Technical advance

**RNA-mediated gene silencing in the cereal fungal pathogen
*Cochliobolus sativus***YUEQIANG LENG¹, CHENGXIANG WU², ZHAOHUI LIU¹, TIMOTHY L. FRIESEN³,
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A high-throughput RNA-mediated gene silencing system was developed for *Cochliobolus sativus* (anamorph: *Bipolaris sorokiniana*), the causal agent of spot blotch, common root rot and black point in barley and wheat. The green fluorescent protein gene (*GFP*) and the proteinaceous host-selective toxin gene (*ToxA*) were first introduced into *C. sativus* via the polyethylene glycol (PEG)-mediated transformation method. Transformants with a high level of expression of *GFP* or *ToxA* were generated. A silencing vector (pSGate1) based on the Gateway cloning system was developed and used to construct RNA interference (RNAi) vectors. Silencing of *GFP* and *ToxA* in the transformants was demonstrated by transformation with the RNAi construct expressing hairpin RNA (hpRNA) of the target gene. The polyketide synthase gene (*CsPKS1*), involved in melanin biosynthesis pathways in *C. sativus*, was also targeted by transformation with the RNAi vector (pSGate1-*CsPKS1*) encoding hpRNA of the *CsPKS1* gene. The transformants with pSGate1-*CsPKS1* exhibited an albino phenotype or reduced melanization, suggesting effective silencing of the endogenous *CsPKS1* in *C. sativus*. Sectors exhibiting the wild-type phenotype of the fungus appeared in some of the *CsPKS1*-silenced transformants after subcultures as a result of inactivation or deletions of the RNAi transgene. The gene silencing system established provides a useful tool for functional genomics studies in *C. sativus* and other filamentous fungi.

INTRODUCTION

RNA-mediated gene silencing or RNA interference (RNAi) has been found to be a common phenomenon in eukaryotic

organisms. In this process, double-stranded RNA (dsRNA), which can be induced by viral RNA, hairpin RNA (hpRNA) or virus-induced gene silencing encoded RNA (Waterhouse and Helliwell, 2003), is degraded into small interfering RNAs (siRNAs). These siRNAs are incorporated into the RNA-induced silencing complex (RISC) to target and degrade the mRNA of genes with the complementary sequence (Liu *et al.*, 2002; McDonald *et al.*, 2005; Nakayashiki and Nguyen, 2008; Waterhouse and Helliwell, 2003). RNAi has been demonstrated to silence genes in a number of fungi (for a review, see Kück and Hoff, 2010). Examples include *Cryptococcus neoformans* (Liu *et al.*, 2002), *Magnaporthe oryzae* (Jeon *et al.*, 2007; Kadotani *et al.*, 2003; Nakayashiki *et al.*, 2005), *Venturia inaequalis* (Fitzgerald *et al.*, 2004), *Histoplasma capsulatum* (Rappleye *et al.*, 2004), *Schizophyllum commune* (de Jong *et al.*, 2006), *Coprinopsis cinerea* (syn. *Coprinus cinereus*) (Namekawa *et al.*, 2005; Wälti *et al.*, 2006), *Mortierella alpina* (Takeno *et al.*, 2005), *Dictyostelium discoideum* (Martens *et al.*, 2002), *Aspergillus* and *Fusarium* species (McDonald *et al.*, 2005), *Bipolaris oryzae* (Moriwaki *et al.*, 2007) and *Ophiostoma novoulmi* (Carneiro *et al.*, 2010). Most of the studies mentioned above used RNAi vectors containing inverted repeats of the target gene or its partial sequence for transformation. Construction of RNAi vectors often involves several cloning steps, and thus is laborious and time-consuming. Nakayashiki *et al.* (2005) developed a pHANNIBAL-like silencing vector, pSilent-1, for RNA silencing studies in filamentous fungi. The applicability of pSilent-1 was demonstrated in several phytopathogenic ascomycete fungi, including *M. oryzae*, *Colletotrichum lagenarium* and *B. oryzae* (Moriwaki *et al.*, 2007; Nakayashiki *et al.*, 2005). However, pSilent-1 is a restriction enzyme-based cloning vector and therefore may not be suitable for the construction of large numbers of RNAi vectors for high-throughput gene silencing studies. Recently, a pHellsgate-like RNAi vector (pTroya) based on Invitrogen's (Carlsbad, CA, USA) Gateway technology has been developed and shown to be useful in *Colletotrichum gloeosporioides* (Shafran *et al.*, 2008). This type of RNAi vector should have wide application in many other plant pathogenic fungi.

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Fig. 1 A flow chart showing the construction of a hairpin RNA (hpRNA)-expressing RNA interference (RNAi) vector using pSGate1. A DNA fragment from a target gene was amplified by polymerase chain reaction (PCR) with primers containing the *attB1* and *attB2* sites. The PCR product was cloned into the plasmid pDONR221 to create an entry clone by BP reaction. The insert in the entry clone then replaced the *ccdB* genes of pSGate1 by LR reaction, resulting in an RNAi construct expressing the hpRNA of the target gene.

