Novel Protein Kinase Induced during Sporangial Cleavage in the Oomycete Phytophthora infestans

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A study of the effect of inhibitors on zoospore development in Phytophthora infestans demonstrated the involvement of protein kinases and calcium and led to the discovery of a gene induced during zoosporogenesis that encoded a protein resembling Ca\(^{2+}\)- and calmodulin-regulated serine/threonine protein kinases. The calcium channel blocker verapamil and the calmodulin antagonist trifluoroperazine inhibited zoosporogenesis and encystment. The protein kinase inhibitors K-252a and KN-93 inhibited zoospore release, encystment, and cyst germination, and K-252a reduced zoospore viability. In contrast, the inhibitors had minor or no effects on sporangia directly germinating in media. Spurred by these findings, a survey of putative protein kinase genes was performed to identify any that were up-regulated during zoosporogenesis. A kinase-encoding gene was identified for which mRNA accumulation was first detected soon after chilling sporangia in water, conditions that induce sporangial cytoplasm to cleave and release zoospores. The transcript persisted in motile zoospores and in germinated cysts but was not detected in other tissues, including hyphae, hyphae placed in water, or directly germinating sporangia. The structure of the predicted protein was novel, as its C-terminal region, which binds calmodulin in related proteins, was unusually short. Concentrations of actinomycin D previously used in experiments that suggested that de novo transcription was not needed for zoosporogenesis or encystment only partially inhibited transcription of the kinase gene, probably due to poor uptake into sporangia.

Dispersal by means of spores is a common feature of the life cycle of many lower eukaryotes. This includes the oomycetes, which encompass many important plant pathogens, including Phytophthora, Pythium, and the downy mildews. Oomycetes lack taxonomic affinity with the so-called true fungi (i.e., ascomycetes and basidiomycetes), instead being related to diatoms and brown algae (2, 15). The asexual sporangia of oomycetes either germinate directly through a germ tube or indirectly by releasing biflagellate zoospores. Many species germinate through either pathway, while others produce only zoospores and some germinate only directly (12, 22, 26). For species displaying both modes of germination, zoospores are considered most important for dispersal and host infection.

One species exhibiting this duality in germination behavior is Phytophthora infestans, which causes late blight of potato and tomato. Sporangia form on sporangiophores that emerge from infected plant tissue or from hyphae on artificial media. As with other oomycetes, the sporangia of P. infestans are multinucleate, nondesiccated, and metabolically active. Germination becomes possible once sporangia detached from sporangiophores encounter liquid. Indirect germination predominates in the absence of nutrients and at cool temperatures, typically below 12°C (30). In contrast, direct germination is favored by higher temperatures and nutrients. Indirect germination takes about 1 h and involves the cleavage of sporangial cytoplasm into multiple zoospores. These emerge from the sporangial apex and swim, displaying several tactic behaviors (8, 20), until encystment occurs in response to chemical or physical stimulation (13). Encystment occurs within seconds, involving the detachment of flagella and cell wall deposition (13). Cysts subsequently elaborate a germ tube that can penetrate and colonize a plant host or grow into new mycelium in artificial culture.

Many elegant microscopic and physiological studies of zoosporogenesis, encystment, and cyst germination have been performed. Changes in cytoskeletal organization and vesicle distribution are documented, as are movements of ions such as calcium (9, 13, 17, 21, 25, 38). In contrast, little is known of the molecular biology of zoospore development or how its stages are regulated. Cleavage, zoospore release, and encystment are reportedly insensitive to actinomycin D, which suggests the involvement of preexisting mRNA or proteins. Later steps, such as cyst germination, are sensitive to such inhibitors (7, 27).

In this study, to better understand the regulation of germination and zoospore development, inhibitors of cellular processes were tested for their effects on direct and indirect germination in P. infestans. One or more stages of zoospore development were inhibited by compounds affecting protein kinases and calcium pathways. This led to a search for protein kinase genes that were differentially expressed during zoosporogenesis, in recognition that protein phosphorylation regulates cellular functions in other species (5). A kinase-encoding gene was identified from which RNA transcripts became detectable soon after placing sporangia in cool water to induce zoospore release. No transcripts were found in uninduced sporangia, directly germinating sporangia, or hyphae. The predicted product of the gene resembled Ca\(^{2+}\)- and calmodulin-regulated protein kinases (CaMKs) but lacked an obvious C-terminal regulatory or protein-association domain typically found in such proteins. The gene was also used to address the effect of actinomycin D on transcription during germination.

