

Internuclear gene silencing in *Phytophthora infestans* is established through chromatin remodelling

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In the plant pathogen *Phytophthora infestans*, nuclear integration of *inf1* transgenic DNA sequences results in internuclear gene silencing of *inf1*. Although silencing is regulated at the transcriptional level, it also affects transcription from other nuclei within heterokaryotic cells of the mycelium. Here we report experiments exploring the mechanism of internuclear gene silencing in *P. infestans*. The DNA methylation inhibitor 5-azacytidine induced reversion of the *inf1*-silenced state. Also, the histone deacetylase inhibitor trichostatin-A was able to reverse *inf1* silencing. *inf1*-expression levels returned to the silenced state when the inhibitors were removed except in non-transgenic *inf1*-silenced strains that were generated via internuclear gene silencing, where *inf1* expression was restored permanently. Therefore, *inf1*-transgenic sequences are required to maintain the silenced state. Prolonged culture of non-transgenic *inf1*-silenced strains resulted in gradual reactivation of *inf1* gene expression. Nuclease digestion of *inf1*-silenced and non-silenced nuclei showed that *inf1* sequences in silenced nuclei were less rapidly degraded than non-silenced *inf1* sequences. Bisulfite sequencing of the endogenous *inf1* locus did not result in detection of any cytosine methylation. Our findings suggest that the *inf1*-silenced state is based on chromatin remodelling.

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INTRODUCTION

In many organisms, including animals, plants, fungi and protists, transcriptional gene silencing (TGS) can occur as a consequence of the introduction of transgenes into the somatic genome (Selker, 1997; Henikoff, 1998; Vaucheret *et al.*, 1998; Cogoni & Macino, 1999; van West *et al.*, 1999). TGS is thought to have evolved as a mechanism to protect the nuclear genome against potentially harmful transforming sequences such as transposons and viruses (Kooter *et al.*, 1999). TGS is reminiscent of paramutation found in plants, and other imprinting phenomena (Hollick *et al.*, 1997). Paramutation is an epigenetic phenomenon in which one allele or locus spontaneously acquires a less transcriptionally active state, which is then imposed on homologous sequences in the genome (Chandler *et al.*, 2000). In plants and animals TGS is often, but not invariably associated with altered methylation patterns and

changes in chromatin structure. Organisms lacking efficient DNA methylation systems, such as *Drosophila*, *Caenorhabditis elegans* and yeast, use chromatin modification to achieve imprinting and to maintain epigenetic states. Several experiments have shown that chromatin remodelling and histone deacetylation processes influence higher-order chromatin structure and can result in transcriptional repression (Tyler & Kadonaga, 1999; Grewal & Elgin, 2002; Vermaak *et al.*, 2003).

Other homology-dependent gene-silencing phenomena are also found; these are thought to occur at a post-transcriptional level in the cytoplasm. Post-transcriptional gene silencing (PTGS) phenomena have one major feature in common, which is that target mRNAs are degraded in a sequence-specific manner. Examples include RNA interference (RNAi) (Fire *et al.*, 1998; Plasterk & Ketting, 2000), co-suppression in plants (van der Krol *et al.*, 1990), quelling in fungi (Cogoni *et al.*, 1996), and virus-induced gene silencing (VIGS) in plants (Ruiz *et al.*, 1998; Lu *et al.*, 2003). It is likely that PTGS phenomena have also evolved as genome defence responses and/or as regulatory mechanisms of gene expression (Baulcombe, 2002).

Abbreviations: 5-AC, 5-azacytidine; BuA, butyric acid; HDAC, histone deacetylase; PTGS, post-transcriptional gene silencing; TGS, transcriptional gene silencing; TSA, trichostatin A.

We have shown previously that transformation of the diploid oomycete plant pathogen *Phytophthora infestans* with constructs of the *infl* elicitor gene resulted in gene silencing of both the transgenes and the endogenous gene (van West *et al.*, 1999). This silencing phenomenon in *P. infestans*, termed internuclear gene silencing, is in some aspects comparable to quelling, found in *Neurospora crassa* (Cogoni *et al.*, 1996; for a review see Nakayashiki, 2005). As in *Neurospora*, gene silencing in *P. infestans* is dominant, and acts *in trans* in heterokaryotic strains, suggesting the involvement of a cytoplasmic diffusible molecule. However, instead of inducing mRNA degradation (PTGS), the diffusible signal in *P. infestans* apparently acts at the transcriptional level (van West *et al.*, 1999). Another distinction from *N. crassa* quelling is that the silenced state is persistent even in non-transgenic homokaryotic progeny. Here we describe evidence that the TGS phenomenon in *P. infestans* is mediated by changes in histone deacetylation.

