Transgene-Induced Silencing of the Zoosporogenesis-Specific
*NIFC* Gene Cluster of *Phytophthora infestans*
Involves Chromatin Alterations

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Clustered within the genome of the oomycete phytopathogen *Phytophthora infestans* are four genes encoding spore-specific nuclear LIM interactor-interacting factors (NIF proteins, a type of transcriptional regulator) that are moderately conserved in DNA sequence. *NIFC1*, *NIFC2*, and *NIFC3* are zoosporogenesis-induced and grouped within 4 kb, and 20 kb away resides a sporulation-induced form, *NIFS*. To test the function of the *NIFC* family, plasmids expressing full-length hairpin constructs of *NIFC1* or *NIFC2* were stably transformed into *P. infestans*. This triggered silencing of the cognate gene in about one-third of transformants, and all three *NIFC* genes were usually cosilenced. However, *NIFS* escaped silencing despite its high sequence similarity to the *NIFC* genes. Silencing of the three *NIFC* genes impaired zoospore cyst germination by 60% but did not affect other aspects of the life cycle. Silencing was transcriptional based on nuclear run-on assays and associated with tighter chromatin packing based on nuclease accessibility experiments. The chromatin alterations extended a few hundred nucleotides beyond the boundaries of the transcribed region of the *NIFC* cluster and were not associated with increased DNA methylation. A plasmid expressing a short hairpin RNA having sequence similarity only to *NIFC1* silenced both that gene and an adjacent member of the gene cluster, likely due to the expansion of a heterochromatic domain from the targeted locus. These data help illuminate the mechanism of silencing in *Phytophthora* and suggest that caution should be used when interpreting silencing experiments involving closely spaced genes.

Strategies for testing the function of genes in eukaryotes often involve transcriptional or posttranscriptional gene silencing methods (TGS and PTGS, respectively), which exploit natural RNA regulation systems of the cell. For example, the canonical RNA interference (RNAi) pathway of PTGS involves introducing or generating within cells double-stranded RNA (dsRNA). This is processed into 20- to 25-nucleotide (nt) small interfering RNAs (siRNAs) by Dicer, which join the RISC complex to direct the cleavage of cognate mRNA through the “slicing” action of Argonaute (28, 43). In contrast, TGS is induced traditionally by introducing extra copies of a gene, which repress both the transgene and the native locus through chromatin remodeling, histone modification, and/or DNA methylation (53). However, distinctions between TGS and PTGS are blurry since PTGS may also lead to chromatin alterations, and dsRNA can also trigger TGS if targeted to a promoter (2, 23, 29). Moreover, in *Schizosaccharomyces pombe* a single Argonaute protein is required for both PTGS and TGS (41). More knowledge about this machinery is needed to both advance gene silencing methods and understand how it participates in the normal regulation of the transcriptome.

Only a few reports exist of the use of silencing methods to test gene function in oomycetes, which are fungus-like eukaryotes that include significant plant and animal pathogens. Genes participating in the biology of spores are of particular interest due to their roles in dissemination and infection (17). In the oomycete genus *Phytophthora*, for example, asexual sporangia are transported between plants by wind or water. At cool temperatures, the sporangia release motile zoospores that can home in and encyst on a host and then breach its barriers using a germ tube or appressorium. Many genes transcribed specifically at these stages have been identified in the potato late blight agent *P. infestans*, as well as related species (24, 39, 42, 47).

The functions of only a few of these genes have been tested. By expressing sense or antisense copies of their open reading frames in transformants of *P. infestans*, a Cdc14 phosphatase, α and β G-protein subunits, and a bZIP transcription factor were silenced and proved important in sporulation, zoospore behavior, or infection (1, 3, 25, 26). Other studies suggested that homology-based silencing in *Phytophthora* is transcriptional and not associated with DNA mutation (20, 52). Despite some success in silencing, several loci have proved to be recalcitrant to the technique (18; G. Senthil and H. Judelson, unpublished data). More information on how silencing occurs would therefore aid functional studies of spore development, as well as other aspects of oomycete biology.

In *P. infestans*, one group of genes expressed specifically during the spore cycle encodes NIF proteins. NIF (Pfam03031) stands for nuclear LIM interactor-interacting factors, where LIM represents a group of homeodomain transcription factors first shown to regulate development in animals. NIF factors contain a phosphatase region and are thought to regulate...
mRNA synthesis by interacting with the C-terminal domain of RNA polymerase II and other components of the transcriptional apparatus (9, 21, 57). In P. infestans, NIFS is expressed during sporulation, whereas NIFC1, NIFC2, and NIFC3 are expressed during zoosporogenesis (46). Other proteins with NIF-like domains are present in hyphae but appear to be membrane translocases (32, 46). It was therefore hypothesized that the NIFS and NIFC proteins are needed to transcribe specific sets of genes at stages of the spore cycle. For example, NIFC-dependent factors might be required for the germination of zoospore cysts or appressorium formation. Interestingly, the three NIFC genes are tightly clustered within the genome, and their promoters share a binding site for a cold-activated transcription factor (46).

In the present study, homology-based silencing methods directed against the NIFC family were used both to test the function of those genes and to better illuminate the mechanism of silencing in Phytophthora. Plasmids expressing inverted repeats of single NIFC genes transcriptionally silenced both the cognate gene and other members of the family, despite moderate divergence in sequence. This was attributed to the establishment of a heterochromatic region, which could spread between loci within the NIFC cluster. Silencing of NIFC genes caused defects in zoospore cyst germination but not other portions of the life cycle.

