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RESEARCH ARTICLE

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Transgenic assessment of *CFP*-mediated cercosporin export and resistance in a cercosporin-sensitive fungus

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Abstract Cercosporin is a toxic polyketide produced by many phytopathogenic members of the fungal genus *Cercospora*. *Cercospora* species, themselves, exhibit the highest level of self-resistance to this almost universally toxic photosensitizer. Although the mechanism of cercosporin self-resistance is multi-faceted, partial resistance does appear to be provided by the encoded product of *CFP* (*c*ercosporin *f*acilitator *p*rotein), a gene recently isolated from the pathogen of soybean, *C. kikuchii*. CFP has significant similarity to the major facilitator superfamily of integral membrane transport proteins. We expressed *CFP* in the cercosporin non-producing, cercosporin-sensitive fungus, *Cochliobolus heterostrophus*, in order to assess the transport activity of CFP and the contribution of CFP to cercosporin resistance in a fungal species free of endogenous toxin production. Expression of the *CFP* transgene in this fungus results in increased resistance to cercosporin due, apparently, to its export out of the fungus.

Keywords Cercosporin resistance · Major facilitator · Heterologous gene expression

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Introduction

Members of the fungal genus *Cercospora* are pathogens of a variety of economically important crops. *C. kikuchii*, the pathogen of soybean, and many other species produce the non-host-specific, phytotoxic polyketide, cercosporin. Cercosporin is a lipid-soluble perylenequinone that, when photoactivated, catalyzes the production of highly reactive oxygen species, principally singlet oxygen (Daub 1982). Singlet oxygen-catalyzed peroxidation of membrane lipids can lead to loss of membrane integrity, cytoplasmic leakage, and cell death (Daub and Ehrenshaft 2000). Thus, as a photosensitizer, cercosporin is highly toxic to a wide variety of organisms, including most plants.

Although a few bacteria and fungi are relatively resistant to cercosporin and other photosensitizers (Daub 1987), *Cercospora* species exhibit the highest degree of cercosporin resistance. Daub and coworkers proposed a model for cercosporin self-resistance (Sollod et al. 1992), in which the toxin is transiently and reversibly reduced by *Cercospora* hyphae. Biochemical studies (Leishman and Daub 1992) and fluorescence microscopy (Daub et al. 1992, 2000) demonstrated that viable hyphae were capable of reducing cercosporin. Genetic support for the model was provided by the observation that cercosporin-sensitive mutants of *C. nicotianae* were unable to reduce cercosporin (Jenns et al. 1995). Genetic complementation of these cercosporin-sensitive mutants resulted in the isolation of two resistance genes. The first gene, *PDX1* (formerly called *SOR1* for *s*inglet *o*xygen *r*esistance *1*; Ehrenshaft et al. 1998, 1999a), is a member of the highly conserved *SNZ* protein family that includes *pyroA* (Osmani et al. 1999), the gene homologue from *Aspergillus nidulans*. *PDX1* and *pyroA* encode a gene that functions in a novel pathway for pyridoxine (vitamin B6) biosynthesis (Ehrenshaft et al. 1999b). Although not involved in toxin reduction, these genes indirectly confer resistance to photosensitizers and singlet oxygen by providing pyridoxine which, as has

recently been shown (Bilski et al. 2000), can chemically quench singlet oxygen. The second gene, *CRG1* (for *c*ercosporin *r*esistance *g*ene *1*; Chung et al. 1999), is required specifically for resistance to cercosporin, but the biochemical function of *CRG1* remains unknown. Recently, two genes were identified from *Saccharomyces cerevisiae* that can provide cercosporin resistance (Ververidis et al. 2001). Over-expression, on high-copy plasmids, of either *Snq2p* or *Cpd1p* in *S. cerevisiae*, provided resistance to cercosporin and other singlet oxygen-generating photosensitizers. Snq2p is a well characterized ABC-type efflux transporter, while *Cpd1p* encodes a novel protein with significant similarity to members of the FAD-dependent pyridine nucleotide-disulfide oxidoreductase family. In summary, these findings support the concept that cellular resistance to cercosporin is mediated by mechanisms that include toxin reduction, singlet oxygen quenching, and toxin export.