METHODS

***P. infestans* strains and culture conditions.** The wild-type *P. infestans* strain 88069 was used in all experiments. *infl* antisense transformants (PY31 and PY37), promoterless *infl*-cDNA transformant (SY21), homokaryotic non-*infl*-transgenic but *infl*-silenced strains (H1, H2, H3, H4 and H5), and a transgenic control strain (Y15) have the 88069 genetic background and were obtained as described by van West *et al.* (1999). All transgenic strains were co-transformed with the geneticin resistance gene *nptII*, except for H1, H2, H3, H4 and H5 which express the hygromycin resistance gene *hpt*. Cultures were grown routinely in the dark at 18 °C on ryecucrose (RS) medium as described previously (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 (van West *et al.*, 1999). When required, trichostatin A (TSA; Calbiochem), butyric acid (BuA; Sigma) and 5-azacytidine (5-AC; Calbiochem) were added to the medium prior to inoculation. Final concentrations were 3.3 µM TSA, 5 mM BuA and 50 µM 5-AC.

Detection of potential DNA methylation in genomic DNA. To detect cytosine methylation in the *infl* gene, the EZ DNA Methylation kit (Zymo Research) protocol was followed according to the instructions supplied by the company. Genomic DNA was treated with bisulfite, resulting in the conversion of unmethylated cytosines to uracil. Methylated cytosines will not undergo conversion. To detect the presence of methylated cytosines, the treated genomic DNA of strains 88069, Y15, PY31, PY37 and H1 was amplified by PCR to allow analysis by DNA sequencing. PCR reactions were carried out using primers (*infl*Fm, 5'-TTTGTGAAGGTGTTATTTT-3'; *infl*Rm, 5'-CATACAAATACACATTAATAC-3') that had been modified to account for conversion of the C into a U, and primers which were not modified (*infl*F, 5'-CTTGTGAAGGCGTCATTCC-3'; *infl*R, 5'-CGTACGAGTACACGTTGAGTAC-3'). PCR products were only obtained in reactions using the modified primers. To improve sensitivity of the PCR reaction and reduce contaminants, a further nested PCR was carried out. Therefore, a second set of modified primers was designed internally to the original primers (*infl*Fmn, 5'-TTTGTGTTAGGATTAGATA-3'; *infl*Rmn, 5'-CAAACCACTAATT-AACACC-3'). Gel purification of the generated nested PCR products was carried out using the QIAquick gel extraction kit protocol following the manufacturer's instructions (Qiagen). The purified products were sequenced employing Applied Biosystems Big-Dye Ver

3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

Nuclear DNaseI digests. To examine changes in chromatin structure of the *infl* locus, we performed nuclear DNaseI digestion studies. Mycelium was grown for 14 days in modified Plich medium (with or without 50 µM 5-AC and 5 µM TSA) and was macerated while frozen in liquid N₂. Exactly 2.0 g of the powder was transferred to a 50 ml vial containing 10 ml nuclease digestion buffer as described by van Blokland *et al.* (1997). Powder and buffer were mixed for 5 min, after which a 400 µl aliquot was retrieved (time point 0). To the remaining suspension, 4.0 µl DNaseI (1 unit µl⁻¹; Roche) was added and this was incubated at 37 °C. Aliquots of 400 µl were taken every 10 min and added to the stop buffer during a 2 h period, and after 2.5, 3 and 4 h. After retrieval, the aliquots were immediately mixed with 80 µl stop buffer (van Blokland *et al.*, 1997) and stored in liquid nitrogen. Genomic DNA was isolated using a phenol/chloroform extraction method (Raeder & Broda, 1985) and dissolved in 100 µl water. A PCR reaction was performed with 1 µl DNA and primers that can amplify the endogenous *infl* gene sequence (primer SJS1, 5'-TTACAGTCGCGACGATGTCG-3'; SJS2, 5'-TCATAGCGACGCACAGTAG-3'). This amplification results in a product of 533 bp from the endogenous *infl* gene only if DNaseI does not digest the corresponding sequence. Controls included PCR with primers SJS3 (5'-TTGGCAACAGATCTCCAAGC-3') and SJS4 (5'-TTCCTTACCGATGAGCGAGG-3') which amplify an 864 bp product of the actin gene (Unkles *et al.*, 1991). Experiments were performed in triplicate.

Southern and Northern analysis. Genomic DNA of *P. infestans* was isolated from mycelium as described by Raeder & Broda (1985) with minor modifications. DNA samples (6 µg) were digested for at least 5 h with 60 units of restriction enzyme (NEB), fractionated on 1 % agarose gels, transferred to nylon membranes and probed as described previously (van West *et al.*, 1998). For small DNA fragment hybridizations (methylation-sensitive restriction enzyme assays), 2.3 kb *Bam*HI-digested genomic DNA containing the endogenous *infl* gene was isolated as described above. Subsequently, the genomic fragments were digested with 10 units of methylation-sensitive restriction enzymes, fractionated on 10 % polyacrylamide/TBE gels (Bio-Rad), transferred to nylon membranes by means of an electroblotting system (Owl Separating Systems), and hybridized to the *infl* probes. DNA templates for probe synthesis were a 354 bp *Nco*I-*Kpn*I fragment of pHIN26 (van West *et al.*, 1999) containing the *infl* coding sequence, 1395 bp of pVW6-9 containing the promoter and coding regions of the *infl* gene, and a 796 bp *Hind*III fragment from pSTA31 (Unkles *et al.*, 1991) containing the actin (*actA*) coding sequence. Probes were radiolabelled with [α -³²P]dCTP by using the Random Primers DNA labelling system (Gibco-BRL). For Northern blotting, total RNA from *P. infestans* was isolated, blotted and hybridized as described previously (van West *et al.*, 1998).

