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Optimization of transgene-mediated silencing in *Phytophthora infestans* and its association with small-interfering RNAs

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ABSTRACT

Methods for silencing genes in *Phytophthora* transformants have been demonstrated previously, but wide variation in effectiveness was reported in different studies. To optimize this important tool for functional genomics, we compared the abilities of sense, antisense, and hairpin transgenes introduced by protoplast, electroporation, and bombardment methods to silence the *inf1* elicitin gene in *Phytophthora infestans*. A hairpin construct induced silencing three times more often than sense or antisense vectors, and protoplast transformation twice as much as electroporation. Using hairpins introduced into protoplasts, 61% of strains were silenced, and transgene copy number was positively correlated with silencing. The utility of bombardment was reduced by the occurrence of heterokaryons containing silenced and non-silenced nuclei, but silenced strains were obtainable from about 20% of primary transformants by single-nuclear purification. Most *inf1*-deficient strains were fully silenced, however some exhibited partial suppression. These produced *inf1*-derived RNAs of about 21-nt which correspond to both the sense and antisense strands of *inf1*, implicating an RNAi-like mechanism in silencing.

1. Introduction

Oomycetes represent a large group of economically and environmentally significant fungus-like eukaryotes. *Phytophthora infestans*, for example, is the cause of the destructive late blight diseases of potato and tomato (Fry and Goodwin, 1997). Genome sequence data for oomycetes have expanded rapidly over the last several years, which provides an enormous resource for understanding their biology (Randall et al., 2005; Tyler et al., 2006). Since oomycetes have not been traditional experimental models, however, tools for their functional genomics are still at an early stage although reasonably efficient gene transfer procedures are established. Improving such methods is critical for studying the biology, pathogenicity, and evolution of these organisms.

Methods for reverse genetics involving gene disruption are not currently feasible in oomycetes due to low rates of homologous recombination during transformation and diploidy. Instead, efforts have involved homology-dependent gene silencing approaches following practices that are now common with many plants, cultured animal cells, and some fungi (Chuang and Meyerowitz Elliot, 2000; Kadotani et al., 2003; Kamath et al., 2003). Van West et al. (1999) were the first to show that transcriptional silencing of a native gene could be induced by introducing sense or antisense copies of that gene into stable transformants of *P. infestans*. The target of those studies was *inf1*, which encodes a 10-kDa secreted protein that is easily detected in broth cultures. Application of the method to other *Phytophthora* genes have subsequently been reported (Blanco and Judelson, 2005; Gaulin et al., 2002; Judelson and Tani, 2007; Latijnhouwers and Govers, 2003; Latijnhouwers et al., 2004). However, these studies reported wide variation in the frequency of silenced transformants, sometimes being very low (<5%). No clear information exists about the optimal way to silence a gene, since the previous studies utilized different target genes, gene transfer methods (zoospore electroporation or protoplast methods), and vector configurations (sense, antisense, or hairpin transgenes).

The molecular basis of silencing in *Phytophthora* is also not well-defined. In other taxa, similar methods result in post-transcriptional or transcriptional silencing (PTGS and TGS, respectively). For example, the RNA interference (RNAi) pathway typically involves introducing a double-stranded RNA that is processed by Dicer into 20- to 25-nt small-interfering RNAs (siRNA), which direct cleavage of the native transcript by the RISC complex and thus cause PTGS (Martienssen et al., 2005; Sontheimer, 2005). In contrast, TGS methods commonly involve introducing additional copies of a gene into transformants, which silences the native locus and transgene through processes such as chromatin remodeling (Vaucheret and Fagard, 2001). However, it is now recognized that the PTGS and TGS pathways may overlap and double-stranded RNA can also trigger TGS (Bernstein and Allis, 2005; Matzke and Birchler, 2005). The nature of silencing reported so far in stable transformants of *Phytophthora* is TGS, based on nuclear run-on assays (Judelson and Tani, 2007; van West et al., 1999). The PTGS machinery also appears to be present based on transient RNAi

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experiments (Whisson et al., 2005). A signal capable of transmitting silencing between nuclei within heterokaryons is also reported, but its molecular nature is undefined (van West et al., 1999).

