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Abstract

Methods to streamline functional studies of large numbers of genes are essential to fully utilize the significant genomic resources now available for fungi. Fusion PCR is often used to join pieces of DNA together, particularly in the construction of DNA fragments for gene replacement in fungi. Here we present high-efficiency primers which reliably direct fusion and amplification to generate constructs for gene knockouts.

Addendum:

The authors communicated this omission post publication:

We inadvertently left out the 3HS sequence with which we had such great success. The 5HS sequence, as seen in Figure 2D, is 5'-AGTCGACGACAACTACCATCGATCTGACG. The 3HS sequence which was missing from the original paper is 5'-ACACTGGTGACGGCTAACCAGAACTGTCA.

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Optimized primers and other critical conditions for efficient fusion PCR to generate knockout vectors in filamentous fungi.

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Methods to streamline functional studies of large numbers of genes are essential to fully utilize the significant genomic resources now available for fungi. Fusion PCR is often used to join pieces of DNA together, particularly in the construction of DNA fragments for gene replacement in fungi. Here we present high-efficiency primers which reliably direct fusion and amplification to generate constructs for gene knockouts.

Introduction

Fusion PCR (FPCR) uses two successive PCR reactions to join together segments of DNA into a linear construct or circular plasmid vector (Bryksin and Matsumura 2010, Ellis *et al.* 2011). In the first PCR, each segment is amplified from an appropriate template with primers containing chimeric regions complementary to one another (Figure 1A). These chimeric overlapping sequences then serve as primers for second PCR in which the amplified DNA segments mixed, allowed to anneal the overlaps, and then extended to form a completely fused construct (Figure 1B). Performing multiple fusions at one time can accomplished by using different overlap regions each fusion, allowing more than two segments to be fused into one (Figure 1C). First developed over twenty years ago, this technique has been used for molecular cloning tasks such as mutagenesis, gene

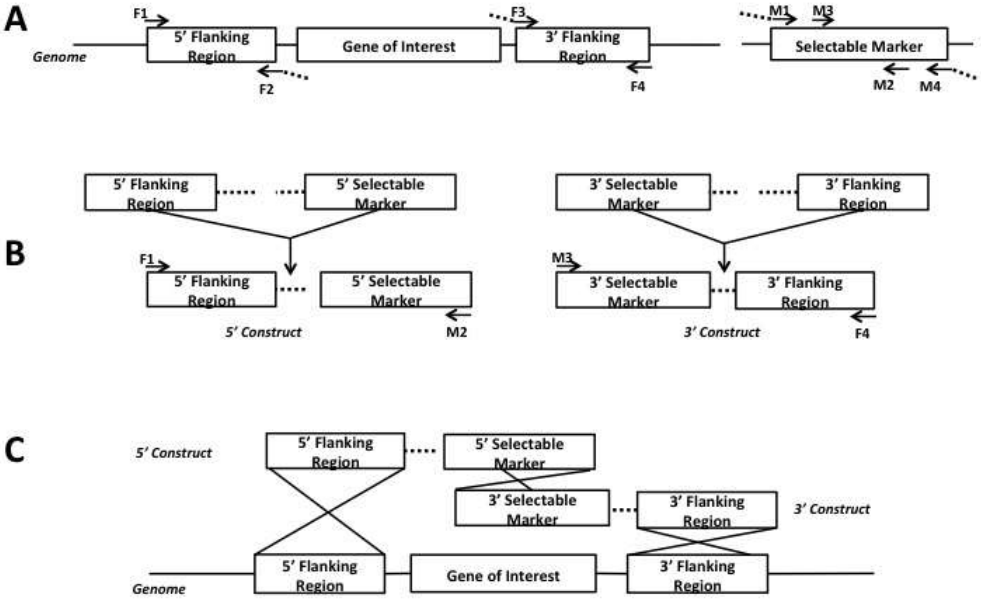

Figure 1. Diagram of a split-marker gene replacement strategy using fusion-PCR
(A) FPCR step 1 - amplification of flanking regions and partial marker segments. Dotted tails indicate the overlap sequences on selected primers which allow fusion in step 2. (B) FPCR step 2 - flanking regions are fused to the marker segments at the overlap sights, forming two constructs. The fusion products are then amplified. (C) Transformation. Three crossovers occur during the gene replacement. Each genomic flanking region crosses over with its complementary sequence in the FPCR constructs. The complementary portions of the marker segments crossover to make the completed selectable marker.

