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Technological advancement

A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum*

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Abstract

Rapid progress in fungal genome sequencing presents many new opportunities for functional genomic analysis of fungal biology through the systematic mutagenesis of the genes identified through sequencing. However, the lack of efficient tools for targeted gene replacement is a limiting factor for fungal functional genomics, as it often necessitates the screening of a large number of transformants to identify the desired mutant. We developed an efficient method of gene replacement and evaluated factors affecting the efficiency of this method using two plant pathogenic fungi, *Magnaporthe grisea* and *Fusarium oxysporum*. This method is based on *Agrobacterium tumefaciens*-mediated transformation with a mutant allele of the target gene flanked by the herpes simplex virus thymidine kinase (*HSVtk*) gene as a conditional negative selection marker against ectopic transformants. The *HSVtk* gene product converts 5-fluoro-2′-deoxyuridine to a compound toxic to diverse fungi. Because ectopic transformants express *HSVtk*, while gene replacement mutants lack *HSVtk*, growing transformants on a medium amended with 5-fluoro-2′-deoxyuridine facilitates the identification of targeted mutants by counter-selecting against ectopic transformants. In addition to *M. grisea* and *F. oxysporum*, the method and associated vectors are likely to be applicable to manipulating genes in a broad spectrum of fungi, thus potentially serving as an efficient, universal functional genomic tool for harnessing the growing body of fungal genome sequence data to study fungal biology.

Keywords: Rice blast; Fusarium wilt; *Magnaporthe grisea*; *Fusarium oxysporum*; *Agrobacterium tumefaciens*; Herpes simplex virus thymidine kinase; Targeted gene replacement; Conditional negative selection

1. Introduction

Better understanding of fungal biology has greatly increased in recent years, as a result of significant advances in fungal genomics including the release of fungal genome sequences and the development of efficient molecular tools. Among these tools, transformation-mediated mutagenesis has undoubtedly been the most widely applied method for studying gene function in fungi. In most filamentous fungi, transformation results from the integration of the transforming DNA into the fungal genome by either non-homologous or homologous recombination. Transformation with a plasmid through a non-homologous recombination pathway has been widely used for random mutagenesis in fungi, as an alternative to chemical or radiation mutagenesis. The integration of a plasmid into a gene provides a convenient molecular tag to rescue and characterize this gene (Mullins and Kang, 2001). Transformation throug h a homologous recombination pathway permits targeted gene replacement and requires a plasmid carrying two DNA fragments from the targeted locus of sufficient length to promote homologous recombination (Hamer et al., 2001). An important advance in fungal

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transformation has been the recent development of *Agrobacterium tumefaciens*-mediated transformation (ATMT). *A. tumefaciens* transfers a segment of the Ti plasmid DNA termed the T-DNA ('transfer' DNA) to the fungal genome (Mullins and Kang, 2001). ATMT has been successfully applied to a wide range of fungal species (Abuodeh et al., 2000; Bundock et al., 1995; Campoy et al., 2003; Chen et al., 2000; Combier et al., 2003; Covert et al., 2001; de Groot et al., 1998; Dobinson et al., 2004; Gardiner and Howlett, 2004; Godio et al., 2004; Gouka et al., 1999; Leclerque et al., 2004; Malonek and Meinhardt, 2001; Mullins et al., 2001; Rho et al., 2001; Sullivan et al., 2002; Zhang et al., 2003; Zwiers and De Waard, 2001).

Fungi with a low frequency of homologous recombination require that a large number of transformants be generated and screened to identify the desired mutant (Mullins and Kang, 2001). To circumvent this time-consuming process, we developed a targeted gene replacement method (termed ATMT-DS) based on ATMT followed by a dual (positive and negative) selection (DS) of transformants. We have evaluated the factors affecting the efficiency of targeted gene replacement using ATMT-DS in two plant pathogenic fungi, *Magnaporthe grisea* and *Fusarium oxysporum*. *M. grisea*, the causal agent of rice blast disease, is the most damaging pathogen of rice. In addition to its economic significance, *M. grisea* is one of the main models for understanding fungal pathogenicity (Kang et al., 2000; Talbot, 2003). The *F. oxysporum* species complex gathers diverse soilborne fungi including plant pathogens causing vascular wilt on more than 100 cultivated plants (Beckman, 1987). We showed that ATMT-DS is an efficient tool for targeted gene replacement in *M. grisea* and *F. oxysporum*, and is likely to be applicable in manipulating genes in other filamentous fungi.

2. Materials and methods

2.1. Strains, media, and ATMT-DS

Agrobacterium tumefaciens strains AGL1 and EHA105 (Klee, 2000) were used to transform *M. grisea* strains KJ201 (Park et al., 2000), and 4091-5-8 (Valent et al., 1986), and *F. oxysporum* O-685 (Mullins et al., 2001). The following fungal and oomycete strains tested for sensitivity to 5-fluoro-2′-deoxyuridine (F2dU) were from our laboratory (*Verticillium dahliae* and *Crinipellis perniciosa*) or our colleagues at Penn State University (University Park, PA), including David Geiser (*Aspergillus oryzae*, *Aspergillus fumigatus*, *Aspergillus nidulans*), Hye-Ji Kim (*Thielaviopsis* sp.), Wakar Uddin (*Rhizoctonia solani*), and Gary Moorman (*Botrytis cinerea*, *Pythium aphanidernatum*, *Pythium ultimum*, *Pythium irregulare*, *Phytophthora cactorum*, and *Phytophthora cinnamomi*). Ganciclovir and F2dU (Sigma), hygromycin B (Calbiochem), and geneticin (Sigma) were dissolved in water and filter-sterilized to prepare stock solutions and stored at −20 °C except hygromycin B (4 °C). For testing sensitivity to ganciclovir and F2dU, *M. grisea* was grown on complete medium (Valent et al., 1986). Potato dextrose agar (Difco) was used for testing other fungi and oomycetes. ATMT was performed as previously described (Mullins et al., 2001). Different amounts of hygromycin B were used for selecting transformants (250 μg/ml for *M. grisea* and 50 μg/ml for *F. oxysporum*). Direct selection of putative gene replacement mutants was carried out by regenerating transformants in the presence of both F2dU (5 or 50 μM) and hygromycin B. Different amounts of geneticin (800 μg/ml for *M. grisea* and 50 μg/ml for *F. oxysporum*) were utilized to identify transformants expressing *neo* (a gene conferring resistance to geneticin). For determining the presence of *HSVtk* (a herpes simplex virus thymidine kinase gene) and mutant allele in transformants, fungal genomic DNA was analyzed by PCR and/or Southern hybridization.