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utilized a dehydrated broth at 10^4/ml. Indirect germination was induced by cooling a sporangial suspension (10^5/ml) to about 10°C, about 1 h after most zoospores were released. Sporulating hyphae were prepared as follows: nonsporulating hyphae were obtained from 5-day-old rye A media for 10 min at 5,000 rpm. Grown at 18°C (A1; The Netherlands) and 1306 (A1; United States). These were generally used to study the effects of inhibitors or as sources of RNA were prepared as follows: nonsporulating hyphae were obtained from 5-day-old rye broth cultures inoculated with 10^4 sporangia/ml. Sporulating hyphae were scraped off polycarbonate membranes laid upon rye agar that had been inoculated with 0.5 mM CaCl2 to zoospores and vortexing for 30 s to induce encystment and then incubation of the cysts in water at 10°C. An average of 74% of sporangia released zoospores in controls. These and values in other categories show averages ± range from two experiments, with at least 200 structures counted for each sample. Percentage of zoospores of fully cleaved, single-unit size; controls averaged 97%. Controls averaged 91%. Percentage of zoospores of fully cleaved, single-unit size; controls averaged 97%. Controls averaged 87%.

**MATERIALS AND METHODS**

**Growth and manipulation of P. infestans.** Isolates used in this study were 88069 (A1; The Netherlands) and 1306 (A1; United States). These were generally grown at 18°C on rye A agar (6) or clarified rye broth prepared by centrifuging rye A media for 10 min at 5,000 × g. Nitrogen-and carbon-starved cultures utilized a defined medium (39) modified by reducing NH4SO4 levels to 0.5 mM and by omitting glucose and reducing fumarate levels to 20 mM, respectively. This reduced growth rates by 70% relative to those found in the complete defined media.

**Tissues used to study the effects of inhibitors or as sources of RNA were prepared as follows:** nonsporulating hyphae were obtained from 5-day-old rye broth cultures inoculated with 10^4 sporangia/ml. Sporulating hyphae were scraped off polycarbonate membranes laid upon rye agar that had been inoculated 8 to 10 days earlier. Sporangia were isolated by flooding 8- to 12-day-old rye agar cultures with water, rubbing the sporangia free with a glass rod, and then separating sporangia from hyphal fragments by passage through 50-μm-pore-sized nylon mesh. To directly germinate sporangia, they were placed in clarified rye broth at 10^8/ml. Indirect germination was induced by cooling a sporangial suspension (10^8/ml) to about 10°C by placing it on ice for about 20 min, followed by incubation in a 10°C chamber for 30 min (for “cleaved” sporangia) or about 90 additional minutes (for zoospores). Zoospores were purified from sporangia by passage through 15-μm-pore-sized mesh. Germinated zoospore cysts were isolated by adding 0.5 mM CaCl2 to zoospores and vortexing for 30 s to induce encystment and then incubation of the cysts in water at 10°C for 6 h.

**Sequence analysis.** An expressed sequence tag (EST) database was generated in collaboration with the Syngenta Agricultural Biotechnology Research Institute (SABRI) (Research Triangle Park, N.C.). This was established using libraries prepared independently from mRNAs extracted from the tissues described above, plus other samples, including some prepared at SABRI. cDNAs were cloned into pfSPORT1 (Invitrogen, Carlsbad, Calif.) prior to 5’ sequencing by SABRI. Starting from a FASTA flat file of all sequences, the ESTs were clustered using the membership of library-specific clusters was then compared for ESTs appearing differentially abundant at a P > 0.80 based on Fisher’s exact test were then tested in RNA blots.
cleavage (zoosporogenesis) was induced by chilling, to sporangia 5 min before placing them in rye media to allow direct germination, to swimming zoospores 5 min before inducing encystment by vortexing in 0.5 mM CaCl₂, and to cysts 10 min after vortexing to study their effects on cyst germination.

Actinomycin D blocked cyst germination and direct germination but had little effect on zoospore release or encystment. It follows that de novo transcription is not required for cleavage or encystment, as previously noted (7, 27). This assumes that actinomycin D enters sporangia with enough rapidity to inhibit transcription. This may not be the case, as described in a later section.

