Evidence for involvement of Dicer-like, Argonaute and histone deacetylase proteins in gene silencing in *Phytophthora infestans*

RAMESH R. VETUKURI^{1,*}, ANNA O. AVROVA², LAURA J. GRENVILLE-BRIGGS³,
PIETER VAN WEST³, FREDRIK SÖDERBOM⁴, EUGENE I. SAVENKOV¹, STEPHEN C. WHISSON² AND
CHRISTINA DIXELIUS¹

¹Department of Plant Biology and Forest Genetics, Uppsala BioCenter, SLU, PO Box 7080, 750 07 Uppsala, Sweden

²Plant-Pathology Programme, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

³Aberdeen Oomycete Laboratory, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

⁴Department of Molecular Biology, Uppsala BioCenter, SLU, PO Box 590, 751 24 Uppsala, Sweden

SUMMARY

Gene silencing may have a direct or indirect impact on many biological processes in eukaryotic cells, and is a useful tool for the determination of the roles of specific genes. In this article, we report silencing in *Phytophthora infestans*, an oomycete pathogen of potato and tomato. Gene silencing is known to occur in *P. infestans*, but its genetic basis has yet to be determined. Genes encoding the major components of the RNA interference (RNAi) pathway, Dicer-like (*Pidcl1*), Argonaute (*Piago1–5*) and RNA-directed RNA polymerase (*Pirdr1*), were identified in the *P. infestans* genome by comparative genomics, together with families of other genes potentially involved in gene silencing, such as histone deacetylases, histone methyltransferases, DEAD helicases, chromodomain proteins and a class 1 RNaseIII. Real-time reverse transcription-polymerase chain reaction demonstrated transcript accumulation for all candidate genes throughout the asexual lifecycle and plant infection, but at different levels of mRNA abundance. A functional assay was developed in which silencing of the sporulation-associated *Pidcl14* gene was released by the treatment of protoplasts with *in vitro*-synthesized double-stranded RNAs homologous to *Pidcl1*, *Piago1/2* and histone deacetylase *Pihda1*. These results suggest that the components of gene silencing, namely Dicer-like, Argonaute and histone deacetylase, are functional in *P. infestans*. Our data demonstrate that this oomycete possesses canonical gene silencing pathways similar to those of other eukaryotes.

INTRODUCTION

Most eukaryotes possess endogenous pathways to degrade viral, transposon or aberrant RNAs that may represent a threat to the genome or transcriptome of an organism (Guru, 2000; Tabara *et al.*, 1999; Vastenhouw *et al.*, 2003; Yang *et al.*, 2004). Some of the RNA degradation pathways are triggered by double-stranded RNA (dsRNA) and involve the generation of small noncoding RNAs generally 18–40 nucleotides long (Jinek and Doudna, 2009). Small noncoding RNAs have been found in many eukaryotes, and play important regulatory roles, for example, by targeting mRNA for degradation, by translational repression or by altering the transcriptional activities of chromatin. Two major classes of small RNA recognized to date, namely short interfering RNA (siRNA) and microRNA (miRNA), differ by their biogenesis and mechanism of action (Moazed, 2009). siRNAs and microRNAs are generated through the catalytic activity of RNaseIII domains in Dicer proteins, which cleave longer dsRNA, such as miRNA precursors, into small RNAs (Bernstein *et al.*, 2001).

In general, to initiate gene silencing, dsRNAs may be formed by transcription from both DNA strands, from inverted repeats or through the action of RNA-dependent RNA polymerase (Rdr) (Cogoni and Macino, 1999; Makeyev and Bamford, 2002). Typically, the resulting dsRNA is processed to siRNA by Dicer. The siRNAs are subsequently unwound and one strand is loaded onto Argonaute (Ago), a member of a protein family characterized by their PAZ/PIWI domains (Höck and Meister, 2008). Ago is a pivotal part of a multi-protein RNA-induced silencing complex (RISC) and cleaves mRNA complementary to incorporated siRNA, through the slicer activity of the PIWI domain. siRNAs can also act to prime further dsRNA synthesis by Rdr, amplifying and reinforcing the silencing through the formation of secondary siRNAs, a process known as

*Correspondence: Email: ramesh.vetukuri@slu.se

transitive RNAi (Alder *et al.*, 2003; Sijen *et al.*, 2001). New findings concerning the mechanisms that produce small RNAs and how dsRNAs are selected and sorted among different protein complexes are steadily growing in this rapidly expanding field of research.

Gene silencing events occur either downstream of transcription (referred to as post-transcriptional gene silencing, PTGS) or upstream of transcription resulting in transcriptional arrest (transcriptional gene silencing, TGS) (Cogoni and Macino, 2000; Verdel *et al.*, 2004; Verdel and Moazed, 2005). Some eukaryotes possess mechanisms for both TGS and PTGS. When TGS is initiated, gene expression is suppressed through either direct methylation of cytosines, as in plants, or deacetylation and methylation of histones, as in fission yeast and the nematode *Caenorhabditis elegans* (Grishok, 2005; Hall *et al.*, 2002; Huettel *et al.*, 2007; Vastenhouw *et al.*, 2006; Volpe *et al.*, 2002).

Phytophthora infestans belongs to the oomycetes, a group of predominantly filamentous organisms that includes many major plant pathogens and some emerging pathogens of animals (Phillips *et al.*, 2008). The oomycetes are phylogenetically separate from the fungi, and are grouped with the golden-brown algae in the stramenopiles (Baldauf *et al.*, 2000; Burki *et al.*, 2007). *P. infestans* causes the devastating late blight disease on its principal solanaceous host plants, potato and tomato (Fry, 2008). This plant pathogen secretes numerous proteins that are important for the colonization of its host plants, a virulence repertoire which assists pathogen adaptation to a changing environment, leading to frequent breakdown of introduced resistance in crop plants.

Gene silencing is known to occur in *P. infestans*, and has been exploited to determine the role(s) of specific genes in the life-cycle or during infection (Ah Fong and Judelson, 2003; Avrova *et al.*, 2008; van West *et al.*, 1999). However, the molecular basis of gene silencing in *P. infestans* is not well understood. Stable transformations using sense and antisense constructs, followed

by nuclear run-on assays, suggest the activation of TGS processes (Judelson and Tani, 2007; van West *et al.*, 1999). The treatment of protoplasts with dsRNA, however, results in transient RNA silencing, most probably via the PTGS machinery (Whisson *et al.*, 2005). The onset of gene silencing in *P. infestans* has so far been shown not to involve cytosine methylation and has been suggested to be mediated through histone deacetylation and the formation of heterochromatin (Judelson and Tani, 2007; van West *et al.*, 2008). As gene silencing processes operate in *P. infestans*, the genes involved in silencing pathways are presumably present in its genome.