Cochliobolus sativus (Anamorph: *Bipolaris sorokiniana*) is the causal agent of several important cereal diseases, including spot blotch, common root rot and black point (Mathre, 1997; Wiese, 1977). Despite the importance of the diseases caused by *C. sativus*, the molecular mechanisms underlying disease development are still poorly understood. Previous studies on *C. sativus* have mainly focused on the identification of pathotypes (Valjavec-Gratian and Steffenson, 1997a), the analysis of genetic diversity (Zhong and Steffenson, 2001a), the cloning of mating-type genes (Zhong and Steffenson, 2001b), genetic studies on virulence (Valjavec-Gratian and Steffenson, 1997b) and the construction of a genetic map (Zhong *et al.*, 2002). An effective gene disruption and gene silencing system has not been developed for *C. sativus*. In this study, an RNAi vector based on the Gateway cloning system (Invitrogen) was modified from pSilent-1 and used to silence two reporter genes [proteinaceous host-selective toxin (HST) gene (*ToxA*) and green fluorescent protein gene (*GFP*)] transformed into *C. sativus* isolates. The RNAi system was further demonstrated by knocking down the polyketide synthase gene (*CsPKS1*), one of the genes involved in the melanin synthesis pathway in *C. sativus*. The RNAi system established will provide an alternative powerful tool for functional genomics studies in *C. sativus* and other filamentous fungi.

RESULTS

Construction of RNAi vectors

To construct an RNAi vector based on the Gateway cloning system, we used pSilent1 (Nakayashiki *et al.*, 2005) as a backbone and cloned the *ccdB* gene, as well as the flanking R1 and R2 recombination sites, from pHellsgate-12 (Wesley *et al.*, 2001) into both sides of the *M. oryzae* intron in pSilent1 to generate pSGate1 (Fig. S1, see Supporting Information). Using

this Gateway-based vector, we constructed three RNAi vectors, pSGate1-*GFP*, pSGate1-*ToxA* and pSGate1-*CsPKS1*, as outlined in Fig. 1. These RNAi constructs were verified by restriction digestion using single enzyme (*XhoI*) or double enzymes (*BglII* and *KpnI*), and confirmed by DNA sequencing (data not shown).

Generation of GFP- and ToxA-expressing transformants of *C. sativus*

To generate GFP-expressing transformants of *C. sativus*, we introduced gGFP (Maor *et al.*, 1998) into the isolate ND93-1 through transformation. Eleven *GFP* transformants were generated. Eight of the transformants showed GFP fluorescence under a fluorescence microscope, but the GFP expression varied among them. Three of the transformants, including GFP-19, showed strong GFP expression (+++) in both spores and mycelia (Fig. 2a).

We also generated *ToxA*-expressing transformants of *C. sativus* by transformation of the isolate ND93-1 with the plasmid pDNA-*ToxA*, which contains the *ToxA* gene (Ciuffetti *et al.*, 1997) from the wheat tan spot pathogen *Pyrenophora tritici-repentis*. The *ToxA* gene encodes a proteinaceous HST, *ToxA*, which is only toxic to certain wheat genotypes susceptible to the HST-producing fungus; isolates of the fungus that do not produce the toxin are either nonpathogenic or weakly virulent (Ciuffetti *et al.*, 1997; Friesen *et al.*, 2006). Three hygromycin-resistant transformants were generated from the transformation experiment. Infiltration of the culture filtrates from these transformants into the leaves of the *ToxA*-sensitive wheat genotypes BG261 and Grandin (Friesen *et al.*, 2006) indicated that all transformants induced the typical necrotic symptoms on these wheat genotypes, suggesting a strong expression of *ToxA* *in vitro*.

Fig. 2 Green fluorescent protein (GFP) expression of the parental *GFP* transformant and the *GFP* RNA interference (RNAi) transformants in *Cochliobolus sativus*. (a) The parental *GFP* transformant, GFP-19, expressing strong GFP fluorescence (+++) in both mycelia and conidial spores. (b–e) Representative *GFP* RNAi transformants showing GFP expression at different levels: 0, without GFP expression; +, with a very faint GFP expression; ++, stronger than + but weaker than the parental *GFP* transformant; +++, the same or stronger expression of GFP compared with the parental *GFP* transformant. Bar, 20 μ m.

Silencing of *GFP* in the *GFP* transformant of *C. sativus*

To test the applicability of RNAi in *C. sativus*, we first attempted to silence the *GFP* gene in the *GFP* transformant, GFP-19, which exhibited the strongest GFP fluorescence among the *GFP* transformants generated. The RNAi construct pSGate1-*GFP* was introduced into GFP-19 by cotransformation with pII99, which contains the gene for resistance to geneticin G418 (Namiki *et al.*, 2001). A total of 48 double transformants was generated, which exhibited different levels of GFP fluorescence observed under a fluorescence microscope (Fig. 2). On the basis of the intensity of GFP fluorescence, these *GFP* RNAi transformants were classified into four categories with 11, 21, 5 and 11 transformants having GFP fluorescence intensity at 0, +, ++ and +++, respectively.

