The *Zymoseptoria tritici* ORFeome: A Functional Genomics Community Resource

Yogesh Chaudhari,1 Timothy C. Cairns,1 Yaadwinder Sidhu,1 Victoria Attah,1 Graham Thomas,1 Michael Csukai,2 Nicholas J. Talbot,3,† David J. Studholme,1,† and Ken Haynes1

1 Biosciences, University of Exeter, Exeter EX4 4QD, U.K.
2 Syngenta, Jealott’s Hill International Research Centre, Bracknell, RG42 6EY, U.K.
3 The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, NR47UH, U.K.

Abstract

Libraries of protein-encoding sequences can be generated by identification of open reading frames (ORFs) from a genome of choice that are then assembled into collections of plasmids termed ORFeome libraries. These represent powerful resources to facilitate functional genomic characterization of genes and their encoded products. Here, we report the generation of an ORFeome for *Zymoseptoria tritici*, which causes the most serious disease of wheat in temperate regions of the world. We screened the genome of strain IP0323 for high confidence gene models, identifying 4,075 candidates from 10,933 predicted genes. These were amplified from genomic DNA, were cloned into the Gateway entry vector pDONR207, and were sequenced, providing a total of 3,022 quality-controlled plasmids. The ORFeome includes genes predicted to encode effectors (n = 410) and secondary metabolite biosynthetic proteins (n = 171) in addition to genes residing at dispensable chromosomes (n = 122) or those that are preferentially expressed during plant infection (n = 527). The ORFeome plasmid library is compatible with our previously developed suite of Gateway destination vectors, which have various combinations of promoters, selection markers, and epitope tags. The *Z. tritici* ORFeome constitutes a powerful resource for functional genomics and offers unparalleled opportunities to understand the biology of *Z. tritici*.

Fungal pathogens kill more people per year than malaria and result in crop destruction or postharvest spoilage that destroys enough food to feed approximately 10% of the human population (Denning and Bromley 2015; Fisher et al. 2012). Technological advances in fungal genomics, transcriptomics, proteomics, metabolomics, bioinformatics, and network analyses, however, now enable pathogenic fungi to be studied as integrated systems, providing opportunities to better understand their biology and develop new control strategies.

Yogesh Chaudhari and Timothy C. Cairns contributed equally to this study

This paper is dedicated to the memory of Ken Haynes, who led the study and was an outstanding fungal biologist as well as an inspirational colleague, friend, and mentor to his fellow co-authors.

Raw sequencing data is available at the Sequencing Read Archive (accession numbers SRX1267196 and SRX1265386).

†Corresponding authors: D. J. Studholme; D.J.Studholme@exeter.ac.uk and, for plasmid requests, N. Talbot: nick.talbot@tsl.ac.uk or weibin.ma@tsl.ac.uk

Current address for Timothy C. Cairns: Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, 300308, China

The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure and two supplementary files are published online.

The author(s) declare no conflict of interest.

Accepted for publication 1 July 2019.

Copyright © 2019 The Author(s). This is an open access article distributed under the CC BY 4.0 International license.
unparalleled opportunities to understand their biology (Cairns et al. 2016; Meyer et al. 2016). Functional genomic approaches, which define the function and interactions of genes and their encoded products at a genome or near-genome level, are increasingly used to dissect host-pathogen interactions, virulence factors, drug resistance, and infectious growth during fungal disease (Chauvel et al. 2012; Jeon et al. 2007; Legrand et al. 2018; Schwarzmüller et al. 2014; Son et al. 2011). However, a significant constraint to conducting functional genomic experiments are high reagent and labor costs, due to the necessity to study thousands or tens of thousands of genes from a given fungal pathogen.

