***CFP*, the Putative Cercosporin Transporter of *Cercospora kikuchii*, Is Required for Wild Type Cercosporin Production, Resistance, and Virulence on Soybean**

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Many species of the fungal genus *Cercospora*, including the soybean pathogen *C. kikuchii*, produce the phytotoxic polyketide cercosporin. Cercosporin production is induced by light. Previously, we identified several cDNA clones of mRNA transcripts that exhibited light-enhanced accumulation in *C. kikuchii*. Targeted disruption of the genomic copy of one of these, now designated *CFP* (cercosporin facilitator protein), results in a drastic reduction in cercosporin production, greatly reduced virulence of the fungus to soybean, and increased sensitivity to exogenous cercosporin. Sequence analysis of *CFP* reveals an 1,821-bp open reading frame encoding a 65.4-kDa protein similar to several members of the major facilitator superfamily (MFS) of integral membrane transporter proteins known to confer resistance to various antibiotics and toxins in fungi and bacteria. We propose that *CFP* encodes a cercosporin transporter that contributes resistance to cercosporin by actively exporting cercosporin, thus maintaining low cellular concentrations of the toxin.

Additional keywords: drug/proton antiporter.

Members of the fungal genus *Cercospora* are pathogens of a variety of economically important crops including soybean and maize. At least 23 *Cercospora* spp. (Assante et al. 1977) produce the host nonspecific polyketide toxin cercosporin. Cercosporin, blood red in color, is a lipophilic, 4, 9-hydroxyperylene-3, 10-quinone derivative with a molecular

weight of 534 (Lousberg et al. 1971; Yamazaki and Ogawa 1972; Okubo et al. 1975). Several observations suggest that cercosporin is a pathogenicity factor, including the finding that UV-induced, cercosporin-deficient mutants of *C. kikuchii* are nonpathogenic on soybean (Upchurch et al. 1991). Cercosporin production is induced by light (Lynch and Geoghegan 1979; Jenns et al. 1989; Ehrenshaft and Upchurch 1991; Rollins et al. 1993) and the toxin itself is light activated (Daub 1982a). In the light, cercosporin is converted to an electronically excited (triplet) state that can react with molecular oxygen by direct energy transfer to yield singlet oxygen (Daub and Hangarter 1983). Singlet oxygen is a highly reactive oxygen species damaging to cell membranes as well as other cellular structures. Singlet oxygen-catalyzed peroxidation of membrane lipids can lead to loss of membrane integrity, cytoplasmic leakage, and eventually cell death (Macri and Vianello 1979; Daub 1982b).

Because cercosporin appears to be a pathogenicity factor, developing ways of suppressing cercosporin production or engineering cercosporin resistance in plants are plausible strategies for combating diseases caused by *Cercospora* spp. Implementing these strategies requires a greater understanding of the molecular genetics of cercosporin biosynthesis and mechanisms of cercosporin resistance. Because cercosporin production is induced by light, work in our laboratory previously focused on the isolation of cDNAs corresponding to mRNAs exhibiting light-enhanced transcript accumulation in the expectation that one or more would be involved in cercosporin metabolism (Ehrenshaft and Upchurch 1991). Of six cDNAs isolated, LE6 (now known as *CFP* [cercosporin facilitator protein]) was the first chosen for targeted disruption analysis since its corresponding transcript showed the greatest light induction (20-fold) and a temporal expression pattern paralleling cercosporin accumulation. Here we report sequence analysis of *CFP* and characterization of its function derived from an examination of *CFP* disruption mutants.

RESULTS

Sequence analysis of the *CFP* gene.

A 1.9-kb cDNA from plasmid pCFP was sequenced as was the 6.5-kb *EcoRI* insert of the genomic DNA clone pgCFP (a 3,312-bp portion of which is shown in Figure 1). A compari-

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Nucleotide and/or amino acid sequence data of *CFP* can be found at the GenBank data base as accession number AF091042.

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son of the cDNA and genomic sequences indicated that *CFP* contains three introns of 52, 50, and 49 bp interrupting an open reading frame (ORF) of 1,821 bp. These introns contain characteristic 5' and 3' splice junctions and an internal lariat consensus sequence observed in other filamentous fungi (Edelmann and Staben 1994). The region around the suspected ATG start site (codon 1174) reveals sequence similarity to Kozak sequences typically found at the translational start site of fungi and other eukaryotes (Edelmann and Staben 1994).

