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A motif within a complex promoter from the oomycete *Phytophthora infestans* determines transcription during an intermediate stage of sporulation

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ABSTRACT

Sporulation in *Phytophthora infestans* is associated with a major remodeling of the transcriptome. To better understand promoter structure and how sporulation-specific expression is determined in this organism, the *Pks1* gene was analyzed. *Pks1* encodes a protein kinase that is induced at an intermediate stage of sporulation, prior to sporangium maturation. Major and minor transcription start sites mapped throughout the promoter, which contains many T-rich stretches and Inr-like elements. Within the T-rich region are several motifs which bound nuclear proteins in EMSA. Tests of modified promoters in transformants implicated a CCGTTG located 110-nt upstream of the transcription start point as a major regulator of sporulation-specific transcription. The motif also bound a sporulation-specific nuclear protein complex. A bioinformatics analysis indicated that the motif is highly over-represented within co-expressed promoters, in which it predominantly resides 100–300-nt upstream of transcription start sites. Other sequences, such as a CATTTGTT motif, also bound nuclear proteins but did not play an essential role in spore-specific expression.

1. Introduction

Many eukaryotic microbes, including true fungi and oomycetes, utilize asexual spores for dissemination and infection. The differentiation of these structures is largely regulated at the level of RNA synthesis by transcription factors that recruit RNA polymerase II to promoters. These promoters are typically bipartite, containing a proximal core region which binds the transcriptional apparatus near the initiation site, and distal activation or repression domains (Lee and Young, 2000). Tissue-specific transcription is thought to be predominantly regulated by the distal sites, although contributions may also be made by core promoters (Smale, 2001). An important element of understanding an organism entails identifying the promoter motifs and transcription factors that regulate its development.

Little is known of transcriptional mechanisms that control sporulation or other developmental processes in oomycetes, a group of important plant and animal pathogens and saprophytes. Compared to well-studied model organisms, oomycetes have a unique evolutionary history as they reside in the kingdom Stramenopila (Baldauf et al., 2000). The extent to which transcriptional regulation in oomycetes resembles that of other eukaryotes remains to be determined. Data from oomycete genomes indicate that their intergenic regions are typically small (<500 nt) and may therefore lack the complexity of many metazoan and plant promoters. Many oomycete promoters contain sequences resembling the Initiator or Inr (YYANWYY), which is one of several core promoter motifs found in higher eukaryotes (Juven-Gershon et al., 2006; McLeod et al., 2004; Smale, 2001).

Phytophthora infestans, which causes the devastating late blight disease of potato, is a promising model for studying both oomycete spores and promoters (Fry, 2008; Judelson and Blanco, 2005). Copious amounts of sporangia are produced which are easily purified for molecular studies, and genome sequence and expression data are available to aid analyses of transcription during development (Judelson et al., 2008). Although its application is complicated by copy number and position effects, a transformation system has proved useful for manipulating genes and promoters associated with development. This has helped to define the binding sites for two stage-specific transcription factors, one acting during early sporulation and another during zoosporogenesis (Ah Fong et al., 2007; Tani and Judelson, 2006).

Sporulation in *P. infestans* begins when vegetative mycelia, in response to environmental and internal cues, produce aerial hyphae and then sporangiophores (Hardham and Hyde, 1997). After cytoplasm, nuclei, and other organelles move into each developing sporangium, a septum forms at its base and a papilla at its apex that is important for germination. Unlike the typically dormant fungal spores, *P. infestans* sporangia are physiologically active and capable of rapid germination. At lower temperatures and high humidity, which are the climatic factors most conducive to epidemics, the sporangium cleaves into six or more flagellated zoospores that exit through the papilla. After an interval of swimming, each zoospore forms a cyst, germ tube, and appressorium. Genes transcribed during

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sporulation appear to participate in both early events in sporangia development and subsequent stages including germination and encystment (Clark et al., 1978; Penington et al., 1989).

To enhance our understanding of both asexual reproduction and gene expression mechanisms in *P. infestans*, this study describes the transcription pattern and promoter structure of the sporulation-induced *Pks1* protein kinase gene, which had been identified as a cDNA in an expression profiling study (Kim and Judelson, 2003). Using transformants expressing the *Pks1* promoter fused to the β-glucuronidase reporter, the gene was shown to be expressed at an intermediate stage of sporulation. Through functional dissection and bioinformatics, the promoter was shown to exhibit a complex structure containing multiple regulatory elements and binding sites for nuclear proteins. In addition, a motif necessary for activating *Pks1* during sporulation was detected which also exists within many similarly-expressed promoters. Such data helps illuminate the pathways regulating asexual reproduction, which in the long-term may lead to targets for crop protection strategies.

2. Materials and methods

2.1. Manipulations of P. infestans

Isolates were cultured on rye-sucrose media at 18 °C in the dark. Strains employed included isolate 1306 and transformants made using the protoplast method (Judelson, 1993). Non-sporulating mycelia were obtained by inoculating clarified rye-sucrose broth with sporangia, followed by 3 days incubation. Sporulating hyphae were from rye-sucrose agar cultures grown for 9–11 days. Sporangia were purified from such cultures by adding water, rubbing with a glass rod, and passing the fluid through a 50-μm mesh to remove hyphal fragments. To induce germination, sporangia were either placed in rye-sucrose broth for 6 h at 18 °C, or water at 4 °C for 1 h to induce zoospores. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

2.2. Cloning and sequencing of Pks1

A cDNA clone corresponding to *Pks1* was hybridized to a bacterial artificial chromosome library (Randall and Judelson, 1999). Positive colonies were subcloned and sequenced.

