Molecular Cell, Vol. 3, 339–348, March, 1999, Copyright ©1999 by Cell Press

Internuclear Gene Silencing in *Phytophthora infestans*

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Summary

Transformation of the diploid oomycete plant pathogen *Phytophthora infestans* with antisense, sense, and promoter-less constructs of the coding sequence of the elicitin gene *inf1* resulted in transcriptional silencing of both the transgenes and the endogenous gene. Since heterokaryons obtained by somatic fusion of an *inf1*-silenced transgenic strain and a wild-type strain displayed stable gene silencing, *inf1* silencing is dominant and acts in *trans*. *Inf1* remained silenced in nontransgenic homokaryotic progeny from the silenced heterokaryons, thereby demonstrating that the presence of transgenes is not essential for maintaining the silenced status of the endogenous *inf1* gene. These findings support a model reminiscent of paramutation and involving a *trans*-acting factor that is capable of transferring a silencing signal between nuclei.

Introduction

Introduction of transgenes into eukaryotic genomes often leads to silencing of expression of both the transgenes and the homologous host genes (Matzke and Matzke, 1995; Baulcombe and English, 1996; Meyer and Saedler, 1996; Pal-Bhadra et al. 1997). As a result, accumulation of specific mRNAs is affected. However, the molecular mechanisms that trigger this so-called homology-dependent gene silencing remain largely unknown.

Gene silencing can be regulated at the transcriptional or posttranscriptional level. Transcriptional silencing in plants and fungi is often found to be correlated with cytosine methylation of promoter sequences and/or coding sequences (Meyer and Saedler, 1996; Park et al., 1996; Schuurs et al., 1997; Selker, 1997). Also condensation of chromatin may play a role (Ye and Signer, 1996; van Blokland et al., 1997). In the case of cosuppression, where homology-dependent gene silencing is regulated at the posttranscriptional level, a high turnover of RNAs is thought to be responsible (Dehio and Schell, 1994; van Blokland et al., 1994; Dougherty and Parks, 1995; English et al., 1996; Metzlaff et al., 1997; Vaucheret et al., 1997). If the transgene is a viral gene, silencing can result in resistance to virus infection. It was proposed that synthesis of aberrant RNA molecules provokes specific degradation of all homologous RNAs in the cytoplasm. The aberrant RNAs may also function as templates for the synthesis of copy-RNA (cRNA) molecules produced by endogenous RNA-dependent RNA polymerases (Sijen et al., 1996; Metzlaff et al., 1997). It has been postulated that these cRNAs bind to the mRNAs and that the so-formed double-stranded RNAs (dsRNAs) will subsequently be degraded by double strand–specific RNases (Lindbo et al., 1993; Dougherty and Parks, 1995). Support for a model in which dsRNA plays an essential role was recently found in *Caenorhabditis elegans*, where efficient silencing of a target gene was accomplished following injection of cells with dsRNA (Fire et al., 1998). This dsRNA interference appears to cross cellular boundaries and requires only a few molecules of dsRNA per affected cell.

Quelling, a transgene-induced gene-silencing phenomenon found in the ascomycete fungus *Neurospora crassa*, resembles posttranscriptional gene silencing in plants (Cogoni and Macino, 1997b). Interestingly, quelling is shown to be a dominant trait in heterokaryotic strains containing a mixture of transgenic and nontransgenic nuclei (Cogoni et al., 1996). Production of aberrant RNA molecules caused by transcription of the transgene is thought to trigger degradation of homologous mRNAs derived from the transformed nucleus and the wild-type nucleus (Cogoni and Macino, 1997a).

The non-cell-autonomous gene silencing recently found in *C. elegans* (Fire et al., 1998) has previously been reported in plants (reviewed by Jorgensen et al., 1998). Palauqui et al. (1997) found silencing in scions of grafted plants consisting of a silenced stock and an initially nonsilenced scion containing the transgene. Such systemic spread of cosuppression, or systemic acquired silencing, was also found by Voinnet and Baulcombe (1997) in transgenic plants producing green fluorescent protein (GFP). After local infection by *Agrobacterium tumefaciens* carrying the GFP reporter gene, the whole plant became GFP silenced. These findings illustrate that a gene-specific diffusible signal is capable of transmitting silencing.

In this study, we describe a novel transcriptional gene-silencing phenomenon in the diploid oomycete *Phytophthora infestans*, an important plant pathogen that is also known as "the Irish potato famine fungus." Despite the fact that *P. infestans* shows filamentous growth, it is considered to be a protoctist eukaryotic organism, and, as such, it is more related to golden-brown algae than to higher fungi. The mycelium of oomycetes is coenocytic, and hence mycelial cells may contain multiple nuclei that can differ genetically resulting in heterokaryotic strains. In the present study, we take full advantage of the unique features of *P. infestans* to address the following questions: (1) is gene silencing dominant in multinucleated cells, (2) can the silenced state be transmitted from nucleus to nucleus, and (3) can stable gene silencing of an endogene occur in a nontransformed nucleus?

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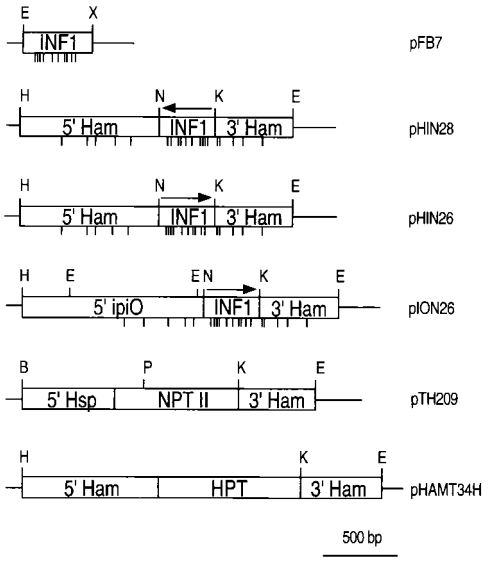

Figure 1. Transformation Constructs

pFB7 is an *inf1* cDNA clone. pHIN26 and pHIN28 contain the *inf1* coding sequence (INF1), either in sense orientation (→) or antisense orientation (←), fused to the promoter (5′ Ham) and terminator (3′ Ham) of the *ham34* gene of *Bremia lactucae*. pION26 contains a transcriptional fusion of the coding sequence of *inf1* in sense orientation and the in planta induced *ipi*O1 promoter (5′ ipiO) of *P. infestans*. pTH209 and pHAM34H contain coding sequences of the selectable antibiotic resistance genes neomycine phosphotransferase (NPTII) and hygromycine B (hyg.B) respectively, flanked by oomycete promoter and terminator sequences. B, BamHI; E, EcoRI; H, HindIII; K, KpnI; N, NcoI; P, PstI; X, XhoI. Restriction sites checked for cytosine and adenosine methylation are indicated in the constructs containing *inf1* (small vertical lines).