Translation of the 1,821-kb *CFP* ORF predicts a protein of 607 amino acids with a molecular mass of 65.4 kDa, an acidic pI of 5.08, and an unusually large number of cysteines (13 residues). BLAST (Gish and States 1993) searches of protein data bases indicate *CFP* is similar to major facilitator superfamily (MFS) drug resistance transporter proteins (Paulsen et al. 1996b), particularly those with 14 transmembrane segments (TMSs), representatives of which are shown in align-

ment (Altshul et al. 1990) in Figure 2. This group, within the MFS, contains transporters or efflux "pumps" that mediate resistance to structurally diverse, typically hydrophobic drugs and toxins, some of which, like cercosporin, are polyketides. Consistent with its sequence similarity to integral membrane MFS transporters, *CFP* is highly (49%) hydrophobic (Fig. 3). Interestingly, *CFP* is predicted to have only 13 TMSs by the program TMpred (Hoffman and Stoffel 1993). Nevertheless, the existence of a fourteenth TMS (double underlined in Figure 3) is suggested by the greater overall similarity of *CFP* to MF transporters with 14, rather than 12, TMSs and by *CFP*'s possession of conserved motifs (D1, H, E, and F) diagnostic of MF transporters with 14 TMSs (Paulsen et al. 1996b).

Disruption of *CFP* and Southern analysis of *CFP* mutants.

To determine whether *CFP* plays a role in cercosporin metabolism, *CFP* (Fig. 4A) was disrupted in wild-type *C.*

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Fig. 1. Genomic DNA sequence of *CFP* and its flanks. *Clal* and *HindIII* restriction sites used in construction of *CFP* disruption vectors are indicated. Deduced 607 amino acid sequence of cercosporin facilitator protein (CFP) is presented below genomic sequence. TM domains predicted by TMpred program (Hoffman and Stoffel 1993) are indicated with a single underline; TM domain predicted by sequence similarity to 14 TM domain MF transporters is double underlined. Introns, identified by a comparison of genomic and cDNA sequences, are shown as numbered gaps in the translation.

10-11, but not from PR (Fig. 5C). Both 10-1 and 10-11 were observed to possess restored ability to produce cercosporin (Fig. 5A) and resistance to cercosporin similar to that of wild-type PR (data not shown).

Virulence of *CFP* disruption mutants on soybean.

To assess virulence, susceptible soybean cv. Lee-68 was inoculated with *CFP* disruptants Bar-1 and Gus-3, wild-type

PR, and *CFP*-complemented Gus-3 disruptants 10-1 and 10-11. Symptoms were allowed to develop for 15 days (Fig. 6) In all cases *CFP* disruptants displayed greatly reduced virulence relative to PR and the *CFP*-complemented disruptant strains (data not shown) were similar in virulence to PR. These results demonstrate that *CFP* is required for wild-type virulence and are consistent with the function of cercosporin as a pathogenicity factor.