We randomly selected four RNAi transformants from each of the four GFP fluorescence categories and carried out Southern blot analyses. gGFP, pSGate1-*GFP* and genomic DNA of the RNAi transformants, as well as the wild-type (WT) strain ND93-1 and the parental *GFP* transformant (GFP-19), were digested with the two enzymes *Xho*I and *Kpn*I and probed with the *GFP* gene. All RNAi transformants carried the 2.3-kb fragment derived from the transcriptional unit of gGFP after digestion with *Xho*I and *Kpn*I (Fig. S2, see Supporting Information). However, the two fragments (0.6 kb and 0.8 kb, respectively) representing the transcriptional unit of hpRNA of the *GFP* gene in the silencing vector pSGate1-*GFP* were only detected in the RNAi transformants exhibiting reduced or no GFP fluorescence expression, except for one (RNAi-47) (Fig. S2). These results indicate that GFP silencing in the double transformants was not caused by a change in the *GFP* gene, but by the presence of the RNAi vector that expressed the intact *GFP* transcriptional unit of hpRNA. RNAi-47 showed some silenced phenotype. This may be a result of the presence of a partial transcriptional unit of *GFP* hpRNA as detected in the Southern blot analysis (Fig. S2).

Quantitative real-time polymerase chain reaction (RT-PCR) was used to measure the mRNA expression of the *GFP* gene in eight representative *GFP* RNAi transformants, which showed various levels of GFP fluorescence. GFP expression was reduced significantly at the transcriptional level in the RNAi transformants that contained the pSGate1-*GFP* vector (Fig. 3). Compared with the parental transformant (GFP-19), RNAi-1 and RNAi-22 had only 1.0% and 1.3% relative expression of GFP, and RNAi-2 and RNAi-20 had 4.6% and 12.1% relative expression of GFP, respectively. RNAi-14 showed strong GFP expression based on the microscopy observation, but had only about 60% of relative expression of GFP compared with the parental *GFP* transformant (Fig. 3). Interestingly, the transcriptional levels in the nonsilenced transformants (RNAi-11 and RNAi-28) were higher than that of the parental *GFP* transformant, GFP-19.

Silencing of *ToxA* in the *ToxA* transformant of *C. sativus*

To silence *ToxA*, pSGate1-*ToxA* was introduced into one of the *ToxA* transformants, designated as ToxA-3, through cotransformation with pII99. Thirty-four double transformants were generated from ToxA-3. Infiltration experiments showed that 18 of these double transformants failed to induce the expanded necrosis symptom on *ToxA*-sensitive wheat plants, whereas the remaining transformants showed the same phenotype as the parental *ToxA* transformant (Fig. 4).

Four *ToxA*-silenced and four nonsilenced transformants were randomly chosen for Southern blot analysis with the *ToxA* gene as a probe. When digested with *Xho*I and *Kpn*I, pDAN-*ToxA* released one fragment insert, whereas pSGate1-*ToxA* produced three fragments. One (the largest) of the three fragments was from the plasmid DNA of pSGate1-*ToxA* because the probe used was a PCR product of *ToxA* amplified from pSGate1-*ToxA*. The other two fragments (0.9 kb and 1.1 kb) were from the transcriptional *ToxA*

Fig. 3 Quantitative real-time polymerase chain reaction (RT-PCR) analysis of green fluorescent protein (GFP) gene expression in the wild-type (WT) isolate, *GFP* transformant (GFP-19) and representative *GFP* RNA interference (RNAi) transformants of *Cochliobolus sativus*. The RNA level of *GFP* is reduced to various degrees in the *GFP* RNAi transformants (RNAi-1, -2, -22, -20, -19 and -14) harbouring pSGate1-*GFP* when compared with the parental *GFP* transformant (GFP-19). The two *GFP* RNAi transformants (11 and 28) without pSGate1-*GFP* inserted into their genomes showed higher RNA expression than the parental *GFP* transformant (GFP-19). The intensity of *GFP* fluorescence observed under fluorescent microscopy is indicated by 0, +, ++ and +++. The error bars indicate the minimum and maximum values of the relative expression of the *GFP* gene.

cassettes of pSGate1-*ToxA*. In the double transformants, the pSGate1-*ToxA* vector was only identified in those that lost the ability to induce necrosis (Fig. S3, see Supporting Information); presumably they had no or reduced expression of *ToxA*. All of the RNAi transformants analysed had the same *ToxA* gene as the parental *ToxA* transformant (ToxA-3), suggesting that silencing of *ToxA* was a result of the presence of hpRNA expressed by the RNAi construct (pSGate1-*ToxA*) introduced via transformation. In the Southern blot hybridization analysis, we found that RNAi transformants derived from the transformation experiments with pSGate1-*ToxA* showed some DNA fragments other than the *ToxA* gene hybridized with the probe (Fig. S3, see Supporting Information). These extra hybridization signals could be a result of multiple insertions of the silencing vector (pSGate1-*ToxA*) into the genomes of the transformants.

