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SHORT COMMUNICATION

Gene-specific disruption in the filamentous fungus *Cercospora nicotianae* using a split-marker approach

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Abstract To determine if DNA configuration, gene locus, and flanking sequences will affect homologous recombination in the phytopathogenic fungus *Cercospora nicotianae*, we evaluated and compared disruption efficiency targeting four cercosporin toxin biosynthetic genes encoding a polyketide synthase (*CTB1*), a monooxygenase/*O*-methyltransferase (*CTB3*), a NADPH-dependent oxidoreductase (*CTB5*), and a FAD/FMN-dependent oxidoreductase (*CTB7*). Transformation of *C. nicotianae* using a circular plasmid resulted in low disruption frequency. The use of endonucleases or a selectable marker DNA fragment flanked by homologous sequence either at one end or at both ends in the transformation procedures, increased disruption efficiency in some but not all *CTB* genes. A split-marker approach, using two DNA fragments overlapping within the selectable marker, increased the frequency of targeted gene disruption and homologous integration as high as 50%, depending on the target gene and on the length of homologous DNA sequence flanking the selectable marker. The results indicate that the split-marker approach favorably decreased ectopic integration and thus, greatly facilitated targeted gene disruption in this important fungal pathogen.

Keywords Gene replacement · Filamentous fungi · Pathogenicity · Plant pathogen · Recombination · Split marker · Toxins · Virulence

Abbreviations

CSPD	Disodium 3-[4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1]decan}-4-yl]phenyl phosphate
BAR	Acetyltransferase gene conferring phosphinothricin resistance
HYG	Phosphotransferase B gene conferring hygromycin resistance
dUTP	2′-Deoxyuracil 5′-triphosphate
DIG	Digoxigenin

Introduction

Cercospora species are important phytopathogenic fungi that have been reported to cause leaf spots on more than 100 plant species (Daub and Ehrenshaft 2000; Daub et al. 2005). Many *Cercospora* species produce a light-activated, nonspecific phytotoxin, cercosporin, which is required for full virulence to invade their hosts and for lesion development (Choquer et al. 2005).

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Cercosporin is synthesized by polypeptides of eight co-regulated and clustering genes (designated *CTB1-8*), encoding a polyketide synthase (CTB1), two *O*-methyltransferases (CTB2 and CTB3 N terminus), a monooxygenase (CTB3 C terminus), an MFS transporter, three oxidoreductases (CTB5, CTB6, and CTB7) and a Zn(II) Cys_6 transcription regulator (CTB8) (Chen et al. 2007a, b; Dekkers et al. 2007). Expression of the *CTB* genes is in part regulated by CRG1 zinc finger transcriptional regulator (Chung et al. 2003a, b). However, the *CRG1* coding sequence is unlinked to the *CTB* gene cluster. Biosynthesis of cercosporin is influenced by light and numerous environmental cues such as ions, nitrogen and carbon sources (You et al. 2008). In addition, considerable research has been devoted to understanding the mechanisms of cellular antioxidant defense that are operated by *Cercospora* fungi to avoid the toxicity of cercosporin and reactive oxygen species (Daub et al. 1992; Sollod et al. 1992; Ehrenshaft et al. 1998, 1999; Chung et al. 1999; Daub and Ehrenshaft 2000). As the CRG1 transcriptional factor regulates genes involved in cercosporin resistance and biosynthesis (Chung et al. 2003a), suppressive subtractive hybridization was used to recover several hundred genes that are differentially expressed between the wild type and a *crg1* null mutant of *C. nicotianae* (Herrero et al. 2007). Determination of the biological functions of those identified genes will be accelerated by the availability of an efficient gene inactivation system.