Inhibitors of calcium pathways (verapamil and trifluoroperazine) strongly inhibited zoospore release. This occurred in a dose-dependent manner for both compounds, but for trifluoroperazine differential effects on cleavage versus zoospore release were noted. At 2.5 μM, 78% of sporangia released their contents, but of these only 4% released zoospores normally. In most cases, an uncleaved multiflagellated cytoplasmic mass was expelled that swam for a few minutes before lysing or encysting. Higher concentrations of trifluoroperazine blocked the formation of all zoospore-like structures.

Verapamil and trifluoroperazine also inhibited encystment but had distinct effects. Verapamil prevented the encystment of most zoospores, leaving the rest in a motile state. Little effect was noted when trifluoroperazine was added to zoospores, but most lysed when vortexed in an attempt to induce encystment. Both compounds also altered the directionality of swimming as reported previously (10).

Both direct germination and cyst germination were modestly inhibited by verapamil and trifluoroperazine, in a dose-dependent fashion. Their effects on cyst germination were less than observed for Phytophthora parasitica, probably due to the 0.5 mM CaCl₂ in our encystment solution, which is reported to attenuate the effects of the inhibitors (38). Another calmodulin inhibitor, W7, had effects that paralleled those of trifluoroperazine (not shown). Verapamil and trifluoroperazine also had modest effects on the direct germination of sporangia in rye media. These effects were less than those observed by Hill et al. (20), who studied germination in water.

The protein kinase inhibitor K-252a strongly affected the zoospore pathway but had little effect on direct germination. Zoospore release was moderately sensitive to K-252a, but most zoospores lysed a few minutes after emergence. K-252a appeared to inhibit encystment, but this was challenging to quantify since many zoospores lysed just prior to or coincident with the encystment stimulus; after vortexing, zoospores appeared lysed or encysted but never motile. Cyst germination was also inhibited in a dose-dependent manner by K-252a, although the compound did not cause cyst lysis. The concentration of K-252a required to observe these phenomena resembled that needed to detect cellular effects in other systems (14), KN-93, which has greater specificity than K-252a for the CaMK II class of eukaryotic kinases (33), also moderately inhibited zoospore release, encystment, and cyst germination. However, KN-93 did not cause lysis of zoospores.

Identification of protein kinase induced during cleavage.

Based on the inhibitor studies, a program to identify protein kinases potentially regulating zoospore development was implemented through a process resembling Digital Differential Display (31). ESTs (S. T. Lam, abstract from Am. Phytopathol. Soc. Meet., August 2001, Salt Lake City, Utah, Phytopathology 91:S158, 2001) were sorted based on the source of mRNA used to construct the corresponding cDNA libraries. These included sporangia, sporangia cleaving into zoospores, germinating zoospore cysts, directly germinating sporangia, hyphae, nutrient-deprived hyphae, and other tissues. The ESTs were first assembled into contigs (EST clusters) within each library, and then contigs from cleaving sporangia and zoospore libraries were assembled against nonzoospore contigs. Contigs specific to cleavage or zoospore libraries that potentially encoded protein kinases were then identified using BLAST against GenBank. Next, Fisher’s exact test was used to determine whether the differential distribution of the underlying ESTs in zoospore versus nonzoospore libraries was statistically significant. By use of a nonstringent cutoff of P > 0.8, three protein kinase-like genes possibly up-regulated during zoospore development were identified. This compares to 220 protein kinase contigs in the original EST libraries.

One candidate proved to be specific to zoospore development based on RNA blot analysis. Hybridization was detected against mRNA from zoospores but not from nonsporulating hyphae, hyphae decorated with sporangia, ungerminated sporangia, or directly germinating sporangia (Fig. 1A). The mRNA detected was 1.3 kb, and BLAST analysis of the existing partial-length cDNA sequence indicated similarity to CaMKs.

Subsequent studies indicated that transcripts from the putative kinase gene started to accumulate very early in zoospore development. This involved flooding culture plates with cool water, rubbing off the spores, incubating the spores at 10°C, and then extracting RNA for analysis. Thirty minutes after water was added, the abundance of the transcript was already at 61% of its maximum level relative to an elongation factor 1 control (Fig. 1B). This was before cytoplasmic changes associated with cleavage were evident by light microscopy and before any zoospores were released (Fig. 1B). A similar pattern of induction was observed in isolates 88069 (Fig. 1) and 1306 (not shown).