In order to obviate this challenge, community-accessible libraries have been developed, which consist of hundreds or thousands of either individual genes, null mutant, or overexpression strains, which ultimately enable facile and high-throughput experimentation by the end user at a minimal expense (Dunlap et al. 2007; Giaever and Nislow 2014; Giaever et al. 2002; Homann et al. 2009; Liu et al. 2008; Noble et al. 2010; Roemer et al. 2003; Winzeler et al. 1999). ORFeomes are collections of open reading frames (ORFs) that are encoded in a library of plasmid vectors. These resources have been generated for several model organisms, including humans, Escherichia coli, Caenorhabditis elegans, and Arabidopsis thaliana (Gong et al. 2004; Lamesch et al. 2004; Li et al. 2008; Matsuyama et al. 2006; Rajagopala et al. 2010; Wiemann et al. 2016). ORFeomes have also been developed for the fungal kingdom, including fission yeast (Matsuyama et al. 2006), budding yeast (Gelperin et al. 2005) and, most recently, the human pathogenic yeast Candida albicans (Legrand et al. 2018). Usually, ORFeomes are compatible with the Gateway cloning technology (Invitrogen), which enables rapid and high-throughput recombinase-based transfer of an ORF coding sequence to generate expression vectors (Alberti et al. 2007; Walthout et al. 2000). Community access to hundreds or thousands of such plasmids in a single library enables highly flexible generation of expression vectors for high-throughput functional genomic experiments.

The filamentous ascomycete fungus Zymoseptoria tritici (previously Mycosphaerella graminicola) (Talbot 2015) causes Septoria tritici blotch, an important foliar disease of wheat (Fones and Gurr 2015). Z. tritici is a significant threat to international food security, and, even with access to some resistant wheat cultivars and frequent fungicide applications, the estimated average yield losses due to this pathogen are still 10% (Fones and Gurr 2015). Even more strikingly, approximately 70% of agricultural fungicides in Europe are deployed to control just this single disease (Goodwin et al. 2011), which likely drives triazole resistance in fungal pathogens of humans as well as plants (Chowdhary et al. 2013). These challenges are compounded by very high levels of genome plasticity and gene flow between populations of Z. tritici (Möller et al. 2018; Zhan et al. 2003).

The first genome sequence of Z. tritici (isolate IP0323) revealed 10,933 predicted genes located on 13 core and eight dispensable chromosomes (Goodwin et al. 2011). Deletion of the dispensable chromosomes confirmed that some are necessary for host specificity (Habig et al. 2017) and enable remarkably high rates of genome plasticity and spontaneous chromosome loss (Möller et al. 2018). Recent construction and analysis of a Z. tritici pangenome suggests a core set of 9,149 genes (Plissonneau et al. 2018). With regards to understanding the mechanistic basis of host infection, studies have characterized the underlying Z. tritici cellular biology and infectious growth (King et al. 2017; Steinberg 2015), transcriptionally deployed secondary metabolite loci (Cairns and Meyer 2015), and components of the secreted effector arsenal (Kettles et al. 2017; Lee et al. 2014; Saintenac et al. 2018; Zhong et al. 2017). However, the vast majority of genes and encoded proteins remain uncharacterized in the laboratory (Talbot 2015).

Recently, there has been a community-wide effort to develop numerous tools, techniques, and resources for Z. tritici. This research toolkit includes mutants in the nonhomologous end joining pathway for highly efficient gene targeting (Sidhu et al. 2015a), optimization of conditional expression systems (Marchegiani et al. 2015), a range of fluorescent translational gene fusion for subcellular localization studies (Kilaru et al. 2017, 2015), optimized virulence assays (Fones et al. 2015), and a suite of Gateway destination vectors suitable for Agrobacterium tumefaciens-mediated transformation (Idnurm et al. 2017; Mehrabi et al. 2015; Sidhu et al. 2015b). These Gateway destination vectors have been validated using a pilot Gateway entry library to generate overexpression mutants, which were phenotypically screened to demonstrate a role of the putative fungal-specific transcription factor–encoding gene almA in vitro hyphal growth (Cairns et al. 2015).
In this study, we report the generation of an improved functional genomics community resource to supplement these tools, by generating a Gateway-compatible Z. tritici ORFeome, which, to our knowledge, is the first such library for a plant-infecting fungus. This library is compatible with numerous Gateway destination vectors that have multiple functionality in Z. tritici, including numerous selection markers, epitope tags, and promoters (Alberti et al. 2007; Mehrabi et al. 2015). For ORFeome construction, we first screened the IP0323 reference genome for high-confidence gene models, yielding 4,075 candidate ORFs from a possible 10,933 predicted genes. These were PCR-amplified from genomic DNA and were cloned into the Gateway entry vector pDONR207. Quality of ORF sequences was verified by a combination of Sanger and Illumina sequencing, yielding 3,022 plasmids that passed quality control checks with 100% sequence verification.

