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TECHNICAL NOTE

Agrobacterium-meditated gene disruption using split-marker in *Grosmannia clavigera*, a mountain pine beetle associated pathogen

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Abstract *Grosmannia clavigera* is a fungal pathogen associated with the mountain pine beetle (*Dendroctonus ponderosae*) which is devastating large areas of western Canada's conifer forests. This fungus also produces a dark melanin pigment that discolors pine sapwood. We have generated the draft genome of *G. clavigera*. However, functional characterization of genes identified in the genome sequence requires an efficient gene disruption method. In this work, we report a gene replacement strategy for *G. clavigera* using the *Agrobacterium*-mediated transformation in conjunction with linear or split-marker deletion cassettes. In addition, we used long flanking regions up to 3 kb from both sides of the targeted genes in our deletion cassettes. We assessed this gene disruption method with two genes from the melanin biosynthesis pathway that produce easily detectable white and red/brown mutant phenotypes: polyketide synthase and scytalone dehydratase. The approach yielded *G. clavigera* gene replacements with homologous recombination rates between 65 and 82%. For filamentous fungi, this is the first report showing that split-markers can be used with *Agrobacterium*-mediated transformation to generate appropriate mutants. This method can now be applied to efficiently identify genes involved in *G. clavigera* fungal pathogenicity and will facilitate understanding how the fungus overcomes the host defence system.

Keywords Ophiostomatoid · Ascomycete · Conifer · Split-marker · Co-transformation · Melanin biosynthesis

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Introduction

Grosmannia clavigera (Robinson-Jeffrey & Davidson) Zipfel, de Beer & Wingfield, is a filamentous ascomycete that is specifically associated with the mountain pine beetle (MPB; *Dendroctonus ponderosae*) (Zipfel et al. 2006). The MPB infests pine forests on a large scale in Western North America; in British Columbia the beetle and its fungal associates have killed 620 million cubic meters or approximately 14 million hectares of lodgepole pine forests (http://www.for.gov.bc.ca/HFP/mountain_pine_beetle/facts.htm). *G. clavigera*, previously reported as *Ophiostoma clavigerum*, is a pathogen that can kill trees in the absence of the beetle when inoculated into lodgepole pines (Solheim and Krokene 1998; Lee et al. 2006; Plattner et al. 2008). With or without the beetle, this fungus overcomes the tree defenses, colonizes and disrupts the transport of water to the crown. As well, it produces a black melanin pigment that discolors the phloem and sapwood of the tree and reduces the commercial value of products made from the wood and wood fibers.

G. clavigera belongs to the ophiostomatoid fungi, which includes over 140 species of wood- or tree-inhabiting fungi that are divided into the genera *Ceratocystiopsis*, *Ceratocystis*, *Grosmannia*, *Ophiostoma* and a number of anamorph genera (Hausner et al. 2000; Zipfel et al. 2006; Massoumi Alamouti et al. 2009). *G. clavigera*'s sexual

phase is found only in nature. It grows faster than other MPB-associated fungi, and is the first symbiotic fungus that was isolated from MPB and described at the population level (Lee et al. 2006). Given the wealth of biological information available on this pathogenic MPB-associate, it is the primary target in our current and ongoing genomics work. We have recently generated a draft genome sequence assembly for *G. clavigera* Kw1407, using a hybrid sequencing and assembly approach (DiGuistini et al. 2009). We are currently annotating the *G. clavigera* Kw1407 genome using ESTs, full-length cDNAs, and transcriptome expression data along with information from other published fungal genomes. However, confirming gene functions and the role of genes in pathogenicity require generating *G. clavigera* mutants in which genes of interest are modified or deleted. Modification and replacement methods need to be optimized for each fungal species, and were not available for *G. clavigera*.

Conventional gene replacement (i.e., gene disruption or gene knockout) in fungi requires two steps: transforming the fungus, and fully or partially replacing the targeted gene with a marker gene through homologous recombination (HR) (Wendland 2003; Weld et al. 2006). The marker gene often confers antibiotic resistance or autotrophy, which allows selecting the modified strains on appropriate media (Wendland 2003; Weld et al. 2006). Efficient high throughput gene disruption strategies have been developed for model organisms like *Saccharomyces cerevisiae* and *Neurospora crassa* (Giaever et al. 2002; Colot et al. 2006). In contrast, while large amounts of gene replacement data have been published for non-model species from the main fungal kingdoms (ascomycetes, basidiomycetes and zygomycetes), transformation methods are limited, vary between species, and often show HR frequency lower than 10%. Developing such methods typically requires screening large numbers of transformants, and the screening can be complicated by multiple insertions (Meyer 2008). While HR frequency can be increased by extending the flanking regions surrounding the targeted gene, such methods are species specific, and require both optimization and sufficient genome sequence information (Walther and Wendland 2008; Meyer 2008). Combined, the above factors impede the development of efficient, large-scale gene replacement methods for filamentous fungi (Meyer 2008). While current sequencing technology can generate large amounts of fungal genome data, transformation and disruption methods still typically have low throughput and rarely target more than a few specific genes.

