Evidence for Dicer-dependent RNA interference in the industrial penicillin producer *Penicillium chrysogenum*

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RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing system that downregulates target gene expression. Here, we provide several lines of evidence for RNA silencing in the industrial β -lactam antibiotic producer *Penicillium chrysogenum* using the *DsRed* reporter gene under the control of the constitutive *trpC* promoter or the inducible *xylP* promoter. The functional RNAi system was verified by detection of siRNAs that hybridized exclusively with gene-specific ^{32}P -labelled RNA probes. Moreover, when RNAi was used to silence the endogenous *PcbclA* morphogene that controls conidiophore development, a dramatic reduction in the formation of conidiospores was observed in 47 % of the corresponding transformants. Evidence that RNAi in *P. chrysogenum* is dependent on a Dicer peptide was provided with a strain lacking *Pcdc12*. In the ΔPcdc12 background, silencing of the *PcbclA* gene was tested. None of the transformants analysed showed a developmental defect. The applicability of the RNAi system in *P. chrysogenum* was finally demonstrated by silencing the *Pcku70* gene to increase homologous recombination frequency. This led to the generation of single and double knockout mutants.

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INTRODUCTION

In filamentous fungi, RNA interference (RNAi) is the most extensively used RNA-based tool for controlled down-regulation of gene expression. In this system, double-stranded RNAs (dsRNAs) trigger the degradation of homologous mRNA post-transcriptionally, leading to decreased gene expression. For this purpose, dsRNAs are processed into small interfering RNAs (siRNAs) by the Dicer polypeptide, an RNaseIII-type endonuclease. Subsequently, these 21–26 bp siRNAs are incorporated into the Argonaute-containing RNA-induced silencing complex (RISC) as single-stranded siRNAs for the identification and degradation of complementary mRNAs (reviewed by Meister & Tuschl, 2004).

Since the discovery of the RNAi phenomenon in *Neurospora crassa* (Romano & Macino, 1992), several studies have provided evidence for RNA silencing machineries in various filamentous fungi and yeasts (reviewed by Nakayashiki & Nguyen, 2008). Compre-

hensive phylogenetic analyses of diverse fungal genomes revealed a difference in the number of paralogous RNA-silencing proteins, such as Argonautes, Dicers and RNA-dependent RNA polymerases (Nakayashiki *et al.*, 2006; Nakayashiki & Nguyen, 2008). In general, filamentous ascomycetes encode each of two Dicer and Argonaute RNA silencing proteins. Remarkably, some of the silencing proteins are missing in a number of ascomycetes and basidiomycetes, for example *Saccharomyces cerevisiae* and *Ustilago maydis* (Nakayashiki *et al.*, 2006). Conversely, in diverse *Aspergillus* species, loss as well as gain of components of the RNA silencing machinery is observed. For example, *Aspergillus oryzae* and *Aspergillus flavus* encode three of both Dicers and Argonautes, while *Aspergillus nidulans* encodes one full-length as well as a truncated copy from both Dicer and Argonaute genes. The full-length genes (*dclB* and *rsdA*) are responsible for RNA silencing induced by dsRNAs, whereas the truncated copies (*dclA* and *ppdB*) belong to the meiotic silencing by unpaired DNA (MSUD) pathway. Thus *A. nidulans* seems to be in the process of losing one of the two copies of Dicer and Argonaute genes (Hammond *et al.*, 2008).

The discovery that RNAi takes place in filamentous fungi subsequently led to the development of different RNAi systems aimed at altering expression of specific genes. The ongoing optimization of existing RNAi systems demonstrates the importance of such tools in deciphering gene

Abbreviations: MSUD, meiotic silencing by unpaired DNA; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; siRNA, small interfering RNA.

Supplementary tables of oligonucleotides and plasmids, and supplementary figures showing amino acid sequence alignments of *Pcdc1* and *Pcdc12* with homologous proteins of other filamentous fungi are available with the online version of this paper.

functions, particularly when gene deletions by homologous recombination occur only at very low frequencies (Caracuel-Rios & Talbot, 2008; Nguyen *et al.*, 2008; Oliveira *et al.*, 2008; Shafran *et al.*, 2008). Several RNAi approaches have been successfully performed using vectors that contain intron or spacer sequences between two inversely oriented target gene fragments to express dsRNA with a hairpin structure (e.g. de Jong *et al.*, 2006; Fitzgerald *et al.*, 2004; Kadotani *et al.*, 2003; Liu *et al.*, 2002; Nakayashiki *et al.*, 2005). These systems provide stable and high silencing frequencies and lead in some cases to completely silenced phenotypes (Nakayashiki & Nguyen, 2008).

Recently, it was shown that RNAi can be used effectively to manipulate a number of biotechnologically relevant ascomycetes (Janus *et al.*, 2007; Oliveira *et al.*, 2008; Takeno *et al.*, 2005; Ullán *et al.*, 2008; Yamada *et al.*, 2007; Zhang *et al.*, 2007). For example, RNAi was used to silence regulators of biosynthetic pathways as well as to down-regulate the expression of genes involved in the synthesis of secondary metabolites such as fatty acids or β -lactam antibiotics. RNAi will further contribute to strain improvements since it will allow direct and easy manipulation of regulators involved in secondary metabolism.