2.2. Vector construction

The *ChGPD-HSVtk* construct (1.8 kb *Eco*RI–*Hin*dIII fragment) in pGEM3Zf (Promega) consists of three modules: the *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase (*ChGPD*) promoter (0.5 kb *Eco*RI–*Bam*HI fragment), the open reading frame (ORF) of *HSVtk* (1.1 kb *Bam*HI–*Sal*I fragment), and the *Neurospora crassa* β-tubulin gene terminator (0.2 kb *Sph*I–*Hin*dIII fragment). The *HSVtk* ORF was amplified by PCR from pPNT (Tybulewicz et al., 1991) using a pair of primers containing appropriate restriction sites at their 5′ end. The promoter and terminator modules were amplified using pSM565 (Bourett et al., 2002) as a template. All the modules were sequenced to verify their sequence.

Plasmid pBHt2-*tk* was constructed by cloning the 1.8 kb *Eco*RI–*Hin*dIII fragment carrying *ChGPD-HSVtk* between *Eco*RI and *Hin*dIII sites of pBHt2 (Mullins et al., 2001). To construct pGKO1, the 1.8 kb *Eco*RI–*Hin*dIII fragment was made blunt by treating it with Klenow fragment in the presence of dNTPs, and cloned between the blunted *Xho*I and *Bst*XI sites of pCAMBIA1300 (http://www.cambia.org.au).

Two genes, *F. oxysporum FoSNF1* (Ospina-Giraldo et al., 2003) and *M. grisea MHP1*, a gene encoding class II hydrophobin (Lee, unpublished data; *M. grisea* genome contig 2.206 and locus ID MG01173.3), were utilized to test factors affecting the efficiency of ATMT-DS. To produce pGKO1-*fosnf1*, a 1 kb fragment corresponding to *FoSNF1* (covering 322 bp of the promoter region and 683 bp of the open reading frame) was amplified from *F. oxysporum* O-685 by PCR using the following primers (Fig. 1): SNF-f (5′-AGCACTAGTAATCTACCCGA

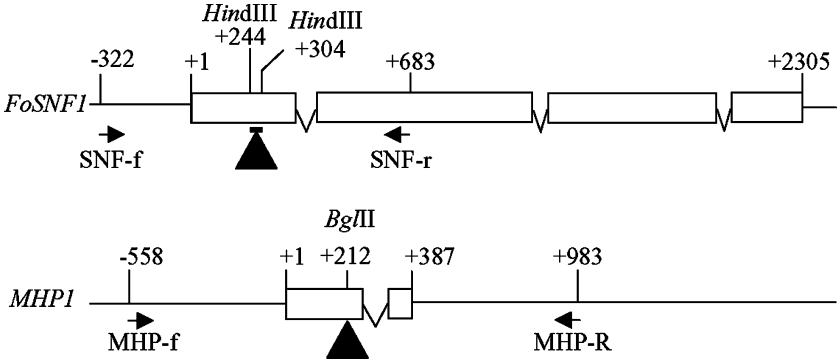


Fig. 1. Schematic diagram of the *F. oxysporum FoSNF1* and *M. grisea MHP1* loci. Exons and introns of the *FoSNF1* and *MHP1* genes are represented by open boxes and hooks, respectively. Numbers indicate the genomic positions of relevant restriction sites/markers relative to the translation start codon (A of ATG as +1). PCR primers that were used to amplify each locus for mutant allele construction were denoted by arrow. The filled triangle indicates the insertion site of the *hpt* gene to create a mutant allele for each gene (between two *Hin*dIII sites for the *FoSNF1* mutant allele and at the *Bgl*II site for the *MHP1* mutant allele).

GGCCAGTC-3′) and SNF-r (5′-AGGCAATTGGG CGATTTTGACGTTGAGA-3′). The underlined seque nces in SNF-f and SNF-r correspond to *Spe*I and *Mfe*I sites, respectively. After cloning the amplified fragment into pGEM-T Easy (Promega), a 60 bp *Hin*dIII–*Hin*dIII fragment of the amplified *FoSNF1* (Fig. 1) was replaced with the 1.4 kb *Hpa*I fragment of pBC1004 carrying *hpt*, a gene encoding hygromycin B phosphotransferase (Carroll et al., 1994). The resulting mutant allele was digested with *Spe*I and *Mfe*I, and cloned between the *Eco*RI and *Xba*I sites of pGKO1 to produce pGKO1-*fosnf1*.

For pGKO1-*mhp1*, a 1.5 kb fragment containing *MHP1* (starting at 558 bp upstream of the start codon and ending at 983 bp downstream from the start codon) was amplified from *M. grisea* 70–15 by PCR using the following primers (Fig. 1): MHP-f (5′-ACGGAATTC TCGACATGGACCGTCTTG-3′) and MHP-r (5′-AG CTCTAGAGTACCAAGCCGCACCACT-3′). The underlined sequences in MHP-f and MHP-r correspond to *Eco*RI and *Xba*I sites, respectively. The *hpt* gene was inserted into a blunted *Bgl*II site (212 bp downstream from the start codon) located in the middle of the amplified *MHP1* locus to generate an *mhp1* mutant allele. The resulting mutant allele was digested with *Eco*RI and *Xba*I, and cloned between the *Eco*RI and *Xba*I sites of pGKO1 to produce pGKO1-*mhp1*. The *mhp1* mutant allele in pGKO1-*mhp1* was isolated as an *Eco*RI–*Hin*dIII fragment and cloned in pGKO2 to produce pGKO2-*mhp1*.