In this study, we compare different methods for inducing silencing in *P. infestans* transformants, using *inf1* as a target. This entailed testing sense, antisense, and hairpin constructs, combined with three methods for transformation involving either Ca^{2+} and polyethylene glycol treatment of protoplasts, zoospore electroporation, and microprojectile bombardment. Hairpin vectors combined with protoplast transformation was found to yield the highest fraction of *inf1*-silenced transformants, with little or no expression detected at the RNA and protein levels in most cases. However, instances of partial suppression were also observed. We also detected 21-nt RNAs derived from *inf1* transcripts in such transformants. This suggests that TGS and PTGS both occur in *Phytophthora*, and that TGS might be triggered after the establishment of a transient PTGS stage.

2. Materials and methods

2.1. Silencing vectors

Constructs incorporated the 376-nt *inf1* coding region (GenBank Accession U50844) in expression plasmid pTOR, which contains the *B. lactucae ham34* promoter and terminator flanking a multiple cloning site, and a gene expressing *npt* for G418-resistance (Torche, 2004). Sense plasmid pINFS was made by amplifying *inf1* using primers SF (5′-CCATCGATCCTCACTCCGTCCACGA) and SR (5′-CGGAATT CGTCAA GTCCACTCATAGC), digesting the product with ClaI and EcoRI, and inserting the fragment into similarly-digested pTOR. Antisense plasmid pINFAS was produced by amplifying *inf1* using primers SR and ASF (5′-ATAAGAATGCGGCCGCTCCTCACTCCGTCCA CGATG) followed by digestion with EcoRI and NotI, and ligation to similarly-digested pTOR. Hairpin construct pINFHP was generated by ligating a 71-bp intron from the *Ste20*-like gene of *P. infestans* (*Pic*20; Tani et al., 2004) into XbaI and NotI sites downstream of *inf1* in pINFS, followed by insertion of an antisense *inf1* fragment into the NotI site. For this the intron was amplified using primers Ste20F (5′-GCTCTAGAGAG AAGGGTAAGCCAAGACATT) and Ste20R (5′-GG ACTAGTAGGACCTAAAGAATCGCCAC), and *inf1* using Not1f (5′-ATA AGAATGCGGCCGCTCCTCACTCCGTCCACGATG) with Not1R (5′-GTC GTAAGCGGCCGCACTCATAGCGACGCACAC).

2.2. Growth and transformation of P. infestans

Isolate 1306 was maintained on rye-sucrose agar at 18 °C. Sporangia undergoing zoosporogenesis were obtained by adding 25 ml of water to a 150-mm culture plate containing sporulating hyphae, rubbing the hyphae using a bent glass rod, running the fluid through a nylon mesh to remove hyphal fragments, and then incubating the sporangial suspension for 60 min at 4 °C.

Transformants were obtained using protoplast, bombardment or electroporation methods. For the protoplast approach, this involved germinating sporangia for 27 h, protoplasting using cellulase (Sigma, St. Louis, MO, USA) and Novozyme 234 (Interspex, Foster City, CA, USA), and treatment with 30 μg of circular plasmid DNA, cationic liposomes, and polyethylene glycol (PEG; Judelson, 1993). For biolistic transformation, 12-h germinated sporangia were bombarded with an aliquot of gold particles treated with 1 μg of DNA, using a burst pressure of 1100 psi (Cvitanich and Judelson, 2003); homokaryotic transformants were later obtained by passing strains through a single-zoospore stage. Electroporation involved treating zoospores concentrated on a LiCl-Percoll gradient with 30 μg of DNA in a 2-mm cell at 550 V, 50 μF (Blanco and Judelson, 2005). Transformants were selected on rye media containing 10 μg/ml G418 and maintained on 25 μg/ml G418.

2.3. Assays for INF1 protein

Cultures were grown for 2 weeks on modified Plich media (van West et al., 1999) and supernatants assayed for protein using the Bradford assay. Equal amounts of protein were then resolved by 15% SDS–PAGE and visualized by silver-staining using 10- to 30-min development times (Sambrook and Russell, 2001). Strains showing reduced production of INF1 were always retested using independent cultures.

2.4. DNA and RNA hybridization analysis

Genomic DNA was isolated as described (Judelson, 1993). Total RNA was extracted using the Qiagen RNeasy Plant Mini kit (Qiagen, Valencia, CA). After electrophoresis on agarose gels and capillary transfer to Hybond-N^+ membranes (GE Healthcare, Piscataway, NJ, USA), hybridizations were performed using ^{32}P-labeled probes prepared by the random primer method, and detected by phosphorimager analysis (Judelson, 1993). Signals were quantified using Quantity One software for Macintosh (Bio-Rad, Richmond, CA, USA). HindIII digests of bacteriophage lambda DNA or an RNA ladder were used as size markers.

