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Note

# Efficient targeted mutagenesis in *Epichloë festucae* using a split marker system

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ABSTRACT

A split-marker system for targeted gene deletion was developed for the model grass endophytic fungus *Epichloë festucae*. Compared to the conventional system that yields up to 25% homologous recombinants, the method resulted in 33–74% targeted deletions in *E. festucae* using as little as 1.5 kb of targeting sequence.



## 1. Introduction

The fungus *Epichloë festucae* (Ascomycota, Clavicipitaceae) is a plant-protective endophytic symbiont of subfamily Pooideae temperate grasses that is broadly used in pastures for biological control against insect herbivores (Schardl, 2010). *E. festucae* Fl1 has recently been used as a model organism for the molecular analysis of mutualistic interactions between fungal endophytes and grasses because it grows relatively faster than many other *Epichloë* strains and is tractable to manipulate genetically by protoplast transformation (Scott et al., 2012).

Gene deletion and phenotypic analysis is one of the most powerful methods for understanding gene function. Replacing the target gene with a selectable marker via site-specific recombination based on homologous recombination is one of the frequently used methods (Gravelat et al., 2012). The first obstacle for using this technique in fungal molecular studies is that for most fungi non-homologous end-joining (NHEJ) is the main process for repairing DNA double-strand breaks (DSB). This process results in high rates of ectopic integration following DNA transformation, even with long homologous sequences (Ninomiya et al., 2004; Xu et al., 2014). To increase homologous recombination, strains with NHEJ deficiency can be generated by disrupting the NHEJ machinery-encoding genes such as *ku70* and *ku80* (Kuck and Hoff, 2010; Gravelat et al., 2012). However, while homologous recombination frequency is increased, strains with NHEJ deficiency show higher sensitivity to DNA damaging chemicals and irradiation compared to wild type strains (Meyer, 2008; Kuck and Hoff, 2010; Liu et al., 2015), requiring re-introduction of the appropriate *ku* gene into the deficient strain – a time consuming process that removes much of the benefit of the approach.

In *E. festucae* Fl1 a marker replacement method is typically used based on homologous recombination using a construct that includes a selectable marker, normally the hygromycin phosphotransferase gene (*hph*), between left and right flanking regions of the targeted gene (For example, Tanaka et al., 2005; Eaton et al., 2008; Johnson et al., 2013, 2015). The proportion of homologous recombination events resulting from this method is normally between 1 and 25% of transformants even with flanking regions of ≥3 kb. With this method the high number of ectopic transformants makes screening laborious (Scott et al., 2007).

Here we describe a pair of vectors for use in *Epichloë* that utilise the split marker strategy, initially developed for *Saccharomyces cerevisiae* (Fairhead et al., 1996, 1998) and successfully used in other fungi such as *Magnaporthe grisea* (Jeong et al., 2007), to greatly increase the number of homologous recombinants among transformants, without requiring any non-target gene mutation and repair. By using two separate overlapping fragments of the selectable marker, neither of which can encode resistance to the antibiotic individually, resistant transformants will only arise from homologous recombination events between the two separate deletion plasmids and the genomic locus of interest.

Two pDONR split marker vectors pSM1 (GenBank accession number: KX904528, Addgene ID 8572) and pSM2 (GenBank accession