In order to address these questions, the *inf1* gene of *P. infestans* was selected as a target gene. This gene encodes the secreted protein INF1, a member of the elicitin family (Kamoun et al., 1997a, 1997b). Elicitins induce defense responses in plants, and recently we demonstrated that INF1 restricts the host range of *Phytophthora infestans* (Kamoun et al., 1998). Since *inf1* is a single locus gene that is highly expressed during vegetative growth in vitro and of which the gene product INF1 is easily detectable, the *inf1* gene appeared ideal for unraveling the mechanism of gene silencing. The novel gene-silencing phenomenon described here involves internuclear transfer of signals from transgenic silenced nuclei to wild-type nuclei leading to stable gene silencing in the wild-type nuclei. Once gene silencing is induced in wild-type nuclei, it is maintained in progeny, even in the absence of nuclei carrying transgenes.

Results

INF1 Is Absent in Culture Filtrates of Antisense and Sense Transformants

To investigate whether we could engineer *P. infestans* mutants deficient in the production of the secreted protein INF1, strain 88069 was transformed with the geneticin resistance construct pTH209 and cotransformed with constructs containing *inf1* in either sense (pION26 or pHIN26) or antisense (pHIN28) orientation (Figure 1). It has been described that integration of multiple copies at the same locus enhances the probability of silencing (Meyer, 1996). Therefore, we aimed at increasing the chance of tandem integrations of the transgenes by linearizing the plasmid DNA with EcoRI (pHIN26 and pHIN28) or HindIII (pION26). Culture filtrates from 56 cotransformants were screened for the absence of INF1. Six out of thirty antisense transformants (PY23, PY31, PY37, PY47, PY53 and PY57) and three out of twenty-six sense transformants (OY1, OY8 and QY1) failed to produce INF1 or produced significantly reduced amounts of INF1 (Figure 2). Twenty control transformants containing only the geneticin resistance construct (Y10 and Y15 are shown in Figure 2), and forty-seven cotransformants (of which ten are shown in Figure 2) produced INF1 in similar amounts as the wild-type recipient strain 88069. Obviously introduction of *inf1* sense and antisense constructs can lead to deficiency in INF1 production in 11% to 20% of the transformants.

Furthermore, we checked whether integration of a promoter-less construct also leads to silencing of *inf1*. Transformants were generated with one or more integrations of plasmid pFB7 containing a full-length *inf1* cDNA sequence. Sixteen cotransformants were obtained and culture filtrates were screened for absence of INF1. Four transformants (SY2, SY6, SY21, and SY27) failed to produce INF1 or produced significantly lower amounts of INF1 (data not shown), demonstrating that promoter sequences in the *inf1* transgene construct are not required to induce gene silencing in *P. infestans*.

inf1 mRNA Is Not Detected in INF1-Deficient Transformants

To determine whether absence of secreted INF1 protein correlates with absence of *inf1* mRNA in the mycelium, total RNA was isolated from in vitro–grown strains and Northern blot analyses were performed. High levels of *inf1* mRNA were detected in the recipient strain 88069, in the INF1-producing cotransformants, and in the control transformants (Y10 and Y15). In contrast, reduced levels of *inf1* mRNA were detected in the INF1-nonproducing antisense, sense (Figure 2), and promoter-less transformants (data not shown). Indeed, endogenous *inf1* mRNA was only observed in a few transformants upon long exposure of the autoradiographs (data not shown). Hybridization of the same blots with a single-stranded antisense *inf1* probe resulted in the same hybridization pattern, whereas with a single-stranded sense *inf1* probe, no antisense transcripts were detected in any of the tested cotransformants (data not shown). Hybridization with a probe of the constitutively expressed actin gene resulted in signals with similar intensity in all lanes, indicating that each lane contained equal amounts of total RNA. We conclude that absence of the INF1 protein is caused by a deficiency in *inf1* mRNA. Apparently, introduction of *inf1* gene constructs has caused silencing of both the endogenous *inf1* gene and the *inf1* transgenes.

To investigate whether the gene silencing observed in the *inf1* silenced transformants is gene sequence specific, expression of the related *inf2b* gene was analyzed by hybridizing the Northern blot with an *inf2b* probe (Figure 2). The *inf2b* gene belongs to a separate class within the elicitin gene family but shares 60% DNA homology to *inf1* over the homologous region of the open

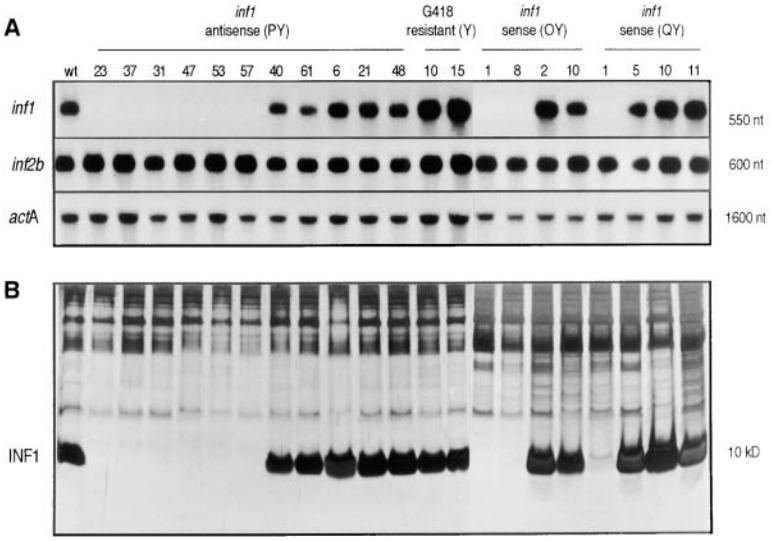

Figure 2. *P. infestans* Transformants Deficient in *inf1* mRNA and INF1 Protein Production

Analysis of *inf1* mRNA production (A) and INF1 protein production (B) in the wild-type recipient strain (wt), in transgenic antisense (PY) and sense (OY and QY) transformants, and in G418-resistant non-cotransformed (Y) strains.