For analyses of small RNAs, these were obtained using the mirVana miRNA kit with the Plant RNA Isolation Aid reagent (Ambion, Austin, TX, USA). RNA was then separated by electrophoresis on 15% polyacrylamide–7 M urea gels, and transferred to Hybond-N^+ by electroblotting at 4 °C in 0.5× TBE for 1 h at 30 V. For radiolabeled DNA probes prepared by the random primer method, the filters were hybridized overnight at 40 °C in 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS, and 0.25 mM EDTA. Membranes were washed four times for 10 min each with 6× SSPE, 0.1% sodium pyrophosphate at room temperature, and then 15 min in 6× SSPE, 0.2% SDS, 0.1% sodium pyrophosphate at 50 °C. DNA oligomers of 23- and 26-nt based on *inf1* were used as size markers. For experiments using single-stranded radiolabeled RNA probes, *inf1* was cloned into pGEMT-Easy. Plasmid DNA was then linearized with the appropriate enzyme, gel-purified, and labeled using the MAXIscript™ *in vitro* transcription system (Ambion). Transcripts were generated using SP6 or T7 RNA polymerase to detect antisense and sense transcripts, respectively. Hybridizations and washes were then executed as described above, except that hybridization was performed at 45 °C and the final wash at 60 °C.

3. Results

3.1. Overview of strategy for testing silencing methods

To examine variables associated with silencing, three vectors were constructed that expressed full-length *inf1* in sense, antisense, and hairpin orientations on a backbone expressing *npt* for G418-resistance (pINFS, pINFAS, and pINFHP, respectively; Fig. 1). Previous studies reported that each configuration can trigger silencing of *Phytophthora* genes in stable transformants, but the optimal method is unknown since all three have not been compared using the same gene. Work in other organisms demonstrated that placing an intron between the sense and antisense portions of a hairpin construct often increases the fraction of silenced transformants (Smith et al., 2000; Wesley et al., 2001). Therefore, a 71-bp intron from the *Pic*20 gene of *P. infestans* was included in our hairpin construct (Tani et al., 2004).

Another dimension to the study was added by using three distinct methods to stably transform *P. infestans* with the plasmids. This involved either Ca^{2+} and PEG-mediated transformation of

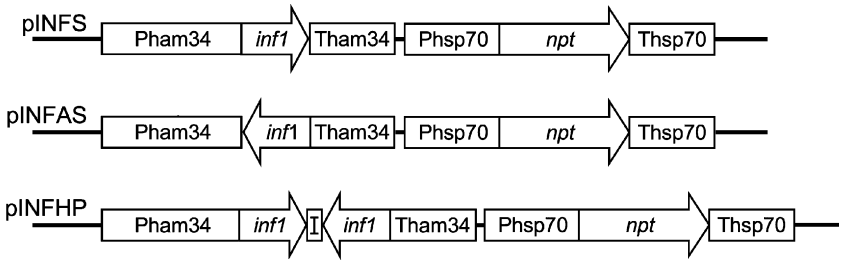

Fig. 1. Plasmids used for silencing *inf1*. In all plasmids, *inf1* is driven by the constitutive *ham34* promoter (Pham34) and terminator (Tham). pINFS contains 376 bp of *inf1* in the sense orientation. pINFAS contains *inf1* in the antisense orientation. pINFHP contains *inf1* in the sense and antisense orientations separated by the 71 bp *Pic*20 intron (I).

protoplasts derived from young hyphae (i.e. young germinated asexual sporangia), biolistic transformation of young hyphae, or electroporation of zoospores. Prior workers reported that silencing can be achieved using the electroporation and protoplast methods (Blanco and Judelson, 2005; Gaulin et al., 2002; Judelson and Tani, 2007; Latijnhouwers and Govers, 2003; Latijnhouwers et al., 2004), although little side-by-side comparative data exist. The efficacy of bombardment for inducing silencing was not previously tested.

The three plasmids were introduced by the three methods into *P. infestans*, and transformants checked for INF1 production by viewing silver-staining gels of secreted proteins; INF1 is normally the predominant protein secreted by *P. infestans*. This was done in multiple, replicated experiments for each combination of plasmid and gene transfer method (Table 1). In total, 1171 transformants were analyzed including controls. To further enhance the validity of the results, several DNA preparations were used for each plasmid to control for potential variation in DNA quality. Also, any transformants appearing to show decreased accumulation of INF1 were re-analyzed using new cultures. This was necessary to correct for fluctuations in the dynamics of INF1 accumulation, which depends on growth phase. Also, for potentially silenced strains, several staining conditions (long and short exposure to silver-staining solution) were employed to help detect weak levels of INF1 protein. Subsets of transformants were also analyzed using RNA blots to correlate the levels of INF1 protein and RNA, as well as to confirm silencing.