Efforts have been made to improve the gene disruption efficiency. Recently, HR yields greater than 50 or 80% have been achieved using either split-marker selection (Fu et al. 2006; Colot et al. 2006; Jeong et al. 2007; You et al. 2009) or mutants deficient in non-homologous end-joining (NHEJ) DNA repair, respectively (Ninomiya et al. 2004; Krappmann 2007; Snoek et al. 2009). While the NHEJ mutants show few obvious growth defects, they seem to be more sensitive to damaging agents like antibiotics or exposure to UV or X-rays (Krappmann 2007). To verify gene function and avoid non-target effects due to the NHEJ mutation, species are sexually propagated, and progeny with mutations in the targeted gene but not in NHEJ regions are selected for functional and physiological characterization (Krappmann 2007). However, this approach cannot be used with asexual fungi or fungi that do not easily mate under laboratory conditions such as *G. clavigera*, whose fruiting bodies are rarely found in nature. The alternative, split-marker approach, uses two constructs that overlap inside the selective marker gene, was initially developed for *S. cerevisiae* (Fairhead et al. 1996) and then for a few filamentous fungi like *Aspergillus nidulans* (Nielsen et al. 2006), *Magnaporthe grisea* (Jeong et al. 2007), *N. crassa* (Colot et al. 2006) and *Cercospora nicotianae* (You et al. 2009). To this point, co-transformation with split-marker constructs has been limited to electroporation and protoplast- mediated transformation, and has not been reported with *Agrobacterium*-mediated transformation (AMT).

In previous work, we showed that homologous recombination is rare in ophiostomatoid fungi, and we developed a number of transformation protocols for such fungi (Wang et al. 1999; Tanguay and Breuil 2003; Loppnau et al. 2004). However, gene replacement was only successful at low HR frequency for *C. resinifera* and *O. piliferum*, (Loppnau et al. 2004; Hoffman and Breuil 2004). In the current work, we developed a gene replacement method for *G. clavigera* by combining AMT, linear marker or split-marker deletion cassettes, and long flanking sequences on both sides of the targeted gene. Using two dihydroxynaphthalene (DHN)-melanin biosynthesis pathway genes, the polyketide synthase (*PKS*) and the scytalone dehydratase (*SD*), that respectively give white and reddish phenotypes when replaced, we showed that HR can occur at high frequency. We also confirmed that the mutants were stable and able to grow in pine seedlings or freshly cut logs.

Materials and methods

Strains, plasmids and media

Grosmannia clavigera wild type Kw1407 (Lee et al. 2003) was grown and maintained on 1% Oxoid malt extract agar (MEA, 33 g malt extract agar and 10 g technical agar per liter), while for the mutants 0.1 μg/ml of hygromycin was added to the medium. *S. cerevisiae* DY1457 (a gift from Dr. James Kronstad, Michael Smith Laboratory, University

of British Columbia) was grown on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) for general purpose and on synthetic complete (SC) medium without uracil for selecting yeast transformants. *Escherichia coli* DH5α was used to propagate and maintain plasmids following standard procedures. *Agrobacterium tumefaciens* GV3101 is a laboratory stock and was used to transform *G. clavigera*. The wild type *A. tumefaciens* was grown in Luria–Bertani (LB) medium with 50 μg/ml gentamycin and 25 μg/ml rifampicin (Loppnau et al. 2004). When *A. tumefaciens* carried the disruption cassettes, an additional 50 μg/ml of kanamycin was added to the media. Plasmid pSCN44 containing the hygromycin *β* phosphotransferase gene (*hph*) and yeast shuttle vector pRS426 were obtained from the Fungal Genetics Stock Center, Kansas City, Missouri. We maintain the plasmid pCambia0380 (CAMBIA, Australia. GeneBank Accession no. AF234290) which we used to make the general construct for *A. tumefaciens*, in our laboratory.

Plasmids and *Agrobacterium* strains generated for gene replacement

In order to develop an efficient gene replacement strategy for *G. clavigera*, we made deletion cassettes with different lengths of flanking regions (1.5 and 3 kb) and different forms of the selective marker (linear or split) using the yeast re-combinational cloning system described by Colot et al. (2006). The complete procedure for generating the deletion constructs is shown in Fig. 1. Sequences for the targeted genes and up to 3 kb flanking regions were obtained from the *G. clavigera* genome sequence assembly (DiGuistini et al. 2009). For each gene, 3 kb of both 5′ flanking regions (Fig. 1, P1, P2) and 3′ flanking regions (Fig. 1, P3, P4) were amplified separately from the wild type genomic DNA. The hygromycin resistance gene plus *trpC* promoter (*hph* cassette) were amplified from plasmid pSCN44 using the primer set P5, P6 (Fig. 1). P1 and P4 are preceded by 22 bp homologous sequences to the yeast