(A) Northern blots containing in each lane 15 μg total RNA isolated from mycelium grown in vitro for 10 days were hybridized with probes derived from *inf1*, *inf2b*, and the actin gene *actA*. Transcript lengths in nucleotides (nt) are indicated on the right.

(B) Proteins present in culture medium of 7-day-old cultures were separated by tricine-SDS-PAGE and visualized by silver staining. The position of the 10 kDa INF1 protein is indicated (INF1).

reading frames (Kamoun et al., 1997a). Equal levels of *inf2b* mRNA were detected in all tested transformants as well as in the wild-type strain, suggesting that expression of the *inf2b* gene was not affected by the presence of the transgenic *inf1* gene sequences. Hence, we conclude that *inf1* gene silencing in *P. infestans* is gene sequence specific.

Silencing Is Not Due to Gene Disruption

To determine whether deficiency in *inf1* mRNA is associated with disruption or displacement of the endogenous *inf1* gene, genomic DNAs of the transformants were analyzed on Southern blots. Hybridization of BamHI-digested DNA with an *inf1* probe resulted in a 2.3 kb hybridizing fragment in all tested transformants and in the recipient strain (Figure 3A). This fragment corresponds to the endogenous single locus *inf1* gene, thereby demonstrating that in all transformants the endogenous *inf1* gene remained intact. Multiple hybridizing fragments, representing integrations of the transgene constructs, were only observed in the cotransformants. Apparently, the observed *inf1* silencing is not based on inactivation due to disruption.

Southern blots, containing genomic DNA digested with enzymes that recognize single restriction sites in the transgenic plasmid DNA, were hybridized with probes specific for the promoters of the transgene constructs (*ham34* for pHIN26 and pHIN28 and *ipi*O for pION26). This resulted in a strong hybridizing band of the size of the linearized plasmids in almost all transformants (Figure 3B), indicating that tandem integration of the constructs had occurred. However, the strong hybridizing band was not observed in the *inf1*-silenced transformants PY31, PY57, (Figure 3B) and OY8 (data not shown), suggesting that these strains contain only one or more single integrations of the construct. Therefore, *inf1* silencing is independent of the number of transgene integrations at a single site.

Silencing Is Not Based on High Turnover of *inf1* mRNA

Absence of *inf1* mRNA in the silenced transformants may result from either reduced transcription of *inf1* or from reduced mRNA stability. To distinguish between these two possibilities, nuclear run-on assays were performed. Nuclei of mycelia of wild-type strain 88069, control transformant Y15, antisense transformant PY37,

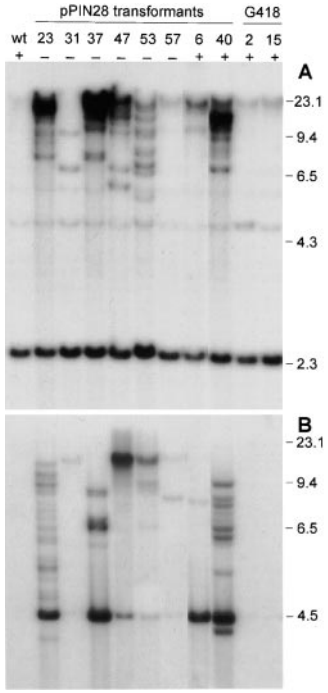

Figure 3. The Endogenous *inf1* Gene Is Not Disrupted in *inf1*-Silenced Transformants

Southern blot analysis of genomic DNA isolated from INF1-producing (+) and INF1-deficient strains (−). BamHI-digested (A) and EcoRI-digested (B) DNA from the wild-type recipient strain (wt), antisense transformants, and G418-resistant non-cotransformed strains were separated by electrophoresis and blotted onto a membrane. The blot in (A) was hybridized with a probe of the *inf1* gene. The 2.3 kb hybridizing band represents the endogenous *inf1* single locus gene. The blot in (B) was hybridized with a probe from the *Ham34* promoter. The 4.5 kb hybridizing band represents tandem integrations of the pHIN28 construct.

sense transformants QY1 and OY1, and a promoterless transformant SY21 were isolated. The transcripts present in the nuclei were extended and ^{32}P[UTP]-labeled. Filters containing single-stranded DNA fragments of *inf1*, *inf2b*, the actin gene *actA*, and the neomycin phosphotransferase gene *nptII* in antisense and sense orientations were hybridized with the nuclear transcripts. As shown in Figure 4A, the control transformant Y15 produced similar steady state levels of nuclear *inf1* RNA as wild-type strain 88069, whereas the silenced strains contained no or very low levels of nuclear *inf1* RNA. Quantification of the hybridization signals showed that the silenced transformant PY37 does not contain sense *inf1* mRNA whereas QY1 contains very few *inf1* primary transcripts, only up to 5% compared to the levels in 88069 and Y15. QY1 also showed small amounts of INF1 protein on silver-stained gels (Figure 2). *Inf1* antisense RNA could not be detected in any of the silenced transformants. *Inf2b* and actin mRNA levels were similar in all strains tested, and antisense RNAs were absent. These results demonstrate that *inf1* silencing in *P. infestans* is not based on a high turnover of *inf1* mRNA. Instead, inhibition of transcription is more likely.

Silencing Is Not Correlated with Hypermethylation of DNA

To investigate a possible role of DNA methylation in transcriptional silencing of *inf1*, genomic DNAs isolated from the wild-type strain, a control transformant (Y15), and four silenced transformants (QY1, OY1, PY37, and SY21) were digested with restriction enzymes suitable to detect cytosine or adenosine methylation and analyzed by Southern blot hybridization with *inf1* probes. The following isoschizomeric restriction enzyme pairs were used: HpaII/MspI (recognizes CCGG), Sau3A/MboI (recognizes GATC), and DpnI/MboI (recognizes GATC). HpaII and Sau3A are sensitive to cytosine methylation, and MboI is sensitive to adenosine methylation. Also, restriction enzymes AluI (recognizes AGCT) and HhaI (recognizes GCGC), which are both cytosine methylation–sensitive, and adenosine methylation–sensitive RsaI (recognizes GTAC) were used. Methylation sites that were tested in *inf1* and in the transformation constructs are shown in Figure 1. By comparing the hybridization patterns of the *inf1*-silenced strains with the wild-type and Y15 strains, no shifts in hybridizing bands representing the endogenous *inf1* gene were noted, suggesting absence of cytosine and adenosine hypermethylation at the sites tested (Figure 4B; data not shown). Similarly, the sizes of the hybridizing bands representing the *inf1* transgenes did not change, indicating that also at the transgenes methylation is absent. From these data, we conclude that hypermethylation of the endogenous *inf1* gene sequence is not involved in the silencing mechanism.