Genetic transformation and targeted gene disruption or replacement provides essential tools to analyze gene functions in filamentous fungi. Numerous fungi have been successfully transformed over the last two decades, due mainly to a great improvement in molecular methodologies for the delivery of DNA constructs carrying various selectable markers. However, gene disruption in some fungal species can be problematic largely due to the predominance of ectopic integrations and the ineffectiveness of homologous recombination (Pratt and Aramayo 2002). Unlike yeasts, integration of foreign genes is considered a rare event in filamentous fungi and presumably influenced by a nonhomologous end joining (NHEJ) mechanism (Ninomiya et al. 2004). To successfully disrupt a gene in filamentous fungi, it often requires at least 0.5–2 kb homologous DNA sequence (Nelson et al. 2003). Some fungal species, such as *Cladosporium fulvum* and *Leptosphaeria maculans* require a minimum of 5–7 kb of flanking DNA to obtain rare disruptants (Segers et al. 2001; Idnurm et al. 2003). Furthermore, integration of foreign genes in filamentous fungi often occurs ectopically and, thus, identification of the desired mutants often requires a large number of transformants to be screened. Impairment of the NHEJ machinery by disrupting the Ku70- and Ku80-coding genes has been shown to significantly increase homologous recombination in fungi (da Silva Ferreira et al. 2006; Haarmann et al. 2008). However, for organisms without genome sequence data, cloning the Ku70- or Ku80-coding gene and creating a parental strain that is defective only in the NHEJ pathway is time consuming and labor intensive.

Cercospora species can be genetically transformed with plasmid vectors containing a selectable marker conferring resistance to antibiotics or herbicides. In *Cercospora* species, the disruption frequency is often lower than 1% among transformants recovered (Ehrenshaft et al. 1998, 1999; Chung et al. 1999; Ehrenshaft and Daub 2001; Chung et al. 2003a, b, c; Wetzel et al. 2004), which has greatly hampered functional analysis of cloned genes in these species. To disrupt or replace a given gene in fungi, one must construct a plasmid harboring a selectable marker gene, such as the hygromycin phosphotransferase B gene, cloned within the ORF of the targeted gene (Pratt and Aramayo 2002). However, the efficiency of gene disruption varies considerably among species, strains and even among isolates of a given species, and systems developed for one microorganism may not be suitable for another. To facilitate targeted gene disruption, a split-marker disruption strategy (Fu et al. 2006) fusing the targeted DNA fragments with truncated, but overlapping, within the selectable marker gene has been successfully adapted in filamentous fungi. This strategy was originally developed for rapid, gap repaired-mediated cloning in *Saccharomyces cerevisiae* (Fairhead et al. 1996). In theory, transformants will not grow on a medium containing the selection agent unless homologous recombination occurs between the overlapping regions of the selectable marker gene. Since the frequency of ectopic integration is decreased markedly, a high frequency of targeted gene disruption via homologous recombination can be achieved by screening fewer transformants. In this study, we evaluated the frequency of targeted gene disruption for four *CTB* genes required for cercosporin biosynthesis in *C. nicotianae* (Chen et al. 2007a). Interruption of any of the *CTB* genes in *C. nicotianae* gave rise to a mutant strain completely lacking the production of the red-pigmented cercosporin (Choquer et al. 2005; Chen et al. 2007a, b; Dekkers et al. 2007). Thus, we took advantage of an easily visualized phenotype of the *CTB* disruptants and the simple extraction method for cercosporin to dissect genetic elements that may impede targeted gene disruption in *C. nicotianae*.

Materials and methods

Microorganisms, culture conditions and cercosporin analysis

Cercospora nicotianae wild-type strain ATCC18366 was used as the DNA recipient host for targeted gene disruption

throughout the study. Fungal strains were cultured in potato dextrose agar (PDA, Difco, Sparks, MD). Cercosporin-deficiency mutants (cr^-) were identified by the lack of production of a red pigment on thin PDA plates as previously described (Chung 2003). Cercosporin was extracted from agar plugs with fungal hyphae with 5 N KOH as described previously (Chung 2003). Cercosporin in the KOH extracts was detected using the spectrophotometer at 480 nm.

Targeted gene disruption

Disruption constructs, pCTB115, pΔctb5 and pΔctb7, harboring an *HYG* gene under the *Aspergillus nidulans trpC* promoter conferring resistance to hygromycin B, and pCTB3/Bar6 containing a *BAR* gene under the *trpC* promoter conferring bialaphos resistance, were created in the previous studies (Choquer et al. 2005; Chen et al. 2007a; Dekkers et al. 2007). The *HYG* or *BAR* gene cassette flanked with different lengths of the *CTB* gene sequence was amplified by PCR with the *CTB* gene-specific primers (Table 1). The resulting PCR products were directly transformed into the protoplasts prepared from the wild type. Disruption frequency (%) is calculated by dividing the number of disruptants by the total number of transformants recovered.