tagging, and gene replacement in a variety of organisms, including fungi (Heckman and Pease 2007, Ho *et al.* 1989, Horton *et al.* 1989, Catlett *et al*. 2003, Szewczyk *et al.* 2006).

FPCR-based gene replacement strategies have been shown to be useful in multiple fungi, including *Saccharomyces cerevisiae*, *Cochliobolus heterostrophus, Fusarium graminearum,* and *Aspergillus nidulans* (Catlett *et al.* 2003, Szewczyk *et al.* 2006; Cavinder *et al.* 2011, Cavinder and Trail 2012). Previously, it was reported that the efficiency of targeted gene replacement could be enhanced by employing a split-marker approach where regions flanking the gene of interest are fused to overlapping partial segments of a selectable marker (Figure 1; Fairhead *et al.* 1998, Catlett *et al.* 2003). For a split marker gene replacement two constructs are generated: a 5' constuct, containing an upstream flanking region and a 5' segment of the selectable marker, and a 3' construct, containing a downstream flanking region and a 3' segment of the selectable marker (Figure 1A and B). These two constucts are used for transformation of the organism in which three crossovers occur: both flanking regions in the genome crossover with their complementary sequences in the two contructs, and the overlapping regions of partial marker segments crossover to form the complete selectable marker (Figure 1C). Thus, the gene of interest is completely replaced with the selectable marker. FPCR was first demonstrated in filamentous fungi as a tool for gene replacement by Catlett *et al.* (2003).

Because FPCR requires only PCR primers and reagents, cloning procedures based on this method can often be quicker, easier, or less expensive than other cloning strategies such as restiction digests and ligations or commercial gene synthesis (Ellis, Adie, and Baldwin 2011). Nonetheless, the efficiency of FPCR reactions can be sensitive to conditions such as primer concentration, template concentration, annealing temperature, the size of the segments to be fused, and the nature of the chimeric overlap sequences (Bryksin and Matsumura 2010, Chai-aim *et al.* 2009). The latter, in particular, can have a serious impact on the success of FPCR because using native sequences as the chimeric overlapping sequences can frequently result in poor fusion or even none at all (Chai-aim *et al.* 2009, Chai-aim *et al.* 2012). Little has been published on the effects different overlapping sequences have on fusion, although previous reports indicated that 15 base pair overlap sequences rich in repeating G and C nucleotides resulted in excellent fusion with broad applicability (Chai-aim *et al.* 2009, Chai-aim *et al.* 2012). Others, however, have suggested that sequences containing high G/C ratios and palindromic elements cause problems in PCR and FPCR reactions (Ellis, Adie, and Baldwin 2011, Zhao *et al.* 2011). We examined the design of high efficiency primers in the generation of split-marker gene replacement constructs, using *Fusarium graminearum* as our target organism. Our results identify primer design strategies and PCR conditions that optimize efficiency in generating gene replacement constructs via FPCR.

Methods

Strains and Primer Design

All studies were performed on *Fusarium graminearum* strain PH-1 (Trail and Common 2000), which was the strain on which the genomic sequence was based (Cuomo et al., 2007). Genomic sequences for PH-1 were obtained from the MIPS *F. graminearum* genome database (http://mips.helmholtz-muenchen.de/genre/proj/FGDB/). Four genes were chosen for comparison of the different strategies of gene disruption. They are designated in the MIPS *Fusarium graminearum* genome database as FGSG_4001, FGSG_4180, FGSG_7376, and FGSG_16930. Eight primers were used to generate FPCR constructs for each gene target (Figure 1). Primers F1 and F2 amplified the upstream flanking region while primers F3 and F4 amplified the downstream flanking region. The flanking amplicons were 500-700 base pairs in length, amplified from genomic DNA (FGSC9075, NRRL31084). All primer sequences were screened for hairpin formation and 3' end primer dimmer formation by the IDT OligoAnalyzer online program (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Primer sequences were selected with G/C contents between 40-60%, melting temperatures of 60˚C, and 1-2 base pair G-C clamps at terminal ends.