Previously, we reported the isolation and molecular characterization of *CFP* (*c*ercosporin *f*acilitator *p*rotein), a light-regulated gene from the soybean pathogen, *C. kikuchii* (Ehrenshaft and Upchurch 1991; Callahan et al. 1999). Gene disruption of *CFP* resulted in dramatically decreased cercosporin production and virulence of the mutant strain on the plant host. Moreover, growth of *CFP* disruptants on medium containing cercosporin was significantly less than that of the wild-type strain. The encoded protein, CFP, is similar to members of the major facilitator superfamily (MFS) of membrane-associated transporter proteins, particularly those containing 14 transmembrane-spanning segments (Paulsen et al. 1996). MFS transporters export molecules from the membrane and/or cytoplasm via proton motive force-antiporter efflux. The major role of the MFS transporters appears to be the export of endogenously produced toxins and antibiotics (Del Sorbo et al. 2000). For example, TOXA, a MFS transporter from the fungal maize pathogen, *Cochliobolus carbonum*, exports the endogenously produced cyclic peptide, HC-toxin (Pitkin et al. 1996) and appears to be essential for self-protection against HC toxin. TRI12, the MFS export pump for the trichothecene family of toxins in *Fusarium sporotrichioides* (Alexander et al. 1999), also appears to contribute to trichothecene self-protection. Based on our previous findings (Callahan et al. 1999), we hypothesized that CFP actively exports cercosporin produced by *Cercospora kikuchii* and also confers partial resistance to cercosporin in that organism. Our objective in this study was to assess the relative contribution of *CFP* to cercosporin resistance in a cercosporin-sensitive, filamentous fungus. *Cochliobolus heterostrophus*, a pathogen of maize (Yoder 1988), was chosen because it is very sensitive to illuminated cercosporin, readily transformable, and amenable to genetic segregation analysis. Expression of *CFP* in *C. heterostrophus* also provides a means of assessing cercosporin export activity in the absence of endogenous cercosporin production. Here we report that expression of *CFP* in *C. heterostrophus* does enhance cercosporin resistance by a mechanism consistent with toxin export.

Materials and methods

Strains and experimental culture conditions

Cochliobolus heterostophus strains C4 (*MAT-2*) and C9 (*MAT-1*) were routinely grown in liquid complete medium (CM) and on CM agar plates, as described by Leach et al. (1982). *Cercospora kikuchii* strain PR was grown in potato dextrose broth (Difco, Detroit, Mich.), as described by Upchurch et al. (1991).

Cercosporin accumulation assay

To prepare standardized *Cochliobolus heterostrophus* inoculum for the cercosporin accumulation assay, 10 mg of fresh weight mycelium from a 3-day culture of each strain were resuspended in 5 ml fresh liquid CM in sterile, capped tubes and vortexed with sterile, 4-mm glass beads. Then, 3 ml of the suspension were pipetted into the wells of a sterile, 12-well Falcon culture plate (Fisher Scientific, Norcross, Ga.). Cercosporin was purified from *Cercospora kikuchii* mycelium as described by Okubo et al. (1975), dissolved in ethanol, and added to each well to provide a toxin concentration of 10 μM. An equivalent volume of ethanol alone was added to the wells of the growth controls. The culture plates were closed, wrapped in foil to exclude light and incubated with gentle rotatory motion (60 rpm) at 23 °C for 48 h. Mycelium was harvested by vacuum filtration and washed on the filter with three, successive 20-ml volumes of 50% ethanol/water. After lyophilization, the tissue was weighed and the entire amount (about 10 mg) was frozen in liquid nitrogen and ground to powder in a microtube with plastic micropestle. The tissue was extracted three times with 1 ml absolute ethanol and the fractions combined. Cercosporin concentration was determined spectrophotometrically at 473 nm, using the molar extinction coefficient of 23,600 (Yamazaki and Ogawa 1972) for cercosporin in ethanol. The concentration of cercosporin accumulated within the fungal mycelium was calculated and expressed as nanomoles per milligram fungal dry weight. The experiment was done in triplicate for each strain and then repeated.

Cercosporin resistance assay

Cercosporin resistance was assayed by measuring colony growth in the presence and absence of cercosporin. Mycelium plugs (5 mm) of C4, C9, 10-5, and *CFP* transgenic strains of equal age were placed, mycelium-side down, in the center of CM agar plates containing 10 μM cercosporin. An equivalent volume of ethanol was added to CM plates for growth controls. Growth was determined by averaging the lengths of two perpendicular diameters per mycelium mass, after incubation for 5 days at 23 °C under continuous white fluorescent light (approximately 80 $\mu E \cdot s^{-1} \cdot m^{-2}$). Growth assays for each strain were done in triplicate and the experiment was repeated.

