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Oligonucleotide sequences forming short self-complimentary hairpins can expedite the down-regulation of *Coprinopsis cinerea* genes

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Abstract

Gene silencing in fungi is often induced by dsRNA hairpin forming constructs the preparation of which can require multiple cloning steps. To simplify gene silencing in the filamentous fungi we have evaluated a high throughput cloning method for target sequences using the homobasidiomycete *Coprinopsis cinerea*, the GFP reporter and a commercially available vector system. The pSUPER RNAi System™, which was developed for mammalian experiments, exploits the human H1 Polymerase III (Pol III) RNA gene promoter and expedites cloning/expression of specific user-defined oligonucleotide sequences to form short self complimentary hairpins. Transformation of *C. cinerea* with pSUPER constructs harboring specific oligonucleotides (19nt stem length) enabled recovery of transformants with reduced transcripts of the GFP transgene, and were less fluorescent in protein assays and microscopic phenotypes. This technological advance should expedite functional genomic studies in *C. cinerea* and has wider potential for utility in other homobasidiomycete and filamentous fungi.

1. Introduction

Coprinopsis cinerea is a model homobasidiomycete used in many studies such as sexual development and meiosis, and also to exploit tools for functional genomics in filamentous fungi (Heneghan et al., 2007; Namekawa et al., 2005; Wälti et al., 2006). The study of gene function in filamentous fungi has been hampered by the lack of efficient tools but RNA mediated gene silencing has recently been successfully achieved in these fungi (Heneghan et al., 2007; Namekawa et al., 2005; Wälti et al., 2006; De Jong et al., 2006). Most of these studies have involved laborious and time consuming multiple-steps to clone target sequence in sense and antisense orientations to yield dsRNA forming hairpin constructs that initiate RNAi mediated gene silencing. Usually, a long hairpin stem length (*ca.* 500 bp) is coupled with fungal promoters to drive transcription in the host cells. In mammalian cells, commercially available silencing vectors harbor promoters to drive expression of siRNAs circumventing the problem of cytotoxicity resulting from the introduction of dsRNA (Paddinson et al., 2002). The vector pSUPER (suppression of endogenous RNA; OligoEngine™, Seattle, WA, USA) directs stable synthesis of siRNA like transcripts using the Pol III H1 RNA gene promoter (Brummelkamp et al., 2002). Although the pSUPER RNAi System™ was designed for mammalian cells, this vector has been used to silence glucuronidase (GUS) in tobacco plants and GFP in chicken cells (Lu et al., 2004; Wang et al., 2006). In studies with plants, the Pol III H1 RNA promoter was shown comparable to the *Arabidopsis* 7SL RNA gene promoter in the expression of specific short hairpin RNAs (Lu et al., 2004). To our knowledge there are no previous reports of use of pSUPER in fungi and it is not known whether the system and short hairpin sequences are effective triggers for gene silencing in these organisms. The pSUPER RNAi System™ enables the cloning of user-specified short oligonucleotides

to form self-complimentary hairpins in a single cloning step that should substantially

expedite down-regulation of multiple target sequences in *C. cinerea*. Through this

manuscript, we describe the use of pSUPER and specific oligonucleotides to down

regulate GFP in protoplast-mediated transformation of *C. cinerea*.

2. Material and methods

2.1. Strains and culture conditions

Escherichia coli strain DH5α (MAX Efficiency®, DH5α™ Competent Cells,

Invitrogen™, Paisley, UK) was used for recombinant cloning. The *Coprinopsis*

cinerea auxotroph strain PG78 (*A6 B42 pab1 trp1.1, 1.6*; (Granado et al., 1997)) was

used for GFP transformations and was the negative non-transformed control.

PG78Green was previously described (Heneghan et al., 2007), expresses GFP and

was used as host strain for pSUPER transformations. *C. cinerea* strains were

maintained at 37ºC on complete medium (CM) (Lewis, 1961) supplemented with 100

μg/L L-tryptophan or 2 μg/L para-aminobenzoic acid as required. CM broth was used

to grow isolates for four days at 37ºC for RNA extraction, fluorimetry and

microscopy.