Silencing of the endogenous gene *CsPKS1* in *C. sativus*

To demonstrate the applicability of RNAi as a tool to knock down endogenous genes in *C. sativus*, we chose to target the *CsPKS1* gene, which encodes a polyketide synthase involved in the initial step of the melanin biosynthesis pathway (Eliahu *et al.*, 2007; Leng and Zhong, unpublished data). As expected, the *CsPKS1* knockout mutant ($\Delta CsPKS1$), which was generated by the split-marker system (Catlett *et al.*, 2003) (data not shown), showed an albino phenotype (Fig. 5), confirming that *CsPKS1* is related to melanin synthesis. When the *C. sativus* isolate ND93-1 was transformed with the *CsPKS1* silencing vector pSGate1-*CsPKS1*, 15 hygromycin-resistant transformants were generated. Two of the

transformants showed the same phenotype as the wild-type, whereas the remaining transformants produced colonies exhibiting various degrees of melanin deficiency (light colour) compared with those of the wild-type (Fig. 5). One of the RNAi transformants consistently showed the albino phenotype, similar to the knockout mutant, even after many subculture transfers, whereas the majority (12/13) of the single-spore-derived white-colour transformants produced black-colour sections on V8 potato dextrose agar (PDA) plates after one or more transfers to new plates (Fig. 5). Single spores from the white and black sectors of each transformant were isolated and plated onto V8 PDA plates. The result indicated that spores from the black sectors produced black colonies similar to the wild-type isolate ND93-1, whereas those from the white sectors generated white colonies, but with black sectors after a few cycles of subculture. This indicated that the majority of the *CsPKS1*-silenced transformants were unstable.

We performed Southern blot analysis of the transformants with the *CsPKS1* gene. A single copy of the endogenous *CsPKS1* gene was detected in all transformants, except for the *CsPKS1* deletion mutant $\Delta CsPKS1$ (Fig. 6). The two parts representing the transcriptional unit of hpRNA of the *CsPKS1* gene in pSGate1-*CsPKS1* were identified in all *CsPKS1* RNAi mutants showing white-colour or light-colour colonies (Fig. 6). This indicated that the silencing of *CsPKS1*, resulting in the white-colour phenotype in the transformants, was a result of the presence of the silencing construct. Genomic DNA samples of the white and black sectors derived from the original white-coloured transformants were analysed using Southern blot hybridization. We found that the transcriptional unit of the hpRNA of the *CsPKS1* gene remained in the white-colour sectors, but was missing in the black sectors.

Fig. 4 Bioassay of culture filtrates from the wild-type (WT) strain ND93-1 [without the proteinaceous host-selective toxin gene (*ToxA*)], the *ToxA* transformant (*ToxA*-3) and the representative *ToxA* RNA interference (RNAi) transformants of *Cochliobolus sativus* on the wheat line BG261 (*ToxA*-sensitive). The culture filtrates from the WT strain and the *ToxA*-silenced double transformants (1, 2, and 3) showed necrosis only at the infiltration site, whereas the culture filtrates from the *ToxA* transformant (*ToxA*-3) and the nonsilenced double transformants (10, 14 and 23) induced extensive necrotic symptoms on the leaves of BG261.

This result indicated that the RNAi transgene introduced could be inactivated or deleted from the genome of the transformants during the subculturing process, resulting in the loss of gene silencing.

DISCUSSION

Although RNAi has been studied in a number of filamentous fungi, its application in *C. sativus* has not been reported. In this study, we demonstrated that both reporter genes (*GFP* and *ToxA*) introduced into the fungus could be silenced by transformation with an RNAi construct expressing hpRNA of the target gene. We further demonstrated that the endogenous *CsPKS1* gene, which is involved in melanin synthesis, was silenced in *C. sativus* when an RNAi construct expressing hpRNA of the gene was introduced. This result indicates that the machinery required for RNAi is present in the fungus. In *C. heterostrophus*, a closely related species of *C. sativus*, gene homologues for dicer and other RNA silencing components were identified in the available

genome sequence (<http://genome.jgi-psf.org/CocheC5-1/CocheC5-1.home.html>). A search of the *C. sativus* sequences generated from a 454 run also indicates the presence of RNAi machinery components (Zhong *et al.*, unpublished data). Furthermore, RNAi was demonstrated in the rice pathogen *B. oryzae* (Moriwaki *et al.*, 2007), which is the asexual stage of *Cochliobolus miyabeanus*. Therefore, *Cochliobolus* species can be added to the group of fungi which are capable of RNA silencing.