Plasmids

Plasmid pCFP (Callahan et al. 1999) contains a cDNA encoding the entire sequence of *CFP* (accession number AF091042), cloned as a 1.9-kb, *Eco*RI-5′-CFP-3′-*Xho*I fragment in pBluescript SK (Stratagene, La Jolla, Calif.). Plasmid pP1CFP was made by ligating, in proper orientation, a 662-bp *Xba*I fragment of pBIT (Straubinger et al. 1992) containing the constitutive, *Cochliobolus heterostrophus* P1 promoter (Turgeon et al. 1987) into a *Xba*I site of pCFP, upstream of the *CFP* start codon. pUCH1 (Turgeon et al. 1993) contains a 2.5-kb fragment of DNA encoding the *Escherichia coli* gene, *hphB*, for resistance to hygromycin B, fused to the

C. heterostrophus P1 promoter inserted into the *Hin*dIII-*Sal*I sites of pUC18. Digestion of pUCH1 with *Eco*RI and *Hin*dIII releases a ca. 1.4-kb DNA fragment containing a portion of the *hphB* sequence that was used for probing Southern blots.

Fungal transformations and genetic crosses

Introduction of both pP1CFP and pUCH1 into *C. heterostrophus* strain C4 was by co-transformation, essentially as described by Callahan et al. (1999). To recover hygromycin-resistant transformants, CM regeneration medium containing 50 μg hygromycin ml^{-1} (Sigma, St. Louis, Mo.), was used for selection. *C. heterostrophus* matings were performed as described by Leach et al. (1982).

Nucleic acid isolation, hybridizations, and PCR

Genomic DNA was isolated from *C. heterostrophus* and *Cercospora kikuchii* by the method of Garber and Yoder (1983). Genomic DNA Southern blot analysis (5 μg $strain^{-1}$), and Northern blot analysis (8 μg total RNA $strain^{-1}$) were performed as described by Sambrook et al. (1989). The RNeasy plant mini-kit (Qiagen, Santa Clarita, Calif.) was used to extract total RNA from lyophilized fungal mycelium. Nytran Plus membranes (Schleicher & Schuell, Keene, N.H.) were used for nucleic acid blots, using procedures supplied by the manufacturer. The Qiaquick gel extraction kit (Qiagen) was use to purify DNA fragments from agarose gels for hybridization probes. ^{32}P-radiolabeled *CFP* insert DNA from pCFP (Callahan et al. 1999) and *hphB* insert DNA from pUCH1 were made with the random-priming oligolabeling kit from Pharmacia (Thousand Islands, Calif.). *CFP* was also detected in fungal genomic DNA by PCR, using *CFP* coding sequence-specific primers. The amplification reactions (25 μl) contained 100 ng genomic DNA, 1× PCR buffer (Promega, Madison, Wis.), 5% glycerol, 240 nM dNTPs, 2 mM $MgCl_2$, and 400 nM primers CFPA (5′-TACAGAGTCTCACGACGT-3′) and PC4 (5′-GCAGAGTGTAGATAGAGGCA-3′). Following an initial denaturation step (95 °C, 1 min), 40 cycles [(94 °C, 1 min)(50 °C, 1 min)(72 °C, 1.5 min)] were performed, using a PTC-100 thermocycler (MJ Research), followed by 5 min at 72 °C.