In this study, we identified nine major gene families potentially involved in gene silencing in *P. infestans* and assessed their expression in mycelium, sporangium, zoospores, germinating cysts and germinating cysts with appressoria, and on plant infection. Furthermore, we used *Pidc14*, encoding a protein phosphatase essential for sporulation (Ah Fong and Judelson, 2003), as a reporter for the release of gene silencing events. The inactivation of *Pidc14* by stable transformation results in a loss of sporulation, a phenotype that is easily identified and assessed. *Pidc14*-silenced lines treated with dsRNAs homologous to the *Pidcl*, *Piagol1/2* and *Pihda1* genes regained sporulation, demonstrating the release of the silenced state, and providing evidence for the involvement of the encoded proteins in gene silencing in *P. infestans*.

RESULTS

Conservation of protein domains in candidate components of the gene silencing pathway in *P. infestans*

Genes encoding the different protein components involved in the silencing pathway were identified in the genome of *P. infestans* through comparative genomics (Table 1). Full gene names, loci and predicted functions are given in Table 2. Unless

Table 1 Candidate components for involvement in gene silencing in *Phytophthora infestans*.

Gene	Predicted protein	Protein domains	Genes
<i>Pidcl1</i>	Dicer-like	2 × RNaseIII, NLS*	1
<i>Piagol</i>	Argonaute	PAZ, PIWI, DUF1785, NLS	5
<i>Pirdr1</i>	RNA-dependent RNA polymerase	RNA-dependent RNA polymerase, helicase C terminal domain, DEAD/DEAH box helicase	1
<i>Pidrb</i>	dsRNA-binding protein	dsRNA-binding motif	2
<i>Pirnh</i>	RNA helicase	DEAD/DEAH box helicase, helicase C-terminal domain	8
<i>Pihda</i>	Histone deacetylase	Histone deacetylase	9
<i>Pihme</i>	Histone methyltransferase	SET	14
<i>Picdp</i>	Chromodomain protein	Chromo	10
<i>Pirms</i>	RNaseIII	RNaseIII	1

Data retrieved from (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans).

*Nuclear localization signals in candidate proteins were predicted using the PredictNLS server (<http://www.predictprotein.org/>).

Table 2 Genes encoding putative components of gene silencing, and predicted Pfam domains.

Gene function	Genome locus	GenBank accession	Domains	<i>Phytophthora infestans</i> gene
Dicer-like Argonaute (Ago)	PITG_09292 PITG_04470 and PITG_04471	EEY55353 EEY67432 EEY67433	2 × RNaseIII, NLS PAZ, PIWI, DUF1785, NLS	<i>Pidc1</i> <i>Piogo1</i> and <i>Piogo2</i>
Argonaute	PITG_01400	EEY61151	PAZ, PIWI, DUF1785	<i>Piogo3</i>
Argonaute	PITG_01444	EEY61192	PAZ, PIWI, DUF1785	<i>Piogo5</i>
Argonaute	PITG_01443	EEY61191	PAZ, PIWI, DUF1785	<i>Piogo4</i>
RNA-dependent RNA polymerase (Rdr1)	PITG_10457	EEY56917	RNA-dependent RNA polymerase, helicase C-terminal domain, DEAD/DEAH box helicase	<i>Pirdr1</i>
dsRNA binding	PITG_12183	EEY59608	Double-stranded RNA-binding motif	<i>Pidrb1</i>
dsRNA binding	PITG_03262	EEY65738	Double-stranded RNA-binding motif, Bicoid-interacting protein 3 (Bin3)	<i>Pidrb2</i>
RNA helicase	PITG_02664	EEY64135	DEAD/DEAH box helicase, helicase C-terminal domain	<i>Pirnh1</i>
RNA helicase	PITG_02856	EEY64303	DEAD/DEAH box helicase, helicase C-terminal domain	<i>Pirnh2</i>
RNA helicase	PITG_01427	EEY61176	DEAD/DEAH box helicase, helicase C-terminal domain	<i>Pirnh3</i>
RNA helicase	PITG_08026	EEY54397	DEAD/DEAH box helicase, helicase C-terminal domain	<i>Pirnh4</i>
Dicer-like RNA helicase	PITG_09951	EEY56422	DEAD/DEAH box helicase, helicase C-terminal domain	<i>Pirnh5</i>
RNA helicase	PITG_00863	EEY58225	DEAD/DEAH box helicase, helicase C-terminal domain	<i>Pirnh6</i>
RNA helicase	PITG_02839	EEY64287	DEAD/DEAH box helicase, helicase C-terminal domain	<i>Pirnh7</i>
RNA helicase	PITG_13976	EEY61994	DEAD/DEAH box helicase, helicase C-terminal domain	<i>Pirnh8</i>
Histone deacetylase	PITG_01897	EEY61575	Histone deacetylase	<i>Pihda1</i>
Histone deacetylase	PITG_08237	EEY54570	Histone deacetylase	<i>Pihda2</i>
Histone deacetylase	PITG_23160	EEY70206	Histone deacetylase	<i>Pihda3</i>
Histone deacetylase	PITG_05176	EEY69010	Histone deacetylase	<i>Pihda4</i>
Histone deacetylase	PITG_15415	EEY63194	Histone deacetylase	<i>Pihda5</i>
Histone deacetylase	PITG_21309	EEY65409	Histone deacetylase	<i>Pihda6</i>
Histone deacetylase	PITG_12962	EEY59827	Histone deacetylase	<i>Pihda7</i>
Histone deacetylase	PITG_01911	EEY61588	Histone deacetylase	<i>Pihda8</i>
Histone deacetylase	PITG_04499	EEY68123	Histone deacetylase	<i>Pihda9</i>
Histone methyltransferase	PITG_04185	EEY67226	SET, bromodomain	<i>Pihme1</i>
Histone methyltransferase	PITG_20502	EEY55136	SET, bromodomain, PHD-finger, F/Y-rich N-terminus	<i>Pihme2</i>
Histone methyltransferase	PITG_05564	EEY69339	SET	<i>Pihme3</i>
Histone methyltransferase	PITG_13838	EEY61878	SET	<i>Pihme4</i>
Histone methyltransferase	PITG_14137	EEY62225	SET	<i>Pihme5</i>
Histone methyltransferase	PITG_02426	EEY63917	SET	<i>Pihme6</i>
Histone methyltransferase	PITG_05692	EEY69457	SET	<i>Pihme7</i>
Histone methyltransferase	PITG_00145	EEY57586	Histone methylation protein DOT1	<i>Pihme8</i>
Histone methyltransferase	PITG_12169	EEY59595	SET	<i>Pihme9</i>
Histone methyltransferase	PITG_02096	EEY63630	SET	<i>Pihme10</i>
Histone methyltransferase	PITG_21689*		SET	<i>Pihme11</i>
Histone methyltransferase	PITG_12718*		SET	<i>Pihme12</i>
Histone methyltransferase	PITG_02699*		SET	<i>Pihme13</i>
Histone methyltransferase	PITG_00474	EEY57887	SET, PHD-finger	<i>Pihme14</i>
Chromodomain protein	PITG_02729	EEY64190	Chromo	<i>Picdp1</i>
Chromodomain protein	PITG_00140	EEY57581	PHD finger, chromo, snf2 family N-terminal domain, helicase C terminal domain	<i>Picdp2</i>
Chromodomain protein	PITG_07902	EEY54293	Chromo	<i>Picdp3</i>
Chromodomain protein	PITG_10023	EEY56485	2 × chromo	<i>Picdp4</i>
Chromodomain protein	PITG_02066 and PITG_02378		Chromo	<i>Picdp5</i> and <i>Picdp6</i>
Chromodomain protein	PITG_05038	EEY68553	Chromo	<i>Picdp7</i>
Chromodomain protein	PITG_14460	EEY62679	Chromo	<i>Picdp8</i>
Chromodomain protein	PITG_15837	EEY63490	Chromo, snf2 family N-terminal domain, helicase C-terminal domain	<i>Picdp9</i>
Chromodomain protein	PITG_06327	EEY69821	Chromo	<i>Picdp10</i>
RNaseIII	PITG_08831	EEY56061	RNaseIII	<i>Pirns1</i>