CFP	M	T	S	P	A	R	S	T	H	T	D	T	E	S	H	D	V	V	K	S	D	S	E	S	K	L	E	L	E	H	S	D	S	D	N	Q	D	E	K	S	40										
TOXA	M	D	-	-	-	-	-	-	-	-	-	-	-	-	E	Q	I	V	S	A	S	S	N	V	K	D	G	V	E	K	Q	P	V	K	D	R	E	D	-	-	26										
SGE1	M	K	S	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4											
QACA	M	I	S	-	-	-	-	-	-	-	-	-	-	-	-	F	F	T	K	T	T	D	M	M	T	S	K	K	R	W	T	A	-	-	-	-	-	-	-	20											
D1																																																			
I D x T v x n v A I P																																																			
CFP	T	V	F	I	V	A	L	S	N	T	I	I	S	T	A	I	P	A	I	T	T	A	F	N	S	T	R	D	I	G	-	W	Y	N	S	G	E	A	L	A	119										
TOXA	A	A	F	L	G	A	L	D	A	T	V	V	A	V	L	T	P	T	L	A	Q	E	F	H	S	V	D	A	V	A	-	W	Y	G	A	I	Y	L	M	96											
SGE1	T	L	F	L	A	A	L	D	I	V	I	V	V	T	L	Y	D	T	I	G	I	K	F	H	D	F	G	N	I	G	-	W	L	V	T	G	Y	A	L	S	55										
QACA	S	L	F	V	V	T	M	D	M	T	I	L	I	M	A	L	P	E	L	V	R	E	L	E	P	S	G	T	Q	Q	L	W	I	V	D	I	Y	S	L	V	66										
CFP	A	T	A	F	Q	L	P	F	G	R	A	Y	L	L	M	D	L	K	W	T	F	L	V	S	L	A	L	Y	L	I	G	S	L	I	C	G	V	A	N	S	159										
TOXA	S	G	T	T	Q	P	L	F	G	K	L	Y	N	E	F	S	P	K	W	L	F	I	T	C	L	I	V	L	Q	L	G	S	L	V	C	A	L	A	R	N	136										
SGE1	N	A	V	F	M	L	L	W	G	R	L	A	E	I	L	G	T	K	E	C	L	M	I	S	V	I	V	F	E	I	G	S	L	I	S	A	L	S	N	S	95										
QACA	L	A	G	F	I	I	P	L	S	A	F	A	D	K	W	G	R	K	K	A	L	L	T	G	F	A	L	F	G	L	V	S	L	A	I	F	F	A	E	S	106										
CFP	S	E	L	L	I	F	G	R	S	I	A	G	V	G	N	A	G	V	F	A	G	V	F	I	I	A	R	N	V	P	L	-	R	K	R	A	L	Y	A	198											
TOXA	S	P	T	F	I	V	G	R	A	V	A	G	I	G	A	G	G	I	L	S	G	A	L	N	I	V	A	L	I	V	P	L	-	H	H	R	A	A	F	T	175										
SGE1	M	A	T	L	I	S	G	R	V	V	A	G	F	G	G	S	G	I	E	S	L	A	F	V	V	G	T	S	I	V	R	E	-	N	H	R	G	I	M	I	134										
QACA	A	E	F	V	I	A	I	R	F	L	L	G	I	A	G	A	L	I	M	P	T	T	L	S	M	I	R	V	I	F	E	N	P	K	E	R	A	T	A	L	146										
C																																																			
g x x x G P x x G G I F																																																			
CFP	G	L	V	G	A	T	F	A	I	A	A	V	L	G	P	V	L	G	G	I	F	T	D	R	I	S	W	R	W	C	L	Y	I	N	L	P	I	G	A	V	238										
TOXA	G	M	I	G	A	L	E	C	V	A	L	I	I	G	P	I	I	G	G	A	I	A	D	N	I	G	W	R	W	C	F	W	I	N	L	P	I	G	A	A	215										
SGE1	T	A	L	A	I	S	Y	V	I	A	E	G	V	G	P	F	I	G	G	A	F	N	E	H	L	S	W	R	W	C	F	Y	I	N	L	P	I	G	A	F	174										
QACA	A	V	W	S	I	A	S	S	I	G	A	V	F	G	P	I	I	G	G	A	L	L	E	Q	F	S	W	H	S	A	F	L	I	N	V	P	F	A	I	I	186										
CFP	R	V	A	I	I	I	F	-	-	-	-	-	-	-	-	-	-	L	L	P	S	R	P	G	E	K	A	-	-	-	-	A	E	V	K	D	L	S	W	263											
TOXA	V	C	A	I	L	L	F	-	-	-	-	-	-	-	-	-	-	F	F	H	P	P	R	S	T	Y	S	-	-	-	-	A	S	G	V	P	R	S	Y	240											
SGE1	A	F	I	I	L	A	F	C	N	T	S	G	E	P	H	Q	K	M	W	L	P	S	K	I	K	I	M	N	Y	D	Y	G	E	L	L	K	A	S	F	214											
QACA	A	V	V	A	G	L	F	-	-	-	-	-	-	-	-	-	-	L	L	P	E	S	K	-	-	-	-	-	-	-	-	L	S	K	E	K	S	H	S	W	208										
E																																																			
D x x G x x L																																																			
CFP	W	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	F	L	K	L	N	P	F	G	S	A	L	L	L	G	S	L	T	C	F	F	L	A	L	Q	W	G	G	G	E	Y	R	W	298
TOXA	S	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	L	G	N	L	D	Y	I	G	A	G	M	I	I	S	S	L	V	C	L	S	L	A	L	Q	W	G	G	T	K	Y	K	W	275
SGE1	W	K	N	T	F	E	V	L	V	F	K	L	D	M	V	G	I	I	L	S	S	A	G	F	T	L	L	M	L	G	L	S	F	G	G	N	N	F	P	W	254										
QACA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	I	P	S	T	I	L	S	I	A	G	M	-	-	-	-	I	G	L	V	W	S	I	K	E	F	S	K	232					

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Fig. 2. Alignments of deduced amino acid sequences of *CFP* from *Cercospora kikuchii* PR, *TOXA* of *Cochliobolus carbonum* (GenBank L48797), *SGE1* of *Saccharomyces cerevisiae* (GenBank I11640), and *QACA* of *Staphylococcus aureus* (GenBank X56628) produced with the CLUSTAL algorithm employed by DNASTar. Shaded amino acids are identical or functionally conserved in three or four aligned proteins. Overall amino acid sequence similarity between *CFP* and *TOXA*, *SGE1*, and *QACA* is 47, 40, and 34%, respectively. Consensus sequences of motifs diagnostic for 14 TMS MF transporters (D1, H, E, and F) (Paulsen et al. 1996a) are indicated above translations. Motif C, common to both 12 and 14 TMS MF transporters, is also indicated.

