Activation of Zoosporogenesis-Specific Genes in *Phytophthora infestans* Involves a 7-Nucleotide Promoter Motif and Cold-Induced Membrane Rigidity

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Infections of plants by the oomycete *Phytophthora infestans* typically result from zoospores, which develop from sporangia at cold temperatures. To help understand the relevant cold-induced signaling pathway, factors regulating the transcription of the zoosporogenesis-specific NIF (nuclear LIM-interactor-interacting factor) gene family were examined. Sequences required for inducing *PinifC3* were identified by analyzing truncated and mutated promoters using the β-glucuronidase reporter in stable transformants. A 7-nucleotide (nt) sequence located 139 bases upstream of the major transcription start point (GGACGAG) proved essential for the induction of *PinifC3* when sporangia were shifted from ambient to cold temperatures. The motif, named the cold box, also conferred cold inducibility to a promoter normally activated only during sexual development. An identical motif was detected in the two other zoosporogenesis-specific NIF genes from *P. infestans* and three *Phytophthora sojae* sojulex orthologues, and a closely related sequence was found in *Phytophthora ramorum* orthologues. The 7-nt motif was also found in the promoters of other zoosporogenesis-induced genes. The presence of a cold box-interacting protein in nuclear extracts of *P. infestans* sporangia was demonstrated using electrophoretic mobility shift assays. Furthermore, zoospore release and cold box-regulated transcription were stimulated by the membrane rigidizer dimethyl sulfoxide and inhibited by the membrane fluidizer benzyl alcohol. The data therefore delineate a pathway in which sporangia perceive cold temperatures through membrane rigidity, which activates signals that drive both zoosporogenesis and cold-box-mediated transcription.

Temperature represents an important environmental factor that affects all organisms. Cellular responses to temperature can be both immediate and long term. The former includes the rapid and transient heat or cold shock responses that are well described in both prokaryotes and eukaryotes (12, 16, 43, 48). Long-term mechanisms are exemplified by the cold acclimation phenomena of plants and fish, which involve altering lipid, osmolyte, protein, or other cellular components (48, 49). Many of these responses aim to enhance survival during stress, but specific temperature regimens may also be integral to normal development. For example, specific temperature treatments stimulate flowering in plants (37), pathogenic development in several fungi and bacteria that colonize animals (22, 30, 34), and apressorium formation in a phytopathogenic fungus (41).

Temperature also influences the germination of spores, particularly in the fungus-like eukaryotic microbes known as oomycetes. Asexual sporangia of the potato late blight pathogen *Phytophthora infestans*, for example, exhibit dual modes of germination when placed in liquid (23). Above 15°C, germ tubes typically emerge directly from sporangia. However, at lower temperatures the cytoplasm of sporangia is conditioned to cleave into six or more uninucleate biflagellated zoospores, which swim in search of a host (13, 42). Germination through zoospores, termed indirect germination, can occur in less than an hour and is not blocked by actinomycin or cycloheximide (10). The contribution of zoospores to disease is believed to be higher than that of directly germinating sporangia except at elevated temperatures, when the wall-lacking zoospores are more prone to desiccation. Therefore, *Phytophthora* sporangia appear to be designed to sense the environment and choose the optimal mode of germination.

Knowledge of the mechanisms regulating responses to cold in oomycetes or any organism is limited. Genes induced by cold temperatures have been described for many species (20, 35, 49) and some of the cognate transcription factors have been identified (6, 8). However, relatively little is known of the upstream components of such temperature perception pathways. An initial sensor of temperature in non-warm-blooded (poikilothermic) species, including plants, microbes, and some animals, has been proposed to be the plasma membrane (32). Its increased rigidity at lower temperatures may influence membrane-associated proteins to transmit signals to downstream targets, such as transcription factors for cold-regulated genes (9). Such membrane-associated proteins may include calcium channels (36), phospholipases (39), histidine kinases (2), and proteins that interact with the cytoskeleton (40).