Immunodetection of CFP

C. kikuchii and *Cochliobolus heterostrophus* proteins were extracted by grinding frozen, lyophilized mycelium in a chilled mortar, with a buffer containing 100 mM Tris-HCl (pH 7.0), 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid disodium salt (pH 8.0), 100 mM dithiothreitol, 10 μg leupeptin ml^{-1}, 0.1% Triton X-100, and 5% polyvinyl polypyrrolodone. Extracts were clarified by centrifugation at 12,000 rpm for 10 min at 4 °C in a microcentrifuge. Proteins were quantified using the DC protein assay (Bio-Rad Laboratories, Hercules, Calif.). Fungal protein (25 μg) was applied to a SDS-polyacrylamine gel (4% stacking, 12% separating gel) and electrophoresed for 45 min in a Mini-Protean II slab cell (Bio-Rad). After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Micron Separations, Westborough, Mass.), using a Transblot semi-dry transfer cell (Bio-Rad), following the manufacturer's protocol. After transfer, the filter was blocked by incubation in 3% non-fat, skimmed milk in TBST buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20] for 2 h at room temperature, with gentle swirling. After the blocking step, the membrane was incubated for 1 h at room temperature in a 1:200 dilution of primary rabbit antisera containing the anti-CFP antibody. The anti-CFP polyclonal antibody was raised against a carboxy terminus portion of CFP (ACREIEDPEKGQSAEIV) by Quality Controlled Biochemicals, Hopkinton Mass. The membrane was then washed five times with TBST, 10 min each wash, with gentle swirling. Following washing, the membrane was incubated for 1 h with a 1:5,000 goat, anti-rabbit secondary antibody conjugated to alkaline phosphatase (Promega, Madison, Wis.). CFP protein was visualized by the color reaction of alkaline phosphatase with the Western blue-stabilized substrate.

Results

Analysis of *C. heterostrophus* transformants

PCR analysis of four of five hygromycin-resistant *C. heterostrophus* putative transformants with *CFP*-specific primers generated an amplicon identical in size (ca. 1,400 bp) to the positive control, *Cercospora kikuchii* PR. As expected, PCR with *Cochliobolus heterostrophus* strains C4 and C9 failed to generate the amplicon. These results (Fig. 1a) were confirmed by Southern analysis with *CFP* as the probe (Fig. 1b). The presence of multiple bands in each *CFP*-positive transformant suggests that *CFP* integrated multiple times into the fungal genome. Southern analysis with the *hphB* probe (Fig. 1c) confirmed that all five *C. heterostrophus* hygromycin-resistant strains contained vector DNA and were thus *hphB* transformants.

CFP$^+$ *C. heterostrophus* transformants express *CFP* transcript and CFP protein

Northern analysis using the *CFP* cDNA probe showed that *CFP* mRNA was readily transcribed from the *C. heterostrophus* P1::CFP construct in *C. heterostrophus*. The 1.9-kb *CFP* transcript was detected in the *Cercospora kikuchii* PR positive control and the four *Cochliobolus heterostrophus CFP*$^+$ transformants, but not in *CFP*$^-$ *C. heterostrophus* wild-type strains C4 and C9 or in transformant 10-5 (Fig. 2a). The ca. 65.4-kDa CFP protein was detected by Western blotting in *Cercospora kikuchii* PR (strong intensity) and the four *CFP*$^+$ *Cochliospora heterostrophus* transformants (10-3, 5-3, 10-1, 5-2) at lower, approximately equal intensity, but not in *CFP*$^-$ transformant 10-5 or in wild-type strains C4 and C9 (Fig. 2b).

CFP$^+$ *C. heterostrophus* transformants accumulate less cercosporin than *CFP*$^-$ strains

We hypothesized that *C. heterostrophus* strains containing the active CFP transporter would accumulate less cercosporin in their mycelium when incubated in growth medium containing cercosporin than *CFP*$^-$ strains, because toxin entering the cell would be pumped out. To test this hypothesis, we measured the amount of cercosporin accumulated within the mycelium of *CFP*$^-$ wild-type strains C4 and C9, the control transformant 10-5, and the *CFP*$^+$ strains 10-3, 5-3, 10-1, and 5-2 cultured in medium containing 10 μM cercosporin. After a 48-h incubation period in the dark, strains C4 and C9 and transformant 10-5 (all *CFP*$^-$) accumulated approximately two to three times the amount of