3.2. Gene silencing is not always absolute

Transformants were categorized as silenced, partially silenced, or non-silenced based on a combination of protein and RNA analysis (Table 1, Fig. 2). Members of each group were generated by all three plasmids, although their fractions varied depending on both the plasmid and transformation method employed. Representative examples obtained using pINFS and pINFHP are shown in Fig. 2.

Classified as silenced were strains showing less than 3% of the wild-type INF1 signal on protein gels based on visual inspection. This represented from 0% to 40% of transformants depending on the plasmid and gene transfer method used (Table 1). As shown for representative transformants such as PHP32, these sometimes contained a faint band on the silver-stained gels, which could be INF1 or another protein (Fig. 2A and B). However, no signal was detected in RNA blots (Fig. 2C and D).

Transformants having INF1 levels between 3% and 20% of wild-type were denoted as partially silenced. Between 2% and 22% fell into this class depending on the plasmid and gene transfer method. As illustrated by transformants such as PHP254 and PHP347, these had weak but evident bands in both protein gels and RNA blots (Fig. 2). When such strains were obtained using pINFHP, a 1.0 kb RNA was frequently seen which matches the expected size of the spliced hairpin RNA. Therefore, some expression of native *inf1* and/or the transgene continues in such partially silenced strains. Distinguishing between this type of partial phenotype and full silencing using the protein gels is not trivial as band intensities vary with the time of development of silver-staining. For instance, transformant PHP347 might appear to be silenced based on a short development period (Fig. 2A, top), but longer incubation indicates it is partially silenced (Fig. 2A, bottom). An *inf1* band is also evident in RNA blots for PHP347, which further confirms its partial phenotype.

The remaining strains, representing the non-silenced class, typically showed close to wild-type levels of INF1 protein. Occasion-

Table 1
Number of transformants with silenced, partially silenced, or non-silenced phenotypes

Gene transfer method	Replicate	Plasmid configuration								
		Sense (pINFS)			Antisense (pINFAS)			Hairpin (pINFHP)		
		Silenced	Partially silenced	Non-silenced	Silenced	Partially silenced	Non-silenced	Silenced	Partially silenced	Non-silenced
Protoplast	I	5	6	43	7	10	26	17	4	18
	II	3	0	12	0	0	15	39	18	12
	III	2	0	10	0	0	13	1	10	14
	IV	1	0	12	2	0	9	48	10	31
	V	0	0	19	4	0	7	–	–	–
	Mean (%)	10.7	2.2	87.1	14.9	4.7	80.4	39.5	21.9	38.6
Bombardment	I	0	0	20	0	1	6	0	4	11
	II	0	4	10	0	1	27	3	5	37
	III	0	0	31	0	0	45	–	–	–
	Mean (%)	0.0	9.5	90.5	0.0	6.0	94.0	3.3	18.9	77.8
Electroporation	I	2	0	51	0	0	39	0	4	15
	II	0	0	11	0	0	16	7	0	14
	III	0	0	11	0	0	19	17	1	19
	IV	0	0	17	0	0	16	6	2	15
	V	–	–	–	–	–	–	5	3	8
	Mean (%)	1.0	0.0	99.0	0.0	0.0	100	27.3	10.2	62.5

