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K.-R. Chung · M. Ehrenshaft · D. K. Wetzel
M. E. Daub

Cercosporin-deficient mutants by plasmid tagging in the asexual fungus *Cercospora nicotianae*

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Abstract We have successfully adapted plasmid insertion and restriction enzyme-mediated integration (REMI) to produce cercosporin toxin-deficient mutants in the asexual phytopathogenic fungus *Cercospora nicotianae*. The use of pre-linearized plasmid or restriction enzymes in the transformation procedure significantly decreased the transformation frequency, but promoted a complicated and undefined mode of plasmid integration that leads to mutations in the *C. nicotianae* genome. Vector DNA generally integrated in multiple copies, and no increase in single-copy insertion was observed when enzymes were added to the transformation mixture. Out of 1873 transformants tested, 39 putative cercosporin toxin biosynthesis (*ctb*) mutants were recovered that showed altered levels of cercosporin production. Seven *ctb* mutants were recovered using pre-linearized plasmids without the addition of enzymes, and these were considered to be non-REMI mutants. The correlation between a specific insertion and a mutant phenotype was confirmed using rescued plasmids as gene disruption vectors in the wild-type strain. Six out of fifteen rescued plasmids tested yielded cercosporin-deficient transformants when re-introduced into the wild-type strain, suggesting a link between the insertion site and the cercosporin-deficient phenotype. Sequence analysis of a fragment flanking the insert site recovered from one insertion mutant showed it to be disrupted in sequences with high homology to the acyl transferase domain of polyketide synthases from other fungi. Disruption of this polyketide synthase gene (*CTB1*) using a rescued plasmid resulted in mutants that were defective in cercosporin production. Thus, we provide the first molecular evidence that cercosporin is synthesized via a polyketide pathway as previously hypothesized.

Keywords Photosensitizer · Plasmid rescue · Active oxygen

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K.-R. Chung · M. Ehrenshaft · D. K. Wetzel · M. E. Daub
Department of Plant Pathology,
North Carolina State University,
Raleigh, NC 27695-7612, USA

M. E. Daub (✉)
Department of Botany,
North Carolina State University,
Raleigh, NC 27695-7612, USA
E-mail: margaret_daub@ncsu.edu
Tel.: +1-919-5133807
Fax: +1-919-5153436

Present address: K.-R. Chung
Citrus Research and Education Center,
University of Florida,
700 Experiment Station Road,
Lake Alfred, FL 33850-2299, USA

Introduction

Biological mutants have been demonstrated repeatedly to be a valuable resource for genetic and molecular analysis in modern biology. The requirements for a successful mutant hunt in fungi, however, can be time-consuming and labor-intensive, and systems developed for one organism may be unsuitable for another. Transposon tagging, used widely and with great success in many bacteria and a few eukaryotic systems, has not been widely developed for mutagenesis in filamentous fungi (Riggle and Kumamoto 1998). As an alternative, plasmid insertion and restriction enzyme-mediated integration (REMI) procedures (Schiestl and Petes 1991; Kuspa and Loomis 1992) have been successfully employed to introduce mutations at a relatively high frequency into some fungal species, and these methodologies and their associated pitfalls have been reviewed (Brown and Holden 1998; Riggle and Kumamoto 1998; Maier and Schäfer 1999). The greatest advantage of these approaches is that they permit random disruption of genes via plasmid insertions, which can subsequently be recovered by plasmid rescue in *E. coli* (Yang et al. 1996). As generally used, however, both plasmid insertion and REMI depend heavily on both an efficient transformation system and on sexual crosses to facilitate the correlation of specific

insertions with the mutant phenotype (Maier and Schäfer 1999). Due to these constraints, these approaches have only been exploited for mutagenesis in a few fungi. Numerous economically important phytopathogenic fungi lack a sexual stage, which precludes efficacious mutagenesis. So far, successful reports of REMI mutagenesis in imperfect fungi include the isolation of toxin-deficient mutants of *Alternaria alternata* (Akamatsu et al. 1997; Tanaka et al. 1999) and promoter tagging in *Aspergillus niger* (Shuster and Connelley 1999).

Cercospora species produce a light-activated perylenequinone phytotoxin named cercosporin, which is an important factor in their ability to parasitize plants (Upchurch et al. 1991; Daub and Ehrenshaft 2000). Cercosporin is unique among the well-characterized fungal toxins because it is a photosensitizer, and is able to absorb light energy and then react with oxygen to generate reactive oxygen species such as superoxide ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) (Daub and Hangarter 1983). Due to its production of $^1O_2$, cercosporin displays nearly universal toxicity to organisms as diverse as mice, bacteria, fungi, and plants. Cercosporin and its structural analogues also inhibit the growth of human tumor cells, inactivate protein kinase C, and are potent anti-viral compounds (Tamaoki and Nakano 1990; Hudson et al. 1994; Diwu 1995).

Cercosporin was first isolated from the soybean pathogen *C. kikuchii* in 1957 (Kuyama and Tamura 1957a, 1957b). Its chemical structure was determined 30 years ago (Lousberg et al. 1971; Yamazaki and Ogawa 1972). However, little progress has been made towards defining the pathway of cercosporin biosynthesis. Early labeling experiments suggested that cercosporin is synthesized through a polyketide pathway via condensation of acetate and malonate subunits (Okubo et al. 1975). Pathway intermediates or enzymes, however, have yet to be identified. The only gene so far characterized that has a role in cercosporin production is *CFP* (*c*ercosporin *f* acilitator *p* rotein), which was isolated from *C. kikuchii* (Callahan et al. 1999). This gene encodes a protein with homology to proteins of the major superfacilitator family that are involved in antibiotic resistance in fungi and bacteria. Disruption of *CFP* results in severely attenuated cercosporin production and increased sensitivity to exogenously added cercosporin (Upchurch et al. 2001, 2002).

The red color of cercosporin makes primary screening for alterations in its synthesis direct and straightforward. We have used this phenotype, therefore, as a plasmid insertion target to test whether these techniques can be adapted for use in asexual *Cercospora* fungi to generate tagged mutants.