*These genes were only predicted in the genome assembly version from 09-2006. These sequences can be found in the current genome locations: PITG_21689, Supercontig1.741:36674–37186; PITG_12718, Supercontig 27:718142–719443; PITG_02699, Supercontig 3:3235760–3237181.

A Dicer proteins **B RNA-dependent RNA polymerase**

Fig. 1 Diagrammatic representation of protein domains predicted by Pfam (23.0) within Dicer-like (a) and RNA-dependent RNA polymerase (b) proteins. Taxa represented were abbreviated as follows: At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Nc, *Neurospora crassa*; Pi, *Phytophthora infestans*; Spo, *Schizosaccharomyces pombe*; Ts, *Thalassiosira pseudonana*. Numbers of amino acids are indicated in parentheses. Protein accession numbers are: (A) Pi Dcl1 (PITG_09292), Dd DrnB (XP647462), Spo Dcr1 (NP588215), Nc Dcl1 (NCU08270), Dm Dcr1 (AAF56056), Ce Dcr1 (AAA28101), Hs Dcr1 (NP803187), At Dcl1 (NP171612), At Dcl4 (NP197532); (B) Pi Rdr1 (PITG_10457), Dd RrpA (XP636093), Spo Rdp1 (CAB11093), Ce Rrf1 (NP492131), At Rdr1 (NP172932), Nc Rdr1 (NCU07534), Ts RdRP1 (EED92869).

specifically stated, support for BLASTP data (e values) resulted from searches of National Center for Biotechnology Information (NCBI) GenBank using *P. infestans* gene sequences as queries.

Only one gene encoded a protein with two RNaseIII domains, and was thus a strong candidate for a Dicer-like protein: *Pidcl1* (Fig. 1A). Other protein domains were not well supported and only poorly conserved domains for an RNA helicase and dsRNA-binding domain were found. On BLASTP search, *Pidcl1* exhibited most significant similarity ($6e^{-27}$) to the DCL4 protein from *Arabidopsis thaliana*. One additional gene (*Pirns1*) was identified as encoding a protein with a single RNaseIII domain. It exhibited similarity only to bacterial class 1 RNaseIII proteins at the

C-terminus, and represented the simplest class of RNaseIII enzymes. *Pirnh5* and *Pirdr1* also showed similarity to Dicer-like sequences, although their domain compositions suggested that these were not Dicer-like proteins. *Pirnh5*, previously suggested to be Dicer-like (Ah Fong *et al.*, 2008), encoded a protein with a predicted DEAD box RNA helicase domain and a poorly conserved dsRNA-binding domain, but no RNaseIII domains. *Pirdr1*, however, encoded a protein with Dicer-like DEAD box RNA helicase and Rdr domains, and corresponded to the only *P. infestans* candidate for RNA-dependent RNA polymerase (Fig. 1B). The only other Rdr identified so far with a similar domain architecture originates from the slime mould, *Dictyostelium discoideum* (Martens *et al.*, 2002).

Five genes, *Piagol1–5*, in the *P. infestans* genome encoded predicted Ago proteins similar to other organisms, and comprised the typical PAZ and PIWI domains, and a domain of unknown function 1785 (DUF1785). No BLASTP similarity to characterized PIWI proteins was observed. *Piagol1* and *Piagol2* showed identical DNA sequences; their encoded proteins also contained a predicted nuclear localization signal (NLS). All five *P. infestans* Ago proteins possessed the catalytic triad of conserved aspartic acid and histidine residues (DDH) found in Ago proteins with slicer activity from other organisms (Baumberger and Baulcombe, 2005; Höck and Meister, 2008).

Pidrb1 and *Pidrb2* were identified as encoding proteins with a dsRNA-binding motif. *Pidrb1* was entirely novel, with no significant BLAST similarity to other known proteins, whereas *Pidrb2* also contained a bicoid interacting domain and exhibited BLASTP similarity to protein methyltransferases ($9e^{-24}$). This is a novel domain architecture not represented in the NCBI sequence databases. In addition to the *Pirnh5* protein with BLASTP similarity to Dicer-like RNA helicase domains described earlier, a further 41 DEAD box RNA helicases were predicted. Of these, *Pirnh1* has been shown previously to be required for zoospore development (Walker *et al.*, 2008). *Pirnh1–8* exhibited BLASTP similarity to RNA helicases MUT14 or RM62 when the *P. infestans* genome was queried with protein sequences of gene silencing components from *C. elegans* and *Drosophila melanogaster*, respectively.

