

A Ligation-PCR Approach for Generating Gene Replacement Constructs in *Magnaporthe grisea*

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The conventional approach for generating gene replacement constructs involves several sequence-specific cloning steps and is time-consuming. A ligation-PCR approach was developed to efficiently generate gene replacement constructs. Two vectors useful for this ligation-PCR approach and another vector suitable for improving the efficiency of knockout mutant screens were constructed.

Flanking sequences longer than 0.5 kb are required to obtain 5-10 % gene replacement transformants in *Magnaporthe grisea* and many other filamentous fungi. Therefore, the direct PCR approach developed for yeast knockout analysis is not generally applicable. We have used a modified ligation-PCR approach (Lau *et al.* 2002 J. Microbiol. Meth. 49: 193-205) for generating gene replacement constructs. As outlined in Figure 1, the upstream and downstream flanking sequences of the target gene are amplified with primers 1F and 2R, and 3F and 4R. Primers 2R and 3F contain the *AscI* and *FseI* (8-bp cutters) sites, respectively. The resulting PCR products are digested with *AscI* or *FseI* and ligated with the hygromycin phosphotransferase (*hph*) gene released from pCX62 or pCX63 (see below) by *AscI* and *FseI* digestion. After ligation, the gene replacement construct can be amplified with primers 1F and 4R or nested primers 5F and 6R.

In pCX62, the *hph* gene is amplified with primers: 5'-AAGAATTCGGCGCGCCGCTGGAGCTAGTGGAGGTCA-3' and 5'-TTGGATCCGGCCGGCCCGGTCGGCATCTACTCTATT-3' from pCB1003 and cloned between the *EcoRI* and *BamHI* sites of pBSKS+ (Stratagene). The *hph* gene also is amplified with primers 5'-AAGAATTCGGCCGGCCGCTGGAGCTAGTGGAGGTCA-3' and 5'-TTGGATCCGGCGCGCCCGGTCGGCATCTACTCTATT-3' and cloned between the *EcoRI* and *BamHI* sites of pBSKS+ as pCX63. Both pCX62 and pCX63 have been sequenced and used to transform *Magnaporthe grisea* and *Fusarium graminearum*. The *hph* gene can be released from pCX62 or pCX63 by digestion with *FseI* and *AscI*. Another 8-bp cutter enzyme, *NotI*, can be used to release the *hph* cassette in the replacement of *FseI* and *AscI* from pCX62 and pCX63, respectively.

To increase the ligation efficiency, the *hph* gene released from pCX62 or pCX63 is dephosphorylated with the shrimp alkaline phosphatase and gel purified. The optimal ratio of the *hph* fragment and the upstream and downstream flanking sequences is 5: 1: 1. We usually use 25, 5, and 5 ng of the *hph* gene, upstream, and downstream fragments, respectively, in a 10 μ l ligation mixture. One microliter of the ligation product is then used in a 50 μ l PCR reaction mixture to amplify the gene replacement construct with the Advantage-2 PCR kit (Clontech).

The resulting PCR product can be used directly for fungal transformation or cloned into pYK11 (Fig. 2B) or other suitable vectors. In pYK11, the bleomycin resistance gene (*ble*) under the *TrpC* promoter control is cloned in the *XmnI* site of pBCKS+. Because the *SmaI* and *PstI* sites of the *ble* gene have been removed by site-directed mutagenesis, all the restriction enzyme sites on the MCS of pBCKS+ except *SalI* can be used for cloning with the blue-white selection. After transformation with gene replacement constructs cloned in pYK11, true knockout mutants will be hygromycin-resistant but bleomycin-sensitive. The ectopic transformants, however, will be resistant to both hygromycin and bleomycin. We have used this ligation-PCR approach to generate gene replacement constructs for six *F. graminearum* genes and over 20 *M. grisea* genes.

