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Construction and high-throughput phenotypic screening of *Zymoseptoria tritici* over-expression strains

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ABSTRACT

Targeted gene deletion has been instrumental in elucidating many aspects of *Zymoseptoria tritici* pathogenicity. Gene over-expression is a complementary approach that is amenable to rapid strain construction and high-throughput screening, which has not been exploited to analyze *Z. tritici*, largely due to a lack of available techniques. Here we exploit the Gateway® cloning technology for rapid construction of over-expression vectors and improved homologous integration efficiency of a *Z. tritici* Δ*ku70* strain to build a pilot over-expression library encompassing 32 genes encoding putative DNA binding proteins, GTPases or kinases. We developed a protocol using a Rotor-HDA robot for rapid and reproducible cell pinning for high-throughput *in vitro* screening. This screen identified an over-expression strain that demonstrated a marked reduction in hyphal production relative to the isogenic progenitor. This study provides a protocol for rapid generation of *Z. tritici* over-expression libraries and a technique for functional genomic screening in this important pathogen.

1. Introduction

Gene disruption or deletion has been an essential technique for understanding the molecular basis of *Zymoseptoria tritici* virulence, including the role of MAP kinase signaling (Cousin et al., 2006; Mehrabi et al., 2006), transcription factor regulation (Kramer et al., 2009; Mirzadi Gohari et al., 2014), transport (Stergiopoulos et al., 2003) and effector biosynthesis (Marshall et al., 2011). In the rice blast fungus *Magnaporthe oryzae*, deletion of every gene encoding the autophagic apparatus clearly demonstrates that gene inactivation is a strategy amenable to functional genomics of plant pathogens (Kershaw and Talbot, 2009), and large-scale gene knock-out libraries have proven invaluable for elucidation of function in model and pathogenic fungi (Goncalves et al., 2011; Schwarzmuller et al., 2014). However gene deletion approaches have certain limitations. For example, essential genes cannot be functionally characterized by deletion. Additionally, for processes where multiple genes of similar function act synergistically, functional redundancy may make time-consuming characterization of null isolates ineffective. Moreover, phenotypic variation of nulls relative to wild-type isolates may be difficult to identify if the gene is not transcribed in standard laboratory culture, or transiently expressed at cryptic stages during infection assays.

Gene over-expression is a complementary approach to gene disruption or deletion. For infectious diseases of plants, researchers have utilized powerful heterologous expression technology facilitated by the availability of genetically tractable vectors and/or host systems. For example, a library of *Cladosporium fulvum* cDNAs was expressed in *Agrobacterium tumefaciens*, and four hypersensitive response-inducing genes were identified following leaf inoculation (Takken et al., 2000). Similarly, *Agrobacterium* mediated over-expression of *Phytophthora infestans* effectors *in planta* has revealed numerous responses by the host, including disease resistant hypersensitivity (Oh et al., 2009; Vleeshouwers et al., 2008). In addition to studies that probe the host/pathogen interface, heterologous expression has been implemented in model organisms, which is exemplified by work defining the *Z. tritici CYP51* gene in *Saccharomyces cerevisiae* (Cools et al., 2010).

In addition, over-expression of genes within the native organism has been used to elucidate basic biology for both model microorganisms and pathogens, especially when combined with high-throughput library construction and screening. Jin and colleagues used 2043 over-expression isolates in conjunction with 3627 transposon insertion mutants to identify the role of mitochondrial function in pseudohyphal growth of *S. cerevisiae* (Jin et al., 2008). In the pathogen *Candida albicans*, screening of a 257

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open reading frame over-expression library identified a novel role in morphogenesis for 11 genes (Chauvel et al., 2012).

The aim of this study was to provide proof of principle for generating a genome-wide *Z. tritici* over-expression library and to develop a high-throughput technique for rapid functional screening *in vitro*. Accordingly, genes enriched amongst putative DNA binding proteins, kinases or GTPases were PCR amplified and cloned into pDONR207 to generate Gateway®Entry plasmids. Genes were subsequently shuttled into the newly described Gateway®Destination vector pYSKH3 (Sidhu et al., 2015). These *A. tumefaciens* adapted vectors were used to place genes under control of the *Z. tritici* translation elongation factor (*tef1*, Mycgr3G92705) promoter at the disrupted *Δku70* locus in strain HLS1000. The pilot library containing 32 *Z. tritici* over-expression strains was used to optimize a high-throughput screening protocol, where *in vitro* grown cells were rapidly and reproducibly pinned, using a Singer Rotor-HDA robot, onto solid agar containing various abiotic stressors that mimic stresses that might be encountered in the host. Using this approach, we identified an isolate that produced markedly less hyphae relative to the isogenic progenitor strain at the colony periphery under several stress conditions. This study provides a robust protocol for rapid generation of *Z. tritici* over-expression libraries and for high-throughput functional genomic screening. We also demonstrate proof of principle that this genome wide functional analysis will enable discovery of novel infection related biology.

2. Methods

2.1. Growth media

Z. tritici synthetic complete (ZTSC), a defined rich growth medium, comprising 6.9 g/l yeast nitrogen base without amino acids (ForMedium, UK), 0.79 g/l complete supplement mix (ForMedium), 20 g/l glucose, 20 g/l bacteriological agar (Lab, UK). Czapex Dox, a defined nutrient limiting medium, comprising 33.4 g/l Czapex Dox (Oxoid, UK), 20 g/l bacteriological agar (Lab).

