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A critical assessment of *Agrobacterium tumefaciens*-mediated transformation as a tool for pathogenicity gene discovery in the phytopathogenic fungus *Leptosphaeria maculans*

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Abstract

We evaluated the usefulness and robustness of *Agrobacterium tumefaciens*-mediated transformation (ATMT) as a high-throughput transformation tool for pathogenicity gene discovery in the filamentous phytopathogen *Leptosphaeria maculans*. Thermal asymmetric interlaced polymerase chain reaction allowed us to amplify the left border (LB) flanking sequence in 135 of 400 transformants analysed, and indicated a high level of preservation of the T-DNA LB. In addition, T-DNA preferentially integrated as a single copy in gene-rich regions of the fungal genome, with a probable bias towards intergenic and/or regulatory regions. A total of 53 transformants out of 1388 (3.8%) showed reproducible pathogenicity defects when inoculated on cotyledons of *Brassica napus*, with diverse altered phenotypes. Co-segregation of the altered phenotype with the T-DNA integration was observed for 6 of 12 transformants crossed. If extrapolated to the whole collection, this indicates that 1.9% of the collection actually corresponds to tagged pathogenicity mutants. The preferential insertion into gene-rich regions along with the high ratio of tagged mutants renders ATMT a tool of choice for large-scale gene discovery in *L. maculans*.

Keywords: *Leptosphaeria maculans*; Plant–fungus interaction; Pathogenicity gene; Agrotransformation; Formal genetics; TAIL-PCR; SON-PCR; Gene tagging

1. Introduction

The first complete eukaryote genome to be sequenced was that of the yeast *Saccharomyces cerevisiae* in 1996 (Goffeau et al., 1996). The importance of the yeast community, the numerous tools developed for large-scale functional genomics (Bader et al., 2003) and the ease for targeted gene mutagenesis in yeast then allowed a systematic mutational analysis of every predicted gene, and enabled the first global studies of eukaryotic gene function and expression.

This first whole-genome sequence of a fungus was unfortunately the ''tree hiding the forest'' for other yeasts and filamentous fungi genomics and gene knowledge, with the first filamentous fungal genome completed in 2003 only (Galagan et al., 2003). Nowadays, however, fungi are massively entering the genome era, with more than 40 fungal genomes completed, and more than 40 others in the process of being sequenced (Galagan et al., 2005). However, these only encompass 27 filamentous ascomycetes (Galagan et al., 2005). In silico analyses predict that the genomes of the ascomycete filamentous fungi contain between 9500 and 16,000 genes for *Aspergillus nidulans* (http://www.broad.mit.edu/annotation/fungi/aspergillus/

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genome_stats.html) and *Stagonospora nodorum* (http://www.broad.mit.edu/annotation/fungi/stagonospora_nodorum/genome_stats.html), respectively. But the accuracy of these annotations is limited by the fact that the majority of species lack significant EST data, therefore preventing correct identification of protein-coding regions or exon–intron boundaries. Comparative genomics between fungal genomes is an additional mean of identifying open reading frames and with an ever-increasing number of fungal genomes being sequenced every year, the possibility to uncover genes that are conserved across many fungal taxa will be enhanced. It remains that, for filamentous fungi, about 20% of predicted genes lack significant matches to known genes from public databases (e.g., Dean et al., 2005). In addition, both small scientific communities for a given fungal species and insufficient tools currently hamper the global studies of gene function. Mainly, systematic generation and analysis of a complete set of deletion mutants is generally prevented by the need for gene disruption cassettes with large homologous flanking regions and by low homologous recombination frequencies as compared to yeast (Wendland, 2003).

An important challenge today consists in the functional analysis of fungal genomes, especially in the case of phytopathogenic fungi for which genes involved in pathogenicity may be species-specific (Rep, 2005; Gout et al., 2006). This goal can only be achieved by using high-throughput random mutagenesis methods linking gene predictions to functions.