**MATERIALS AND METHODS**

**Growth and transformation of P. infestans.** Developmental stages were obtained as described previously (3); other details are provided in the footnotes to Table 1. In particular, zoosporogenesis was induced by liberating sporangia from hyphal mats by rubbing the plates with water, followed by passage of the liquid through 50-μm-pore-size nylon mesh to remove hyphal fragments and incubation of the sporangia at 10°C for 60 to 90 min. Transformations were performed by using the protoplast method and G418 selection (19), using circular plasmids pCISIA, pCIS2A (Fig. 1A), and pCIPH. Plasmids pCISIA and pCIS2A were constructed in pTOR (49) by inserting open reading frames from NIFC1 and NIFC2, respectively, in sense and antisense orientations separated by a 25-nt intron from the Ste20-like PEC20 gene (47). To construct pCHIP, two complementary oligonucleotides (5′-CGAAGAGAAGTTGCCATGAACTG-3′ and 5′-CTTTGCTCAGTGCCCACTTCTG-3′) were inserted into pTOR. The resulting construct contains a 21-nt duplex region, separated by a 9-nt loop, that matches a region unique to the 5′ end of the NIFC1 gene (Fig. 1C).

**RNA and DNA hybridization analysis.** RNA was extracted, after grinding in liquid nitrogen, using the Qiagen plant minikit (Qiagen, Valencia, CA). Total DNA was extracted as described previously (19), except for nuclear DNA which was purified as described below. Hybridizations were performed as described previously (3). Radiolabeled probes were generated from cDNA clones of MUSC (8), elongation factor-1α (EF-1), and protein kinase KNN1 (GenBank accession number CV052935); a cloned portion of a P. infestans Gypsy-like retroelement (16); or sequences amplified by PCR using primers for NIFC1 (5′-ATAGTACGACGGCCACCGAACT-3′ and 5′-GGTTGTCGCTATCCTCCGGA-3′), referred to as to and +329 in Results); NIFC2 (+1 and +318; 5′-CAAGTTCGTTGCTATCGTGC-3′ and 5′-GGCTCAATAGTGTTGCCAAA-3′), NIFC3 (+14 and +382; 5′-CTGAAATACCACTCCTACCGAATG-3′ and 5′-GGGAAATCTCGGACAGAGAG-3′), and NIFS (+23 and +255; 5′-GGACGAGACCTCTGATGTAACAC-3′ and 5′-GGGCATCCAGTGCATGAAACA-3′), as NIFS amplitves behave as locus-specific probes in high-stringency hybridizations. RT-PCR. For preliminary screens of transformants, 0.2 μg of DNase-treated RNA was reverse transcribed and amplified for 30 cycles using the Superscript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) and primer sets described in Results. Other reactions were performed by two-step reverse transcription-PCR (RT-PCR) using cDNA primed with oligo(dT). Controls lacking reverse transcriptase were included to ensure that amplicons were not derived from genomic or contaminating DNA.

**Nuclease accessibility assays.** Nuclei from cold-treated sporangia (36, 47) were treated with DNase I according to a protocol adapted from Weinmann et al. (55). After the nuclei were resuspended in 100 mM NaCl 50 mM Tris (pH 8.0), 3 mM MgCl2, 0.15 mM m-spermine, 0.5 mM spermidine, and 1 mM CaCl2, DNase I was added, followed by incubation for 15 min at 3°C. Preliminary assays tested a range of concentrations (0.001 to 0.1 U per 10 μl nuclei), although most experiments used 0.01 U per 10 μl nuclei. Reactions were stopped with 5 mM EDTA and incubated for 16 h at 37°C in 25 μg of protease K and 1% sodium dodecyl sulfate. DNA was then extracted once with 1:1 phenol-chloroform and once with chloroform, treated with RNase A for 1 h, precipitated with ethanol, quantitated, and analyzed by blot hybridization or PCR.

A 20 ng of DNA from the nucleus treatments was used as a template in “hot start” quantitative PCR (qPCR) using SYBR green and an iCycler (Bio-Rad, Richmond, CA). Primer pairs amplified the EF-1 gene (5′-GGACGAGACCTCTGATGTAACAC-3′ and 5′-GGGCATCCAGTGCATGAAACA-3′), the NIFS gene (5′-ATAGTACGACGGCCACCGAACT-3′ and 5′-GGCTCAATAGTGTTGCCAAA-3′), referred to as to and +329 in Results), as NIFS amplitves behave as locus-specific probes in high-stringency hybridizations. RT-PCR. For preliminary screens of transformants, 0.2 μg of DNase-treated RNA was reverse transcribed and amplified for 30 cycles using the Superscript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) and primer sets described in Results. Other reactions were performed by two-step reverse transcription-PCR (RT-PCR) using cDNA primed with oligo(dT). Controls lacking reverse transcriptase were included to ensure that amplicons were not derived from genomic or contaminating DNA.
primer set and ranged between 0.89 and 0.95%. Reactions using primers for the Gypsy-like retroelement and EF-1 were used as normalization controls. **Methylation assays.** qPCR was performed with 20 ng of genomic DNA and primers \( /H11002 \) 620 and \( /H11002 \) 61 or primers NIFC1F and NIFC1R (5\(-\)AGAATGATGACGATGCCGACCAC and 5\(-\)AGAAGTTAGCATCCCACTGTTG). Template DNA was either undigested or treated with a 100-fold excess of restriction enzyme. These were as follows, with their recognition sequences in the amplicon shown in parentheses with sites sensitive to methylation followed by the superscript “m”: AclI (AACmGTT), BsaBI (GAmTNNNNAmTC), BsiEI (CmGRYCmG), ClaI (ATCmGAT), and HpaII (CC mGG).