Bacteria were grown in LB medium (Formedium) supplemented with kanamycin salt at 50 μg/ml (Sigma, UK) or gentamicin at 50 μg/ml (Sigma) where appropriate.

All strains were routinely stored at −80 °C in 50% (v/v) glycerol.

2.2. Plasmids used in this study

All plasmids were stored at −20 °C prior to use. Gateway®Entry vectors were constructed using pDONR207 (Invitrogen, UK) which contains a gentamicin resistance gene for selection in *Escherichia coli*. This plasmid also contains a *ccd*B gene flanked by *att*P sequences for Gateway® mediated recombination. For construction of over-expression vectors, we used pYSKH3 (Sidhu et al., 2015). This vector confers kanamycin resistance for selection in *E. coli* and *A. tumefaciens*. pYSKH3 contains a *ccd*B gene (coordinates 14,856–16,558) flanked with *att*R sites for Gateway® mediated recombination and selection. With regards to gene over-expression in *Z. tritici*, the Gateway® *ccd*B gene is flanked by an upstream sequence encoding (5′-3′): 26 bp t-border repeat (8733–8758), 1000 bp homology region to 5′UTR of the *Z. tritici Ku70* locus (8854–9853), 2361 bp functional *Ku70* gene (9854–12,214), 1382 bp of hygromycin resistance cassette (12,243–13,625) and 1200 bp translation elongation factor promoter (13,656–14,855). The 3′ region of the *ccd*B gene is flanked by a stop codon, 212 bp *CYC1* terminator (16,634–16,873), 800 bp region of 3′ UTR of *Ku70* locus (16,980–17,779) and a 26 bp t-border repeat (18,094–18,119, see Fig. 1A). Derivatives of pYSKH3 in which the *ccd*B gene was replaced with a DNA sequence encoding a *Z. tritici* gene were named pCCKH and numbered 1-32 (Supplementary Table S1).

2.3. Strains used in this study

Z. tritici strain HLS1000 (Sidhu et al., 2015) was used throughout. In this IPO323 derivative, the *Ku70* gene (Mycgr3G85040) of *Z. tritici* has been replaced with a G418 cassette using *A. tumefaciens* mediated transformation.

E. coli One Shot® *ccd*B Survival™ 2 T1^R was used for propagation of pDONR207 (Invitrogen, UK) and pYSKH3 (Sidhu et al., 2015). All Gateway®Entry and modified destination vectors were propagated in DH5α (Invitrogen, UK).

The kanamycin sensitive *A. tumefaciens* strain EHA105 (Hood et al., 1993) was used for *Z. tritici* transformation.

2.4. Construction of Gateway Entry vectors

For PCR amplification of each gene of interest, forward primers were designed to include the *att*B1 site (ggggacaagtttgtacaaaaaagcaggcttg and the first 20 bp of the gene, and reverse primers to include the *att*B2 site (ggggaccactttgtacaagaaagctgggtc) and the last 20 bp of the gene, excluding the stop codon. Predicted gene models were derived from the JGI (Goodwin et al., 2011). Primers were synthesised by Sigma (Table S1). PCR was conducted using Phusion® High-Fidelity DNA Polymerase (NEB) with a 65 °C primer annealing temperature and an extension of 0.5 min/kb, using *Z. tritici* IP0323 genomic DNA as template. PCR amplicons of predicted sizes were confirmed by gel electrophoresis, PEG purified, suspended in 10 μl TE buffer (40 mM TRIS base, 20 mM glacial acetic acid, 0.1 mM EDTA, pH8). For construction of Gateway®Entry vectors, 150 ng of pDONR207 was mixed with 2.5 μl of purified PCR product, 1 μl of Gateway® BP Clonase™ with TE buffer added to a total volume of 10 μl. Reactions were incubated at 25 °C for 24 h then treated with Proteinase K (Invitrogen, UK) following the manufacturer's instructions. *E. coli* strain DH5α was transformed with 5 μl of each reaction mixture. Transformants were selected on LB supplemented with gentamicin (50 μg/ml). Colonies were grown over-night in LB medium with selection, and plasmids extracted using Plasmid Mini Kit (Qiagen, UK). In order to confirm recombination of the *ccd*B gene with the gene of interest, plasmids were digested with *Bsr*GI (NEB, UK), and digest reactions analyzed by gel electrophoresis. Putative Gateway®Entry vectors were Sanger Sequenced (Eurofins, UK) using primer GOXF (tcgcgttaacgctagcatgga).

2.5. Generation of Z. tritici over-expression plasmids

For construction of over-expression vectors pCCKH 1-32, 200 ng of *Z. tritici* Gateway®Entry plasmids were mixed with 200 ng of pYSKH3, 1 μl of LR Clonase™ with TE buffer added to a total volume of 10 μl. Reactions were incubated at 25 °C for 24 h then treated with Proteinase K (Invitrogen) following the manufacturer's instructions. Reaction mixtures were transformed into *E. coli* strain DH5α as described above except transformants were selected on LB medium supplemented with kanamycin (50 μg/ml). Following plasmid extraction, plasmids were restriction mapped using either *Eco*RV, *Eco*RI or *Bam*HI (NEB). Confirmed over-expression vectors were named pCCKH 1-32. *Z. tritici* transformation with pCCKH plasmids 1-32 was conducted as described (Sidhu et al., 2015).

2.6. PCR confirmation of Z. tritici over-expression isolates

Genomic DNA was isolated from each putative transformant and integration of the over-expression construct at the *Δku70*::G418 locus determined by two PCR reactions (Figs. 2A