Fungal Genetics and Biology 79 (2015) 166–173

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Conditional promoters for analysis of essential genes in *Zymoseptoria tritici* ☆

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ARTICLE INFO

Article history:
Received 22 January 2015
Revised 12 March 2015
Accepted 19 March 2015

Keywords:
Inducible–repressible promoter
Fungal wheat pathogen
Septoria tritici blotch
Mycosphaerella graminicola

ABSTRACT

Development of new fungicides, needed for sustainable control of fungal plant pathogens, requires identification of novel anti-fungal targets. Essential fungal-specific proteins are good candidates, but due to their importance, gene deletion mutants are not viable. Consequently, their cellular role often remains elusive. This hindrance can be overcome by the use of conditional mutants, where expression is controlled by an inducible/repressible promoter. Here, we introduce 5 inducible/repressible promoter systems to study essential genes in the wheat pathogen *Zymoseptoria tritici*. We fused the gene for enhanced green-fluorescent protein (*egfp*) to the promoter region of *Z. tritici* nitrate reductase (P*nar1*; induced by nitrogen and repressed by ammonium), 1,4-β-endoxylanase A (P*ex1A*; induced by xylose and repressed by maltodextrin), L-arabinofuranosidase B (P*laraB*; induced by arabinose and repressed by glucose), galactose-1-phosphate uridylyltransferase 7 (P*gal7*; induced by galactose and repressed by glucose) and isocitrate lyase (P*icl1*; induced by sodium acetate and repressed by glucose). This was followed by quantitative analysis of cytoplasmic reporter fluorescence under induced and repressed conditions. We show that P*nar1*, P*laraB* and P*ex1A* drive very little or no *egfp* expression when repressed, but induce moderate protein production when induced. In contrast, P*gal7* and P*icl1* show considerable *egfp* expression when repressed, and were strongly induced in the presence of their inducers. Normalising the expression levels of all promoters to that of the α-tubulin promoter P*tub2* revealed that P*laraB* was the weakest promoter (~20% of P*tub2*), whereas P*icl1* strongly expressed the reporter (~250% of P*tub2*). The use of these tools promises a better understanding of essential genes, which will help developing novel control strategies that protect wheat from *Z. tritici*.

1. Introduction

Effective management of fungal pathogens requires in-depth knowledge of their cell biology and host invasion strategies. Sophisticated sequencing methods have provided a large wealth of genes and predicted proteins, which need to be functionally analyzed to gain insight into their cellular roles. The generation of gene mutants, such as knock-out, knock-down and over-expression mutants, has greatly helped understanding protein function in eukaryotic cells (Meyer et al., 2011). Examples are the systematic deletion of genes in the budding yeast *Saccharomyces cerevisiae* (Giaever et al., 2002) and the genome-wide knock-down of proteins by RNAi silencing methods in the worm *Caenorhabditis elegans* (Kamath et al., 2003). However, a major limitation of these approaches is that cellular function of essential genes and their protein products cannot be analyzed (Meyer et al., 2011). This caveat can be overcome by the generation of conditional promoter mutants, where the expression of a gene of interest depends on the experimental condition. Such substrate-induced promoter mutants have been used in a wide range of fungi. Examples include galactose-inducible promoters of galactose-1-phosphate uridylyltransferase in *Cryptococcus neoformans* (Ruff et al., 2009), the sodium acetate inducible isocitrate lyase promoter in *Magnaporthe grisea* (Wang et al., 2003), or

Abbreviations: P, promoter; *nar1*, nitrate reductase; *ex1A*, 1,4-β-endoxylanase; *laraB*, L-arabinofuranosidase B; *gal7*, galactose-1-phosphate uridylyltransferase 7; *icl1*, isocitrate lyase; *tub2*, α-tubulin; *sdi1*, succinate dehydrogenase 1; aa, amino acid; eGFP, enhanced green fluorescent protein; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; MT, microtubule; ROI, region of interest.

