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MicroCommentary

Silencing the crowd: high-throughput functional genomics in *Magnaporthe oryzae*

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Summary

A new high-throughput RNA-silencing system has been developed for use in the rice blast fungus *Magnaporthe oryzae*, allowing rapid generation of transformants in which individual genes have been silenced. Development of this system will allow large-scale functional analysis of genes in the fungus to define the cellular processes required for plant infection and disease symptoms. Functional analysis of 37 genes predicted to be involved in calcium signalling was carried out by RNA silencing to validate the new strategy and has provided new insight into the role of calcium-mediated signal transduction in plant pathogenic fungi.

Introduction

The growing availability of genome sequence information from a wide range of microbial eukaryotes has provided an unparalleled opportunity to adopt a holistic or system-based approach to studying the flow of biological information within cells (Brown, 2003). Such genomic approaches have been applied widely in model organisms, such as the budding yeast *Saccharomyces cerevisiae* (Eisenberg *et al*. 2000; Giaever *et al.,* 2002), and have addressed the inherent complexity of biological systems, allowing insight into the interplay of large numbers of gene products and predicting the consequences of this communication to the physiology of a cell (McGary *et al*., 2007). Introducing such high-throughput approaches to the study of pathogenic eukaryotic microorganisms is an exciting prospect, because they will allow a system-level approach to investigating the morphogenetic and physiological adaptations employed by pathogenic microbes to cause disease. In this issue of *Molecular Microbiology,* a novel high-throughput gene-silencing system is described in the rice blast fungus *Magnaporthe oryzae* (Quoc *et al*., 2008), a plant pathogenic microorganism responsible for the most devastating disease of cultivated rice (Talbot, 2003). The paper describes a novel high-throughput means of gene functional analysis using RNA interference (RNAi), which provides an alternative to the targeted disruption or deletion of genes by homologous recombination. This technique is based on the well-known RNAi methodology in which generation of a double-stranded RNA (dsRNA) molecule acts as a template for Dicer ribonuclease III, producing short interfering RNAs (siRNAs). These siRNAs are incorporated into the RNA-inducing silencing complex (RISC) and serve as sequence-specific guides that target corresponding mRNA molecules for destruction. RNAi has been used to great effect to study gene function in the model metazoan *Caenorhabditis elegans* (see for example Kamath *et al*., 2003) and in plants, where virus-induced gene silencing has been very successfully deployed as a means of addressing the function of specific genes (Lu *et al*., 2003; Baulcombe, 2004). The novelty of the approach utilized by Quoc and colleagues, however, is the development of vectors that allow rapid gene silencing to be carried out routinely for analysis of very large sets of genes.

Previous work by the authors has clearly established that RNAi occurs in *M. oryzae* (Kadotani *et al*., 2004). The fungus has two Dicer-like proteins, encoded by the *MoDcl1* and *MoDcl2* genes respectively, and *MoDcl2* is known to be necessary for generation of siRNAs in hairpin dsRNA-mediated RNA silencing (Kadotani *et al*., 2004; Murata *et al*., 2007). Previous attempts to induce gene silencing in *M. oryzae* utilized a vector in which two asymmetric cloning steps were necessary to produce a hairpin RNA template. The vector, pSilent-1, utilized in these studies, also contained an intron sequence, which was spliced from the hairpin RNA to generate a dsRNA that was processed by the RISC apparatus to generate siRNAs in the fungus (Nakayashiki *et al*., 2005). The two-step cloning procedure limited the utility of gene silencing using pSilent-1 (Quoc *et al*., 2008). In the new

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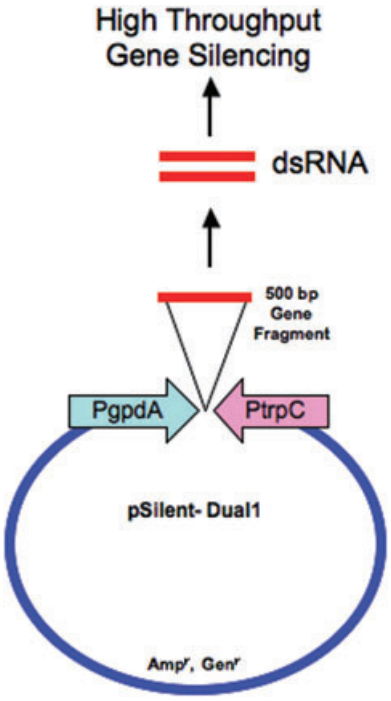

Fig. 1. Diagram of the RNA-silencing vector, pSilent-Dual1, constructed by Quoc *et al*. (2008). Two promoter sequences from the filamentous ascomycete fungus *A. nidulans,* the trpC promoter and the glyceraldehyde-3-phosphate dehydrogenase gpdA promoter, were cloned in a convergent arrangement separated by a multicloning site. Gene fragments of 500 bp can be inserted into the vector and constructs transformed into a recipient fungus which will express a corresponding dsRNA, a template for RNAi-mediated gene silencing. The vector has a geneticin-resistance cassette for selection in fungi. The vector allows high-throughput genome-wide RNAi in filamentous fungi.

study, a vector, pSilent-Dual, was generated that carries two convergent promoters, the TrpC promoter and glyceraldehyde-3-phosphate dehydrogenase promoter (gpdA) from *Aspergillus nidulans*. Both promoters have been widely used to drive constitutive gene expression in a large number of filamentous ascomycete species. A multicloning site has been inserted between the two promoters (see Fig. 1), and the vector therefore allows a single step cloning for generation of an RNAi construct. The authors have also used the green fluorescent protein-encoding gene GFP to generate pSD1-GFP, which allows expression of a chimeric RNA and assessment of gene-silencing efficiency by utilizing a recipient strain of *M. oryzae* that produces GFP and therefore fluoresces green, by epifluorescence microscopy. In this way, the degree of silencing could be visually assessed by the loss of GFP signal, prior to testing the expression level of individual target genes using quantitative real-time PCR or RNA gel blot analysis.