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The *E. coli* hygromycin phosphotransferase gene *hph* conferring resistance to hygromycin was used as the selectable marker, under the control of the trpC promoter and terminator from *A. nidulans* (amplified from plasmid pCB1004; Carroll, Sweigard and Valent 1994) and was amplified in two overlapping pieces using primers M1x M2 and M3 x M4 (Figure 1A). M1 and M2 primed the 5' selectable marker segment, while M3 and M4 primed the 3' selectable marker segment. There was a 1.1 kB overlap between the 5' and 3' selectable marker segments for mediating homologous recombination during transformation (Figure 1C).

Fusion PCR

Homologous overlapping sequences were incorporated into the 5' ends of primers F2, M1, F3, and M4 (Figure 1A). F2 overlaps were homologous to M1 overlaps, and F3 overlaps were homologous to M4 overlaps. This allowed the 5' flanking region to be fused to the 5' marker segment, and the 3' flanking region to be fused to the 3' marker segment (Figure 1B). We compared 3 different strategies for designing the overlapping sequences (Figure 2). F2 primers (Figure 1) were composed of 15C, 5CGC, and 5HS (designated 5HS for "5' heterogeneous sequence", see below) overlaps, while M1 primers received the complementary overlaps. F3 primers contained 15G, 5GCG, and 3HS overlaps (designated 3HS for "3' heterogeneous sequence") and M4 primers contained sequences complementary to F3 primers. To control for the annealing sequences of the primers, for each gene target the non-overlap primers (F1, F4, M2, M3) and the primer stems of the overlap primers (F2, F3, M1, M4) were the same for all three trials. Figure 2 shows examples of primers for the 5' construct of gene target FGSG_4001. This method was used for all four aforementioned genes to compare the G-C and heterogeneous sequence overlaps. For each fusion per gene target we tested a 15G/15C overlap, a 5CGC/5GCG overlap, and either a 5HS or 3HS overlap.

Figure 2. Examples of F2 and M1 primers for gene target FGSG_4001
(A) Primer stems only (no overlaps). (B) The bold/underlined sequences were added to the primer stems to create NSOs that incorporate the sequence of each primer into the overlap. (C) 15G/15C overlap added to primer stems. (D) 5HS overlap added to primer stems.

Generation of knockout constructs by FPCR was accomplished in a two-step procedure. In Step 1, marker and flanking regions were amplified from pCB1004 and *F. graminearum* genomic DNA templates, respectively, using Phusion DNA polymerase (New England Biolabs, which has been previously shown to optimize the efficiency and error rate of FPCR reactions (Bryksin and Matsumura 2010). The 5' flanking regions were amplified with F1 and F2 primers, the 3' flanking region with F3 and F4 primers, the 5' marker segement with M1 and M2 primers, and the 3' marker segment with M3 and M4 primers (Figure 1A). These reactions were mixed as per manufacturer's directions. An initial denaturation at 98˚ for 30s - 3 min (longer for genomic DNA, shorter for pCB1004) was followed by 31 cycles of 98˚for 10s (denaturation), 60-64˚ for 30s

(annealing), and 72˚C for 50s (extension). After the final cycle a 10 min final extension at 72˚ finished the program. Although our primers had melting temperatures of 60˚ C, we tested annealing temperatures of both 60˚C and 64˚C to accomodate the manufacturer reccomendations for reactions with the Phusion DNA polymerase. Step 1 reactions were separated on 0.6% agarose gels. Bands were excised and purified with the Wizard SV gel and PCR clean-up kit (Promega). The purified amplicons were then used in step 2 for fusion.