In this study, we provide a detailed molecular characterization of functional RNAi in *P. chrysogenum*. This system is based on the previously described vector pREDi that carries the *DsRed* gene as a reporter for direct monitoring of gene silencing on solid media (Janus *et al.*, 2007). The vector was modified by using the *xylP* promoter from *P. chrysogenum*, which is induced when xylose is the sole carbon source in the medium (Zadra *et al.*, 2000). The applicability of this inducible RNAi system was further tested with the endogenous *PcbrlA* morphogene controlling conidiophore development, chosen as the specific pheno-

typic reporter. Based on the recently published *P. chrysogenum* genome sequence (van den Berg *et al.*, 2008), we also identified components of the RNAi machinery that were functionally characterized using a knockout strain. Finally, the applicability of the RNAi system was demonstrated by transient downregulation of *Pcku70* gene expression in order to improve homologous recombination efficiencies.

METHODS

Fungal strains, culture conditions and transformation. All *P. chrysogenum* strains used in this study are listed in Table 1. Strains were cultivated at 27 °C in complete liquid (120 r.p.m. or as surface culture) or on solid CCM or minimal (MM) medium supplemented with glucose or xylose (Haas *et al.*, 1997; Minut *et al.*, 1982).

Transformation of *P. chrysogenum* strains was performed using conventional transformation procedures with modifications as described by Windhofer *et al.* (2002). Cultures were inoculated with $2.5\text{--}5 \times 10^6$ spores ml⁻¹ and grown at 27 °C and 120 r.p.m. for 72 h in CCM medium. Fungal protoplasts were transformed with plasmid DNA or with linear PCR fragments. Transformants were grown on selective CCM or MM media, depending on the experimental conditions, containing the following antibiotic concentrations: nourseothricin, 150 µg ml⁻¹; phleomycin, 40 µg ml⁻¹; pyrithiamine, 7 µg ml⁻¹.

Identification of *P. chrysogenum* genes and sequence analysis. The recently published genome database from *P. chrysogenum* served as source for the identification of genes involved in RNAi (van den Berg *et al.*, 2008). Similarly, the gene encoding the *PcbrlA* protein, a homologue of the *A. nidulans* BRLA polypeptide, was identified (Adams *et al.*, 1988). Fungal protein sequences (*A. nidulans* and *N. crassa*) were used as query sequences in searches with the basic local alignment search tool BLAST (Altschul *et al.*, 1990). Protein sequence data from *A. nidulans* and *N. crassa* were taken from the Broad Institute (<http://www.broad.mit.edu/annotation/fungi/fgi/index.html>), and sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997). For domain identification, the predicted

Table 1. Recipient and transgenic *P. chrysogenum* strains used in this study

Strain	Characteristics	Source
P2niaD18	Penicillin producer; <i>niaD</i> ⁻	Hoff <i>et al.</i> (2008)
P2: BrlAi_xyl	<i>xylP(p)::PcbrlAs::PcbrlAas::trpC(t), gpd(p)::nat1</i>	This study
P2: BrlAi	<i>trpC(p)::PcbrlAs::PcbrlAas::trpC(t), ptrA(p)::ptrA</i>	This study
P2: KU70i	<i>xylP(p)::Pcku70s::Pcku70as::trpC(t), ptrA(p)::ptrA</i>	This study
P2: KU70i: ΔPclaeA	<i>xylP(p)::Pcku70s::Pcku70as::trpC(t), ptrA(p)::ptrA; ΔPclaeA::trpC(p)::ble</i>	This study
P2: KU70i: ΔPclaeA: ΔPcvelA	<i>xylP(p)::Pcku70s::Pcku70as::trpC(t), ptrA(p)::ptrA; ΔPclaeA::trpC(p)::ble; ΔPcvelA::trpC(p)::nat1</i>	This study
P2red	<i>gpd(p)::DsRed::trpC(t), pcbAB/C(p)::ble</i>	This study
P2red: DsRedi	<i>gpd(p)::DsRed::trpC(t), pcbAB/C(p)::ble; trpC(p)::DsReds::DsRedas::trpC(t), gpd(p)::nat1</i>	This study
P2red: DsRedi_xyl	<i>gpd(p)::DsRed::trpC(t), pcbAB/C(p)::ble; xylP(p)::DsReds::DsRedas::trpC(t), gpd(p)::nat1</i>	This study
ΔPcku70	<i>ΔPcku70::trpC(p)::nat1</i>	Hoff <i>et al.</i> (2009)
ΔPcdcl2	<i>ΔPcku70::trpC(p)::nat1; ΔPcdcl2::trpC(p)::ble</i>	This study
ΔPcdcl2: BrlAi	<i>ΔPcku70::trpC(p)::nat1; ΔPcdcl2::trpC(p)::ble; trpC(p)::PcbrlAs::PcbrlAas::trpC(t), ptrA(p)::ptrA</i>	This study

P. chrysogenum protein sequences were used to perform the NCBI conserved domain search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>; Marchler-Bauer *et al.*, 2007).

Construction of RNAi vectors. *Escherichia coli* K-12 XL1-Blue (Stratagene) was used for general plasmid construction and maintenance (Bullock *et al.*, 1987). Oligonucleotides and recombinant plasmids are listed in Supplementary Tables S1 and S2, respectively.

All PCR fragments generated in this investigation for the construction of diverse plasmids were subcloned into plasmid pDrive (Qiagen) and sequenced (Eurofins MWG Operon). Plasmid pREDi was previously described (Janus *et al.*, 2007) and served for construction of pREDi_{xyl}. For this purpose, a *xylP* promoter fragment was generated by PCR using primers Pxyl_s and Pxyl_a with genomic DNA from *P. chrysogenum* as template. This amplicon was ligated into *EcoRI*/*Clal*-restricted pREDi to replace the constitutive *trpC* promoter from *A. nidulans* by the *xylP* promoter from *P. chrysogenum*.