Materials and methods

Fungal strains and transformation

A wild-type strain of *C. nicotianae* (ATCC 18366) was used as the recipient host for all transformation and mutagenesis experiments. Protoplasts were prepared and transformed as described previously (Ehrenshaft et al. 1995, 1998) with the fungal plasmid vectors pBARKS1 (see below), pBARGPE1 (Pall and Brunelli 1993) and pUCATPH1 (Lu et al. 1994). Both pBARKS1 (4.5 kb) and pBARGPE1 (5.5 kb) carry the Ignite/Basta (bialaphos)-resistance (*BAR*) gene (Avalos et al. 1989) under the control of the *Aspergillus nidulans trpC* promoter. In addition, pBARGPE1, which was originally designed for gene expression, carries a constitutive *gpdA* promoter (Punt et al. 1988) from *A. nidulans*. The plasmid pUCATPH1 (5.1 kb) carries a hygromycin resistance gene under the control of the *trpC* promoter and terminator. Approximately 1×10^7 protoplasts in 100 µl of STC (1.2 M sorbitol, 10 mM TRIS-HCl pH 7.5, 50 mM $CaCl_2$) were gently mixed with 20 µg of circular or linearized plasmid with or without restriction enzymes. The mixture was placed on ice for 20 min before addition of polyethylene glycol (PEG) and completion of the regular transformation and plating protocols. Linearized vector DNA was prepared by digestion with restriction enzymes at 37°C in a 100-µl volume, followed by extraction with phenol/chloroform, precipitation with ethanol, and resuspension in 20 µl of sterile water. Transformants were selected on medium containing 50 µg/ml bialaphos or 250 µg/ml hygromycin as appropriate. The assay for resistance to cercosporin was performed as described by Jenns et al. (1995). Tests for auxotrophy were conducted in minimal medium as previously described (Jenns et al. 1989).

Screening of toxin-deficient mutants

Because cercosporin is red in color, production of cercosporin can be detected easily on agar medium. All bialaphos- and hygromycin-resistant transformants were transferred onto potato dextrose agar (PDA) medium (Difco, Detroit, Mich.) and incubated in the light for 4–5 days at 28°C. To maximize resolution, PDA medium was prepared fresh, and plates contained less than 15 ml of medium. Transformants were selected for lack or altered timing of red pigment production, and examined further. Quantitative assays for cercosporin production were performed by extracting plugs from mycelial cultures with 5 N KOH as described previously (Jenns et al. 1989; Chung 2003). Toxin-altered strains were tested six to ten times by growth on thin PDA plates to confirm stability of the mutant phenotype. Individual mutants were designated as cercosporin toxin biosynthesis (*ctb*) mutants, followed by a number (*ctb* -1, *ctb* -2, etc.). Plasmids were designated like the mutants from which they were rescued, and different plasmids from the same mutant were distinguished by letter suffixes (pCTB14A, pCTB14B, etc). Disruption data are indicated as the number of cercosporin-deficiency mutants relative to the total number of transformants tested.

Southern hybridization analyses

Fungal DNA was purified as described by Woloshuk et al. (1989) or using a DNeasy Plant Mini kit (Qiagen, Valencia, Calif.). Standard procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern blotting (Sambrook and Russell 2001). The hybridization probes were labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, Ind.) using PCR as described by Chung et al. (1996). Primers (sense: 5′-TCTGCACCATCGTCAACCAC-3′, and anti-sense: 5′-AAACCCACGTCATGCCAGTT-3′) specific for the phosphinothricin acetyltransferase gene (*BAR*) in pBARKS1 were used to amplify and label the 575-bp *bar* fragment. The primers (sense: 5′-CTGCTCGACGCTACTGC-3′, and anti-sense: 5′-GCACAACCATGCAGACCTTT-3′) derived from the *Xho* I fragment of pCTB1 were used to amplify and label the 700-bp partial acyl transferase (AT) domain of the polyketide synthase gene (*CTB1*) from *C. nicotianae*. Conditions for PCR labeling of probes and Southern hybridization were as previously described (Chung et al. 1999). After hybridization, membranes were washed in 0.1×SSC and 0.1% SDS at 68°C for 1 h. Immunological detection of labeled probe using CSPD Ready-to-Use lumigenic substrate for alkaline

phosphatase was conducted according to the manufacturer's recommendations (Roche Molecular Biochemicals). Oligonucleotide primers for PCR were synthesized by the Molecular Genetics Facility, North Carolina State University (Raleigh, N.C.) and Genosys Biotechnologies (Woodlands, Tex.).

Plasmid rescue and DNA manipulation

Genomic DNA flanking plasmid insertion sites in putative mutants was recovered by the method of Yang et al. (1996) with some modifications. Total DNA (5–10 μg) was partially digested with a restriction enzyme that did not cleave in the transforming vector or with an enzyme that only digested the vector at one site in a region not required for plasmid replication in *E. coli*. The digested DNA was purified with phenol/chloroform, precipitated with ethanol, and ligated in a 100-μl reaction with T4 DNA ligase (Promega, Madison, Wis.). Three microliters of this ligation mixture was transformed into the *E. coli* strain DH10B (Life Technologies BRL, Gaithersburg, Md.) by electroporation (BioRad, Hercules, Calif.). Transformants were grown on medium containing ampicillin, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl- β-D-thiogalactopyranoside), and white colonies were screened for the presence of inserts. The rescued plasmid DNA was purified using the Wizard DNA kit (Promega). DNA sequencing was conducted at the Molecular Genetics Facility, University of Georgia (Athens, Ga.). Database searches and comparisons were performed using the BLAST network service at the National Center of Biotechnology Information (Altschul et al. 1997). A search for functional motifs was performed in the PROSITE database using the ExPASy Molecular Biology Server (http://us.expasy.org).

