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

M. Ehrenshaft, A. E. Jenns, and M. E. Daub

Department of Plant Pathology, North Carolina State University, Raleigh 27695 U.S.A.
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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.

*Corresponding author: M. Ehrenshaft; E-mail: M_EHRENSHAFT@NCSU.EDU*

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thetis 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
NcoI fragment cloned in pGEM5Zf(+). To construct a dis-
rupted version, this clone was digested at the unique SmaI site
and ligated to a PvuII fragment from pUCH1 (Turgeon et al.
1987) containing promoter I from Cochliobolus heterospor-
thus and the hygromycin B phosphotransferase gene from E.
coli. C. nicotianae wild-type strain ATCC 18366 was trans-
formed 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-car-
tene (Fig. 1). In contrast, the wild-type and transformant VH1,
a hygromycin-resistant, carotenoid-producing strain trans-
formed with pUCH1 alone (Table 1) accumulated approx-
imately 24 ng of β-carotene per milligram fungal dry weight.

The disruption construct was also used to transform two C.
icotianae mutant strains sensitive to cercosporin. These cer-
cosporin-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 trans-
formants 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.

Southern hybridization analysis.

To confirm that the endogenous phytoene dehydrogenase
gene was replaced by our disrupted version, Southern hy-
bridization analysis was performed. HindIII-digested total
genomic DNA was probed with a fragment of the phytoene
dehydrogenase gene spanning the SmaI 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 HindIII fragment which hybridized to the probe
(Fig. 2A and B). The disrupted, carotenoid-minus mutants,
however, all had 1.4- and 6.6-kb HindIII fragments hybridiz-
ing 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 HindIII
fragment which hybridized to the probe, while the disruption
mutants all had 1.4- and 6.6-kb HindIII hybridizing fragments
(data not shown).

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<tr>
<th>Table 1. Cercospora nicotianae strains used in this study</th>
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* Growth in the presence of 10 μM cercosporin as a percent of growth of control.

570 / Molecular Plant-Microbe Interactions
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 CS5 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, HindIII-digested genomic DNA was electrophoresed, transferred to Magnagraph membrane, and probed with the phytoene dehydrogenase BamHII-EcoRV restriction fragment shown in B. WT, wild-type; VH1, transformed with pUC18; 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, BamHII, D, DdelI; H, HindIII; RV, EcoRV; S, Smal; Sa, Sallf; (S), Smal site destroyed by ligation to PvuII 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 pUC18; 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 5, 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 \( \beta \)-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).

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 1982a; 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 \( \beta \)-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, ruled 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 (Krisnys 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 was 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 μE/m2/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 CaCl₂ 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 |
|---|---|---|---|---|---|
| **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 |

* 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 (α = 0.05) from that of their carotenoid-containing parental strains.
β-glucuronidase (Sigma Chemical Co., St. Louis, MO), 0.6 M NaCl, 10 mM CaCl₂ and 20 mM Na₂HPO₄. 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 CaCl₂) centrifuged, and resuspended in a small volume of 80% STC plus 20% polyethylene glycol (MW 3,350) and frozen at −80°C until use. From 10⁶ to 10⁸ 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 CaCl₂ 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 lyopholized. After determining the dry weight, 1.5 ml of extraction solvent (7:3:3:1 hexane/toluene/aceton/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 filtrates) 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 A₆₅₀, 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 HindIII, 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 (Ehrensheat 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 x 10⁴ per milliliter before being atomized onto leaves of 7- to 8-wk-old plants of Nicotiana 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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