At the moment, three main insertional mutagenesis methods are used. The first consists in the transformation of fungal protoplasts by a plasmid in a buffered medium, typically containing polyethylene glycol (PEG). An improved efficiency of this technique named REMI (Restriction Enzyme Mediated Integration) was obtained when the transformation is performed in the presence of a restriction enzyme. These two techniques have been extensively used in the past to tag pathogenicity genes or to functionally study genes in numerous fungal phytopathogens (reviewed by Mullins and Kang, 2001). The second method was developed after the finding that transposable elements are present in fungal genomes. DNA transposons (Class II transposons) excise from DNA and reinsert directly at a new genomic position (Daboussi and Capy, 2003). Transposition of autonomous DNA transposons (*Restless* from *Tolypocladium inflatum* and *impala* from *Fusarium oxysporum*) led to efficient gene tagging in fungi (Kempken and Kuck, 1998; Daboussi and Capy, 2003). Moreover, *impala* has been shown to transpose efficiently in three other fungal species in addition to *F. oxysporum*, which suggests that this autonomous element could permit the development of gene tagging in many fungi (Daboussi and Capy, 2003). Finally, the third method relies on the use of *Agrobacterium tumefaciens* (*A. tumefaciens*-mediated transformation, ATMT). This plant pathogenic bacterium which is a widespread tool of DNA transfer to plants has been used since 1996 to transform yeast (Bundock and Hooykaas, 1996). Since then, many papers reported about successful transformation of filamentous fungi with *A. tumefaciens* including plant pathogenic or symbiotic fungi, mushrooms, industrial fungi and biological control fungi (reviewed by Michielse et al., 2005). It was shown that this technique offers several advantages such as versatility in choosing which starting material to transform, a high efficiency of transformation and a high percentage of single T-DNA insertion. Moreover, a random distribution of T-DNA insertions was suggested when analysing position of 54 T-DNA integrations in the *S. cerevisae* genome (Bundock et al., 2002).

Leptosphaeria maculans is a dothideomycete responsible for major economic losses in the main oilseed rape (*Brassica napus*) growing areas of the world (Fitt et al., 2006). The numerous resources and tools available in this fungal species (for a review, Rouxel and Balesdent, 2005), along with the current sequencing initiative at Genoscope (http://www.genoscope.cns.fr/externe/English/Projets/Projet_DM/organisme_DM.html), necessitated that tools for high-throughput gene disruption are available both for pathogenicity genes discovery and future functional annotation of the genome. In this species, pathogenicity genes have been initially identified by using the REMI random inactivation technique (Idnurm and Howlett, 2002, 2003) and, more recently, the successful transformation of *L. maculans* by *A. tumefaciens* was reported (Gardiner and Howlett, 2004; Meyer et al., 2004; Eckert et al., 2005b).

However, before using ATMT as a high-throughput gene function discovery tool, we investigate here the accuracy of ATMT in *L. maculans*, in terms of number of insertions in the genome, distribution in the genome, preservation of the T-DNA ends and ability to generate reliably altered phenotypes in the fungus. For this purpose, we generated a collection of 3000 transformants of *L. maculans* and investigated the ratio of biologically altered phenotypes (mainly in terms of plant pathogenicity) in half of this collection. Genetic investigation of co-segregation between the selection marker and the altered phenotype was used to evaluate the ratio of altered phenotypes corresponding to tagged genes. Finally, the pattern of T-DNA insertions in the genome, along with T-DNA ends preservation was studied by analysing 135 T-DNA junction sequences.

2. Materials and methods

2.1. Fungal isolates

The reference isolate v23.1.3 (Attard et al., 2002), in the process to be sequenced by Genoscope, was chosen as a recipient strain for ATMT. The sexually compatible sister isolate v23.1.2 (Attard et al., 2002) or the v23.1.3 near-isogenic, sexually compatible, isolate v41.1.2, corresponding to a 5th generation of back-crosses with the recurrent parent v23.1.3 (Huang et al., 2006) were used as parents for crosses with transformants.