**Run-on assays.** Nuclei from cold-treated sporangia were incubated with \(^{32}P\)UTP as described previously (22), omitting the Kirby solution. About 3 \( \times 10^5 \) cpm were obtained per preparation and hybridized to filters containing 50 ng of DNA from each target gene.

**DNA sequence analysis.** Sequences of the \( \text{NIF} \) loci, as well as DNA flanking those genes, were identified previously (46). These matched sequences were generated for \( P. \text{infestans} \) by the Broad Institute (www.broad.mit.edu). Regions flanking the \( \text{NIF} \) cluster were searched for genes by comparison to \( P. \text{infestans} \) ESTs (35) and annotations in the \( P. \text{sojae} \) genome (genome.jgi-psf.org) and by similarity searches against GenBank. This identified a putative protein kinase.
gene corresponding to EST PVrpvb_9420 (GenBank accession number CV952935, referred to here as KNN1).

RESULTS

Introduction of silencing plasmids into *P. infestans*. Homology-based gene silencing was used to help assess the roles of the zoosporogenesis-induced NIFC genes. To enable this, two plasmids were made containing NIFC1 and NIFC2 sequences. These genes were selected for the experiments since in phylogenetic analyses of NIF-like proteins from *Phytophthora*, NIFC1 clustered with NIFC3 and NIFS, whereas NIFC2 resided in a different clade (46). The plasmids that were constructed contained open reading frames of NIFC1 and NIFC2 in sense and antisense orientations, separated by an intron and placed behind the strong constitutive *ham34* promoter (Fig. 1A). Although published studies only report the use of sense or antisense constructs to silence genes in *P. infestans*, our laboratory has observed that inverted repeat constructs, including the open reading frame of the Cdc14 gene, also can trigger silencing (A. Ah Fong and H. S. Judelson, unpublished data).

Due to potential redundancy in function between the three NIFC genes, and possibly also with NIFS, it was recognized that the silencing of a single NIFC gene using one of these plasmids might not have a strong effect. However, it was thought possible that a single NIF transgene might silence multiple members of the family, since the four genes are similar in DNA sequence. In plants, sequences diverged by about 10% are still able to silence each other (7, 50). The three variable domains are present near their 5’/H11032 ends of the genes and more variable domains are present near their 5’ ends (Fig. 1C).

In all, 13 and 5 transformants were generated using pC1SIA and pC2SIA, respectively. These were then examined by RNA blot analysis with gene-specific hybridization probes derived from the diverged 5′ ends of the genes, and RNA from sporangia subjected to a cold treatment, which normally stimulates zoosporogenesis and NIFC expression (46). The results demonstrated silencing of NIFC1 in 4 of the 13 transformants made using pC1SIA and of NIFC2 in 2 of 5 transformants obtained with pC2SIA (28% of all transformants). This is illustrated in Fig. 2A for representative transformants generated using pC1SIA, where T3, T6, and T11 appeared to be silenced for NIFC1. In no transformant were larger RNA bands detected consistent with the transcription of a hairpin RNA, suggesting either that the transgenes silenced themselves or that their expression was impaired by position effects.

Concurrent transcriptional silencing of NIFC loci. The silenced strains described above were studied further to assess whether other members of the NIF family had also been turned off. Blot analysis of RNA from cold-treated sporangia indicated that in most cases all three NIFC genes were silenced (Fig. 2B). Of the four NIFC1-silenced strains obtained using pC1SIA, all were also silenced for NIFC2 and NIFC3. Of the two NIFC2-silenced strains generated using pC2SIA, one was silenced for NIFC2 only (transformant T22) and the other (T21) for all three NIFC genes. In contrast, NIFS always escaped silencing.

Included in this analysis as a control was transglutaminase *M81C*, which is another zoosporogenesis-specific gene (8). *M81C* was used to ensure that the absence of NIFC transcripts was not an artifact of poor induction of the zoosporogenesis pathway. Since *M81C* was expressed in the transformants, their lack of NIFC mRNA is presumably due to silencing. This was shown in independent experiments by comparing mRNA levels in cold-treated and untreated sporangia.

Whether genes flanking the NIFC cluster were affected was also examined. Based on sequence analysis of a BAC clone containing the cluster (46), which was later confirmed by genome sequence data, the closest neighboring gene is 2.3 kb left of NIFC2 and encodes a protein kinase, *KNN1* (Fig. 1B). RNA blot analysis indicated that this gene is expressed normally in the silenced lines, based on comparison to the EF-1 control.
As controls were zoosporogenesis-induced genes M81C and adenylate kinase PIC5 (AK).

Runs on assays performed using nuclei from chilled sporangia of wild-type progenitor strain 1306 (W) and the silenced transformant T3 obtained with pC1SIA (S). Target genes on the filters included NIFC1 (C1), NIFC2 (C2), NIFC3 (C3), and NIFS (S). Used as controls were zoosporogenesis-induced genes M81C (M81) and adenylate kinase PIC5 (AK).

FIG. 3. Nuclear run-on assay of silenced and wild-type strains. Radiolabeled transcripts were generated using nuclei from chilled sporangia of wild-type progenitor strain 1306 (W) and the silenced transformant T3 obtained with pC1SIA (S). Target genes on the filters included NIFC1 (C1), NIFC2 (C2), NIFC3 (C3), and NIFS (S). Used as controls were zoosporogenesis-induced genes M81C (M81) and adenylate kinase PIC5 (AK).