2.2. Construction of pSUPER-GFP plasmids

The vector pSUPER.basic (OligoEngine™) was used to clone specific oligonucleotide

sequences with homology to GFP. The 720 bp eGFP (enhanced green fluorescence

protein) sequence (Clontech Laboratories, Mountain View, CA, USA) and iRNAi

version 2.0 software were used to identify forward and reverse oligonucleotide pairs

(Sigma® Genosys, Sulfolk, UK). Three different regions of the GFP mRNA were

targeted, GFP51 (5' GCTGGACGGCGACGTAAAC 3'), GFP63 (5'

CGTAAACGGCCACAAGTTC 3') and GFP128 (5' CCCTGAAGTTCATCTGCAC

3'), where numbers refer to the nucleotide position relative to the ATG codon. Three

vectors were assembled, pSUPER-GFP51, pSUPER-GFP63 and pSUPER-GFP128.

The sequence of the forward oligonucleotide includes the unique 19 nt target

sequence in both sense and antisense orientation, separated by a 9 nt spacer sequence

(TTCAAGAGA), which generates the hairpin loop. The oligonucleotides were also

designed to give overhanging ends suitable for direct ligation with *Bgl*II-*Hind*III

digested vector. To anneal forward and reverse oligonucleotides, 1 µl of each (3

mg/µl) was mixed with 48 µl of annealing buffer (10 mM Tris pH 7.5-8.0, 50 mM

NaCl and 1 mM EDTA) in a 50 µl total reaction volume and incubated at 94ºC 4 min,

90ºC 4 min, 80ºC 4 min, 70ºC 4 min, 37ºC 20 min, room temperature 20 min before

storing at 4ºC. Annealed oligonucleotides (2 µl of annealing reaction) were ligated

directly with pSUPER *Bgl*II-*Hind*III digested, according to the manufacturers

instructions. The pSUPER-GFP ligations were transformed into *E. coli*, and

ampicillin resistant colonies screened for recombinants using PCR with M13 primers

(Table 1) and/or restriction digestion using *Hin*dIII and *Eco*RI of the oligonucleotide.

PCR screening was performed using 2.5 U of Taq polymerase (Invitrogen™), 1 X

reaction buffer, 1.5 mM MgCl$_2$, 10 mM dNTPs, 0.4 µM of each forward and reverse

primer in a 25 µl reaction volume with the following thermal cycling parameters:

95ºC for 3 min; 30 cycles of 95ºC for 30 sec, 50ºC for 30 sec, 72ºC for 30 sec; and a

final extension of 72ºC for 10 min.

2.3. Transformation

Plasmid DNA for *C. cinerea* transformation was prepared using QIAprep Spin

Miniprep Kit (Qiagen®, Germantown, MD, USA) according to the manufacturers

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instructions. Protoplast cotransformations were performed as previously described

(Binninger et al., 1987), using *ca*. 2 µg of pST17 (1µg) with *ca*. 5 µg pSUPER-GFP

plasmid. Pab+ transformants were sub-cultured onto CM agar.

2.4. PCR based methods

Fungal DNAs were prepared using a Chelex®100 Resin (Bio-Rad Laboratories,

Headquarters, Hercules, CA, USA) miniprep method (Challen et al., 2003). Pab+

transformants were PCR screened using two pairs of primers (SUPER_F727,

SUPER_ R841 and SUPER_F727, SUPER_R1492, Table 1) that amplify the H1

promoter or the hairpin region, respectively. PCR reactions were performed as

described above.

2.5. Quantitative RT-PCR (Q-PCR)

Total RNA was extracted from freeze-dried mycelium using phenol-chloroform-

isoamylalcohol (25:24:1) and precipitated with LiCl (2M) as previously described

(Sreenivasaprasad, 2000]. Approximately 2 µg of total RNA was treated with DNase

(Promega Corporation, Madison, WI, USA) and cDNA was synthesized using random

hexamer primers (Applied Biosystems, Foster City, CA, USA) and SuperScript® II

reverse transcriptase (Invitrogen™). Absence of contaminating genomic DNA in

cDNAs was confirmed by RT-PCR using primers (CcBtub_F and CcBtub_R, Table

1) that span an intron of the *C. cinerea* β tubulin gene. GFP transcripts were

quantified using quantitative RT-PCR (Q-PCR) using primers qGFP_F and qGFP_R

(Table 1) designed outside the hairpin sequence as previously described (Heneghan et

al., 007), negative controls were water (no cDNA) and the non-transformed PG78.