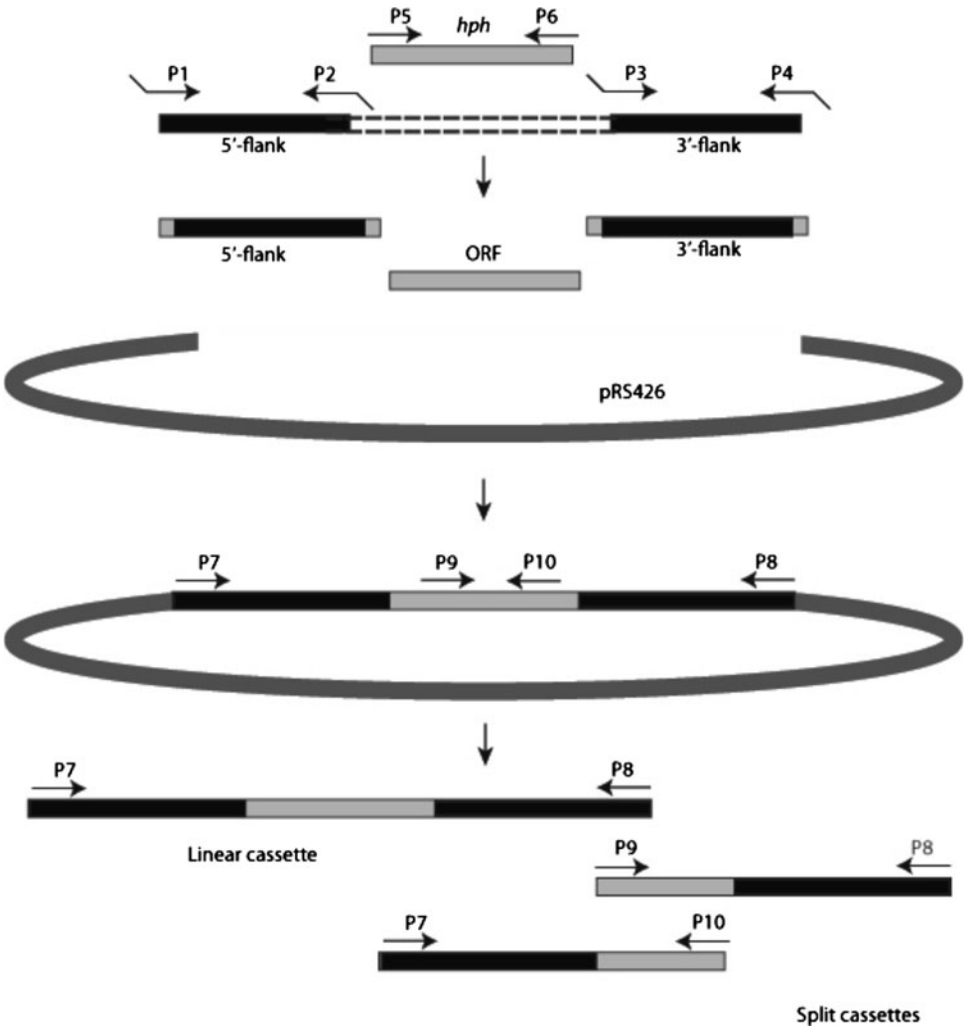

Fig. 1 Schematic diagram for generating deletion cassettes. The 3′ and 5′ flanking regions and *hph* cassette were amplified with the indicated primers. The separate fragments along with pre-cut vector pRS426 were assembled into one circular construct using yeast re-combinational machinery. The linear and split cassettes were amplified from the yeast DNA by PCR and were further linked to *Agrobacterium* vector separately

vector pRS426 while P2 and P3 are sharing short homologous regions with the *hph* primers P5 and P6, respectively. The three independent fragments were co-transformed into the yeast wild type (strain BY1457) along with the gaped vector pRS426 that was digested by E*co*RI and X*ho*lI. The yeast re-combinational machinery assembled the three DNA fragments into one with the following order: 5′ flank-*hph*-3′ flank. The linear cassettes (5′ flank-*hph*-3′ flank) and each split cassettes (5′ flank-2/3 *hph* upstream sequence and 2/3 *hph* downstream sequence-3′ flank) were separately retrieved from the yeast DNA by PCR and further cloned into *E. coli—A. tumefaciens* shuttle vector pCambia0380 using conventional restrictive digestion and T4 ligation method. *A. tumefaciens* was transformed with plasmids carrying the deletion cassettes, Three *A. tumefaciens* strains were generated for each gene; one strain was transformed with a linear cassette, while two other strains were transformed with two separate split cassettes. The resulting bacteria were ready to transform *G. clavigera*. The *Agrobacterium* strain containing the linear cassette was used to transform *G. clavigera*, while for the split cassette two *Agrobacterium* strains were used to co-transform *G. clavigera* and to confer hygromycin resistance. Linear and split-marker cassettes were made with 1.5 and 3 kb flanking regions. All PCRs were carried out using Phusion high-fidelity DNA polymerase (New England Biolabs). Table 1 summarizes the primers sequences and their applications, while Table 2 lists the plasmids/strains generated in this work.

Agrobacterium-mediated transformation (AMT)

We transformed *G. clavigera* using the method described by Loppnau et al. (2004), with a modified spore preparation as follows. The asexual spores were harvested by flooding sterile distilled water onto a 7-day culture grown on 1% MEA. The spore suspension was filtered through a cell strainer with a 40 µm screen (BD Biosciences) to remove fragments of mycelia. 100 µl of fungal spores at a concentration of 10^6/ml were spread onto cellophane membranes overlaid on 1% MEA plates. The spores were incubated at room temperature for 24 h or until the majority of them have developed germ tubes. The germinated spores were co-cultivated with *A. tumefaciens* carrying linear cassettes as described previously (Loppnau et al. 2004). However, for the co-transformation of the split-marker cassettes, we separately grew in induced media two *A. tumefaciens* strains that carried the split cassettes, and then pre-mixed the two cultures at a 1:1 ratio before co-cultivating the mixture with the germinated fungal spores. The cellophane sheets were transferred onto selective medium. White or reddish transformants were selected and transferred on fresh hygromycin MEA plates.

Characterization of mutants

To confirm the stability of a phenotype we conducted single conidium isolations and a series of 4–5 transfers on fresh non-selective media. We extracted the DNA of the stable transformants following the protocol of Möller et al. (1992). Using PCR with specific primers for *hph*, integrating regions and targeted genes (*PKS* or *SD*), we verified the presence or absence of these fragments in the wild type and the transformants. To determine the copy number of the *hph* gene we used southern blots with the Digoxigenin labeling and detection system (High Prime DNA Labeling and Detection Starter Kit I, Roche Applied Science). Genomic DNA (10 µg) digested with 10 units of the restriction enzyme X*ho*lI (New England Biolabs) was separated on 0.8% agarose gel. DNA was alkaline transferred to Zeta-Probe GT membranes (Bio-Rad). The hybridization and detection procedure was carried out following the manufacturer's protocol.