Silencing is associated with reduced mRNA synthesis. Run-on assays performed using nuclei from cold-treated sporangia indicated that silencing was attributable to strongly reduced transcription of each NIFC gene (Fig. 3). This was performed using wild-type P. infestans and transformant T3, which was silenced for all NIFC genes. Signals were detected for the three NIFC genes and controls using [32P]UTP-labeled transcripts from wild-type nuclei but not from the silenced transformant. The controls used in this experiment were the zoosporogenesis-specific M81C gene and another zoosporogenesis-specific gene, the PIC5 adenylate kinase (47).

Silencing causes defects in cyst germination. Four strains silenced for the three NIFC genes were compared to four controls to check for alterations in growth, progression through the spore cycle, and pathogenicity. The only feature for which a significant difference existed (P < 0.05) in cyst germination, which was reduced two-thirds compared to the wild type (Table 1). Cysts not able to germinate did not form aberrant structures. The cysts that did germinate were fully competent at making appressoria and infecting tomato leaflets. In a tomato infection assay in which equal numbers of zoospores were placed on each leaflet, the disease rating obtained with silenced transformants was slightly less than that of controls, but this likely reflects the reduction in infective propagules due to reduced cyst germination. Although not examined quantitatively, there were no obvious defects in zoospore motility or attraction to glutamate in a capillary tube swim-in assay. In contrast to the defect in cyst germination, direct sporangial germination (which involves the elaboration of germ tubes from sporangia without an intermediate zoospore stage) was unaffected.

FIG. 4. DNase I accessibility assays. Nuclei from the silenced transformant (S, corresponding to T3 in Fig. 3) and wild-type progenitor 1306 (W) were treated partially with DNase I, and then the DNA was purified, restriction digested, and hybridized with radiolabeled probes. (A) XhoI-digested DNA hybridized with a gene-specific probe for NIFC3 and, after stripping the filter, for the Gypsy-like retroelement (GY). (B) BstEII-MseI digest hybridized with gene-specific probes for NIFC1, NIFC2, NIFS, protein kinase KN1 (PK), and the Gypsy-like retroelement (GY). In both panels A and B, the same filter was used for each hybridization after stripping of the prior probe. The locations of the detected XhoI, MseI, or BstEII-MseI bands in the NIF cluster are illustrated in Fig. 1B.

Silencing alters chromatin at the NIFC cluster. When we considered the mechanism responsible for the concurrent silencing of the three NIFC genes, it was striking that NIFS had escaped inactivation since, as shown in Fig. 1C, it has approximately the same sequence identity with NIFC1 and NIFC2 (80 and 71%, respectively) as NIFC1 and NIFC2 have with NIFC3 (83 and 70%). If a diffusible RNA-based signal triggered silencing, for example, then NIFS should have also been inactivated by pC1SIA or pC2SIA. A hypothesis was therefore developed in which the inactivation of all three NIFC genes resulted from a silenced chromatin domain spanning the cluster. This domain could have been nucleated by the primary target of silencing, such as NIFC1 in experiments using pC1SIA, with NIFS evading silencing due to its distance from this nucleation site.

To test whether silencing had actually altered chromatin at the NIFC cluster, a DNase I accessibility assay was performed. Nuclei from a strain silenced for the cluster (transformant T3) and wild type were purified from chilled sporangia and treated with DNase I. Genomic DNA was then extracted, digested with restriction enzymes, and hybridized with probes specific for each NIF gene as well as control loci. The restriction digests (XhoI or MseI-BstEII treatments) and probes were chosen such that hybridization would focus on the transcribed regions of the genes (see Fig. 1B).

As shown in the XhoI digest in Fig. 4A, NIFC3 was more resistant to DNase I in silenced nuclei than wild-type nuclei, based on the stronger intensity of the 1-kb band in the former. As a control, both silenced and wild-type samples were hybridized with a probe from a medium-copy Gypsy-like retroelement (16) to show that the two DNA samples were similar otherwise; this element does not cross-hybridize with the retroelement located near the NIFC cluster. The status of chromatin at the Gypsy-like control loci are unknown but presumably would be the same in silenced and wild-type nuclei.

Similar results were observed for the other NIFC genes,
based on an MseI-BstEII digest of DNA from DNase I-treated nuclei (Fig. 4B). For example, 0.35- and 1.1-kb bands specific to NIFC1 and NIFC2, respectively, were stronger in the silenced nuclei than wild-type nuclei. In contrast, similar intensities were observed for NIFS which had escaped silencing and in the Gypsy-like control. In addition, the KNN1 locus (located 2.3 kb left of NIFC1) appeared unchanged, which is consistent with the data showing that its mRNA levels are unaffected by NIFC silencing (Fig. 2C).

Defining boundary of altered chromatin by qPCR. To better determine the scope of chromatin alterations, DNA from nuclei that had been isolated from cold-treated sporangia and treated partially with DNase I was subjected to qPCR. The concept is that chromatin that is more relaxed would yield faster amplification kinetics, since DNase I would more rapidly degrade the template (37). Used in these assays were primer pairs within and flanking the NIFC genes, which are labeled in Fig. 5A based on their distances from the start codon of the closest NIFC locus. Also marked in Fig. 5A are the major transcription start points for NIFC1, NIFC2, and NIFC3 (117, 95, and 134 nt upstream of their start codons, respectively) and their polyadenylation sites (133, 136, and 27 nt downstream of their stop codons).

