

## NOTES

# Marker Fusion Tagging, a New Method for Production of Chromosomally Encoded Fusion Proteins<sup>∇</sup>

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**A new gene-tagging method (marker fusion tagging [MFT]) is demonstrated for *Neurospora crassa* and *Magnaporthe oryzae*. Translational fusions between the hygromycin B resistance gene and various markers are inserted into genes of interest by homologous recombination to produce chromosomally encoded fusion proteins. This method can produce tags at any position and create deletion alleles that maintain N- and C-terminal sequences. We show the utility of MFT by producing enhanced green fluorescent protein (EGFP) tags in proteins localized to nuclei, spindle pole bodies, septal pore plugs, Woronin bodies, developing septa, and the endoplasmic reticulum.**

The use of homologous recombination to delete or tag chromosomally encoded genes is a key tool in modern biology. Where homologous recombination has been exploited, integrating DNA fragments are selected using marker genes under the control of their own constitutive promoters. As a result, the production of chromosomally encoded fusion proteins has largely been restricted to tagging genes at their 3' ends, where selectable markers are unlikely to have a major impact on gene expression (5, 6, 15).

We reasoned that expressed genes might be tagged at any position by integrating a promoterless tag-fused selectable marker directly into the gene of interest to produce a chromosomally encoded fusion protein. We began by creating an in-frame fusion of the hygromycin B resistance gene (*hyg<sup>r</sup>*) and the enhanced green fluorescent protein (EGFP) (Fig. 1a). Fusion PCR was then used to generate integration fragments (Fig. 1b and c) flanked by in-frame sequences from a set of *Neurospora* target genes encoding proteins selected for their distinctive localization patterns and loss-of-function phenotypes. We tagged the fungal spindle pole body (SPB) protein ApsB (12) at the N terminus [*apsB::hyg<sup>r</sup>-gfp(N)*], the soft protein (SO), which localizes to septal plugs (3), between residues 40 and 68 [*so::hyg<sup>r</sup>-gfp(Δ40–68)*], and the VIB-1 nuclear protein, which has been associated with programmed cell death (2) at the C terminus [*vib-1::hyg<sup>r</sup>-gfp(C)*].

Six primers (P1 to P6) are required for PCR synthesis of fusion cassettes (Fig. 1b and c). Primer P1 is located about 700 bp upstream of the integration site, while P6 is located 700 bp downstream from the integration site. P2 and P5 are located immediately at the integration site, with a 20-bp overlap with the *hyg<sup>r</sup>-gfp* fragment. P3 and P4 are complements of P2 and P5, respectively. Primers 2 and 3 and 4 and 5 define the reading

frame of the translational fusion. In the first step, 3 fragments are produced using the Expand long template PCR kit (Roche Bioscience) with the following PCR protocol: step 1, initial denaturation (94°C for 2 min); step 2, denaturation (94°C for 30 s); step 3, annealing (52°C for 30 s); step 4, elongation (68°C for 1 min); step 5, repeat from step 2 to step 4 30 times; and step 6, fragment finishing (72°C for 5 min). PCR products were checked and gel purified using the GFX gel purification kit (GE Healthcare). Fusion PCR with these three fragments was carried out using primers 1 and 6 and the same PCR protocol, with an elongation time of 2 min 30 s (step 4). Fusion PCR products were checked on gels, ethanol precipitated, and used directly for transformation of *Neurospora crassa* conidia.

Vogel's N synthetic medium was used for growth on solid medium (1). Transformation of *N. crassa* conidia (FGSC 9720) was carried out by electroporation as previously described (11) and plated on selective plates with 100 μg/ml of hygromycin B. Transformants were then screened for GFP signal. Primers P7 and P8, which fall outside of the integration fragment, are used in conjunction with primers 9 and 10 to confirm proper integration (Fig. 1d).