In previous work we identified 70 genes induced during zoosporogenesis in *P. infestans* (46), a process which is induced conveniently in the laboratory by shifting sporangial suspensions from ambient to cold temperatures. Most such genes are up-regulated within minutes of cold treatment, before cytoplasmic reorganization is apparent within sporangia. Three of the zoosporogenesis-specific genes (*PinifC1*, *PinifC2*, and *PinifC3*) encode protein phosphatases known as nuclear LIM interactor-interacting proteins (NIFs). These are believed to control transcription by altering phosphorylation of the C-terminal domain of RNA polymerase, or by interacting with other regulators (18, 51). The *PinifC* genes appear to be con-
trolled by phosphoinositols since their transcription is arrested by the phospholipase C inhibitor U-73122 and 2-aminoethoxy-diphenylborate, which blocks calcium channels gated by inositol trisphosphate (45, 46). Therefore, the PinifC genes are promising targets for unraveling the mechanisms of cold perception in P. infestans.

This report demonstrates that a reduction in membrane rigidity stimulates both zoosporogenesis and expression of the PinifC genes. By analyzing truncated, chimeric, and mutated promoters, a 7-nucleotide (nt) motif sufficient for driving zoosporogenesis-induced transcription was identified, and a protein activity that binds the motif was documented.

**MATERIALS AND METHODS**

**Growth of P. infestans.** Cultures of P. infestans isolate 1306 were maintained at 18°C, and developmental stages were obtained as described previously (25). Brieﬂy, sporangia were puriﬁed by rubbing hyphal mats with a glass rod in water, followed by passage through 50-μm mesh to remove hyphal fragments. Cleavage (zoosporogenesis) was induced by placing sporangia in 10°C water for the times indicated in Results; in general, by 60 min cytoplasmic cleavage was visible in most sporangia, although zoospores had emerged from fewer than 10%.

**Analysis of promoters in transgenic P. infestans.** Stable transformants were obtained using a protoplast method and stained histochemically for β-glucuronidase (GUS) activity (24). This involved derivatives of pOGUS (14) into which contains an nptII gene and pOGUS also contains an nptII gene for G418 selection.

Promoter fragments were obtained by PCR using the oligonucleotides listed in Table 1. The structures of the amplicons were veriﬁed by DNA sequencing. To generate the ~254, ~151, and ~65 PinifC3 fragments, PCR employed primer NLIC3RC with C3F-254, C3-151, and C3-65, respectively. The M2 promoter was ampliﬁed using primers M82F and M82R. The 60-nt and 7-nt regions of the PinifC1 promoter were cloned into pGEMT-EZ (Promega, Madison, WI), and se-
experimental portion of our study, the significance of interspecies conservation at some sites will be discussed in more detail later. However, it is notable that only partial conservation exists near TSP1 and that the TSP2 region is well conserved within \textit{P. infestans} (gray shading in Fig. 1) but not the other species. Neither region contains a consensus sequence present at the transcription start sites of some, but not all, genes from \textit{P. infestans} (31).

**Deletion analysis of the PininfC1/PininfC3 promoter region.** Truncated fragments of the PininfC1/PininfC3 intergenic region were used to determine whether that interval contained shared motifs required to express the two genes or two independent promoters. Fragments of approximately 350 nt were isolated from each end of the region, fused to the GUS reporter, and stably transformed into \textit{P. infestans}. GUS was then scored histochemically in sporangia that had been placed at 10°C for 40 min to activate zoosporogenesis. In such “cleaving sporangia,” GUS expression was enabled by both the right and left fragments of the intergenic region in three of six and 4 of 12 transformants, respectively (Fig. 2A and B).

The failure of some transformants in these and later experiments to express GUS presumably reflects the general nature of transformation in \textit{P. infestans} and not any feature specific to the particular PininfC3 promoter variant being tested. Even when the complete 1.4-kb intergenic region between PininfC1 and PininfC3 was used as a promoter, for example, only half of the transformants expressed GUS. A similar result was obtained using constitutive promoters such as ham34 and hsp70. Previous studies indicated that the failure to express GUS from constitutive promoters was usually due not to rearrangement or truncation of the transgene, but instead to position effects (27).

