	This work
DH1	pdh disruption	no	100	This work
DH9	pdh disruption	no	108	This work
DH25	pdh disruption	no	98	This work
DH37	pdh disruption	no	105	This work
DH48	pdh disruption	no	113	This work
CS8	Cercosporin sensitive	yes	0	Jenns et al. 1995
CS8B.19	pdh disruption of CS8	no	0	This work
CS10	Cercosporin sensitive	yes	66	Jenns et al. 1995
CS10.15	pdh disruption of CS10	no	70	This work
CS10.20	pdh disruption of CS10	no	58	This work

[a] Growth in the presence of 10 μM cercosporin as a percent of growth of control.

Cercosporin sensitivity of carotenoid-minus mutants.

To assess the contribution of carotenoids to resistance to endogenously synthesized cercosporin, cultures were grown in PDB either in continuous light or in continuous darkness. Light induces both cercosporin synthesis and activation. Carotenoids were extracted from light-grown cultures (Fig. 1), while cercosporin concentrations and dry weight were determined for all samples (Fig. 3). All strains accumulated high levels of cercosporin in the light, but no detectable cercosporin when incubated in continuous darkness (Fig. 3A). Neither light nor high levels of cercosporin, however, reduced growth of any of the strains tested (Fig. 3B); approximately equal amounts of fungal tissue accumulated in light- and dark-grown cultures of control and β–carotene-minus disruption mutants.

A similar experiment was also performed with CS10 and the two CS10-derived carotenoid-minus disruption mutants. CS10, however, is also altered in cercosporin production (Jenns and Daub 1995) and neither it nor its derivative strains accumulated cercosporin during the course of the experiment. We did determine, however, that none of the CS10 strains were light sensitive as all grew equally well in the light as in the dark (data not shown). This experiment was not performed with CS8 and its carotenoid-minus disruption mutant because CS8 does not survive induction of cercosporin synthesis under the light conditions used (Jenns et al. 1995).

The carotenoid-minus disruption mutants were also tested for resistance to exogenously supplied cercosporin. Mycelial plugs were grown under continuous light on PCG, a medium on which they produce no detectable cercosporin, amended with 10 or 50 μM cercosporin. After 4 days, change in colony diameter was determined and normalized to that of the non-cercosporin-containing controls (Fig. 4). Medium composition can affect cercosporin sensitivity of *Cercospora* species (Sollod et al. 1992) and while growth of neither the wild-type strain nor the vector-transformed control (VH1) was inhibited by PCG containing 10 μM cercosporin, slight growth inhibition was seen with 50 μM cercosporin. CS10, as previously reported (Jenns et al. 1995), exhibited a 40 to 50% reduction