Since cercosporin production in *C. kikuchii* is induced by light, we had hypothesized that transcription of genes involved in cercosporin metabolism would also be light induced. Consequently, we cloned cDNAs of transcripts that accumulated to higher levels in the light than in the dark (Ehrenshaft and Upchurch 1991). This led to the identification of several light-induced genes. Of these, one now designated

Here we report that targeted disruption of *CFP* has multiple effects on cercosporin metabolism. Though not diminished in growth or viability, *CFP* disruptants produce 95% less cercosporin than does the wild type. Consistent with earlier studies

CFP SAGRVVAVLVV-----FAVSFIGWLVLQY-FQGDE 327
TOXA GDGRVVALLV-----FGVLFILSASGHQY-WKGEK 304
SGE1 NSGIIICFFTVGPILLLLFCAYDFHFLSLSGLHYDNKRIK 294
QACA EGLADI----IPWVVIIVLAITMIVIVFVKR-----NLSSSSD 263

CFP ATLPFNVAKQRRTVGGASITYTLHLASAAGFLVIYIYLPWFQA 367
TOXA ALFPTRLRLRQRGFLLSLFLNGLCFGGVQYAALYIYLPWFQA 344
SGE1 PLLTWNINIASNCGIFTSSIITGFLSCFAAYELQSAIYLVQLYQL 334
QACA PMLLDVRLLFKKRSFSAGTIAAFMTMFAMASVLLLASQWLQV 303

CFP VRSDSAEAAGLKLGLGIVISLTLSSIAAGGAVVKIGYYYPF 407
TOXA IKGETRVLGAGIQMLPIVGAIIGVNIVAGITISFTGRLAPF 384
SGE1 VFKKKPTLASIHLWELSIIPAMIATMAIAYLNSKYGIKPA 374
QACA VEELSPFKAGLYLLPMAIGDMVFAPIAAPGLAARFG---PK 340

CFP IYAGTVLCSIGSGLLYTITLDTTPQW-DIIGYSIVFAIGIG 446
TOXA IVIATVLA SVSGLLYTFTPTKSA-RIIGYQLIYGAGSG 423
SGE1 IVFG-VLFCGIVSGSGLFTLI-NGELS-QSIGYSILPGIAFG 411
QACA IVLPSGIGIAAIGMFIMYFFGHPLSYSTMALALILLVGAGM 380

F

CFP VSLEQSNVAVQTVLPDAQIPAGTSLVLFVRL--LGSAIPG 484
TOXA AGVQQAFIGAQAALDPADVITYASASVLLM-----NSMSG 457
SGE1 SIFQATLLSSQVQITSDDPDFQNKFI EVTAFNSFAKSLGF 451
QACA ASLAVA--SALIMLETPTSKAGNAAAVEESMYDLGNVFGV 418

CFP PIGQSVLQTTL-----ASRLGTEVAEQAYGGTGATEIRSK 519
TOXA VITLCVCQNLF-----TNRI--NALTEVLPGVTKETLQSG 490
SGE1 AFGGNMGAMIF-----TASLKNQMRSSQLNIPQFTSVETL 486
QACA AVLGSLS SMLYRVFLDISSFS SKGIVGDLAHVAEESVVG 458

CFP LDNIFGAGTPEARD-ALDAF--NDSVTKI FMVAIIVSCLSA 557
TOXA FAFRLRSTLTPEAFGVAIQTF--NSAIQDAFLVAIVLSCASV 529
SGE1 LAY--STEHYDGPQS SLSKFINTAIHDFYCA--LGC--- 519
QACA VEVAKATGIKQLANEAVTSFNDAFVATALVG GTIMIIIS 498

CFP LPLPLIELKSVKREKRDNEDAKEGKKTNGTTREIEDPEKG 597
TOXA LGWPFLSWASVKGQKKMNK 548
SGE1 YALSFF-FGIFTSSKKKTTSIAK----- 540
QACA VVYLLIP-----KSLD----- 509

CFP QSAEIVGKAV 607
TOXA 548
SGE1 ---KQQ 543
QACA ---ITKQK 514

that suggested that cercosporin is a pathogenicity factor, *CFP* disruptants, presumably by virtue of their reduced cercosporin production, are greatly reduced in virulence to soybean. Furthermore, disruptants are sensitive to cercosporin when it is exogenously supplied. That cercosporin production, resistance, and virulence phenotypes of the *CFP* disruptants are solely attributable to *CFP* disruption was confirmed by genetic complementation of *CFP* disruptant strain Gus-3 with a wild-type copy of *CFP* that restored cercosporin production, virulence, and resistance.