Previous studies have indicated that the most efficient gene silencing can be achieved by the introduction of a silencing construct containing duplicated sequences of a gene or a partial gene arranged as inverted repeats separated by a spacer (Goldoni *et al.*, 2004; Kadotani *et al.*, 2003; Smith *et al.*, 2000). A series of Gateway-based vectors, such as pHellgate, have been developed for high-throughput gene silencing in plants (Helliwell *et al.*, 2002; Wesley *et al.*, 2001). However, such vectors have not been available in fungi until recently, when Shafran *et al.* (2008) developed a Gateway-based RNAi vector (pTroya) and demonstrated its applicability in *Colletotrichum gloeosporioides*. In the current study, we developed the RNAi vector, pSGate1, similar to pTroya, and further demonstrated its high efficiency for the construction of vectors for gene silencing experiments. pSGate1 will allow for large-scale gene silencing studies, especially when cDNA libraries are constructed with the entry vectors such as pDONR221 (Cho *et al.*, 2008). cDNA clones can be used to develop RNAi constructs easily because only one step (LR reaction) is required to transfer the cDNA insert from the entry vector into pSGate1, resulting in RNAi constructs expressing hpRNA of the target gene.

Our studies showed that 77% of the *GFP* RNAi transformants revealed some degree of silencing of the *GFP* gene, and approximately 23% of the *GFP* RNAi mutants showed complete silencing of the target gene. In addition, in the *ToxA* silencing experiments, 53% of the *ToxA* RNAi transformants lost the ability to induce necrosis in *ToxA*-sensitive wheat plants. These results are in agreement with those reported previously. For example, Fitzgerald *et al.* (2004) showed that transcripts of the silenced gene (trihydroxynaphthalene reductase gene, *THN*) were almost undetectable in *Venturia inaequalis*. In other studies, silencing of the targeted gene was partial, as reported in *Neurospora crassa* (Goldoni *et al.*, 2004), *M. grisea* (Nakayashiki *et al.*, 2005), *Coprinus cinereus* (Wälti *et al.*, 2006) and *Aspergillus* species (Hammond and Keller, 2005; Mouyna *et al.*, 2004). This partial silencing may make phenotyping complicated and difficult. However, cosilencing of the target gene with a reporter gene such as *GFP* may resolve the problem (Fitzgerald *et al.*, 2004; Nakayashiki and Nguyen, 2008.).

We found that some of the nonsilenced RNAi transformants showed a higher relative expression of *GFP* than the parental *GFP* transformant at the transcriptional level. The same phenomenon was also observed by Fitzgerald *et al.* (2004) when using RNA-mediated gene silencing to silence the *THN* gene. They

Fig. 5 Colony morphology of the wild-type (WT) strain ND93-1, the polyketide synthase gene (*CsPKS1*) deletion mutant ($\Delta CsPKS1$) and the representative *CsPKS1* RNA interference (RNAi) mutants cultured on V8 potato dextrose agar (PDA) plates. The WT ND93-1 produces a dark black–brown colony, whereas the *CsPKS1* deletion mutant shows a white colony. The *CsPKS1* RNAi mutants showed various degrees of reduction in pigmentation compared with the WT strain. Black sectors were observed in some of the *CsPKS1* RNAi mutants, as shown for RNAi-1 and RNAi-13. RNAi-13W and RNAi-13B were subcultures from single spores of the white and black sectors of RNAi-13, respectively.

Fig. 6 Southern blot analysis of the wild-type (WT) isolate ND93-1, the polyketide synthase gene (*CsPKS1*) deletion mutant $\Delta CsPKS1$ and the *CsPKS1* RNA interference (RNAi) mutants. Genomic DNA of the *CsPKS1* RNAi mutants, as well as the WT strain ND93-1 and the *CsPKS1* deletion mutant $\Delta CsPKS1$, was digested with *XhoI* and *KpnI*, subjected to electrophoresis, blotted onto a nylon membrane and probed with the *CsPKS1* gene. The full arrow indicates the fragment (5.1 kb) carrying the single-copy *CsPKS1* gene present in the WT isolate. The broken arrows indicate the two fragments representing the transcriptional unit of hairpin RNA of the *CsPKS1* gene in pSGate1-*CsPKS1*. *CsPKS1* RNAi mutants 13 and 15 produced black sectors (13B and 15B) among the white colonies (13W and 15W). The transcriptional unit of hairpin RNA of the *CsPKS1* gene was not detected in the black sectors. The extra fragment (~1.8 kb) detected in *CsPKS1* RNAi mutants 1 and 15W was caused by the insertion of the partial transcriptional unit of the *CsPKS1* gene from pSGate1-*CsPKS1* into the other location of the fungal genome.

found that some nonsilenced transformants exhibited higher expression of the targeted *THN* gene compared with the wild-type at the transcriptional level based on RT-PCR analysis. The reason for this is unknown, but merits further investigation.