Gene Silencing in Heterokaryons of *P. infestans*

To determine whether gene silencing is dominant in heterokaryotic strains, forced heterokaryons of *inf1*-silenced transformants and a nonsilenced wild-type strain were

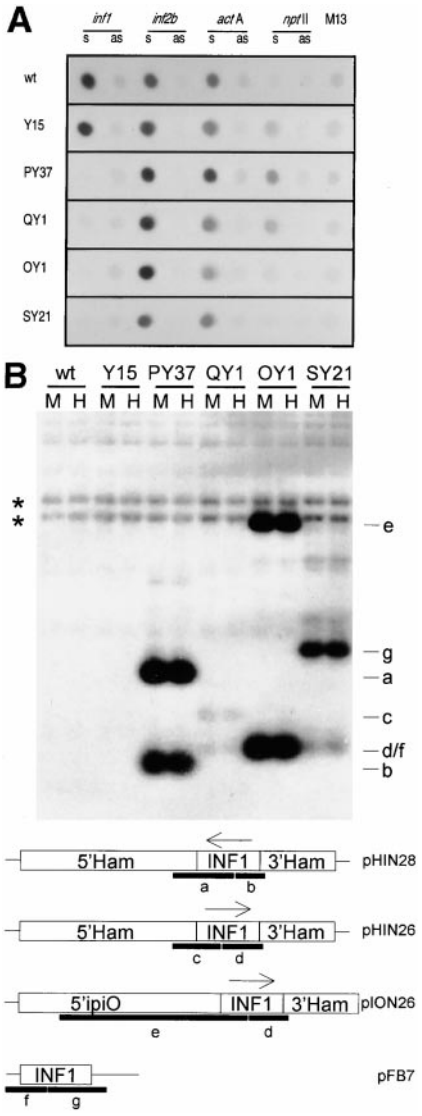

Figure 4. *Inf1* Silencing Is Regulated at the Transcriptional Level and Not Due to Hypermethylation

(A) Transcriptional activity of *inf1* analysed by nuclear run-on assays. Autoradiographs of dot-blot filters, containing per dot 1 μg of gene-specific single-stranded DNAs hybridized to ^{32}P-labeled nascent RNA synthesized in vitro in nuclei isolated from 5-day-old mycelia of wild-type strain 88069, non-cotransformant Y15, antisense transformant PY37, sense transformants QY1 and OY1, and promoter-less transformant SY21. The gene-specific single-stranded DNAs synthesized from M13-based recombinant phages hybridize to sense (s) or antisense (as) RNAs derived from *inf1*, *inf2b*, *actA*, and *nptII*. The filters contain M13 DNA as a control.

(B) Analysis of cytosine methylation at the *inf1* locus. Southern blot analysis of genomic DNA isolated from INF1-producing and INF1-deficient strains. DNA from the wild-type recipient strain 88069 (wt), a non-cotransformed strain (Y15), an antisense transformant (PY37), two sense transformants (QY1 and OY1), and a promoter-less transformant (SY21) were digested with the isoschizomeric restriction enzymes MspI (M) and HpaII (H), separated by electrophoresis, blotted, and hybridized with a *inf1* probe. HpaII does not digest when a internal C residue in the recognition site CCGG is methylated. Endogenous *inf1* DNA fragments are indicated with an asterisk (*). The strong hybridizing bands a–g correspond to DNA fragments indicated by a–g in the transformation constructs shown at the bottom.

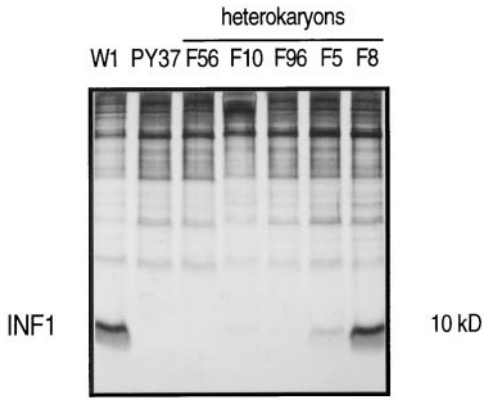

Figure 5. INF1 Deficiency in Heterokaryons

Analysis for the presence or absence of INF1 protein in culture filtrate of heterokaryons (F3, F5, F8, F10, F56, and F96) obtained by fusion of a hygromycin B–resistant INF1-producing strain (W1) and a G418-resistant *inf1*-silenced transformant (PY37). The heterokaryons are G418 and hyg.B resistant. Proteins present in culture medium of 7-day-old cultures were separated by tricine-SDS-PAGE and visualized by silver staining. The position of the 10 kDa INF1 protein is indicated (INF1).

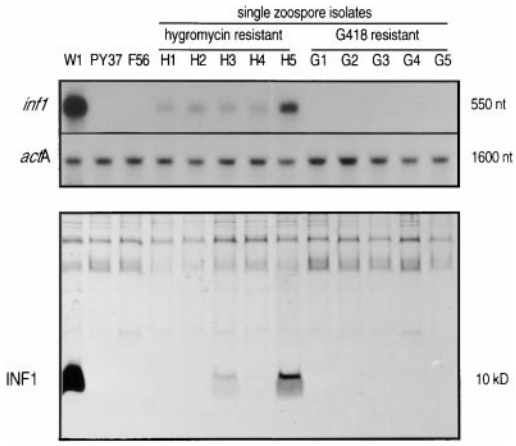

Figure 6. *Inf1* Silencing in Homokaryotic Progeny from a Silenced Heterokaryon

Analysis of *inf1* mRNA production (upper panel) and INF1 protein production (lower panel) in homokaryotic single zoospore isolates (H1–H5, G1–G5) derived from the INF1-deficient heterokaryon F56. F56 was obtained by fusion of a hyg.B-resistant INF1-producing strain (W1) and a G418-resistant *inf1*-silenced transformant (PY37). F56 is G418 and hygromycin B resistant, whereas G1–G5 are G418 resistant, hyg.B sensitive, and H1–H5 are G418 sensitive, hyg.B resistant.

(Upper panel) Northern blots containing in each lane 15 μg total RNA isolated from mycelium grown in vitro for 10 days were hybridized with probes derived from *inf1* and *actA*. Transcript lengths in nucleotides (nt) are indicated on the right.