In Step 2, the fusions of 5' flanking regions to 5' selectable marker segments, and 3' flanking regions to 3' marker segments were performed (Figure 1B) to produce the 5' and 3' constructs for each gene, respectively. Reactions were first mixed as follows for each construct: 10 ng-1.2 µg each of flanking region and marker segment DNA (these serve as the template in this reaction), in a 50 µL with the other components as per manufacturer's directions for Phusion DNA polymerase. Because some FPCR-based applications have been reported to be sensitive to both the concentration of template used in this step and the concentration ratios of the segments to be fused, we tested several different ratios and concentrations between 10ng and1.2 µg for each piece. These reactions were submitted to 30s at 98˚C initial denaturation followed by 8 cycles of: 10s at 98˚C denaturation, 30s at 64-68˚C annealing, and 1min at 72˚C extension. Once again, the program concluded with a 10 min, 72˚C, final extension time. Note that the annealing step in this reaction was the annealing of the overlap sequences between the flanking and marker segments and not the annealing of primers since none were included in the reaction mix. We also tested reaction conditions where the overlaps were allowed to both anneal and extend at 72˚C, by simply eliminating the separate annealing step.

Products of the above fusions were used to make the final merged constructs. The following mix was made: 10 µL of 5X Phusion HF buffer, 0.25 µM final concentration of each primer, 200 µM final concentration of dNTP mix, 0.5 µL of Phusion enzyme, and DNase-free water up to a total volume of 50 µL. These mixes were added to each reaction. The primers used for 5' constructs were F1 and M2; for 3' constructs they were M3 and F4. A final program, to amplify the fused constructs, was run with a 30s at 98˚C initial denaturation followed by 31 cycles of 98˚C for 10s, 60-64˚C for 30s, and 72˚ C for 1 min. A final extension of 10 min concluded the program. These reactions were separated by electrophoresis on 0.6 % agarose gels and DNA purified with the Wizard SV gel and PCR clean-up kit.

Transformation and screening of transformants

Transformation of *F.graminearum* strain PH-1 with the merged constructs was accomplished by the polyethylene glycol/protoplast method, as previously described (Hallen-Adams, Cavinder, and Trail 2011). Putative transformants were screened on V8 agar amended with 450 µg/mL hygromycin. Hygromycin-resistant transformants were then grown in 5-8 mL carboxymethylcellulose (CMC) broth to produce condia (Cappellini and Perterson 1965). Spores were germinated on water agar and single-spore isolates were recovered to ensure each transformant was a true-breeding strain. Mycelia from each strain were grown in 10 mL YES broth, frozen, and lyophilized. Genomic DNA was extracted from the lyophilized tissue. PCR amplification of the locus of interest was then used to determine whether constructs had correctly integrated into the locus and replaced the target gene.

Results

Evaluation of conditions affecting the success of amplification by primers

We compared three strategies for designing primers that would drive merges. In the first strategy (Figure 2B) we used the native gene sequences (Native Overlap Sequences; NSO) as our overlapping sequences. Prior to this study, this had been our method for generating knockouts. The second strategy (Figure 2C) employed two overlapping sequences containing repeating strings of G and C nucleotides previously reported to enhance merge efficacy (Chai-aim *et al.* 2009, Chai-aim *et al.* 2012). In the third

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strategy (Figure 2D), we developed two novel sequences with a heterogeneous and non-repeating mix of all four nucleotides. We designed primers using NSO for 11 target genes, as part of an ongoing gene knockout project, immediately prior to this study. We chose four additional genes for targeted replacement to compare the G-C rich and heterogeneous sequence (HS) overlap methods (Figure 2C and D). The four genes were FGSG_4001, FGSG_4180, FGSG_7376, and FGSG_16930. Step 1 of FPCR was the amplification (by standard PCR conditions) of marker and flanking segments (Figure 1A). For all primer pairs, annealing temperatures of 64°C produced successful amplifications for the majority of trials as compared to 60°C (data not shown).