Sequence analysis of *CFP* indicates that it encodes a protein similar to several members of the MFS of transporter proteins (Marger and Saier 1993; Paulsen et al. 1996b), particularly those in the drug resistance family (Fig. 3), examples of which include TOXA, the putative HC-toxin transporter from *Cochliobolus carbonum* (Pitkin et al. 1996), SGE1, which confers crystal violet resistance in *Saccharomyces cer-*

visiae (Ehrenhofer-Murray et al. 1994), QACA, which confers multidrug resistance in *Staphylococcus aureus* (Paulsen et al. 1996a), and TRI12, the recently discovered putative trichothecene transporter in *Fusarium sporotrichioides* (Alexander et al. 1997). Members of this family are known to confer resistance to drugs and other toxins through an active export mechanism that is driven by proton motive force. While most of these transporters (including SGE1 and QACA) are involved in exporting toxins encountered by the organism in its external environment, others appear to export endogenously synthesized toxins (TOXA, TRI12, and now *CFP*).

The drug resistance family of the MFS includes two evolutionarily distinct clusters each with unique consensus motifs and topologies; those with 14 TMSs and those with 12 TMSs (Paulsen et al. 1996b). Although computer analysis predicted 13 TMSs in *CFP*, *CFP* has all motifs (D1, H, E, F in Figure 2) identified as diagnostic of 14 TMS MF transporters, and lacks

Fig. 3. Hydrophobicity plot of cercosporin facilitator protein (*CFP*) (Kyte and Doolittle 1982). Hydrophobic amino acids are plotted as positive values; hydrophilic amino acids as negative values. TM domains predicted by TMpred program are indicated with a single underline; TM domain predicted by sequence similarity to 14 TM domain MF transporters is double underlined.

Fig. 4. Constructs used to disrupt *CFP* and Southern analysis of wild-type and three disruptant strains of *Cercospora kikuchii*. **A**, Restriction map of the 6.5-kb *EcoRI* genomic fragment containing *CFP* excised from *pgCFP*. Arrowheads indicate relative positions of polymerase chain reaction (PCR) primers O2 and O3. **B**, Restriction map of *pgΔCFP::BAR* (minus *pGEM3zf+* vector) used to produce *CFP* disruption strains Bar-1 and Bar-2. **C**, Restriction map of *pgCFP::UIDA* construct used to produce *CFP* disruption strain Gus-3. Arrowheads indicate positions of PCR primers O3 and Otct. **D**, Southern analysis of *EcoRI*-digested genomic DNA probed with the radiolabeled 6.5-kb *EcoRI* fragment from *pgCFP*. Lane 1, *C. kikuchii* strain PR; lane 2, Bar-1; lane 3, Bar-2; and lane 4, Gus-3. Consistent with predictions for *CFP* disruptants, the wild-type 6.5-kb *EcoRI* fragment hybridizing with the *CFP* probe (lane 1) is replaced in transformants Bar-1 and Bar-2 by 2.1- and 5.0-kb bands (lanes 2, 3) and in Gus-3 by 3.9- and 9.7-kb bands (lane 4).

those associated exclusively with 12 TMS MF transporters. For this reason and because of CFP's greater overall similarity to 14 TMS transporters we propose that CFP possesses 14 TMSs and is responsible for cercosporin export in *C. kikuchii*—hence the name cercosporin facilitator protein. CFP also possesses motif C (Fig. 2), which is common to both 12 and 14 TMS MF drug/proton antiporters but not found in MF symporters. CFP is the first putative MF transporter of a fungal polyketide to be described.

Since sequence analysis indicates that CFP is an MFS transporter protein its role in cercosporin production appears to be regulatory rather than biosynthetic. The low levels of cercosporin accumulating in *CFP* disruptants (less than 5% of wild type) suggest that functional CFP is required for a fully functional cercosporin biosynthetic pathway. Hypothetically, when the cellular cercosporin concentration reaches a level above a critical theoretical threshold in the absence of export, as may happen in *CFP* disruptants, biosynthetic enzymes may be inhibited by end product (cercosporin) feedback. Alternatively, the transcription of cercosporin biosynthetic pathway genes may be down-regulated in response to perceived intracellular accumulation of cercosporin. Such a phenomenon occurs in *Pseudomonas syringae*, in which mutation of the gene encoding the syringomycin ABC-type drug transporter SyrD down-regulates the transcription of syringomycin biosynthetic genes (Quigley et al. 1993). Interestingly, *SyrD* mutants display a phenotype similar to that of *CFP* disruptants; they produce much less toxin (syringomycin) than does the wild type and are sensitive to it when it is exogenously supplied. *SyrD* disruptants are also dramatically reduced in virulence.