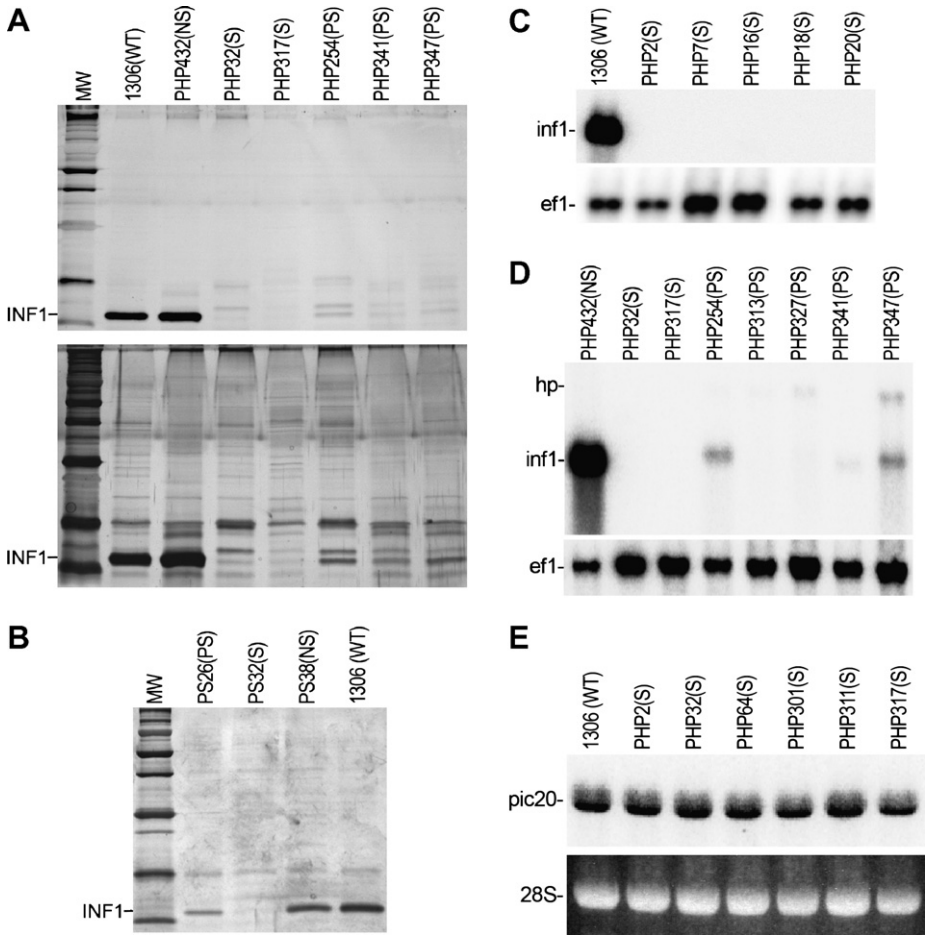

Fig. 2. Analysis of *inf1* expression in transformants. Strains are named based on the transformation method employed (P, protoplast) and the type of plasmid employed (HP, hairpin; S, sense). Shown in parentheses after the name is the phenotypic category (non-silenced, NS; partially silenced, PS; or silenced, S). (A) Silver-stained gel of secreted proteins from wild-type progenitor 1306 or transformants obtained using the hairpin plasmid. The gel in the upper panel was developed for 10 min in silver-staining solution, and the lower panel is a gel developed for 30 min. The position of the INF1 band is labeled. (B) Silver-stained gel from 1306 and transformants obtained using sense plasmid. (C and D) RNA blot analysis of silenced transformants harboring hairpin *inf1* sequences, hybridized as indicated with probes for *inf1* or elongation factor-1 (*ef1*) as a loading control. RNA is from vegetative hyphae, and indicated is a 1.0 band consistent with the size expected for the hairpin transcript (hp). (E) RNA from 1306 and *inf1*-silenced transformants obtained using the hairpin construct, hybridized with a probe for the *Pic*20 gene. RNA is from sporangia undergoing zoosporogenesis, and an image of ethidium bromide-stained 28S rRNA is shown as a loading control.

ally a few strains appeared to have only about 50% of wild-type INF1 when first tested. However, when retested they showed normal levels of the protein.

As a control for the above experiments, transformants obtained using an empty plasmid (pTOR) or pTOR expressing other transgenes were checked for *inf1* expression. No cases of full or partial silencing were obtained in 84 control transformants from electroporation. However, one case of full silencing was observed in 146 control transformants obtained using the protoplast method; this phenotype was reproduced in four independent experiments. Since this background rate of gene inactivation is far below that reported for the *inf1* sense, antisense, or hairpin constructs the general conclusions of our optimization studies are not altered. However, this finding points out the danger of inferring results from experiments in which the rate of silencing is low; a cause-and-effect relationship may not exist between silencing, the transgene construct, and any observed phenotypes.

The *Pic*20 gene, which was the source of the intron used in hairpin plasmid pINFHP, was not co-silenced along with *inf1* within transformants obtained using that plasmid. This was demonstrated by RNA blot analysis of six *inf1*-silenced strains, using sporangia undergoing zoosporogenesis since *Pic*20 expression is specific to that stage. As shown in Fig. 2E, each of the *inf1*-silenced strains express *Pic*20 at levels similar to that of the wild-type progenitor.