Results

Transformation

A wild type strain of *C. nicotianae* was transformed with either circular or pre-linearized pBARKS1 or pBARGPE1 plasmid DNA with or without added restriction enzymes. All pre-linearized and circular DNAs were first extracted with phenol/chloroform and then precipitated with ethanol to inactivate and remove the enzyme prior to transformation. Transformation was then performed with none, or with 10 or 50 U of one of the linearizing restriction enzymes (*Bam*HI, *Eco* RI, *Eco* RV, *Sac* I, and *Xho* I) added to the reaction mixture, and bialaphos-resistant colonies were recovered. Results are summarized in Table 1. In the absence of enzyme in the transformation mixture, pre-linearizing of either plasmid decreased the transformation frequency. With the addition of enzyme, pre-linearizing of plasmids either did not affect or slightly increased the transformation frequency as compared to transformation frequencies with circular plasmids. Overall, however, the use of restriction enzymes during the transformation procedure significantly decreased the transformation frequency (by up to 90% or more), with the lowest rates of transformation occurring with the highest levels of enzyme, suggesting that restriction enzymes may have a deleterious effect on protoplast viability.

Isolation of cercosporin toxin biosynthesis (*ctb*) mutants

Because cercosporin is red in color, toxin-deficient mutants can be easily detected on PDA agar medium by looking for changes in pigmentation (Fig. 1A). The wild-type fungus and most transformants displayed the distinct dark-red colony color that is due to secretion and accumulation of cercosporin around the hyphae in culture. Transformants that exhibited any change in color, color pattern, or timing of cercosporin production were scored as cercosporin toxin biosynthesis (*ctb*)

Table 1 Transformants of*C. nicotianae* recovered from transformations with and without addition of restriction enzyme using the plasmids pBARKS1 and pBARGPE1

Enzyme	Quantity (U)[a]	pBARKS1				pBARGPE1	
		Circular[b]		Linear[c]		Circular	Linear
		(Exp. 1)	(Exp. 2)	(Exp. 1)	(Exp. 2)		
*Bam*HI	0	324	231	235	147	476	289
	10	33	9	57	18	78	73
	50	26	6	3	11	4	7
*Eco*RI	0	324	231	103	136	476	297
	10	30	23	107	81	218	175
	50	9	7	32	26	38	52
*Eco*RV	0	324	231	92	87	476	226
	10	10	9	14	25	36	14
	50	12	6	4	7	1	1
*Sac*I	0	324	231	125	71	nd[d]	nd
	10	41	9	54	13	nd	nd
	50	6	8	6	12	nd	nd
*Xho*I	0	324	231	136	163	476	343
	10	57	14	138	70	135	158
	50	2	8	47	32	36	69

[a]The indicated quantity (expressed in units of activity) of enzyme was mixed with the DNA, and then used for transformation
[b]Numbers of transformants recovered using circular plasmid DNA together with enzyme added as indicated to the transformation mix
[c]Numbers of transformants recovered using pre-linearized plasmid DNA together with enzyme added as indicated to the transformation mix. Plasmids were linearized with the same enzyme as used for transformation
[d]nd, not determined

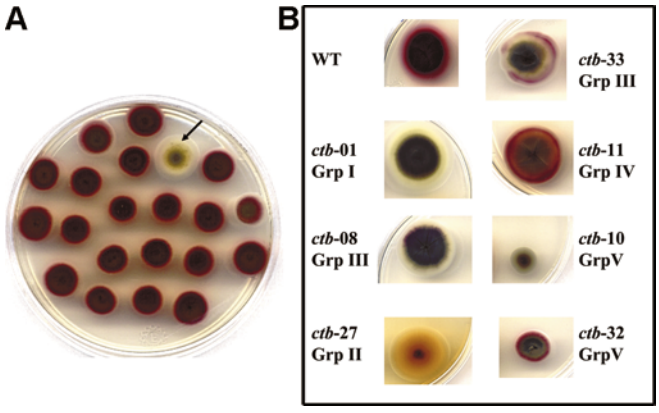

Fig. 1A, B Screening and phenotypic characterization of cercosporin-deficient mutants of *C. nicotianae*. **A** Production of cercosporin is indicated by the secretion of red pigment on potato dextrose agar plates. A putative cercosporin-deficient mutant is indicated by the *arrow*. **B** Colonies of selected cercosporin toxin biosynthesis (*ctb*) mutants. The wild-type (WT) strain produced uniform red pigmentation (cercosporin). Putative insertional mutants altered in cercosporin production lacked visible red pigmentation (*ctb* -1, 10), produced alternate pigments (*ctb* -27), or had reduced amounts or altered patterns of production (*ctb* -33, 11, 8, 32)

mutants (Fig. 1B). Mutant recovery was highly dependent on the combination of plasmids and restriction enzymes used (Table 2). No mutants were found upon screening of 1500 transformants transformed with plasmid pUCATPH1 treated with four different enzymes (data not shown). No putative mutants were recovered using circular pBARKS1 or pBARGPE1 plasmids without added enzyme. By contrast, mutants were recovered using pre-linearized pBARKS1 or pBARGPE1 plasmid either with or without the addition of restriction enzymes, as well as with circular plasmids with the addition of restriction enzymes. In total, 39 mutants were recovered from 1873 colonies derived from transformation with either circular or linearized pBARKS1 or pBARGPE1 DNA. Most of these mutants (32) were derived from transformation in the presence of restriction enzymes (REMI mutagenesis); however, seven mutants were recovered without the use of added restriction enzymes (insertional mutagenesis).

Confirmation that the addition of restriction enzyme was required for the generation of *ctb* mutants transformed with circular plasmids came from an independent study that used screening for cercosporin production to determine the mutagenesis rate. Over 10,000 *C. nicotianae* colonies derived from transformation with undigested, circular DNA from five different transposon-containing plasmid constructs were screened for alterations in cercosporin production phenotype, and not a single mutant was found (D. K. Wetzel, unpublished data). This additional study confirmed that transformation of *C. nicotianae* with circular plasmid constructs in the absence of restriction enzymes is not an effective means of generating stable mutants.