The amino acid sequence of CFP has 13 cysteine residues. Proteins with as few as five cysteine residues have been considered "cysteine-rich" (Pineiro et al. 1994). The relatively high cysteine content of CFP may suggest additional aspects of function. Plant proteins containing five or more cysteine residues have been implicated in the modulation of redox po-

Table 1. Cercosporin production by wild-type PR and *CFP* disruption strains

Strain	Cercosporin (nmol/mg dry wt) ^a	Cercosporin relative to PR Lt (%) ^b
PR Lt	30.1 ± 10.4	100
PR Dk	3.3 ± 1.4	11
Bar-1 Lt	0.8 ± 0.5	3
Bar-2 Lt	1.3 ± 0.8	4
Gus-3 Lt	1.2 ± 0.9	4

^a Mean and standard error for two experiments.

^b Lt = grown in continuous light for 4 days; Dk = grown in continuous dark for 4 days.

Table 2. Sensitivity of *Cercospora kikuchii* wild-type PR and *CFP* disruptants to cercosporin

Strain ^a	Colony diameter (mm) ± cercosporin ^b	Growth (%) ± cercosporin	Growth (%) inhibition
PR	14.1 ± 0.8 / 14.4 ± 0.7	98.3	1.7
Bar-1	8.0 ± 0.8 / 15.0 ± 0.5	53.3	46.7
Bar-2	8.1 ± 0.9 / 15.0 ± 0.8	54.2	45.8
Gus-3	8.5 ± 0.9 / 14.8 ± 0.9	57.6	42.4

^a Wild-type *C. kikuchii* PR and *CFP* disruptant strains grown in continuous light for 4 days with and without (±) 10 mM cercosporin.

^b Mean and standard error for two experiments.

Fig. 5. Genetic complementation of the *CFP* disruptant Gus-3. **A**, Potato dextrose agar plate showing cercosporin production (red-brown pigment) by *Cercospora kikuchii* wild-type strain PR and *CFP*-complemented strains 10-1 and 10-11. Cercosporin was not visually detected in Gus-3 mycelium. **B**, Agarose electrophoretic gel showing polymerase chain reaction (PCR) products amplified from PR, Gus-3, 10-1, 10-11 (lanes 2–5, respectively), with primers O2 and O3, designed to detect intact *CFP* (see Figure 4C). Lane 1 is *Hind*III-digested lambda molecular weight markers. **C**, PCR products amplified with primers O3 and Otct, designed to detect the disrupted copy of *CFP* engineered in transformant Gus-3 (see Figure 4D). Lanes are as in **B**.

Fig. 6. Soybean cultivar Lee-68, 15 days after inoculation with **(A)** water, **(B)** wild-type *C. kikuchii* PR, **(C)** cercosporin facilitator protein (CFP) disruptant Bar-1, and **(D)** *CFP* disruptant Gus-3.

tentials of various compounds (Pineiro et al. 1994). To date, however, there is no evidence for CFP involvement in cercosporin reduction. Even if the cysteine content of CFP has no connection to reductive capabilities, the high cysteine content of CFP and TOXA, which contains nine cysteine residues, merits further attention.

Genes encoding enzymes required for the biosynthesis of several fungal and bacterial toxins are clustered in the genomes of the producing organism. In several instances genes encoding toxin transporters have also been found within these clusters. For example, MF transporters TOXA (*C. carbonum*) and TRI12 (*F. sporotrichioides*) are clustered with the HC-toxin and trichothecene biosynthetic pathway genes, respectively, while the ABC transporter SyrD is clustered with the syringomycin biosynthetic pathway genes (Pitkin et al. 1996; Alexander et al. 1997; Quigley et al. 1993). Sequence similarity analysis of several kilobases of CFP-flanking DNA, however, has not revealed the presence of any genes known to be involved in polyketide metabolism, suggesting that the cercosporin biosynthetic genes are not closely linked to CFP (M. S. Rose, unpublished data).