3.3. Most silencing occurs with hairpin construct and protoplast method

The protoplast method appears to be the procedure of choice for introducing silencing cassettes, as the highest rate of INF1 deficiencies was obtained with this compared to bombardment or electroporation (Table 1). This was observed with each of the three vector configurations. For example, on average 21.7% of protoplast-derived transformants were fully silenced compared to 1.1% and 9.4% from bombardment and electroporation, respectively. Partially silenced transformants were obtained using all gene transfer methods, although the ratio of partially to fully silenced strains was highest in those from bombardment.

The hairpin construct appears to be the best option for achieving silencing. When averaged over the three transformation methods, 23.4%, 3.9%, and 5.0% of strains were fully silenced using the hairpin, sense, and antisense plasmids, respectively. When the hairpin construct was employed with the protoplast method, 39.5% of transformants were fully silenced and 21.9% partially silenced. Curiously, despite the propensity of the hairpin to yield

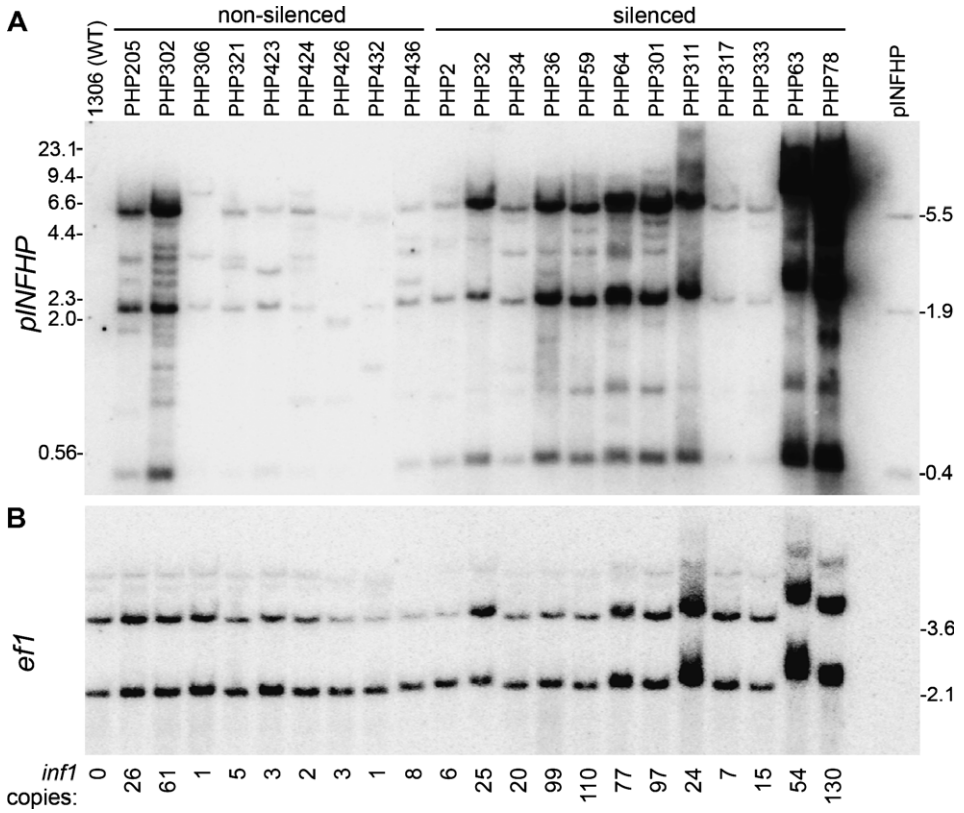

Fig. 3. Blot analysis of DNA from hairpin-derived transformants generated by the protoplast method. (A) XhoI digest probed with pINFHP. DNA is from transformants obtained with pINFHP, grouped based on whether they are silenced or non-silenced; wild-type progenitor strain 1306; and an amount of pINFHP equal to that present in ten copies per nucleus. (B) Same blot hybridized with probe for elongation factor-1α. Relative numbers of *inf1* copies per nucleus are shown at the base of the panel.

more silenced transformants, it also results in a higher ratio of partially to fully silenced strains.