We tested whether different overlaps had an effect on the success of amplification of the two flanking regions and the two selectable marker segments for our four chosen genes. Each amplification reaction contained one primer without an overlap and one primer with an overlap (Figure 1). Table 1 summarizes the success of these amplification reactions related to the type of overlap primer used. In total, 12 out of 20 amplifications using a primer with a G-C rich overlap had weak amplification or failed to amplify completely. When the G-C rich overlaps on these primers were replaced by 5HS and 3HS overlaps, all of the reactions returned strong amplification. Figure 3 shows results from separation of fragments by agarose gel electrophoresis for these amplifications. After receiving these results, we created constucts for 15 additional genes using the 5HS and 3HS overlaps, with similarly successful results. In addition, the application of NSOs to amplify flanking segments for 15 target genes, which are part of an ongoing gene knockout project, also resulted in successful amplification for all segments.

Table 1. Summary of results of FPCR step 1 reactions in comparison of overlaps

Type of overlap	Results of FPCR step 1 reactions (out of 5 reactions/overlap)		
	Strong Amplification	Weak Amplification	No Amplification
15C	3	1	1
15G	0	0	5
5CGC	3	1	1
5GCG	2	0	3
5HS	5	0	0
3HS	5	0	0

Evaluation of conditions affecting fusion of amplified segments

For our four genes, the amplified segments of the 5’ flanking regions to 5’ selectable marker segments, and 3’ flanking regions to 3’ marker segments were merged to produce the 5’ and 3’ constructs for each gene, respectively (Figure 1B). The resulting fusion products were designated as 5’ and 3’ constructs, respectively. Since many of the segment amplifications involved primers with G-C rich overlaps that failed, only 3 pairs of flanking regions and corresponding marker segments were available to be fused. Of these, all three returned strong bands in agarose gel electrophoresis with sizes corresponding to completed constructs (Figure 4). Similarly, all fusion reactions with 5HS or 3HS overlaps yielded strong bands. Results of fusions with NSO containing segments were highly variable.

Allowing overlaps to anneal to each other at 68° in the first PCR program of fusion (using an 8 cycle program, see Methods section) consistently yielded stronger fusion than annealing at 64°, resulting in stronger bands corresponding to the desired product (Figure 4). Removing the annealing step in this program, and allowing the overlaps to both anneal and extend at 72°, provided indistinguishable results from programs with the 68° annealing step. Using more than 8 cycles did not improve the specificity of the fusion, and using more

than 10-15 cycles increased the amount of side-reactions as viewed by extra and unexpected bands on the gels. In the second amplification step (the 31 cycle amplification, see Methods section) primer annealing