RESULTS

Methylation of the endogenous *infl* gene in silenced strains

Modification of DNA and changes in chromatin structure provide direct and powerful mechanisms to regulate gene expression and can influence the transcriptional machinery by bringing about transcriptional repression. Cytosine methylation of DNA is one of many modifications influencing the transcriptional activity of a gene. To investigate whether the *infl* locus exhibits DNA cytosine

methylation, we carried out a methylation-sensitive restriction analysis of the *inf1* gene (GenBank accession no. AY830090). Previously, the coding sequence in the *inf1*-silenced strains was analysed using methylation-sensitive restriction enzymes, but no cytosine or adenosine methylation could be detected, neither in the endogenous gene nor in the transgenes (van West *et al.*, 1999). Genomic DNA isolated from the wild-type (88069) and four silenced strains (PY31, PY37, SY21 and H1) was digested with several methylation-sensitive restriction enzymes and analysed by Southern analysis using the *inf1* promoter and coding regions as probes. The following restriction enzymes were used: *AluI*, *BstUI*, *Fnu4HI*, *MnII*, *MspI*, *Sau3A* and *Sau96AI*, representing 28 potential methylation sites in the promoter sequence. By comparing the hybridization patterns of 88069 with the *inf1*-silenced strains, no shifts in hybridizing bands were noted, suggesting absence of methylation at all tested sites (Fig. 1 and data not shown).

To investigate further whether cytosine methylation is involved in gene silencing in *P. infestans*, we performed expression studies with *inf1*-silenced strains grown in the presence of 5-AC (Fig. 2). This cytosine analogue can inhibit methyltransferases when incorporated into DNA (Kass *et al.*, 1997). *P. infestans* isolates 88069 (wild-type strain), a control transformant (Y15), two transgenic *inf1*-silenced transformants (PY31 and SY21), and one non-transgenic *inf1*-silenced transformant (H1) were examined (van West *et al.*, 1999). 5-AC was added to the growth medium at different concentrations (up to 200 μ M) and cultures were allowed to grow for 21 days. Considerable reduction in colony radial growth of all *P. infestans* isolates was observed at concentrations of 5-AC higher than 50 μ M, suggesting toxicity of the drug towards *P. infestans*. However, little growth reduction was noted at concentrations lower than 50 μ M (data not shown), and therefore we used 50 μ M 5-AC to determine its effect on *inf1* gene expression in the silenced strains. Expression was analysed by Northern analysis and relative mRNA levels were quantified by phosphoimaging. The amount of *inf1* mRNA in the wild-type strain grown in the absence of 5-AC was set at 100% and the level of actin mRNA expression in each sample was used to correct for differences in loading.

Growth in the presence of 50 μ M 5-AC resulted in a significant increase of *inf1* expression in several of the silenced and non-silenced strains as determined by Northern analyses with phosphoimaging (Fig. 2). *inf1* mRNA levels in the wild-type strain grown in the presence of 5-AC increased 2.3-fold compared to the control level. The antisense transformant PY31 showed an expression level of 20%, compared to 0.5% in untreated mycelium, representing a 40-fold increase after treatment with 5-AC. The non-transgenic, *inf1*-silenced strain H1 exhibited an 80-fold increase, compared to the untreated mycelium. The relative amounts of *inf1* mRNA were low, upon treatment with 5-AC in SY21 (0.5%), when compared to the

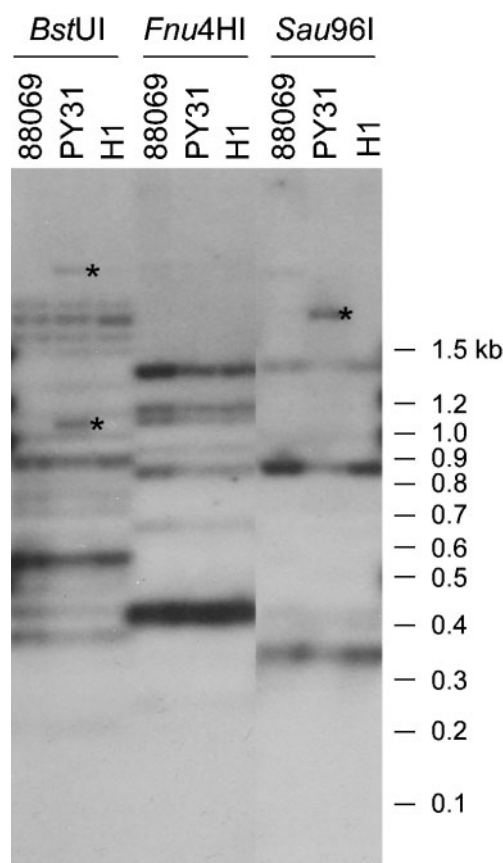


Fig. 1. Can cytosine methylation at the *inf1* locus be detected? Methylation-sensitive restriction analysis by TBE-agarose gel electrophoresis and Southern blotting of genomic DNA isolated from the wild-type strain 88069 (wt), a transgenic *inf1*-silenced strain (PY31) and a non-transgenic *inf1*-silenced strain (H1). Genomic DNA was digested with *BstUI*, *Fnu4HI* and *Sau96I* and then separated on a gel. A 1395 bp fragment containing the promoter and coding regions of the *inf1* gene was used as probe. Unique transgenic *inf1* DNA fragments from the coding region [derived from the transformation vector pHIN28 (van West *et al.*, 1999)] are indicated with an asterisk (*), the other hybridizing bands represent products derived from both the endogenous and transgenic *inf1* gene sequences.