Several data suggested that mRNA accumulation initiated within minutes of adding water to sporangia. The timing was difficult to precisely assess, since variation was observed between experiments, and obtaining a true “zero time point” was impossible, since 5 min is normally required to harvest sporangia from culture plates. Little transcript was detected in most preparations of freshly harvested sporangia, but in a few the kinase mRNA was already at one-third of its maximum level. Figure 1B shows the most common result, where the transcript is ~1% of its maximum. Such discrepancies likely reflect variation in the swiftness at which sporangia were harvested or differences between batches of sporangia as some release zoospores faster than others. It is notable that the transcript was never detected in RNA from sporulating cultures (containing unseparated hyphae and sporangia, harvested without water).

The abundance of the kinase mRNA peaked during cleavage and zoospore release and fell afterwards (Fig. 1C). In cysts germinated in water for 8 h, the abundance of the transcript had dropped 40% from its peak. This may understate the decline in mRNA abundance after cyst germination, as only...
FIG. 1. Expression of kinase transcript in *P. infestans*. RNA was extracted from isolate 88069, electrophoresed, blotted, and probed with a 32P-labeled fragment of the kinase gene (PK). Blots were then stripped and hybridized with an elongation factor 1 probe (EF1). (A) RNA from nonsporulating hyphae (HY), sporulating hyphae (SH), purified sporangia (SP), zoospores released in 10°C water (ZO), or sporangia undergoing direct germination in rye media (DG). (B) Left panel shows blot using RNA from nonsporulating hyphae (HY), sporangia freshly harvested in water (0 [min]), or RNA from sporangia held in 10°C water for 30, 60, 90, or 120 min. Right panel shows quantitation of PK mRNA and zoospore development during the same experiment. Indicated are the ratio of PK and EF1 signals, as determined by phosphorimager analysis and expressed as percentage of maximum PK abundance (open circles); percentage of sporangia showing visible cytoplasmic cleavage but not having released zoospores (triangles); percentage of empty sporangia, reflecting those that released zoospores (closed circles); and percentage of sporangia not showing any differentiation (squares). RNA was extracted from both sporangia and zoospores in the samples. (C) RNA from cleaving sporangia (60 min in 10°C water [CL]), zoospores (ZO), and cysts germinated for 8 h in 10°C water (GC). (D) RNA from cleaving sporangia (CL) and hyphae placed in nitrogen or carbon-limited media or water (NS, CS, and HS).
55% of cysts germinated in the experiment shown, which is a fraction typical for *P. infestans*.

The induction of the gene appeared to be a zoosporogenesis-specific response and not simply the consequence of starvation resulting from placing spores in water. The latter scenario needed to be considered since oomycete sporangia are metabolically active. However, the kinase transcript was not present in hyphae incubated in media lacking nitrogen or carbon or in water (Fig. 1D). Also, the gene was not induced in sporangia held at 24°C for 1 h in water (S. Tani and H. Judelson, unpublished data).

**Gene structure.** A single copy of the gene seemed to be present within the genome based on studies of independent BAC (bacterial artificial chromosome) clones and blot analysis of genomic DNA. On each of several BACs analyzed, the gene resided on a 6-kb HindIII fragment of a BAC containing the protein kinase gene. Digestions employed BamHI (B), EcoRI (E), HindIII (H), or PstI (P). Size standards shown in the right margin were determined using a 1-kb ladder (Invitrogen).

The gene appeared to be expressed as an intron-lacking transcript bearing 5’ and 3’ untranslated regions of 50 and 45 nucleotides (nt), respectively. This was determined by isolating and sequencing several full-length cDNAs that were compared to the genomic clone. The sequence CCAGATTCCACCA TTT was noted 96 nt upstream of the transcription start site, which weakly resembles the GCTCATTYNCAATTT motif found in most oomycete promoters although it is farther upstream than average (28). An A-T-rich region similar to a consensus polyadenylation signal was detected 20 nt upstream of the poly(A) tail.