Growth of mutants in logs and seedlings

To determine if the mutants were still able to grow on pine wood or in pine seedlings, mycelial plugs from actively growing colonies of the wild type and mutants were inoculated into freshly cut pine logs (about 60–80 years old lodgepole pines from Merrit, BC, Canada) and in 5-year-old lodgepole pine seedlings maintained in a green house. For log inoculation, mycelia plugs of the wild type, *PKS* and *SD* mutants, and non-inoculated agar (control) were placed into holes cut through the bark and phloem using a 0.5 cm cork borer, the bark was replaced over the mycelia to close the inoculation hole. Each log received only one row of inoculations that was wrapped with duct tape. The logs were incubated at room temperature for 3 weeks. Then the logs were debarked, the discolored or reaction areas were measured and re-isolations were carried out. The seedling inoculations were performed using the same procedure. We used an inoculation density of 16 inoculation points per seedling. Symptom development and seedling deaths were observed after 3–4 weeks. Debarking, lesion measurements and fungal re-isolation were carried out to confirm the cause of symptoms.

Result

Characterizing genes in the *G. clavigera* DHN-melanin biosynthesis pathway

In the DHN-melanin pathway, fungi use polyketide synthase (*PKS*) to convert acetyl- or malonyl-coA into 1,3,6,8,-tetrahydroxynaphthalene. This intermediate is reduced by the

Table 1 Primers used in this study

Name[a]	Sequence 5′-3′	Amplification purpose
*hph*F (P6, Fig. 1)	GTCGGAGACAGAAGATGATATTGAAGG AGC	*hph* cassette
*hph*R (P5, Fig1)	GTTGGAGATTTCAGTAACGTTAAGTGGAT	*hph* cassette
GC-*SD*-5F (P1, Fig. 1)	***GTAACGCCAGGGTTTTCCCAGTCACGACG***CGCATGACAGGTCACAATT CA	*SD* 5′ flank region[b]
GC-*SD*-5R (P2, Fig. 1)	***ATCCACTTAACGTTACTGAAATCTCCAAC***TAGA TCCCATATGGCAGGGG	*SD* 5’ flank region
GC-*SD*-3F (P3, Fig. 1)	***CTCCTTCAATATCATCTTCTGTCTCCGACTTC***GAGG ACTGGGAATAAGGC	*SD* 3′ flank region
GC-*SD*-3R (P4, Fig. 1)	***GCGGATAACAATTTCACACAGGAAACAGC***CGTCGTT TGCGTTGATCTTTC	*SD* 3′ flank region
GC-*PKS*-5F (P1, Fig. 1)	***GTAACGCCAGGGTTTTCCCAGTCACGACG***GTCGCTTCGA CGACCTTTCTT	*PKS* 5′ flank region
GC-*PKS*-5R (P2, Fig. 1)	***ATCCACTTAACGTTACTGAAATCTCCAAC***GAACTTTCTGGGT CAAGGCGT	*PKS* 5′ flank region
GC-*PKS*-3F (P3, Fig. 1)	***CTCCTTCAATATCATCTTCTGTCTCCGACTTC***CTGGCAGCAGCTA ACATC	*PKS* 5′ flank region
GC-*PKS*-3R (P4, Fig. 1)	***GCGGATAACAATTTCACACAGGAAACAG***CAGAGCAAGCTCGGTGT GTGAG	*PKS* 5′ flank region
hphR-ol-HindIII (P10, Fig. 1)	CGAAGCTTTCCAGAAGAAGATGTTGGCGA	Split deletion cassette[c]
hphF-ol-AvrII (P9, Fig. 1)	AGCCTAGGGCGAAGAATCTCGTGCTTTCA	Split deletion cassette
SD- FW-HindIII-1.5 (P7, Fig. 1)	CGAAGCTTAAAAACAGAACCCCTCCCTCC	Linear cassette
SD-RV-AvrII-1.5 (P8, Fig. 1)	AGCCTAGGATGCATCGTCAAACACCAACA	Linear cassette
SD-FW-HindIII -3 (P7, Fig. 1)	AGAAGCTTGTTCCCACCACCTGTCACCTT	Split and linear cassettes
SD-RV-avrII-3 (P8, Fig. 1)	ATCCTAGGGCAGGCTACCGAGTGGTGAAG	Split and linear cassettes
PKS-FW-HindIII- 1.5 (P7, Fig. 1)	GCAAGCTTGACAAACCGCCTCCCTTCTAA	Linear cassette
PKS-RV-avrII 1.5 (P8, Fig. 1)	AGCCTAGGACACGCAGCTGCCAACTTT TA	Linear cassette
PKS-FW-HindIII -3 (P7, Fig. 1)	CGAAGCTTTCTCTCGCTGACAAGACACCC	Split and linear cassettes
PKS-RV-avrII 3 (P8, Fig. 1)	AGCCTAGGGCAGAAAAGCGGAATGAGACA	Split and linear cassettes
PKS-innerFW (P11, Fig. 2)	CAG GCT GGC ATT CCT TCT CTT	Confirm mutants
PKS-innerRV (P12, Fig. 2)	CAT GTG CCG GAT CAT CAT CTT	Confirm mutants
SD-innerF	TCTCGGTCTTCATTCTGCTGC	Confirm mutants
SD-innerR	ATCGTACTCGAACCAGCGGAT	Confirm mutants
PKS far5 (P13, Fig. 2)	CCGTCAAAAGGATGAAAAGGC	Confirm mutants
PKS far3 (P16, Fig. 2)	AGACGTGTTCGTTATGGGGCT	Confirm mutants
SD far5	CCTTTTGCGCCTTGCTCTT	Confirm mutants
SD far3	TGCTGACTCATCCAACAGCAA	Confirm mutants