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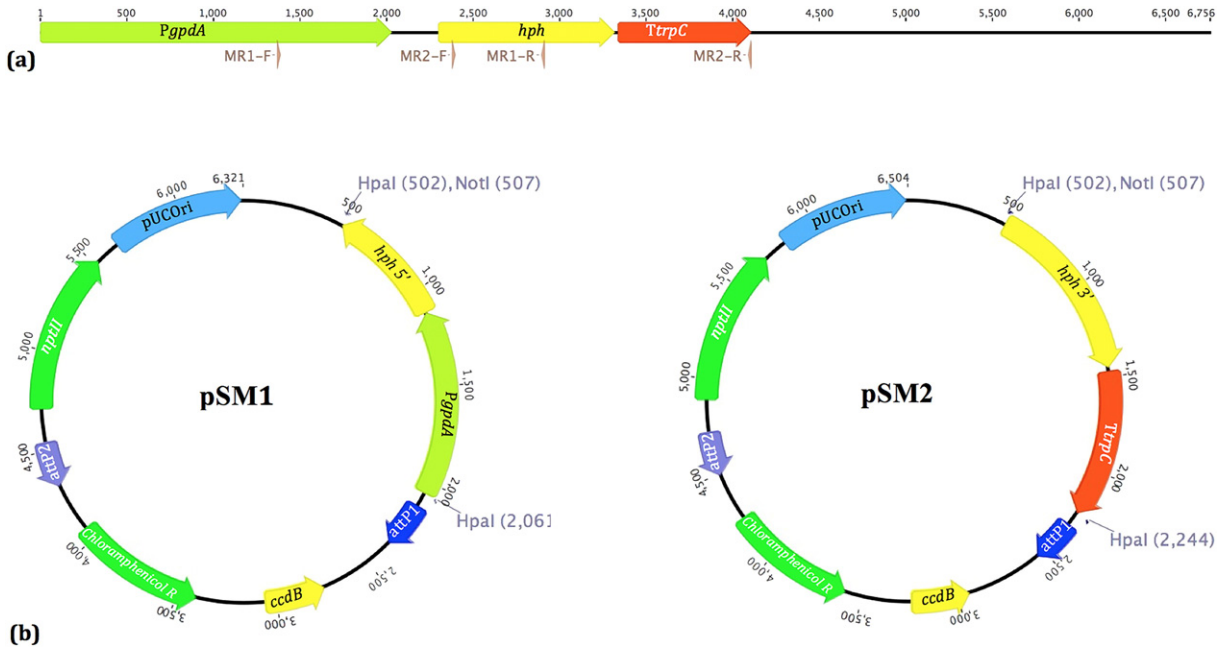


**Fig. 1.** Physical maps of split marker vectors. (a) Linear pAN7-1 used to prepare *hph* fragments, showing primers used to amplify overlapping fragments cloned into pDONR 221. (b) pDONR vectors pMR1 and pMR2. P*gpdA* = *Aspergillus nidulans gpdA* gene promoter; T*trpC* = tryptophan synthetase C gene terminator; *hph* = hygromycin resistance gene; *nptII* = neomycin phosphotransferase gene II; *hph 3′* = 3′ end fragment of *hph*; *hph 5′* = 5′ end fragment of *hph*; pUC Ori = origin of replication (pUC-type); *ccdB* = control of cell death, lethal gene that targets DNA gyrase; attP1 = attP recombination site 1; attP2 = attP recombination site 2.

number: KX904529, Addgene ID 85753) were generated by ligating 5′ (612 bp) and 3′ (942 bp) fragments respectively, overlapping by 534 bp, of the *hph* gene from pAN7-1 (Punt et al., 1987, Fig. 1a) into a unique *Hpa*I site in pDONR221 (Fig. 1b) adjacent to the attP site. These two fragments were amplified from pAN7-1 using primers MR1-F, MR1-R, MR2-F and MR2-R with *Hpa*I site overhangs (Supplementary Table 1). In one of each pair of primers (MR1-R and MR2-F) a *Not*I site was also engineered to facilitate linearization of the plasmids.

In order to test the utility of these plasmids, we generated deletion constructs for an *E. festucae* gene called *ldcA* (LysM domain containing, EfM3.029340 in the *E. festucae* Fl1 genome database (http://www.endophyte.uky.edu)) predicted to encode a small secreted LysM-domain-containing protein. To generate deletion vectors pMR3 and pMR4, 5′ (2731 bp) and 3′ (2861 bp) flanking regions (Fig. 2a) of the *ldcA* gene were amplified (cycle 1 (1×), 98 °C for 3 min; cycle 2 (35×), 98 °C for 10 s, 61 °C for 30 s, 72 °C for 1.5 min; cycle 3 (1×), 72 °C for 10 min) from genomic DNA extracted from freeze dried mycelium as described by Samarrai Al and Schmid , 2000, using primers MR5-F, MR5-R, MR6-F and MR6-R with the appropriate attB sites (Supplementary Table 1). The PCR products were cloned into vectors pSM1 and pSM2 using Gateway® BP Clonase® II enzyme and transformed into Invitrogen One Shot® TOP10 cells according to manufacturer's instructions and selected on 50 μg/mL kanamycin.