☆ All material and protocols described here are available upon request.

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http://dx.doi.org/10.1016/j.fgb.2015.03.024
1087-1845/

the arabinose-inducible, glucose repressible *crg* promoter in *Ustilago maydis* (Bottin et al., 1996). An essential requirement for conditional promoters is that they are tightly repressed in the absence of an inducer (Vu et al., 2013). On the other hand, controlled over-expression of entire genes or DNA encoding domains of interest also provides a useful way of gaining insight into their cellular role (Schuster et al., 2011).

Here, we establish 5 homologous inducible/repressible promoters in the wheat pathogen *Zymoseptoria tritici*. We identify the promoter region of nitrate reductase homologue (P*nar1*) in *Z. tritici*, which induced by nitrate and repressed by ammonium in *U. maydis* (Banks et al., 1993). Secondly, we use the promoter of L-arabinofuranosidase B homologue (P*laraB*) in *Z. tritici* that is induced by arabinose and repressed by glucose in *Aspergillus niger* (v d Veen et al., 1993). We establish use of the promoter of a 1,4-β-endoxylanase A homologue (P*ex1A*) in *Z. tritici*, which is induced by xylose and repressed by maltodextrin in *Aspergillus awamori* (Gouka et al., 1996). Moreover, we express eGFP under the promoter of a galactose-1-phosphate uridylyltransferase homologue (P*gal7*) in *Z. tritici*, which is induced by galactose and repressed by glucose in *C. neoformans* (Ruff et al., 2009). Finally, we use the promoter of a homologue of isocitrate lyase (P*icl1*), induced by sodium acetate and repressed by glucose in *M. grisea* (Wang et al., 2003). Using enhanced green-fluorescent protein (eGFP) as a reporter, we show that all 5 promoter systems derive eGFP expression when induced in liquid culture. Comparison with auto-fluorescence in control strains reveals that P*nar1*, P*laraB* and P*ex1A* are "shut down" under OFF conditions, whereas significant background fluorescence of eGFP was found when the reporter was placed behind P*gal7* and P*icl1*. Comparing the relative expression strength of all promoters with that of the α-tubulin promoter shows that P*laraB*, P*nar1* and P*ex1A* drive relatively weak expression under induced conditions (ON conditions), whereas P*gal7* and, in particular, P*icl1* are stronger promoters. When α-tubulin was expressed under the P*ex1A* promoter, cells grew normally, but growth was significantly impaired when P*ex1A* was repressed. This demonstrates the use of conditional promoters in functional analysis of essential genes in *Z. tritici*.

2. Materials and methods

2.1. Bacterial and fungal strains and growth conditions

Escherichia coli strain DH5α was used for the maintenance of plasmids. *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) was used for maintenance of plasmids and subsequently for *A. tumefaciens*-mediated transformation of *Z. tritici*. *E. coli* and *A. tumefaciens* were grown in DYT broth or DYT agar at 37 °C and 28 °C respectively. The fully sequenced *Z. tritici* wild-type isolate IPO323 (Kema and van Silfhout, 1997) was used as recipient strain for the genetic transformation experiments. The isolate was inoculated from stocks stored in glycerol at −80 °C onto solid YPD agar (yeast extract, 10 g/l; peptone, 20 g/l; glucose, 20 g/l; agar, 20 g/l) and grown at 18 °C for 4–5 days.

2.2. Identification of enzymes in Z. tritici and bioinformatics

To identify conditional promoters, we obtained the sequences of *U. maydis* Nar1 sequence (XP_759994.1), *Aspergillus fumigatus* LaraB sequence (KEY83958.1), *A. awamori* Ex1A sequence (CAA55005.1), *C. neoformans* Gal7 sequence (XP_568349.1) and *M. oryzae* Icl1 sequence (XP_003712381.1) from the NCBI server (http://www.ncbi.nlm.nih.gov/pubmed). We used these to screen the published sequence of *Z. tritici* (http://genome.jgi.doe.gov/Mycgr3/Mycgr3.home.html) using the provided BLASTP function and compared the best hits in EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The domain structure was analyzed in PFAM (http://pfam.xfam.org/search/sequence). The promoter sequences upstream of the open reading frame were obtained from the JIG server (http://genome.jgi.doe.gov/Mycgr3/Mycgr3.home.html).

