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A systematic analysis of T-DNA insertion events in *Magnaporthe oryzae*

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Abstract

We describe here the analysis of random T-DNA insertions that were generated as part of a large-scale insertional mutagenesis project for *Magnaporthe oryzae*. Chromosomal regions flanking T-DNA insertions were rescued by inverse PCR, sequenced and used to search the *M. oryzae* genome assembly. Among the 175 insertions for which at least one flank was rescued, 137 had integrated in single-copy regions of the genome, 17 were in repeated sequences, one had no match to the genome, and the remainder were unassigned due to illegitimate T-DNA integration events. These included in order of abundance: head-to-tail tandem insertions, right border excision failures, left border excision failures and insertion of one T-DNA into another. The left borders of the T-DNA were frequently truncated and inserted in sequences with micro-homology to the left terminus. By contrast the right borders were less prone to degradation and appeared to have been integrated in a homology-independent manner. Gross genome rearrangements rarely occurred when the T-DNAs integrated in single-copy regions, although most insertions did cause small deletions at the target site. Significant insertion bias was detected, with promoters receiving two times more T-DNA hits than expected, and open reading frames receiving three times fewer. In addition, we found that the distribution of T-DNA inserts among the *M. oryzae* chromosomes was not random. The implications of these findings with regard to saturation mutagenesis of the *M. oryzae* genome are discussed.

Keywords: Agrobacterium-mediated transformation; Insertional mutagenesis; Inverse PCR

1. Introduction

Genome sequences are now available for at least 40 filamentous fungi, and additional genomes are currently in production (Galagan et al., 2005; Xu et al., 2006). Along with a complete genome sequence comes the potential for large-scale studies of gene function, using such techniques as systematic gene knock-outs, RNAi-mediated gene-silencing, and transcriptional and protein profiling (reviewed in Xu et al., 2006). In *Saccharomyces cerevisiae*, the systematic deletion of genes was facilitated by the fact that gene targeting occurs almost 100% of the time when linear gene replacement constructs have ends with as little as 20–40 bp of identity to the target site (Baudin et al., 1993). Unfortunately, gene targeting is much less efficient in most filamentous fungi, and typically requires more than 400 bp of homology at each end of the construct (Asch and Kinsey, 1990; Bird and Bradshaw, 1997; Casqueiro et al.,

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1999; Gonzalez et al., 1999; Nelson et al., 2003; Wendland, 2003). As such, systematic gene deletion projects are likely to be much more challenging in these organisms and, in most cases, alternative approaches will be needed.

One strategy that would appear to be suitable for such endeavors is the creation of large libraries of random insertional mutants, which can then be screened for insertions in genes of interest. Random insertional mutagenesis is a well-established technique for gene discovery in filamentous fungi (Gold et al., 2001; Kahmann and Basse, 1999). Large-scale insertional mutant screens were made possible with the development of restriction-enzyme mediated insertional (REMI) mutagenesis, in which a restriction-enzyme is introduced into protoplasts, along with the transforming DNA. The advantages of REMI over standard (DNA only) transformation procedures are that it results in higher transformation frequencies (Lu et al., 1994; Schiestl and Petes, 1991; Shi et al., 1995) and tends to increase the proportion of single-copy insertions (Brown et al., 1996; Bölker et al., 1995; Itoh and Scott, 1997; Kahmann and Basse, 1999). However, REMI has some disadvantages that compromise its suitability for whole genome approaches to the analysis of gene function. First, the use of site-specific enzymes introduces non-randomness into the integration process, so that multiple enzymes must be used to allow mutational saturation of the genome. Second, although REMI increases the proportion of single-copy integrations, it still produces an appreciable number of transformants with tandem arrays of vector sequences, which makes it difficult to rescue and characterize the disrupted genes (Bölker et al., 1995; Itoh and Scott, 1994, 1997; Shi et al., 1995; Thon et al., 2000). Finally, REMI can cause large chromosomal deletions at the site of transgene insertion, making it difficult to identify the gene responsible for the observed phenotype (Itoh and Scott, 1994, 1997; Sweigard et al., 1998).