This work, as well as research in *Cercospora nicotianae*, suggests that resistance to cercosporin in *Cercospora* spp. may be due to multiple mechanisms. A number of studies have described a strong correlation between reduction of cercosporin and cercosporin resistance (Daub et al. 1992; Leisman and Daub 1992; Sollod et al. 1992; Jenns et al. 1995). Recent work in *C. nicotianae* (Daub et al. 1998; Ehrenshaft et al. 1998, 1999) identified two genes that complement cercosporin-sensitive mutants (Jenns et al. 1995; Jenns and Daub 1995). The first gene, *SOR1* (singlet oxygen resistance), rescues five independently isolated mutants from sensitivity to cercosporin and other singlet-oxygen-generating photosensitizers to resistance. The second gene, *CGR1* (cercosporin resistance gene), restores cercosporin resistance to a mutant that is partially sensitive to cercosporin but is unaltered in resistance to other photosensitizers. Although mutations in *CGR1* and CFP produce similar cercosporin-sensitive phenotypes, they are distinct sequences. The mechanisms by which *SOR1* and *CGR1* contribute to cercosporin resistance remain to be clarified. We propose that CFP confers resistance by actively exporting cercosporin from the fungal cell. Although we have as yet no direct measurement of CFP-driven cercosporin efflux in *C. kikuchii*, such a mechanism is indicated by the structural similarity of CFP to MFS drug transporters and the phenotypic similarities of CFP and *SyrD* mutants. To further characterize the role of CFP in cercosporin resistance and to determine its utility as a source of transferable toxin resistance, we intend to express CFP in other toxin-sensitive organisms.

MATERIALS AND METHODS

Fungal strains, culture conditions.

Cercospora kikuchii wild-type strain PR was provided by J. B. Sinclair, University of Illinois, Urbana. *C. kikuchii* strains were grown in either PDB (Difco, Detroit, MI) or on potato dextrose agar (PDA) at 23°C under either continuous white fluorescent light (approximately $80 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) or in darkness. Liquid cultures were shaken at 180 rpm (Innova illuminated incubator shaker; New Brunswick Scientific, Edison,

NJ) in 125-ml Erlenmeyer flasks containing 50 ml of PDB. Dark-grown cultures were obtained by wrapping flasks with thick, plastic-lined, black cloth and aluminum foil. Pure cultures were maintained by hyphal tip transfer.

Plasmids.

pCFP, previously designated pcLE6 (Ehrenshaft and Upchurch 1991), contains a 1.9-kb cDNA of a full-length transcript cloned as an *EcoRI-XhoI* fragment in pBSISK⁺ (Stratagene, La Jolla, CA). To obtain a genomic clone, a genomic library of *C. kikuchii* DNA was constructed in the cosmid vector pCKB4 (Upchurch et al. 1991) by standard techniques. Each clone was stored individually in the wells of microtiter plates. After replication of the clones to nitrocellulose filters, colony blots were made by standard techniques and probed with ³²P-labeled insert DNA from pCFP. Analysis of the approximately 50-kb hybridizing cosmid indicated that a single 6.5-kb *EcoRI* fragment contained the hybridizing DNA plus 5' and 3' flanking sequences. This fragment was subcloned into pGEM3Zf⁺ (Promega, Madison, WI), resulting in pgCFP. Disruption plasmid pgΔCFP::BAR is pgCFP in which the 2.5-kb *Clal-HindIII* fragment containing the 5' end of CFP is replaced with the 3.1-kb *Clal-HindIII* BAR expression cassette subcloned from plasmid pNB1 (Upchurch et al. 1994). The BAR gene encodes phosphinothricin acetyltransferase, which enables transformed fungi to grow in the presence of the selective agent bialaphos (Straubinger et al. 1992). The 2.5-kb *Clal-HindIII* genomic DNA deleted in pgCFP::BAR was demonstrated by Northern analysis to encode no other transcript but that of CFP (M. S. Rose, unpublished result). Disruption plasmid pgCFP::UIDA was constructed by gel purifying the 6.5-kb *EcoRI* genomic DNA insert of pgCFP followed by treatment with T4 DNA ligase to circularize the fragment. Following heat inactivation of the ligase, the circular molecule was linearized with *HindIII* and ligated into the *HindIII* site of pNOM102. pNOM102 contains UIDA, which encodes a β-glucuronidase (GUS) able to cleave the substrate methyl umbelliferyl glucuronide (MUG) (Sigma, St. Louis, MO) to confer a (GUS⁺) fluorescent phenotype that can be visually scored (Roberts et al. 1989). pUCHI contains *hph*, a gene encoding hygromycin phosphotransferase that enables transformed fungi to grow in the presence of the selective agent hygromycin B (Turgeon et al. 1987).

Transformation.