We found that most of the white RNAi transformants derived from the pSGate1-*CsPKS1* transformation experiments generated black sectors after one or more subcultures. Southern hybridization indicated that these black sectors lost the transcriptional units of the RNAi construct and thus recovered the function to produce melanin. Loss or modification of the RNAi vector in silenced fungal transformants has also been reported by other researchers (Henry *et al.*, 2007; McDonald *et al.*, 2005). McDonald *et al.* (2005) reported that, although the RNA silencing phenotype was stable in the three fungi (*Aspergillus flavus*, *A. parasiticus* and *Fusarium graminearum*) studied, there was some loss of silencing in one replication of an *A. parasiticus* transformant (McDonald *et al.*, 2005). They explained that the inverted repeat transgene (IRT) could be lost during normal growth. Support for this hypothesis comes from the fact that up to 10% of the progeny from a sexual cross of *A. parasiticus* lose an IRT carried by both parents (McDonald *et al.*, 2005). Henry *et al.* (2007) also observed that the vector used for RNAi was lost or modified in the transformed fungus *A. fumigatus* over successive transfers, resulting in the loss of gene silencing. In our study, we did not find any loss of RNAi for the other two genes (*GFP* and *Tox4*) studied. Therefore, this RNAi instability may not be a

Table 1 Primers used in this study.

Primer name	Primer sequence (5'–3')*
F-Xba I-12	TTCAGATCTCACAGCTTGGATCCTAGACCAC
R-Xba I-12	GAACAGATCTCAGGACGAAATTAATCCCATGGTCA
<i>attB1</i> -GFP-1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT GACGTAACGCCACAAGTT
<i>attB2</i> -GFP-1R	GGGGACCACTTTGTACAAGAAAGCTGGGT GAAGTCCAGCAGGACCATGT
<i>attB1</i> -ToxA-1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT ACCGTCCGGCTAACCGTCCG
<i>attB2</i> -ToxA-1R	GGGGACCACTTTGTACAAGAAAGCTGGGT CGTTCCGGTTGTCGTTCCGG
<i>attB1</i> -PKS1-1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT GCTTTCGCCCAGAGAAGAAGT
<i>attB2</i> -PKS1-1R	GGGGACCACTTTGTACAAGAAAGCTGGGT GATCTCGGAGACATCAACAC
GFP-2F	CACCTCGTGACCACTTC
GFP-2R	CACCTTGATGCCGTTCTTCT
ToxA-F	CTACAGGAGCGGCAGGGAAGC
ToxA-R	GCTGCATTCTCCAATTTTCACG
RT-GFP-F	CAAGGGCGAGGAGCTGTT
RT-GFP-R	AAGTCGTGCTGCTTCATGTG
RT-Actin-F	GTATGGGCCAAAAGGACTCA
RT-Actin-R	CACGCAGCTCGTTGAGAAG

*Restriction sites are shown in italic and *att* sites are shown in bold.

universal phenomenon and probably is gene dependent. Interestingly, all of the genes targeted and exhibiting instability for RNAi are involved in pathways for the synthesis of melanin or other secondary metabolites (Henry *et al.*, 2007; McDonald *et al.*, 2005; the present study). It is not known whether silencing of these types of genes by RNAi is more unstable than for other types of genes in fungi. Further studies are needed on RNAi instability and its mechanisms.

In summary, we have demonstrated that RNAi is a powerful tool for studying the gene function of *C. sativus*. The Gateway-based RNAi vector described in our study and others may provide a high-throughput system for large-scale functional genomics of *C. sativus* and other filamentous fungi.

EXPERIMENTAL PROCEDURES

Fungal isolates, growth conditions and media

The *C. sativus* isolate ND93-1 was used as the recipient for all transformation experiments. The isolate and its transformants were grown on V8 PDA (150 mL V8 juice, 850 mL H₂O, 10 g PDA, 10 g agar and 3 g CaCO₃) in a cycle of 14 h of light and 10 h of darkness. An appropriate antibiotic compound was added to V8 PDA for the growth of transformants. The silica gel method described by Windels *et al.* (1988) was used for long-term storage of *C. sativus* and its transformants. For DNA/RNA isolation and protoplast preparation, *C. sativus* was grown in potato dextrose broth (PDB) (4 g potato extract, 20 g dextrose and 1000 mL H₂O) at ambient temperature.

Plasmids and vector construction

The plasmids, gGFP and pDAN-*ToxA*, were used to generate GFP- and ToxA-expressing transformants of *C. sativus*, respectively.

gGFP carries the *sGFP* gene and hygromycin-resistance gene under the control of the *Aspergillus nidulans gdp* promoter and *trpC* terminator (Maor *et al.*, 1998). pDAN-*ToxA* was constructed as described previously (Friesen *et al.*, 2006). pDAN-*ToxA* contains a *cpc-1::hygromycin-resistance gene* cassette from pLP605KO (Panaccione *et al.*, 2001) and the *ToxA* gene (Ciuffetti *et al.*, 1997) amplified from the *P. tritici-repentis* isolate 86–124.