The Z. tritici ORFeome described in this study is available to the research community. This resource can be rapidly utilized to interrogate the broadest aspects of Z. tritici biology, including identification of novel drug targets, mechanisms of drug detoxification and resistance, and pathogen virulence factors, which may ultimately enable development of new disease control strategies.

For ORFeome construction, E. coli One Shot ccdB Survival 2 T1R were used for propagation of pDONR207 (Invitrogen) and destination vectors. All Gateway entry and expression vectors were propagated in E. coli DH5α (Invitrogen).

For generation of Gateway entry vectors, this study utilized the Gateway donor vector pDONR207 (Invitrogen), which contains a gentamicin resistance gene, for selection in E. coli. This plasmid also contains a ccdB gene flanked by attP sequences for Gateway-mediated recombination using the BP reaction.

Generation of Gateway entry vectors was conducted as described previously (Cairns et al. 2015). For PCR amplification of each gene of interest, forward primers were designed to include the attB1 site (ggggacaagtttacaaaaaagacgcgctg) and the first 20 bp of the gene and reverse primers to include the attB2 site (ggggacactttgacaagagcctgc) and the last 20 bp of the gene. The stop codon was excluded to enable c-terminal epitope tagging by the end user. Primers were synthesized by Sigma-Aldrich UK and are listed in Supplementary File S1. PCRs were conducted using Phusion high-fidelity DNA polymerase (NEB) with a 65°C primer annealing temperature, an extension of 30 s/kb, using Z. tritici IP0323 genomic DNA as template. PCR amplicons of predicted sizes were confirmed by gel electrophoresis, were polyethylene glycol– purified, and were suspended in 10 μl Tris-EDTA (TE) buffer (40 mM TRIS base, 20 mM glacial acetic acid, 0.1 mM EDTA, pH 8). For construction of Gateway entry vectors, 150 ng of pDONR207 was mixed with 2.5 μl of purified PCR product, 0.5 μl of Gateway BP Clonase with TE buffer added to a total volume of 10 μl. Reactions were incubated at 25°C for 12 to 24 h and were then treated with Proteinase K (Invitrogen), following the manufacturer instructions. E. coli DH5α was transformed with 5 μl of each reaction mixture. Luria Bertani (LB) supplemented with gentamicin (50 μg/ml) was subsequently used to select transformants, which were grown overnight in LB medium with selection, and plasmids were extracted using Plasmid mini kit (Qiagen). Plasmids were indexed and stored in 96-well plates and at −20°C.

In order to confirm replacement of the ccdB gene with the ORF encoding sequence and to confirm high fidelity PCR amplification, a total of 688 Gateway entry vectors were randomly selected and Sanger-sequenced (Eurofins) using primer GOXF (tcgcgttaacgctagcatgga). Quality control reactions are summarized in Supplementary File S2, which also gives plate co-ordinates for plasmid requests. A second quality-control experiment was conducted using two rounds of Illumina HiSeq 2500 sequencing of 3,396 pooled Gateway entry vectors. Raw sequencing data are available at the Sequencing Read Archive (Leinonen et al. 2011) (accession SRX1267196 and SRX1265386).

Evaluation of the 10,933 predicted genes in the Z. tritici IP0323 reference genome (Goodwin et al. 2011) returned 4,075 high-confidence gene models that had unambiguous start and stop codons (data not shown). This analysis was conducted prior to RNA-seq analysis and comparative genomic analyses (Grandaubert et al. 2015), which have since improved Z. tritici gene models. High-confidence gene models were complemented with a total of 1,345 priority ORFs that had been requested during consultation with members of the Z. tritici research community. These latter ORFs were included in the ORFeome construction project even if they failed our gene model quality control. An overview of the ORFeome construction project is shown in Supplementary Figure S1. PCR amplification utilized
genomic DNA as template, which was chosen over cDNA, in order to maintain alternative splice variants (Grützmann et al. 2014) during downstream ORFeome expression in Z. tritici. Additionally, in order to enable c-terminal epitope-tagging of encoded ORFs using a variety of destination vectors (Alberti et al. 2007), primers were designed to omit the native stop codon. If expression of the encoded ORFs with a 3’ stop codon is desired, we have developed numerous destination vectors for this purpose (Sidhu et al. 2015b).