To generate two binary vectors, pDHt-KS and pDHt-SK (identical except the orientation of their MCS), a 300 bp *Pvu*II–*Pvu*II fragment of pDHt (Mullins et al., 2001) containing the multiple cloning site (MCS) was replaced with a 0.8 kb *Hpa*I–*Stu*I fragment isolated from pGreenII0000 (Hellens et al., 2000).

For constructing pNHTK and pTKHN, three selectable markers, *neo* (1.2 kb *Bam*HI–*Sal*I fragment), *hpt* (1.4 kb *Sal*I–*Eco*RI fragment), and *ChGPD-HSVtk* (1.8 kb *Eco*RI–*Hin*dIII fragment), were initially cloned between *Bam*HI and *Hin*dIII sites of pBluescript SK (Stratagene) in the order of *Spe*I–*Bam*HI-*neo-hpt-ChGPD-HSVtk-Hin*dIII, resulting in pSK1697. The 4.4 kb *Spe*I–*Hin*dIII fragment of pSK1697 was cloned between the *Spe*I and *Hin*dIII sites of pDHt-SK and pDHt-KS to generate pNHTK and pTKHN, respectively.

Selected restriction sites on the *ChGPD-HSVtk* construct in pGEM3Zf were mutagenized using QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instruction. The modified *ChGPD-HSVtk* construct (as a blunted *Eco*RI–*Hin*dIII fragment) was cloned into a blunted *Sac*I site of pDHt-KS, resulting in pGKO2. To allow for cloning of mutant allele into pGKO2 without relying on available restriction sites, we constructed pGKO2-Gateway as follows: the *ccdB* (control of cell death B) and chloramphenicol resistance genes flanked by the *λattP* sites in pDONR201 (Invitrogen) were amplified by PCR using the following primers: 5′-TCG CTCTAGAAATAATGATTTTATTTGAC-3′ and 5′-TC GCAAGCTTGCTGGATGGCAAATAATGAT-3′ (the underlined sequences correspond to *Xba*I and *Hin*dIII sites, respectively). The resulting product (2.3 kb) was first cloned in pGEM-T Easy for sequence verification and was subsequently cloned between the *Xba*I and *Hin*dIII sites of pGKO2.

3. Results

3.1. Herpes simplex virus thymidine kinase (HSVtk) functions as a negative selection marker in diverse fungi

ATMT-DS is designed to counter-select ectopic transformants using a gene conferring lethality when expressed in transformants (the negative selection marker). This marker flanks a mutant allele that contains a positive selection maker such as the hygromycin B resistance gene. Both markers are located between LB and RB of the T-DNA. Ectopic transformants express

both the negative and positive selection markers, while transformants resulting from gene replacement lack the negative selection marker. Therefore, the negative selection should eliminate ectopic transformants (Fig. 2), facilitating the identification of transformants resulting from gene replacement since they are resistant to this negative selection.

We tested two genes, *Dtx-A* encoding diphtheria toxin subunit A and *HSVtk* encoding a viral thymidine kinase, as potential negative selection markers for fungi. Although *Dtx-A* has been successfully utilized as a negative selection marker in plants (Czako and An, 1991; Terada et al., 2002) and mammalian cells (Yagi et al., 1990), *Dtx-A* expressed using two different fungal promoters did not appear to be toxic to *M. grisea* and *F. oxysporum* (data not shown). The *HSVtk* gene product converts nucleoside analogs, such as ganciclovir and F2dU, to toxic compounds and has been utilized as a conditional negative selection marker in diverse organisms (Capecchi, 1989; Chen et al., 2002; Duraisingh et al., 2002; Sachs et al., 1997).

Transformants of *A. fumigatus*, *B. cinerea*, *M. grisea*, and *F. oxysporum* generated using pBHt2-*tk* (Fig. 3), a binary vector carrying the *HSVtk* gene under the control of the *C. heterostrophus* glyceraldehyde-3-phosphate dehydrogenase (*ChGPD*) gene promoter and the *N. crassa* β-tubulin gene terminator on the T-DNA, exhibited sensitivity to ganciclovir (with the exception of

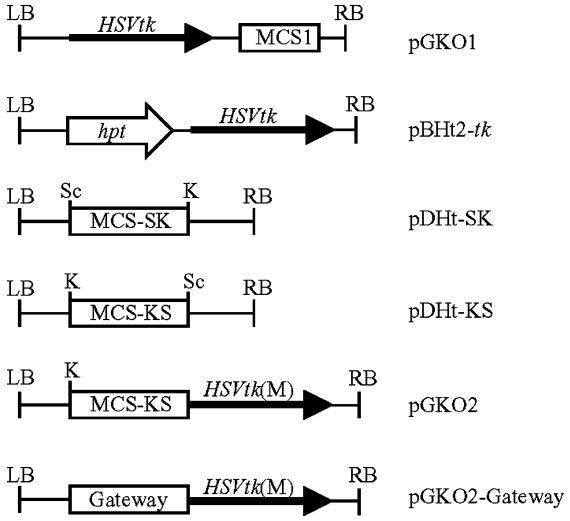


Fig. 3. Schematic diagrams of the T-DNA regions in the binary vectors constructed in this study. The LB and RB of the T-DNA are denoted by vertical lines. The orientation of transcription from *hpt* and *HSVtk* is indicated by arrow (5′–3′). MCS1 corresponds to the multiple cloning site of pCAMBIA1300. The multiple cloning site of pGreenII0000 cloned in pDHt was designated as MCS-SK or MCS-KS depending on its orientation: *Kpn*I (K) and *Sac*I (Sc) sites are shown to indicate the orientation of the MCS relative to other markers. A modified version of *HSVtk* obtained by site-directed mutagenesis is denoted as *HSVtk*(M). Gateway corresponds to the *ccdB* and chloramphenicol-resistance genes flanked by the *λattP* sites. Drawings are not to scale.