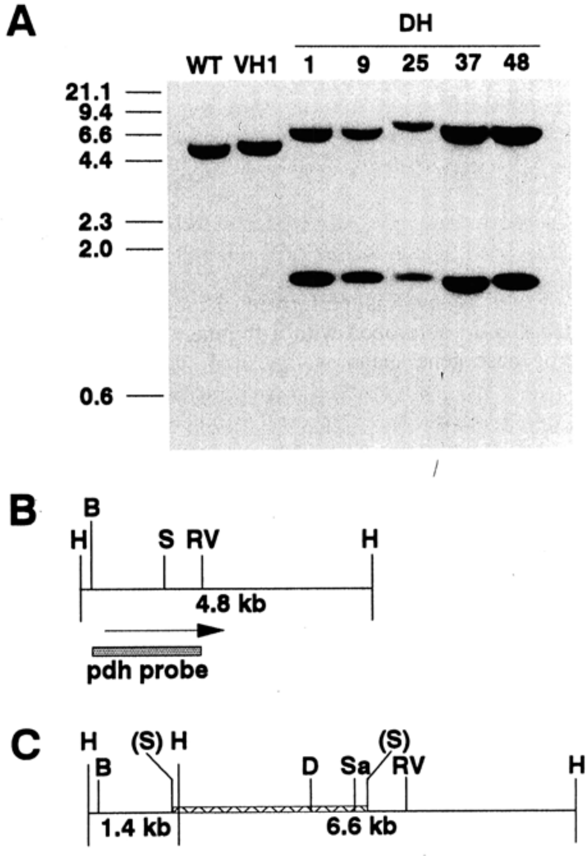

Fig. 2. Southern hybridization analysis of wild-type and transformed strains of *Cercospora nicotianae*. **A,** *Hin*dIII-digested genomic DNA was electrophoresed, transferred to Magnagraph membrane, and probed with the phytoene dehydrogenase *Bam*HI–*Eco*RV restriction fragment shown in **B.** WT, wild-type; VH1, transformed with pUCH1; DH1, DH9, DH25, DH37, and DH48 are carotenoid-minus disruption mutants derived the wild-type strain. The numbers to the left are in kilobase pairs. **B,** Restriction map of the endogenous phytoene dehydrogenase gene, with the protein coding region (arrow) and probe used in **A** (stippled box) shown. **C,** Restriction map of the phytoene dehydrogenase gene interrupted with the hygromycin-resistance cassette (cross-hatched box). B, *Bam*HI; D, *Dde*I; H, *Hin*dIII; RV, *Eco*RV; S, *Sma*I; Sa, *Sal*Ii, (S), *Sma*I site destroyed by ligation to *Pvu*II fragment.

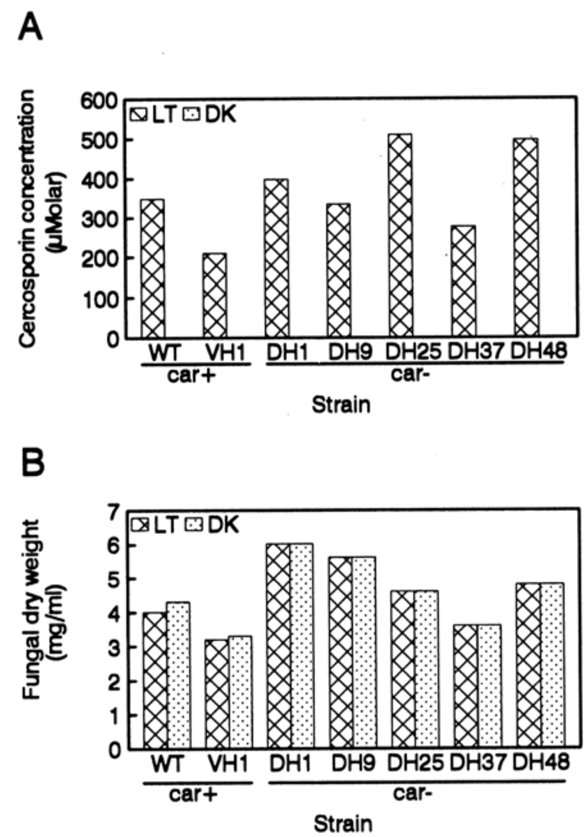

Fig. 3. Cercosporin accumulation and growth of *Cercospora nicotianae* strains under cercosporin-producing (light) and non-producing (dark) conditions. Fungal cultures grown in PDB for 4 days in continuous light or continuous darkness were measured for (A) cercosporin accumulation and (B) dry weight accumulation. WT, wild-type; VH1, transformed with pUCH1; DH1, DH9, DH25, DH37, and DH48 are carotenoid-minus derivatives of the wild-type strain. No cercosporin was detected in any of the dark grown cultures. Data shown are the average of three independent experiments each with three replicates. Analysis of variance indicated no significant difference in the growth of carotenoid-minus disruption mutants as compared to that of the carotenoid-containing controls ($P = 0.05$).

in growth on 10 µM cercosporin (Fig. 4B). None of the carotenoid-minus disruption mutants, however, were any more sensitive to cercosporin than their respective carotenoid-containing parents (Fig. 4A and B).