(Lower panel) Proteins present in culture medium of 7-day-old cultures were separated by tricine-SDS-PAGE and visualized by silver staining. The position of the 10 kDa INF1 protein is indicated (INF1).

generated by protoplast fusion. Protoplasts of geneticin- (G418-) resistant, *inf1*-silenced transformants QY1, PY23, or PY37 were fused with protoplasts of W1, a hygromycin B– (hyg.B-) resistant transformant. W1 is a derivative of the wild-type strain 88069 obtained after transformation with pHAMT34H, a vector containing the hygromycin phosphotransferase gene (*hpt*) (Judelson et al., 1991). W1 produces normal levels of INF1 protein (Figure 5). In total, 100 heterokaryotic somatic fusion products of PY37 and W1 were selected and analyzed for INF1 production. Seven heterokaryotic isolates failed to produce INF1, whereas 34 showed reduced levels of INF1 protein when compared to the levels produced by the wild-type strain or W1. F56 and F96 are examples of heterokaryons that fail to produce INF1 (Figure 5). Silencing in the heterokaryons remained stable during vegetative growth on medium containing both G418 and hyg.B for at least 9 months (data not shown). Similar results were obtained upon fusion of PY23 with W1, and QY1 with W1 (data not shown). In all cases, the heterokaryons represented the whole range of INF1 phenotypes: some produced no INF1 at all, whereas others produced reduced or similar amounts as the wild type. These results demonstrate that *inf1* gene silencing in heterokaryotic strains can be dominant and act in *trans*.

Internuclear Transfer of the Silenced State

To investigate whether the silenced state can be transmitted from nucleus to nucleus, single unicellular and uninucleate zoospore cultures were obtained from the silenced heterokaryons. Zoospores from heterokaryons F56 and F96 (fully silenced), from heterokaryon F10 (partly silenced), and from the parental strains PY37 and W1 were plated onto medium without antibiotics and on medium containing either G418, hyg.B, or both G418 and hyg.B. No colonies resistant to both antibiotics were recovered, suggesting that karyogamy had not occurred in these heterokaryons. In all cases, equal numbers of colonies grew on plates containing hyg.B and on plates containing G418. In general, the total number of colonies growing on these plates matched the total number of colonies growing on plates without antibiotics, indicating a nuclear distribution of 1:1 of the two nuclear types in the heterokaryotic mycelia.

Subsequently, the homokaryotic strains derived from the heterokaryons F56, F96, and F10 were screened for INF1 protein production and *inf1* gene expression. All *inf1* transgenic (G418-resistant) homokaryotic strains derived from the silenced heterokaryotic strains F56, F96, and F10 remained fully silenced (homokaryons G1–G5 derived from F56 are shown in Figure 6). In the G418-resistant single zoospore isolates G1–G5, the *inf1* mRNA levels were less than 0.1% of the wild-type level, which is comparable to the reduction found in the heterokaryotic strain F56 and the parental strain PY37. Surprisingly, silencing was also observed in all homokaryotic isolates resistant to hyg.B (homokaryons H1–H5 derived from F56 are shown in Figure 6). In these single zoospore isolates, *inf1* mRNA levels were reduced varying from 3% (H1) to 18% (H5) of the wild-type level and little or no INF1 protein was found.

In order to confirm that karyogamy did not occur in the heterokaryons, BamHI-digested genomic DNA of the parental strains W1 and PY37, heterokaryotic strain F56, and its derived homokaryotic single zoospore isolates (H1–H5 and G1–G5) were hybridized to a probe of the *hpt* gene (Figure 7B). One hybridizing band was observed in

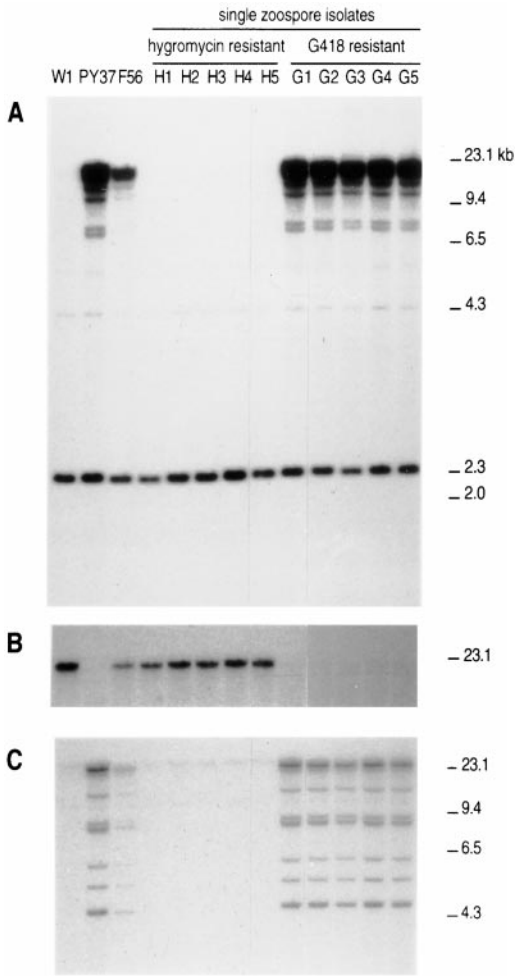

Figure 7. *Inf1* Silencing in the Absence of *inf1* Transgenes

Southern blot analysis of genomic DNA isolated from homokarytic hyg.B-resistant (H1–H5) and G418-resistant (G1–G5) progeny from the INF1-deficient heterokaryon F56. F56 was obtained by somatic fusion of INF1-producing strain W1 and *inf1*-silenced transformant PY37. BamHI-digested DNA was separated by electrophoresis, blotted onto a membrane, and hybridized with a ^{32}P-labeled probe of (A) *inf1*, (B) the hyg.B resistance gene *hpt*, and (C) the G418 resistance gene *nptII*.

(A) The 2.3 kb hybridizing fragment, present in all lanes, represents the endogenous *inf1* single locus gene. Other hybridizing fragments, visible in lanes containing genomic DNA of PY37, F56, and G1–G5, represent *inf1* transgenes.

(B) The 23 kb hybridizing fragment contains the transgenic *hpt* gene(s) present in the hyg.B-resistant strains W1 and F56 and the homokaryons H1–H5.