To determine the efficacy of using RNAi to study the biology of *M. oryzae* at a genomic scale, the authors selected a physiological process and underlying signal transduction network, and test its function by high-throughput gene silencing. They chose to investigate the role of calcium-mediated signal transduction in infection-related development and fungal pathogenicity of *M. oryzae*. The rice blast fungus elaborates a specialised cell, an appressorium, to gain entry to a rice leaf (Talbot, 2003) The appressorium is a swollen, dome-shaped cell that differentiates from the end of a germ tube shortly after germination of a fungal spore on the rice leaf surface. A narrow penetration hypha forms at the base of the appressorium and ruptures the plant cuticle, entering an epidermal cell by invagination of the plasmalemma. The fungus then grows within the rice leaf, forming branched invasive hyphae that ramify throughout the leaf tissue, prior to disease symptom expression (Howard and Valent, 1996).

Calcium signalling has previously implicated in a range of fungal morphogenetic processes and during cellular differentiation (see for example Brand *et al*., 2007). The recent availability of full genome sequences for the filamentous fungi *Neurospora crassa* and *M. oryzae* has allowed definition and comparative analysis of genes that putatively encode calcium-signalling proteins with the predicted repertoire defined in the model yeast *S. cerevisiae* (Zelter *et al*. 2004). This information was used by Quoc and colleagues to characterize 37 genes involved in calcium-signalling pathways. These included genes predicted to encode Ca^{2+}-permeable channel proteins, calcineurin, calcium- and calmodulin-binding proteins, Ca^{2+} exchangers, phospholipase C, calpactin I heavy chain, calmodulin and Ca^{2+} pumps. Transformants showing gene silencing were then tested for hyphal growth and development, sporulation, appressorium formation and pathogenicity.

So, what has been learned from this study about the role of calcium signalling in the rice blast fungus? First, it seems likely that calmodulin, the primary transducer of cytosolic Ca^{2+}, is essential for viability in the fungus. A transformant exhibiting strong calmodulin RNA silencing showed very severe growth defects and was unable to cause disease. This is consistent with previous studies in yeast and *A. nidulans* (Rasmussen *et al*., 1990) and with pharmacological studies employing calmodulin antagonists (Lee and Lee, 1998). The regulatory B subunit of the calcium-dependent serine/threonine protein phosphatase, calcineurin, was also shown to be necessary for fungal pathogenesis and important for conidiation and appressorium morphogenesis (Quoc *et al*., 2008), whereas the catalytic calcineurin A subunit-encoding gene appeared to be largely dispensable for these developmental transitions. This contrasts with studies in *A. fumigatus* and *Candida albicans*, where calcineurin A mutants have impaired growth and are avirulent (Bader *et al*., 2003;

Steinbach *et al*., 2006). The Ca^{2+}-binding proteins, calreticulin, calnexin and calpactin, were all shown to be necessary for pathogenicity, as were four of the 12 predicted Ca^{2+} pump-encoding genes in the *M. oryzae* genome. Six further Ca^{2+} pump mutants were also unable to sporulate, preventing an assessment of their virulence, but highlighting the importance of these P-type ATPases in fungal development. Silencing of two predicted phospholipase C genes also prevented sporulation by *M. oryzae.*

When considered together, the study has provided an overview of calcium signalling in the rice blast fungus that should act as a platform for more detailed cell biological studies. The use of RNAi as a means of providing a functional assessment of gene families, particularly those that may have essential cellular functions, is therefore extremely valuable.

The limitations of the approach have also been considered, however. For example, the possibility that homologous genes might be co-silenced by RNAi was investigated. Some co-silencing was observed in 22 out of 30 cases where a gene being silenced had a close homologue. This is vital information, because it demonstrates the care that is needed when evaluating the phenotypes observed in the study. Off-target effects will need to be addressed in some of these strains in future, for instance, to determine the precise role of particular Ca^{2+}-signalling components. There is also clearly a need to select gene fragments with great care to avoid areas of strong nucleic acid sequence similarity with other loci in the genome, when planning future RNAi studies. Notwithstanding these limitations, there is likely to be considerable take-up of RNA silencing to study the biology of this plant pathogenic fungus and indeed many others. When considered alongside the rapid advances in targeted gene deletion strategies in *M. oryzae*, using mutants lacking the non-homologue DNA end-joining pathway (Villalba *et al*., 2008; M.J. Gilbert and N.J. Talbot, unpublished) and the large-scale insertional mutagenesis collections available for this fungus (Jeon *et al*., 2007), it is now becoming feasible to adopt a high-throughput approach to gene functional analysis. We are therefore poised for rapid progress in understanding the biology of rice blast disease and defining the gene set necessary for its ability to infect, colonize and debilitate its plant host: a fascinating prospect.

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