Preparation of fungal protoplasts and transformation

Preparation of fungal protoplasts (>10^7 ml^{-1}) and transformation using polyethylene glycol (PEG)/$CaCl_2$ were performed as described previously (Chung et al. 2002). Transformants were selected in a regeneration medium containing 250 μg ml^{-1} hygromycin (Roche Applied Science) or 50 μg ml^{-1} bialaphos (Phytotechnology Lab., Lenexa, KS), and tested for cercosporin production.

Molecular analysis

Standard protocols were used to perform endonuclease digestion, electrophoresis, Southern blotting and hybridization of DNA. Fungal DNA was isolated using a DNeasy Plant Mini kit (Qiagen, Valencia, CA); plasmid DNA was purified using a Wizard DNA purification kit (Promega, Madison, WI). DNA hybridization probes were synthesized by PCR with gene-specific primers to integrate digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN) as previously described (Chung et al. 2003b, c). Immunological detection of the probe using a CSPD lumigenic substrate for alkaline phosphatase was performed following the manufacturer's instructions (Roche).

Table 1 Oligonucleotide primers used in the study

Primer	Sequence (5′–3′)	Gene
CTB1W	ggctacggcataggccagaa	*CTB1*
CTB1X	tcacggagacaggtcttaccgc	*CTB1*
CTB1Y	ccacgtcggcgaaacttgtg	*CTB1*
0315L	ggcagtctcacagctcttgag	*CTB1*
0315R	ccgggtaagaggtgcagtttcg	*CTB1*
P3	actccaggtccacgtgaagc	*CTB1*
Q1	tcgtaggtgggaccaacgtc	*CTB1*
Q7	gtgagcatagcgaacgccat	*CTB1*
hyg3	ggatgcctccgctcgaagta	Hygromycin phosphotransferase B gene (*HYG*)
hyg4	cgttgcaagaactgcctgaa	Hygromycin phosphotransferase B gene (*HYG*)
TrpCP	gacagaagatgatattgaaggagcac	*Aspergillus nidulans trpC* promoter
HygT	gctcttgttcggtcggcatctac	Terminator of *HYG*
CTB3E	cactctagttaggcgcttgcactcaga	*CTB3*
CTB3H	aggagcggattcgatgccctcatg	*CTB3*
P40	cagctacgatgagtccgaggc	*CTB3*
P42	cctcggtctcacaggtcaac	*CTB3*
bar1	tctgcaccatcgtcaaccac	Phosphinothricin acetyltransferase gene (*BAR*)
bar2	aaacccacgtcatgccagtt	Phosphinothricin acetyltransferase gene (*BAR*)
TF4	ccatgaagcgagatgc	*CTB7*
ORD-3	cgtataccaaatcccatgtcgtac	*CTB7*
CTB7F	ccgcatagtgtcccgac	*CTB7*
CTB7R	tccggtaagtgacacagtcggggaa	*CTB7*
ctb7x	tggcagacagtccccgtatc	*CTB7*
ctb7z	gccccaacatgatggtgaatc	*CTB7*

Results and discussion

In the present study, we disrupted four *CTB* genes that reside in a cluster in *C. nicotianae* (Fig. 1a) to evaluate how genetic loci and their sizes will affect efficiency of homologous recombination. The minimum flanking sequence required for efficient homologous integration has never been determined in *C. nicotianae*. In each experiment, we were able to repeatedly identify the cercosporin non-producing mutants (cr^-) after transforming a wild-type strain with most of the constructs, providing an opportunity to determine the genetic factors that might have a profound effect on targeted gene disruption in this fungal species. All putative cr^- mutants were streaked three times for single colony on medium to eliminate false positive. All cr^- mutants recovered were very stable and no spontaneously reverted strains were identified for the duration of the experiment.

Disruption of the *CTB1* gene

The *CTB* gene cluster contains eight genes (Fig. 1a) that have been previously shown to be essential for the production and accumulation of cercosporin (Chen et al. 2007a). As described below, successful disruption of the *CTB* genes in the wild type was identified for the strains that failed to accumulate the red pigment, exemplified by the *CTB1*, *CTB3* and *CTB7* disruptants (Fig. 1b).

