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* 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 appressorium 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-

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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 wild-type or mutagenized portions of the *PinifC1*, *PinifC3*, and *M82* promoters were inserted into the ApaI and ClaI sites upstream of GUS. pOGUS also contains an *npt* 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-255, C3-151, and C3-65, respectively. The *M82* promoter was amplified using primers M82F and M82R. The 60-nt and 7-nt regions of the *PinifC3* promoters destined for insertion upstream of *M82* were amplified using C3-92Rp and C3-151 M, and M82C3-7F and M82R, respectively.

For site-directed mutagenesis of *PiNifC3*, a two-stage PCR strategy was used. The first involved separate reactions in which fragments extending leftward and rightward of the mutagenized sites were amplified, which were joined in the second stage using oligonucleotides NLIC1-2RA and NLIC3RC. The first/second-stage primer sets used to generate p151M, p141M, p131M, p121M, p111M, and p101M were NLIC12RA/C3151MR and NLIC3RC/C3151MF; NLIC12RA/C3141MR and NLIC3RC/C3141MF; NLIC12RA/C3131MR and NLIC3RC/C3131MF; NLIC12RA/C3121MR and NLIC3RC/C3121MF; NLIC12RA/C3111MR and NLIC3RC/C3111MF; and NLIC12RA/C3101MR and NLIC3RC/C3101MF, respectively.

RNA analysis. RNA was extracted and blotted as described previously (25). Radiolabeled probes were generated from full-length cDNA clones or gene-specific fragments were amplified using primer pair NIFC1SF-NIFC1SR, NIFC2SF-NIFC2SR, or NIFC2SF-NIFC2SR. Signals were detected by phosphorimager analysis. Random amplification of cDNA end (RACE) products were generated with a kit (Invitrogen, Carlsbad, CA) using reverse transcription at 42°C and 50°C, cloned into pGEMT-EZ (Promega, Madison, WI), and sequenced. Reverse transcription-PCR was performed with primers GUSF and GUSR against DNase-treated RNA using a one-step kit (Invitrogen) and 35 cycles of PCR; in such assays, no bands were observed in controls lacking reverse transcriptase.

Sequence analyses. Sequences were assembled using Seqman for Macintosh (DNASTAR, Madison, WI). Promoter alignments were performed using ClustalW. *Phytophthora ramorum* and *Phytophthora sojae* sequences were from assembly 1.0 of their draft genome sequences, which are available online from the Joint Genome Institute of the U.S. Department of Energy (Walnut Creek, CA).

Electrophoretic mobility shift assays. Nuclear extracts were prepared using sporangia from 15 150-mm petri plates which were flooded with cold water, incubated at 10°C for 40 min to induce cleavage, homogenized in liquid nitrogen, suspended in buffer containing 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES)–KOH, pH 7.0, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 25% glycerol, and 0.5% Triton X-100, and processed as described but without Percoll centrifugation (33). DNA probes were amplified from *PinifC3* using primers NLIC360F and NLIC360R. After end-labeling with ^{32}P, 10,000 cpm was mixed with 10 μg of nuclear protein in binding buffer (125 mM HEPES, pH 7.9, 100