Phenotype of cercosporin production mutants

The 39 *ctb* mutant strains altered in cercosporin production exhibited various phenotypes, ranging from no detectable cercosporin to reduced production; representative examples are shown in Fig. 1B. Some transformants produced yellow, green, or dark pigments rather than the red-colored cercosporin. Some produced

Table 2 Frequency of cercosporin toxin biosynthesis (*ctb*) mutants generated by transformation of *C. nicotianae* using plasmid pBARKS1 or pBARGPE1 with or without added restriction enzymes

Plasmid[a]	Enzyme added[b]	pBARKS1		pBARGPE1	
		Frequency	Mutants	Frequency	Mutants
Circular	None	0/324 (0%)	–	0/476 (0%)	–
Pre-linearized/*Bam*HI	None	2/235 (0.85%)	(*ctb* 1, 5)	0/289 (0%)	–
Pre-linearized/*Eco*RI	None	0/103 (0%)	–	0/297 (0%)	–
Pre-linearized/*Eco*RV	None	5/92 (5.4%)	(*ctb* 6–10)	0/226 (0%)	–
Pre-linearized/*Sac*I	None	0/125 (0%)	–	nd[c]	–
Pre-linearized/*Xho*I	None	0/136 (0%)	–	0/343 (0%)	–
Pre-linearized/*Bam*HI	*Bam*HI	2/60 (3.3%)	(*ctb* 11–12)	0/80 (0%)	–
Pre-linearized/*Eco*RI	*Eco*RI	7/139 (5.0%)	(*ctb* 13–19)	0/227 (0%)	–
Pre-linearized/*Eco*RV	*Eco*RV	7/18 (38.8%)	(*ctb* 20–26)	1/15 (6.7%)	(*ctb* 39)
Pre-linearized/*Sac*I	*Sac*I	4/60 (6.7%)	(*ctb* 27–30)	nd	–
Pre-linearized/*Xho*I	*Xho*I	2/185 (1.1%)	(*ctb* 31–32)	0/227 (0%)	–
Circular	*Bam*HI	2/59 (3.4%)	(*ctb* 2, 4)	2/82 (2.4%)	(*ctb* 33–34)
Circular	*Eco*RI	0/39 (0%)	–	0/256 (0%)	–
Circular	*Eco*RV	0/22 (0%)	–	0/37 (0%)	–
Circular	*Sac*I	1/47 (2.1%)	(*ctb* 3)	nd	–
Circular	*Xho*I	0/59 (0%)	–	4/171 (2.3%)	(*ctb* 35–38)
Total mutants		32	–	7	–

[a]The number of cercosporin-deficient transformants identified and the total number of transformants recovered are listed for each experiment

[b]Either 0, 10 or 50 U of the indicated enzyme was added to the transformation mixture

[c]nd, not determined

low amounts of cercosporin or only synthesized the compound at the margin of the colony. Cercosporin was also purified and quantified from cultures. When assayed on day 7, the wild-type strain accumulated an average of 38.5 nmol of cercosporin per agar plug (6 mm in diameter), while the putative mutants accumulated less than ~65% of wild-type amounts. The 39 *ctb* mutants were tentatively divided into five groups primarily based on cercosporin production, pigmentation, and growth rate. Group I mutants (*ctb* -1, 6, 14, 29, 30, 35 and 36) did not produce cercosporin and never showed red pigmentation in PDA medium, but produced a green pigment. Group II mutants (*ctb* -27 and 28) did not produce cercosporin, but produced a yellow pigment. Group III mutants (*ctb* -7, 8, 12, 13, 15–23, 33, 34 and 39) showed red coloration only at the margin of the colony. Group IV mutants (*ctb* -2, 3, 4, 5, 11, 24, 25, 31, 37 and 38) produced significantly reduced (at least 35%) amounts of cercosporin compared to wild type. Group V comprises mutants (*ctb* -9, 10, 26 and 32) that showed reduced amounts of cercosporin and lower growth rates. None of the mutants were auxotrophs, and all mutants displayed the normal wild-type level of cercosporin resistance (data not shown). All of these mutant phenotypes were consistently observed in the laboratory for over a year, indicating that the mutations are stable.

Integration events

The number of copies of integrated pBARKS1 or pBARGPE1 in transformants was determined by Southern-hybridization using the selection marker *BAR* gene as a probe (Fig. 2A–D). In each case, the genomic DNA from the *ctb* mutants was digested with the enzyme used for linearization and/or transformation (Fig. 2A, *Bam* HI; B, *Xho* I; C and D, *Eco* RV). In general, a REMI event is defined as integration of a digested plasmid into a matching restriction site in the genome. If a plasmid is integrated into the host genome at its homologous restriction sites (via REMI), digestion of the genomic DNA of the transformant with the enzyme used for transformation should yield a fragment identical in size to the original vector. Hybridization revealed that most transformants contained multiple pBARKS1 or pBARGPE1 inserts, both longer and shorter than the original vectors (Fig. 2A–D), indicating that either one or both of the terminal restriction sites were destroyed. In some transformants, a 4.5-kb band (indicated by the arrowhead in Fig. 2D) or a 5.5-kb band (as indicated by the arrows in Fig. 2A and B) similar in size to plasmids pBARKS1 and pBARGPE1, respectively, was identified, suggesting that at least some copies of plasmids had integrated via the compatible ends of the enzyme recognition sites. There was no difference in number of integration sites or tendency to lose the vector restriction sites between pre-linearized and circular plasmid. The same DNA was also hybridized to a probe for the cercosporin resistance gene *CRG1* (Chung et al. 2003) and fragments of the expected sizes were detected, indicating that the genomic DNA had been successfully digested prior to electrophoresis (data not shown).