Because growth of CS8 is completely inhibited at 10 µM cercosporin, lower concentrations were used to determine if the carotenoid-minus disruption derivative of CS8 exhibited increased sensitivity. At 1, 0.1, and 0.01 µM cercosporin, CS8 mycelial plugs grew, respectively, 13, 50, and 83% of control growth. At these same concentrations, mycelial plugs of the CS8 carotenoid-minus disruption mutant grew 8, 63, and 87% of control growth. These values were not significantly different (at $P = 0.05$) from those of CS8.

Sensitivity to other singlet oxygen-generating photosensitizers.

To determine if loss of carotenoids increased the sensitivity of *C. nicotianae* to singlet oxygen-generating photosensitizers other than cercosporin, cultures were grown on PCG medium containing 100 µM rose bengal, 50 µM hematoporphyrin, 100 µM methylene blue, 100 µM toluidine blue, or 100 µM eosin yellow (Table 2). As reported previously (Jenns et al. 1995) both the wild-type strain and CS10 were resistant to most photosensitizers. Hematoporphyrin and rose bengal partially inhibited growth of both the wild-type strain and CS10, while the other photosensitizers did not. Lack of β–carotene did not alter the resistance level; none of the carotenoid-minus mutants were any more sensitive to these photosensitizers than their parental, carotenoid-producing strains. The photosensitizer sensitivity tests were not performed on the CS8 strains because growth of CS8 is known to be completely inhibited by the photosensitizers used (Jenns et al. 1995).

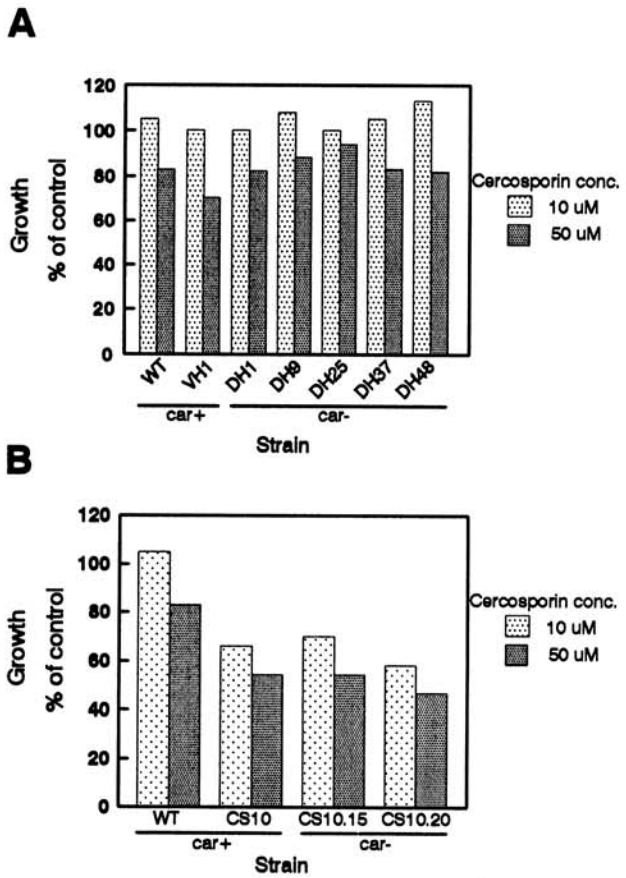

Fig. 4. Effect of exogenously applied cercosporin on growth of *Cercospora nicotianae* strains. Mycelial plugs were grown for 4 days in continuous light on PCG medium with 10 or 50 µM cercosporin or with 0.05% acetone as a control. Colony diameters were measured, and growth is shown as percentage of growth of the solvent control. **A,** WT, wild-type; VH1, transformed with pUCH1; DH1, DH9, DH25, DH37, and DH48 are carotenoid-minus disruption mutants derived the wild-type strain. **B,** WT, wild-type; CS10, partially cercosporin-sensitive strain; CS10.15 and CS10.20 are carotenoid-minus disruption mutants derived from CS10. Data shown is the average of three independent experiments each performed with three replicates. Analysis of variance of the data confirmed that the growth of carotenoid-minus disruption mutants was not significantly different (at P=0.05) than that of their respective carotenoid-containing parents.

Pathogenicity.