CTB1 contains a 7,036-bp ORF that encodes a putative fungal type-I polyketide synthase in *C. nicotianae* (Choquer et al. 2005). To disrupt the *CTB1* gene, the pCTB115 plasmid was used as a template in PCR to generate all DNA fragments with various lengths of homologous sequence (Fig. 1c). The pCTB115 plasmid contains the *HYG* gene cassette under the control of the *A. nidulans trpC* gene promoter that is flanked with a 2.5-kb fragment representing the 5′ end region of *CTB1* and a 1.6-kb fragment of 3′*CTB1* region. It was demonstrated that the addition of restriction endonucleases enhances the rate of recovery of transformants in *C. nicotianae* (Chung et al. 2003b). To determine whether or not endonucleases will promote targeted gene disruption, transformation of the wild-type strain of *C. nicotianae* with circular pCTB115 plasmid alone or with 10 U *Nru*I endonuclease yielded low frequency of cr^- mutants among transformants; transformation of the *C. nicotianae* wild type with pCTB115 mixed with an *Xba*I endonuclease gave rise to an enhanced efficiency for recovery of cr^- mutants. The restriction endonuclease in storage buffer was added directly into transformation cocktail. There is no restriction site for either *Nru*I or *Xba*I within the pCTB115 plasmid. Transformation of the *C. nicotianae* wild type with a linear construct comprised 1.3-kb *CTB1* sequence flanking near either ends of the *HYG* cassette

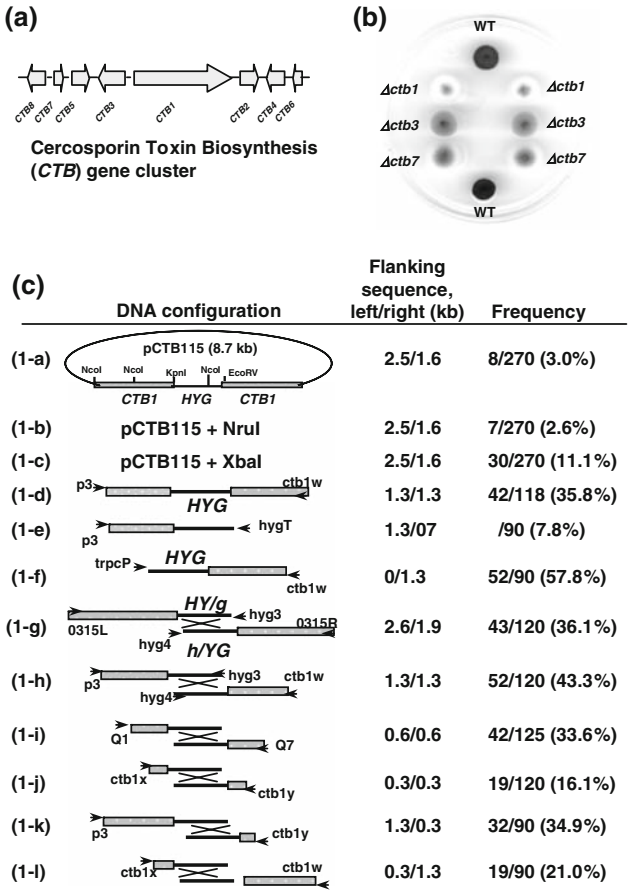

Fig. 1 **a** Physical map of the cercosporin toxin biosynthetic (*CTB1*–*CTB8*) gene cluster in *C. nicotianae*. **b** Production of the red-pigmented cercosporin and other pigments by strains of *C. nicotianae* on potato dextrose agar plates. **c** Targeted gene disruption of the *CTB1* gene in *C. nicotianae*. The disruption plasmid, pCTB115, was constructed by inserting a hygromycin phosphotransferase B gene (*HYG*) into *CTB1*. DNA fragments with various flanking sequence on the *left* and *right* border, separated by the *slash*, were obtained by PCR with primers as indicated. The split-marker fragments contain a mixture of DNA with overlapping, but truncated, *HYG* gene. The symbol (X) represents homologous recombination between the two DNA fragments. The number of cercosporin-deficient transformants identified and the total number of transformants recovered are indicated for each construct

(construct 1-d) resulted in high frequency for recovery of cr^- mutants (Fig. 1c).

A novel liner minimal element (LME) construct, containing a selectable marker gene fused with partial target gene sequence at only one end, was developed to inactivate genes with an incredibly high frequency in *Alternaria brassicicola* (Cho et al. 2006). Transformation of the *C. nicotianae* wild type with the 5′-end *CTB1* fused with the *HYG* fragment (construct 1-e) yielded very few cr^- mutants, whereas transformation of the *HYG* fused with the 3′-end *CTB1* fragment (construct 1-f) produced numerous cr^- mutants.