Furthermore, nine genes were predicted to encode histone deacetylases (*Hda*), two of which, *Pihda1* and *Pihda9*, were highly similar to *Hda6* from *A. thaliana*, a component of TGS (Probst *et al.*, 2004). The histone methyltransferase (*Hme*) gene family, encoding 14 proteins with either SET domains (13) or histone methylase domains (1), and exhibiting BLASTP homology to Hmes from other eukaryotes, were also identified in the *P. infestans* genome (*Pihme1–14*). For some of the predicted Hmes, additional domains were also discovered (Table 2).

Finally, 10 genes encoding nine unique chromatin-modifying (chromo) domain proteins (Cdps) were found. Seven of these contained the chromodomain as the only identifiable domain.

Picdp1 exhibited BLASTP similarity to heterochromatin protein 1 of *Drosophila yakuba*. Additional domains were predicted for Picdp9 and Picdp10 (Table 2).

Position of *P. infestans* Dicer-like and Rdr sequences amongst other eukaryotes

Dicer-like and RNA-dependent RNA polymerase are major components of the RNAi pathway (Cerutti and Casas-Mollano, 2006). A comparison of the domain architectures of Pidcl1 and Pirdr1 with corresponding proteins from other organisms revealed a high degree of diversity among the Dicer-like, relative to RdR, proteins (Fig. 1A,B). As a result, Pidcl1 was selected for further sequence analysis by the assessment of its relatedness to Dicer proteins from other organisms (Fig. 2). The phylogenetic tree that includes the *Phytophthora* Dicer sequences broadly agrees with similarities/differences in the domain architecture of Dicer-like proteins from different species. Hence, the Dicer-like

Fig. 2 Protein sequence relationships (linearized) of Dicer-like proteins. Phylogenetic analyses were conducted in MEGA4 and only predicted functional protein domain sequences of *Phytophthora infestans* were used to align with similar sequences in other organisms. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Branches corresponding to partitions reproduced in less than 40% bootstrap replicates are collapsed. Sources and accessions of protein sequences are shown in Table S1.

protein sequences from *P. infestans*, *P. ramorum* and *P. sojae* were most closely related as expected. No Dicer homologue was identified from the diatom *Thalassiosira pseudonana*, a representative of another phylogenetic grouping from the same evolutionary lineage as the oomycetes, or from the red alga *Galdieria sulphuraria*. The Dicer sequences were highly diverse in distantly related species such as *D. melanogaster*, *C. elegans* and *Homo sapiens*. The Pidcl1 domain architecture most closely resembled that of *Schizosaccharomyces pombe* Dcr1 and *Neurospora crassa* Dcl1 (Fig. 1A). In line with this observation, Dicer-like protein sequences from *Phytophthora* species showed more similarity to Dicer proteins of the amoebozoan *D. discoideum*, and representatives of yeast and fungi, *S. pombe* and *N. crassa*, than to Dicer proteins from plants and other higher eukaryotes (Fig. 2).

Transcripts of candidates of all gene categories accumulated in pre-infection stages and in planta

We used real-time reverse transcription-polymerase chain reaction (RT-PCR) to carry out transcript profiling of 51 *P. infestans* genes encoding candidate proteins in the gene silencing pathway. From our assessment of several candidates for endogenous controls described in the Experimental Procedures, *actin A* (*PiactA*) (Table S3) was found to be a comparatively stable control gene. Although *PiactA* showed a 2.7-fold increase in transcript abundance in sporangia and a four-fold decrease in germinated cysts, compared with its level in mycelium, spiking of cDNA samples with a known nonendogenous gene confirmed *PiactA* transcript levels to be the least variable in pre-infection stages (Fig. 3I), with other genes tested showing up to 10–15-fold variation between the stages. Taking into account *PiactA* variability in mRNA levels, changes in transcript abundance of other genes relative to *PiactA* of less than three-fold in sporangia and four-fold in germinating cysts were not considered to be significant (Fig. 3; Fig. S1; Table S3).

PiactA transcripts are known to be highly abundant throughout the *P. infestans* lifecycle, providing a baseline for the calculation of the relative abundance of other mRNAs. Transcripts from all genes analysed in this study accumulated in all stages of

Fig. 3 Transcript abundance [real-time reverse transcription-polymerase chain reaction (RT-PCR)], relative to *actinA* (*PiactA*) transcripts, of candidate genes encoding components of the silencing pathway in *Phytophthora infestans*. Representatives of most abundant gene transcripts in each gene family are shown: (A) *Pidcl1*; (B) *Piogo1/2*; (C) *Pirdr1*; (D) *Picdp1*; (E) *Pidrb1* and *Pidrb2*; (F) *Pihda1*; (G) *Pirnh1* and *Pirnh2*; (H) *Pihme1–3*, *Pihme5*. For *PiactA* (I), mRNA levels are shown relative to the spiked *GFP* gene, representing actual cDNA concentration in pre-infection stages. Less abundant transcripts from families in (D) and (F)–(H) are shown in Fig. S1. The transcript profiles are shown in pre-infection stages (A, germinating cysts with appressoria; C, germinating cysts; S, sporangia; Z, zoospores) and at 24 (B24), 48 (B48) and 72 h (B72) post-inoculation of potato relative to the family member exhibiting the highest mRNA level in cultured nonsporulating mycelium (M). All calculations and statistical analyses were carried out as described in Avrova *et al.* (2003). Error bars represent confidence intervals calculated using three technical replicates for each sample within the RT-PCR assay. The abundance of mRNA for each gene is shown as a proportion of the *actin A* (*PiactA*) gene on the right y-axis of each graph. Amplifications repeated on independent occasions with different starting RNA and cDNA samples resulted in similar transcript accumulation profiles for all genes studied in this work.