Table 1 continued

Name[a]	Sequence 5′-3′	Amplification purpose
hphR RV con (P14, Fig. 2)	ACTGTCGGGCGTACACAAATC	Confirm mutants
hphF FW con (P15, Fig. 2)	GTGGGCTGATCTGACCAGTTG	Confirm mutants

[a] Text in parentheses indicates positions and generic names of primers in Figs. 1 and 2

[b] Italics indicate overlapped regions for the yeast recombination cloning

[c] Underlined texts indicate restriction enzyme recognition sequences

Table 2 Plasmids containing deletion cassettes generated in this study

Name of plasmids	Target gene	Full or split	kb of flank	Structure	Total size (kb)
pks-f3	*PKS*	Full	3	5′ flank-*hph*-3′ flank	7.3
pks-f1.5	*PKS*	Full	1.5	5′ flank-*hph*-3′ flank	4.5
pks-s3a	*PKS*	Split	3	5′ flank-2/3*hph* upstream	4.0
pks-s3b	*PKS*	Split	3	2/3*hph*-downstream 3′ flank	4.0
sd-f3	*SD*	Full	3	5′ flank-*hph*-3′ flank	7.6
sd-f1.5	*SD*	Full	1.5	5′ flank-*hph*-3′ flank	4.5
sd-s3a	*SD*	Split	3	5′ flank-2/3*hph* upstream	4.0
sd-s3b	*SD*	Split	3	2/3 *hph* downstream-3′ flank	4.0

1,3,6,8,-tetrahydroxynaphthalene reductase into scytalone, which is converted into 1,3,8-trihydroxynaphthalene by scytalone dehydratase (*SD*). A second reductase, 1,3,8-trihydroxynaphthalene reductase, then produces vermelone, which is finally oxidized and polymerized to form DHN-melanin (Bell and Wheeler 1986). We identified genes involved in the DHN-melanin biosynthesis pathway in *G. clavigera* using EST and genome sequence resources described by DiGuistini et al. (2007, 2009). We aligned putative transcripts from our EST assembly to the draft genome sequence using BLAT (Kent 2002). The alignments indicate that genes of *G. clavigera* involved in DHN-melanin biosynthesis are dispersed across the genome rather than clustered in one genomic region. In *G. clavigera*, the *PKS* gene is 6,696 bp long, consists of a 6,522 bp open reading frame (ORF) with a 59 bp and a 115 bp intron. Like other ophiostomatoid melanin *PKS*s, it contains five functional motifs: beta-ketoacyl synthase, acyl transferase, two acyl carrier proteins, and a thioesterase (Loppnau et al. 2004). The 905 bp *SD* gene has a 624 bp ORF interrupted by two introns of 86 bp and 195 bp. Table 3 summarizes pairwise comparisons of these two genes with orthologs from *M. grisea*, *C. resinifera* and *O. floccosum*.

Table 3 Pairwise comparison of *PKS* (bold) and *SD* (italics) ORFs with other fungi (% identity)

	G. clavigera	*O. floccosum*	*C. resinifera*	*M. grisea*
G. clavigera	100	*77.23*	*60.22*	*59.90*
O. floccosum	**N/A**	100	*60.68*	*57.91*
C. resinifera	**61.83**	**N/A**	100	*60.25*
M. grisea	**68.95**	**N/A**	**65.04**	100

Efficiency of AMT

First, we used the linear cassettes to optimize the AMT in *G. clavigera*. We found that the best fungal material for AMT was fresh germinated spores obtained after 20 h incubation at ~22°C on MEA at a spore concentration of 3–5 × 10^5 cells per plate. Plates incubated for only 6 h or for more than 48 h had 50–100 times less transformants. Next, we used linear deletion cassettes with different lengths of flanking regions (Table 2) to optimize the AMT procedure in *G. clavigera* and to verify whether the efficiency of the transformation process and HR were sensitive to the lengths of the genes and the flanking regions of the targeted genes. When we used the 3 and 1.5 kb flanking regions to generate mutants, for both *PKS* and *SD*, the 3 kb region produced less transformants but with a higher rate of expected white or reddish mutants (Table 4). The targeting locus for *PKS* was 6.7 kb and only 0.8 kb for *SD*. The HR frequency improvement from 1.5 to 3 kb flanking regions was slightly higher for *PKS* (36% to 59%) than for *SD* (31–41%); however, the differences were not statistically significant. In addition, we found that induction of *A. tumefaciens* culture

Table 4 Transformation and disruption efficiency of different flanking region

Target gene	1.5 kb flank			3 kb flank		
	Total[a]	HR[b]	Efficiency (%)	Total	HR	Efficiency (%)
PKS	75	27	36	41	24	58
SD	81	25	30	29	12	41

Values were collected from three independent experiments and calculated as transformants or mutants/10^5 spores

[a] Total transformants

[b] Transformants giving the correct deletion, screened based on color change

was essential for a successful transformation; when the bacterium was grown only in the complete medium (LB), we obtained few transformants (~5 from a total of 20 plates).