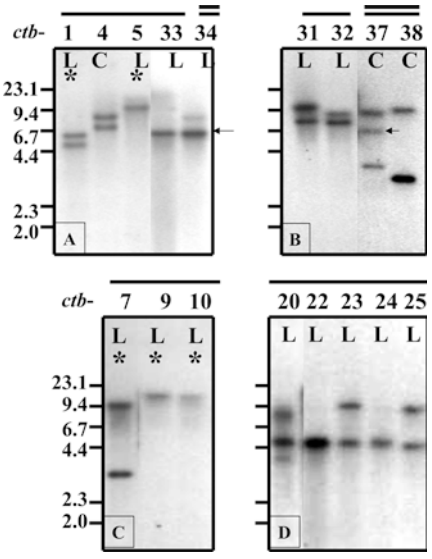

Fig. 2A–D Southern hybridization analysis of *C. nicotianae* cercosporin-deficient (*ctb*) mutants. Fungal DNA from cercosporin-deficient mutants (the mutant number is indicated at the *top* of each lane) obtained by transformation with either linearized (L) or circular (C) of pBARKS1 (indicated by the *single horizontal lines* at the *top* of each gel) and pBARGPE1 (indicated by *double lines*). The *asterisks* indicate transformants that were obtained from pre-linearized plasmid without the addition of extra restriction enzymes (non-REMI events). DNA was digested with *Bam* HI (**A**), *Xho* I (**B**), or *Eco* RV (**C, D**), the enzymes used for linearization and/or transformation. The digested DNA was hybridized to a PCR-generated *BAR* probe. Multiple integration sites were detected in most samples. The bands of similar size to either the 4.5-kb pBARKS1 (indicated by an arrowhead) or the 5.5-kb pBARGPE1 (indicated by arrows) are likely to be the products of integration via restriction sites. The size markers were *Hin* dIII fragments of bacteriophage lambda DNA, and their sizes are indicated in kb

Plasmid rescue and targeted gene disruption

Because *Cercospora* lacks a sexual stage, genetic segregation cannot be used to correlate a specific insertion with a mutant phenotype. To identify the disrupted genes responsible for the cercosporin-deficient mutant phenotype, we adopted the strategy of using rescued plasmids (containing *C. nicotianae* genomic DNA adjacent to the vector sequences) as gene disruption vectors. In this approach, rescued plasmids were introduced into the wild-type *C. nicotianae* strain, and transformants were screened for alterations in cercosporin production.

To recover plasmid sequences and genomic DNA flanking the integration sites, total fungal DNA from seven putative mutants (*ctb* -1, 6, 10, 14, 28, 29, and 35) was digested with *Eco* RI, self-ligated, and then transformed into *E. coli*. The rescued plasmids were propagated in *E. coli* cells. At least ten plasmids from

each mutant were purified and first examined in an agarose gel. The rescued plasmid DNA was generally much larger than the original plasmids (data not shown), indicating that they indeed carried some *C. nicotianae* genomic DNA. Only plasmids larger than the original pBARKS1 or pBARGPE1 were selected, and plasmids of the same size from one mutant were considered to be identical. In total, 15 plasmids were rescued from the seven different *ctb* mutants. Only one plasmid was recovered from mutants *ctb* -1, *ctb* -10 and *ctb* -28; multiple plasmids were recovered from mutants *ctb* -6, *ctb* -14, *ctb* -29 and *ctb* -35. Plasmids were designated by the name of the mutant, followed by a letter in cases where multiple plasmids were recovered, e.g. pCTB14A.

The rescued plasmids were individually transformed into the wild type *C. nicotianae* strain, and bialaphos-resistant transformants were screened for the cercosporin-deficient phenotype. Cercosporin-deficient mutants were recovered with low efficiency using six of the rescued plasmids. These plasmids and the frequency of recovery of disruption mutants (number of cercosporin-deficient transformants identified over the total number of transformants recovered) were: pCTB1 (1/264, 2/55, 2/77), pCTB10 (2/194, 0/74, 2/31), pCTB14A (1/189), pCTB28 (2/174), pCTB29C (1/14), and pCTB35C (1/9). No cercosporin-deficient mutants were recovered using the plasmids pCTB29B (0/88), pCTB35D (0/93), pCTB35E (0/90), pCTB35F (0/90), and pCTB35G (0/94). Transformation with the rescued plasmids pCTB14B, pCTB14C, pCTB6A, and pCTB6D resulted in only small numbers of transformants (< 40); thus no conclusions could be drawn in these cases. The frequencies of gene disruption seen in these experiments are similar to those in previously reported disruption experiments in *C. nicotianae* (Chung et al. 1999, 2003). This approach of using rescued plasmids for direct disruption in the wild type was able to distinguish which insertion event was associated with the change in phenotype in the mutants *ctb* -14, *ctb* -29, and *ctb* -35. In each case, only one of the plasmids rescued from these strains, pCTB14A, pCTB29C, or pCTB35C, was able to disrupt cercosporin synthesis.

Sequencing of flanking DNA and identification of polyketide synthase gene (*CTB1*) sequences

Recreating cercosporin-deficient phenotypes using rescued plasmids suggested that the *C. nicotianae* genomic fragment present in these plasmids was likely to include sequences necessary for toxin production. We therefore sequenced the flanking DNA fragment in plasmid pCTB1 rescued from mutant *ctb* -1, which is completely deficient in cercosporin production. The plasmid was digested with restriction enzymes that are present in the multiple cloning sites of pBARKS1, and a 0.8-kb *Xho* I fragment was subcloned and sequenced. This sequence codes for part of a protein with high homology to polyketide synthases from several fungi including *Aspergillus nidulans, A. fumigatus*, *A. parasiticus*, *Colletotrichum lagenarium*, *Gibberella fujikuroi,* and *Nodulisporium* sp. (Fig. 3). The predicted 239 amino acids specified by this sequence, part of the putative polyketide synthase (*PKS*) gene in *C. nicotianae* (named *CTB1*), shares 30–35% identity and 50–57% similarity to the region required for acyl transferase (AT) activity in the other fungal polyketide synthases. Interestingly, the AT domain encoded by the rescued fragment shares no similarity to that of the PKS encoded by *TOX1* of *Cochliobolus heterostrophus* (data not shown).

To determine the copy number of *CTB1* in *C. nicotianae*, Southern analysis was performed using restriction enzymes that do not cut within the recovered sequence. Only a single band was identified in all digests (Fig. 4). A faint band above 4.4 kb was observed in the *Hind* III digest, which probably results from incomplete digestion. The data thus indicate that the *C. nicotianae* genome contains a single copy of *CTB1*.