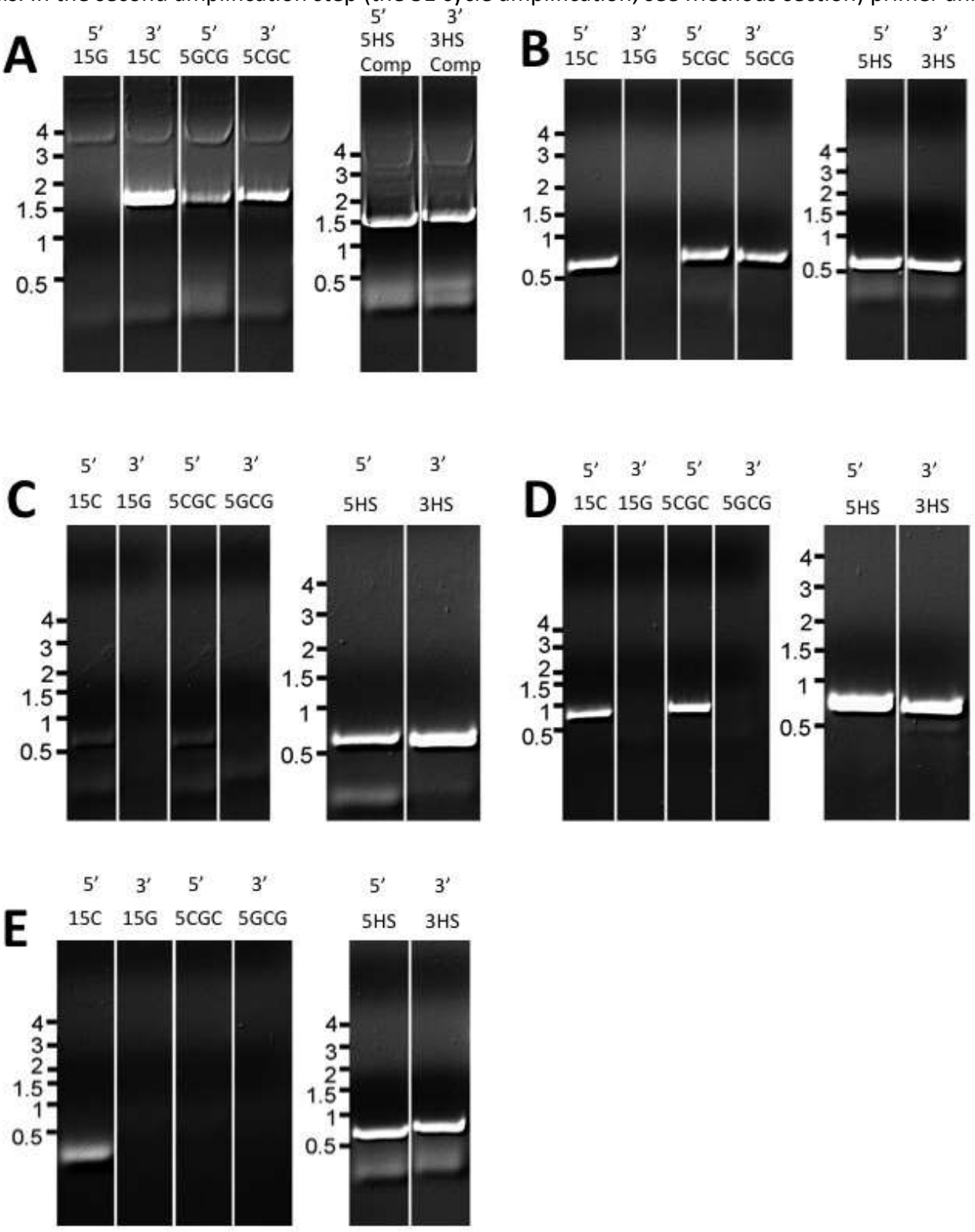

Figure 3. FPCR step 1 amplifications of flanking and marker segments with different overlaps.
5′ or 3′ indicates the identity of the segment amplified (e.g. 5′ marker or 3′ marker) 15C, 15G, 5CGC, 5GCG, 5HS, and 3HS indicate the overlap sequence on the overlap primer used for the amplification. 5HS comp and 3HS comp refer to the 5HS and 3HS complement sequences on the M1 and M4 primers (A) Marker segments (B) FGSG_4001 flanking segments (C) FGSG_4180 flanking segments (D) FGSG_7376 flanking segments (E) FGSG_16930 flanking segments

temperatures of 64° proved preferable to 60° as viewed by stronger bands when reactions products were resolved by agarose gel electrophoresis.

We also tested several different concentrations and ratios of marker segment and flanking region DNA in the first reaction mix of step 2 for the four genes examined. Despite concentrations of each piece ranging from 10 ng-1.2 µg and marker:flanking segment ratios ranging from 12:1 to 1:12, we did not notice a difference in the success of these reactions between different conditions as seen by band size during separation on agarose gels.