2.3. Gene expression analysis

Staining for β-glucuronidase (GUS) was performed as described (Judelson, 1993). For blot studies, total RNA was resolved on formaldehyde gels and hybridized with ^{32}P-labeled probes. These were generated by polymerase chain reaction using primer pairs for *Pks1* (5′-TAGGATCCTTCTCTTCCAACGTAAG and 5′-CGGCGGCCGCGA AATCTTGATCCG AACCGA), GUS (5′-AAACCCCAACCCGTGAAAT-CAAAA and 5′-ATCGGTGTGAGCGTCGCAGAA CA), or elongation factor 1-α (EF-1; 5′-ATTCTGGTGGTCGCTTCGGGTGTGG and 5′-GA CGAA ACCACGACGCAGCTCCTTG) and labeled by the random primer method. To map transcription start sites (TSSs), RACE was performed using a kit from Invitrogen (Carlsbad, CA, USA) with specific primers QX68R (5′-GCGTGTTTTGCTGCCAGAA and QX69R (5′-CCTCACTCAACACCGAGTCCTTA). Products were resolved on 2% agarose, cloned into pGEM-T Easy (Promega, Madison, WI, USA), and sequenced.

2.4. GUS plasmid construction

Promoter fragments were generated by polymerase chain reaction (PCR) using Platinum Taq DNA polymerase High-Fidelity (Invitrogen) and primers with *Apa*I sites, and cloned into pOGUS which contains a promoterless GUS gene along with neomycin phosphotransferase (*nptII)* for G418 selection. For 5′ deletion constructs, plasmids are named based on the size of the promoter as counted upstream from the TSS (+1); p877 contains 877 nt of sequences upstream of the TSS plus the 125-nt 5′ untranslated region, for example. These were amplified using reverse primer RP1 (5′-AAGGGCCC GATAGCTTCGGACTTAG) and upstream primers 5′-AAGGGCCCGGGATACTATTGATGCT for p877, 5′-AAGGGCCCAC CCGAACGACCCCAAGA for p525, 5′-AAGGGCCCAATGGTAG GTG CTCTTAG for p225, 5′-AAGGGCCCTTTGCGTTGCCGTTGTGC for p119, 5′-AAGGGC CCTTGTGCCATTTGTTTT for p107, 5′-AAGGG CCCTTTTTCCTCGTCCCA for p95, 5′-AAGG GCCCCGTCCCATTTTCTCA for p87, 5′-AAGGGCCCTTCTCATGCTGCTCGTAT for p78, or 5′-AA GGGCCCAAAAGTGTTCTCCATCG for p50. To mutate the CGTTG motifs, PCR was performed using RP1 with MR1 (5′-TTGGGCCCT TTTGACGGTCCGTTGTGCCATTTGTT), MR2 (5′-TTGGGCCCTTTT GCGTTGCATGACTGCCATTTGTTTTTCC), or MR12 (5′-TTGGGC CCTTTTGACGGTCATGACTGCCATTTGTTTT TCC) using p119 as template. To mutate the CAGTTTGTT motif, PCR used RP1 and NC (5′-TT GGGCCCTTTTGCGTTGCCGTTGTGCACGGGTGGTTTCCTCGTCCCAT) against p119. Other mutations described in the text were made by two stage PCR using outer primers matching wild-type *Pks1* and inner mutagenic primers.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear protein isolation and EMSA were performed as described (Ah Fong et al., 2007), except that heparin agarose was not used for the extractions. Briefly, EMSA involved mixing 5 μg of nuclear protein with 1 μg poly dI-dC and 1.6 ng of ^{32}P-labeled probe for 30 min on ice, followed by electrophoresis at room temperature. For competition assays, protein was incubated with unlabeled DNA for 30 min and then the labeled probe. Double-stranded oligonucleotides listed in Results were used as probe and cold competitors. Bands were quantified using a phosphorimager.

2.6. Bioinformatics surveys

Genes induced or repressed during sporulation were identified from microarray data (Judelson et al., 2008). The corresponding promoters, and total promoters, were extracted from the *P. infestans* genome database developed by the Broad Institute of MIT and Harvard (www.broad.mit.edu). Motif searches were performed using a combination of text editors and custom perl scripts, and background frequencies were calculated based on the base composition of *P. infestans* promoters.

3. Results

3.1. Pks1 encodes a protein kinase induced upon spore maturation

Our previous macroarray study identified a cDNA clone named *pisp18* that corresponds to a gene induced during sporulation (Kim and Judelson, 2003). To identify the structure of the full-length gene, a genomic clone was obtained from a BAC library and sequenced. The gene is renamed here as *Pks1* (*P. infestans* protein kinase in sporangia 1), as its sequence matched a serine/threonine protein kinase. Subsequently, *Pks1* was defined in the *P. infestans* genome sequencing project underway at the Broad Institute of MIT and Harvard as gene PITG_10884. DNA blot analysis indicated that *Pks1* is a single-copy gene (not shown).

Pks1 encodes an intron-lacking open reading frame of 541 amino acids with similarity to the AGC group of the eukaryotic protein kinase superfamily. AGC kinases typically control essential cellular processes such as growth, cell cycle progression, differentiation,