Three independent RNA extractions were performed for each sample tested.

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Expression levels of GFP were normalized against 18S rRNA using primers q18S_F

and q18S_R (Table 1).

2.6. Microscopy and Fluorimetric GFP quantification

Transformants were observed microscopically using an Olympus IX70 microscope

(105 objective) coupled with an Olympus U-RFL-T UV pack and an Olympus camera

LG-PS2 to capture the photomicrographs. GFP activity was fluorimetrically

quantified as previously described (Heneghan et al., 2007) using three independent

protein extractions for each strain.

3. Results and discussion

In this work we tested the utility of pSUPER and short hairpin (19 nt stem) forming

oligonucleotides to initiate gene silencing in *C. cinerea*. The commercial pSUPER

RNAi System™ facilitates high throughput targeting of gene sequences, as it only

involves ordering of appropriate forward and reverse oligonucleotides, annealing and

cloning into the vector. However, as the pSUPER RNAi system was developed for

mammalian cells, it was not known whether the Pol III H1 RNA gene promoter

incorporated in the vector would permit expression of short hairpin sequences in the

fungal host, or even if the relatively short 19 nt stem hairpins would trigger down-

regulation in this organism. Based on observations of short interfering RNAs in *C.

cinerea* hairpin transformants (Costa 2007) we designed oligonucleotides forming

duplexes from 19 nt target sequences. However, as the length of the target sequence is

specified by the user, the system has added flexibility in that it can easily

accommodate different sized oligonucleotide to optimise selection of short-interfering

RNAs in different fungal species. In mammalian cells it has been shown that 29 nt

hairpins stems can be more efficient triggers of down-regulation than the 19 nt stems

generated using the pSUPER RNAi System™ (Siolas et al., 2005); for optimal

efficiency it would therefore be important to test a selection of oligonucleotides for

different organisms.

Three vectors targeting different GFP regions (pSUPER-GFP51, pSUPER-GFP63 and

pSUPER-GFP128) were successfully introduced into *C. cinerea* PG78Green using

protoplast mediated co-transformation. Rates of co-transformation, where the

pSUPER-GFP plasmid was detected by PCR, ranged between 25%, (pSUPER-GFP51

and pSUPER-GFP63) and 15% (pSUPER-GFP128) and are similar to those reported

elsewhere (Kües et al., 2001). From 61 pab+ transformants, 13 (T2, T46, T51, T56,

T58, T59, T61, T64, T64, T82, T119, T121 and T171) were found to contain the

complete hairpin expression cassette. In 8 of the pab+ transformants the promoter

region could be detected but not the hairpin expression sequences. When transforming

with closed circular plasmid, the integration of partial constructs and/or

rearrangements are not unusual in *C. cinerea* (Heneghan et al., 2007; Namekawa et

al., 2005; Wälti et al., 2006).

In fluorimetric analysis, 11 of 21 transformants (harboring hairpin sequences) had

reduced GFP activity compared with controls (PG78Green, T187; Figure 1A). There

were no obvious differences in silencing efficacy of different constructs. Four

transformants were selected for further analysis (Fig. 1A); three with reduced GFP

activity (T55, T61 and T82) and one where expression was not altered (T2), which

were compared with the non-transformed PG78 and two GFP expressing controls

(PG78Green, T187). In microscopy, two transformants (T55 and T61) exhibited

reduced fluorescence (Fig. 2). In Figure 1B, GFP transcripts of three transformants

(T2, T55 and T82) are compared with controls (PG78, PG78Green, T187).