Conidia of the wild type and the carotenoid-minus disruption mutant DH25 were used to inoculate *N. tabacum* plants. Time to lesion development and the number and size of lesions produced by these two strains were indistinguishable (data not shown). Additionally, both strains produced lesions that continued to expand and coalesce until the infected leaves collapsed.

DISCUSSION

Cercosporin damages cells primarily through generation of singlet oxygen, which causes peroxidation of membrane lipids resulting in increased permeability and, ultimately, cell death (Daub 1982b; Daub 1987a). Carotenoids play an indispensable role in quenching singlet oxygen in photosynthetic systems and in defense against singlet-oxygen-producing compounds (Bellus 1979; Krinsky 1979; Young 1991). Furthermore, there is evidence that carotenoids can play at least a minor role in protection against cercosporin. Killing of cultured tobacco cells by cercosporin was delayed in the presence of bixin, a carotenoid carboxylic acid (Daub 1982a). Daub and Payne (1989) found that a carotenoid-overproducing strain of *Phycomyces blakesleeanus* was more resistant to cercosporin than the wild-type, although a 10,000-fold increase in carotenoid accumulation resulted in only a two- to fourfold increase in resistance. In addition, a recent study of four rice cultivars demonstrated that resistance to *Cercospora oryzae* infection and to purified cercosporin was correlated with carotenoid content, and that chemical inhibition of carotenoid synthesis resulted in greatly increased cercosporin sensitivity (Batchvarova et al. 1992).

An earlier study used three carotenoid synthesis inhibitors effective in plants and microbes, but failed to reduce carotenoid accumulation in *C. nicotianae* (Daub and Payne 1989). As an alternate approach, we used targeted gene disruption to create carotenoid-minus mutants. Transformation of a wild-type strain, a partially cercosporin-sensitive strain and a completely cercosporin-sensitive strain with our disrupting construct resulted in the recovery of carotenoid-minus disruption mutants in 10, 10, and 1.5%, respectively, of transformants screened. Southern hybridizations confirmed that the interrupted phytoene dehydrogenase gene had replaced the endogenous copy, and carotenoid analysis indicated that β–carotene was not produced in the disruption mutants.

When grown on medium containing cercosporin, the carotenoid-minus derivatives of all three *Cercospora* strains were phenotypically identical to their parents in resistance. The carotenoid-minus disruption mutants of the wild-type strain were also identical to their parent when induced to produce their own toxin (Fig. 3). Additionally, the wild-type and CS10 disruption mutants were indistinguishable from their parent strains in resistance to other photosensitizers. These disruption mutants also grew as well in continuous light as in continuous darkness, indicating they had no general sensitivity to light. Finally, one of the carotenoid-minus disruption mutants of the wild-type was used in pathogenicity tests and was found to be identical to the wild-type in ability to cause disease. Thus we have been unable to define any function for β-carotene in *Cercospora* species, although this pigment is constitutively produced in these fungi (Ehrenshaft and Daub 1994). Carotenoids have frequently been implicated as photoreceptors, and β–carotene does absorb in the wavelengths that induce cercosporin synthesis. Our data, however, rule out the possibility that β–carotene is the photoreceptor for induction of cercosporin synthesis as carotenoid-minus fungi clearly retain the ability to respond to light as a cue for induction of cercosporin synthesis (Fig. 3).

A certain percentage of the chlorophyll excitations that occur in photosynthetic tissue fail to funnel the energy through the electron transport chain and, instead, create triplet state chlorophyll (Sandmann and Boger 1989). In the absence of carotenoids, which can directly quench triplet state chlorophyll or can quench the singlet oxygen generated by it, the photosynthetic apparatus is destroyed (Young 1991). The efficacy of many herbicides is based on their ability to block carotenoid synthesis (Sandmann and Boger 1989; Young 1991). In biological systems there are no known quenchers of singlet oxygen more potent than carotenoids (Krinsky 1989). Despite the parallels between photosynthetic organisms and *Cercospora* fungi, i.e., both synthesize photosensitizers and carotenoids, their mechanisms for circumventing the toxicity of singlet oxygen are clearly different.