Other deletions narrowed the region required for PininfC3 transcription to an 86-nt interval. Transformants employing promoter fragments with 5' endpoints 254 and 151 nt upstream of the transcription start site, but not one with the promoter fragment truncated to 65, produced GUS enzyme in cleaving sporangia (Fig. 2A and B). These transformants were also examined using RNA blotting, since enzyme levels may underestimate transcript accumulation during the rapid process of cleavage (45). In transformants utilizing either the 254 or 151 promoter deletions, strong mRNA induction was observed,
similar to that obtained using the full 1.4-kb region (Fig. 2C). No GUS mRNA was observed using the −65 promoter (not shown).

Conserved blocks within these regions were revealed by promoter alignments (Fig. 1). Several are within the −65 to −151 interval of PinifC3, which, as described above, is sufficient to confer stage-specific transcription. These represent potential binding sites for a cold-activated transcription factor(s). They probably bind between −151 and −92 since little conservation exists downstream of the latter. Conserved blocks also reside within the −151 to −254 region, such as the AAAAAATA at −206. The functions of such blocks are unclear, as their elimination did not block induction during cleavage. In theory, they may contribute quantitatively to transcript abundance. However, no trends in expression levels were noted in comparisons of transformants utilizing the full 1.4-kb intergenic region as a promoter versus the −254 or −151 fragment, although such analyses are difficult since in P. infestans neither the copy number nor the integration site of transgenes can be controlled. Integration-independent approaches for measuring promoters such as transient assays (26, 31), although more amenable to quantitative analysis, are not suited to studies of developmentally regulated promoters such as those of the PinifC genes.

**Site-directed mutagenesis of PinifC3.** Based on the deletion and alignment studies, it seemed that bases required for activating transcription during cleavage might reside between −151 and −92. Therefore, a mutagenesis scheme was undertaken in which blocks of 10 bases spanning that interval were sequentially altered (Fig. 3). To maximize the nature of the mutations, adenines were replaced with cytosines, and guanines were replaced with thymines. Only changes in the −141 to −131 region yielded transformants that failed to transcribe GUS in cleaving sporangia, based on both histochemical staining and reverse transcription-PCR. Notably, this region contains a GGACGAG motif that is absolutely conserved in P. infestans and P. sojae and conserved at six of seven bases in P. ramorum. Alterations in other sites did not result in phenotypes appreciably different from that with the full 1.4-kb promoter, such as constitutive expression and drastic quantitative variation.

**Using promoter chimeras to define the region needed for induction.** To better define the bases required for activating transcription during cleavage, and to test whether they act independently of other conserved portions of the PinifC3 promoter (such as those upstream of −151 or surrounding TSP2),
a chimeric promoter approach was employed. This involved mating-induced gene M82 (17). Native M82 exhibits low expression in hyphae, strong expression in mating cultures, and very low expression in asexual sporangia and cleaving sporangia.

Initially, the 60-nt block between −151 and −92 was inserted at site −560 of the M82 promoter in its native 5′-to-3′ orientation. All GUS-expressing transformants (four of five total transformants) accumulated GUS mRNA in cleaving but not undifferentiated sporangia; this is shown in Fig. 4 for two strains containing plasmid M82(C3-60S)::GUS. A similar result was observed when the 60-nt region was in the opposite orientation, using plasmid M82(C3-60A)::GUS. It is therefore concluded that sequences within the 60-nt region determine cold inducibility, act in a position- and orientation-independent manner, and act through a mechanism dominant to the normal mode of M82 regulation.

Next, the conserved GGACGAG within the 10-nt region shown by site-directed mutagenesis to be critical for induction was tested in an M82 chimera. The relevant plasmid, M82(C3-7S):GUS, resulted in cleavage-specific induction of the reporter in two of three transformants (Fig. 4). The 7-nt motif is therefore the presumed binding site for the transcription factor that activates PinifC3 when sporangia are shifted from ambient to cold temperatures. It was consequently named the cold box.