TABLE 1. Oligonucleotides used for PCR

Name	Sequence (5′ to 3′)
C3101MF	GATTGTCAAGGTTAGTGAGACAAGGCCCTTCCATG
C3101MR	AAGGGCCTTGTCTCACTAACCTTGACAATCAAAAC
C3111MF	GTAAGGTTTTTCGGTGACCTTGGCTGTCTCCAAG
C3111MR	AGACAGCCAAGGTCACCGAAAAAACCTTACCACCC
C3121MF	CTCGAGGGTGTGCCTTGGGGGATTGTCAAGTGGC
C3121MR	TTGACAATCCCCCAAGGCACACCCTCGAGTCTCG
C3131MF	GCGGACGAGAAGATCTTTGTGTAAGGTTTTGATTG
C3131MR	AAAACCTTACACAAAGATCTTCTCGTCCGCTCTCG
C3141MF	CGAGGTCAGATATTCATCTCCTCGAGGGTGGTAA
C3141MR	CACCCTCGAGGAGATGAATATCTGACCTCGCCGTT
C3151MF	GGAGGAACGGATCTTGACTCGCGGACGAGACTCGA
C3151MR	TCTCGTCCGCGAGTCAAGATCCGTTCCTCCCTTAT
C392Rp	TTGGGCCCGAGACAGCCACTTGACAATC
C3F151	AAGGGCCCCGAGGTCAGAGCGGACGAGAC
C3F254	AAGGGCCCACCTGCCTCAGCGTAATTGTA
C3F65	AAGGGCCCCAAACTCGCTTTCAACGCACTG
M82C37F	AAGGGCCCGGACGAGTCTTCGGCAGCATTACCAC
M82F	AAGGGCCCTCTTCGGCAGCATTACCA
M82R	CCATCGATGGTTAAGGGTGGTCTTGGAA
NIFC1SF	ATGATGACGATGCCGACCAC
NIFC1SR	GCTTTGCTGTTATCTTCGGC
NIFC2SF	CAAGTCCAGTGGTCTATCGC
NIFC2SR	GCTCAATAGTGGTGCCCAAA
NIFC3SF	CTGAATATCACCTACCCACTG
NIFC3SR	GGAAACTGGCGGCAAGAGACT
NLIC12RA	AAGGGCCCGTCCGAAAAGGGGTCTTGG
NLIC360F	CCCAAGCTTTATGGATAAGGGAGGAACGG
NLIC360R	GCTCTAGAAAGGGCCTTGGAGACAGC
NLIC3RC	CCATCGATTCTTTACTGAGATTGTCTCGTTA
GUSF	TAGAAACCCCAACCCGTGAA
GUSR	GCAATAACATACGGCGTGAC

mM KCl, 25 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 50% glycerol) for 15 min at 4°C, and electrophoresed in 4% acrylamide. For competition assays, unlabeled DNA was added 15 min before the labeled probe. Competitors were from either the native *PinifC3* promoter, the mutated version present in plasmid p141M, or pBluescript II SK+ (Invitrogen).

RESULTS

Mapping transcription start points. As described earlier (45), *PinifC1*, *PinifC2*, and *PinifC3* are clustered within *P. infestans* and appear to be transcribed exclusively during zoosporogenesis. The *PinifC1* and *PinifC3* open reading frames are in opposite orientations, separated by a 1.4-kb intergenic region. *PinifC2* resides 1.2 kb downstream of *PinifC1*. As a first step towards characterizing their promoters, transcription start sites were mapped by sequencing cDNAs primed by oligo(dT) or oligonucleotides binding near their translational start codons (5′ RACE).

The 5′ termini of the reaction products mapped to two regions, named TSP1 and TSP2. In *PinifC1*, *PinifC2*, and *PinifC3*, TSP1 is 116, 85, and 123 nt upstream of the ATG codon, respectively, while TSP2 is at 74, 42, and 75 nt, respectively. These data may reflect in vivo heterogeneity in initiation sites as is common in many species (21, 29), or artifacts during reverse transcription due to a high cytosine content between TSP1 and TSP2. Since nearly all 5′ RACE products ended at TSP2, including those assays performed at elevated temperatures, for simplicity TSP2 will be referred to as +1 in this article.

An alignment of sequences upstream of the ATG codons in the three *P. infestans* genes is shown in Fig. 1, along with the sites of TSP1 and TSP2. The figure also aligns promoters from putative *P. ramorum* and *P. sojae* homologues, which were extracted from draft genome data. Although the sequences from *P. ramorum* and *P. sojae* were not available during the

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                Distance from:
                TSP  ATG
PinifC1  -271  -349  TGTCTCGGTGTAGGTGCGCATGTAAATTGCACAGGTT----GAGCAGGTAAAAAATAAGCACAA---CGTCATTTGCA--------------------
PinifC2  -217  -255  GGTGGAAT----------GCACAGGTTTGGAAGGAT---CGCC----AAAAATAAACCG----CGTCCATGTTAA----------
PinifC3  -252  -329  CCTCAGCGTAATTGTATTAATAGAAGATGAGAATTAA----TGACGTATAAAAATAGGCGCAA---CGTCATCG--------