(C) Hybridizing fragments contain transgenic *nptII* sequences present in the G418-resistant strains PY37 and F56 and the homokaryons G1–G5.

the hyg.B-resistant homokaryotic single zoospore strains and in F56 and W1, but not in the silenced parental strain PY37 or the G418-resistant homokaryotic single zoospore isolates (G1–G5). Hybridization with a probe derived from the *nptII* gene revealed hybridizing bands in G1–G5, F56, and PY37, but not in the parental strain W1 or the single zoospore isolates H1–H5 (Figure 7C). The endogenous *inf1* gene visualized by hybridization with the *inf1* probe was detected in all strains, whereas transgenic *inf1* sequences could only be found in the G418-resistant strains (Figure 7A). Southern blot analysis of the single zoospore homokaryotic strains derived from F10 and F96 gave similar results (data not shown). Apparently, karyogamy did not occur in the heterokaryotic strains tested.

To investigate whether mutations in the DNA sequence of the endogenous *inf1* gene are responsible for loss of INF1 production in the silenced strains, the nontransgenic *inf1*-silenced single zoospore isolates resistant to hyg.B (H1–H5) appeared to be ideal. In those strains, the endogenous *inf1* gene can be amplified by PCR from genomic DNA without interference of transgenic *inf1* sequences. Sequence analysis of PCR fragments corresponding to endogenous *inf1* derived from four silenced homokaryotic strains (H1, H3, H4, and H5) did not reveal any consistent base pair mutation (data not shown).

These results demonstrate that *inf1* in wild-type nuclei remains silenced in the absence of transgenic *inf1* sequences, indicating that internuclear transfer of the silenced state must have occurred.

Discussion

Here, we describe a novel gene-silencing phenomenon in the oomycete *Phytophthora infestans*. Stable silencing of an endogenous target gene was achieved following transformation with antisense, sense, and promoter-less gene constructs. Efficient silencing was also manifested in heterokaryotic mycelia obtained by protoplast fusion of a transgenic silenced strain and a nonsilenced strain, suggesting the involvement of a *trans*-acting silencing signal. Furthermore, we discovered that the presence of nuclear transgenic sequences is not essential to retain silencing of an endogenous gene: homokaryotic strains, obtained from uninuclear spores from silenced heterokaryotic strains, maintained the silenced phenotype even in the absence of transgenes.

Inf1 Silencing Is Gene Sequence Specific

The introduction of various DNA constructs of the *inf1* target gene into the *P. infestans* genome resulted in a complete suppression of INF1 protein production in up to 20% of the cotransformants, and this suppression is the result of absence of *inf1* mRNA. Similar to what has been found in other systems (Matzke and Matzke, 1995), this gene silencing in *P. infestans* seems to be gene sequence specific. In the silenced transformants, only accumulation of *inf1* mRNA is affected, but not the mRNA levels of a related *inf2b* elicitin gene.

Since only a portion of the cotransformants (up to 20%) shows silencing, introduction of *inf1* transgenes in itself does not seem to be the trigger for gene silencing nor is tandem integration of transgenes. While in most silenced transformants, the transgene was integrated in tandem repeats with high copy numbers, we obtained several silenced transformants with single integrations. In all silenced transformants, the endogenous *inf1* gene is still intact and sequence analyses of the endogenous *inf1* coding sequence in silenced homokaryotic strains revealed no specific (point) mutations, demonstrating

that the observed gene silencing is not the result of mutations or gene disruptions.

Transcriptional *inf1* Silencing Is Not Due to De Novo Methylation

Nuclear run-on assays demonstrated that *inf1* silencing occurs at the transcriptional level. A feature, often found to be associated with transcriptional silencing in plants as well as in filamentous fungi, is cytosine methylation of repeated sequences (Selker, 1990; Rossignol and Faugeron, 1994; Meyer, 1996; Schuurs et al., 1997). This DNA methylation can either be the cause or the consequence of gene inactivation. Here, we show that the endogenous and transgenic *inf1* sequences are not hypermethylated and conclude that methylation can not be responsible for the *inf1* silencing.

Internuclear Gene Silencing

A major advantage of using a fungus or a fungus-like organism for dissecting gene-silencing mechanisms is that individual nuclei can easily be separated and multiplied by regenerating mycelium from single vegetative spores. Subsequently, fusion of protoplasts derived from different homokaryotic strains will result in stable heterokaryotic strains carrying nuclei with different characteristics. Here, we report efficient gene silencing in heterokaryons of *P. infestans* containing *inf1* transgenic and nontransgenic nuclei, thereby demonstrating that silencing is dominant and acts in *trans* to silence the target gene in both transformed and untransformed nuclei. Moreover, *inf1* silencing is stably maintained in homokaryotic strains obtained following nuclear separation of the silenced heterokaryons, even in the absence of *inf1* transgenes. Since karyogamy could not be demonstrated, it is unlikely that the silenced state of the *inf1* gene is transmitted from one nucleus to the other by specific DNA–DNA interactions. Also transitory interactions between DNA of *inf1* transgenic and nontransgenic nuclei during simultaneous mitotic divisions seem unlikely, since the nuclear envelope and nuclear matrix remain fully intact throughout mitosis in most fungal and oomycete species (Heath, 1980). We propose a novel-silencing phenomenon in which a diffusible silencing factor is involved in inducing stable gene silencing, and we call this phenomenon internuclear gene silencing.

Even though a silencing phenomenon seemingly similar to what we observed in heterokaryons of *P. infestans* was found in the ascomycete fungus *Neurospora crassa*, the mechanisms are not the same. While analyzing the mechanism of quelling in *N. crassa*, Cogoni et al. (1996) found that fusion of a wild-type orange-colored strain and an *al-1*-silenced strain with the "albino" phenotype, resulted in "albino" heterokaryons. However, after nuclear separation of the quelled heterokaryons, the recovered homokaryotic strains containing only wild-type nuclei were orange again, and *al-1* was not silenced anymore. This demonstrated that, in contrast to *P. infestans*, the silenced state in *N. crassa* is not heritably transmitted from nucleus to nucleus. The presence of transgenes in quelled strains of *N. crassa* seems to be a prerequisite for maintenance of the silenced state. Furthermore, quelling is regulated at the posttranscriptional level, and, therefore, quelling in *N. crassa* and internuclear gene silencing in *P. infestans* must be based on different mechanisms.

Internuclear silencing in *P. infestans* is also clearly different from yet another silencing phenomenon observed in ascomycetes and that is MIP (methylation induced premeiotically), a process extensively studied in *Ascobolus immersus* (Colot et al., 1996). MIP involves transfer of DNA methylation between homologous alleles, is most likely based on DNA–DNA interaction, and takes place during a particular stage in the sexual cycle when the haploid nuclei are in a common cytoplasm. In contrast to MIP, internuclear gene silencing involves neither methylation nor DNA–DNA contact and is an event occurring in diploid nuclei during asexual stages of the life cycle.