An RNAi vector (pSGate1) based on the Gateway cloning system was developed through modification of pSilent1 (Nakayashiki *et al.*, 2005). To develop pSGate1, one of the *ccdB*-containing recombination cassettes in pHellsgate-12 (Wesley *et al.*, 2001) was released by *XhoI* and cloned into the *XhoI* site in pSilent1, resulting in pSilent1-*ccdB*. To clone the other recombination cassette from pHellsgate-12 into pSilent1-*ccdB*, the primers F-*Xba* I-12 and R-*Xba* I-12 (Table 1) were used in PCR amplification with pHellsgate-12 as a template. The PCR product was cloned into the plasmid pCR 4-TOPO (Invitrogen) following the manufacturer's manual. The insert was subsequently released from the TOPO vector with *Bgl*III and *Kpn*I and cloned into the *Bgl*III/*Kpn*I sites of pSilent1-*ccdB* to produce the Gateway-based RNAi vector, pSGate1 (Fig. S1, see Supporting Information).

Three RNAi constructs (pSGate1-*GFP*, pSGate1-*ToxA* and pSGate1-*CsPKS1*) were developed using pSGate1 to silence the respective genes for GFP, ToxA and a polyketide synthase in *C. sativus*. The flow chart for making these RNAi constructs is presented in Fig. 1. To construct pSGate1-*GFP*, a fragment of *GFP* (609 bp) was amplified from gGFP by PCR using the primer pair *attB1*-GFP-1F/*attB2*-GFP-1R (Table 1). The PCR (50 µL) contained 30 ng of fungal genomic DNA, 5 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 × reaction buffer [20 mM Tris-Cl, 2.0 mM MgSO₄, 50 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, pH 8.8] and 2.5 U of Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA), and was performed

using a Mastercycler PTC-100 (MJ Research, Ramsey, MN, USA). The thermal cycling conditions were as follows: initial denaturation (95 °C, 2 min), followed by 35 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 30 s) and extension (72 °C, 1 min), and then one final cycle of extension (72 °C, 10 min). The PCR fragment was cloned into the pDONR221 vector (Invitrogen) using the BP clonase II enzyme mix (Invitrogen) to create an entry vector pDONR221-*GFP*. The *GFP* fragment was then cloned into pSGate1 through LR recombination between pDONR221-*GFP* and pSGate1 to generate the silencing vector pSGate1-*GFP*. To construct pSGate1-*ToxA*, the primer pair attB1-*ToxA*-1F/attB2-*ToxA*-1R (Table 1) was used to amplify a 976-bp fragment from DNA of the *P. tritici-repentis* isolate 86–124, and cloned into pDONR221. The resulting entry vector pDONR221-*ToxA* was recombined with pSGate1 in an LR reaction to produce pSGate1-*ToxA*, as described above for pSGate1-*GFP*. To silence the *CsPKS1* gene in *C. sativus*, the internal *CsPKS1* fragment (765 bp) was amplified by PCR using primers attB1-PKS1-1F and attB2-PKS1-1R (Table 1), with *C. sativus* genomic DNA as a template. This amplified fragment was cloned into pDONR221 to generate the entry clone pDONR221-*CsPKS1*. pDONR221-*CsPKS1* was recombined with pSGate1 in an LR reaction to produce pSGate1-*CsPKS1*.

The plasmid pII99 (Namiki *et al.*, 2001) containing the gene for resistance to geneticin (G418, Sigma-Aldrich, St. Louis, MO, USA) was used for cotransformation with the RNAi construct (pSGate1-*GFP* or pSGate1-*ToxA*) when the GFP- or ToxA-expressing transformants, which carry the hygromycin-resistance gene, were used as recipients.

Fungal transformation

Fungal transformants were obtained via polyethylene glycol (PEG)-mediated transformation. Protoplast preparation was performed according to Zhong *et al.* (2002) and transformation was carried out according to the procedure of Liu *et al.* (2004)

Detection of GFP and ToxA expression

The GFP fluorescence of *C. sativus* transformants was detected using an Olympus BX51 phase/fluorescence microscope (Olympus, Center Vally, PA, USA). The GFP fluorescence was excited at 488 nm and detected using a fluorescein isothiocyanate (FITC) optical filter. The fluorescent images were recorded by a CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The intensity of GFP fluorescence observed in the GFP transformants was classified into one of four categories: 0, no GFP; +, very faint GFP; ++, stronger than + but less than +++; +++, strongest expression of GFP (Fig. 2). The *GFP* RNAi transformants generated were also grouped into the four categories based on their GFP intensities observed under the microscope (Fig. 2).

ToxA expression was detected by infiltration of culture filtrates onto the ToxA-sensitive wheat differentials BG261 and Grandin. To prepare culture filtrates, 1×10^7 conidia of the wild-type isolate or the transformants were inoculated into 75 mL of Fries Medium (ammonium tartrate, 5.0 g/L; ammonium nitrate, 1.0 g/L; magnesium sulphate, 0.5 g/L; potassium phosphate-dibasic, 1.3 g/L; potassium phosphate-monobasic, 2.6 g/L; sucrose, 30.0 g/L; yeast extract, 1.0 g/L) and incubated with shaking at 100 rpm for 3 days, followed by 3 weeks of stationary growth in the dark at room temperature (23–25 °C). Finally, 2 mL of culture filtrate were collected from each isolate and used for leaf infiltration. Infiltration and symptom recording were conducted according to the method described by Liu *et al.* (2004). Based on the reaction, the parental isolate and transformants were divided into two groups: 0, no ToxA; 1, with ToxA.