Psnif1   -449  CGCATCGTTCGGGATAAGTGCACAGGCTTAGAAGAGTGAAACGCACTGTCAGGTGAAGGATAGCCGTGGCAGCAGCACC
Psnif2   -318  GGTGGAATTACTCGCTA----GCACAGGCACAGAAGAAT----CGCAACGCATCGCAAAAATAAACCT--CCACACCAAAGGCAACGATAGGGTGATG---------
Psnif3   -443  AGTTCGATTCGGGATAAGTGCACAGGCTTAGACGAGTGAAACGCATCGCAAAAATAAATGCAACACTGTCAATGATAGCAGTGGCAGCAGTTGGGCA---
Prnif1   -357  GACTTCCTTCGT------TGCACAGGTTTAGAAGA-----ACTAATCGCAAAAATAAATCCAACGCCATCCAGGATAGCAAACACAACAAAGGA------
Prnif2   -348  GGTGGAATTCTTTCCAG-CGCACAGGTTAAGAAGAGT----TGGAACGCAAAAATAAACGCAA---CGTCGTCA--------------------

                                                                   -151        cold box
PinifC1  -201  -279  AACAGTTGGCTCATTG-----GATGAAAGAAGGAACGGGTTGCCTTACAGAT--CGAGGCCAGGACGAGATTCGGAG----------GCGATAAGA
PinifC2  -175  -213  CATCTTTTGTTCTTGAG-------AATCAAGGTAGAACGGATTGCCTGCCGCCT--TTGTGTCGG--AGGACGAG-----------------GTGCCAAGT
PinifC3  -182  -259  GCTGGGTTGGATTATGG-------ATAAGGGAGGAACGG--------------CGAGGTCAGAGCGGACGAGACTCGGAG----------GTGGTAAGG

Psnif1   -349  AATACACGGATGCGAGCGGAGAAATCTCGGGTGAATGGGTTGCCATACTCCACACGAGGTCAC--CGGACGAGATTCTGAGGATGCAAGTTGTGTCAAGT
Psnif2   -235  ATGAGCTGGCTTAAAG------GATCAAGGGGGAACGGGTTGCTCGACACCT--TGAGGTCCG--TGGACGAGACTTTGAGGGAG-C----GTGTCAAGT
Psnif3   -346  GCCGCATGGATGCGTGCGG-AGATTTCTCGGGTGGATGGGTTGCCGGACACCTT-CGACGTGAC--CGGACGAGACTTCTGAGGATGCAAGTTGTGTCAAGT
Prnif1   -274  TGCCGTACAGGTGATAG----GGAATCATGGAGGGACGGGTTGCGCTGCCCTAACGCT--CGAGGTCAT--CGGGCGAGACGTGGAGGGAG-AAATAGTGTCAAGT
Prnif2   -282  AG------GATAGACGCTGGAAGGTCAAGGAGGGACGGGTTGCGCAACACGT--CGAGGCCAT--CGGGCGAGACGTGGAGGGAG-AAATAGTGTCAAGT

                                   -92                     -65               TSP1
PinifC1  -118  -196  TTGTGTTTGTCGCGTGG---AAGCACAGCGGGCCATGAACGACATCCATTTGAAAACTTGCTCCCAAGACACCACTCTACACAAGCCAGCACCCCTCC
PinifC2  -103  -141  GTTTGCAGT--CCACTG------TCAGGATATCGATACGCGCTGCTACTGAAG----AGGTATTCTCAGTTTGGACA---ACACGACCGCACCGCTCTGC
PinifC3  -115  -192  TTTTGATTGTCAAGTGG---CTGTCTC--CAAGGCCCTTCCATATATCCATTCGAAAACTCGCTTTCAACGCACTGCACTACCCACCCCCCCCCTCCCC

Psnif1   -249  GGGCCGTGGTGCCGTTGACCGGGTTG-GTTTTCGACTCCCGACGCTGCCGTTTCCCAGCTTAGTATCACATTTGAAGTCAAGCTCCCACCCAACACAGCAC
Psnif2   -150  GTGTGCAGT--CCGGGGCCGTCGGAGTTTCCGTCGGCCACCGGTC--GTT----AATTATTATCACTTTGGAGG---ACTAGCCGCACTGC---C
Psnif3   -251  GGGCCGTGGTGCCGTTGACCGGGTTG-GTTTTCGACTCCCGACGCTGCCGTTTACCAGCTTAGTATCACATTTGAAGTCAAGCTCCCACCAACACAGCAC
Prnif1   -183  GTGTGCTGG-----CCGTTGCTGCGCGTTCGAGTTTGCGGTGCTC----CTTCCGTTT---GATTTATTATCGCATTCGAGC---ACTAGCCAGCACCGCACCAC
Prnif2   -193  GTGTGCTGG-----CCGTTGCCCTTCGAGTTTGCGGTGCTCCTTCCGTT---TG----ATTTATTATCGCATTCGAGC---ACTAGCCAGCACCGCACCAC