Presence of the cold box in other cleavage-associated genes. Whether P. infestans genes coinduced with the PinifC family also contained the 7-nt motif was assessed. This involved obtaining 350 nt of sequences upstream of nine P. infestans genes (pic2, pic7, pic9, pic11, pic15, pic19, pic21, pic23, and pic27) (46), which were searched for the motif in the sense and antisense directions. The latter was appropriate since both orientations functioned in the chimeric promoter studies (Fig. 4). The 7-nt motif was detected four times, in four of the nine genes. This is significantly higher (P < 0.03) than would be expected for random DNA of the same G+C content (50.5% G+C), and higher (P < 0.05) than expected based on the frequency at which GGACGAG was found in either orientation in 20 Mb of random P. infestans DNA (once every 5,200 nt). The genome resources for P. infestans are currently insufficient to determine the frequency of the heptamer in its promoters; however, analyses of 75 kb of putative promoter sequences extracted from the draft genome sequence of P. sojae revealed that the heptamer is actually underrepresented in such regions, being present once every 11,800 nt.

To validate the importance of finding the 7-nt motif in the four zoosporogenesis-induced P. infestans genes, an attempt was made to check whether their P. ramorum or P. sojae homologues also contained the sequence in the same position relative to the ATG codon. This was difficult to calculate in most cases since homologues, based on BLASTN searches, were absent from both P. ramorum and P. sojae. However, one exception was pic23, in which GGACGAG was detected in the putative promoter regions of each of the P. infestans, P. ramorum (gene model fgenesh1_pg_C_ scaffold_29000029), and P. sojae (gene model estExt_fgenesh1_pg_C_130067) orthologues. Moreover, the 7-nt sequence was at approximately the same position in each of the three genes (−119, −135, and −125, respectively).

These results suggest that comparative promoter analysis may be useful for Phytophthora spp., but the findings should be considered preliminary. This is because the number of genes sampled was small, the transcription start sites of the P. ramorum and P. sojae genes are unmapped, and whether the genes from the latter two species are induced during cold treatment or zoosporogenesis is not known.
Nuclear factor that binds the cold box. Electrophoretic mobility shift assays detected protein-binding activities that potentially regulate the PinifC loci. This involved nuclear extracts from cleaving sporangia and radiolabeled DNA corresponding to bases /H11002 151 to /H11002 92 of PinifC. Two prominent shifted bands were detected (Fig. 5). Both appear to define specific DNA-protein interactions since they were eliminated effectively by unlabeled PinifC fragments but not by a nonspecific competitor of similar G/C content (54%) amplified from pBluescript II SK(+). The graphs below the gel show quantitations of bands a and b (left and right panels, respectively), using increasing excess wild-type DNA competitor (circles), cold-box-mutated competitor (squares), and nonspecific competitor (diamonds).

Mechanisms for temperature perception in sporangia. Temperature determines whether germination occurs by release of zoospores (which predominates below 15°C), or directly by elaboration of germ tubes through the sporangial wall. In other biological systems, membrane fluidity is proposed to be a primary sensor of temperature, acting by modifying the activity of membrane-associated proteins. The latter might regulate the cold-box-binding protein.

To investigate whether the mode of germination in P. infestans correlates with membrane fluidity, the effects of dimethyl sulfoxide (DMSO) and benzyl alcohol were measured. These compounds fluidize and rigidify plasma membranes, respectively, and have been used to study temperature-regulated processes in several organisms (38, 40).

At every temperature tested, 35 mM DMSO (0.25%) stimulated zoospore release compared to that of the controls (Fig. 6). The effect was most striking at 18°C, when direct germination normally predominates, but DMSO caused 86% of germination events to involve zoospores. A similar trend was noted at higher and lower concentrations of DMSO; however, only the 35 mM data are reported here since it gave a noticeable effect and at that level forms only a minor component (1.4 mM) of the plasma membrane based on a log P(octanol-water partition coefficient) for DMSO of /H11002 1.35. In contrast to the effect of DMSO, 10 mM benzyl alcohol inhibited zoospore release at every temperature and stimulated direct germination at 18°C. However, benzyl alcohol did not enable direct germination to occur below 15°C.