The results confirmed that silencing had modified DNA within the cluster to a less accessible configuration, reflecting more tightly packed chromatin (Fig. 5B). For example, average normalized Cq values using the primer pair −61/−282, which amplifies a fragment between 61 and 282 nt upstream of the NIFC2 start codon, were 22.4 and 26.0 for DNase I-treated silenced and wild-type templates, respectively. This corresponds to 10.2-fold-greater amplification from silenced nuclei, based on an empirically determined amplification efficiency (E) of 0.93 for this amplicon. Other targets within the cluster also showed faster amplification from the silenced template. For example, primer pair +1/+329, which hybridizes within the NIFC1 open reading frame, displayed a 7.8-fold difference between silenced and wild-type nuclei. The region of altered chromatin also involved the intergenic region between NIFC1 and NIFC3, which showed a 5.3-fold difference based on primer set −661/−501. Consistent with its escape from silencing, a primer pair specific to NIFS (+35/+255) did not exhibit a significant difference between silenced and wild-type nuclei.

The left boundary of altered chromatin appeared to be a few hundred nucleotides upstream of the NIFC2 start codon. This is because primer pair −728/−993 showed similar PCR kinetics against silenced and wild-type templates compared to primer pair −384/−620, which amplified 5.7 times more efficiently from the silenced strain. Therefore, the boundary of chromatin alterations is approximately 400 nt upstream of the start codon. This equals 305 nt upstream of the transcriptional start point and 166 nt upstream of the “cold box” transcription factor binding site required for cold-induced NIFC expression (45).

At the right side of the NIFC1 cluster, chromatin showed a graded return to a normal configuration. Primer pair +757/+901, which targets the 3’ end of the NIFC3 open reading frame, amplified its target 2.9 times faster from the silenced strain. More distal fragments such as one defined by primer pair +1305/+1666, which binds 404 to 765 nt downstream of the NIFC3 stop codon, displayed kinetics similar to those of the wild-type and silenced templates.

Silencing is not associated with DNA methylation. No increase in 5-cytosine or N6-adenine methylation upon silencing was indicated by qPCR assays targeted to the NIFC2 promoter (Fig. 6A) or the NIFC1 open reading frame (Fig. 6B). Previous tests for methylation in P. infestans involved traditional DNA blot experiments (20, 52), but qPCR was used here as a more sensitive test. This entailed using template DNA that was undigested or cleaved with enzymes sensitive to methylation at cytosine (CpG sites within AclI, HpaII, ClaI, or BsiEI sites) or adenine (BsaBI); methylation would protect the DNA and enable products to form in PCR. No significant amplification occurred from silenced or wild-type DNA treated with enzymes blocked by CpG methylation in either the promoter (Fig. 6A) or transcribed regions (Fig. 6B). A tiny amount of amplifiable template was detected in the terminal cycles of
most restriction enzyme-treated reactions, equaling <0.02% of undigested controls based on \( C_T \) calculations. However, this is likely insignificant and may simply reflect incomplete digestion of the genomic DNA due to technical issues.

The assay for adenine methylation, using BsaBI, did suggest that a low level of that modification existed within the \( NIFC1 \) open reading frame (Fig. 6B). However, there was no significant difference between wild-type and silenced templates. Based on \( C_T \) values, ca. 0.3% of adenines may be methylated; for comparison, the curve in Fig. 6B marked by triangles represents a control reaction in which the template concentration was 1% of the uncut controls. Determining whether the persistent template represents authentic methylation or partial digestion due to technical issues is left to future studies, since the goal here was to assess whether methylation levels change upon silencing. A search of \( Phytophthora \) sequence databases does indicate that these species encode N6-adenine methylases, however, which may participate in DNA replication and repair (38).

Gene-specific hairpin construct also silences multiple loci.

A hypothesis presented above was that an expanding heterochromatic domain initiated at one \( NIFC \) locus silenced the entire cluster. However, it is also possible that each of the three genes had interacted individually with the full-length inverted repeat transgenes in pC1SIA or pC2SIA due to their sequence similarity. To help distinguish between these possibilities, a plasmid expressing a short hairpin RNA (shRNA) matching only a 21-nt region of \( NIFC1 \) (“HP” in Fig. 1C) was introduced into transformants. It should be noted that there is no precedent in oomycetes for silencing using a shRNA transgene, although one study describes a transient dsRNA-mediated knockdown method (56). However, similar constructs induce silencing in other taxa (48).

Fifty-two transformants (HT1 to HT52) were obtained using the shRNA construct (pC1HP), and RNA from their cold-treated sporangia were assayed for \( NIFC1 \) expression by RT-PCR. Only HT11 proved to be silenced for \( NIFC1 \). This frequency of silencing (2%) was less than observed using the full-length hairpin in pC1SIA (31%), suggesting that longer transgenes induce silencing more efficiently in \( Phytophthora \). However, this is a tentative inference since not all parts of a gene are equally susceptible to RNAi (12).

To test whether HT11 was also silenced for other members of the \( NIF \) family, RT-PCR was performed (Fig. 7). The results indicated that HT11 was silenced for both \( NIFC1 \) and \( NIFC2 \), whereas \( NIFC3 \) and \( NIFS \) remained expressed. Included in this experiment as controls were the untransformed wild-type progenitor strain, and silenced and unsilenced transformants obtained using the full-length inverted repeat plasmid pC1SIA described previously (T3 and T2, respectively).

In consideration of the low rate of silencing using the shRNA construct and the relatively low transformation efficiency of \( Phytophthora \), attempts to test more transformants to determine whether pC1HP could ever silence all three \( NIFC \) genes were discontinued. Nevertheless, the results are consistent with the ability of an altered chromatin domain initiated at one gene to spread along the chromosome.