                        TSP2
PinifC1   -21   -99  --GCTACA-------CAACAACAAAAACTACAGGTCACCACATCAGTTCCAAGACCCCTTTCGGACAGGACTCT--AAGCGTATCCAGACGCAGCGCTAC
PinifC2   -18   -56  --ATCACCCTCC----CAATAGCAACACTCAACTAGGACCCGCATTCCAACACA-C-----TCCAGCA--------------------
PinifC3   -20   -97  --CCAACAGTTACAGCAACAACAACAATTCCAGCTCGCAACTCCAGTTCAAGACCCTCGTTCGACCAGAACTTTTCAAGCGTCTCGCAGC-TAACGAGAC

Psnif1   -149  CAACCAACCAACCACCACCACCAACCCGTTCGAAACCTCGGAAGTCACGTCAAGAGGGACGCAGGCCCCTTTTCGGACAGGACTCGAAAGCGTACCGGTAC
Psnif2    -65  --ACCTCCGCCCACAGCGCACCGTCCGCACCAGCTTTCTAAGCCACCACCAAGAGGGACGCAGGCCCCTTTTCGGACAGGACTCGAAAGCGTACCGGTAC
Psnif3   -152  CAACCACCCAATCACCACCACCAACCTCGGAAACCTCGGGAAGTCACGTCAAGAGGGACGCAGGCCCCTTTTCGGACAGGACTCGAAAGCGTACCGGTAC
Prnif1    -95  --GCCACAC--AACAGCAGCTAGT--CTTTAGACGATTTTCCAGGGACCTCACAAG-----CAAGGCCCCGTTCAGACAA-ACTC-AAAGCGTCTCGATAC
Prnif2   -105  --ACCACACAAC-AGCAGCTAGTCTTTAGACGATTTTCCAGG--GACCTCACAAG-----CAAGGCCCCGTTCAGACAAACTCAAAGCGTCTCGATACGC
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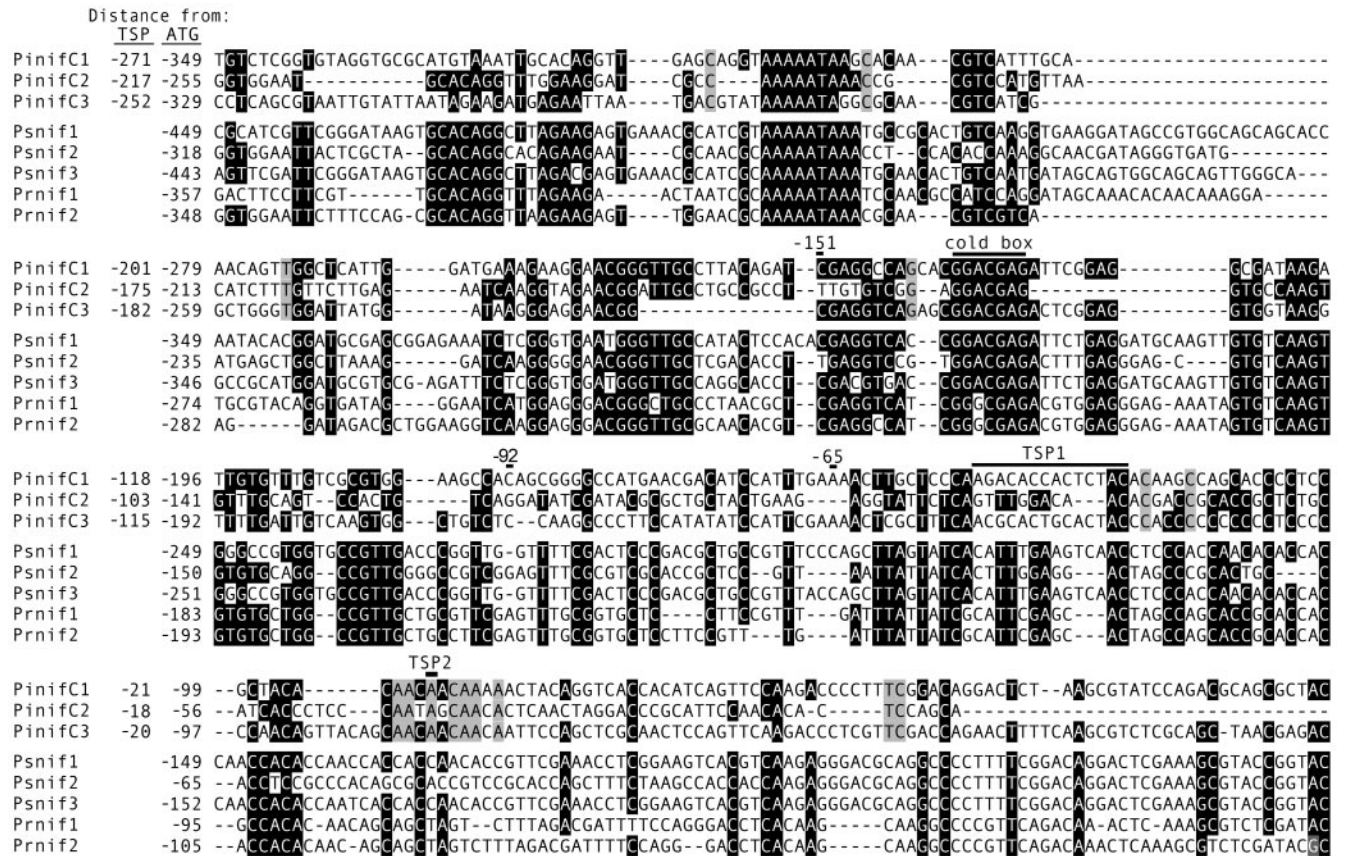
FIG. 1. Promoters of *Phytophthora* NIF genes. Shown (top to bottom) are alignments of portions of the promoters of the *PinifC1*, *PinifC2*, and *PinifC3* genes of *P. infestans*, three putative homologues from *P. sojae*, and two from *P. ramorum*. The last five are extracted from sequences upstream of gene models estExt_fgenesh1_pg.C_260137, estExt_fgenesh1_pg.C_260134, estExt_fgenesh1_pg.C_260135, fgenesh1_pg.C_scaffold_35000061, and fgenesh1_pg.C_scaffold_35000062 from assembly 1.0 of their respective genome projects. Numbers in the left margin indicate distances from transcriptional and/or translational start points; only the latter are predicted for the *P. sojae* and *P. ramorum* genes. Black shading represents >70% identity among the eight genes, and gray shading indicates identity in *P. infestans* only. Indicated at the top of each panel for the *P. infestans* genes are approximate areas of predicted transcription start sites (TSP1 and TSP2), deletion endpoints used in functional studies of *PinifC3*, and the 7-nt cold box required for gene induction during zoosporogenesis. For *PinifC1* and *PinifC2*, a majority of 5′ transcript termini appeared to be within TSP1, while for *PinifC3* most were at TSP2.

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 *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 *P. infestans* (31).

Deletion analysis of the *PinifC1*/*PinifC3* promoter region. Truncated fragments of the *PinifC1*/*PinifC3* 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 *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 ex-