wild-type strain. However, compared to the untreated mycelium of SY21, expression in this transformant increased six times.

Following these results we decided to perform bisulfite treatment of genomic DNA and sequence a large section of the endogenous *inf1* locus to detect any potential methylated cytosines. We sequenced an area of the promoter of up to 265 bp upstream from the ATG start codon and a further 300 bp downstream in the coding region from bisulfite-treated genomic DNA from the wild-type strains 88069 and Y15, and the *inf1*-silenced strains PY31, PY37 and H1. Remarkably, we were not able to

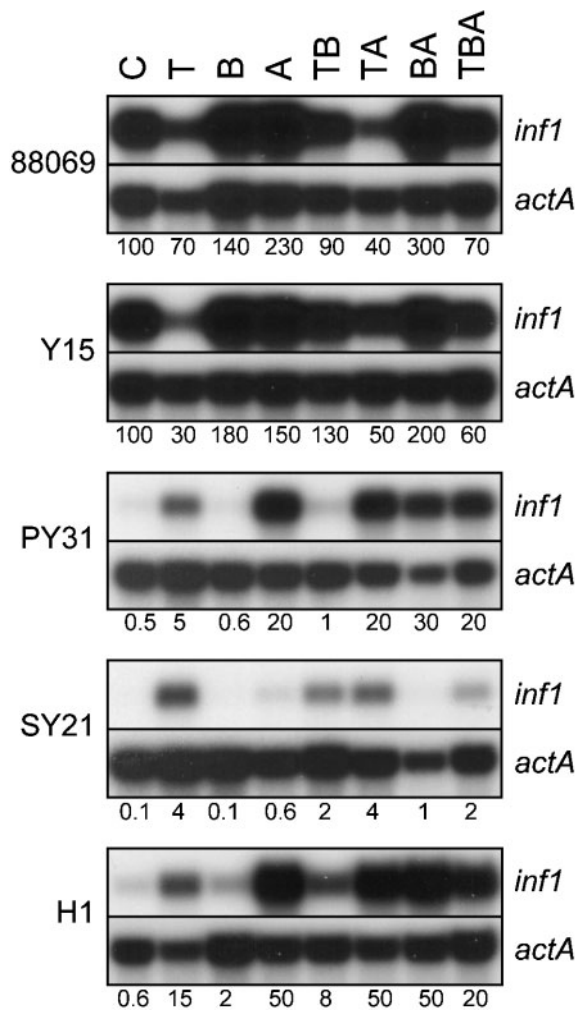


Fig. 2. Reversion of *inf1* gene silencing in silenced strains of *P. infestans*. Analysis of *inf1* mRNA production in wild-type strain (88069), in a transgenic antisense strain (PY31), in a cDNA transformant (SY21), in a non-transgenic silenced strain (H1) and in a G418-resistant non-silenced strain (Y15), after addition of a DNA methylation inhibitor [50 μ M 5-AC, (A)] or HDAC inhibitors [5 mM BuA (B), or 5 μ M TSA (T)], or combinations of two of these chemicals (TB, TA and BA), or all three (TBA). Northern blots, containing 15 μ g total RNA isolated from mycelium grown *in vitro* for 21 days, were hybridized with *inf1* and actin (*actA*) probes. Relative amounts of *inf1* mRNA corrected for loading errors (using the actin hybridizations) and compared to the level of *inf1* mRNA of untreated wild-type *P. infestans* (100%) are given below the autoradiographs.

detect any methylated cytosine residues in all tested DNA samples (data not shown).

These data indicate that, although 5-AC reverses *inf1* silencing, methylation of cytosines does not take place in the *inf1* locus.

Relief of *inf1* silencing with histone deacetylase (HDAC) inhibitors

In general, acetylated regions of chromatin are transcriptionally active, whereas regions that are hypoacetylated are inactive (Katan-Khaykovich & Struhl, 2002). Enzymes such as HDACs are believed to repress transcription by removing the acetyl groups from the N-terminal tails of the core histones of the chromatin. Several reports demonstrate that inhibition of HDACs by drugs, such as trichostatin-A (TSA) and BuA, results in reactivation of transcriptionally silenced genes (Selker, 1998; Ayer, 1999).