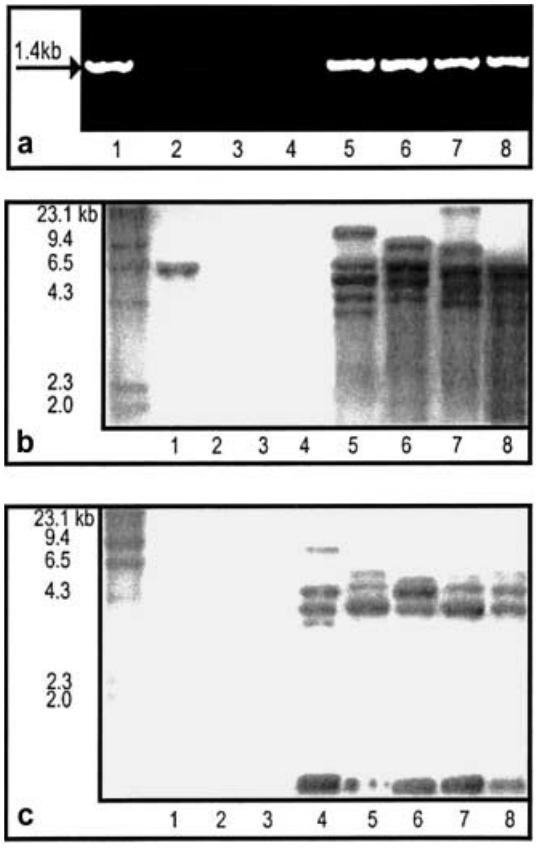

Fig. 1a–c Genomic analysis of *CFP* transgenic *Cochliobolus heterostrophus* strains. Lane designations for panels **a**, **b**, and **c** are as follows: *lane 1 Cercospora kikuchii* PR (*CFP* positive control), *lanes 2 and 3 Cochliobolus heterostrophus* wild-types C4 and C9, respectively, *lanes 4–8 C. heterostrophus* transformants 10-5, 10-3, 5-3, 10-1, and 5-2, respectively. **a** PCR analysis of fungal strains with the *CFP*-specific primers CFPA and PC4. **b** Southern analysis of *Eco*RI-digested genomic DNA of the *C. heterostrophus* transformant strains, using the ^{32}P-labeled, ca.1.9-kb cDNA from pCFP (Callahan et al. 1999). The *CFP* probe hybridizes to a 6.5-kb *Eco*RI band of *Cercospora kikuchii* in *lane 1*. **c** Southern analysis of a separate blot of *Eco*RI-1-digested genomic DNA of the *Cochliobolus heterostrophus* transformant strains, using the ^{32}P-labeled, ca. 1.4-kb *hph* gene fragment from pUCH1 (Turgeon et al. 1993). *Hin*dIII-digested lambda DNA molecular weight markers are *to the far left* in panels **b** and **c**

extractable toxin within their mycelium, compared with transformants expressing *CFP* (Table 1).

CFP$^+$ *C. heterostrophus* transformants are more resistant to cercosporin than *CFP*$^-$ strains

Resistance to cercosporin was estimated by measuring the radial growth of *CFP*$^-$ and *CFP*$^+$ strains on cercosporin medium as a percentage of growth on medium lacking cercosporin. Growth inhibition data (Table 2) show that the growth of the three *CFP*$^-$ strains (C4, C9, 10-5) was inhibited by approximately 60% (range: 60.8–63.1% inhibition), whereas growth of the *CFP*$^+$ strains (10-3, 5-3, 10-1, 5-2) was inhibited by approximately 40% (range: 33–42% inhibition). On medium lacking

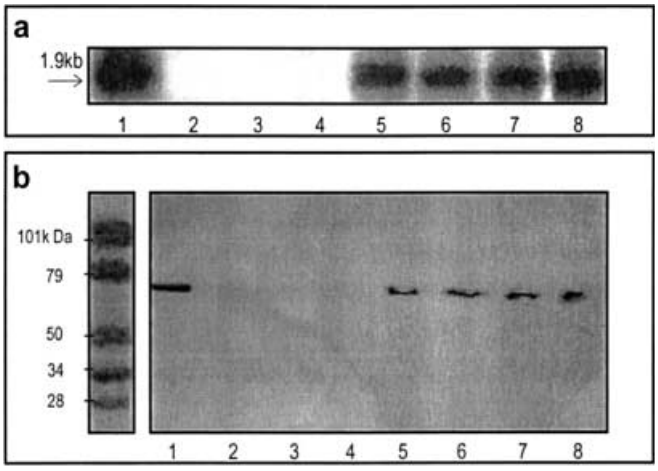

Fig. 2a, b *CFP* expression analysis of transgenic *C. heterostrophus* strains. Lane designations for panels **a** and **b** are as in Fig. 1. **a** Northern blot analysis of *CFP* transcript accumulation in *C. heterostrophus* transformant strains, using the ^{32}P-labeled, ca.1.9-kb cDNA from p*CFP*. **b** Western blot analysis of CFP accumulation in *C. heterostrophus* transformant strains, using the CFP-peptide specific antibody (see Materials and methods). Protein molecular markers are depicted *at the left* of panel **b**