Analysis of the pCTB1 plasmid and disruption mutants

To characterize further the pCTB1-derived disruption mutants, we first sequenced the recovered plasmid. Inverted primers derived from the 0.8-kb *Xho* I DNA sequence were used to recover and sequence the entire flanking sequence and junction regions in plasmid pCTB1 (Fig. 5). Sequencing analysis indicated that the flanking region contained a DNA fragment of 1575 bp. Relative to the original pBARKS1 vector (4.5 kb) (Fig. 5A), a 0.6-kb DNA fragment (*Spe*I-*Pst*I) was deleted in the rescued plasmid, resulting in a truncated pBARKS1 with a size of 3.9 kb (Fig. 5B). Further analysis revealed that the *Xho* I and *Sal* I sites in the polylinker were also missing, whereas the rest of the sites in the polylinker, including those for *Eco* RI and *Eco* RV, were still present (Fig. 5B). These results confirmed the *CTB1* sequence in the recovered plasmid, and also indicated that the insertion event resulted in deletions in the plasmid.

Four independent disruption mutants obtained by transforming the rescued pCTB1 plasmid into the wild type strain were then characterized further. As with the original *ctb* -1 mutant, the disruption mutants were completely defective in cercosporin production (Fig. 6A). Southern analysis using the 0.8-kb *Xho* I fragment as a probe revealed that they contained a disrupted version of *CTB1* (Fig. 6B). Hybridization of *Eco* RI + *Eco*RV-digested DNA from the wild-type isolate resulted in a single fragment of 4 kb (Fig. 6B, lane 1). A restriction map of the 4-kb genomic region in relation to the plasmid probe is shown in Fig. 6C. Hybridization of DNA from the *ctb* -1 mutant identified a single, but larger band of 5.5 kb (Fig. 6B, lane 2), indicating that the mutant harbored the expected altered version of *CTB1* due to plasmid insertion [the truncated 3.9-kb pBARKS1 plus 1.6 kb of flanking DNA (Fig. 5B)]. Additional mutants derived from transformations with

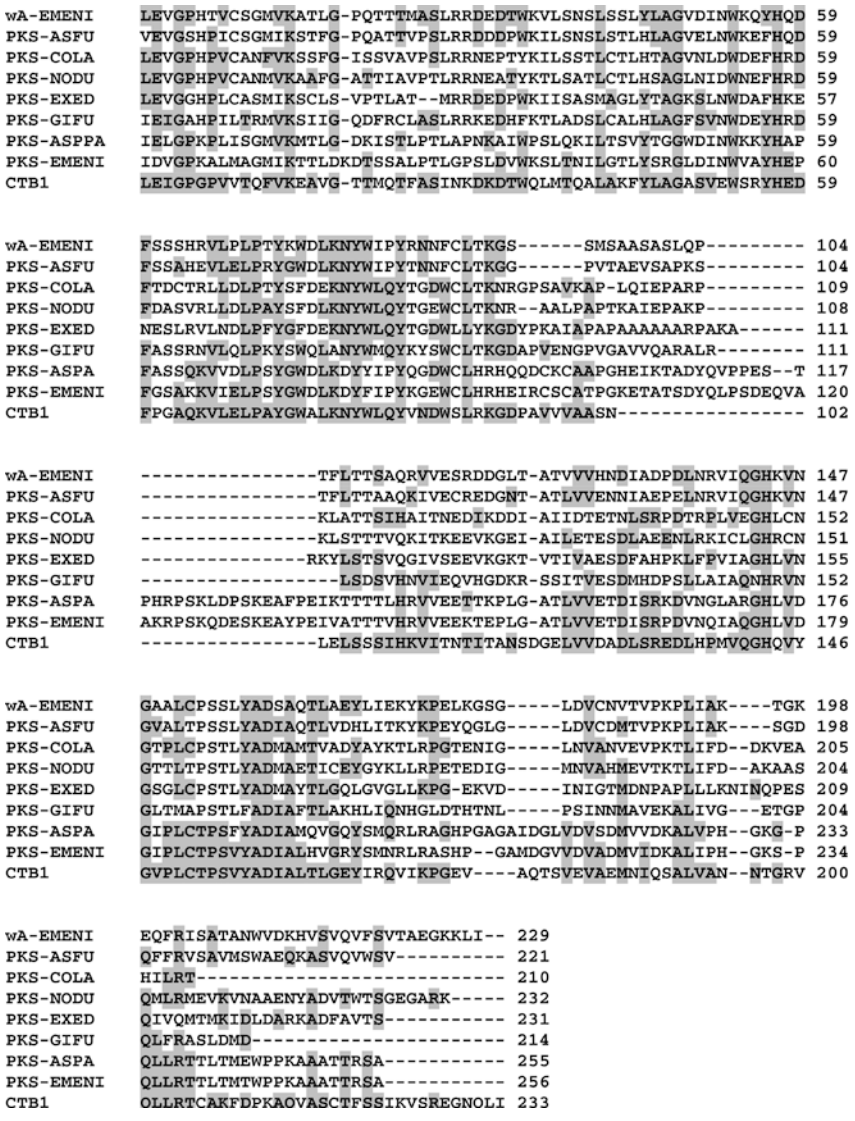

Fig. 3 Comparison of partial amino acid sequences of the putative polyketide synthase deduced from the DNA fragment contained in the rescued plasmid from the *C. nicotianae* tagged *ctb* -1mutant (CTB1) with other polyketide synthase (PKS) sequences. Amino acid sequences shown are those of the conidial green pigment synthase from *Aspergillus nidulans* (wA-EMENI, Accession No. Q03149), and PKS sequences from *A. fumigatus* (PKS-ASFU, Y17317), *Colletotrichum lagenarium* (PKS-COLA, S60224), *Nodulisporium* sp. (PKS-NODU, AF151533), *Fusarium fujikuroi* (PKS-GIFU, AJ278141), *A. parasiticus* (PKS-ASPA, Q12053), and *A. nidulans* (PKS-EMENI, Q12397). Amino acid identities to those of the predicted *C. nicotianae* protein are *shaded*

pCTB1 contained hybridizing bands of 5.3 kb or 6.3 kb (Fig. 6B, lanes 3–6), indicating that they also contained a disrupted version of *CTB1*. Since insertion was performed using a single flanking region in pCTB1, the mechanisms involved in the formation of the 5.3- and 6.3-kb hybridizing bands remain uncertain.