We used HDAC inhibitors to determine whether reactivation of the *inf1*-silenced state could be achieved in *P. infestans*. Therefore, we analysed expression of *inf1* in several *inf1*-silenced strains grown in the presence of TSA (3.3 μ M) and BuA (5 mM) (Fig. 2). Similar to the experiments described above for 5-AC, we first determined the highest concentration of the drugs that had little or no toxic effects by colony radial growth assays (data not shown). Addition of BuA resulted in some increase of expression of the *inf1* gene in the wild-type and control (Y15) strains (1.4- and 1.8-fold, respectively). Similarly, little or no increases in expression levels were observed in the *inf1*-silenced strains SY21, PY31 and H1 after treatment with BuA.

Addition of TSA to the culture medium resulted in reduced *inf1* expression in the wild-type and control strains, whereas an increase of *inf1* expression was noted in all silenced strains. For example, in the *inf1* cDNA transformant (SY21), expression increased more than 40-fold, and a 25-fold increase was noted in the non-transgenic *inf1*-silenced strain (H1) (Fig. 2).

To investigate whether combinations of the drugs work synergistically in reactivating *inf1* expression, we performed expression studies with combinations of 5-AC, TSA and BuA. Analysing the results of these experiments did not reveal any obvious synergistic activities as none of the combined treatments resulted in an increased *inf1* expression level when compared to the highest level that was measured for the mycelial samples that were treated with only one inhibitor compound. The only exception is in mycelium of PY31 when this was treated with both BuA and TSA. However, in this case, only a 50% increase was observed when compared to PY31 mycelium treated with TSA alone.

We also investigated whether exposure to other chemicals involved in DNA synthesis and degradation had any effect on reactivating *inf1* gene expression. The DNA synthesis inhibitor hydroxy-urea was tested at concentrations of up to 50 mM, but no reactivation of *inf1* gene expression was found. Similarly, concentrations of up to 100 μ M of the nuclease inhibitor aurintricarboxylic acid (ATA) did not result in reactivation (data not shown).

From these experiments we can conclude that growth of the *inf1*-silenced strains in the presence of TSA leads to

reversion of the *inf1*-silenced state, suggesting that the silenced state is, at least partly, the result of hypoacetylated histones.

Changes in chromatin structure

To investigate whether silencing had led to changes in the chromatin structure at the endogenous *inf1*-silenced locus, we performed DNaseI digestion experiments of isolated nuclei. It has been demonstrated that densely packed heterochromatin is less accessible to nucleases (Grewal & Elgin, 2002). Nuclear digests were performed with three *inf1*-silenced strains, PY31, PY37 and H1, and wild-type strain 88069 as a control. A time-course experiment was performed by taking samples from the DNaseI-treated suspensions every 10 min over a 2 h period, and after 2.5, 3 and 4 h. Subsequently, the genomic DNA was isolated and PCR was performed to test for the presence of the intact *inf1* gene sequence (Fig. 3a). We used a primer combination that only amplified the complete endogenous *inf1* gene sequence. Transgenic sequences could not be amplified. As a control, we also used primers to amplify the actin gene. After 30 min, the control actin gene could not be amplified in DNaseI-treated DNA that was isolated from the wild-type and all *inf1*-silenced strains. *inf1* could not be amplified in the wild-type strain after 30 min. However, *inf1* could be amplified in all tested *inf1*-silenced strains up to 90 min after DNaseI treatment. These findings indicate

that the chromatin present in the endogenous *inf1* locus is more densely packed in the *inf1*-silenced strains compared to the wild-type and control strains.

We then decided to investigate whether nuclear DNaseI treatment of isolated nuclei from *inf1*-silenced lines isolated from mycelium grown in the presence of TSA and 5-AC would result in increased digestion efficiency. We found for PY31, PY37 and 88069 (Fig. 3b and data not shown) that the actin gene could be amplified up to 20 min after DNaseI treatment, which is identical to non-treated nuclei of these strains (Fig. 3a). However, the *inf1* gene could only be amplified up to 20 min after DNaseI treatment for all three tested strains (Fig. 3b and data not shown), whereas in the non-treated experiment (Fig. 3a), the *inf1* gene could be amplified up to 90 min following DNaseI treatment. This provides further evidence that the *inf1* locus is indeed densely packed in the *inf1*-silenced lines and that adding TSA and 5-AC to the growth medium allows the chromatin to remodel back to a less condensed state.

Reactivated transgenic strains revert back to the silenced state, while non-transgenic strains do not

To investigate whether reactivated *inf1* expression was maintained in the silenced strains after treatment with 5-AC and TSA, we performed experiments in which we grew transgenic *inf1*-silenced strains (PY37 and SY21) and a