FIG. 6. qPCR test for DNA methylation based on amplification of DNA digested with methylation-sensitive enzymes. (A) \( NIFC2 \) promoter showing binding sites for primers \(-620 \) and \(-61 \) nt from the start codon (arrows), transcriptional start point (TSP, at \(-95 \)), cold box (\(-234 \); [45]), and restriction sites. Beneath the map are qPCR profiles for 20 ng of uncut template DNA from wild-type or silenced transformant (W and S, respectively; reproduced in the top and bottom panel) and template digested with HpaII and AciI (top box) or ClaI and BsiEI (bottom box). (B) \( NIFC1 \) open reading frame showing sites for primers NIFC1F and NIFC1R and restriction sites and a qPCR graph for DNA that was uncut or digested with HpaII or BsaBI prior to amplification. Triangles represent a reaction using 0.2 ng of uncut template DNA compared to 20 ng for the other samples.
transcription based on run-on assays and associated with more tightly packaged chromatin. Although no evidence of NIFC transcription was detected in the silenced transformants, the inverted repeat constructs may have initially induced PTGS, which then transmuted into TGS. This is consistent with findings in other taxa, where both PTGS and TGS can induce heterochromatin when their RNA-RNA (or RNA-DNA) recognition systems interact with histone or DNA methyltransferases, chromatin architecture proteins, Piwi-like Argonaute proteins, RecQ homologues, or other mediators of chromatin structure (2, 11, 27, 33).

Unlike the case in many eukaryotes where cytosine methylation is an important activator of chromatin-modifying complexes, this was not associated with TGS in Phytophthora. Now that complete genome sequences are available for P. infestans and other members of the genus, the lack of 5-methylcytosine demonstrated here and suggested in earlier reports (20, 52) can be explained by the absence in the genome of homologs of known cytosine methyltransferase (DNMTs). The occurrence of chromatin modifications in Phytophthora, despite the absence of cytosine methylation, is consistent with the known lack of significant cytosine methylation in yeast, studies of methylation mutants of Arabidopsis, and the observation that 5-methylcytosine-deficient Neurospora grows normally (10, 31, 34, 40).

Our PCR assays did detect N6-methyladenine in the silenced transformants; K.-S. Kim and H. S. Judelson, unpublished results. Several distinct mechanisms by which silencing may have spread through the NIFC cluster can be posited. In theory, DNA-DNA interactions along the lines of the RIP and MIP phenomenon of filamentous fungi could be responsible (14). However, this seems unlikely since RIP and MIP are traditionally restricted to the sexual cycle, are favored when endogenes and transgenes are linked (which is unlikely in P. infestans since the rate of homologous integration greatly exceeds the frequency of silencing), and since the NIFC genes should be immune to RIP or MIP since they exist normally as a family. Therefore, the spread of silencing through the NIFC cluster is more likely to be due to RNA-RNA or RNA-DNA interactions, or characteristics of the heterochromatin propagation machinery (11).

One plausible RNA-based mechanism for the silencing of multiple NIFC genes invokes autonomous interactions between RNA signals emitted by the transgene and each member of the gene family. Cross-silencing is to be expected, since limited mismatches between silencing transgenes and targets do not block TGS in other species (7, 50). Occasional escapes from cross-silencing are anticipated since silencing is not 100% efficient, which explains the phenotype of transformant T22. The immunity of NIFS contradicts this model; however, this might be due to its binding of an antisilencing factor that maintains open chromatin. This could simply be a transcription factor or histone variant (15, 30). Indeed, our attempts to silence NIFS have failed to date (based on the analysis of 28 transformants; K.-S. Kim and H. S. Judelson, unpublished results), so that gene may be less amenable to silencing.

However, additional models (which may not be mutually exclusive) must be proposed to explain how the NIFC1-specific shRNA from pC1HP silenced the adjacent NIFC2 gene in
transformant HT11. One possibility is “transitive RNAi.” This has been described in plants, nematodes, and fungi and involves the generation of siRNAs distant from the site targeted by the original dsRNA by a RNA-dependent RNA polymerase. This process involves the propagation of siRNAs and is distant from the site targeted by the transgene.

Another model to explain cross-silencing within the NIFC cluster and the immunity of NIFS involves the propagation of heterochromatin from the site targeted by the transgene. Heterochromatin-generating proteins activated by the PTGS machinery are believed to move along chromosomes until reaching an “insulator” sequence, which delimits heterochromatin from transcriptionally active regions. The presence of an insulator left of the NIFC cluster may explain why KNN2 was unaffected by NIFC silencing. Transcription factors regulating KNN2 may also have blocked the proliferation of heterochromatin, as shown for bromodomain-containing factors in budding yeast. No change in chromatin was also observed further than 1 kb to the right of the NIFC cluster, where a retroelement was found. This might also be explained by an insulator. Alternatively, that region might have already been in condensed chromatin since transposons preferentially recruit the heterochromatin machinery.

In the present study, the failure of chromatin alterations to spread to the flanking KNN1 gene means that the cyst germination defect associated with silencing can be attributed to the absence of NIFC function. However, silencing studies of other loci should be interpreted cautiously since intergenic regions in Phytophthora are frequently only a few hundred nucleotides in size, and the presence of insulators between genes cannot be assumed. We also suggest that other gene manipulation studies in Phytophthora might benefit from more research into insulators, such as the element that may flank the NIFC cluster. When incorporated into transformation plasmids, insulators may guard against position effects which frequently impair overexpression and gain-of-function studies.