Discussion

In this work we have demonstrated the feasibility of using plasmid-tagging mutagenesis to generate *C. nicotianae* mutants altered in the ability to produce the pathogenicity factor cercosporin. Mutagenesis via plasmid insertion and REMI can produce multiple insertions. In organisms with a sexual cycle, backcrossing of a putative tagged mutant with a non-mutant isolate is generally used to determine which insertion is responsible for the altered phenotype. In organisms such as *C. nicotianae*, which lacks a sexual stage, this route is obviously unavailable. Direct disruption using the rescued plasmids as described in other fungal systems (Bölker et al. 1995; Kuspa and Loomis 1992; Riggle and Kumamoto 1998) provides an alternative means to correlate plasmid insertion with the altered phenotype of interest. However, the strategy has not been widely used, probably because of the low frequency of homologous integration, as we also observed in *C. nicotianae*. Disruption of wild-type genes in *C. nicotianae* usually results in a low frequency of disruptants (< 5% of the total number of transformants recovered; Ehrenshaft et al. 1995, 1998; Chung et al. 1999; Ehrenshaft and Daub 2001), suggesting that homologous integration is not a favored event in *C. nicotianae*. The rarity of this event may explain why we only recreated cercosporin production mutants with low frequency from 6 rescued plasmids, although we transformed 15 rescued plasmids into the wild type.

In many other fungi, transformation with linearized plasmids and with restriction enzymes enhances transformation frequency (Lu et al. 1994; Shi and Leung 1995; Akamatsu et al. 1997; Granado et al. 1997). In contrast, the highest transformation frequencies

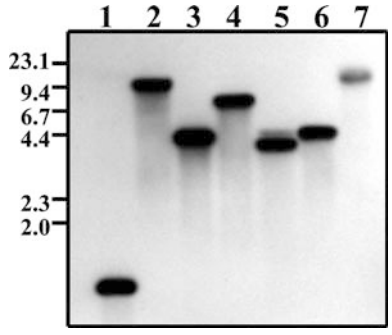

Fig. 4 Southern analysis of *CTB1* in *C. nicotianae*. Genomic DNA was purified and digested with *Bam* HI (lane 1), *Cla* I (lane 2), *Eco* RI (lane 3), *Eco* RV (lane 4), *Hin* dIII (lane 5), *Pst* I (lane 6), or *Xba* I (lane 7), none of which cleaves within the 0.8-kb *Xho* I fragment of *CTB1*. The digested DNA was hybridized to a PCR-labeled probe specific for the *Xho* I fragment subcloned from pCTB1. The faint band in lane 5 probably results from incomplete digestion

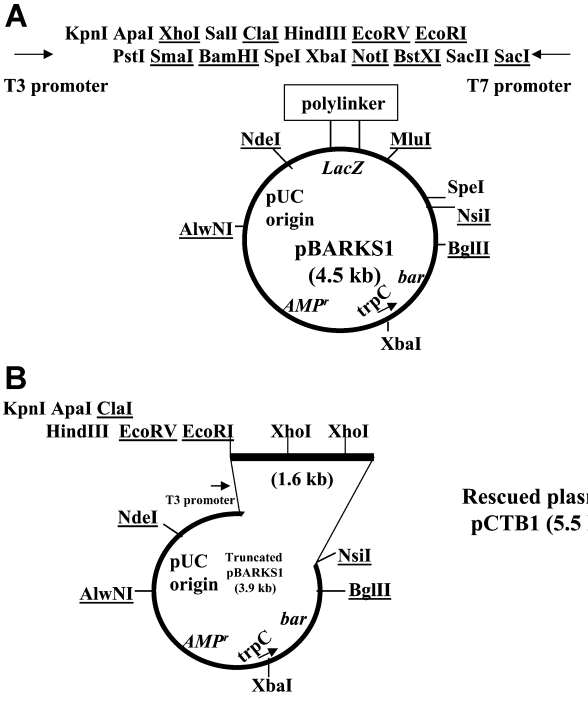

Fig. 5A, B Maps of the transformation vector pBARKS1 (**A**) and the rescued plasmid pCTB1 from the *ctb* -1 tagged mutant (**B**). **A** Restriction map of transformation vector pBARKS1 used to create insertional mutants of *C. nicotianae*. Unique restriction sites are *underlined*. *AMP*, ampicillin resistance gene; PtrpC, *trpC* promoter of *A. nidulans*; *BAR*, bialaphos resistance gene. **B** Map of the rescued plasmid pCTB1 with a truncated pBARKS1 and a flanking region containing a partial fungal polyketide synthase gene (*CTB1*) from *C. nicotianae*. Sequencing analysis uncovered a deletion of the *Spe* I-*Pst*I fragment of pBARKS1 in the rescued plasmid. Both the *Xho* I and *Sal* I sites in the pBARKS1 polylinker were also missing in the rescued plasmid

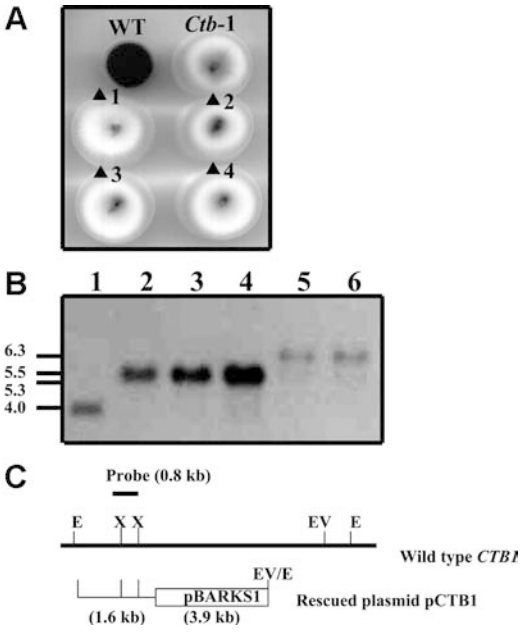

Fig. 6A–C Gene disruption in the wild type strain using the pCTB1 rescued plasmid from the *ctb* -1 mutant. **A** Phenotypic characterization of cercosporin toxin (dark pigment) production by the *C. nicotianae* wild type (WT) strain, the original insertional mutant *ctb* -1, and the disruption mutants (▲1–4) resulting from transformation of the wild type strain with pCTB1. **B** Southern-analysis of disrupted strains. Fungal DNA from the wild-type strain (lane 1), the insertional mutant *ctb* -1 (lane 2) and the disruption mutants generated by transformation with the rescued plasmid pCTB1 (lanes 3–6). DNA was digested with *Eco* RI and *Eco* RV. Blots were hybridized to the PCR-generated *CTB1* probe (0.8-kb *Xho* I fragment subcloned from pCTB1) and washed at high stringency. The sizes of the hybridizing bands are indicated in kb. **C** Restriction enzyme map of the genomic region around *CTB1* in the *C. nicotianae* wild type. Enzyme abbreviations: E, *Eco* RI; X, *Xho* I; EV, *Eco* RV. The 0.8-kb *Xho* I fragment used as a DNA probe is also indicated