Effects of DMSO and benzyl alcohol on gene expression. The influence of these chemicals on PinifC expression was consistent with a model in which transcription during zoosporeogenesis is induced by the effect of cold on membrane fluidity. DMSO enhanced the level of mRNA from the three PinifC genes in sporangia kept at 12°C for 30 min, while benzyl alcohol suppressed expression (Fig. 7A). As illustrated in Fig. 7B, the same result was observed for GUS driven by a chimeric M82-PinifC promoter in plasmid M82(C3-60S)::GUS. It follows that membrane rigidification induces transcription by acting through the cold box.

DISCUSSION

For more than a century, it has been recognized that temperature is a critical factor in the epidemiology of potato late...
of a phospholipase, kinase, or other enzyme may be induced by conformational changes resulting from a transition of the plasma membrane from a liquid crystalline to a more rigid state, or by altered access to activators due to reduced phospholipid "flip-flop" between inner and outer leaflets (5).

The best-understood examples of the effect of membrane fluidity on signaling proteins involve phospholipases C and D in eukaryotes and histidine kinases in bacteria (39, 44). In Phytophthora spp., such enzymes or other membrane-associated proteins such as Ca\(^{2+}\) channels may participate in detecting cold, and indeed there is evidence that multiple mechanisms for transducing the temperature signal exist. Our previous study of the effect of inhibitors on zoosporogenesis-specific transcription supported the presence of four signaling mechanisms; these involved an inositol trisphosphate-regulated Ca\(^{2+}\) channel, an inositol trisphosphate-independent Ca\(^{2+}\) channel, a diacylglycerol-dependent pathway, and a Ca\(^{2+}\)-plus-phospholipid-independent pathway (46). Multiple membrane-associated effectors may therefore exist, and participants independent of the membrane such as cold-induced microtubule disassembly cannot be discounted (1). A logical next step in identifying such effectors could involve determining how the transcription factor that binds the GGACGAG cold box becomes activated.

The cold box was identified based on functional tests performed prior to the availability of genome sequence data for P. ramorum and P. sojae. Nevertheless, a retrospective look at the feasibility of employing phylogenetic footprinting to identify binding sites for transcription factors in the genus is useful. In other systems, regulatory modules within promoters have been predicted by searching for regions that stand out from a non-conserved background (19). The best results are obtained using species of moderate overall genome similarity (60 to 70%), as in comparisons between humans and rodents or between Saccharomyces species (11, 28). Predictions are more difficult between close species, such as primates, since distinguishing functional from passive conservation is challenging (3). Our analyses suggest that the three Phytophthora species are suitably distant to perform phylogenetic footprinting, since the blocks of conservation evident in the alignment in Fig. 1 encompassed 51% of the promoter region. This compares with average identities in overall coding sequences between P. infestans and P. ramorum and between P. infestans and P. sojae of about 82% (unpublished data).

However, phylogenetic footprinting in Phytophthora spp. will benefit from more-accurate transcript mapping and expression data for all three species, and comprehensive genome data from P. infestans (fortunately, public funding for an eightfold draft sequence of P. infestans was recently obtained). For example, while the 7-nt cold box was 100% conserved between P. infestans and P. sojae, there was one mismatch in P. ramorum. This could indicate divergence in the specificity of the cold-box-binding protein. Alternatively, the NIF genes from P. ramorum may be expressed differently than the P. infestans genes; unfortunately, our ability to test this is impaired by quarantine restrictions concerning this invasive pathogen in California (15). Another limitation on comparative studies is a dearth of functional data on oomycete promoters, especially binding sites for general transcription factors. The PinifC genes contain the first stage-specific promoters to be dissected; how-
ever, even for constitutive promoters, only a few functional studies have been performed (7, 31).

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