periments to express GUS presumably reflects the general nature of transformation in *P. infestans* and not any feature specific to the particular *PinifC3* promoter variant being tested. Even when the complete 1.4-kb intergenic region between *PinifC1* and *PinifC3* 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 *PinifC3* 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 understate transcript accumulation during the rapid process of cleavage (45). In transformants utilizing either the −254 or −151 promoter deletions, strong mRNA induction was observed,
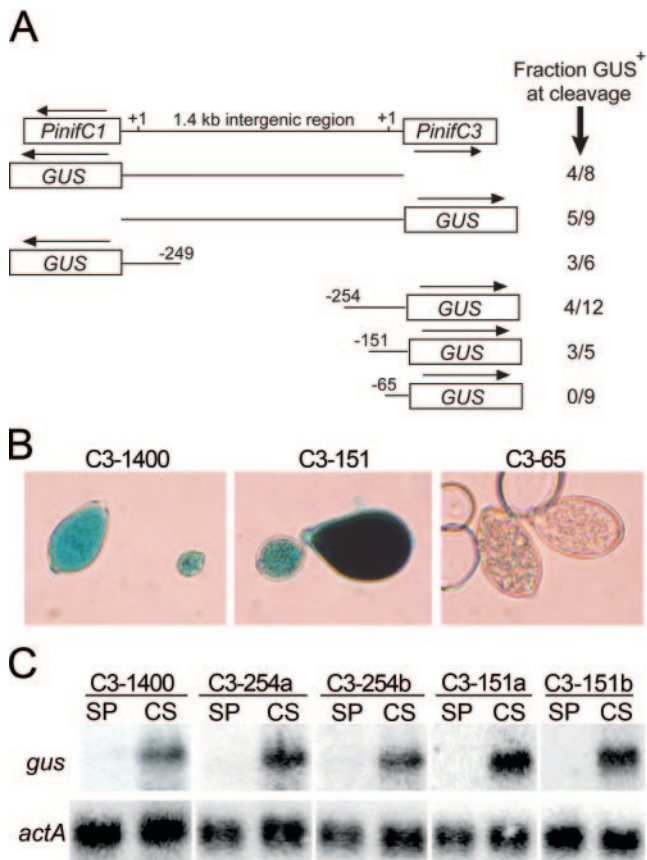
FIG. 2. Deletion analyses of *PinifC* promoters. (A) Arrangement of *PinifC1* and *PinifC3* in the *P. infestans* genome, and GUS fusions made with fragments of their intergenic region. Indicated to the right for each fusion are the fraction of total transformants expressing GUS in cleaving sporangia; transformants in the negative class failed to express GUS at any life stage. Constructs represent, top to bottom, those with the entire 1.4-kb intergenic region, C1-1400::GUS or C3-1400::GUS; one with the 249-nt 5′ region of *PinifC1*, C1-249::GUS; and those with the 254-, 151-, and 65-nt regions 5′ to *PinifC3*, named C1-254::GUS, C1-151::GUS, and C1-65::GUS, respectively. (B) Representative transformants obtained using C3-1400::GUS and C3-151::GUS, which produce GUS in cleaving sporangia, and C3-65::GUS, which fails to express the reporter. (C) Blot analysis of RNA from freshly harvested undifferentiated sporangia (SP) and sporangia treated at 10°C for 40 min to induce cleavage (CS), hybridized with a probe for the GUS gene (*gus*) and actin (*actA*). The strains shown are a transformant expressing GUS from C3-1400::GUS, two strains with C3-254::GUS, and two strains with C3-151::GUS.

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 AAAAATA 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),
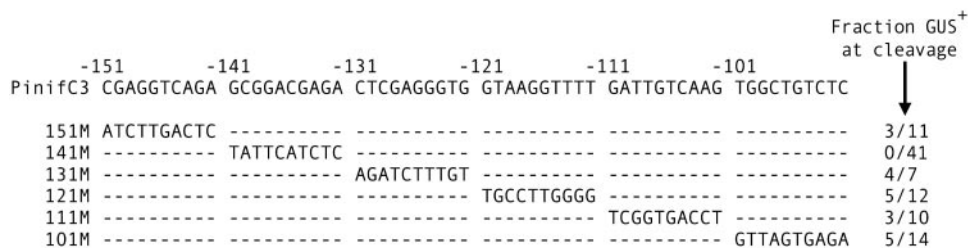