obtained in this study were obtained by transformation of *C. nicotianae* with circular, undigested plasmid DNA (Table 1). Pre-linearization of plasmid DNA with any of the enzymes used in this study reduced the number of transformants recovered. If additional enzyme was added during the transformation process itself, the frequency decreased even further, with proportionally greater decreases resulting with increased levels of enzyme. This finding suggests that restriction enzymes may have a detrimental effect on *C. nicotianae* protoplast viability or regeneration, a phenomenon that has also been observed in other fungi (Shi and Leung 1995; Akamatsu et al. 1997; Granado et al. 1997; Sánchez et al. 1998). Although the overall transformation frequency in *C. nicotianae* decreased when linearized plasmids and restriction enzymes were utilized, the use of pre-linearized plasmids and restriction enzymes apparently can promote plasmid integration and the production of mutations.

Our results are similar to those of other studies in which factors such as the type and conformation (linear vs. circular) of plasmids, the type and amount of restriction enzymes, and the precise transformation protocols have been shown to affect REMI tagging (Maier and Schäfer 1999). In REMI events, the restriction enzyme is believed to bind to the ends of linearized plasmid DNA, and to promote integration into the

genome plasmid via a nonhomologous end-joining mechanism (Manivasakam and Schiestl 1998). However, the ability of a plasmid to be integrated into the genome via compatible endonuclease restriction sites is highly species-dependent, and the mechanisms of REMI integration and mutagenesis remain unclear (Maier and Schäfer 1999).

In our study, most transformants contained multiple vector copies, irrespective of the vector configuration used (linear vs. circular) or the presence of enzyme in the transformation mix. This result contrasts with those in other fungi (*M. grisea*, *U. maydis*, and *C. heterostrophus*), in which the addition of restriction enzymes increased the number of single-copy integration events (Lu et al. 1994; Bölker et al. 1995; Shi and Leung 1995). Southern hybridization indicated that at least one copy of the vector was integrated into the fungal genome via compatible ends created by the respective enzyme. Most transformants also contained hybridizing bands larger than pBARKS1 or pBARGPE1, suggesting that one or both restriction sites were destroyed upon integration. Sequence analysis of the rescued pCTB1 plasmid confirmed that restriction enzyme sites present in the original pBARKS1 plasmid were missing in the rescued plasmid. Other integration events may have occurred via illegitimate recombination of tandemly-repeated or inverted-repeat configurations. Hybridizing fragments smaller than pBARKS1 or pBARGPE1 probably resulted from deletion and/or recombination. Plasmid insertion can cause deletions and translocations, as observed in many other fungi (Turgeon et al. 1995; Yun et al. 1998; Ito and Scott 1997; Sweigard et al. 1998; Linnemannstöns et al. 1999). Since the restriction enzymes significantly reduced the transformation rate, we cannot rule out the possibility that some of our mutants may be derived from deletion or rearrangement caused by enzymes.

The frequency of recovery of cercosporin synthesis mutants in this study was very high. Cercosporin biosynthesis is affected by numerous environmental cues as well as by developmental stage; these include light intensity and duration, temperature, medium composition, medium volume, culture age, growth stage, and developmental stage (vegetative vs. reproductive) (Jenns et al. 1989; Ehrenshaft and Upchurch 1991, 1993; Daub and Ehrenshaft 2000). A recent study also demonstrated that production of cercosporin is affected by calcium/calmodulin signaling in *C. nicotianae* (Chung 2003). In this present study, cercosporin-deficient phenotypes could be due to mutations in genes that alter fungal responses to light, nutrients, temperature or other environmental factors, or that alter growth and development, and only a fraction would be expected to be mutant in genes involved in the biosynthetic pathway itself. For our analysis, we recovered a plasmid from a mutant that was completely deficient in toxin production, as this type of mutant was the most likely to yield genes directly involved in biosynthesis. Fungal polyketide synthase genes are very large (typically 6–8 kb encoding over 2000 amino acids with several function domains) (Yang et al. 1996), and it is logical to assume that their rates of mutation would be higher than for other, smaller genes. A high frequency (~40%) of spore morphogenetic mutants has been observed with REMI in *M. grisea* (Shi et al. 1998). We are now using the same protocol and strain to generate REMI mutants that are sensitive to cercosporin. In this work, our mutation frequency is much lower (7 mutants out of 33,020 screened), confirming our hypothesis that the high frequency of *ctb* mutants recovered is probably due to the very large number of factors that affect cercosporin synthesis.

One of the most significant aspects of this study is the confirmation that cercosporin biosynthesis involves a polyketide synthase. It was proposed as long ago as 1975 (Okubo et al. 1975) that cercosporin is synthesized via the polyketide pathway. These studies were limited, and were based on structure and on some labeling experiments. Unfortunately, no further experimentation followed, leaving a large gap in our understanding of the biosynthesis and regulation of this important phytotoxin. In this study we have provided initial molecular evidence that a polyketide synthase is involved in cercosporin synthesis, confirming the conclusions of the 1975 study. Currently, we are cloning and sequencing the full-length *CTB1* gene. Genetic disruption of the entire *CTB1* gene and functional complementation will be used to further elucidate the role of polyketide synthase in the biosynthesis of cercosporin.

In summary, the use of plasmid insertion and REMI mutagenesis in *C. nicotianae* and related organisms should prove valuable in dissecting toxin biosynthesis and other physiological processes that are important for pathogenesis and other important biological processes in this and related fungi.

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