*E. festucae* Fl1 protoplasts were prepared as described by Young et al. (2005) and transformed with 5 μg of each plasmid based on Vollmer and Yanofsky (1986) with modifications (Itoh et al., 1994). After linearizing pMR3 and pMR4 using restriction enzyme *Not*I (New England BioLabs), 5 μg of each were added to 100-μL protoplast aliquots followed by 2 μL of 50 mM spermidine and 5 μL of 0.5% heparin. The solution was mixed gently and incubated on ice for 30 min. A 900-μL aliquot of 40% PEG solution was added and the mixture incubated at room temperature for 20 min. Aliquots of 200 μL of this transformation solution were each mixed with 4 mL of molten 0.8% regeneration media (0.8% agar, 2.4% potato dextrose broth, 0.8 sucrose, pH 6.5 with NaOH) and overlayed onto petri dishes containing 1.5% regeneration media. The plates were incubated at 22 °C overnight and the following day plates were overlaid with 4 mL of 0.8% regeneration media (cooled to 50 °C) containing hygromycin (Invitrogen) to give a final concentration of 200 μg/mL for the entire plate. The overlaid plates were incubated at 22 °C for 15–20 day. Hygromycin resistant transformant colonies that grew after this time were transferred to potato dextrose agar (PDA) plates with 200 μg/mL hygromycin and subcultured from the edge of the colony three times for nuclear purification (Young et al., 2005).

Transformants were screened by PCR using primers MR7-F & MR7-R (Supplementary Table 1) that amplify the region from left flank to right flank, giving a size difference between wild type and mutant loci, and MR8-F & MR8-R for determining the presence of the *hph* gene. Additional primer pairs MR9-F/MR9-R and MR10-F/MR10-R were used to examine the integration of whole right and left flanking sequences, respectively. In addition, primer pairs MR8-F/MR9-R and MR10-F/MR8-R were used to confirm correct site-specific replacement by amplifying from outside of the right and left flanking sequences respectively to the *hph* gene (Supplementary Table 1 and Fig. 2b).

Southern hybridization was used to confirm deletion and absence of additional ectopic integrations of the deletion construct using a probe within the right flanking sequence amplified with primers MR11-F and MR11-R (Fig. 2c). Genomic DNA was extracted from each transformant using the method of Samarrai Al and Schmid (2000) and around 3 μg of *Eco*RV-digested DNA was separated by agarose gel electrophoresis and then transferred to a positively charged nylon membrane (Hybond-N+; Amersham) by capillary transfer. DNA was fixed on the membrane by UV cross linking for 1 min. The membrane was hybridised with a DIG-labelled probe generated from the MR11-F/MR11-R PCR product using the DIG high prime DNA labeling and Detection Starter Kit II (Roche). The hybridization was done at 42 °C overnight in DIG Easy Hyb buffer after which the membrane was washed and incubated with primary and secondary antibodies as per the manufacturer's instructions. To visualise the results, the membrane was exposed for 10 min to a Fuji LAS-4000 Imaging System (Fujitsu Life Sciences).

Results of PCR (Fig. 2b) and Southern hybridization (Fig. 2c) screening revealed that 8 out of 12 (67%) tested hygromycin-resistant transformants had the *ldcA* gene replaced with the hygromycin cassette with no deletions or rearrangements at the locus and no additional ectopic integrations in any tested transformants. To confirm that the method was broadly applicable, we used the system to delete five