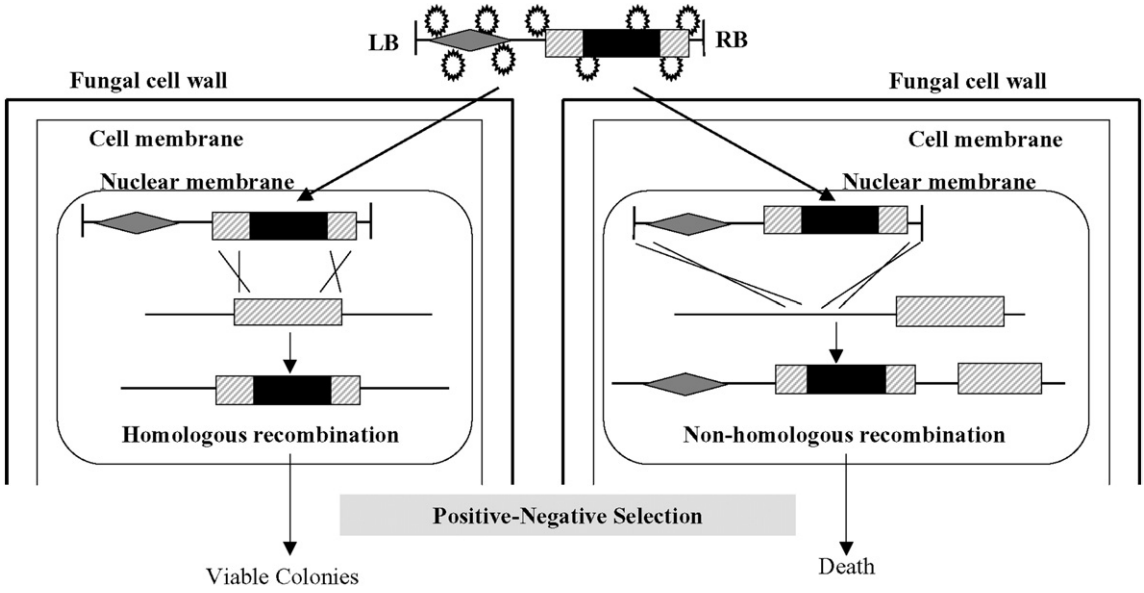


Fig. 2. Schematic diagram of ATMT-DS. *A. tumefaciens* cells, carrying a binary vector that contains a mutant allele (disrupted by a positive selection maker, such as *hpt* conferring resistance to hygromycin B; marked as the filled box) and *HSVtk* (encoding thymidine kinase that converts nucleoside analogs such as 5-fluoro-2′-deoxyuridine (F2dU) to a compound toxic to fungi; denoted by the diamond) on the T-DNA, are co-incubated with fungal cells in the presence of acetosyringone (AS), a chemical inducer of virulence genes of *A. tumefaciens*. During co-cultivation, DNA situated between the left border (LB) and right border (RB) of the T-DNA is transported into fungal nuclei (probably as a complex with *A. tumefaciens* Vir proteins denoted by the circles). Homologous recombination between the native gene and the mutant allele on the T-DNA leads to the loss of *HSVtk*. If the T-DNA integrates into a random location in the fungal genome via non-homologous recombination, both *hpt* and *HSVtk* will be expressed. Targeted gene replacement mutants can be selected by subjecting transformants to both the positive (hygromycin B) and negative (F2dU) selection agents.

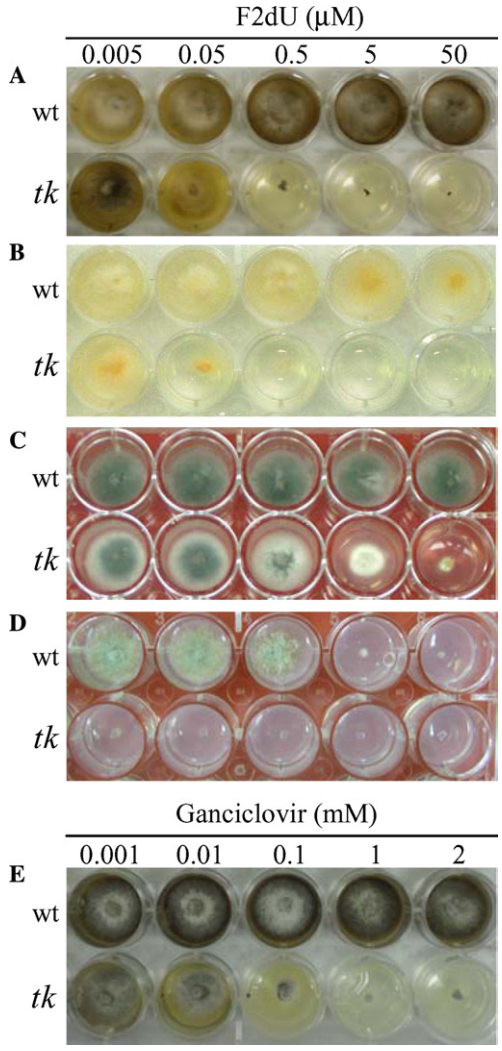


Fig. 4. Growth of *M. grisea*, *F. oxysporum*, *A. fumigatus*, and *B. cinerea* in the presence of 5-fluoro-2′-deoxyuridine (F2dU) or ganciclovir. Wild-type strains (wt) and transformants with *ChGPD-HSVtk* (*tk*) of *M. grisea* (A and E), *F. oxysporum* (B), *A. fumigatus* (C), and *B. cinerea* (D) were grown in the presence of F2dU (A–D) or ganciclovir (E) at concentrations ranging from 5 nM to 50 μM (F2dU) or 1 μM to 2 mM (ganciclovir).