Gene disruption efficiency using split-marker

To compare the transformation and HR efficiencies between the linear and split-marker deletion cassettes, we used 3 kb flanking regions on each side of the target gene. The linear cassette contained both flanking regions and the *hph* cassette. The split-marker cassettes had two constructs. One contained the 5′ flanking region and two-thirds of *hph* (upstream), the other included two-thirds of *hph* (downstream) and the 3′ flanking region of the targeted gene. The 500 bp overlap region in selective marker gene was in the center of the *hph* cassette (see Fig. 1). Table 5 shows the data collected from three independent transformation experiments. Linear cassettes gave ~20 times higher transformation efficiency than split cassettes; 40–55% of the transformants produced with the linear cassettes were targeted mutants. In contrast, the split-marker cassettes gave lower transformation efficiency, but with higher HR rate than the linear cassettes (*PKS* 82%, *SD* 65%). To confirm that the transformants had the correct gene integration, we selected ten transformants of the appropriate color from both linear and split-marker cassettes and extracted the DNA from these transformants. First, using specific primers and PCR, we confirmed the presence of *hph*, correct integration of the 3′ and 5′ flanking regions, and the absence of the targeted gene (Fig. 2a). All 20 selected *PKS* transformants showed the expected amplicons, i.e., a *hph* band (1.4 kb), bands for the integrating regions (3.2 kb), and no band for the targeted gene (Fig. 2b). Similar amplification patterns were obtained for *SD* mutants (20 mutants, data not shown). Then, using southern blots, we assessed the copy number of *hph* cassette and correct integration in six mutants obtained from each construct. Only a single *hph* copy was found in the 24 mutants that we selected (*PKS* and *SD* mutants for both constructs). No mutant showed evidence of extra ectopic integration (Fig. 2c).

Table 5 Gene disruption efficiency between split and linear cassettes

Target gene	Linear cassettes			Split cassettes		
	Total[a]	HR[b]	Efficiency (%)	Total	HR	Efficiency (%)
PKS	714	380	53	34	28	82
SD	1,186	474	39	46	30	65

Values were collected from three independent experiments and calculated as transformants or mutants/10 plates

[a] Total transformants

[b] Transformants giving the correct deletion, based on color change

Analysis of the *PKS* and *SD* mutants

The disruption of different genes in the melanin pathway resulted in fungal phenotypes with different colors than the black wild type. For example, deleting *PKS* resulted in an albino phenotype, while deleting *SD* resulted in the accumulation of an intermediate product, scytalone and a reddish fungal colony (Fig. 3). We observed that when the *SD* and *PKS* mutants were grown from opposite sides on the same plate, the mycelia of the albino *PKS* mutant turned black in area where mycelia of both mutants were in contact. This result suggests that the scytalone produced by the *SD* mutant was used by the *PKS* mutant to restore the complete melanin pathway (Fig. 3), confirming that the *SD* and *PKS* genes targeted in this work were involved in melanin biosynthesis. Except for changes of the color phenotype, we observed no marked differences between the mutants and wild type in morphology, sporulation, or in vegetative growth (data not shown).

We also compared the growth of the wild type and the mutants on freshly cut logs. After 3 weeks incubation at room temperature, the logs were debarked and re-isolation was carried out. The wild type induced a brown to black reaction (lesion) in the phloem and sapwood of about 5 cm in length (averaged from four independent experiments), while the mutants and agar control produced very weak or no significant reaction. Despite this difference of color phenotype, we successfully re-isolated both wild type and mutants from the phloem and sapwood up to 15 cm away from the inoculated point. As well, we compared the pathogenicity of the wild type and mutants on 5-year-old seedlings at high inoculation concentrations (16 inoculation plugs per seedling). Both the wild type and *PKS* mutants killed the seedlings within 4 weeks, while the control (agar plug) had no obvious effect on the seedlings.