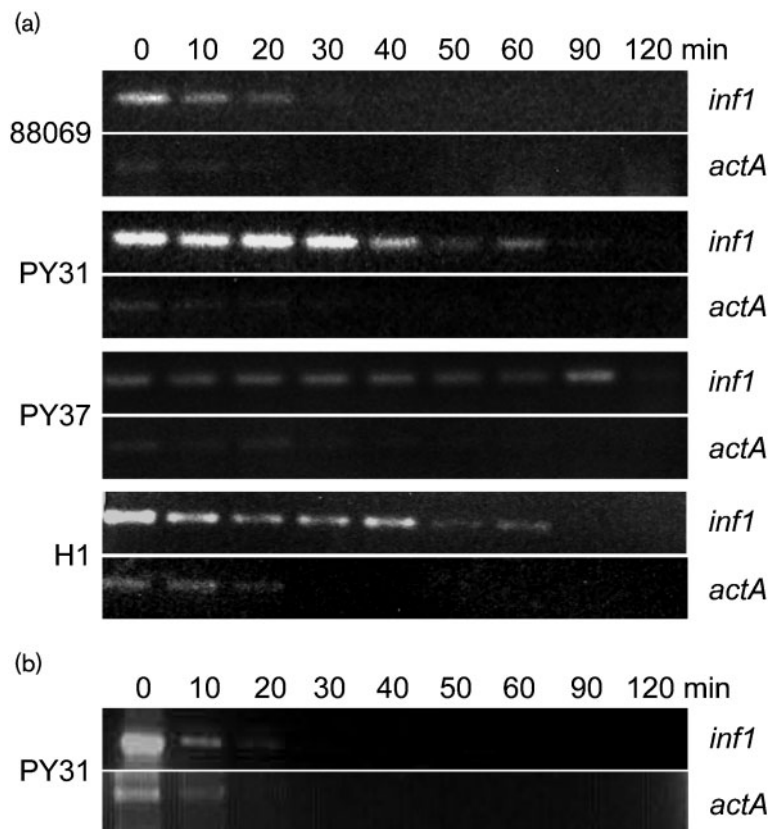


Fig. 3. Genomic DNA at the endogenous *inf1* locus of *inf1*-silenced strains is less accessible to DNaseI digestion. Genomic DNA of *inf1*-silenced lines was isolated from identical amounts of nuclei that were treated with DNaseI over a time-course of 120 min. The nuclei were isolated from mycelium that had been grown in Plich medium without (a) and with 50 μ M 5-AC and 5 μ M TSA (b). PCR was performed on the isolated DNA with *inf1*- and actin-specific primers and the amplified products were run on a 1% agarose gel. The presence of the amplified product indicates that DNaseI had not restricted the *inf1* or actin gene sequences. The *inf1*-silenced strains show a delay in DNaseI digestion of the endogenous *inf1* gene compared to the non-silenced wild-type (a), whereas the 5-AC- and TSA-treated strain PY31 (b) does not show a delay in DNaseI digestion, suggesting that treatment with these compounds resulted in less condensed DNA in the *inf1* locus.

non-transgenic *inf1*-silenced strain (H1) in the presence of 5-AC and TSA on solidified RS-medium for 21 days. Subsequently, pieces of mycelia were transferred to fresh liquid medium with and without the drugs for a further period of growth for 21 days. Total RNA was isolated and *inf1* expression was analysed by Northern blotting (Fig. 4a). Untreated mycelium was used as a control. The *inf1* transgenic strains PY37 and SY21 showed an increase of *inf1* expression during growth in medium containing 5-AC and TSA, whereas *inf1* expression decreased again after transfer to medium without the drugs. Interestingly, the *inf1* non-transgenic silenced strain (H1) also resumed *inf1* expression upon growth in medium containing the drugs, but expression of the *inf1* gene continued after transfer to fresh medium in the absence of these drugs. Furthermore, we noticed that the non-transgenic, *inf1*-silenced strains gradually reverted to the wild-type state of INF1 protein production during subculturing over a period of 18 months. We investigated this in more detail by performing Northern analysis of several of these strains which had been subcultured and grown for 18 months, and compared these with the same isolates that had been stored in liquid N₂ soon after they had been generated (Fig. 4b). All tested strains showed a higher *inf1* expression level when compared to the same isolate stored in liquid N₂, indicating a loss of the silenced state over time.

These findings demonstrate that the presence of *inf1* transgenic sequences is required to maintain the *inf1*-silenced state.

DISCUSSION

Here we describe evidence for chromatin remodelling and histone deacetylation controlling gene expression in the oomycete *P. infestans*. Our findings suggest that silencing of the *inf1* gene is accomplished by a hypoacetylated state of histones at the *inf1* locus, resulting in the formation of heterochromatin. Cytosine methylation does not seem to occur, although the cytosine methylation inhibitor 5-azacytidine is able to reverse the silenced state temporarily.

DNA methylation is important for regulating genome functions and differential control of gene expression in eukaryotes such as plants, fungi and mammals. In total we have tested almost 50 methylation-sensitive restriction sites within the *inf1* gene without detection of any methylated cytosines (van West *et al.*, 1999; this study). Furthermore, methylation-sensitive sequencing of the *inf1* locus did not reveal any methylated cytosine in the *inf1*-silenced strains. These results are in line with observations made by Judelson & Whittaker (1995), who found that transgenes in *P. infestans* became inactivated without noticeable methylation of the transgenic DNA sequences.