Plasmid pBRLAi_{xyl} was designed by first introducing an annealed pair of complementary oligonucleotides (Nde₁ and Nde₂) into the *ApaI*-*BglII* restriction sites of plasmid pS-NAT1 (Janus *et al.*, 2007) to insert a single *NdeI* restriction site. The resulting plasmid pS-NAT1-*NdeI* was then digested using *EcoRI* and *Clal* for insertion of the above-mentioned *xylP* promoter to generate pS-NAT_{xyl}. In a next step, two 596 bp *Pcb1A* gene fragments, *Pcb1A*_{sense} and *Pcb1A*_{anti}, were amplified using primers Pcb1A-1 and Pcb1A-2 as well as Pcb1A-3 and Pcb1A-4 with genomic DNA of strain P2niaD18 as template. The *Pcb1A* fragments were then inserted in inverse orientation into the *SnaBI*-*HindIII* (*Pcb1A*_{sense}) and *NdeI*-*BglII* (*Pcb1A*_{anti}) restriction sites of plasmid pS-NAT_{xyl}, generating plasmid pBRLAi_{xyl}.

For silencing experiments with strain Δ*Pcdcl2*, plasmid pBRLAi was constructed as follows. pPTRII (Supplementary Table S2), carrying the AMA1-based elements together with the pyrithiamin-resistance gene *ptrA* from *Aspergillus oryzae* (Gems *et al.*, 1991; Kubodera *et al.*, 2000, 2002), was modified by introducing complementary annealed oligonucleotides (Nhe₁ and Nhe₂) into the *KpnI*-*HindIII* restriction sites. This construction generated a single *NheI* site in plasmid pPTRII-*NheI*. In a second step, pS-NAT was digested with *EcoRI* and *Clal* and the resulting 1246 bp fragment, corresponding to the *trpC* promoter of *A. nidulans*, was used for ligation with the *EcoRI*/*Clal*-restricted 6478 bp fragment of pBRLAi_{xyl}. The resulting plasmid pBRLAi_{trpC} was further cut with *XbaI* and the 3395 bp fragment spanning the whole *Pcb1A* silencing cassette was ligated into the *NheI* restriction site of plasmid pPTRII-*NheI* to generate pBRLAi.

For silencing of the *Pcku70* gene, we generated plasmid pKU70i. For this purpose, two 552 bp *Pcku70* gene fragments, *Pcku70*_{sense} and *Pcku70*_{anti}, were generated using primers Pcku70-1 and Pcku70-2 as well as Pcku70-3 and Pcku70-4 with genomic DNA of strain P2niaD18 as template. The resulting *Pcku70* fragments were inserted in inverse orientation into the *XhoI*-*SnaBI* (*Pcku70*_{sense}) and *StuI*-*BglII* (*Pcku70*_{anti}) restriction sites of plasmid pS-NAT1, generating plasmid pS_KU70i. The above-mentioned *xylP* promoter was ligated into the *EcoRI*-*Clal* restriction sites, generating plasmid pKU70i_{xyl}. Using restriction enzyme *XbaI*, the whole *Pcku70*-RNAi cassette was isolated from plasmid pKU70i_{xyl} and ligated into the *NheI* restriction site of plasmid pPTRII-*NheI* to generate pKU70i.

Generation of knockout strains. Plasmid pKOPcDCL2 (Supplementary Table S2) was constructed to substitute the *Pcdcl2* gene in *P. chrysogenum*. Fragments of about 1 kb of the 5'- and 3'-flanking regions of *Pcdcl2* were amplified using primers Pcdcl2-5s and Pcdcl2-5a and Pcdcl2-3s and Pcdcl2-3a, respectively, with genomic DNA from P2niaD18 as template. After subcloning into pDrive

(Qiagen), the *MluI*-*BamHI* 5'-fragment and the *XhoI*-*XbaI* 3'-fragment were ligated into the corresponding restriction sites of plasmid pD-Phleo (Hoff *et al.*, 2009), expressing the *ble* gene under control of the *trpC* promoter of *A. nidulans*. The *Pcdcl2* knockout cassette was amplified by PCR using primers Pcdcl2-5s and Pcdcl2-3a with plasmid pKOPcDCL2 as template. The resulting 2.8 kb PCR fragment was used for transformation of Δ*Pcku70* (Hoff *et al.*, 2009) to generate Δ*Pcdcl2*.

Plasmid pKOPcLAEA carries the phleomycin-resistance gene flanked by 1.5 kb sequences from the upstream and downstream region of the *PclaeA* gene (B. Hoff, unpublished). This plasmid served as template to generate a 3.8 kb PCR fragment to construct a Δ*PclaeA* knockout strain. Plasmid pKOPcVELA_{Nat} is a derivative of pKOPcVELA in which the phleomycin-resistance gene has been substituted by the nourseothricin-resistance cassette (Hoff *et al.*, 2009).

Preparation of nucleic acids, hybridization protocols and PCR.

Bulk DNA and RNA were isolated as described previously (Jekosch & Kück, 2000); mycelia were grown at 27 °C and 120 r.p.m. for 3 days in liquid CCM or MM medium or as static liquid cultures for 4 days in MM medium supplemented with glucose or xylose. RNA for mRNA detection and cDNA synthesis was isolated using the RNeasy Lipid Tissue kit (Qiagen) according to the manufacturer's instructions. Agarose gels, Southern and Northern blotting as well as hybridizations were performed using standard methods (Sambrook & Russell, 2001). The filters were hybridized with [α -³²P]dCTP-labelled DNA probes as indicated in Results.