The CS8 mutant used in this study is from a group of six *C. nicotianae* mutants isolated for sensitivity to cercosporin. The mutation in five of these cercosporin-sensitive strains, including CS8, renders them completely sensitive to 10 μM cercosporin as well to the five other photosensitizers used in this study (Jenns et al. 1995). This indicates that the mechanism *Cercospora* species have for cercosporin resistance may be generally effective against a broad range of photosensitizers. None of cercosporin-sensitive *Cercospora* mutants are altered in β–carotene production (Jenns and Daub 1995). The mechanism by which *Cercospora* species resist cercosporin and other photosensitizers, therefore, appears to be a more potent defense against these singlet oxygen generators than carotenoids. We are currently transforming a genomic library constructed from DNA from the wild-type cercosporin-resistant strain into the two cercosporin-sensitive mutants (CS8 and CS10) discussed in this work. Functional complementation of the cercosporin-sensitive phenotype should not only allow us to identify genes essential for cercosporin resistance, but should also help elucidate the mechanism involved. Once identified we should be able to determine the feasibility of expressing this broad resistance to photosensitizers in other organisms.

MATERIALS AND METHODS

Strains and media.

C. nicotianae strain ATCC 18366 and cercosporin-sensitive mutants (CS8 and CS10) derived from strain ATCC 18366 (Jenns et al. 1995) were used as controls and transformation recipients. Stock cultures were maintained on malt agar (Jenns et al. 1989). Liquid shake cultures were grown in 50 ml of potato-dextrose broth (PDB) (Difco Laboratories), at 200 rpm, at 25°C, in either continuous darkness or continuous light with an average intensity of 100 $\mu E/m^2/s$.

Fungal transformation.

C. nicotianae was transformed by a modification of the method described for *Cercospora kikuchii* (Upchurch et al. 1991a). Cultures grown for 5 days in shake culture in complete medium (CM) broth (Jenns et al. 1989) were ground for 2 10-s bursts at high speed in a Waring blender, and 50 ml of this macerate was used to inoculate 200 ml of fresh CM broth. After approximately 24 h mycelium was recovered by centrifugation, washed in a solution containing 1 M NaCl, 10 mM $CaCl_2$ and recentrifuged. Approximately 1-g aliquots were digested for 2 h at 30°C in 20 ml of a solution containing 100 mg of Novozym (Novo Nordisk Biolabs), 200 μl of

Table 2. Sensitivity of *Cercospora nicotianae* strains to photosensitizers

	Photosensitizer[a]				
Strain	Rose bengal 100 μM	Hematoporphyrin 50 μM	Methylene blue 100 μM	Toluidine blue 100 μM	Eosin yellow 100 μM
WT	53	71	108	108	100
VH1	59	76	94	108	100
DH1	67	73	121	119	107
DH9	63	62	105	121	95
DH25	59	68	113	100	94
DH37	59	78	108	113	106
DH48	47	78	100	95	95
CS10	57	66	100	100	107
CS10.15	57	55	100	93	100
CS10.20	64	63	96	133	100

[a] In each of three independent experiments three replicate mycelial plugs from each strain were grown on the photosensitizer specified or a control. Numbers are mean growth on the photosensitizer as a percent of growth of controls. Analysis of variance of the data confirmed that the growth of carotenoid-minus disruption mutants was not significantly different (at $P = 0.05$) from that of their carotenoid-containing parental strains.