Transformation of *C. kikuchii* has been described (Upchurch et al. 1994). Transformants were selected on minimal regeneration medium with either 20 or 50 μg of bialaphos or hygromycin B (Sigma, St. Louis, MO) per ml, respectively. Introduction of pgCFP into strain Gus-3 was by co-transformation with pUCHI. Introduction of gCFP::UIDA into PR was by co-transformation with the BAR plasmid pBPIT (Straubinger et al. 1992).

Nucleic acid manipulations.

DNA restriction digests and Southern blot analyses were performed as described by Sambrook et al. (1989). The Gene-clean II kit from Bio101 (La Jolla, CA) was used to purify DNA fragments from agarose gels. *C. kikuchii* genomic DNA was isolated from mycelium harvested from 5-day-old liquid cultures as described by Garber and Yoder (1983). Nitrocel-

lulose or Nytran Plus membranes (Schleicher and Schuell, Keene, NH) were used for nucleic acid blots following protocols supplied by the manufacturer. ³²P-radiolabeled probes were made with the random priming oligolabeling kit from Pharmacia (Thousand Islands, CA).

DNA sequencing and analysis.

pCFP and gCFP were sequenced with the Sequenase kit supplied by U.S. Biochemical (Cleveland, OH). Custom primers for sequencing were supplied by Integrated DNA Technologies (Coralville, IA). Nested deletion clones also used for sequencing were made with Erase-a-base (Promega, Madison, WI). Sequence analysis and management software used included MacVector (IBI, New Haven, CT), DNASTAR (DNASTAR, Madison, WI) and Gene Works (IntelliGenetics, Mountain View, CA). Transmembrane sequences were predicted with TMPred (Hoffman and Stoffel 1993). Searches for sequence similarity in the NCBI data bases were performed with BLAST (Gish and States 1993; Altschul et al. 1990). The GenBank accession number for CFP is AF091042.

PCR detection of CFP complementation.

PCR amplifications to detect intact CFP contained genomic DNA from selected strains, 1× PCR buffer with 2 mM MgCl₂ (Stratagene, La Jolla, CA), 240 nM dNTPs, 200 nM concentrations of primers "O2" (5'-GGATGATTTTGGAGAGCGA GAGCGAGTA-3') and "O3" (5'-ACGTGGTAGGTGGGC CGCTGATAGTC-3'), and 2 U of *Taq* DNA polymerase (Stratagene). Detection of disrupted CFP was as above except primer "O2ct" (5'-GTAAGCGCCCACTCCACATCTCCA-3') was substituted for primer O2. Following an initial denaturation step of 95°C for 1 min, 35 thermocycles of 94°C, 1 min; 60°C, 1 min; 72°C, 2.5 min were performed with a PTC-100 thermocycler (MJ Research, Watertown, MA) followed by a final 10-min, 72°C incubation.

Cercosporin production assay.

Mycelium suspensions were inoculated in PDB and incubated in the light for 4 days. Entire cultures (mycelium plus medium) were blended in a Waring blender for two 20-s pulses. A 10-ml aliquot of the suspension was treated with 1 vol of 5 N KOH (Jenns et al. 1989) in darkness for 4 h and clarified by low speed centrifugation. The pelleted mycelium was lyophilized and used for dry weight determination. Cercosporin concentrations in the supernatants were determined spectrophotometrically (Yamazaki and Ogawa 1972). Three culture flasks were assayed for each strain in the experiment and the entire experiment was repeated.

Cercosporin sensitivity assay.

Mycelium plugs, 5 mm in diameter, were inoculated mycelium side down onto divided petri plates containing PDA with one half of each plate amended with cercosporin dissolved in acetone to a final concentration of 10 µM, and the other half amended with an equal volume of acetone (Daub 1987). Radial growth was measured in two perpendicular directions after 4 days of incubation in light or darkness. Growth inhibition was calculated from measurements of each strain on cercosporin medium as a percentage of its growth on medium lacking cercosporin. Four colonies of each strain were measured for the experiment and the experiment was repeated.

Fungal pathogenicity on soybean.

One half gram of washed, dark-grown mycelium of each strain tested was placed in 20 ml of sterile water and blended in a Waring blender twice (two 20-s pulses). Mycelium suspensions were then sprayed, with a Preval atomizer (Yonkers, NY), onto the underside of leaves of 5-week-old soybean plants (cv. Lee-68). To promote infection, the plants were then placed in plastic bags and in reduced light for 48 h prior to being placed on benchtops in the greenhouse. Disease severity was assessed after 14 days (Upchurch et al. 1991). Six plants were inoculated with each fungal strain and the entire experiment was repeated.

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