the *P. infestans* lifecycle, but at different levels relative to *PiactA* mRNA levels. The majority of the analysed genes exhibited mRNA levels in the range of 1–5% of the *PiactA* mRNA level. However, the members of gene families with the greatest mRNA levels reached 5–15% of the *PiactA* mRNA level (Fig. 3). The abundance of mRNAs for *Picdp1*, *Pihda1*, *Piogo1/2* and *Pirnh1* reached values of 0.7-, 0.8-, 6.5- and 14-fold of the level of *PiactA* mRNA, respectively. Transcripts of the single copy gene encoding *Pidcl1* were present at a low level (0.1–2% of *PiactA* mRNA) throughout the asexual lifecycle, with marked decreases in transcript abundance in zoospores and germinating cysts (Fig. 3A). Transcripts of *Pirnh1* accumulated markedly in the zoospore stage (Fig. 3G). Some less abundant transcripts also exhibited greater accumulation in specific stages (Fig. S1): *Piogo5* at 24 h post-inoculation (hpi) (Fig. S1D), *Picdp5/6* in cultured mycelium and at 24 hpi (Fig. S1H), and *Pihme8* in sporangia, zoospores and at 24 hpi (Fig. S1N). Taken together, the relative increases in transcript accumulation for most genes, compared with cultured mycelium, were modest and ranged from approximately two- to three-fold (*Pidcl1*, *Piogo1/2* and *Piogo4*, *Pirnr1*, *Pidrb2*, *Pirms1*, *Picdp9*, *Pihda1* and *Pihda3*, *Pihme5* and *Pihme9*, *Pirnh5* and *Pirnh7*) to five- to seven-fold (*Pirnh1*, *Piogo4*, *Pihda9*). Amplifications were repeated on three independent occasions, with different starting RNA and cDNA samples resulting in similar transcript abundance profiles for all the genes studied in this work.

Moreover, to determine whether the mRNA levels of gene silencing components were specifically influenced by the presence of dsRNA, or whether mRNA levels were more closely associated with events in the *P. infestans* lifecycle, protoplasts were exposed to *in vitro*-synthesized *Piinf1* dsRNA and single-stranded RNA (ssRNA). As transient silencing can persist in *P. infestans* for up to 15 days after dsRNA application (Whisson *et al.*, 2005), samples were taken at 2 and 15 days after treatment. No significant correlation between *Piinf1* dsRNA- or ssRNA-treated samples and the mRNA levels of any candidate gene encoding silencing components was identified by real-time PCR analysis (data not shown).

Release of *Picdc14* silencing through transient RNA silencing of Dicer-like, Ago and Hda

To examine whether selected gene silencing components were functional in *P. infestans*, a transient RNA silencing approach was employed. We hypothesized that the silent state of a gene may be briefly released if *Pidcl1*, *Piogo1/2* or *Pihda1* transcript levels are knocked down by transient RNAi. *Pihda1* was selected as histone modification has been implicated previously in silencing in *P. infestans*, whereas Dicer and Ago proteins are essential components of RNA silencing and their role in gene silencing in *P. infestans* has not been investigated previously.

To this end, *Picdc14* was chosen as a reporter for the release of silencing because inactivation of this gene leads to an easily assessable absence of sporulation phenotype (Ah Fong and Judelson, 2003). As a first step, stable silencing of *Picdc14* was established by transformation of *P. infestans* with an inverted repeat construct specific for this gene. As expected, the transformant Ns exhibited the loss of sporulation phenotype compared with the wild-type (Fig. 4A,B). Real-time RT-PCR experiments revealed an almost complete absence of the *Picdc14* transcript (Fig. 5A). These results demonstrated that the Ns line was silenced for *Picdc14*.

In a second step, protoplasts of the Ns transformant were treated with dsRNA homologous to *Pidcl1*, *Piogo1/2* or *Pihda1* to compromise the mRNA levels of these silencing genes.

The colonies regenerated after dsRNA treatments were examined microscopically at 6, 14 and 21 days post-treatment (dpt). The analysis was carried out at three time points, because the effects of transient RNA silencing in *P. infestans* have been reported previously to diminish after 2 weeks (Whisson *et al.*, 2005). At 14 dpt, two of 10 (*Pidcl1*), one of 12 (*Piogo1/2*) and six of 24 (*Pihda1*) regenerated lines showed an obvious sporulation phenotype (Fig. 4D–F) compared with the untreated Ns line and with the control lines treated with non-homologous dsRNA, which did not recover sporulation (Fig. 4B,C). This transient silencing frequency is consistent with previous observations (Avrova *et al.*, 2008; Grenville-Briggs *et al.*, 2008; Whisson *et al.*, 2005). In total, 136 control lines were regenerated from protoplasts treated with nonhomologous dsRNA, and none exhibited the restoration of sporulation phenotype. These data suggest that the recovery of sporulation in the Ns lines is generated by transient silencing of *Pidcl1*, *Piogo1/2* or *Pihda1*, and not because of a nonspecific low-frequency alleviation of *Picdc14* silencing.

Real-time RT-PCR was used to investigate correlations between the mRNA levels for *Pidcl1*, *Piogo1/2* or *Pihda1* and the observed phenotypes at 14 dpt. We found that the recovered sporulation phenotype in regenerated lines always correlated with low transcript levels of silencing genes regardless of the three genes analysed (*Pidcl1*, *Piogo1/2* or *Pihda1*). Transcript levels were reduced 3.7- and 8.3-fold (*Pidcl1* lines D1 and D2, respectively; Fig. 5B), 10-fold (*Piogo1/2* line A1; Fig. 5C) and more than 10-fold (*Pihda1* line H5; Fig. 5D). Conversely, all other lines with target gene mRNA levels reduced by two-fold or less (Fig. 5) did not show the sporulation phenotype. Taken together, our results demonstrate that the silencing of any of the three examined genes which encode components of the RNA silencing pathway, Dicer, Ago and Hda, leads to the release of the silent state, providing evidence for the role of these genes in silencing.

Fig. 4 Transient RNA silencing of *Pidcl1*, *Piago1/2* and *Pihda1* genes in the Ns (*Picdc14*-silenced) transformant. Individual regenerated lines derived from the Ns transformant were photographed 14 days after treatment of protoplasts with dsRNA homologous to *Pidcl1*, *Piago1/2* and *Pihda1*. (A) Wild-type line showing numerous sporangia. (B) Ns transformant showing absence of sporangia (no dsRNA treatment). (C) Ns transformant line showing absence of sporangia after treatment with nonhomologous dsRNA (control). (D–F) Sporulating regenerated protoplast lines of Ns transformant after treatment with dsRNA homologous to *Pidcl1* (D), *Piago1/2* (E) and *Pihda1* (F).

DISCUSSION

We have demonstrated that predicted genes in the *P. infestans* genome sequence encompass homologues for all components of a functional gene silencing pathway. This includes the well-characterized Dicer-like, Ago and RNA-dependent RNA polymerase, and the broader families of chromodomain and histone modifiers implicated in gene silencing in numerous other organisms. This study represents the most detailed analysis of the RNA silencing pathway for any organism of the stramenopile lineage, and is also the first demonstration that proteins similar to Dicer, Ago and Hda are essential for gene silencing in oomycetes.