PCR amplifications were carried out with either HotMaster DNA polymerase (Eppendorf) or *Taq* DNA polymerase (Eppendorf) according to the manufacturer's instructions.

Quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR) was performed as described previously (Nowrousian *et al.*, 2005), with the modifications described by Janus *et al.* (2007).

Detection of siRNAs. Total RNA was separated on 17.5% polyacrylamide (19:1) gels containing 7 M urea in 0.5 × TBE. To prepare samples, 15 µg total RNA was mixed with 1 vol. 100% formamide and incubated at 95 °C for 5 min. Electrophoretically separated RNA was blotted onto a Hybond-N+ (Amersham) membrane and hybridization was conducted as described by Dalmay *et al.* (2000) with the following modifications. Using the MAXIsript kit (Ambion), single-stranded ³²P-labelled RNA probes were transcribed *in vitro* using pDrive_{DsRed} or pDrive_{brlA} as templates (Supplementary Table S2) and hybridizations were done at 37 °C.

RESULTS

The *DsRed* gene is a suitable reporter to detect RNAi in *P. chrysogenum*

Previously, we developed a *DsRed*-based RNAi system for the cephalosporin C producer *Acremonium chrysogenum* (Janus *et al.*, 2007). Because of its easy applicability and high silencing efficiency, we applied this system to *Penicillium chrysogenum*. In a first step, a strain expressing the *DsRed* reporter gene was generated by transforming P2niaD18 with plasmid pRN1-Phleo, thereby allowing selection of transformants on phleomycin-containing media. A single transformant showing a stable red-coloured phenotype was named P2red, and used for

transformation with the silencing vector pREDi carrying a *DsRed*-silencing cassette with two inverse-oriented *DsRed* gene fragments (Janus *et al.*, 2007). A total of 97 transformants were obtained, showing a red (9.3%), pink (39.2%) or colourless phenotype (51.5%). We predicted that the colourless transformants would carry the most silenced *DsRed* gene.

For further analysis, Northern blot hybridization and the detection of siRNAs were used to molecularly characterize fungal transformants. We randomly selected five transformants (P2red:DsRedi T1, T2, T5, T14, T18) having differentially coloured phenotypes on solid media and examined these colonies together with the P2red recipient as control. After RNA isolation, Northern blot analysis was performed with *DsRed* and γ -actin as probes. To detect only the full-length *DsRed* transcript and not RNAs derived from the pREDi vector, a 240 bp fragment was used to detect the 3' end of *DsRed* as previously described (Janus *et al.*, 2007). In comparison to the recipient strain P2red, Northern blot analysis revealed a reduction in the *DsRed* transcript level in transformants having a pink or colourless phenotype (P2red:DsRedi T2, T5, T14, T18) (Fig. 1a). However, red-coloured transformants, e.g.

P2red:DsRedi T1, showed hybridizing bands with almost the same intensity as the recipient strain. The most drastic reduction in *DsRed* transcript levels was seen in transformants P2red:DsRedi 14 and T18, both of which have colourless mycelia. The γ -actin probe was used as a loading control and gave almost identical signals with RNA from all transformants tested. As further evidence of dsRNA-induced RNAi, the accumulation of *DsRed*-specific siRNAs was analysed to confirm that the decrease in *DsRed* transcript levels correlated with an increase of siRNAs. Interestingly, *DsRed* siRNA accumulation was detected in all transformants that were found to have a decreased level of *DsRed* transcript (Fig. 1b). For example, transformants P2red:DsRedi T2 and T18 showed the most marked accumulation of *DsRed*-specific siRNAs (Fig. 1b). As expected, no signal was observed with the *DsRed* probe in P2red:DsRedi T1 and recipient P2red, both exhibiting a red-coloured phenotype.

Inducible silencing of the *DsRed* reporter gene

To further validate the existence of an RNAi system, we generated an inducible interference construct for controlled gene silencing during different stages of fungal development. We first constructed the inducible *DsRed*-silencing vector pREDi_{xyl} carrying the *P. chrysogenum* *xylP* promoter, which can be induced by xylan or xylose and repressed by glucose (Zadra *et al.*, 2000). After transformation of vector pREDi_{xyl} into recipient strain P2red, we obtained 41 transformants, which were subsequently transferred onto solid MM medium supplemented with either xylose (inducing conditions) or glucose (repressing conditions) as the main carbon source. Phenotypic characterization of the mycelium revealed that 83% (34 transformants) of all transformants showed a xylose-induced colour switch. Fig. 2(a) shows three randomly selected transformants (P2red:DsRedi_{xyl} T6, T13, T18), the recipient strain P2red, and the constitutively silenced transformant P2red:DsRedi T2 growing on agar plates containing MM medium with glucose or xylose as the main carbon source. As can be seen, all three transformants showed a reduction in *DsRed* synthesis during growth on xylose-containing medium. In contrast, *DsRed* silencing was not induced in these transformants upon growth on glucose-containing medium, as shown by the red colour of the colonies. Furthermore, the constitutively silenced control strain P2red:DsRedi T2 is colourless on plates containing either glucose or xylose, and conversely, the red colour of the recipient strain P2red was not significantly altered during growth on glucose- or xylose-containing media. Selected transformants were further characterized by qRT-PCR, Northern blot analysis and detection of siRNAs. As can be seen from the qRT-PCR results in Fig. 2(b), the level of the *DsRed* transcript was clearly reduced in transformants P2red:DsRedi_{xyl} T13, T18 growing on xylose, whereas in the control strains P2red and P2red:DsRedi T2 the level of transcripts was not significantly different. These data were further

Fig. 1. Expression of the *DsRed* reporter gene in *P. chrysogenum* transformants (P2red:DsRedi T1, T2, T5, T14, T18) and recipient P2red. White, grey and black stars indicate white, pink and red fungal colonies, respectively. (a) Northern blot analysis to follow silencing of *DsRed* gene expression on the mRNA level. Hybridization was performed using a *DsRed* gene fragment as probe, with γ -actin as loading control. (b) Northern blot analysis of *DsRed*-specific siRNAs in different transformants and the recipient P2red. A *DsRed* oligonucleotide (25 nt) was used as molecular size marker and predominant RNAs served as loading control.