FIG. 3. Site-directed mutagenesis of 60-nt region of *PinifC3*. Illustrated are the −92 to −151 portions of the wild-type gene and six mutant versions; dashes represent no change from the wild-type sequence. Indicated to the right are the fraction of total transformants expressing GUS in cleaving sporangia.
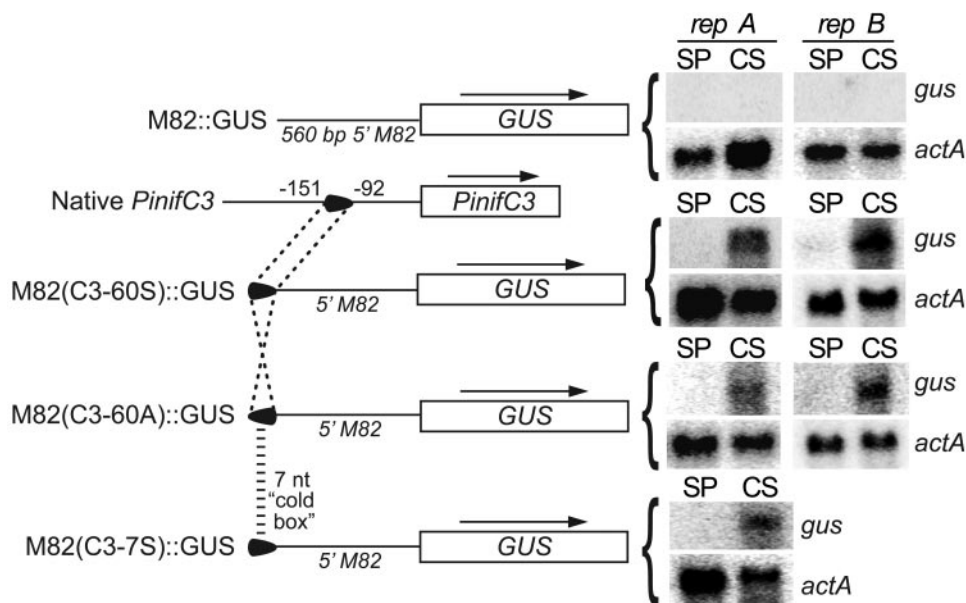
FIG. 4. Analysis of chimeric promoters. Shown on the left are, top to bottom, schematics of the *M82* promoter fused to GUS; *PinifC3*, including the 60-nt region; a fusion between GUS, the *M82* promoter, and the 60-nt region in the sense orientation; a fusion between GUS, the *M82* promoter, and the 60-nt region in the antisense orientation; and a fusion between GUS, the *M82* promoter, and the 7-nt cold box. On the right are blots of RNA from representative strains transformed with each plasmid (*repA* and *repB*), using the same tissues and probes as in Fig. 2.

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.
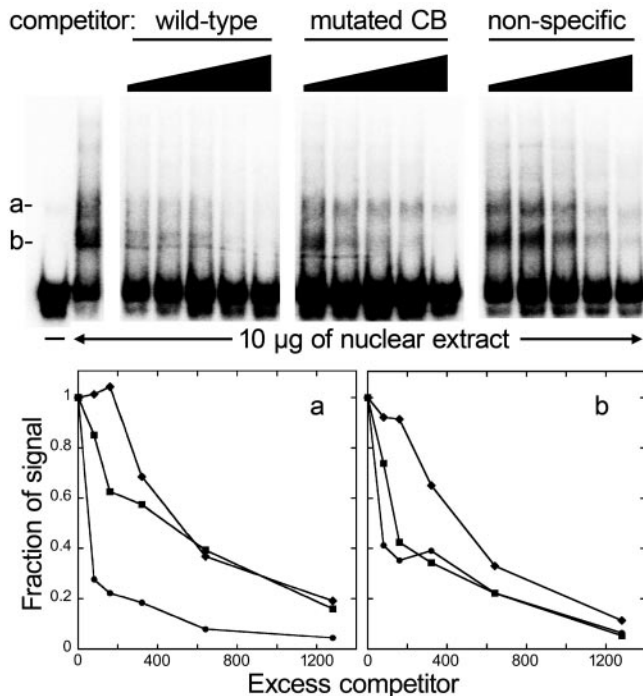
FIG. 5. Electrophoretic mobility shift assay using the *PinifC3* promoter. Labeled double-stranded DNA from the −151 to −92 region of *PinifC3* was mixed with 10 μg of nuclear protein from cleaving sporangia and increasing amounts of unlabeled competitor DNA representing an approximately 80- to 1,280-fold excess. The competitors are, left to right, the normal −151 to −92 region (wild type); the −151 to −92 region containing an altered 7-nt cold box (mutated CB); and the pBluescript II SK(+) multiple cloning site (nonspecific). 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).