3.4. Transgene copy number and silencing are correlated

Transformants generated by the protoplast method using pINFHP were examined to explore the relationship between transgene integration events and silencing. This involved blot analysis of DNA from 12 silenced and 9 non-silenced transformants, digested with XhoI. This cleaves the plasmid into 5.5, 0.4, and 1.9 kb bands with the *inf1* cassette residing on the latter. The blots were probed with pINFHP (Fig. 3A), and then with elongation factor-1α as a loading control (Fig. 3B). Relative copy numbers were assessed by comparing lanes containing transformant DNA with a lane containing the single copy-equivalent of pINFHP, and comparing the pINFHP and elongation factor-1α signals to correct for uneven loading. Native *inf1* could not be used for normalization since it is on a high molecular weight band not easily seen in this exposure.

The results indicate a positive correlation between copy number and silencing. Nearly all transformants contain tandemly repeated copies of pINFHP based on the presence of the 5.5, 0.4, and 1.9 kb bands. This is similar to findings from a previous study of transformation, in which circular DNA usually integrated into long arrays at a single chromosomal site (Judelson, 1993). The exceptions include non-silenced transformants PHP436, which only contains part of the plasmid, and PHP432 which contains about one copy. In silenced transformants the mean estimated copy number is 51 with a standard deviation of 44, while in non-silenced strains this is 11 ± 20. There are exceptions to this trend, however. For example, PHP302 has a copy number of 61 yet is non-silenced.

DNA analysis also indicated that the native *inf1* gene had not been disrupted in the transformants. In blot analyses using a probe for native *inf1*, bands of the normal size were seen in all 22 transformants described above (not shown). A similar conclusion was provided by a PCR assay for homologous integration between the *inf1* transgene and the native locus, using primers targeting the *ham34* promoter and *inf1*. Taken together, the data indicate that the deficiencies in INF1 expression are the result of silencing and not gene disruption.

3.5. Partially silenced transformants from bombardment are heterokaryons

That microprojectile bombardment yielded almost no silenced transformants was surprising since it is applied to germinated sporangia, like the protoplast method, and since prior DNA blot analyses indicated that integration events in protoplast and bombardment transformants are similar (Cvitanich and Judelson, 2003). However, it was interesting that up to 19% of the bombardment-derived transformants were partially silenced. As it is known that bombardment typically yields heterokaryotic transformants, unlike the protoplast and electroporation methods that typically yield homokaryons, the possibility was considered that some bombarded strains are heterokaryons of silenced and non-silenced nuclei. To test this, transgenic homokaryons were generated from partially silenced transformants; this was done about 7 months after the initial transformation event. This involved plating zoospores on G418-containing media, and then assaying for INF1 production.

This proved that many partially silenced strains were indeed heterokaryons of silenced and non-silenced nuclei. This is illus-

trated in Fig. 4A for transformant BHP172. INF1 expression is evident in culture supernatants obtained 1 and 7 months after transformation (lanes BHP172[1] and BHP172[7]). However, of the zoospore-derived lines, one continues to show INF1 expression (BHP172-1) while four others are silenced (BHP172-2 to BHP172-5). Similarly, transformant BHP120 expressed INF1 at both 1 and 7 months, while all five homokaryotic derivatives were INF-minus (Fig. 4B). In both cases, the fraction of INF-minus derivatives appears higher than would be expected based on the intensity of the INF1 band in the heterokaryon. This is likely because most untransformed nuclei were non-silenced, and would be lost when the zoospore-derived lines were grown on G418 media.