PG78Green and T187 showed the highest levels of GFP transcription. The non-transformed PG78 had no detectable transcripts. Transcripts were significantly down-regulated in T55 and T61, these transformants also exhibited reduced fluorescence in fluorimetric and microscopy tests. Transcripts in T2 and T82 did not differ significantly to PG78Green. For T2 this was consistent with fluorimetry and microscopy observations. Observations were more disparate for T82, which had reduced fluorimetric values but did not appear different when observed by microscopy.

4. Conclusion

The use of short hairpin sequences to mimic siRNAs and down-regulate fungal gene expression represents a significant technological advance. Longer stem hairpins (*ca.* 500 bp) have been used in several other fungi (Rappleye et al., 2004; Goldoni et al., 2004; Tanguay et al., 2006), and we had previously observed that shorter hairpins (162 bp) could initiate down-regulation of GFP in *C. cinerea* (Heneghan et al., 2007). Although gene silencing mediated by such hairpin constructs has been successful, the preparation of the custom built constructs has proved laborious and time consuming. With the pSUPER RNAi System™, oligonucleotides can be simply designed using a variety of different software, ordered from one of the many commercial service providers, and then cloned via a single step with pSUPER.

To our knowledge this is the first time that short, duplex forming, oligonucleotides have been used down-regulate genes in a filamentous fungus. Our observations that 19 nt stem hairpins and the pSUPER RNAi System™ can initiate down-regulation of a target gene in a homobasidiomycete should be of broader academic interest.

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Considering the burgeoning of fungal genome sequence projects, the utility of this approach to expedite high-throughput functional gene analyses in a broader range of filamentous fungi, should promote wider evaluation. Further experiments would however be required to determine optimal hairpin lengths for different filamentous fungi.

Competing interests statement

The authors declare that they have no competing interests.

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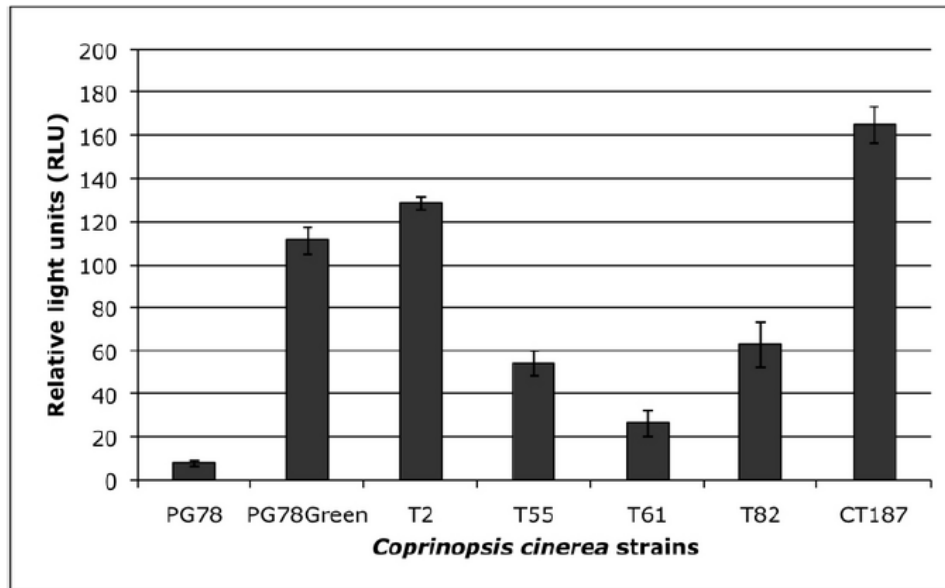
FIGURE LEGENDS

Figure 1 GFP expression in pSUPER transformants: (A) Fluorimetric determination of GFP activity in total protein extracts. (B) Q-PCR analysis of GFP transgene transcripts. Negative control, PG78, positive controls, PG78Green and T187. Error bars are the standard error of the mean of biological replicates.