**Protein structure.** The gene was predicted to encode a protein of 399 amino acids, which resembled CaMKs from other species (Fig. 3). The strongest match against sequences in GenBank was against a predicted calcium-dependent protein kinase (CDPK) from *Arabidopsis thaliana* (GenBank accession no. S46283; BLAST E = 10^{-41}), followed by a CDPK from the protozoan *Toxoplasma gondii* (GenBank accession no. AF043629; E = 10^{-37}). The *P. infestans* protein includes each of the 12 kinase subdomains diagnostic of serine/threonine kinases, including the ATP-binding and catalytic motifs (Fig. 3A and B). Twenty-one amino acids previously shown to be invariant or nearly invariant in other protein kinases are each conserved in the predicted *P. infestans* protein (16) (Fig. 3C). Twelve phosphorylation sites were predicted by PROSITE.

The relationship between the *P. infestans* gene product and CaMKs was strengthened by performing alignments between the kinase subdomains of representative proteins. The eukaryotic serine/threonine kinase subfamily can be classified into seven major groups (AGC, CaMK, CK1, CMGC, G Cycl, PTK, and STE) plus several other protein kinase groups (OPKs) (16). Amino acid alignments between the *P. infestans* protein and three representatives of each major group, plus 10 OPKs, indicated strongest affinity with the CaMKs (not shown).

However, strong affinity to any particular group of CaMKs was not revealed by alignment and tree-building studies (Fig. 4). This analysis included representative animal and fungal CaMKs, plant and protozoan CDPKs, Ca2+- and/or Ca2+- and calmodulin-regulated visinin-like kinases, plus proteins not directly regulated by calcium or calmodulin but resembling such kinases, including the calcium-dependent kinase-related kinases, phosphorylase kinase, SNF1-related kinases, phosphoenolpyruvate carboxylase kinases (PEPCKs), the DUN1 checkpoint kinase, and PEPCK-related kinases (PEPRKs). Best affinity was observed against the CaMK, CCaMK, and protozoan CDPK groups, but low bootstrap numbers preclude firm conclusions.

While the data suggested a relationship between the *P. infestans* protein and Ca2+- or calmodulin-regulated kinases, the *P. infestans* protein is novel in structure. The region N-terminal to the kinase subdomains is 91 amino acids long, which is larger than usually observed (18). In addition, in the *P. infestans* protein the region C terminal to the kinase subdomains is only 28 amino acids compared to 41 to >200 amino acids in other proteins (5, 18). Such C-terminal regions generally include autoinhibitory domains, a calmodulin binding domain(s), and association domains for other proteins (5, 18). Motifs for such functions (CaM domains, elongation factor 1 hands, and polyglutamine tracts) were not detected in the *P. infestans* protein, although these are known to be poorly conserved.

**Effect of actinomycin D on transcription of the kinase gene.**
The failure of actinomycin D to block cleavage and encystment has been used to suggest that these steps do not require de novo transcription (7, 27). To directly test how effectively this inhibitor blocks mRNA accumulation in sporangia, the kinase gene was probed against RNA from sporangia incubated in water at 10°C for 60 min with 0, 10, and 100 μg of actinomycin D/ml. For actinomycin D treatments, sporangia were harvested from cultures flooded with the inhibitor to ensure that it rapidly contacted the sporangia. Even at 100 μg of actinomycin D/ml, expression levels were 24% of normal (Fig. 5). Considering that other stages of development such as cyst germination are totally blocked by 1 μg of this compound/ml, passage of the inhibitor into sporangia likely occurred too slowly to block the transcription of rapidly induced genes such as the kinase. Tests of higher concentrations of actinomycin D were deemed impractical due to cost and solubility issues and the possibility of nonspecific effects.

DISCUSSION

The kinase is a marker for the earliest changes in gene expression that are known to occur during oomycete zoosporogenesis. Transcripts accumulated quickly after exposing sporangia to cool water, which initiates the cleavage of sporangial cytoplasm into individual zoospores. The kinase appears to be induced much earlier than the only other gene known to be activated in the zoospore pathway, which encodes a proline biosynthesis enzyme from P. parasitica (1). Levels of mRNA...
from the *P. parasitica* gene peak 4 h after zoospores are liberated, compared to those of the *P. infestans* kinase, which climax prior to release. Kinases play diverse regulatory roles by modifying targets such as transcription factors, metabolic enzymes, ion channels, and cytoskeletal components (5); consequently, it is reasonable to propose that the kinase regulates an early stage of the zoospore pathway.