Interestingly, for a long time, several organisms including yeast, *C. elegans* and *Drosophila* have been reported to lack detectable methylation of their genomes (Lyko, 2001). For example, *C. elegans* does not seem to have a conventional DNA methyltransferase-like gene (Bird, 2002). However, it was discovered that at certain stages in the developmental process of *Drosophila*, methylated cytosine bases are

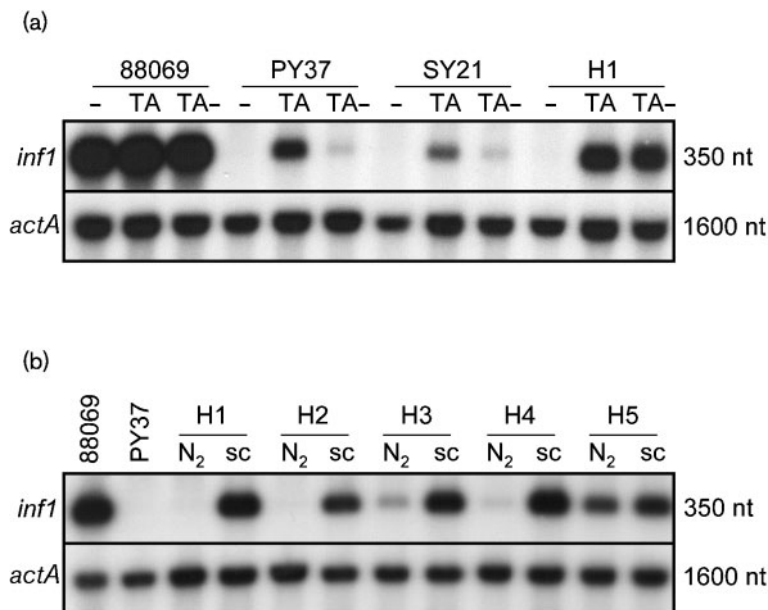


Fig. 4. Stable reversion of *inf1* gene silencing only occurs in non-transgenic *inf1*-silenced strains after treatment with TSA and 5-AC, or over time. (a) Analysis of *inf1* mRNA production in mycelium of wild-type strain (88069), in transgenic antisense strains (PY37), in a cDNA transformant (SY21) and in a non-transgenic silenced strain (H1) before treatment (-), after addition of 50 μ M 5-AC and 5 μ M TSA (TA) and after transfer again to medium without 5-AC and TSA (TA-). Only the non-transgenic silenced line H1 reverses to wild-type levels of *inf1* expression after treatment with 5-AC and TSA. (b) Analysis of *inf1* mRNA production in mycelium of wild-type strain (88069), in transgenic antisense strains (PY37) and in five individual non-transgenic silenced strains (H1-5) after storage in liquid nitrogen (N₂) and after subculturing for 18 months (sc). Northern blots, containing 15 μ g total RNA, were hybridized with *inf1* and actin (*actA*) probes. Transcript lengths are indicated in nucleotides (nt). All non-transgenic silenced lines revert to wild-type levels of *inf1* expression after prolonged subculturing.

present. Apparently, methylation occurs in *Drosophila* at unusual target sequences not including traditional CpG sites (Lyko *et al.*, 2000). At present we have not been able to identify a good DNA methyltransferase candidate gene in the available genome sequences of *P. infestans*, based on BLAST searches (www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html).

Interestingly, addition of 5-AC to the growth medium resulted in reactivation of *infl* gene expression in silenced strains. The fact that we were unable to demonstrate any DNA methylation using restriction analysis or bisulfite sequencing suggests that the inhibitor must function in a different way to release silencing. Perhaps one explanation could be that the incorporation of the 5-AC residue in the *infl* locus enables the generation of a more open conformation of the *infl* gene, which results in an increase of *infl* expression. Alternatively, there is good evidence that 5-AC can also inhibit histone methylation (Wada *et al.*, 2005; Wozniak *et al.*, 2006). Therefore, adding 5-azacytidine into the culture medium may have had a strong effect in reducing methylation of the histones in the *infl* locus, resulting in the release of silencing.

Acetylation, deacetylation, ubiquitination, phosphorylation and methylation of histones have all been implicated in activation and silencing of transcription (Grewal & Elgin, 2002; Fischle *et al.*, 2003). The different histone modifications and the corresponding enzymic systems have been studied extensively, and this has resulted in a complex picture of how eukaryotic cells establish and maintain local and global patterns of chromatin modification. It is generally accepted that hyperacetylated histones are associated with activated genome regions, whereas deacetylation of histones leads to repression and silencing (Fischle *et al.*, 2003).

Here we set out to investigate whether we are able to reverse *infl* gene silencing by inactivating HDACs. In many eukaryotic systems, long-term treatment with BuA, TSA or other HDAC inhibitors can lead to complete shifts from predominantly non-acetylated to largely multi-acetylated histones (Waterborg, 1998 and references therein). Addition of TSA to the growth medium of *infl*-silenced strains resulted in reactivation of *infl* gene expression in the tested strains (Fig. 1), demonstrating that *infl* silencing in *P. infestans* is correlated with a hypoacetylated state of histone molecules.