Fig. 2. Evidence for an inducible RNAi system using the heterologous *DsRed* reporter gene. (a) Phenotypic analysis of transformants T6, T13 and T18 carrying pREDi_xyl, transformant T2 carrying pREDi and the corresponding recipient P2red on solid MM medium containing glucose or xylose as main carbon source. (b) qRT-PCR for detection of the *DsRed* transcript and (c) Northern blot analysis of *DsRed*-specific siRNAs from strains as indicated. Strains were cultivated in MM medium containing glucose (+G) or xylose (+X).

confirmed by Northern hybridizations using a *DsRed*-specific fragment as probe (data not shown). In Fig. 2(c) the detection of *DsRed*-specific siRNAs with a size of about 25 nt is shown. Recombinant strains P2red:DsRedi_xyl T6, T13 and T18 grown in MM medium with glucose as the main carbon source did not show any accumulation of *DsRed* siRNAs, whereas siRNAs were clearly visible in cases when strains were grown in xylose-supplemented medium.

Growth of two control strains under both conditions delivered signals with almost identical intensities in P2red:DsRedi T2, whereas no signals were detectable in the P2red recipient. In conclusion, the detections of siRNA as shown in Fig. 2(c) coincide very well with the phenotypic characterizations.

Inducible silencing of the endogenous *PcbrlA* morphogene

To examine the effect of the functional inducible RNAi system on the expression of an endogenous gene, an interference construct was generated to inhibit specifically the expression of *PcbrlA* from *P. chrysogenum*. This gene is a homologue of the *Aspergillus nidulans brlA* gene, which is responsible for conidiophore development (Adams *et al.*, 1988). Inactivation or disruption of this gene resulted in reduced conidiation in various *Aspergillus* species (Barton & Prade, 2008; Clutterbuck, 1969; Mah & Yu, 2006; Yamada *et al.*, 1999). To silence *PcbrlA* gene expression, we generated vector pBRLAi_xyl (Supplementary Table S2) containing the *P. chrysogenum xylP* promoter and two inversely oriented *PcbrlA* gene fragments separated by intron 2 from the *Magnaporthe oryzae* cutinase gene. After transformation of this vector into the wild-type strain P2niaD18, we obtained 19 different transformants. All transformants were then tested for conidiophore development during growth on solid MM medium supplemented with either glucose or xylose. When grown on xylose-containing solid medium, nine of the transformants obtained showed delayed conidiospore formation. As shown in Fig. 3(a), all randomly selected transformants P2:BRLAi_xyl T6, T7, T11 and T15, and the recipient strain P2niaD18, developed green conidiospores on MM medium supplemented with glucose. However, when xylose was used as the carbon source, the silencing vector pBRLAi_xyl was induced, and transformants were unable to form conidiospores and thus appeared colourless. In contrast, the wild-type P2niaD18 developed conidiospores under both carbon growth conditions. These phenotypic characterizations were confirmed by Northern blot analysis as shown in Fig. 3(b). The *PcbrlA* transcript was not detectable in strains grown on xylose, whereas the control strain P2niaD18 produced a *PcbrlA* transcript on both tested carbon sources. Consistent with these results was the detection of *PcbrlA*-specific siRNAs in the silenced transformants. As can be seen in Fig. 3(c), all transformants accumulated *PcbrlA* siRNAs when grown in xylose-containing medium, whereas no signals were obtained from strains that were grown in MM medium containing glucose. As expected, the recipient P2niaD18 did not produce a signal at all. This finding demonstrates that induction of the *xylP* promoter by xylose results in expression of the silencing vector pBRLAi_xyl and correlates with the generation of siRNAs with homology to *PcbrlA*. These results agree well with our phenotypic characterization of conidiophore development as well as Northern blot analysis of silenced transformants.

Fig. 3. Evidence for an inducible RNAi system using the homologous *PcbriA* developmental gene. (a) Phenotypic analysis of conidiospore development of transformants T6, T7, T11 and T15 carrying pBRLAi_xyl and the corresponding recipient P2niaD18 on solid MM medium supplemented with glucose or xylose. (b) Northern blot hybridization with a *PcbriA* gene fragment as probe, with γ -actin as loading control. (c) Detection of siRNAs specific for *PcbriA*. RNA was obtained from strains as indicated. A 25 nt *PcbriA* oligonucleotide was used as molecular size marker and predominant RNAs served as loading control. Strains were grown MM medium containing glucose (+G) or xylose (+X) as main carbon source.