A total of 4,896 PCR reactions were conducted in 51 96-well plates, which yielded 4,174 amplicons of the predicted molecular weight, as determined by gel electrophoresis (data not shown). A total of 3,396 of these genes were successfully cloned into pDONR207, using the Gateway BP reaction, as determined by bacterial growth on selection agar, for which plasmids were extracted. Over 650 Gateway entry plasmids were randomly selected for sequence verification using Sanger sequencing, with 99.5% passing quality control. A second quality-control experiment was conducted, in which all 3,396 ORFs were pooled and were sequenced using an Illumina HiSeq 2500. When combined with Sanger sequencing experiments, a total of 3,022 Z. tritici ORFs passed quality control checks. Genes that are represented in the quality-controlled Z. tritici ORFeome (n = 3,022) are plotted as a function of chromosomal locus and cover both core and accessory chromosomes (Table 1). All 3,396 plasmids are available to end users, with the caveat that plasmids that failed our quality control need be sequence-verified by the end user. A summary of ORFeome coverage for 3,022 quality-controlled ORFs among predicted effector-encoding genes, secondary metabolite biosynthetic genes, various other functional groups, chromosomal loci, and differentially expressed genes during infection (Rudd et al. 2015) is provided in Table 1. These data indicate that the ORFeome will be applicable for functional genomic experiments to test diverse hypotheses regarding, for example, gene function, expression, or genomic location.

In summary, we have generated an ORFeome library of Z. tritici that covers high-confidence gene models from the isolate IP0323 (Goodwin et al. 2011) for functional genomic analyses. The ORFeome contains 3,022 sequence verified clones. Genes represented in this

### Table 1. Summary information for the Zymoseptoria tritici ORFeome

<table>
<thead>
<tr>
<th>Predicted category</th>
<th>IP0323 genome</th>
<th>ORFeome</th>
<th>Coverage in ORFeome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. genes</td>
<td>10,933</td>
<td>3,022</td>
<td>27.6</td>
<td>Goodwin et al. 2011</td>
</tr>
<tr>
<td>Secreted proteinsc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Signal peptide</td>
<td>909</td>
<td>538</td>
<td>59.1</td>
<td>Aken et al. 2016; Sperschneider et al. 2018</td>
</tr>
<tr>
<td>Effector P</td>
<td>1,438</td>
<td>410</td>
<td>28.5</td>
<td></td>
</tr>
<tr>
<td>Chromosomal loci</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core chromosome</td>
<td>10,278</td>
<td>2,900</td>
<td>28.2</td>
<td>Goodwin et al. 2011; Cairns and Meyer 2017</td>
</tr>
<tr>
<td>Accessory chromosome</td>
<td>654</td>
<td>122</td>
<td>18.6</td>
<td></td>
</tr>
<tr>
<td>Subtelomericc</td>
<td>2,501</td>
<td>644</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td>Secondary metabolite cluster</td>
<td>682</td>
<td>171</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>Differentially expressed during infectiona</td>
<td></td>
<td></td>
<td></td>
<td>Rudd et al. 2015</td>
</tr>
<tr>
<td>1 dpi</td>
<td>626</td>
<td>194</td>
<td>30.9</td>
<td></td>
</tr>
<tr>
<td>4 dpi</td>
<td>769</td>
<td>256</td>
<td>33.2</td>
<td></td>
</tr>
<tr>
<td>9 dpi</td>
<td>812</td>
<td>271</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td>14 dpi</td>
<td>637</td>
<td>206</td>
<td>32.3</td>
<td></td>
</tr>
<tr>
<td>21 dpi</td>
<td>718</td>
<td>203</td>
<td>28.2</td>
<td></td>
</tr>
<tr>
<td>Exemplar GO terms</td>
<td></td>
<td></td>
<td></td>
<td>Aken et al. 2016</td>
</tr>
<tr>
<td>DNA binding (GO:0003677)</td>
<td>491</td>
<td>76</td>
<td>15.4</td>
<td></td>
</tr>
<tr>
<td>GTPase activity (GO:0003924)</td>
<td>82</td>
<td>12</td>
<td>14.6</td>
<td></td>
</tr>
<tr>
<td>Protein kinase activity (GO:0004672)</td>
<td>301</td>
<td>53</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>Transmembrane transport (GO:0055085)</td>
<td>950</td>
<td>145</td>
<td>15.2</td>
<td></td>
</tr>
</tbody>
</table>