Is Internuclear Gene Silencing in *P. infestans* Related to Paramutation?

Interestingly, the internuclear silencing observed in *P. infestans* is a *trans*-inactivation phenomenon that shares similarities with paramutation. Paramutation is an epigenetic phenomenon involving either allelic interactions or interactions between homologous unlinked loci (e.g., endogenes and transgenes) and resulting in persistent changes in expression even after the interacting alleles or genes segregate in the progeny (Meyer et al., 1993; Hollick et al., 1997). Alleles that are sensitive to paramutation are termed "paramutable," and alleles that incite paramutation are termed "paramutagenic." Examples of paramutation are so far limited to plant genes, but there are indications that paramutation occurs in a wide variety of biological systems (Hollick et al., 1997).

We speculate that in the *inf1*-silenced *P. infestans* heterokaryons the duplicated *inf1* gene sequences in the transgenic nucleus represent the paramutagenic loci (or silencer loci) and that the endogenous *inf1* gene loci in the transgenic nuclei and in the wild-type nuclei represent the paramutable loci. These endogenous *inf1* gene loci have become *trans*-inactivated and converted into paramutant, *inf1*-silenced loci. Following separation of the transgenic and wild-type nuclei, the paramutant *inf1* loci in the homokaryotic single zoospore isolates containing a wild-type nucleus retain the reduced level of expression. Among the heterokaryons and homokaryons, we found variation in INF1 protein production ranging from 82% to 97% reduction. Similarly, variation in expression of paramutant loci has been found in plants (Meyer and Saedler, 1996).

The most extensively studied examples of paramutation concern plant genes that determine flower or seed color, two phenotypes that, when mutated, are easily recognized in sexual progeny of the primary mutants (reviewed by Hollick et al., 1997). In general, paramutation is defined as an epigenetic change that is meiotically inheritable. Nevertheless, the paramutation itself takes place in somatic cells and besides being meiotically stable, the paramutant loci are also stable during mitosis. Silencing of *inf1* in *P. infestans* is incited in somatic cells by introduction of homologous transgenes. The presumed *inf1* paramutation in nontransgenic nuclei also

occurs in somatic cells. As demonstrated, the paramutant *inf1* loci are stable during mitosis, and the silenced phenotype is maintained after the interacting genes are inherited separately in asexual progeny. Whether the epigenetic change at the *inf1* locus is inherited in sexual progeny and is meiotically stable remains to be determined.

The molecular mechanisms underlying paramutation are not clear. In fact, every example of paramutation is different and each example might reflect a distinct mechanism (Hollick et al., 1997). For instance, some cases seem to be associated with DNA methylation while others are not. One intriguing aspect of the mechanism is the basis of the allelic interaction. Our findings imply that the *inf1* silenced state is transmitted in *trans* without direct DNA–DNA contact between the paramutagenic *inf1* locus and the paramutable loci. In the heterokaryons, karyogamy could not be demonstrated, and in coenocytic mycelium where the nuclear envelope remains intact during mitosis (Heath, 1980), pairing of chromosomes present in different nuclei seems very unlikely. Also in maize, where paramutation occurs at several loci, there is no evidence for chromosomal pairing in somatic cells (Heslop-Harrison and Bennett, 1990). It has been postulated that protein factors produced by transposable elements mediate *trans*-interactions that cause heritable changes in gene activity in genes located in the vicinity of transposable elements (Martienssen, 1996; Matzke et al., 1996; Hollick et al., 1997). In such a model, physical contact between paramutagenic and paramutable loci is not required. Instead, a *trans*-acting factor mediates silencing from one locus to the other. The heritable change that influences neighboring gene activity could well be a conformational change in chromatin structure.

We conclude that the strongly reduced transcription in the homokaryotic nontransgenic *P. infestans* strains has to be the consequence of paramutation causing an inheritable change in *inf1* expression possibly brought about by changes in chromatin structure.

What Is the *trans*-acting Silencing Factor?

If indeed changes in chromatin structure bring about the heritable change in *inf1* expression, the proposed *trans*-acting silencing factor that moves from nucleus to nucleus might be a protein. In *Drosophila* and yeast, it has been shown that changes in chromatin structure and histone modifications are responsible for the silenced state of several genes and these processes involve many different proteins that constitute chromatin-silencing factors (reviewed by Pirrotta, 1997, 1998; Sherman and Pillus, 1997). Examples are the Polycomb group (PcG) proteins that interact with several genes in *Drosophila* as well as in vertebrates (Pirrotta, 1998) and the Sir1p-4p proteins from yeast, which establish and maintain silencing at the silent mating type loci and at the telomeres. Sir2p is known to affect levels of histone deacetylation, while Sir3p and Sir4p are limiting structural components important for remodeling and establishing silenced chromatin (Sherman and Pillus, 1997).

However, to account for the sequence specificity of the internuclear gene silencing, the *trans*-acting silencing factor must be able to recognize the paramutable *inf1* alleles. It is feasible that one of the components of the *trans*-acting silencing factor is *inf1* RNA by which the target gene is recognized. Indeed, interactions between RNA and genomic DNA, combined with propagation of changes along the chromatin, have been suggested to play a role in transcriptional gene silencing, not only in plants (Wassenegger et al., 1994; Matzke and Matzke, 1995) and fungi (Schuurs et al., 1997) but also in mammalian X chromosome inactivation (Latham, 1996) and in gene inactivation in *C. elegans* (Fire et al., 1998).

Based on our results and in conjunction with the gene silencing concepts described above, we speculate that the proposed diffusible *trans*-acting silencing factor in *P. infestans* is either a protein, an aberrant RNA molecule, or a complex consisting of RNA and protein. At first, RNA-mediated interference at the level of chromatin structure or transcription seems unlikely, since in the run-on assays no (aberrant) *inf1* transcripts have been detected. However, the proposed silencing factor does not need to be produced in large amounts. Even very low quantities could be effective, since the molecule would act at the DNA level (Fire et al., 1998). Moreover, if the silencing factor is a small RNA molecule, it can be easily transported from nucleus to nucleus to facilitate changes in DNA structure of the target gene in nontransgenic nuclei in heterokaryotic strains. However, as long as the *trans*-acting silencing factor is not characterized, its exact nature remains a matter of speculation.