Two genes for Dicer homologues in the *P. chrysogenum* genome

Based on the recently published *P. chrysogenum* genome sequence (van den Berg *et al.*, 2008), we identified major components of the RNAi machinery in this fungus. Using the *A. nidulans* (AN10380.3) and *N. crassa* (NCU06766.3) amino acid sequences for Dicer-like proteins, we identified two possible homologues (Pc21g06890 and Pc12g13700), named *Pcdcl1* and *Pcdcl2*, in the *P. chrysogenum* genome

database. The *Pcdcl1* and *Pcdcl2* genes encode proteins of 1495 and 1393 amino acids, respectively (Supplementary Figs S1 and S2). The NCBI conserved-domain search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>; Marchler-Bauer *et al.*, 2007) revealed that both proteins contain all characteristic domains of Dicer proteins, i.e. the N-terminal DEAD-like helicase (pfam00270, cd00046), a helicase C (cd00079), two C-terminal RNaseIII domains (cd00593), as well as the Duf283 domain. The latter domain shows a folding similar to the double-stranded RNA-binding domain (pfam03368; Dlakic, 2006). Alignments of all sequences showed that Pc21g06890 (*Pcdcl1*) has a high sequence similarity to the *N. crassa* DCL-1 protein (NCU08270.3; about 40 %) and to the truncated version of the *A. nidulans* DclA protein (AN0119.3; about 12 %). Thus, it is likely that this protein plays a role in the MSUD pathway. In contrast, Pc12g13700 (*Pcdcl2*) shows a higher homology to Dicer proteins that are responsible for RNAi (quelling), such as the *N. crassa* DCL-2 polypeptide (NCU06766.3; about 25 %) and the *A. nidulans* DclB protein (AN10380.3; about 37 %). The alignments of the *P. chrysogenum* *Pcdcl1* and *Pcdcl2* proteins to corresponding sequences of closely related fungi, such as *Neosartorya fischeri* (gil119478976|reflXP_001259517.1; gil158705692|splA1D9Z6.1) and *Apergillus fumigatus* (Afu5g11790; Afu4g02930), revealed an identity of between 55 % and 57 % and 52 % and 55 %, respectively (Supplementary Figs S1 and S2). Based on this *in silico* analysis, we chose the *Pcdcl2* gene for construction of a knockout strain for specific disruption of Dicer-directed RNAi.

A *Pcdcl2* knockout strain lacks RNAi silencing

In order to confirm a functional hairpin-induced silencing process in *P. chrysogenum*, a *Pcdcl2* disruption strain was generated by homologous recombination in a Δ Pcku70 background (Hoff *et al.*, 2009). A gene-replacement cassette was generated containing the phleomycin-resistance marker flanked on both sites by approximately 1 kb of 5' and 3' genomic sequences from the *Pcdcl2* locus (Fig. 4a). A 2.8 kb PCR fragment was used for deletion of the *Pcdcl2* gene (Fig. 4a). Resulting transformants were screened for phleomycin and nourseothricin resistance to identify positive colonies. Subsequently, genomic DNA was isolated from five different colonies and used as template to amplify the 5'- and 3'-flanking regions (Fig. 4a). Since primers P1 to P4 are located outside the gene-replacement cassette or within the phleomycin-resistance marker, the corresponding PCR fragments can only be obtained when homologous recombination of the transforming DNA has occurred. PCR analysis clearly showed that the *Pcdcl2* gene had been successfully disrupted in all five transformants (data not shown). DNA from conidiospore isolates (Δ Pcdcl2 T3.4 and Δ Pcdcl2 T3.6) derived from Δ Pcdcl2 T3 served as source for a PCR analysis. As evidenced by the 1.3 kb and 1.2 kb PCR fragments shown in Fig. 4(b), homologous recombination had taken place in both

Fig. 4. Disruption of the *Pcdcl2* gene in *P. chrysogenum*. (a) Schematic representation of the *Pcdcl2* locus before ($\Delta Pcku70$) and after homologous integration ($\Delta Pcdcl2$) of a phleomycin-resistance cassette (*ble*). P1 to P6 represent oligonucleotides used in different PCR experiments to confirm *Pcdcl2* gene disruption. Probes used for Southern blot analysis are indicated by grey bars. *HindIII* sites relevant for interpretation of the Southern hybridization blot are given in black. (b) Agarose gel electrophoresis showing PCR amplicons generated with primer pairs as indicated using genomic DNAs from the strains shown. (l) indicates PCRs without DNA as control. (c) Southern blot analysis of *HindIII*-restricted genomic DNA from strains as indicated. Hybridization was done with a radiolabelled fragment of the 5'-flanking region of the *Pcdcl2* gene.

isolates, whereas the recipient strain $\Delta Pcku70$ did not produce any amplicon. A second PCR was performed using primers P5 and P6 for amplification of the *Pcdcl2* ORF. As shown in Fig. 4(b), the expected 720 bp PCR fragment was obtained in the recipient $\Delta Pcku70$, but was absent in both knockout strains. This result further confirms that *Pcdcl2* has been deleted in $\Delta Pcdcl2$ T3.4 and $\Delta Pcdcl2$ T3.6. To exclude any heterokaryotic state of the transformant, Southern blot analysis was performed using the 5'-*Pcdcl2* flanking region as probe. A 3.2 kb fragment was detected for P2niaD18 and $\Delta Pcku70$, and a single 2.6 kb fragment in the two $\Delta Pcdcl2$ single-spore isolates (Fig. 4c), thus confirming homologous recombination at the *Pcdcl2* locus.