DNA fragments from constructs (1-g) to (1-l) contain the split-*HYG* gene marker flanked with asymmetric lengths of

the truncated *CTB1* DNA fragment (Fig. 1c). In all constructs, the 5′ *CTB1* was fused with the 3′ *HYG* fragment and the 5′ *HYG* was fused with the 3′ *CTB1* fragment. All DNA fragments were amplified by PCR and directly transformed into the wild type. The results revealed that transformation of the *C. nicotianae* wild type with two split, but overlapping, DNA fragments yielded *cr*$^-$ mutants with varied frequencies, depending on the lengths of homologous DNA at either end of *HYG* (Fig. 1c). It appears that disruption frequency increased as the lengths of flanking *CTB1* sequence on one end or both ends increased (Fig. 1c).

Disruption of the *CTB7* gene

The *CTB7* gene (1,401 bp) encodes an FAD/FMN-dependent oxidoreductase for the cercosporin biosynthesis (Chen et al. 2007b). The pΔctb7 plasmid contains the *HYG* gene cassette surrounded by truncated 2.5- and 1.6-kb fragments of *CTB7* (construct 7-a). Transformation of the *C. nicotianae* wild type with a DNA fragment, harboring the *HYG* cassette flanked with various lengths of the truncated *CTB7* DNA fragment (constructs 7-b–7-e), resulted in *cr*$^-$ mutants at varied frequencies, which ranged from 0 to 10% (Fig. 2). Reducing the flanking sequence apparently decreased the disruption efficacy. No transformants were identified when the lengths of the flanking region were both reduced to 0.6 kb. DNA fragments containing homologous *CTB7* sequence at only one end (construct 7-f or 7-g) did not result in any *cr*$^-$ mutants. Co-transformation of the *C. nicotianae* wild type with two split-*HYG* fragments flanked with the truncated *CTB7* near either end (construct 7-h) resulted in abundant *cr*$^-$ mutants, whereas disruption frequency dropped sharply as the flanking *CTB7* sequence on both ends was reduced (construct 7-i to 7-k). Transformation of the *C. nicotianae* wild type with two split-*HYG* fragments in which one contains a 0.7-kb 5′*CTB7* and the other contains a 1.6-kb 3′*CTB7* (construct 7-l) failed to yield any *cr*$^-$ mutants (Fig. 2).

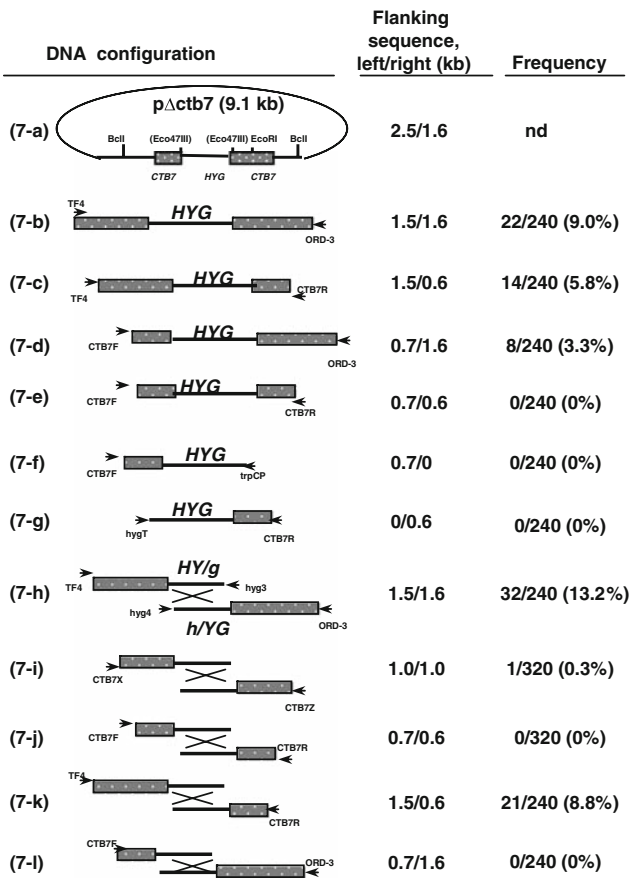

Fig. 2 Targeted gene disruption of the *CTB7* gene in *C. nicotianae*. The disruption plasmid, pΔctb7, was constructed by inserting a hygromycin phosphotransferase B gene (*HYG*) under the *trpC* promoter into *CTB7*. The split-marker fragments contain a mixture of DNA with overlapping but truncated *HYG* gene. *nd* Not determined. The number of cercosporin-deficient transformants identified and the total number of transformants recovered are indicated for each experiment