Many of the disadvantages of REMI are overcome by using *Agrobacterium tumefaciens*-mediated transformation (ATMT) methods. *A. tumefaciens* is a Gram-negative, pathogenic bacterium that transfers a segment of DNA, known as the T-DNA, into plant cells. The T-DNA becomes integrated into the plant genome, making it an ideal vehicle for the delivery of recombinant genes. In plants, T-DNA integration occurs via illegitimate (non-homologous) recombination (Gheysen et al., 1991; Mayerhofer et al., 1991). This property has been exploited to generate large libraries of T-DNA insertion mutants in Arabidopsis (Alonso et al., 2003; Forsbach et al., 2003; Sessions et al., 2002; Szabados et al., 2002) and rice (An et al., 2003; Jeon et al., 2000). The utility of this approach is illustrated by the fact that, as of 2003, insertions had been identified in at least 21,700 of the estimated 29,454 Arabidopsis genes (Alonso et al., 2003).

In 1995, Bundock and coworkers reported that *A. tumefaciens* can also be used for transformation of *S. cerevisiae*. Soon after, *A. tumefaciens*-mediated transformation (ATMT) was reported for several species of filamentous fungi (de Groot et al., 1998). This was a boon to filamentous fungal research for several reasons. First, ATMT procedures tend to be more streamlined than previously used transformation methods, which generally required the formation and manipulation of osmotically sensitive protoplasts. More importantly, however, ATMT tends to result in higher transformation frequencies (Amey et al., 2002; de Groot et al., 1998; Fitzgerald et al., 2003; Flowers and Vaillancourt, 2005; Meyer et al., 2003; Michielse et al., 2005), and does not seem to be so sensitive to the experimental variation that is frequently associated with protoplast transformation methods (Flowers and Vaillancourt, 2005; Meng, Patel and Farman, unpublished data). ATMT has been used to transform well over 50 different fungal species (Michielse et al., 2005), including a number that were recalcitrant to transformation by other methods (Chen et al., 2000; Covert et al., 2001; Degefu and Hanif, 2003; Malonek and Meinhardt, 2001).

ATMT has been a major benefit to fungal insertional mutagenesis projects and has already led to the identification of genes controlling a variety of different traits (Elliott and Howlett, 2006; Idnurm et al., 2004; Rogers et al., 2004; Walton et al., 2005). However, because most studies have focused on the identification and characterization of specific mutants, it is not yet known whether ATMT results in a random distribution of insertions within chromosomes, or if it shows a preference for certain sequences or chromosomal regions. Without this information, it is not known if ATMT will allow saturation mutagenesis of fungal genomes. Characterization of 54 transformants of *S. cerevisiae* (Bundock et al., 2002) revealed that all strains contained a single T-DNA insertion, which had integrated via non-homologous recombination. Twenty-six percent of insertions had occurred in ORFs, 24% were in upstream elements, 6% were in downstream regions and 41% were intergenic. Based on their data, the authors concluded that *S. cerevisiae* does not normally exhibit bias toward T-DNA insertion into particular genes, sequence motifs or chromosomal regions. One recipient strain was an exception to this rule, however, because it showed preferential insertion of T-DNAs into a chromosome arm with an unusually high density of Ty elements.

By comparison, most studies of T-DNA insertion patterns in filamentous fungi have been rather limited in scope. Southern hybridization has been widely used to show that filamentous fungi usually experience simple T-DNA insertion events, suggesting integration at a single chromosomal location (de Groot et al., 1998; LeClerque et al., 2004; Rho et al., 2001; Tsuji et al., 2003). However, there is fairly limited information on the chromosomal distribution of the T-DNAs because, in most studies, only a few flanking sequences were rescued. Recently, Blaise and coworkers reported an analysis of T-DNA insertion patterns in 135 *Leptosphaeria maculans* transformants (Blaise et al., 2007). However, the lack of a genome sequence for this fungus limited the types of analyses that could be performed. In addition, the interpretation of the data is open