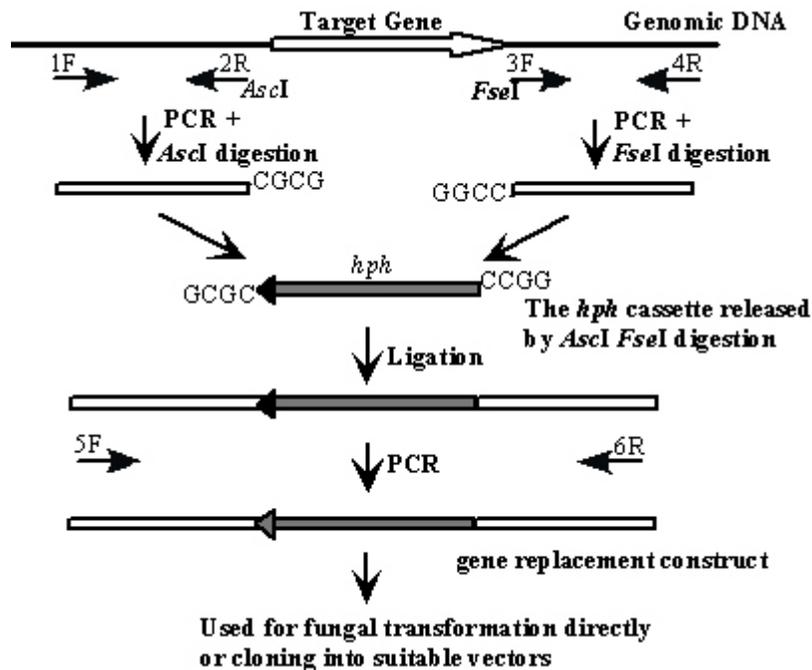


Figure 1. The ligation-PCR procedure for generating gene replacement constructs

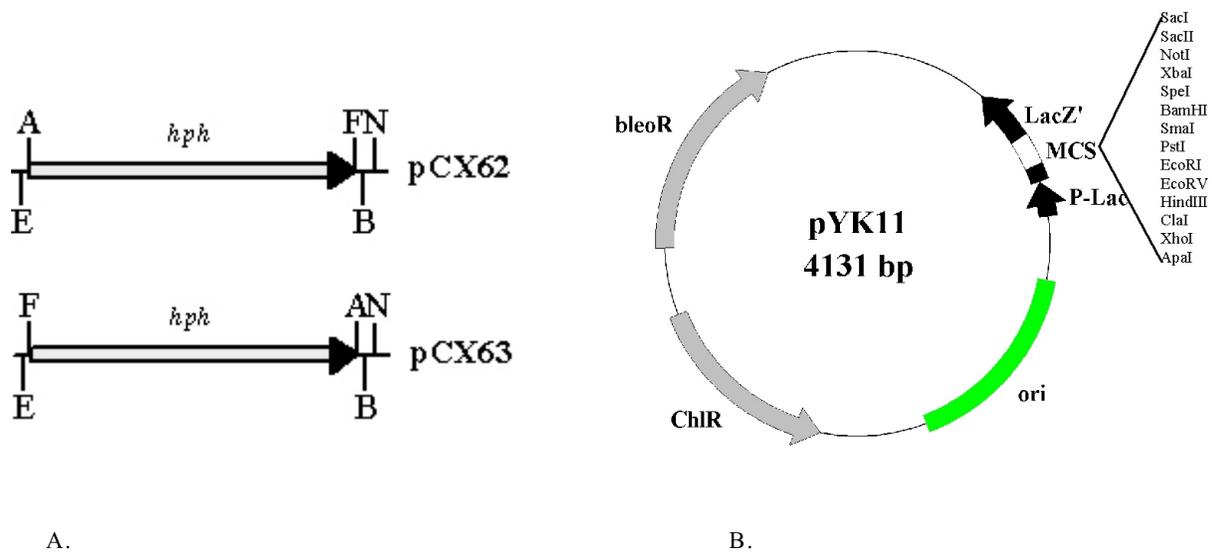


Figure 2. Vectors pCX62, pCX63, and pYK11. A. The *hph* gene is amplified and cloned between *EcoRI* and *BamHI* sites of pBCKS+ in pCX62 and pCX63. Restriction enzymes are: A, *Ascl*; B, *BamHI*; E, *EcoRI*; F, *FseI*; and N, *NotI*. B. The modified *ble* gene is cloned into the *XmnI* site on pBCKS+. Unique restriction enzyme sites on the multiple cloning site (MCS) of pYK11 are listed.