In all three cases, the properly integrated tags were recovered under stringent hygromycin B selection (100 μg/ml) and produced GFP fluorescence patterns consistent with localization to the SPBs, pore plugs and nuclei, respectively (Fig. 2a). These data show that hygromycin B resistance can be engendered from chromosomally encoded fusion proteins localized to distinct cellular compartments. Moreover, both *vib-1* and *so* mutants display reduced colonial growth, while tagged strains grow at wild-type rates, suggesting that these tagged proteins are functional (Fig. 2b). To enhance the utility of this system we also constructed in-frame *hyg<sup>r</sup>* fusions to the mCherry fluorescent protein and triple-hemagglutinin (HA) epitope tag (Fig. 1a). Thus, when useful tags are identified, an additional pair of primers (primers 4 and 5) allow the integration of other tags at the same position. Plasmids bearing the *hyr<sup>r</sup>-egfp*, *hyg<sup>r</sup>-*

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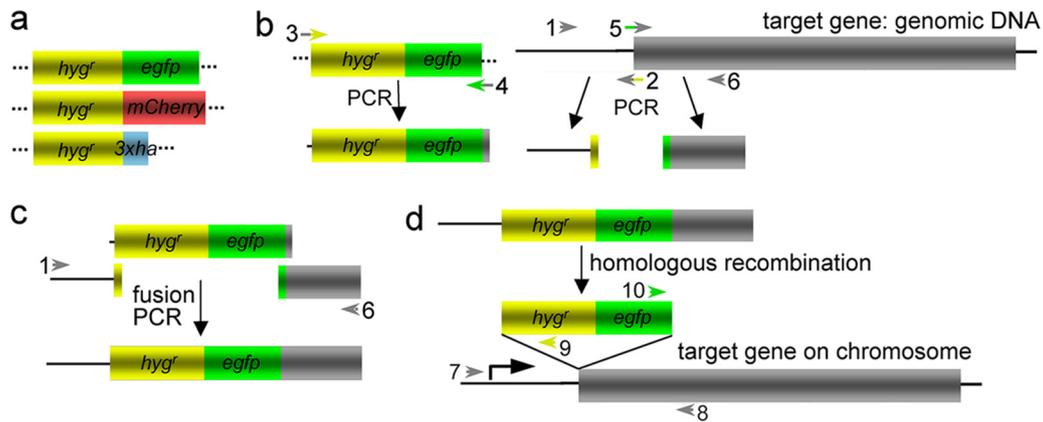


FIG. 1. How MFT works. (a) The fusion tags *hyg<sup>r</sup>-gfp*, *hyg<sup>r</sup>-mCherry*, and *hyg<sup>r</sup>-3×HA* are shown schematically. (b) For each integration fragment, three DNA fragments are amplified using a total of 6 primers. For simplicity, the figure depicts the production of an N-terminal tag. Primers 1 and 2 amplify genomic DNA corresponding to the left flank, primers 3 and 4 amplify the plasmid-borne fusion tag, and primers 5 and 6 amplify the right flank. Primers 2 and 3 and 4 and 5 are complementary to each other and determine the fusion junction. When the tag is integrated to the N terminus or within the gene of interest, the stop codon at the end of the tag is excluded from primers 4 and 5 but is included for C-terminal tags. (c) The three fragments are purified and fused using primers 1 and 6. (d) Transformation followed by homologous recombination results in gene tagging. Primers 4 and 5 can be varied when other tags are desired at the same position. Primers 7, 8, 9, and 10 are used to confirm proper integration of the tag.

3×HA and *hyg<sup>r</sup>-mCherry* cassettes can be obtained from the Fungal Genetics Stock Center (FGSC; <http://www.fgsc.net/>).

We also tagged previously uncharacterized *Neurospora* proteins: NCU06705 contains a conserved domain of unknown function (DUF1777), NCU03679 carries a single predicted transmembrane domain and represents a gene family unique to the Pezizomycotina, and NCU03897 is a conserved RNA-binding protein encoding multiple KH domains. The yeast homolog (Scp160) of the last protein is known to be polysome associated and localized to the nuclear envelope and endoplasmic reticulum (4). Tags were recovered for all of these

genes, and these localized to cytoplasmic punctate structures (NCU06705), developing septa (NCU03679), and a perinuclear compartment suggestive of the endoplasmic reticulum (NCU03897) (Fig. 2a). The punctate structures observed in the NCU06705-tagged strain appeared uniform in size and did not colocalize with DAPI (4',6-diamidino-2-phenylindole)-stained nuclei, suggesting that these are cytoplasmic. Together, these results further demonstrate our ability to recover fusion tags in proteins localized to diverse cellular compartments.