B. cinerea) and F2dU. Sensitivity to F2dU was much greater than that to ganciclovir (Fig. 4 and data not shown). For instance, the effective concentration of F2dU for completely inhibiting the growth of *M. grisea* was approximately 0.5 μM, while 1 mM ganciclovir was needed to achieve the same growth inhibition. Ganciclovir failed to inhibit the growth of *B. cinerea* transformants even at 2 mM, while 5 nM of F2dU was sufficient to inhibit their growth (Fig. 4D). *HSVtk* transformants of *A. fumigatus* were much less sensitive to F2dU than were *B. cinerea*, *M. grisea*, and *F. oxysporum* transformants, requiring 50 μM F2dU for significant growth inhibition. In contrast, wild-type strains of these and other fungal and oomycete species, including ascomycetes (*A. oryzae*, *A. nidulans*, *Thielaviopsis* spp., and *V. dahliae*), basidiomycetes (*R. solani* and *C. perniciosa*), and oomycetes (*P. aphanidernatum*, *P. ultimum*, *P. irregulare*, *P. cactorum*, and *P. cinnamomi*), did not exhibit sensitivity to F2dU or ganciclovir at the concentrations that completely blocked the growth of *HSVtk* transformants derived from *A. fumigatus*, *B. Cinerea*, *F. oxysporum*, and *M. grisea* (data not shown), suggesting the applicability of *HSVtk* as a negative selection marker for ATMT-DS in diverse fungal and oomycete species.

3.2. Targeted gene replacement in *F. oxysporum* and *M. grisea* genes using ATMT-DS

We utilized two genes, *F. oxysporum FoSNF1*, a gene playing a critical role in carbon catabolite repression and pathogenicity (Ospina-Giraldo et al., 2003), and *M. grisea MHP1*, a gene encoding class II hydrophobin (Lee, unpublished data), to test ATMT-DS (Fig. 1). To determine the degree of enrichment of gene replacement mutants via ATMT-DS, we first isolated hygromycin B-resistant transformants in the absence of F2dU and subsequently analyzed for their sensitivity to F2dU and the presence of target mutation (Table 1). In addition, to determine if bacterial and/or fungal strain-specific

Table 1
Efficiency of targeted gene replacement via ATMT-DS in *M. grisea* and *F. oxysporum*

Clones used	Fungal strain[a]	*A. tumefaciens* strain	HR[b]	HFR[c]	TGR[d]	False positive (FP)[e]
pGKO1-*fosnf1*	O-685	AGL1	51	11	10 (20%)	1 (9%)
		EHA105	34	6	3 (9%)	3 (50%)
pGKO1-*mhp1*	KJ201	AGL1	70	26	18 (26%)	8 (31%)
		EHA105	49	33	25 (51%)	8 (24%)
	4091-5-8	AGL1	31	10	2 (6%)	8 (80%)
		EHA105	40	8	0 (0%)	8 (100%)

[a] *Fusarium oxysporum* strain O-685 was transformed using pGKO1-*fosnf1*, and *M. grisea* strains KJ201 and 4091-5-8 were transformed using pGKO1-*mhp1*.
[b] Total number of hygromycin B-resistant (HR) transformants isolated from two to four independent transformations.
[c] The number of HR transformants resistant to 5 μM F2dU (HFR).
[d] The number and percentage of targeted gene replacement (TGR) mutants among HR.
[e] The number and percentage of false positive (FP) transformants (resistant to both hygromycin B and F2dU but do not carry the targeted gene replacement) among HFR.

factors affect the efficiency of targeted gene replacement, we introduced gene disruption vectors pGKO1-*fosnf1* and pGKO1-*mhp1* into two different *A. tumefaciens* strains, AGL1 and EHA105 (Klee, 2000), and also employed two strains of *M. grisea*, KJ201 (Park et al., 2000) and 4091-5-8 (Valent et al., 1986). With *F. oxysporum*, AGL1 yielded a higher gene replacement frequency than did EHA105 (20 vs. 9%). With *M. grisea*, AGL1 was better than EHA105 in generating gene replacement mutants in 4091-5-8 (6 vs. 0%), but produced fewer gene replacement mutants in KJ201 than did EHA105 (26 vs. 51%). With both AGL1 and EHA105, the frequencies of gene replacement in KJ201 were significantly higher than those in 4091-5-8.

In both fungal species, some hygromycin B and F2dU-resistant transformants had intact *MHP1* or *FoSNF1* and likely resulted from ectopic integrations of the T-DNA that failed to express *HSVtk*. The frequency of such false positive (FP) ranged from 9–50% in *F. oxysporum* to 24–100% in *M. grisea* (Table 1). In *M. grisea*, KJ201 yielded lower frequencies of FP than did 4091-5-8. To determine whether FP transformants resulted from the truncation of *HSVtk* during ectopic integration of the T-DNA, a set of 28 FP transformants, including three from *F. oxysporum* and 25 from *M. grisea*, was analyzed by PCR and Southern hybridization (Fig. 5). All FP transformants from *F. oxysporum* (3/3) and KJ201 (13/13), and most FP transformants from 4091-5-8 (9/12) corresponded to ectopic transformants with various truncations of LB and *HSVtk* (Fig. 5). One 4091-5-8 FP had extensive truncations at both the LB and RB. The remaining two FP transformants from 4091-5-8, however, had intact LB and *ChGPD-HSVtk* but were insensitive even to 50 μM F2dU (data not shown), suggesting that the expression of *HSVtk* was suppressed probably due to the chromosomal context of inserted T-DNA.