A model to explain how cleavage is triggered and how the kinase is induced can be proposed based on the fact that sporangia must be exposed to both water and cool temperatures. The primary effect of water is likely not hydration of the sporangium, since these are not desiccated like typical spores of true fungi. Instead, water may release a germination inhibitor. Chilling may reduce membrane fluidity and thus alter the activity of membrane-associated proteins, such as G proteins or phospholipase C. Similar changes are invoked to explain chilling responses in plants (4, 32). Within seconds, chilling could result in the synthesis of inositol triphosphate and the release of intracellular calcium through inositol triphosphate receptor-gated channels. This could initiate changes in the cytoskeleton leading to cleavage and could activate transcription factors needed to express genes encoding proteins such as the kinase.

Although accumulation of kinase mRNA begins before cleavage is evident, the protein may not function until a later stage, such as encystment, cyst germination, or appressorium formation. To address when the kinase might act, we have generated transformants in which the gene is constitutively expressed.
expressed, using the strong ham34 promoter (21) (unpublished results). Of four transformants tested that expressed the full-length kinase cDNA, none appeared different from the wild type in terms of their rates of hyphal growth, sporulation, cleavage, encystment, cyst germination, or appressorium formation. This may indicate that the kinase does not assert its role until a substrate or regulatory subunit is synthesized during or after cleavage.

Previous studies using inhibitors such as actinomycin D led to the conclusion that cleavage and encystment do not require de novo RNA synthesis, apparently excluding the involvement of the kinase in those developmental stages (7, 27). However, we observed that even 100 μg of actinomycin D/ml only partially inhibited transcription. Our data do not necessarily contradict the previous conclusions, since at that concentration the abundance of the kinase mRNA was depressed by 76% while cleavage and encystment were reduced by only partially inhibited transcription. Our data do not necessarily contradict the previous conclusions, since at that concentration the abundance of the kinase mRNA was depressed by 76% while cleavage and encystment were reduced by 76%.

Further analysis of the P. infestans kinase is required to determine its substrates and mode of regulation. In other species, some kinases are dedicated to a single substrate while others are multifunctional, and some are regulated solely by transcription while others are controlled by regulatory subunits, calmodulin, or phosphorylation (5). The P. infestans protein has the strongest sequence similarity to Ca2+-and calmodulin-regulated kinases. However, compared to such kinases, the C-terminal domain of the P. infestans protein appears too small to have conventional sites for calmodulin binding. The gene thus resembles phosphoenolpyruvate carboxylase kinases of plants and the Dictyostelium myosin light-chain kinase (19, 34), which also lack sizable C-terminal domains and have kinase domains resembling the Ca2+- and calmodulin-regulated group but are not regulated by Ca2+ or calmodulin. Plant PEPCKs are controlled only at the level of transcription, while the Dictyostelium kinase is regulated by transcription and phosphorylation. As the P. infestans protein described here represents the first kinase characterized from any oomycete, it is interesting to speculate as to the relative importance of transcriptional versus posttranscriptional regulation of such proteins within that taxon.

The expression pattern of the kinase and the effects of inhibitors imply that the gene plays an important role in differentiation, but proof must await the development of a procedure for inactivating its function. Gene knockouts made using integrative disruption are not feasible in Phytophthora due to rare homologous recombination between transforming and chromosomal DNA. However, several groups report that sense or antisense transgenes can effect transcriptional silencing of the corresponding native gene at frequencies of 10% or higher (Lassaad Belbahri and Felix Mauch, personal communication) (35). We attempted this with the kinase gene but without success (unpublished results). Over 100 transformants expressing sense or antisense kinase sequences were analyzed, but in each the endogenous gene maintained its wild-type expression pattern and zoospore development (cleavage, release, encystment, and cyst germination) appeared normal. There are several possible explanations for why the kinase gene was recalcitrant to silencing. Only a small number of Phytophthora loci have been subjected to silencing, and not all may be equally susceptible due to variation in the structures of their mRNAs or the native chromatin environment of the gene. Also, those genes that have been silenced are all expressed during normal hyphal growth in contrast to the kinase, which is only expressed during a brief developmental stage. The kinase might thus be refractory to silencing, which presumably requires a period of time during which RNA-RNA or RNA-DNA interactions must occur. Also, kinase expression is specific to a phase of development when mitosis does not occur. This may restrict the formation of a silenced state since some proteins involved in silencing act primarily during division (3, 24, 36). Our future studies must address not only the kinase per se but also methods for manipulating genes with similar patterns of expression.

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