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**REFERENCES**

1. Ah Fong, A., and H. S. Judelson. 2003. Cell cycle regulator Cdc14 is expressed during sporulation but not hyphal growth in the fungus-like oomycete Phytophthora infestans. Mol. Microbiol. 50:487–494.
2. Bernstein, E., and C. D. Allis. 2005. RNA meets chromatin. Genes Dev. 19:1635–1655.
3. Blanco, F. A., and H. S. Judelson. 2005. A bZIP transcription factor from Phytophthora interacts with a protein kinase and is required for zoospore motility and plant infection. Mol. Microbiol. 56:538–548.
4. Bley, A., H. Van Houdt, and A. Depicker. 2006. Down-regulation of endogenous genes mediated by a transitive silencing signal. RNA 12:1631–1639.
5. Clark, M. C., D. L. Melanson, and O. T. Page. 1978. Purine metabolism and differential inhibition of spore germination in Phytophthora infestans. Can. J. Microbiol. 24:1032–1038.
6. Dillen, A. 2003. The specifics of small interfering RNA specificity. Proc. Natl. Acad. Sci. USA 100:6289–6291.
7. Elkind, Y., R. Edwards, M. Mavandad, S. A. Hedrick, O. Ribak, R. A. Dixon, and C. J. Lamb. 1990. Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc. Natl. Acad. Sci. USA 87:9057–9061.
8. Fabritius, A. L., and H. S. Judelson. 2003. A mating-induced protein of Phytophthora infestans is a member of a family of elicitors with divergent structures and stage-specific patterns of expression. Mol. Plant-Microbe Interact. 16:926–935.
9. Fernandez, A. O., C. W. Campagnoni, K. Kampf, J. M. Feng, V. W. Handle, V. Schonmann, E. R. Bongarzone, S. Reyes, and A. T. Campagnoni. 2004. Identification of a protein that interacts with the goll-myelin basic protein and with nuclear LIM interactor in the nuclear system. J. Neurosci. Res. 75:43–57.
10. Foss, H. M., C. J. Roberts, K. M. Clasys, and E. U. Selker. 1993. Abnormal chromosome behavior in Neurospora mutants defective in DNA methylation. Science 262:1757–1741.
11. Grewal, S. I., and D. Moazed. 2003. Heterochromatin and epigenetic control of gene expression. Science 301:798–802.
12. Holen, T., M. Amarzguioui, M. T. Wiiger, E. Babaie, and H. Prydz. 2002. Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Res. 30:1757–1766.
13. Hsieh, J., and A. Fire. 2000. Recognition and silencing of repeated DNA. Annu. Rev. Genet. 34:187–204.
14. Irelan, J. T., E. U. Selker. 1996. Gene silencing in filamentous fungi: RIP1, RIP2, and quelling. J. Gen. Virol. 78:313–324.
15. Jambunathan, N., A. W. Martinez, E. C. Robert, N. B. Aogochukwu, M. E. Ibs, S. L. Dugas, and D. Donze. 2005. Multiple bromodomain genes are involved in restricting the spread of heterochromatin at the Saccharomyces cerevisiae HMR-TRNA boundary. Genetics 171:913–922.
16. Judelson, H. S. 2002. Sequence variation and genomic amplification of a family of Gypsy-like elements in the oomycete genus Phytophthora. Mol. Biol. Evol. 19:1313–1322.
17. Judelson, H. S., and F. A. Blanco. 2005. The species of Phytophthora: weapons and plant destroyer. Nat. Rev. Genet. 3:477–58.
18. Judelson, H. S., and R. Roberts. 2002. Novel protein kinase induced during sporangial cleavage in the oomycete Phytophthora infestans. Eukaryot. Cell 1:697–699.
19. Judelson, H. S., R. M. Tyler, and R. W. Michemore. 1991. Transformation of the oomycete pathogen, Phytophthora infestans. Mol. Plant-Microbe Inter. 4:602–607.
20. Judelson, H. S., and S. L. Whitaker. 1995. Inactivation of transgenes in Phytophthora infestans is not associated with their deletion, methylation, or mutation. Curr. Genet. 28:571–579.
21. Kaido, D., H. Yashiroda, A. Toh-E, and Y. Ikukichi. 2002. Yeast Whi2 and Psl1-phosphatase form a complex and regulate STRE-mediated gene expression. Genes Cells 7:347–358.
22. Kanazawa, A., M. O’Dell, R. P. Hellens, E. Hitchin, and M. Metzlafl. 2000. Mini-scale method for nuclear run-on transcription assay in plants. Plant Mol. Biol. Rep. 18:377–383.
23. Kanazawa, A., M. O’Dell, R. P. Hellens, E. Hitchin, and M. Metzlafl. 2000. Mini-scale method for nuclear run-on transcription assay in plants. Plant Mol. Biol. Rep. 18:377–383.
24. Kanazawa, A., M. O’Dell, R. P. Hellens, E. Hitchin, and M. Metzlafl. 2000. Mini-scale method for nuclear run-on transcription assay in plants. Plant Mol. Biol. Rep. 18:377–383.
25. Kimm, K. S., and H. S. Judelson. 2003. Sporangiium-specific gene expression in the oomycete phytopathogen Phytophthora infestans. Eukaryot. Cell 2:1327–1335.
26. Lal, S., S. A. Maretigui, and B. D. Goto. 2004. A G-protein subunit is involved in sporulation formation. Eukaryot. Cell 2:971–977.
27. Lal, S., S. A. Maretigui, and B. D. Goto. 2004. A G-protein subunit is involved in sporulation formation. Eukaryot. Cell 2:971–977.
28. Martienssen, R. A., M. Zariatigui, and D. B. Goto. 2005. RNA interference and heterochromatin in the fusion yeast Schizosaccharomyces pombe. Trends Genet. 21:450–456.
29. Matzke, M. A., and J. A. Birchler. 2005. RNAi-mediated pathways in the nucleus. Nat. Rev. Genet. 6:24–35.
30. Meneghini, M. D., M. Wu, and H. D. Madhani. 2003. Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. Cell 112:275–276.
31. Mittelsten Scheid, O., A. V. Probst, K. Afsar, and J. Paszkowski. 2002. Two regulatory levels of transcriptional gene silencing in Arabidopsis. Proc. Natl. Acad. Sci. USA 99:13659–13662.
32. Mokranjac, D., S. A. Paschen, C. Kozany, H. Prokisch, S. C. Hoppins, F. E. Testa, T. Torto, M. Zhang, L. Zheng, E. Mueller, J. Windass, A. Binder, and J. Testa, T. Torto, M. Zhang, L. Zheng, E. Mueller, J. Windass, A. Binder, and J. Testa. 2002. Yeast Whi2 and Psll-phosphatase form a complex and regulate STRE-mediated gene expression. Genes Cells 7:347–358.
P. R. J. Birch, U. Gisi, F. Govers, N. A. R. Gow, F. Mauch, P. van West, M. E. Waugh, J. Yu, T. Boller, S. Kamoun, S. T. Lam, and H. S. Judelson. 2005. Large-scale gene discovery in the oomycete *Phytophthora infestans* reveals likely components of phytopathogenicity shared with true fungi. Mol. Plant-Microbe Inter. 18:229–243.