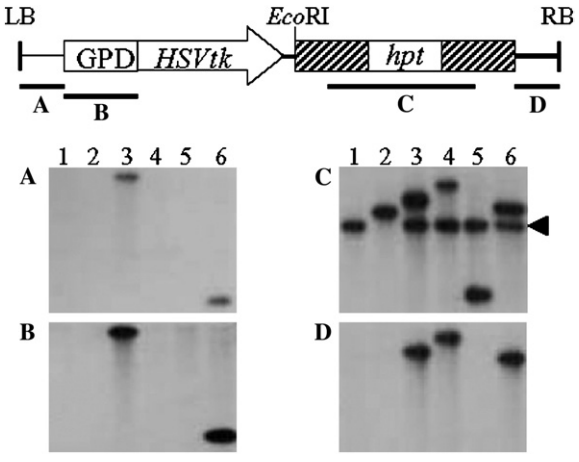


Fig. 5. Southern analysis of *M. grisea* ATMT-DS transformants. The hatched box interrupted by *hpt* denotes the *mhp1* mutant allele cloned in pGKO1. The diagram of the T-DNA was not drawn to scale. *Eco*RI-digested genomic DNA of wild-type 4091-5-8 strain (lane 1) and its transformants, including one gene replacement mutant (lane 2), one ectopic transformant (lane 3), and three different types of false positive transformants (lanes 4–6), was hybridized with each of the four probes shown underneath the T-DNA diagram: (A) 0.3 kb fragment covering the region between the LB and the *ChGPD* promoter, (B) 0.4 kb fragment covering the *ChGPD* promoter, (C) 2.9 kb fragment covering both *hpt* and parts of the *MHP1* locus, and (D) 250 bp fragment covering the region between the RB and the mutant allele. The arrow in Fig. 5C marks the wild-type *MHP1* gene, which is absent in the gene replacement mutant (lane 2).

Table 2
Leakiness of direct negative selection during the regeneration of transformants

Fungal strain[a]	Direct selection[b]		F2dU-insensitive transformants[c]
	F2dU (μM)	Number of transformants	
O-685	0	155	42 (27%)
	5	128	38 (30%)
	50	83	27 (33%)
4091-5-8	0	138	78 (57%)
	5	164	62 (38%)
	50	41	31 (76%)

[a] *Fusarium oxysporum* strain O-685 and *M. grisea* strain 4091-5-8 were transformed using pGKO1-*fosnf1* and pGKO1-*mhp1*, respectively.

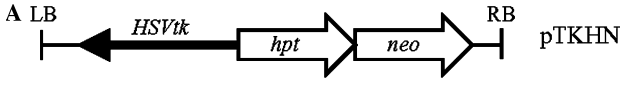
[b] Total number of transformants isolated from selection plates containing both hygromycin B and F2dU (0, 5 or 50 μM). Seven plates were used for each treatment.

[c] The number and percentage of primary transformants that grew on a new plate containing 5 μM F2dU.

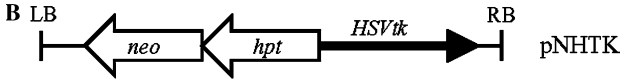
When putative gene replacement mutants were directly selected by regenerating transformants of O-685, KJ201, and 4091-5-8 in the presence of both hygromycin B and 5 μM F2dU, in all strains, the negative selection was leaky: a significant fraction of transformants (71%, 20%, and 82% in O-685, KJ201, and 4091-5-8, respectively) exhibited sensitivity to F2dU when transferred to fresh media containing the same concentration of F2dU (data not shown). During selection, *A. tumefaciens* cells began to lyse, possibly releasing nucleosides counteracting the F2dU toxicity. If so, increasing the concentration of F2dU should reduce the leakiness of negative selection. However, even 50 μM F2dU did not alleviate the problem, as a significant number of O-685 and 4091-5-8 transformants still remained sensitive to 5 μM F2dU (Table 2). During this experiment, we also noticed that in both fungi the presence of 50 μM F2dU consistently reduced (2- to 4-fold) the number of transformants compared to the number obtained with 0 or 5 μM F2dU, suggesting that high F2dU interfere with the efficiency of transformation.

3.3. Stability of HSVtk depends on fungal strains and its location in the T-DNA

In pGKO1-*fosnf1* and pGKO1-*mhp1* gene replacement vectors, *HSVtk* was located at LB of the T-DNA.



Fungal strain	HR	Loss of *HSVtk*	Loss of *neo*	Loss of both
O-685	83	3 (4%)	2 (2%)	0 (0%)
4091-5-8	173	22 (13%)	29 (17%)	5 (3%)
KJ201	98	8 (8%)	24 (24%)	1 (1%)



Fungal strain	HR	Loss of *neo*	Loss of *HSVtk*	Loss of both
O-685	136	5 (4%)	1 (1%)	1 (1%)
4091-5-8	163	31 (19%)	4 (2%)	2 (1%)
KJ201	113	34 (30%)	1 (1%)	0 (0%)

Fig. 6. Stability of *neo* and *HSVtk* located at either LB or RB of the T-DNA. Each fungal strain (*F. oxysporum* strain O-685 and *M. grisea* strains 4091-5-8 and KJ201) was transformed using (A) pTKHN and (B) pNHTK. The total number of hygromycin B-resistant (HR) transformants were analyzed, and the number and percentage of HR sensitive to geneticin and F2dU (Loss of *neo*), resistant to F2dU and geneticin (Loss of *HSVtk*), and sensitive to geneticin and resistant to F2dU (loss of both markers) were indicated in the tables.

We have observed a significant number of transformants with truncations of LB including *HSVtk* (Fig. 5). To evaluate the stability of markers according to their location in the T-DNA, we constructed two vectors carrying *neo* and *HSVtk* on both sides of the *hpt* positive selection marker (Fig. 6). In the pTKHN vector, *HSVtk* is located near LB and *neo* near RB. In the pNHTK vector, *neo* is located near LB and *HSVtk* near RB. Hygromycin B-resistant transformants of *F. oxysporum* and *M. grisea* obtained with either pNHTK or pTKHN (Fig. 6) were scored for their resistance to F2dU (loss of *HSVtk*) and geneticin (presence of *neo*). *HSVtk* was more easily lost or inactive when located at LB (4–13%) than at RB (1–2%) in the three fungal strains tested (Fig. 6).