As further evidence of the functionality of the RNAi machinery in *P. chrysogenum*, single-spore isolates from both the $\Delta Pcdcl2$ deletion strain and the wild-type strain

P2niaD18 were transformed with silencing plasmid pBRLAi, carrying two inversely oriented *PcbrlA* gene fragments. In a first step, transformants were selected on the basis of their conidiophore formation on agar plates. As exemplified in Fig. 5, conidiophore development comparable to the wild-type was found for 100 and 98 tested transformants derived from recipients $\Delta Pcdcl2$ T3.4 and $\Delta Pcdcl2$ T3.6, respectively. In contrast, 65 out of 94 derivatives from P2niaD18 showed reduced conidiophore development, as can be recognized phenotypically by their colourless mycelium. In the remaining 29 P2niaD18 derivatives, mycelial sectors were green, thus indicating partial silencing in these transformants. For a more detailed analysis on the molecular level, we investigated the occurrence of siRNAs. As expected, *PcbrlA*-specific siRNAs were found in the P2niaD18 derivatives, whereas derivatives of the Dicer knockout lacked siRNAs (data not

Fig. 5. Phenotypic analysis of conidiophore development on solid medium. Shown are the recipient P2niaD18 and Δ Pcd70, as well as the two Dicer knockout strains carrying the hairpin-expressing vector pBRLAi.

shown). The phenotypic and molecular characterization allow us to conclude that *Pcdcl2* is a prerequisite to trigger the RNAi pathway in *P. chrysogenum*.

Application of the RNAi system for efficient homologous recombination

The evidence of Dicer-dependent RNAi prompted us to examine the applicability of the silencing system by increasing the efficiency of homologous recombination in *P. chrysogenum*. As described in Methods, plasmid pKU70i (Supplementary Table S2) carries two inversely oriented

Pcku70 gene fragments; this plasmid was used for transformation of the wild-type strain. The resulting transformant P2:KU70i with reduced expression of the *Pcku70* gene was used further as a recipient for homologous recombination experiments. In order to test the efficiency, three independent transformation experiments were conducted to replace target genes by a resistance marker. In the first two approaches, a 3.8 kb fragment from plasmid pKOPcLAEA carrying the phleomycin-resistance gene flanked by 1.5 kb sequences of the *PclaeA* gene was transformed into P2:KU70i under *xylP*-inducing conditions. PCR (Fig. 6a) and Southern hybridization analysis (data not shown) from a total of 18 transformants revealed two strains where the *PclaeA* gene was substituted by the phleomycin-resistance gene. In order to demonstrate further the efficiency of the transiently silenced *Pcku70*-system, we generated a double mutant. One of the above-described knockout strains, P2:KU70i: Δ PclaeA, was used for the third and final homologous recombination experiment. A 3.4 kb PCR fragment from plasmid pKOPcVELA_Nat carrying the nourseothricin-resistance gene and adjacent 5'- and 3'-sequences from the *PcvelA* gene was used for transformation of strain P2:KU70i: Δ PclaeA. We obtained 21 transformants, which were screened by PCR for homologous recombination. As shown in Fig. 6(b), isolate P2:KU70i: Δ PclaeA: Δ PcvelA lacks the *PcvelA* gene completely, thus verifying the construction of a double-knockout strain. These data were further confirmed by Southern hybridization (data not shown). Growth of the double mutant on non-selective medium lacking pyrithiamine led to a loss of the self-replicating plasmid pKU70i, and resulted in a mutant strain devoid of any Δ ku70 background.

Fig. 6. PCR analysis to verify a single (Δ PclaeA) and double (Δ PclaeA: Δ PcvelA) knockout strain. (a) Primer pairs were chosen to verify homologous recombination by amplifying specifically the 5', 3' and internal region of the *PclaeA* gene. (b) The primer pair detects the internal region of *PcvelA* and thus verifies the loss of the gene in P2:KU70i: Δ PclaeA: Δ PcvelA.

DISCUSSION

We chose the industrial fungus *Penicillium chrysogenum* as our model to demonstrate how a Dicer-dependent RNAi system can be used for genetic manipulation of this β -lactam antibiotic producer. RNAi is a sequence-specific post-transcriptional gene silencing process that is triggered in fungi by the highly conserved RNAi pathway, which uses siRNAs as specificity determinants (Nakayashiki & Nguyen, 2008). Here, we used four independent experimental approaches to demonstrate the occurrence of siRNAs in *P. chrysogenum*, thereby providing strong evidence for the presence of an RNAi pathway in this filamentous fungus. siRNA has already been shown to accumulate during gene silencing not only in ascomycetes such as *N. crassa*, *M. oryzae* and *A. nidulans* but also in the zygomycete *Mucor circinelloides* (Barton & Prade, 2008; Goldoni *et al.*, 2004; Kadotani *et al.*, 2004; Nicolás *et al.*, 2007). In some cases, inducible promoter systems, similar to those described here, were used to provide unequivocal evidence that siRNA formation is dependent on activation of RNAi by dsRNA. Inducible RNAi systems were for example described using the *cbhB* promoter from the cellobiohydrolase gene, which can be repressed by glucose (Bromley *et al.*, 2006; Khalaj *et al.*, 2007); the authors repressed *alb1* expression to change the pigmentation of conidiospores. Quite recently, another inducible RNAi system was successfully established for *A. nidulans*. Barton & Prade (2008) used the alcohol dehydrogenase promoter, which can be induced on threonine-containing media, to silence the *brlA* gene. The *brlA* homologue from *P. chrysogenum* also served as a morphological marker in our studies, which we extended by the quantified analysis of 19 transformants. Randomly selected transformants served further for analysis of siRNAs, which appeared only in transformants that were grown on xylose-containing medium. These inducible promoters, which can be activated by the choice of carbon source, have the key advantage that they enable the conditional silencing of gene expression, thereby allowing the study of different stages of development (Barton & Prade, 2008).