* Open reading frames (ORFs) cloned into pDONR207 that passed quality control (n = 3,022) were assigned various functional categories and are reported both as number of genes and as a percentage of the predicted total for the IP0323 genome.
* Percent of genome total.
* Putative effector-encoding genes were predicted from amino acid coding sequences, using default parameters, in the Effector P prediction algorithm (Sperschneider et al. 2018). ORFs encoding predicted signal peptides and exemplar Gene Ontology (GO) terms were retrieved using the Ensemble Biomart pipeline (Aken et al. 2016).
* Subtelomeric genes were defined as those residing within 300 kb of the chromosome end. Genes predicted to reside in secondary metabolite biosynthetic gene clusters were identified from AntiSMASH and SMURF predictions (Cairns and Meyer 2017).
* Differentially expressed genes were defined from transcriptional profiling by Rudd and coworkers (2015), with the number of genes significantly upregulated in planta at various days postinfection (dpi) relative to in vitro growth on Czapek-Dox media reported. The total predicted number of genes in the IP0323 genome belonging to each functional category is also shown.
library are putatively involved in a diverse range of processes, including secreted proteins and putative effectors, biosynthesis of secondary metabolite toxins, drug detoxification, signal sensing and transduction, and regulation of gene expression, among many others (Table 1), and will therefore facilitate functional genomic experiments for diverse aspects of *Z. tritici* biology.

Our strategy prioritized high-confidence gene models for ORFeome construction over a genome-wide cloning approach. While this has resulted in a partial ORFeome for *Z. tritici* IP0323, we believe that a focus on accurate gene models will avoid large-scale future updates of this resource. For example, the first *C. elegans* ORFeome (Reboul et al. 2003) underwent various revisions and additions due to improved gene model predictions (Lamesch et al. 2004). More importantly, high-confidence gene models will likely limit expression of incorrect ORFs during cost and labor-intensive experiments by end users.

ORFeomes for several model organisms are amplified intron-free sequences from cDNA libraries (Lamesch et al. 2004; Wiemann et al. 2016). In contrast, we amplified ORF sequences from genomic DNA in order to maintain the opportunity to generate alternative splice variants (e.g., due to intron skipping) in subsequent *Z. tritici* overexpression or localization experiments. While the extent of alternative splicing in fungi is not comprehensively determined, an estimated 6.1% of *Z. tritici* genes have splice variants (Grützmann et al. 2014). Alternative splicing is thought to predominantly occur for genes required for virulence, multicellularity, and dimorphic switching (Grützmann et al. 2014). Our ORFeome will therefore facilitate the study of splice variants that may have critical impacts on infection, or, alternatively, encode promising drug targets in this pathogen.

It should be noted that the ORFeome was constructed using isolate IP0323 (Goodwin et al. 2011). While this strain is commonly used by the research community, IP0323 was isolated in 1984 and is sensitive to crop protection chemistries to which current field isolates have evolved resistance. Thus, the resource may be limited for understanding resistance to such antifungals.

We have previously demonstrated that the application of a pilot (*n* = 32) collection of putative DNA-binding protein–encoding genes in entry vectors can enable medium-throughput gene functional analyses in *Z. tritici* (Cairns et al. 2015). The ORFeome generated in this study will drastically increase the throughput of these capabilities for the research community. We predict that functional genomics in *Z. tritici* will enable systems-level understanding of a diverse range of processes, including but not limited to growth and development, sensing and signal transduction, virulence, host-pathogen interactions, toxin biosynthesis, drug resistance, and chemical-genetic interactions. Such advances may ultimately lead to novel fungicide development and development of novel resistant wheat cultivars.

**Literature Cited**