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 −151 to −92 of *PinifC3*. Two prominent shifted bands were detected (Fig. 5). Both appear to define specific DNA-protein interactions since they were eliminated effectively by unlabeled *PinifC3* fragments but not by a nonspecific competitor of similar G+C content (54%) amplified from pBluescript II SK(+). The shift band of higher mobility (band b) was eliminated nearly equally by 60-nt DNAs representing the wild-type sequence and one with a mutated cold box. In contrast, the species of lower mobility (band a) was eliminated at least 20 times more effectively by the wild-type sequence than one with a mutated cold box. Therefore, band a likely results from association with the cold-box-binding transcription factor.

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
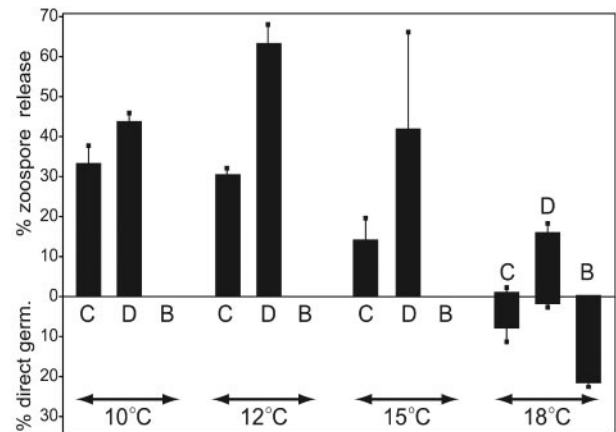

FIG. 6. Influence of modifiers of membrane rigidity on *P. infestans* germination. Sporangia (10^4/ml in water) were incubated for 30 min at the indicated temperatures in water as a control (C), 35 mM DMSO (D), or 10 mM benzyl alcohol (B). The average percentage of sporangia germinating directly or through zoospores was recorded; error bars represent standard deviations based on four independent replicates.

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 −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 zoosporogenesis 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
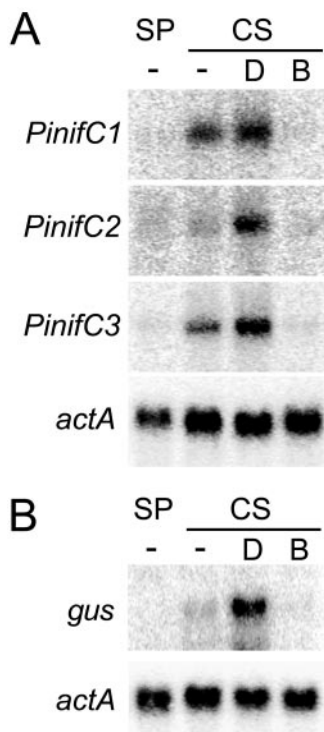
FIG. 7. Influence of modifiers of membrane rigidity on transcription. (A) Blot of RNA from undifferentiated sporangia (SP) and cleaving sporangia (CS) treated with 35 mM DMSO (D), 10 mM benzyl alcohol (B), or no chemical (−). Blots were hybridized with specific probes for *PinifC1*, *PinifC2*, *PinifC3*, or *actA*. (B) Same as panel A except that the RNA was from a transformant expressing M82 (C3-60S)::GUS, and a probe for GUS (*gus*) was employed.

blight (reference 4 and references therein). Early researchers noted that a low temperature (or at least the avoidance of warmer temperatures) was important for causing sporangia to release zoospores (13). Computer programs used to forecast late blight often incorporate measurements of the time that potato fields are at low temperature and give a higher score if conditions are optimal for zoospore emergence (47, 50). Since such programs are used by growers to plan fungicide applications, understanding how cool temperatures affect sporangia is of practical as well as fundamental importance.

This paper presents the first data suggesting that an increase in membrane rigidity is responsible for triggering the cellular and molecular events that characterize zoosporogenesis. Since zoospores can be produced in the absence of de novo transcription or translation (10), zoosporogenesis-induced genes such as the *PinifC* loci are probably required not for zoosporogenesis but instead for later stages, such as those between cyst germination and appressorium development. Nevertheless, zoosporogenesis and *PinifC* loci undoubtedly are regulated by common signals. Since previous research suggested that phospholipid messengers were required for both zoosporogenesis and *PinifC3* expression (46), the signaling pathway may involve a membrane-bound phospholipase. The nature of that enzyme remains obscure since we have been unable to convincingly show that inositol trisphosphate accumulates in chilled sporangia (unpublished). Nevertheless, oscillations in the activity

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 nonconserved 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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