We next sought to apply marker fusion tagging (MFT) in the plant pathogenic fungus *Magnaporthe oryzae*. We chose

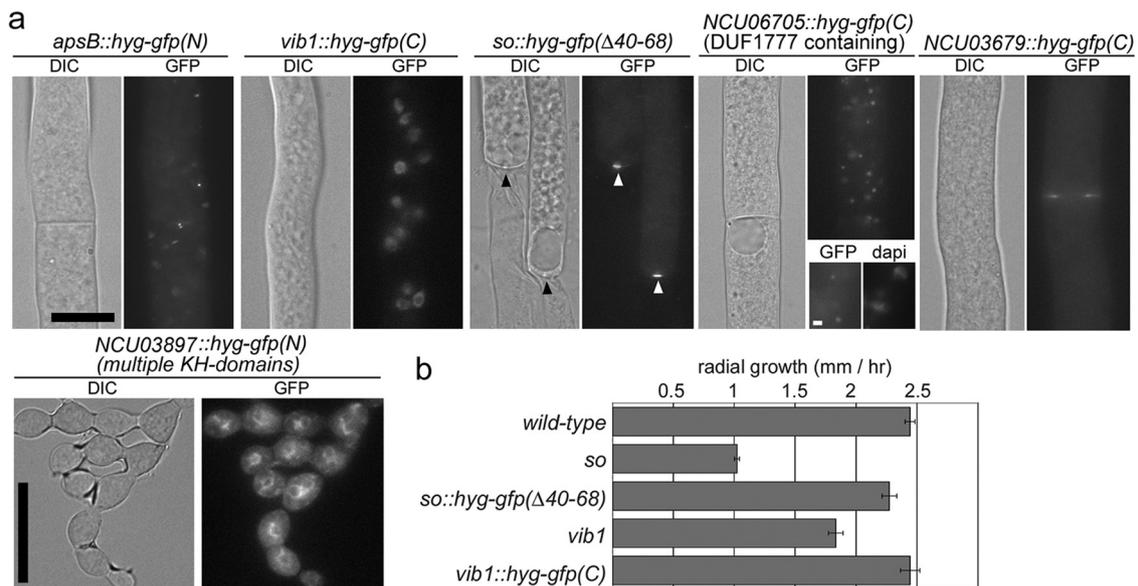


FIG. 2. Localization of *hyg<sup>r</sup>-gfp*-tagged proteins. (a) Parentheses in the gene name indicate the position of the *hyg<sup>r</sup>-gfp* tag. Both bright-field (differential interference contrast [DIC]) and fluorescence (EGFP) images are shown. Bar, 10  $\mu$ m. To show that punctate structures produced by the NCU06705 tag are cytoplasmic, the hyphae were fixed and counterstained with DAPI to reveal nuclei. Bar, 2  $\mu$ m. (b) Growth rates of the indicated strains are shown with standard deviations.