Figure 2 Microscopic observations of GFP expression in *C. cinerea* transformants using bright field and UV light. Samples (A) to (E): PG78, PG78Green, T187, T55 and T61. (100x magnification)

Table 1– Designation, sequence and purpose of primers used in this study.
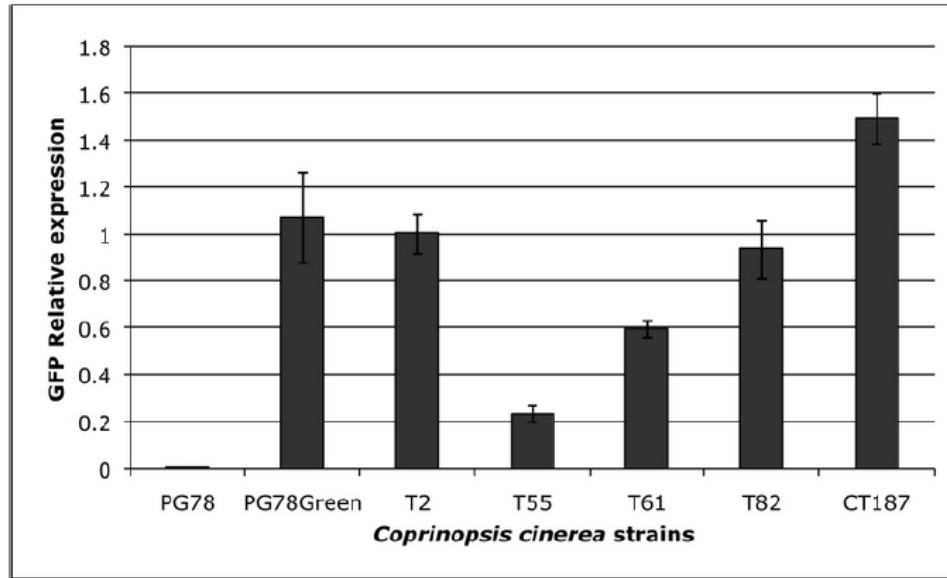
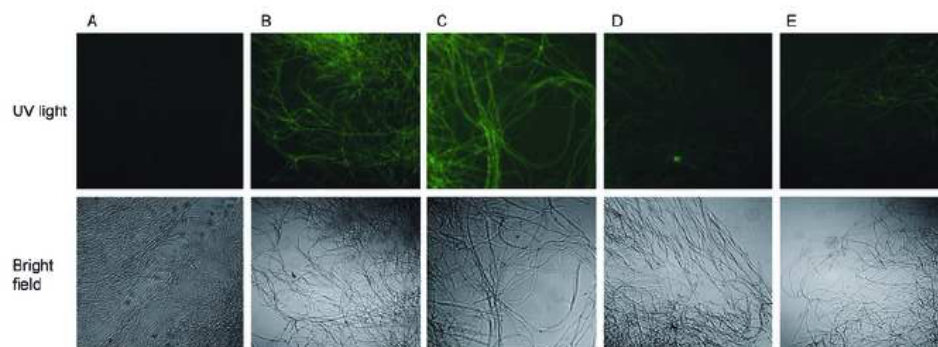| Designation	Primer sequence	Purpose
M13_F	FOR 5'-GTAAAACGACGGCCAGT-3'	M13 sequences to distinguish *E. coli* recombinants by PCR
M13_R	REV 5'-AACAGCTATGACCATG-3'	
SUPER_F727	FOR 5'-TCAACCCGCTCCAAGGAATC-3'	To detect the H1 promoter
SUPER_R841	REV 5'-TCGCCACCTCTGACTTGAGC-3'	
SUPER_R1492	REV 5'-AATATTGCAGGGCGCCACTC-3'	To detect entire construct (use with SUPER_F727 above)
CcBtub_F	FOR 5'-TGTCTCCGATGAGCATGGTA-3'	To discriminate between genomic DNA and cDNA
CcBtub_R	REV 5'-GAGGCAGCCATCATGTTCTT-3'	
qGFP_F	FOR 5'-CTGCTGCCCGACAACCA-3'	To detect GFP transcript by Q-PCR
qGFP_R	REV 5'-TGTGATCGCGCTTCTCGTT-3'	
q18S_F	FOR 5'-GCCTGTTTGAGTGTCATTAAATTCTC-3'	To normalize GFP transcript levels by Q-PCR.
q18S_R	REV 5'-CTGCAACCCCCACATCCA-3'	

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Figure 1A

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Figure 1B

Figure 2