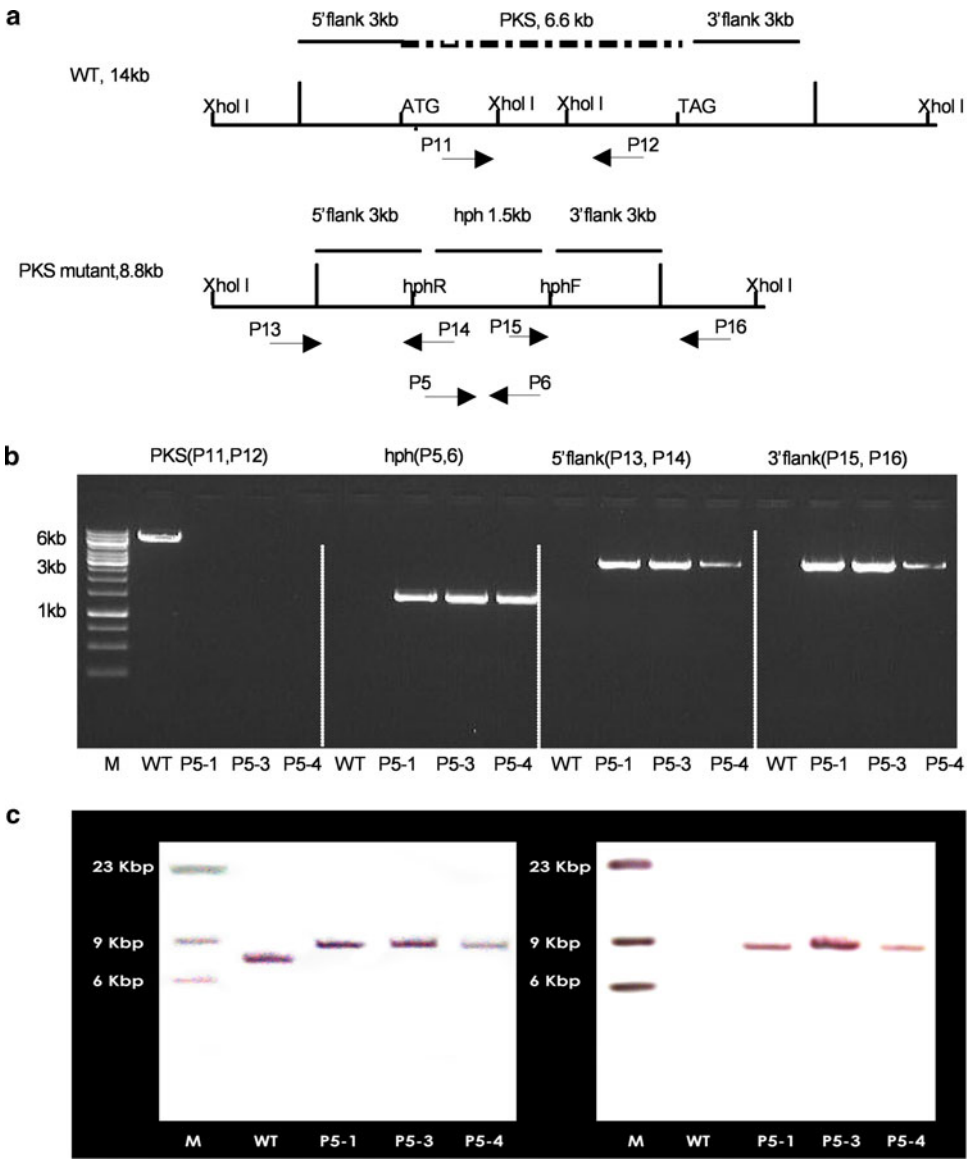

Fig. 2 Molecular characterization of *PKS* mutants. **a** Predicted DNA structures of *PKS* locus in wild type (WT) and mutant (not to scale). **b** PCR amplification of *PKS* (6.6 kb), *hph* (1.5 kb), 5′ flank region (3.2 kb), 3′ flank (3.2 kb) region using primers indicated in a from WT and *PKS* mutants P5-1, P5-3, P5-4. P13 and P16 were extracted from the sequence beyond the flanking regions used for HR, P14 and P15 are located in *hph* cassette, therefore, no amplicons were expected from wild type, when the integration is accurate, the size of amplicons are expected to be 3.2 kb. **c** Southern analysis to confirm the *PKS* mutants. 10 µg genomic DNA from WT and mutants (P5-1, P5-2, P5-3) was digested by X*ho*lI and hybridized with a *hph* probe (*right*) and a probe from the 5′ flanking region (*left*). Based on the draft genome sequence, the predicted hybridized fragment size for *PKS* is 8.8 kb when only one copy of the *hph* gene is present in the mutant genome. The WT gives a band of 7.6 kb when hybridized with the 5′ flanking region probe and no band with the *hph* probe

Discussion

The aim of gene replacement is to substitute a targeted gene by a selective marker gene through HR. However, obtaining successful gene replacements requires first developing an efficient transformation system for the target species. For filamentous fungi the most commonly used transformation methods include, but are not limited to: protoplasts, electroporation, biolistic and AMT (Meyer 2008). The efficiency of these methods varies with the fungal species, and biological, chemical or physical parameters must be optimized for each species (Meyer 2008). In previous work with ophiostomatoid species we found that both protoplast transformation and AMT gave reasonable transformation rates with no sign of mitotic instability but with multiple integration events (Wang et al. 1999, 2001; Tanguay and Breuil 2003). However, using AMT we obtained only very low numbers of mutants for *O. piliferum* and *C. resinifera* that were deleted in specific target genes (Hoffman and Breuil 2004; Loppnau et al. 2004). For both model and non-model fungi, AMT has been shown to have high transformation efficiency and low copy numbers of insertion for a range of fungal species (Michielse et al. 2005). Consistent with this, in our current work with *G. clavigera*, after optimizing a number of parameters we obtained 1,000 times more transformants than with other ophiostomatoid species that we have previously studied. The higher transformation rate with *G. clavigera* could be due to using a more appropriate ratio of *A. tumefaciens* cells to fungal spores or more appropriate germination conditions for fungal spores. It is also possible that our success in transformation rate and HR frequency (higher than 40%) was the result of having longer flanking gene regions (3 kb) in our deletion cassettes than in previous work.

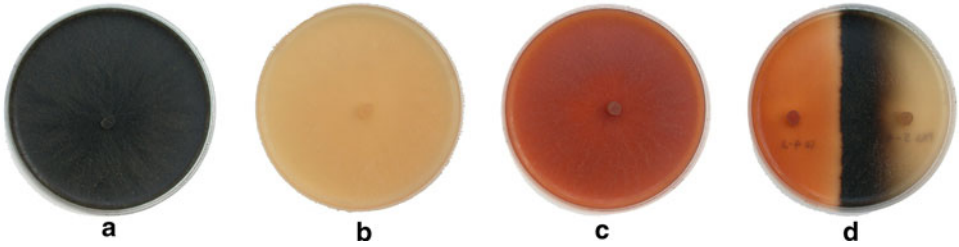

Fig. 3 Mutant phenotypes on MEA plates. **a** Wild type Kw1407, **b** *PKS* mutant 5-4, **c** *SD* mutant 4-3, **d** dual culture of **b** and **c**