β-glucuronidase (Sigma Chemical Co., St. Louis, MO), 0.6 M NaCl, 10 mM $CaCl_2$ and 20 mM Na_2HPO_4. After digestion, the protoplast suspension was filtered sequentially through cheesecloth, glass wool, and a 30 micron Nitex filter, and recovered by centrifugation. The protoplasts were washed once in STC (1.2 M sorbitol, 10 mM Tris pH 7.5, 10 mM $CaCl_2$) recentrifuged, and resuspended in a small volume of 80% STC plus 20% polyethylene glycol (MW 3,350) and frozen at –80°C until use. From 10^6 to 10^8 protoplasts were used per transformation. Protoplasts were incubated with 2 μg of DNA at room temperature for 20 min, after which 1 ml of 50% polyethylene glycol (MW 3,350), 10 mM Tris, pH 7.5, 10 mM $CaCl_2$ was added and gently mixed and the protoplasts incubated for an additional 20 min. For regeneration, protoplasts were plated in minimal medium (Jenns et al. 1989) plus 1.2 M sorbitol. After 24 h, plates were overlaid with the same regeneration medium containing 200 μg/ml hygromycin B (Sigma Chem. Co.). After 7 to 10 days, colonies were transferred to malt agar plus 200 μg/ml hygromycin B. For CS8 transformants regenerated in the presence of bixin, a carotenoid carboxylic acid which is more water soluble than β–carotene, bixin was added to a final concentration of 100 μM to both the original regeneration medium and the overlay.

β-Carotene extraction.

To screen for β–carotene production, transformants were inoculated into 50 ml of PDB cultures using five 3-mm plugs taken from the perimeter of colonies grown on malt agar. After 3 days of incubation in continuous darkness, the original agar plugs were discarded, and the remaining tissue harvested by filtration and lyophilized. After determining the dry weight, 1.5 ml of extraction solvent (7:3:3:1 hexane/toluene/acetone/ethanol) was added to each sample. The tissue was crushed in the solvent, vortexed, and extracted overnight in the dark at room temperature. The solvent was recovered by two consecutive centrifugations (15 min and 5 min) at 4°C in a microfuge. The absorption spectrum between 350 and 600 nm was then recorded. Once putative β-carotene-minus transformants were identified, large scale β-carotene extractions were performed as previously described (Daub and Payne 1989) using 200 mg of lyophilized tissue samples from light-grown cultures.

Cercosporin and dry weight determinations.

Cercosporin concentrations in liquid cultures were determined as previously described (Jenns et al. 1989). Briefly, cultures (mycelium plus filtrate) macerated for 30 s at high speed in a Waring blender were mixed with an equal volume of 5 N KOH, incubated for 1 h, and the supernatants recovered after centrifugation. Cercosporin concentrations were quantified at A_{480}, the absorption maximum of cercosporin in base. Mycelia were harvested by filtration, washed, and dry weights determined after lyophilization.

Cercosporin and photosensitizer sensitivity.

Control and carotenoid-minus *C. nicotianae* strains were tested for sensitivity to cercosporin, rose bengal, hematoporphyrin, methylene blue, toluidine blue, and eosin yellow by plating mycelial plugs on either side of two-compartment Petri plates containing PCG medium (Sollod et al. 1992). Medium on one side of the plate was amended with photosensitizers at concentrations indicated while the other side served as a control. Cercosporin and hematoporphyrin were dissolved in acetone; controls for those tests consisted of PCG medium with 0.05% acetone, a concentration equal to that in the photosensitizer-containing medium. Plates were incubated at 25°C in the light and radial growth was measured after 4 days.

Southern blotting analysis.

DNA was extracted as previously described (Woloshuk et al. 1989). Samples were digested with *Hin*dIII, electrophoresed through 0.7% agarose, and transferred to Magnagraph membrane (Maniatis et al. 1982). A phytoene dehydrogenase probe was labeled to high specific activity using a kit from Pharmacia according to the manufacturer's instructions, and used as previously described (Ehrenshaft and Daub 1994).

Infection of *Nicotiana tabacum*.

Cultures of the wild type and one of the carotenoid disruption mutants (DH25) were induced to sporulate by culture on a V8 juice plus soybean medium (Jenns et al. 1989). Cultures were incubated at 20°C for 7 days under continuous light. Conidia were harvested in water and adjusted to approximately 5 × 10^4 per milliliter before being atomized onto leaves of 7- to 8-wk-old plants of *Nicotianae tabacum* cultivar 'Burley 21.' Inoculated plants were incubated initially at 100% RH for 4 days followed by growth under standard greenhouse conditions. Number and size of lesions were recorded at 7 and 14 days.

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