Southern hybridization

Genomic DNA was isolated according to Zhong *et al.* (2002) and digested with one or more of the appropriate restriction enzymes. The digests were fractionated on a Tris-acetate-ethylenediaminetetraacetic acid agarose gel and transferred to Hybond N+ (Amersham Biosciences, Piscataway, NJ, USA). The PCR products generated by GFP-2F/GFP-2R and ToxA-F/ToxA-R from gGFP and pDAN-*ToxA*, respectively, were used as the probes for the detection of *GFP* and *ToxA* in the transformants, respectively. For the detection of *CsPKS1* in *C. sativus*, a PCR fragment amplified from *C. sativus* with the primer pair attB1-PKS1-1F and attB2-PKS1-1R was used as a probe. The hybridization and detection procedures were performed according to the method described previously by Zhong *et al.* (2002).

Quantitative RT-PCR analysis

For RT-PCR, total RNA was extracted from mycelia grown in PDB (BD, Franklin Lakes, NJ, USA) using a PureLink™ RNA Mini Kit (Invitrogen) and purified by treatment with DNase I (Invitrogen). The reverse transcription reaction was performed on 2 µg of total RNA using the SuperScript® III First-Strand Synthesis System (Invitrogen). cDNA was diluted 20 times and used as the template for quantitative RT-PCR, which was performed with the AB7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers RT-GFP-F and RT-GFP-R (Table 1) were used for PCR. For each cDNA sample, three replications were performed. Each reaction mixture (20 µL) contained 5 µL of the cDNA template, 10 µL of SYBR® Green PCR Master Mix (Applied Biosystems) and 0.3 µL of each primer (10 mM). All samples were normalized using RT-Actin-F and RT-Actin-R primers (Table 1) as a control, and the values were expressed as the change (increase/decrease) relative to the levels of the control sample.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 A schematic map of the Gateway cloning system-based RNA interference (RNAi) vector, pSGate1. The *ccdB* cassettes were derived from pHellsgate-12 (Wesley *et al.*, 2001) and cloned into the silencing vector pSilent1 (Nakayashiki *et al.*, 2005). Unique restriction sites are indicated. AmpR, ampicillin-resistant gene; HphR, hygromycin-resistant gene; intron, intron 2 of the cutinase (CUT) gene from *Magnaporthe oryzae*; PtrpC, *Aspergillus nidulans trpC* promoter; TtrpC, *A. nidulans trpC* terminator.

Fig. S2 Southern blot analysis of the green fluorescent protein (GFP) RNA interference (RNAi) transformants in *Cochliobolus sativus*. gGFP, pSGate1-GFP and genomic DNA of the GFP RNAi transformants, as well as the wild-type (WT) strain ND93-1 and the parental GFP transformant (GFP-19), were digested with *XhoI* and *KpnI*, subjected to electrophoresis, blotted onto a

nylon membrane and probed with ³²P-labelled polymerase chain reaction (PCR) products of GFP amplified from gGFP. The full arrow indicates the fragment (2.3 kb) containing the entire transcriptional unit of the GFP gene in gGFP. The broken arrows indicate the two fragments (0.6 kb and 0.8 kb, respectively) representing the transcriptional unit of hairpin RNA of the GFP gene in the GFP silencing vector pSGate1-GFP. The GFP expression level of each transformant is indicated by 0, +, ++, or +++.

Fig. S3 Southern blot analysis of the parental *ToxA* transformant (ToxA-3) and the representative *ToxA* RNA interference (RNAi) transformants. pDAN-*ToxA*, pSGate1-*ToxA*, genomic DNA of the *ToxA* RNAi transformants, as well as the wild-type (WT) strain ND93-1 and the *ToxA* transformant (ToxA-3), were digested with *XhoI* and *KpnI*, subjected to electrophoresis, blotted onto a nylon membrane and probed with ³²P-labelled polymerase chain reaction (PCR) products from the *ToxA* gene in pDAN-*ToxA*. The full arrow indicates the fragment (6.0 kb) containing the entire transcriptional unit of the *ToxA* gene in pDAN-*ToxA*. The broken arrows indicate the two fragments (0.9 kb and 1.1 kb, respectively) representing the transcriptional unit of hairpin RNA of the *ToxA* gene from pSGate1-*ToxA*. *ToxA* RNAi transformants 1, 2, 3 and 4 harbour pSGate1-*ToxA* and express low or no ToxA, whereas *ToxA* RNAi transformants 6, 10, 14 and 23 contain no pSGate1-*ToxA* and produce ToxA based on bioassay of the culture filtrates infiltrated into the leaves of the ToxA-sensitive wheat line BG261.

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