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Development of a high throughput transformation system for insertional mutagenesis in *Magnaporthe oryzae*

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Abstract

Towards the goal of disrupting all genes in the genome of *Magnaporthe oryzae* and identifying their function, a collection of >55,000 random insertion lines of *M. oryzae* strain 70-15 were generated. All strains were screened to identify genes involved in growth rate, conidiation, pigmentation, auxotrophy, and pathogenicity. Here, we provide a description of the high throughput transformation and analysis pipeline used to create our library. Transformed lines were generated either by $CaCl_2$/PEG treatment of protoplasts with DNA or by *Agrobacterium tumefaciens*-mediated transformation (ATMT). We describe the optimization of both approaches and compare their efficiency. ATMT was found to be a more reproducible method, resulting in predominantly single copy insertions, and its efficiency was high with up to 0.3% of conidia being transformed. The phenotypic data is accessible via a public database called MGOS and all strains are publicly available. This represents the most comprehensive insertional mutagenesis analysis of a fungal pathogen.
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1. Introduction

Magnaporthe oryzae, an ascomyceteous fungus and causal agent of rice blast disease, has been developed as a model organism to study host–microbe interactions (Talbot, 2003; Valent and Chumley, 1991). In addition to its significant threat to worldwide rice production, blast disease impacts many other gramineous species such as finger millet, barley and wheat (Borromeo et al., 1993; Urashima et al., 1993). The disease cycle starts when a conidium lands on a leaf surface, germinates and sensing the surface, initiates formation of a penetration structure known as an appressorium (Dean, 1997). Infection begins after the accumulation of turgor pressure in the appressorium, leading to mechanical penetration by an infection peg, which is followed by the differentiation of infectious hyphae in the host cell (Howard et al., 1991; Howard and Valent, 1996; Talbot, 1995; Valent, 1990). Following intracellular growth by the pathogen, the disease cycle is completed by the production of spores from lesions on the leaf surface and their release to re-initiate infection. A full understanding of initiation and progression through the *M. oryzae* disease cycle may allow identification of targets to control the disease.

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One approach to defining genes important for disease development is to identify mutants defective in pathogenicity. Insertional mutagenesis approaches have been successfully used with *M. oryzae* in order to identify several pathogenicity genes (Balhadere et al., 1999; Sweigard et al., 1998). In these cases, a limited number of insertion strains were generated using REMI (restriction enzyme-mediated insertion) and screened for defects in pathogenicity resulting in 32 mutants, with 20 of them tagged by the transforming DNA. In order to identify additional genes important for the ability of the fungus to cause disease, we have chosen to saturate the *M. oryzae* genome with random insertions of a defined DNA fragment. Through this approach, any genes of interest can be recovered because they are tagged.

We have generated a collection of >55,000 strains with DNA insertions in this fungus with a predicted gene set of ~11,000, to be screened for defects in pathogenicity, metabolism, morphology and conidiation as well as other phenotypic traits. Generation of this large collection of insertion lines allowed us to compare different transformation methods and to optimize both transformation and processing of strains for purification and phenotypic analyses. In the present study, we compare the efficiency of two different transformation methods, *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Covert et al., 2001; de Groot, 1998; Meyer et al., 2003; Mullins et al., 2001; Mullins and Kang, 2001) and standard protoplast transformation (Sweigard et al., 1995), both for ease of generating insertion lines, and analyzing tagged genes. We demonstrate that ATMT is more efficient for high throughput insertion strain generation and downstream analysis of the insertions, and present the development of a highly efficient pipeline for processing transformed lines. We show that by optimizing the ratio of *A. tumefaciens* and *M. oryzae* cells, and the time of co-cultivation between the cells, transformation can occur in up to 0.3% of the conidia. We also present initial phenotypic analyses of the strains for defects in growth and pathogenicity and molecular analysis of insertion sites and distribution. This large-scale insertional mutagenesis study is the first effort for a phytopathogenic fungus that aims to target the full genome and may serve as a model for other projects with similar goals.

2. Materials and methods

2.1. Fungal strains, growth conditions and media

Magnaporthe oryzae strain 70-15 (Chao and Ellingboe, 1991) was obtained from A. Ellingboe (University of Wisconsin) and distributed from a single stock in the Dean laboratory. It was stored on paper filters with desiccation, at −20 °C. For production of mycelium, cultures were grown on complete solid media (Crawford et al., 1986) at 28 °C with no light. For production of conidia, fungal cultures were grown at 25 °C under constant fluorescent light on either supplemented complete medium (SCM), a modified complete medium that allows *M. oryzae* cultures to sporulate (Talbot et al., 1993), or oatmeal agar (OA) (Valent et al., 1991).

2.2. Protoplast transformation and constructs

Transformations were performed as described (Sweigard et al., 1995) with the following modifications. Mycelial cultures were grown in modified Iwasaki medium (Chida and Sisler, 1987) in place of complete medium to reduce melanization. The number of protoplasts per transformation was decreased to 0.1 ml of 1×10^7 protoplasts/ml, which were mixed with 1 μg of purified DNA fragment. The regeneration top and bottom agars were modified by inclusion of 20% sucrose instead of 1 M sorbitol, as this helped to reduce the background growth. Hygromycin B (HygB) (Calbiochem, LaJolla, CA) was added to the bottom agar at a concentration of 300 μg/ml. The top agar-plating medium contained 1.5% agarose (BioWhitaker Molecular Applications) instead of 2% low melting point agarose and HygB at 100 μg/ml. Control experiments, to determine transformation efficiency, were assessed using the vector pCB1004 (Carroll et al., 1994) as the transforming DNA. For creation of insertion library strains, linear fragments of DNA were prepared from vectors pAM1120, pAM1270, pAM1273 and pAM1274, or the vectors pB-GC-Hyg or pB-AT-Hyg (see below).

Colonies were picked from transformation plates to oatmeal agar containing HygB (300 μg/ml) and grown for 5–7 days before streaking conidia onto CM agar (Crawford et al., 1986) with 300 μg/ml HygB or 4% water agar with 100 μg/ml HygB. Single germinating conidia were transferred to CM with HygB (200 μg/ml), and represent unique cultures for each transformed line.

2.3. Construction of transformation vectors

Several constructs were used for protoplast transformation. One set of vectors consisted of pCB1004 (Carroll et al., 1994) and three derivatives, pB-AT-Hyg and pB-GC-Hyg, which contain the hygromycin phosphotransferase (Hyg^R) cassette of pCB1004 with ~100 bp of AT-rich and GC-rich flanking sequences, respectively, and pAM1120. The second set of transformation vectors was pAM1270, pAM1273 and pAM1274 (Fig. 1a). Each contained the Hyg^R cassette of pCB1004 flanked by two inward facing promoterless fluorescent protein genes, EGFP and DsRed (Clontech). Like the first set of vectors, they differed in the ends of their transforming fragment, with pAM1273 and pAM1274 containing ~100 nt of AT-rich or GC-rich sequences on each end, respectively, while pAM1270 lacked these additional sequences.

The pCB1004 derivatives pB-AT-Hyg and pB-GC-Hyg were constructed by annealing pairs of oligonucleotides that were complementary at their 3′ ends, and then filling them in by PCR to create a ~200 bp fragment. For the

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