Table 1 Cercosporin accumulation in *Cochliobolus heterostrophus* *CFP*$^-$and *CFP*$^+$ strains grown in the dark in liquid complete medium (CM) containing10 μM cercosporin. Genotype data are given in Fig. 1. Data are derived from two growth experiments, each with three replications

Strain	Genotype	nmol cercosporin mg^{-1} fungal dry weight
C4	*hph*$^-$/*CFP*$^-$	3.00 ± 0.45
C9	*hph*$^-$/*CFP*$^-$	2.97 ± 0.42
10-5	*hph*$^+$/*CFP*$^-$	3.10 ± 0.54
10-3	*hph*$^+$/*CFP*$^+$	1.52 ± 0.72
5-3	*hph*$^+$/*CFP*$^+$	1.04 ± 0.57
10-1	*hph*$^+$/*CFP*$^+$	1.00 ± 0.52
5-2	*hph*$^+$/*CFP*$^+$	0.97 ± 0.67

cercosporin, growth was reduced for *CFP*$^+$ transformants 5-3, 10-1, and 5-2; but the growth of *CFP*$^+$ transformant 10-3 approximated that of wild-types C4 and C9 on this medium.

Transformant 10-3 (*MAT-2*) was crossed to wild type strain C9 (*MAT-1*) and random ascospores were selected and grown out on CM medium. Of the resulting progeny, 12 were analyzed for the presence of *CFP*, using PCR and Western blotting, and for *hphB* by Southern analysis (data not shown). Progeny and their parents were assayed for cercosporin sensitivity, as described above. Percentage growth inhibition data (Table 3) indicated that all progeny expressing CFP and parent 10-3 were less sensitive to cercosporin (range of growth inhibition: 36.1–48.8%) than were progeny lacking *CFP* and parent C9 (range of growth inhibition: 56.5–67.0%). The 1:1 co-segregation of *CFP* and partial resistance to cercosporin strongly suggests that *CFP* is responsible for the increased cercosporin resistance exhibited by transformant 10-3. Interestingly, Southern analysis of the

Table 2 Cercosporin resistance of *C. heterostrophus* CFP^- and CFP^+ strains. Data are derived from two growth experiments conducted in the light, each with three replications. Growth is given as the mean and standard error of colony diameter. Percentage growth inhibition is determined by calculating the mean growth of the strain on CM containing cercosporin as a percentage of its mean growth on CM without cercosporin

Strain	Growth (mm) Without cercosporin	Growth (mm) With 10 μM cercosporin	% Growth inhibition
C4	78.5 ± 5.2	30.8 ± 2.3	60.8
C9	71.3 ± 3.7	26.3 ± 2.2	63.1
10-5	78.2 ± 4.9	29.7 ± 2.0	62.2
10-3	75.3 ± 4.6	43.7 ± 3.3	42.0
5-3	56.2 ± 3.2	34.2 ± 2.5	39.2
10-1	57.9 ± 4.1	37.0 ± 3.2	36.1
5-2	55.7 ± 3.5	37.1 ± 2.3	33.4

progeny with the *hphB* probe showed that CFP^- progeny were also $hphB^-$ and all CFP^+ progeny were $hphB^+$. The co-segregation of CFP^+ with $hphB^+$ occurred even though progeny ascospores were picked randomly and plated on non-selective medium.