The final evidence for an RNAi pathway was provided by investigating a Dicer knockout strain that is unable to induce downregulation of the *PcbrlA* morphogene. Similarly, Dicer knockout strains were recently described to prevent RNAi silencing in *Cryphonectria parasitica*, *A. nidulans*, *N. crassa* and *M. oryzae* (Catalanotto *et al.*, 2004; Hammond *et al.*, 2008; Kadotani *et al.*, 2004; Nguyen *et al.*, 2008; Segers *et al.*, 2007).

For induction of an RNAi pathway two different vector types, namely hairpin-expressing and opposing-dual promoter systems, have recently been used in different fungal species. Opposing-dual promoter systems carry two inversely oriented promoters that transcribe a single fragment of the target gene to produce sense and antisense RNA. The advantage of such a system is the one-step construction of RNA silencing vectors with just a single,

non-oriented cloning step. This strategy is beneficial for high-throughput screenings in which the function of multiple gene fragments is tested. In a careful study this vector was successfully used in *M. oryzae* to test genes involved in calcium signalling pathways. However, compared with hairpin expression vectors, this approach showed a reduced silencing efficiency (Nguyen *et al.*, 2008). In a similar approach, Ullán *et al.* (2008) observed in *P. chrysogenum* a silencing efficiency of only 20 % using the opposing-dual promoter system. Construction of hairpin expression vectors requires two oriented cloning steps and is therefore mainly applicable in small-scale analyses (reviewed by Nakayashiki & Nguyen, 2008). The successful application of a hairpin expression vector based on the *DsRed* reporter gene was recently demonstrated in *A. chrysogenum* (Janus *et al.*, 2007), and further developed here for *P. chrysogenum*. In our first approach, we generated transformants of which 90 % showed a white or pink phenotype, which is indicative of a functional RNAi system. Remarkably, about half of the transformants showed a null phenotype, an effect which was not observed when opposing-dual promoter systems were used to downregulate the penicillin biosynthesis genes (Ullán *et al.*, 2008).

The RNAi system described here is particularly valuable when for example biosynthesis genes are present in multi-copy numbers. *P. chrysogenum* producer strains are known to contain 6 to 14 copies of the clustered penicillin biosynthesis genes (Fierro *et al.*, 2006). We have recently demonstrated that Δ Pcku70 strains can be used to delete single gene copies from the penicillin cluster; however, a simultaneous deletion of all amplified gene copies was impossible (Hoff *et al.*, 2009). Using an alternative RNAi approach, Ullán *et al.* (2008) demonstrated recently the successful expressional downregulation of the multi-copy penicillin biosynthesis genes. Similarly, a single copy of an RNAi vector was sufficient to silence three copies of the strongly expressed α -amylase gene in *A. oryzae*, leading to a decrease of enzymic activity to 10 % of control levels (Yamada *et al.*, 2007). In another attempt, downregulation of three *MnSOD* genes, encoding manganese-containing superoxide dismutases, with a single silencing vector, containing exon 1 of the *MnSOD1* gene, was demonstrated in *Phanerochaete chrysosporium* (Matityahu *et al.*, 2008). These examples show the usefulness of RNAi systems for the functional analysis of multi-copy genes or gene families sharing adequate sequence homology.

The applicability of RNAi to studying fungal metabolism was demonstrated recently for the zygomycete *Mortierella alpina*, the commercial producer of arachidonic acid. Silencing of the Δ 12-desaturase gene in *M. alpina* led to enhanced fatty acid production. Another positive effect was that different fatty acid compositions and contents were observed in the transgenic strains compared with the recipient (Takeno *et al.*, 2005). Another impressive example of how RNAi can be used to regulate fungal physiology was shown for the rice-blast fungus *M. oryzae*.

Nguyen *et al.* (2008) tested the expression of 37 calcium-signalling-related genes and showed that they were involved in hyphal growth, sporulation and pathogenicity. Finally, the RNAi system has already been successfully used to silence expression of β -lactam biosynthesis genes in different fungal producer strains (Janus *et al.*, 2007; Ullán *et al.*, 2008). A novel useful application of the RNAi system is presented here, with the transient downregulation of the *Pcku70* gene, which is involved in non-homologous end-joining. Deletion of the *Pcku70* gene in *P. chrysogenum* was recently shown to result in the generation of strains with a higher sensitivity to osmotic stress compared to the recipient strain (Hoff *et al.*, 2009). In fungi with a functional mating system, such *ku* deletions can be replaced by the wild-type gene simply by conventional crossing experiments. However, most industrially relevant fungi do not have a sexual cycle that allows *ku* disruption to be rectified. Alternatively, the transient expression system with an inducible, non-integrative RNAi vector as shown here offers the possibility to silence *ku* gene expression for a time-restricted homologous recombination and integration event in appropriate recipient strains. Although the targeting efficiency obtained is less than in a Δ Pcku70 strain (Hoff *et al.*, 2009) the observed frequencies of homologous recombination are clearly above values that were observed with the wild-type strain (e.g. Casqueiro *et al.*, 1999; Hoff *et al.*, 2009; Naranjo *et al.*, 2004; Snoek *et al.*, 2009). The use of the non-integrative RNAi vector leads to the construction of single and double mutants that will be a valuable tool for further functional and physiological studies. In summary, RNAi is a useful tool to manipulate fungal secondary metabolism, e.g. to improve producer strains or to test components of as yet incompletely understood signalling or biosynthetic pathways.

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