The analysis of the assembled sequence supercontigs of the *P. infestans* genome revealed only a single candidate for a Dicer-like protein (*Pidcl1*) with two RNaseIII domains, and four unique Agos (*Piago1/2–5*). It may be speculated that the four candidate Agos may have, at least partially, specialized roles in *P. infestans*, as is the case in some other organisms such as *A. thaliana*

(Vaucheret, 2008). Consistent with the previous observation of gene silencing in *P. infestans*, which may act at the DNA level through heterochromatin formation, both *Pidcl1* and the highly expressed *Piago1/2* genes encode proteins that possess predicted NLSs. No NLS was predicted for *Pirdr1*, and *Pirdr1* differs from many other RdRs in its domain architecture by the inclusion of a Dicer-like RNA helicase domain. Rdr in *D. discoideum* also shows a similar domain architecture, and it has been suggested to be the result of domain swapping between the Dicer and Rdr encoding genes (Martens *et al.*, 2002). However, the protein sequences of *P. infestans* and *D. discoideum* RdRs are phylogenetically distinct (data not shown), suggesting that this protein organization arose independently. In this regard, Rdr may be a Rosetta stone protein and give insight into the protein–protein interactions involved in RNA silencing in this organism. Rosetta stone proteins are described as functionally disparate proteins that are linked in a common pathway and fused into a single translated unit (Date, 2008). This suggests that, in *P. infestans*, Rdr functions with an RNA helicase and, possibly, with the Dicer

Fig. 5 Relative transcript levels of *Picdc14* and genes encoding candidate silencing proteins in the release of gene silencing in *Phytophthora infestans*. (A) Comparison of *Picdc14* transcript abundance in wild-type and the Ns (nonsporulating, *Picdc14*-silenced) transformant. (B–D) Transcript abundance of *Pidcl1*, *Piago1/2* and *Pihda1* in regenerated protoplast lines 14 days after treatment of protoplasts of the Ns transformant with dsRNA homologous to *Pidcl1* (D1–D4 lines) (B), *Piago1/2* (A1–A3 lines) (C) and *Pihda1* (H1–H7 lines) (D). All calculations and statistical analyses were carried out as described in Avrova *et al.* (2003). Error bars represent confidence intervals calculated using three technical replicates for each sample within the [real-time reverse transcription-polymerase chain reaction (RT-PCR)] assay.

assessment of the evolutionary relationships among the latter revealed that the *Phytophthora* Dicer was more closely related to fungal Dicers than to Dicers of plant or animal origin. These observations are in agreement with the findings of Cerutti and Casas-Mollano (2006). They do not rule out a polyphyletic origin of Dicer-like proteins or the existence of multiple Dicer-like proteins in a eukaryotic ancestor.

Although some transposable elements exhibit differential transcript accumulation in *P. infestans* (Judelson *et al.*, 2008), and some genes encoding candidate silencing components are located in gene-sparse genomic regions similar to effectors (Rafaele *et al.*, 2010), few if any of the candidate genes assayed here exhibited strong stage-specific accumulation of transcripts. Furthermore, exogenous application of dsRNA had no effect on transcript accumulation from genes encoding components of gene silencing. This suggests that the activity of the gene silencing pathway is most probably linked to events in the asexual and infection lifecycle of *P. infestans*.

Previous studies have demonstrated that gene silencing in *P. infestans* does not involve the methylation of DNA (van West *et al.*, 2008). In support of this finding, genes encoding components of gene silencing involved in DNA methylation, such as cytosine methyltransferases and RNA polymerase IV, were not found in the *P. infestans* genome. Also not found in the *P. infestans* genome were genes encoding homologues of two important proteins involved in gene silencing in other organisms. Firstly, genes encoding proteins involved in miRNA biogenesis, such as Droscha (Lee *et al.*, 2003) only present in animals, were not found. No reports of miRNAs from oomycetes have been published to date. The other category comprises gene homologues of *ERI1*. Apart from its role in the destruction of siRNA, *ERI1* is also involved in 5.8S ribosomal RNA processing (Ansel *et al.*, 2008; Gabel and Ruvkun, 2008). The absence of this protein may explain in part why silencing may persist almost indefinitely in some *Phytophthora* species in the absence of the primary silencing signal (Gaulin *et al.*, 2007; van West *et al.*, 1999).

Gene silencing in *P. infestans* has been postulated to be mediated by balancing reactions of histone acetylation,

ribonuclease. Some support for this speculation comes from *S. pombe*, in which Rdr and Dicer-like (with helicase) have been shown to function in a nuclear protein complex (Colmenares *et al.*, 2007; Motamedi *et al.*, 2004).

Although *P. infestans* Ago proteins were broadly similar in primary sequence and domain organization, compared with other organisms, Dicer-like proteins were more diverse. An

deacetylation and methylation (van West *et al.*, 2008). The last two processes are directed by Hdac and Hme, respectively. The removal of acetyl groups from histone tails by Hdac is a means of establishing gene silencing by chromatin structural modifications, leading to the inhibition of replication and transcription (Grunstein, 1990). Changes in chromatin structure have been found in *P. infestans* transformants silenced, individually, for *Piinf1* elicitor and *PinifC* transcriptional regulator genes (Judelson and Tani, 2007). Here, we found the accumulation of transcripts encoding candidate Hdac and Hme in all lifecycle stages of *P. infestans*. A similar observation was made for the genes encoding the chromodomain family of proteins. In studies on *S. pombe*, it was reported that chromodomain proteins play an important role in the formation of repressive chromatin (Sadaie *et al.*, 2004). In organisms in which DNA methylation has not been found so far, for example *D. melanogaster* and *S. pombe*, gene silencing is associated with histone deacetylation and histone methylation (Tamaru and Selker, 2001). We speculate that this may also be the case in *P. infestans*, although it remains to be determined which Hda, Hma and Cdp family members are involved in this process.