Discussion

In *Cercospora* species, resistance to the photosensitizer cercosporin involves multiple mechanisms of defense. Daub and colleagues have shown that the fungal maintenance of cercosporin in a chemically reduced state suppresses the production of singlet oxygen and that pyridoxine production can provide resistance to singlet oxygen, via chemical quenching (Daub and Ehrenshaft 2000). In this report, we provide evidence supporting a role for the MF-like protein, CFP, in a third type of resistance. Based on the observation that disruption of *CFP* in *C. kikuchii* resulted in an approximate 50% reduction in growth of the fungus on medium containing cercosporin, we proposed that CFP confers cercosporin resistance in *C. kikuchii* by lowering cellular concentrations of the toxin via toxin export (Callahan et al. 1999). Because CFP may both regulate cercosporin production (Callahan et al. 1999) and export the toxin in *C. kikuchii*, we decided to assess the relative contribution of *CFP* to cercosporin export and resistance in a fungus lacking endogenous cercosporin production. We chose *Cochliobolus heterostrophus* strain C4 as the recipient for *CFP* because this organism is sensitive to illuminated cercosporin at low concentrations (1–5 μM). In contrast, the cercosporin sensitivity tests we conducted revealed that the model organism, *Aspergillus nidulans*, was not as sensitive as *C. heterostrophus* (data not shown). Another model organism, the yeast *S. cerevisiae*, was not chosen as a recipient because it has been reported to be relatively resistant to cercosporin (Daub 1987) and to possess multiple defense mechanisms against oxidative stress (Muller 1996).

Here, we report that heterologous expression of *CFP* can limit the accumulation of toxin (presumably by its transport activity) and provide a level of resistance to cercosporin in primary CFP^+ transformants of *C. heterostrophus*. Further, this resistance co-segregates with the presence of *CFP*, strongly suggesting that *CFP* is responsible for the increased cercosporin resistance exhibited by the CFP^+ parent. Although it is well documented that members of the ABC family of transporters (structurally unrelated efflux pumps directly energized by ATP hydrolysis) can enhance resistance to environmental toxicants in heterologous organisms (Mickisch et al. 1991; Raymond et al. 1994; Evans et al. 1995; Volkman et al. 1995; George et al. 1996; Del Sorbo et al. 2000), to our knowledge, this is the first report that a MFS transporter can act as transferable source of resistance to a fungal toxin involved in plant pathogenesis.

Table 3 Cercosporin resistance of the CFP^- and CFP^+ progeny of the C9×10-3 mating. CFP^+ and $hphB^+$ genotypes were determined by PCR, Western blot, and Southern analyses. Data are derived from two growth experiments conducted in the light, each with three replications

Strain	Genotype	% Growth inhibition
C9	*MAT-1*, CFP^-, $hphB^-$	63.1
10-3	*MAT-2*, CFP^+, $hphB^+$	42.6
P11	CFP^-, $hphB^-$	67.0
P12	CFP^-, $hphB^-$	65.6
P4	CFP^-, $hphB^-$	62.2
P14	CFP^-, $hphB^-$	62.2
P6	CFP^-, $hphB^-$	61.8
P8	CFP^-, $hphB^-$	56.5
P3	CFP^+, $hphB^+$	48.8
P15	CFP^+, $hphB^+$	46.5
P9	CFP^+, $hphB^+$	46.5
P13	CFP^+, $hphB^+$	46.2
P10	CFP^+, $hphB^+$	39.5
P7	CFP^+, $hphB^+$	36.1

While our data clearly show that expression of *CFP* in *C. heterostrophus* confers increased resistance to cercosporin, three of four transformants (5-3, 10-1, 5-2) also showed reduced growth on medium lacking cercosporin. Thus, expression of *CFP* may cause pleiotropic effects in the fungus, perhaps through interaction(s) with constituents of the *C. heterostrophus* membrane. As our understanding of the functions of MFS proteins, particularly CFP, is rudimentary, it is difficult to postulate why this may occur. Because the growth of one CFP^+ transformant (10-3) with resistance was unaffected, growth and resistance to cercosporin do not appear to be linked.

It is interesting that neither *CFP* transcript nor CFP protein accumulation in the transformants was correlated with the degree of resistance expressed by the transformants. One explanation could be that the level of resistance conferred by *CFP* is maximally around 20% and further expression of the protein does not provide increased resistance. An alternative explanation is that differences in toxin resistance among the transformants expressing CFP may be due to differences in their ability to post-translationally modify CFP or to efficiently localize it.

That *CFP* can partially suppress cercosporin accumulation by 30–50% and enhance cercosporin resistance by ca. 20% in a heterologous fungus presumably lacking

such a specific resistance mechanism indicates that CFP, at least in part, directly functions to confer cercosporin resistance in *Cercospora kikuchii*. As a transferable source of resistance, CFP may therefore have potential for engineering cercosporin and/or *Cercospora* resistance in crop plants.

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