Disruption of the *CTB3* and *CTB5* genes

The *CTB3* gene (2,731 bp) encoding a polypeptide with dual *O*-methyltransferase/monooxygenase domains is also required for cercosporin production in *C. nicotianae* (Dekkers et al. 2007). To disrupt the *CTB3* gene, the pCTB3/Bar6 plasmid (construct 3-a), harboring the *BAR* gene cassette responsible for bialaphos herbicide resistance under control of the *A. nidulans trpC* promoter was constructed (Fig. 3a). Transformation of the *C. nicotianae* wild type with the split *BAR* fragments, in which one contains a 2.1-kb flanking sequence of the 5′-*CTB3* and the other contains a 0.3-kb flanking sequence of the 3′-*CTB3* (construct 3-b) failed to obtain any *cr*$^-$ mutants. In contrast, transformation of the wild type with the split *BAR* fragments containing 1-kb flanking sequence of *CTB3* on both ends (construct 3-c) generated *cr*$^-$ mutants at a frequency as high as 32% (Fig. 3a).

The pΔctb5 plasmid, containing 1.2-kb flanking sequence on both ends of *HYG*, was prepared for disruption of the *CTB5* gene (1,380 bp) encoding a putative NADPH-dependent oxidoreductase for cercosporin biosynthesis in *C. nicotianae* (Fig. 3b). Transformation of the *C. nicotianae* wild type with circular pΔctb5 alone or with a *Bam*HI endonuclease at 10 U generated *cr*$^-$ mutants at low frequency. Transformation of the wild type using pΔctb5 mixed with an *Nhe*I endonuclease increased the overall disruption efficiency. There is no restriction site for either *Bam*HI or *Nhe*I within the pΔctb5 plasmid. Transformation of the *C. nicotianae* wild type with a linear DNA fragment containing a functional *HYG* gene cassette within the *CTB5* ORF resulted in low frequency for recovery of *cr*$^-$ mutants. Transformation of the *C. nicotianae* wild type with the

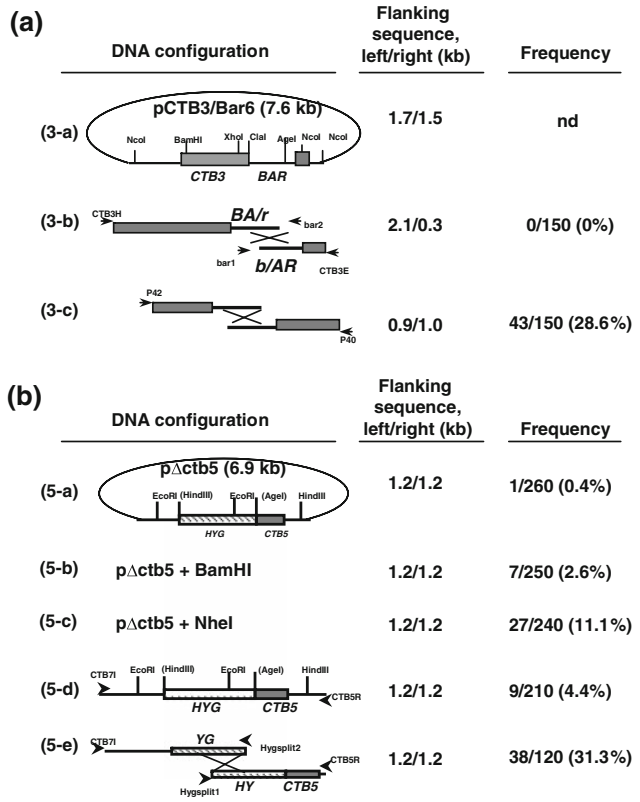

Fig. 3 **a**, **b** Targeted gene disruption of the *CTB3* and *CTB5* genes in *C. nicotianae*. The disruption plasmids, pCTB3/Bar6 and pΔctb5, were constructed, respectively, by inserting a phosphinothricin acetyltransferase gene (*BAR*) under the *trpC* promoter into *CTB3* and a hygromycin phosphotransferase B gene (*HYG*) into *CTB5*. The split-marker fragments contain a mixture of DNA with overlapping, but truncated, *BAR* or *HYG* gene

split-*HYG* fragments with 1.2-kb flanking sequence of *CTB5* on both ends yielded *cr*$^-$ mutants at high frequency (Fig. 3b).