Typically, HR complicates generating targeted mutants in filamentous fungi. It is well documented that the length of the target gene flanking regions plays a key role in HR efficiency, regardless of the transformation process used (Ma et al. 2009; You et al. 2009; Walther and Wendland 2008). For yeasts, especially *S. cerevisiae*, 100% efficient gene replacement can be achieved using as short as 40 to 50-bp flanking regions. For filamentous fungi several hundred to several thousand base pair flanking regions are necessary to produce reasonable numbers of desired mutants (Meyer 2008), despite these long flanking regions HR frequency remain relatively low, usually less than 10%. For example, for *M. grisea*, upstream flanking region of 1.5 kb and downstream flanking region of 0.7 kb of the targeted gene resulted in 4.9% HR rate (Villalba et al. 2008). For *Podospora anserina*, the HR rate was very low at ~0.5% with flanking regions of 1 kb (El-khoury et al. 2008). Large variations in HR rates have been reported in the genus *Aspergillus*. *A. fumigatus*, *A oryzae* and *A. sojea* require 1–1.4 kb flanking regions to produce 1.4–10% HR gene replacements, while *A. nidulans* showed up to 38% HR frequency with only 0.5-kb flanking regions (Krappmann 2007). However, the targeted loci can also influence the HR efficiency; for example, in *Botrytis cinerea*, flanking regions of 0.5–1 kb can result in 0 or 91% HR (Choquer et al. 2008). Despite these highly variable results, researchers agree that for conventional linear cassettes increasing HR rate is easily obtained with longer flanking regions (Zwiers and De waard 2001; Walther and Wendland 2008). However, using longer flanking regions requires substantial genome sequence information. For fungi for which reference genome sequences are not available, few reports show flanking regions larger than 1.5 or 2 kb. In the work described here, we used a draft genome sequence_ assembly we recently generated (DiGuistini et al. 2009) to retrieve flanking regions up to 3 kb long. We showed that for *G. clavigera* good transformation efficiency and HR frequencies up to 55% were obtained with flanking regions of 1.5–3 kb. While we showed a slightly higher HR efficiency with *PKS* than with *SD*, at this point we can not conclude whether the length of the targeted gene (*PKS* 6.7 kb vs. *SD* 0.8 kb) or the surrounding upstream and downstream regions of the gene, particularly the GC content, contributed to the difference in HR efficiency.

Split-marker, another promising approach for increasing HR frequency, has been successfully used with *M. grisea*, *N. crassa*, *C. neoformans*, and some others (Colot et al. 2006; Fu et al. 2006; Jeong et al. 2007). This approach works well with *Cochliobolus heterostrophus*, which showed a HR rate of ~100% (Catlett et al. 2003). With *M. grisea*, using 500 bp flanking regions resulted in 2 out of 5 transformants being the targeted deletion mutants, in contrast to 0 out of 120 transformants for linear cassette (Jeong et al. 2007). For *N. crassa*, the average HR rate was 44% with the split-marker approach using 3 kb flanking regions (Colot et al. 2006) while for *Cercospora nicotianae* HR varies from 8.8 to 43%, depending on the targeted locus and flanking regions, but the number of targeted mutants significantly increased in comparison to the linear cassette (You et al. 2009). Our results were consistent with the above results. We obtained higher HR rates for the deletion of large (*PKS* 82%) and small (*SD* 65%) genes. The additional crossover in *hph* (i.e., overlapped region) resulted in fewer transformants (20 times less). Despite this, the ~10 transformants that we obtained allowed us to continue our investigation. Both split-marker and AMT have been shown to have rare multiple insertions (Zwiers and De Waard 2001; Michielse et al. 2005; Jeong et al. 2007; You et al. 2009). In our work, we minimized multiple copy insertions by combining these two methods, and, in our screen, found no such mutants. This is a desirable outcome for gene replacement. To our knowledge, this is the first report of combining AMT and split-marker for filamentous fungi. This method is efficient and reproducible and will allow us to generate more mutants within a reasonable time.

Like many other filamentous fungi, the ophiostomatoid fungi produce melanin. Ophiostomatoid melanin is synthesized by the DHN-pathway and discolors the sapwood of trees or logs. We confirmed that *G. clavigera* produces melanin through the DHN-pathway. We found that the absence of melanin did not seem to affect the growth and sporulation of this fungus; both the black wild type and white *PKS* mutants colonized and eventually killed seedlings when they were inoculated at a high density. While melanin has been reported as a virulence factor in plant pathogens like *M. grisea* and *Colletotrichum* spp. (Kubo et al. 1996; Howard and Valent 1996), to this point we have been unable to demonstrate a similar role for *G. clavigera* melanin, and the

ecological role of this pigment remains uncertain. In ongoing work we are investigating an intriguing observation: the *PKS* mutant inoculations did not induce a lesion in the pine logs, while the wild type did.

In conclusion, we developed an efficient gene replacement strategy for *G. clavigera*, a recently sequenced fungal pathogen associated with the mountain pine. We used *Agrobacterium*-mediated transformation (AMT) in combination with deletion cassettes containing either linear or split-marker flanked by long segments of DNA adjacent to the targeted genes. The method was efficient and we obtained two types of mutants with *PKS* or *SD* deletion affecting the black melanized phenotype of our wild type, *G. clavigera*. The gene disruption strategy described here along with the recently developed genome information will allow us to functionally identify genes involved in pathogenicity and understand how *G. clavigera* can bypass or overcome tree defenses.

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