There was no apparent positional difference for *neo* loss in *M. grisea* while in *F. oxysporum* the stability of *neo* followed the same trend as *HSVtk*. In *F. oxysporum*, the loss of *neo* was two times higher when the marker was located at LB compared to RB. The fact that *neo* was significantly more prone to loss of function than *HSVtk* at both LB and RB locations in *M. grisea* is puzzling.

3.4. Construction of improved targeted gene replacement vectors

To facilitate ATMT-DS mediated targeted gene replacement, we constructed a number of improved vectors (Fig. 3). The *ChGPD-HSVtk* construct on pGKO1 contains one or more of the following restriction sites: *Bam*HI, *Eco*RV, *Pst*I, *Sac*I, *Sal*I, and *Sma*I. Most of these sites (except *Eco*RV) are also present in the multiple cloning site of pGKO1, significantly reducing the number of available cloning sites. We removed these restriction sites from the *ChGPD-HSVtk* construct via site-directed mutagenesis, and at the same time, improved codons at the mutated sites based on the fungal codon usage (http://www.kazusa.or.jp/codon). To further expand the number of available restriction sites for cloning, we replaced the original multiple cloning site of the binary vector pDHt (Mullins et al., 2001) with the multiple cloning site from pGreenII0000 (Hellens et al., 2000), resulting in pDHt-KS and pDHt-SK with 15 unique restriction sites.

The stability of *HSVtk* was significantly higher at RB than that at LB (Fig. 6). To reduce the generation of FP transformants caused by loss of the *HSVtk* gene during T-DNA integration, the mutated *ChGPD-HSVtk* construct was cloned at the *Sac*I site (located near RB) in the multiple cloning site of pDHt-KS to generate pGKO2 (Fig. 3). The frequency of FP with pGKO2 was compared with that with pGKO1 using *M. grisea*. We cloned *mhp1* in pGKO2 to produce pGKO2-*mhp1*. After transforming *M. grisea* strain KJ201 with pGKO1-*mhp1* and pGKO2-*mhp1*, we counted the number of FP transformants among randomly picked transformants (132 transformants for each construct). With pGKO1-*mhp1*, 56% of the transformants resistant to both hygromycin B and F2dU (14/25) were FP, whereas with pGKO2-*mhp1*, the frequency of FP was only 6% (2/35). A derivative of pGKO2, termed pGKO2-Gateway, was also produced by introducing the Gateway system (Stratagene), designed to facilitate the movement of DNA fragments between vectors through the use of lambda recombinase instead of restriction enzymes and ligase, into pGKO2 (Fig. 3).

4. Discussion

A significant number of fungal genomes have been sequenced or are currently being sequenced (Galagan et al., 2003; Goffeau et al., 1996; Wood et al., 2002; http://www.genomesonline.org/). In many fungi, determining gene function through the generation of null mutants by targeted gene replacement is limited by the low efficiency of homologous recombination. Therefore, the development of a technique to circumvent this limiting factor is critical for fungal functional genomics. ATMT has many advantages for gene manipulation in fungi, including high transformation efficiency (de Groot et al., 1998; Mullins et al., 2001; Rho et al., 2001), increased frequency of homologous recombination (Bundock et al., 1999; Dobinson et al., 2004; Michielse et al., 2005; Zwiers and De Waard, 2001), and ability to transform intact spores and hyphae (de Groot et al., 1998). To further improve targeted gene replacement based on ATMT, we constructed and tested a binary *Agrobacterium* vector (pGKO1) carrying as a negative selection marker

against ectopic transformants the *HSVtk* gene. This gene was originally used in animal cells to facilitate targeted gene replacement (Capecchi, 1989).

In *N. crassa*, a different negative selection marker, the *N. crassa mat a-1* gene conferring heterokaryon incompatibility in the strains carrying the *mat A* gene, was utilized to enhance the efficiency of gene replacement through protoplast-mediated transformation (Pratt and Aramayo, 2002). While the *N. crassa mat a-1* gene allowed a significant enrichment of gene replacement mutants, its utility is limited because this marker confers toxicity only to certain strains of *N. crassa*. In contrast, *HSVtk* appears to function as a universal, conditional negative selection marker. In addition to the four fungal species tested in our study (Fig. 4), transformants of *N. crassa* (Pratt and Aramayo, 2002; Sachs et al., 1997), *Leptosphaeria maculans* (Gardiner and Howlett, 2004), and the human pathogenic basidiomycete *Cryptococcus neoformans* (Y. Chang and J. Kwon-Chung at NIH, personal communication) that express *HSVtk* also exhibited sensitivity to F2dU. Our survey suggested the lack of an enzyme equivalent to *HSVtk* in many other fungi and oomycetes, further supporting the utility of *HSVtk* as a universal negative selection marker. Only a wild-type strain of *B. cinerea* exhibited noticeable sensitivity to F2dU (Fig. 4).

A targeted gene replacement strategy identical to ATMT-DS was recently applied to disrupt genes in *L. maculans* (Gardiner and Howlett, 2004). Our work further extended this study as follows: (i) we demonstrated that *HSVtk* could function as a conditional negative selection marker in diverse fungal/oomycete species. Given that diverse fungi have now been successfully transformed via ATMT, ATMT-DS can be broadly adopted to disrupt fungal genes; the only modification that might be needed would be to replace the *ChGPD* promoter driving the expression of *HSVtk* with an appropriate promoter for certain target fungi. Due to the modular structure of the *HSVtk* construct, such a modification should be simple. (ii) We showed that the stability of *HSVtk* varied according to its location in the T-DNA and the fungal species/strains transformed (see below for more discussion). (iii) By employing two different *A. tumefaciens* strains and two strains of *M. grisea*, we tested if bacterial and/or fungal strain-specific factors affect the efficiency of targeted gene replacement through ATMT-DS (Table 1).