The split-marker approach decreases ectopic integration

To determine if integration of split-marker fragments occurred specifically at the targeted *CTB1* gene locus and to assess if the split-marker approach would reduce ectopic integration (non-homologous integration), ten putative *cr*$^-$ mutants were randomly selected from transformants transformed with split-*HYG* fragments (construct 1-c). Hybridization of wild-type genomic DNA cleaved with *Eco*RV and *Nco*I to a 5′ *CTB1* probe resulted in two expected hybridizing bands of 4.1 and 1.4 kb in sizes (Fig. 4a). In contrast, hybridization of the *Eco*RV/*Nco*I-digested genomic DNA from *cr*$^-$ mutants also displayed two expected bands of 2.1 and 1.4 kb due to insertion of the *HYG* gene within *CTB1*. Of the 10 *cr*$^-$ mutants examined, four of them were targeted gene disruption mutants clearly derived from homologous recombination specifically at the *CTB1* gene, whereas the other six mutants had multiple hybridizing bands in addition to the 2.1 and 1.4-kb bands (Fig. 4a). The transforming DNA in the latter was likely resulting from ectopic integration or tandem insertion at the integration site. Similar analyses were conducted to examine ten randomly selected *cr*$^-$ mutants that were transformed with a whole PCR fragment containing partial *CTB1* at either end of *HYG* (construct 1-d), revealing that only one *cr*$^-$ mutant (#1 in Fig. 4b) was derived from homologous integration. The other nine mutants, displaying multiple hybridizing signals larger or smaller than 2.1 kb, had ectopic or tandem integrations (Fig. 4b). Southern-blot analysis of genomic DNA in randomly selected *CTB3*, *CTB5* or *CTB7* disruptants also revealed that at least two *cr*$^-$ disruptants displayed integration profiles clearly resulted from homologous recombination after transformation of the *C. nicotianae* wild type with the split-marker approach, whereas other disruptants had profiles from both ectopic and homologous integrations (Fig. 4c–e).

As it was evidenced from the present study, the frequency of *CTB* disruptants differed greatly among the transforming DNA constructs, highly depending on the gene targeted and the length of the homologous sequences. Although the size of the sequence being disrupted varied among constructs, it appears that the longer homologous sequences within the construct often resulted in higher frequencies of disruption in *C. nicotianae* even though the *CTB* clustering genes are involved in cercosporin biosynthesis. It also appears that circular plasmid constructs resulted in a low rate of disruption. Transformation of plasmid constructs with certain, but not all, restriction endonucleases slightly elevated the disruption frequency, as evidenced in the disruption of both *CTB1* and *CTB5* genes. Transformation with linear DNA fragments, obtained from disruption constructs by PCR (whole PCR fragments), improved disruption frequency as tested in *CTB1* and *CTB7*, but not *CTB5*, genes. As shown in the disruption of the *CTB1* and *CTB7* genes, the length of the homologous DNA sequence present in the whole PCR fragment affected the disruption frequency variably. When the split-marker approach was used for disruption, recovery of *cr*$^-$ mutants markedly increased for all *CTB* genes tested. As the homologous DNA sequence was decreased from one or both fragments, disruption frequency deceased to varied degrees, depending on the gene of the target. Thus, it is essential to have sufficient lengths of the flanking DNA sequence (at least 0.8 kb on both ends are needed) when using the split-marker approach for targeted gene disruption in *C. nicotianae*. Although the split-marker approach also led to ectopic or tandem integrations in addition to the gene-specific disruption, we were able to identify *cr*$^-$ mutants with a clean disruption at the target gene allele by screening fewer transformants in each case. Thus, it appears that the