We developed a novel strategy to determine the involvement of candidate proteins in gene silencing in *P. infestans*. Oomycetes are diploid and their sexual stages are frequently difficult to manipulate, complicating studies using mutagenesis. We therefore proposed to first stably silence a *P. infestans* gene that yields an unambiguous phenotype on silencing, and then attempt to inhibit silencing by introducing dsRNAs homologous to genes encoding candidate proteins involved in the silencing pathway. In this instance, we selected the *Picdc14* gene as a reporter for the release of silencing as it yields a loss of sporulation phenotype on silencing (Ah Fong and Judelson, 2003). Using this strategy, our transient knockdown studies on components of the silencing pathway have shown that proteins encoded by *Pidcl1*, *Piagol1/2* and *Pihda1* are involved in gene silencing in *P. infestans*. We also searched the *P. infestans* genome for candidate genes that may be affected by off-target silencing, but no such genes could be predicted for *Pidcl1*, *Piagol1/2* or *Pihda1*. Our results for the silencing of *Pihda1* are consistent with the findings of van West *et al.* (2008), where treatment of a *Piinf1*-silenced transformant with the Hda inhibitor trichostatin A led to a reversal of gene silencing. Earlier studies on a wide variety of model organisms have clearly shown that Ago proteins are major components of complexes [RISC and RNA-induced transcriptional silencing (RITS)] that facilitate gene silencing events (Kim *et al.*, 2006; Sigova *et al.*, 2004). Studies in *A. thaliana*, *S. pombe*, *D. melanogaster*, *C. elegans* and human cells have all shown that siRNAs have the ability to modulate gene silencing (Morris, 2005). *S. pombe*, which lacks the epigenetic mechanism of DNA methylation, utilizes Ago1 to guide the methylation of histones and

heterochromatin formation. Furthermore, it has been shown that an RNA-Ago-1 complex can function to slice gene-associated RNAs, resulting in the silencing of the targeted gene in *S. pombe* (Irvine *et al.*, 2006). Although here we have demonstrated that the silencing of *Pidcl1*, *Piagol1/2* and *Pihda1*, individually, is associated with a release of the *Picdc14* gene silencing phenotype, it remains to be demonstrated whether this is a result of changes in the abundance of siRNAs homologous to *Picdc14*. Ah Fong *et al.* (2008) identified siRNAs only in transgenic lines that were partially silenced for *Piinf1*; no *Piinf1* siRNAs were identified in lines exhibiting complete silencing. Similarly, we have previously stably silenced the pathogenicity effector *Piavr3a* in *P. infestans* (Bos *et al.*, 2010), but it has not been possible to detect siRNAs to *Piavr3a* (R. R. Vetukuri, unpublished data).

The genome size of *P. infestans* is two- to four-fold greater than those of the other sequenced *Phytophthora* species, *P. sojae* and *P. ramorum* (Haas *et al.*, 2009). One reason for the increased size of the *P. infestans* genome (240 Mb) is the large number of transposable element and repeat sequences (74% of the genome size). Transcripts from transposable elements may accumulate to high levels in *P. infestans* (Judelson *et al.*, 2008) and are associated with pathogenicity effector family expansion and genome reorganizations (Haas *et al.*, 2009; Raffaele *et al.*, 2010). Uncontrolled activities of transposable elements are, however, not beneficial; thus, appropriate recognition and control mechanisms have most probably evolved in *P. infestans* as in other eukaryotes (reviewed in Malone and Hannon, 2009). Furthermore, the genome of *P. infestans* also codes for large numbers (>700) of effector proteins; effectors in this instance are defined as secreted pathogen proteins and other molecules that modulate plant defence circuitry and enable parasitic colonization of plant tissue. Whether small RNAs play a role in effector gene activation or regulation, or alternatively repress defence responses on host invasion, is an open question. This and other gene regulation processes in oomycetes, and their host interactions, remain to be examined further. The elucidation of small RNA biogenesis pathways and their mechanisms of action may contribute to such advancement in this group of economically important eukaryotic plant pathogens.

EXPERIMENTAL PROCEDURES

Identification of *P. infestans* genes encoding silencing components

The assembled sequence supercontigs of the *P. infestans* T30-4 genome were searched for conserved Pfam domains using the gene index facility of the *P. infestans* genome database (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans). Searches were carried out for RNaseIII, Paz, Piwi,

RNA-dependent RNA polymerase, DEAD helicase, chromo-domain, bromodomain, Hda and Hme, methylase, SET and dsRNA-binding domain proteins. To verify protein similarity, *P. infestans* sequences were also searched against NCBI GenBank nonredundant proteins using the BLASTP algorithm with default settings. Recovered genes with domains identified by Pfam (<http://pfam.sanger.ac.uk/>) and their GenBank accession numbers for candidate *P. infestans* genes involved in gene silencing are listed in Tables 1 and 2.

Phylogenetic analysis of *P. infestans* Dicer-like proteins

Sequences of Dicer-like proteins from other model organisms were recovered from sequence databases (Table S1). Other taxa were selected to give a broad phylogenetic representation of eukaryotic organisms. Orthologues of Dicer-like proteins from oomycetes *P. sojae* and *P. ramorum* were identified by BLASTP search (default search parameters) of the predicted proteomes in the Virginia Microbial Database (<http://vmd.vbi.vt.edu/>) using *P. infestans* protein sequences as queries. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007) using only predicted functional protein domain sequences in alignments between different organisms. The relatedness of the proteins was inferred using the neighbour-joining method (Saitou and Nei, 1987). Support for the relatedness of sequence clades was derived from the percentage of replicate phylogenetic trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) (Felsenstein, 1985).

The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takezaki *et al.*, 1995), and drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

Growth of *P. infestans*, potato plants and plant inoculation

P. infestans strain 88069 was maintained on rye medium supplemented with 2% sucrose (Caten and Jinks, 1968) at 20°C. The susceptible potato cv. Bintje was chosen to study late blight disease development. *P. infestans* lifecycle stages of cultured nonsporulating mycelium, sporangia, zoospores, germinating cysts and germinating cysts with appressoria were prepared as described by Grenville-Briggs *et al.* (2005). Samples of potato leaves inoculated with *P. infestans* were taken at 24, 48 and 72 hpi as described in Grenville-Briggs *et al.* (2005). Leaf

samples were frozen in liquid nitrogen and stored at –70°C prior to RNA extraction.

RNA isolation and cDNA synthesis

Total RNA for gene expression analysis was extracted from frozen samples using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with the inclusion of the on-column DNase step. The yield and integrity of the RNA were assessed using a NanoDrop Micro Photometer (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis. First-strand cDNA was synthesized from 20 µg of total RNA by oligo(dT) priming using the first-strand cDNA synthesis kit (GE Healthcare, Uppsala, Sweden).