Although ATMT-DS facilitated the rapid identification of gene replacement mutants in *F. oxysporum* and *M. grisea* through the efficient counter-selection of ectopic transformants, we encountered two limiting factors for ATMT-DS. First, we identified FP transformants in both *F. oxysporum* and *M. grisea* that are resistant to hygromycin B and F2dU but do not result from targeted gene replacement (Table 1). These FP transformants mainly resulted from ectopic integrations of the T-DNA truncated at its LB leading to the loss of *HSVtk* (Fig. 5). Although RB was not immune to truncation, when *HSVtk* was located near RB, the frequency of its loss (or inactivation) was significantly lower (ranging from <1% in *F. oxysporum* to 2% in *M. grisea* 4091-5-8) than that near LB (ranging from 4% in *F. oxysporum* to 13% in *M. grisea* 4091-5-8) (Fig. 6). In plants, when truncations in inserted T-DNAs occur, they appear more extensive at LB than RB (Tzfira et al., 2004).

Differential stability of the T-DNA borders is likely caused by different molecular changes occurring at LB and RB during T-DNA integration (Tzfira et al., 2004). In plants, T-DNA integration seems to be initiated by micro-homology dependent annealing of the LB sequence or an adjacent region to a region in the host genome. The T-DNA sequence at the 3′ side of the annealed region is trimmed by exonucleases prior to the ligation of the T-DNA to the host DNA, suggesting that initial annealing through a region in the middle of the T-DNA can bring about extensive T-DNA deletion. The RB side of T-DNA single-strand, which is covalently attached to the *A. tumefaciens* VirD2 protein, may also anneal to the host genome but be protected by VirD2 until this end is ligated (presumably mediated by VirD2 and/or unknown host ligases attracted by VirD2) to the host DNA. Considering that ATMT of fungi seems to involve host and bacterial factors that are also important for successful ATMT of plants (Michielse et al., 2005), integration and truncation of the T-DNA in fungi may also be caused by the same mechanism observed in plants.

To reduce the number of FP transformants, we constructed a new binary vector, pGKO2 (Fig. 3). Targeted gene replacement using pGKO2 indeed significantly reduced the frequency of FP in *M. grisea* (56% FP with pGKO1-*mhp1* vs. 6% FP with pGKO2-*mhp1*). Vector pGKO2-Gateway was also constructed to facilitate the cloning of fungal genes for targeted mutagenesis. Although this improvement should alleviate the problem of FP in *M. grisea* and *F. oxysporum*, in fungal species that exhibit a high rate of T-DNA truncations and a low homologous recombination frequency, FP transformants still remain a significant problem. This problem seems particularly severe in *L. maculans*, since the use of a binary vector carrying *HSVtk* located at both LB and RB still led to a significant number of FP transformants (Gardiner and Howlett, 2004). In the long run, solving this problem will require a better understanding of the mechanism underpinning ATMT and the gene replacement using the T-DNA. Further studies on such a mechanism will not only help us improve ATMT-DS to allow efficient gene replacements even in fungi like *L. maculans* but also complement efforts to understand the mechanism of ATMT in plants (Gelvin, 2003; Tzfira et al., 2004). Studies for determining the bacterial and fungal

factors that affect ATMT have been conducted using *Saccharomyces cerevisiae* (Bundock et al., 1995; Piers et al., 1996; Risseeuw et al., 1996; Roberts et al., 2003; van Attikum et al., 2001; van Attikum and Hooykaas, 2003) and *Aspergillus awamori* (Michielse et al., 2004).

Another problem, although minor, is the leakiness of the negative selection during the regeneration of transformants. Considering that even 50 μM F2dU failed to effectively select against F2dU-sensitive transformants during regeneration, it seems unlikely that nucleosides released from dead *A. tumefaciens* cells are responsible for the leakiness. Alternatively, we hypothesize that the expression of *HSVtk* driven by the *ChGPD* promoter might be suppressed during regeneration. If so, using a different fungal promoter might solve the problem. For now, screening transformants for their sensitivity to F2dU after their regeneration is sufficient for solving this problem.

A number of factors seem to affect the efficiency of isolating gene replacement mutants through ATMT-DS (Table 1 and Fig. 6). As discussed above, the position of *HSVtk* on the T-DNA is one such factor. Interestingly, the stability of *HSVtk* located at LB was significantly higher in *F. oxysporum* (2- to 3-fold) than in *M. grisea* (Fig. 6). This result is consistent with those obtained with pGKO1-*fosnf1* and pGKO1-*mhp1* gene replacement vectors (Table 1), as we observed significantly less FP transformants in *F. oxysporum* than in *M. grisea*. Therefore, the truncation of LB during T-DNA integration appeared more frequent in *M. grisea* than in *F. oxysporum*. In *M. grisea*, the stability of *HSVtk* located at LB was significantly higher in KJ201 than 4091-5-8 (Table 1 and Fig. 6). These results suggest that a significant role of the host genotype in the control of this aberrant integration process. Regardless of *A. tumefaciens* strains used, the frequency of gene replacement in KJ201 was significantly higher than that in 4091-5-8. Slight sequence differences between the mutant allele and its chromosomal copy in 4091-5-8 might have caused the reduced gene replacement frequency; the *mhp1* allele used for mutagenesis is identical to that of KJ201, but exhibits a number of polymorphic sites (31 out of 1540 bp) to that of 4091-5-8 (data not shown). Considering that a number of factors can potentially affect the efficiency of targeted gene replacement via ATMT-DS, for a new fungal species to be mutagenized, prior to launching a large-scale mutagenesis experiment, carefully evaluating such factors for their role in the efficiency of gene replacement is recommended.

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