Interestingly, addition of BuA alone did not result in an increase of *infl* gene expression in the transgenic *infl*-silenced strains. Only very little reactivation could be observed in the non-transgenic *infl*-silenced strain, possibly indicating that BuA is a poor inhibitor of HDAC in *P. infestans*. Similar results have been found in *Chlamydomonas reinhardtii* (Waterborg, 1998). In this alga, TSA was also able to induce hyperacetylation of histones, whereas BuA was ineffective (Waterborg, 1998). Combinations of two or three inhibitors added to the growth medium resulted in no clear synergistic reactivation of *infl* expression.

To determine whether reactivation of *infl* expression is stable after treatment with TSA and 5-AC, we subcultured the treated strains on media without TSA and 5-AC. This resulted in restoration of silencing in all strains with the exception of the non-transgenic silenced strains. Therefore, the presence of transgenic *infl* sequences was essential to re-establish the silenced state. Furthermore we noticed that silencing was lost in the non-transgenic *infl*-silenced strains over prolonged subculturing.

These findings demonstrate that the acetylation status of histones is inherited during vegetative growth, but that it can be modulated by external or internal factors. It seems that the cell has a regulatory system that allows it to monitor the acetylation status of its histones. Experiments in yeast have suggested the presence of a system that maintains a dynamic balance of acetylation of histone proteins (Vogelauer *et al.*, 2000; Katan-Khaykovich & Struhl, 2002). It was found that acetylation and deacetylation reactions occur continuously, thereby generating a steady-state level of global or bulk histone acetylation. This homeostasis can be disturbed locally by the recruitment of histone acetylases or HDACs to DNA-binding proteins linked to the chromatin (Struhl, 1998). It is thought that once recruitment and activity is finished, the cell rapidly restores its initial acetylation status by allowing globally acting enzymes to restore the acetylation equilibrium (Katan-Khaykovich & Struhl, 2002).

Modification of gene expression through alteration of chromatin structure has been found in many eukaryotic organisms, including nematodes (Ryner & Swain, 1995; Kelly & Fire, 1998), *Drosophila* (Weissmann & Lyko, 2003), plants (van Blokland *et al.*, 1997), mammals (Bird & Wolffe, 1999), yeasts and fungi (Roth, 1995; Selker, 1998). A common method to demonstrate a more condensed state of chromatin at a particular locus is to treat intact nuclei with nucleases in a time-course experiment and to assess the rate of DNA degradation. If the DNA is less accessible for nucleases, a delay in DNA degradation is observed (Grewal & Elgin, 2002). We observed such a delay in DNA degradation in both the transgenic and non-transgenic *infl*-silenced strains compared to non-silenced strains. These results demonstrate that *infl* silencing is associated with a condensed chromatin state of the *infl* locus.

We demonstrated previously that the *infl*-silenced state could be transmitted in heterokaryotic strains from transgenic *infl*-silenced nuclei to wild-type nuclei (van West *et al.*, 1999). Furthermore, using run-on assays we demonstrated that *infl* silencing is regulated at the transcriptional level and therefore we could not explain the observed internuclear gene-silencing phenomenon as a post-transcriptional silencing process.

Whisson *et al.* (2005) demonstrated that efficient silencing is obtained in *P. infestans* when protoplasts are treated with dsRNA of a target gene. Typically, about 15 days post-treatment silencing appears to reach maximum levels and normal gene expression levels resume gradually thereafter.

At present it is unclear whether this silencing process is based upon mRNA degradation or whether transcriptional silencing processes play a role. Indeed, several studies indicate that eukaryotes may require dsRNA and siRNA (small interfering RNA) to induce TGS (Cerutti, 2003; Vermaak *et al.*, 2003; Morris *et al.*, 2004). Evidence from fission yeast shows that repetitive sequences found at the centromeres produce dsRNAs that are involved in the formation and maintenance of heterochromatin (Vermaak *et al.*, 2003; Volpe *et al.*, 2002; Reinhart & Bartel, 2002).

We hypothesize that integration of homologous transgenes in the genome of *P. infestans* results in the formation of aberrant RNA molecules (van West *et al.*, 1999). These aberrant RNA molecules may be dsRNA molecules, or they may be used as a template to generate new dsRNA via an RNA-dependent RNA polymerase (Dalmay *et al.*, 2000; Beclin *et al.*, 2002). The dsRNA molecules may be cleaved by a homologue of Dicer into siRNAs. Database searches against genome sequences from *P. infestans* (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html) do suggest that Dicer and RNA-dependent RNA polymerase candidate genes may be present. It is then speculated that the generated siRNAs induce changes in the chromatin structure of homologous sequences, which has already been described in several systems (Nykänen *et al.*, 2001; Mette *et al.*, 2000; Dalmay *et al.*, 2000; Vaistij *et al.*, 2002; Brodersen & Voinnet, 2006). It is thought that the siRNA may act as a guide to activate enzymes involved in modulating the methylation status of histones and/or to activate HDACs, which would remove the acetyl groups on the homologous DNA sequence (Pickford & Cogoni, 2003). Once the siRNAs are formed in the cell, they may be imported into other nuclei in heterokaryotic cells, where, subsequently, a similar condensed chromatin silenced state is induced. We are currently testing this model.

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