For the 11 gene targets for which we used the NSO, immediately prior to this study, only four yeilded at least 2 confirmed gene replacement strains. However, for the 15 gene targets (using SH to generate the knockout constructs) we performed transformation experiments using completed 5' and 3' constructs (Figure 1C). Transformations yielded between 5-20 recombinant transformants for each of the 15 genes. We analyzed putative transformants by PCR amplification to separate ectopic insertional mutants from true deletion mutants. We screened transformants until we confirmed at least 2 true deletion strains for 12 of the 15 genes. Three genes yielded only ectopic transformants. We repeated transformation experiments three times each for these genes, with similar results, before concluding that the deletion of these genes may produce lethal mutations. The phenotypic analysis of the knockout transformants will be published separately.

Discussion

We observed very inconsistent results when using the native gene sequences in designing overlaps for deletion constructs. While some fusions worked quite well, others produced unacceptably low yields of fusion product and many failed to yield any product. Of 11 genes we attempted to knockout by the NSO method immediately preceding this study, only four were efficiently knocked out. This supported the hypothesis that the specific overlap sequence used can have a significant impact on the success of FPCR applications. It also indicated that strategies which utilize the same overlaps each time, regardless of what segments are being fused, may provide an advantage through their consistency.

We tested two such strategies, the G-C rich overlaps and the HS overlaps, and indeed found that they produced consistent fusion across multiple constructs. Nonetheless, with the G-C rich overlaps we observed other problems which may make them unsuitable for creating gene replacement constructs. While it had been previously shown that overlaps such as the 15C/15G and 5CGC/5GCG sequences promote strong and specific fusion products, we hypotheisized that adding long repeats of G and C nucleotides to the tail of an oligonucleotide in this way could cause the formation of primer secondary structures that could inhibit standard PCR amplification. The great difficulty we had with amplifications using such primers supports this hypothesis. Primer synthesis companies often warn against ordering primers with stretches of 6 or more G nucleotides in particular, because such oligonucleotides can be extremely difficult to synthesize reliably, and it is noteworthy that none of the amplifications with 15G primers worked at all. Many of the amplifications with 15C, 5CGC and 5GCG primers also failed to produce product. The issues we had in amplifying with these primers continued even when we altered the annealing temperatures and primer concentrations used. Perhaps more telling, though, is that amplification with these primers worked every time when the same gene regions were amplified with overlaps containing a more heterogenous mix of nucleotides (5HS and 3HS). The G-C overlaps did facilitate strong and specific fusion as previously reported, although the heterogeneous sequence overlaps produced fusions of equally good quality. Thus, due to their non-interefering nature in amplification and promotion of strong fusion, we see our novel 5HS and 3HS sequences as far more desirable options for FPCR, at least in a low complexity application like the creation of split-marker gene replacement constructs. Although we designed constructs for *F.graminearum*, these overlaps and our overall strategy should work equally well in FPCRs for other fungi that can be transformed with targeted gene replacement

constructs. It is worth noting, however, that if these overlaps were used for FPCR applications such as gene tagging, which requires maintenance of the reading frame, they would need to be altered.

We expected primer and template concentrations to be important factors for the success of both steps of the FPCR. Surprisingly, neither seemed to have a significant impact on outcomes. Even varying the ratio of marker segment DNA to flanking segment DNA between 12:1 and 1:12 seemed to have little effect on producing strong fusion products. It may be that since our application represents a fairly simple FPCR, having only one fusion per reaction and using relatively small and similarly sized segments of DNA, it is insensitive to such conditions. This is encouraging when trying to assess the overall utility of this method because fewer limitations on certain conditions should make these methods more easily adaptable.