SYBR green real-time RT-PCR assays

Specific primer pairs (Table S2) were designed and their amplification efficiency was optimized as described in Avrova *et al.* (2003). cDNA from *P. infestans* nonsporulating mycelium was used as a template for the optimization of all primer pairs. Apart from *PiactA*, four additional genes that exhibited less than 25% variation between stages in microarray experiments (Judelson *et al.*, 2008) were selected as potential endogenous reference genes. The pre-infection stage cDNA was spiked by adding amounts of pSAM plasmid (GenBank accession EU257519), containing the *GFP* gene encoding the green fluorescent protein, proportional to cDNA concentration. It was not possible to accurately spike the cDNA for infection time points as these represent a mixture of *P. infestans* and potato cDNA with no accurate way of measuring the proportion of *P. infestans* cDNA within them. Transcript levels of *PiactA* (AAA33749), *Mago nashi RNA-binding protein* homologue (PITG_20194), *Ubiquitin protein ligase* (PITG_07230), *Exosome ribonuclease* (PITG_18034) and *Protoporphyrinogen oxidase* (PITG_01514) were measured against *GFP* levels, representing actual cDNA concentration (Table S3). Transcript levels of *PiactA* were the least variable and this gene was adopted as the endogenous internal control gene. All calculations and statistical analyses were carried out as described in Avrova *et al.* (2003). The amplification efficiencies of all genes of interest and the endogenous control were shown to be equivalent, allowing the use of the comparative C_t method for the relative quantification of *P. infestans* gene transcript levels in all life-cycle stages and inoculated plant samples in relation to their transcript levels in nonsporulating mycelium. Amplifications were repeated on three independent occasions with different RNA and cDNA samples. Numbers for each gene within the encoded protein families were given on the basis of the mRNA abundance in cultured mycelium. That is, the most abundantly

expressed was given the number 1. The transcript abundance of all other silencing gene family members was calculated as a proportion of the most abundant in cultured mycelium.

***In vitro* synthesis of dsRNA and treatment of *P. infestans* protoplasts**

Protoplasts of *P. infestans* strain 88069 were prepared as for transformation (Grouffaud *et al.*, 2008; Judelson *et al.*, 1991). dsRNAs homologous to *Piinf1* (AAB49807), *Pidcl1* (PITG_09292), *Piagio1/2* (PITG_04470) and *Pihda1* (PITG_01897) (Table S4) were prepared using an Ambion Megascript RNAi kit (Applied Biosystems, Foster City, CA, USA). The control template in the kit was used to prepare control dsRNA. Treatment of protoplasts with *Piinf1* dsRNA was carried out as described previously (Whisson *et al.*, 2005). The experiments were repeated three times. The treatment of protoplasts with dsRNA homologous to *Pidcl1*, *Piagio1/2* and *Pihda1* was carried out as follows: 10 µL dsRNA (4 µg/µL) in MT buffer (1 M mannitol, 10 mM Tris/HCl pH 7.5, 20 mM CaCl₂) was mixed with 10 µL Lipofectin reagent (Invitrogen, Carlsbad, CA, USA); 20 µL of protoplasts (10⁷ in 1 mL) were added to dsRNA or MT with Lipofectin and kept at 20°C in the dark for 24 h. The treated protoplasts were diluted in 50 mL pea broth and 2 mL was dispensed into each well of a 24-well plate and allowed to regenerate for a further 4 days. Individual regenerated colonies were removed and plated on rye medium and allowed to grow for 10 days. The colonies were examined using an inverted epifluorescence microscope (Leica, Wetzlar, Germany) at time points 6, 14 and 21 dpt, and photographs were taken (Leica LAS AF Lite software). Samples of mycelium were frozen immediately, and stored at –70°C until required for RNA isolation. These transiently silenced colonies were too small to yield sufficient materials for additional analyses, such as nuclear run-on assays or Northern blot hybridization.

Construction of the *Pidcl14* silencing vector and transformation of *P. infestans*

The *Pidcl14* gene was cloned as an inverted repeat hairpin construct, with sense and antisense copies separated by a 71-bp intron, from the *Ste20*-like gene of *P. infestans* (Ah Fong *et al.*, 2008; Tani *et al.*, 2004). The sense and antisense copies were PCR amplified (primers, see Table S5). PCR conditions were 98°C for 2 min, followed by 35 cycles of 98°C for 10 s, 65°C for 30 s and 72°C for 1 min, followed by an extension step of 72°C for 10 min. Each 50 µL PCR contained 1 U Phusion DNA polymerase (Finnzymes, Espoo, Finland), 10 µL of 5 × reaction buffer, 200 µM of each deoxynucleotide triphosphate (Fermentas GmbH, St. Leon-Rot, Germany), 0.5 µM forward and reverse primers and 10 ng of *P. infestans* genomic DNA. PCR products were gel

purified, digested (*Sfi*I for sense; *Xba*I and *Asc*I for antisense) and directionally ligated, using T4 DNA ligase (Promega, Madison, WI, USA), into the pSTORA vector (from H. Judelson, University of California, Riverside, CA, USA), with the sense copy cloned into the *Sfi*I site and the antisense copy cloned into the *Xba*I and *Asc*I sites, respectively. Insert orientation and integrity were confirmed by DNA sequencing (Macrogen Inc., Seoul, South Korea).

Stable transformation of *P. infestans* for the generation of Ns (nonsporulating, *Pidcl14* silenced) transformants was carried out using a modified polyethylene glycol–CaCl₂–Lipofectin protocol (Grouffaud *et al.*, 2008; Judelson *et al.*, 1991). Ns transformants were maintained on rye sucrose agar plates containing 10 mg/L G418 (Avrova *et al.*, 2008; Whisson *et al.*, 2007).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Relative transcript abundance (real-time reverse transcription-polymerase chain reaction) of all candidate genes encoding components of the RNA silencing pathway in *Phytophthora infestans*.

Table S1 Dicer-like sequences from different organisms used for phylogenetic analyses.

Table S2 Oligonucleotide primer sequences used for real-time reverse transcription-polymerase chain reaction of genes encoding components of gene silencing in *Phytophthora infestans*.

Table S3 Transcript abundance of candidate *Phytophthora infestans* endogenous control genes relative to levels of GFP in different cell types compared with their abundance in mycelium.

Table S4 Oligonucleotide primer sequences used for dsRNA synthesis.

Table S5 Oligonucleotide primer sequences used for cloning of *Phytophthora infestans* genes.

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