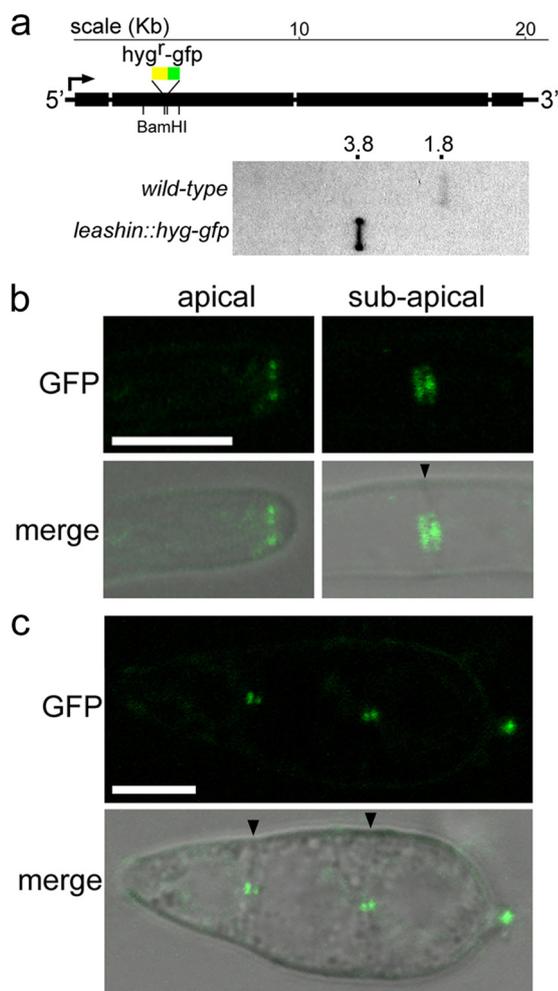


FIG. 3. MFT in *Magnaporthe oryzae*. (a) The diagram depicts the structure of the *Magnaporthe leashin* locus and the position of the MFT tag. Thick lines represent exons. The scale is indicated in kilobases. Lower panels show Southern blotting (10) using a BamHI digest of genomic DNA. Proper integration of the tag results in deletion of two BamHI sites and an increase in the size of a diagnostic BamHI fragment. (b) Localization of *Leashin<sup>mg</sup>::hyg-gfp* in vegetative hyphae. Hyphae were grown on solid medium and visualized by laser-scanning confocal microscopy. Representative apical and subapical hyphae are shown. (c) Localization of *Leashin<sup>mg</sup>::hyg-gfp* in conidia. In both panels b and c, the arrowheads indicate the position of the septum. Bar, 5 μm.

the homolog of the recently identified Woronin body tethering protein, Leashin (8). *Magnaporthe leashin* (MGG\_01625.6) encodes a 19,500-bp open reading frame predicted to encode a 6,500-amino-acid protein (Fig. 3a). An MFT integration fragment (*leashin<sup>mg</sup>::hyg-gfp*Δ3601-4149) was produced by fusion PCR as described above and subcloned into a plasmid for *Agrobacterium*-mediated transformation, and transformants were selected using 200 μg/ml hygromycin B. The correct integration of the *leashin<sup>mg</sup>::hyg-gfp* tag was verified by Southern blotting (Fig. 3a). In the *leashin<sup>mg</sup>::hyg-gfp* strain, GFP signal is observed at both sides of the septal pore and from a region just beneath the growing hyphal apex (Fig. 3b), both positions where Woronin bodies are known to occur (7, 10). We also examined germinating conidia and found discreet foci of GFP signal emanating

from the center of conidial cell walls, suggesting that these cell walls are perforate and harbor Woronin bodies (Fig. 3c).

MFT can be applied to genes that are expressed under conditions of selection and localized to a compartment where the Hyg<sup>r</sup> protein is capable of inactivating hygromycin B. The HYG<sup>r</sup> protein is 341 amino acids in length and has a predicted size of 38 kDa. In many cases in which GFP (27 kDa) is tolerated, it is likely that HYG<sup>r</sup>-GFP (65 kDa) will also be tolerated. This is demonstrated for the SOFT and VIB-1 fusions presented here (Fig. 2). However, in some cases this may not be the true. As with any form of epitope tagging, fusion proteins can interfere with normal protein function. Thus, whenever functional tags are sought, these need to be carefully compared with wild-type and deletion strains to assess their effect on protein function.

Genome-wide tagging studies in the yeast *Saccharomyces cerevisiae* suggest that between 60 and 80% of genes are expressed during vegetative growth (5, 6), suggesting that a reasonable proportion of genes can be studied by MFT. Here we show that cytoplasmic and nuclear proteins can be tagged by MFT. In addition, we have successfully tagged a number of *Neurospora* genes that do not produce visible GFP fluorescence (data not shown), suggesting that relatively-low-abundance proteins can produce levels of HYG<sup>r</sup> activity sufficient for the selection of primary transformants.

MFT provides an efficient method for deletion analysis that is especially useful where large multidomain proteins are under investigation. Previously, we used MFT to produce epitope and GFP tags at the large (>30 kb) *Neurospora leashin* locus. MFT-derived deletions further allowed us to test specific models of *leashin* evolution (8). Tagging of *Magnaporthe leashin* (Fig. 3) shows that this method is not restricted to *Neurospora* and can probably be applied in a variety of cellular systems where homologous recombination is available.

As with MFT, site-specific recombination systems also allow integration at any position (13, 14, 16); however, these methods require additional genetic engineering and secondary recombination events for the excision of selectable markers. In MFT, a single recombination event allows tagging of chromosomally encoded genes at any position and the production of deletion alleles that maintain N- and C-terminal sequences. The hygromycin B resistance gene is widely used as a selectable marker, suggesting that MFT may be useful in a variety of other organisms. The HYG<sup>r</sup> protein inactivates hygromycin B by ATP-dependent phosphorylation (9), and our results demonstrate that this activity can function in fusion proteins produced at various levels and localized to distinct cellular compartments (Fig. 2 and 3). Other selectable markers may also function as fusion proteins, potentially expanding the context in which this method can be applied.

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