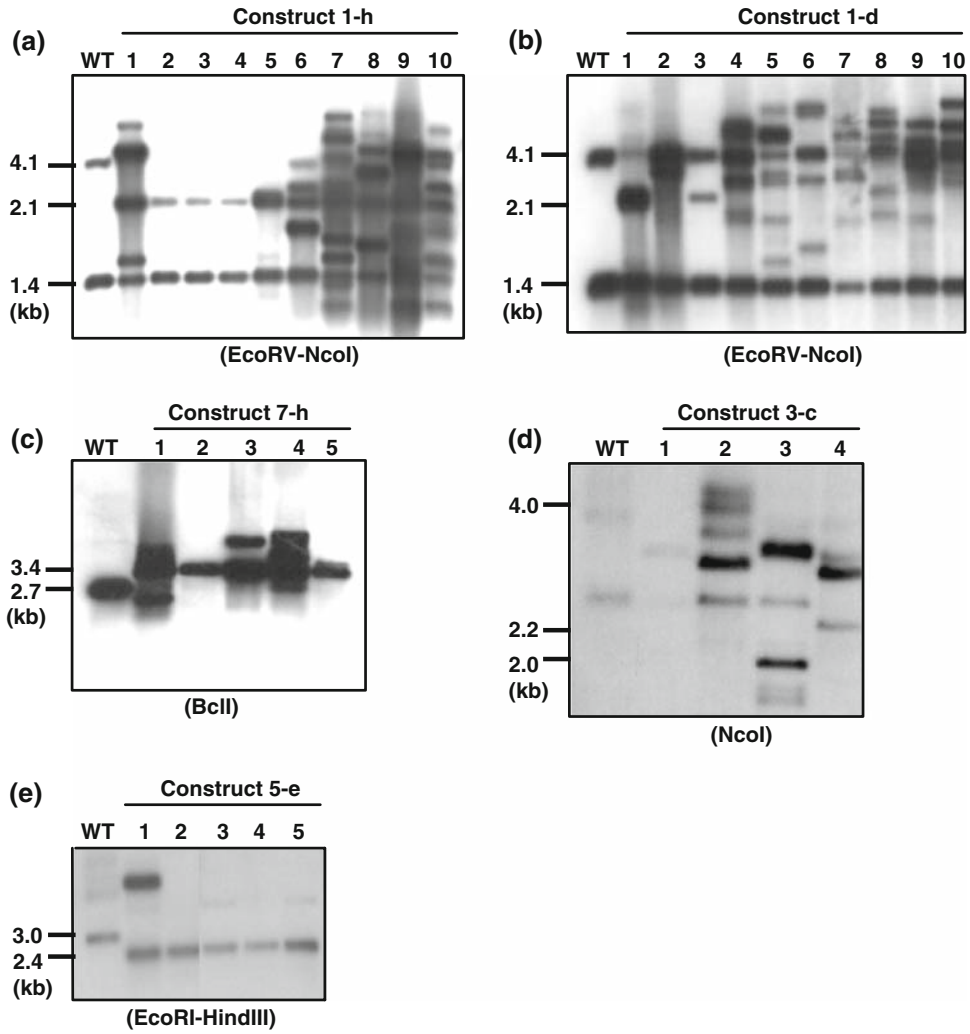

Fig. 4 **a**–**e** Southern-blot analyses of genomic DNA from the cercosporin non-producing mutants of *Cercospora nicotianae*, obtained from transformation experiments with the *CTB1* split-marker DNA fragments (**a**), the entire PCR fragment with truncated *CTB1* (**b**), or with the *CTB7* (**c**), *CTB3* (**d**), and *CTB5* (**e**) split-marker fragments as indicated on the *top* of each panel, *WT* wild-type strain

split-marker approach led to an increase in the homologous integration frequency.

Compared to the disruption targeting at *CTB1*, disruption of the *CTB5* or *CTB7* gene yielded lower disruption frequencies, indicating an allele-dependent disruption. It also suggests that the size of the targeting gene may influence disruption frequency, as the larger genes such as *CTB1* (7 kb) have higher rates of disruption than other smaller genes such as *CTB5* and *CTB7* (1.4 kb). Further, it was demonstrated that the split-marker approach led to a decrease in ectopic integration as evident by Southern-blot analysis, thereby promoting gene-specific disruption. Efficient gene disruption strategies along with the other molecular techniques available for manipulating *C. nicotianae* shall facilitate functional genomic analysis for this important fungal pathogen.

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