36. Randall, T. A., and H. S. Judelson. 1999. Construction of a bacterial artificial chromosome library of *Phytophthora infestans* and transformation of clones into *P. infestans*. Fungal Genet. Biol. 28:160–170.

37. Rao, S., E. Procko, and M. F. Shannon. 2001. Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the IL-2 gene. J. Immunol. 167:4494–4503.

38. Ratel, D., J. L. Ravanat, F. Berger, and D. Wion. 2006. N6-methyladenine: the other methylated base of DNA. Bioessays 28:309–315.

39. Shan, W.-X., J. S. Marshall, and R. Hardham Adrienne. 2004. Gene expression in germinated cysts of *Phytophthora nicotianae*. Mol. Plant Pathol. 5:317–330.

40. Shilatifard, A. 2006. Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu. Rev. Biochem. 75:243–269.

41. Sigova, A., N. Rhind, and P. D. Zamore. 2004. A single Argonaute protein mediates both transcriptional and posttranscriptional silencing in *Schizosaccharomyces pombe*. Genes Dev. 18:2359–2367.

42. Skalamera, D., A. P. Wasson, and A. R. Hardham. 2004. Genes expressed in zoospores of *Phytophthora nicotianae*. Mol. Genet. Genom. 270:549–557.

43. Sontheimer, E. J. 2005. Assembly and function of RNA silencing complexes. Nat. Rev. Mol. Cell. Biol. 6:127–138.

44. Sutter, N. B., D. Scalzo, S. Fiering, M. Groudine, and D. I. Martin. 2003. Chromatin insulation by a transcriptional activator. Proc. Natl. Acad. Sci. USA 100:1105–1110.

45. Tani, S., and H. S. Judelson. 2006. Activation of zoosporogenesis-specific genes in *Phytophthora infestans* involves a 7-nucleotide promoter motif and cold-induced membrane rigidity. Eukaryot. Cell 5:745–752.

46. Tani, S., K. S. Kim, and H. S. Judelson. 2005. A cluster of NIF transcriptional regulators with divergent patterns of spore-specific expression in *Phytophthora infestans*. Fungal Genet. Biol. 42:42–50.

47. Tani, S., E. Yatzkan, and H. S. Judelson. 2004. Multiple pathways regulate the induction of genes during zoosporogenesis in *Phytophthora infestans*. Mol. Plant-Microbe Interact. 17:330–337.

48. Taxman, D. J., L. R. Livingstone, J. Zhang, B. J. Conti, H. A. Iocca, K. L. Williams, J. D. Lich, J. P. Ting, and W. Reed. 2006. Criteria for effective design, construction, and gene knockdown by shRNA vectors. BMC Biotechnol. 6:7.

49. Torche, S. 2004. Isolation et etudes d'expression de genes potentiellement lies a la pathogenicite chez *Phytophthora porri*. Ph.D. thesis. University of Fribourg, Friborg, Switzerland.

50. Trevanion, S. J., R. T. Furbank, and A. R. Ashton. 1997. NADP-malate dehydrogenase in the C4 plant *Flaveria bidentis*: cosense suppression of activity in mesophyll and bundle-sheath cells and consequences for photosynthesis. Plant Physiol. 113:1153–1165.

51. Van Houdt, H., A. Bleys, and A. Depicker. 2003. RNA target sequences promote spreading of RNA silencing. Plant Physiol. 131:245–253.

52. Van West, P., S. Kamoun, J. W. Van't Klooster, and F. Govers. 1999. Internuclear gene silencing in *Phytophthora infestans*. Mol. Cell 3:339–348.

53. Vaucheret, H., and M. Fagard. 2001. Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet. 17:29–35.

54. Wei, G. H., D. P. Liu, and C. C. Liang. 2005. Chromatin domain boundaries: insulators and beyond. Cell Res. 15:292–300.

55. Whisson, S. C., A. O. Avrova, P. van West, and J. T. Jones. 2005. A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*. Mol. Plant Pathol. 6:153–163.

56. Whisson, S. C., A. O. Avrova, P. van West, and J. T. Jones. 2005. A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*. Mol. Plant Pathol. 6:153–163.

57. Yeo, M., S. K. Lee, B. Lee, E. C. Ruiz, S. L. Pfaff, and G. N. Gill. 2005. Small CTD phosphatases function in silencing neuronal gene expression. Science 307:596–600.