Annealing temperature had a much more significant effect on amplification. Lower annealing temperatures produced inferior fusion products with lower yields and less specificity. We found excellent results with amplification annealing temperatures of 64° and overlap annealing temperatures of 68-72°, however the optimal temperatures may be lower if an enzyme other than Phusion is used. The Phusion enzyme is only one of several high-fidelity polymerases, and another such enzyme may yield equivalent success. It is our recommendation that for any FPCR annealing temperatures be raised to the highest level for which fusion and priming are still possible (although not exceeding the temperature of extension). Higher temperatures increase not only the specificity of priming, but also the specificity of overlaps binding to one another in step 2. This is important because under less specific conditions (with lower temperatures) the DNA segments being fused may bind to each other in undesirable ways that create unwanted side products. This effect can be directly seen when the fusion reactions are run out on a gel, as a large amount of unwanted bands and a lack of amplification on the desired band.

Using 5HS and 3HS overlaps and higher annealing temperatures, we were able to consistently produce strong fusion products. While our fusions with the heterogenous sequence overlaps (and the G-C rich overlaps as well) did have a few weak undesirable bands (Figure 4), the conditions used apparently did not

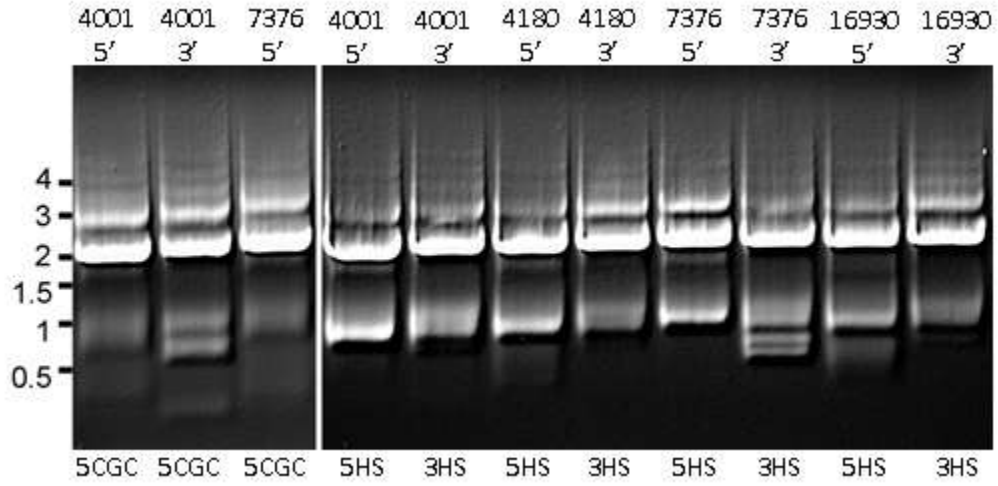

Figure 4. Separation by gel electrophoresis of merge reactions of amplified segments of the 5' or 3' flanking regions to selectable marker gene using either G-C rich (left panel) or heterogeneous sequence overlaps (HS, right panel). Numbers at left indicate size in kB. Gene designation is indicated above each lane. For each reaction, merge product size is approximately 2 kB. Products of amplification at 64 C for both Step 1 and Step 2, and fusion temperature of 68 C in Step 2.

produce enough interference from side reactions to inhibit the strong amplification of the desired products. The desired products were easily extracted from gels with the Promega Wizard SV gel and PCR clean-up kit, with any possible loss in the quantity/quality of DNA unnoticable in the following steps. When transformed

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into *F.graminearum* we recovered an excess of recombinant strains for each gene target, which we confirmed as gene replacements by PCR. Of 24 genes subjected to knockout by the HS method since this study, 20 have yielded verified knockouts. Because of the overall speed, low cost, and repeatablity of this method, it should be exceptionally useful for producing deletion mutants in many filamentous fungi. Adapting the method we used for *F. graminearum* to other fungi may be as easy altering the length of the flanking regions (to match the specific recombinatorial requirements of each fungus) and using a different selectable marker if required.

Addendum

The authors communicated this omission post publication:

We inadvertently left out the 3HS sequence with which we had such great success. The 5HS sequence, as seen in Figure 2D, is 5'-AGTCGACGACAACTACCATCGATCTGACG. The 3HS sequence which was missing from the original paper is 5'-ACACTGGTGACGGCTAACCAGAACTGTCA.

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