Experimental Procedures

Phytophthora Strains and Culture Conditions

P. infestans strain 88069 was used in all transformation experiments. Cultures were routinely grown in the dark at 18°C on rye agar medium supplemented with 2% (w/v) sucrose (RS medium) as described before (van West et al., 1998). Mycelium for isolation of DNA and RNA was obtained by growing cultures of *P. infestans* in liquid RS medium or modified Plich medium (containing per liter 0.5 g KH_2PO_4, 0.25 g $MgSO_4$, 1.0 g asparagine, 1.0 mg thiamin, 0.5 g yeast extract, 10 mg β-sitosterol, and 25 g glucose).

Plasmid Constructions and Transformation of *P. infestans*

Plasmid pFB7, containing a 557 bp *inf1* cDNA, was used as a promoter-less transformation construct (Kamoun et al., 1997b). Plasmids pION26 and pHIN26, which contain the coding region of *inf1* fused to the *ipi*O1 promoter (Pieterse et al., 1994) or the *ham34* promoter (Judelson et al., 1991), respectively, were constructed as follows. A 354 bp fragment of the complete *inf1* coding sequence was generated by polymerase chain reaction (PCR) using oligonucleotides PIET25 (5′-CCGATATCCATGGACTTTCGTGCTCTGTTCGC-3′) and PIET26 (5′-GGCCCCGGGTACCTCATAGCGACGCACACGTAG-3′) and plasmid pFB7. Following digestion, the amplified fragment was inserted in NcoI- and KpnI-digested pPIN13 (van West et al., 1998) or pHAMT35G (Judelson et al., 1991), which resulted in pION26 and pHIN26, respectively. Plasmid pHIN28 was obtained as described by Kamoun et al. (1998). Stable *P. infestans* transformation was conducted according to van West et al. (1998) using linearized plasmid DNA.

M13 recombinant phages used for generation of single-stranded DNA probes for the detection of sense and antisense *inf1*, *inf2b*, *actA*, and *nptII* RNA were constructed using standard techniques. Basically, full-length coding sequences of *inf1*, *inf2b*, *actA*, and *nptII* were amplified using complementary PCR primers extended with appropriate restriction sites. The amplified fragments were inserted in pGEM-T (Promoga) and recloned in M13mp18 RF and M13mp19 RF (GIBCO BRL).

Protoplast Fusion

Protoplasts from two parental transformants either resistant to hygromycin B (hyg.B) or resistant to geneticin (G418) were obtained and

regenerated as described by van West et al. (1998). The parental protoplast suspensions were diluted to an equal concentration (10^7–10^8 protoplasts per ml). To 1 ml of the protoplast mix, 1 ml of 50% PEG 3350 (Sigma) in 20 mM $CaCl_2$ and 10 mM Tris/HCl (pH 7.5) were added very slowly. Selection of heterokaryons was performed on RS medium containing 10 μg/ml G418 and 50 μg/ml hyg.B. Heterokaryons able to grow under these conditions were further subcultured on RS medium containing 5 μg/ml G418 and 25 μg/ml hyg.B. Uninuclear zoospores were collected as described by van West et al. (1998). To obtain homokaryotic isolates, 100 μl of a diluted zoospore suspension (1×10^2 per ml) was gently spead on RS medium containing 5 μg/ml G418 or 25 μg hyg.B. Colonies appeared within 4–10 days and were propagated on RS medium containing the appropriate antibiotic.

Southern and Northern Blot Analysis

Genomic DNA of *P. infestans* was isolated from mycelium as described by Raeder and Broda (1985) with minor modifications. Genomic DNA for methylation studies was digested with a 5–10 times excess of restriction enzyme. Total RNA from *P. infestans* was isolated, blotted, and hybridized as previously described (van West et al., 1998).

DNA templates for probe synthesis were a 354 bp NcoI–KpnI fragment and a 920 bp HindIII–NcoI frament of pHIN26 containing the *inf1* coding sequence and the *Ham34* promoter sequence respectively, a 796 bp HindIII fragment from pSTA31 containing the *actA* coding sequence, and a 343 bp PCR fragment containing the *inf2b* coding sequence. Probes were radiolabeled with [α-^{32}P]dATP by using a Random Primers DNA Labeling System (GIBCO BRL).

Protein Analysis

Protein concentrations in culture filtrates of *P. infestans* isolates were determined by the Bradford method with bovine serum albumin as a standard (Bradford, 1976). Equal amounts of protein were electrophoresed on tricine sodium dodecyl sulfate-polyacrylamide gels and the separated proteins were silver stained as described before (Kamoun et al., 1998).

Nuclei Isolation and Nuclear Run-ons

Suspensions of 2.10^5 sporangia per ml were mixed with two times concentrated ALBA medium (van West et al. 1998). After growth for 5 days, mycelial pellets were harvested by filtration and frozen in liquid nitrogen. From approximately 3 g of frozen mycelia, sufficient nuclei can be obtained to perform a nuclear run-on assay. The isolation of nuclei and the run-on assays were conducted as described by van Blokland et al. (1994). Aliquots of labeled nuclear RNA were taken for scintillation counting to determine the incorporation of labeled nucleotides, which varied from 1×10^6 to 2.2×10^6 cpm per batch of nuclei.

One microgram of the single-stranded DNAs prepared from the M13 recombinant phages (diluted in 200 μl of 10 × SSC) was applied to Hybond N^+ membranes (Amersham) using a dot blot apparatus (Biorad). The membranes were hybridized with the radioactive labeled nuclear run-on RNA (1×10^6 cpm) as described by van Blokland et al. (1994).

Transcription levels of the endogenous *inf1* gene were quantified by determining the amount of radioactive labeled RNA hybridizing to the membranes with a Fujix Bio-imaging analyser (BAS 2000). To normalize for incorporation differences between separate run-ons, we determined the ratio of the signals of the *inf1* sense probe to that of the *actA* sense probe and used that as internal control. The percentage of *inf1* transcripts in the silenced transformants was determined by dividing the *inf1* to *actA* signal ratios of the *inf1*-silenced transformants by the average *inf1* to *actA* signal ratio of 88069 and Y15, multiplying this by 100%, and subtracting M13 background hybridization (5%).

Acknowledgments

We are grateful to Tony van Kampen, Grardy van den Berg-Velthuis, and Koen de Groot for technical assistance. Pierre J. G. M. de Wit, Ab van Kammen, and Marcel Prins are acknowledged for critical reading of the manuscript and Neil Gow for giving P. v. W. the opportunity to prepare the manuscript. This work was financially supported by NWO-SLW project 805–22–461 (P. v. W) and by NWO-SLW/STW project 805–22–767 (S. K.).

Received November 2, 1998; revised January 12, 1999.

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