2.3. Molecular cloning

All vectors in this study were generated by in vivo recombination in the yeast *S. cerevisiae* DS94 (MATα, *ura3-52*, *trp1-1*, *leu2-3*, *his3-111*, and *lys2-801* (Tang et al., 1996) following published procedures (Raymond et al., 1999; Kilaru and Steinberg, 2015). For all the recombination events, the fragments were amplified with 30 bp homologous sequences to the upstream and downstream of the fragments to be cloned (see Table 1 for primer details). PCR reactions and other molecular techniques followed standard protocols (Sambrook and Russell, 2001). All restriction enzymes and reagents were obtained from New England Biolabs Inc (NEB, Herts, UK).

2.4. Construction of vectors pCPnar1eGFP, pCPex1eGFP, pCPlaraB-eGFP, pCPgal7-eGFP, pCPicl1-eGFP and pHex1A-tub2

Vector pCP*Pnar1*eGFP contains *egfp* under the control of *Z. tritici nar1* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. A 13,083 bp fragment of pCeGFPTub2 (digested with *Pml*I; Schuster et al., 2015) and 1000 bp *Z. tritici nar1* promoter (amplified with SK-Sep-112 and SK-Sep-113; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCP*nar1*eGFP. Vector pCP*ex1*eGFP contains *egfp* under the control of *Z. tritici ex1A* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. A 13,083 bp fragment of pCeGFPTub2 (digested with *Pml*I) and 1000 bp *Z. tritici ex1A* promoter (amplified with SK-Sep-114 and SK-Sep-115; Table 1) were recombined in *S. cerevisiae* to obtain the vector pC*Pex1*eGFP. Vector pCP*laraB*eGFP contains *egfp* under the control of *Z. tritici laraB* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. A 13,083 bp fragment of pCeGFPTub2 (digested with *Pml*I) and 1,000 bp *laraB* promoter (amplified with SK-Sep-118 and SK-Sep-119; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCP*laraB*eGFP. Vector pCP*gal7*-eGFP contains *egfp* under the control of *Z. tritici gal7* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. A 13,083 bp fragment of pCeGFPTub2 (digested with *Pml*I) and 1000 bp *Z. tritici gal7* promoter (amplified with SK-Sep-126 and SK-Sep-127; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCP*gal7*-eGFP. Vector pCP*icl1*-eGFP contains *egfp* under the control of *Z. tritici icl1* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. A 13,083 bp fragment of pCeGFPTub2 (digested with *Pml*I) and 1000 bp *Z. tritici icl1* promoter (amplified with SK-Sep-120 and SK-Sep-121; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCP*icl1*-eGFP. Vector pHP*ex1A*Tub2 contains the *Z. tritici ex1A* promoter (*Pex1A*) fused to *tub2* gene for replacement of endogenous *tub2* promoter in *Z. tritici* with *ex1A* promoter using hygromycin as a selectable marker. A 9533 bp fragment of pCeGFPTub2 (digested with *Bam*HI and *Hind*III), 1149 bp *tub2* promoter (amplified with SK-Sep-217 and SK-Sep-218; Table 1), 1806 bp hygromycin resistance cassette (amplified with SK-Sep-136 and SK-Sep-137; Table 1), 1000 bp *ex1A* promoter (amplified with SK-Sep-225 and SK-Sep-226; Table 1), 5′ end of 1002 bp *tub2* gene (amplified with SK-Sep-221 and SK-Sep-222; Table 1) were recombined in *S. cerevisiae* to obtain the vector pHP*ex1A*tub2.