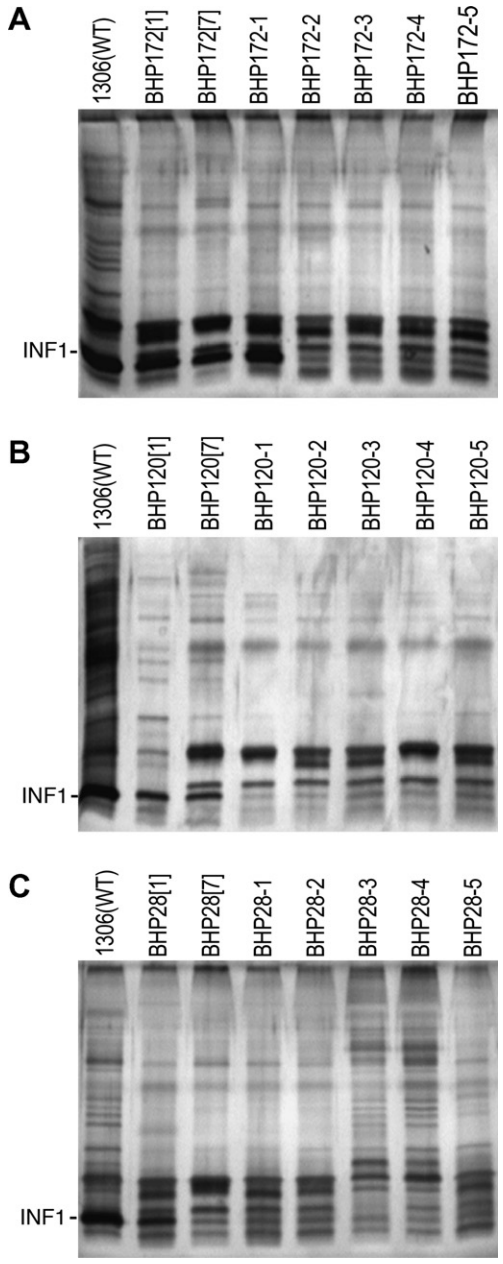

Fig. 4. Single-zoospore derivatives of partially silenced strains obtained by bombardment with pINFHP. Shown are silver-staining profiles of extracellular proteins from the wild-type progenitor 1306, primary transformants tested 1 and 7 months after the transformation event (possible heterokaryons), and zoospore-derived, G418-resistant strains (presumed homokaryons). (A) Partially silenced primary transformant BHP172 at 1 and 7 months post-transformation (BHP172[1] and BHP172[7], respectively), and G418-resistant single-zoospore derivatives (BHP172-1, which is non-silenced, and BHP172-2 to BHP172-5 which are silenced). (B) Partially silenced primary transformant BHP120 and five single-zoospore derivatives, all of which are silenced. (C) Partially silenced primary transformant PHP28, which converted to full silencing by 7 months. All single-zoospore transformants are fully silenced.

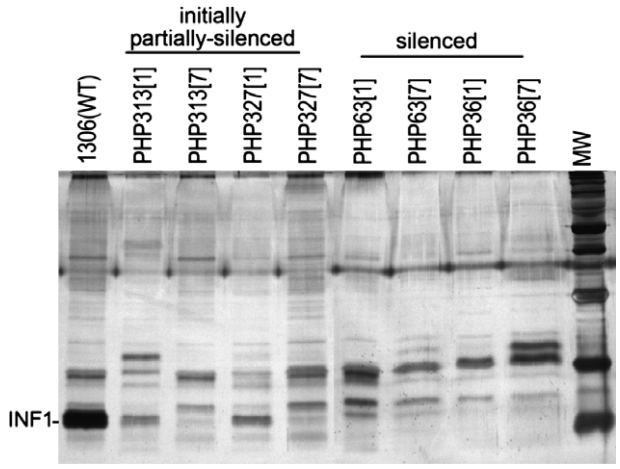

Fig. 5. Phenotypic stability of protoplast-derived transformants obtained using pINFHP. Shown are the wild-type progenitor strain 1306; transformants scored initially as partially silenced (PHP313[1], PHP327[1]) but which became fully silenced by 7 months post-transformation (PHP313[7], PHP327[7]); and transformants scored initially as silenced (PHP63[1], PHP36[1]) which maintained that phenotype at 7 months (PHP63[7], PHP36[7]).

The phenotypes of several bombardment-derived transformants appeared to spontaneously convert from partial to full silencing over time. This is illustrated in Fig. 4C for BHP28, in which the INF1 band is much stronger in the 1-month culture (BHP28[1]) than the 7-month culture (BHP28[7]). This could be due to the spread of the silencing signal as proposed by van West et al. (1999). As expected, all G418-resistant zoospore derivatives of BHP28[7] were also silenced.

3.6. Silencing induced by hairpin construct is stable

The stability of silenced phenotypes was investigated in protoplast transformants obtained using the hairpin construct. Of eight strains scored as being silenced 1 month after being transformed, all maintained the same phenotype after 6 additional months of growth (four serial transfers). This is illustrated for PHP36 and PHP63 (Fig. 5, right).

In contrast, partially silenced transformants tended to show more silencing over time. After 6 months of additional growth, three of five strains examined appeared to transition from the partially silenced to the fully silenced class. This is shown for BHP313 and PHP327 in Fig. 5 (left). For example, after 1 month PHP313 was partially silenced while little if any INF1 protein was detected after 7 months (PHP313[7]).

3.7. Silencing involves the formation of siRNA

Prior studies indicated that the suppressed expression of silenced genes in *Phytophthora* transformants is due to reduced *de novo* transcription, but the molecular signals triggering this are unknown (Judelson and Tani, 2007; van West et al., 1999). We therefore tested whether small-interfering RNAs (siRNAs) similar to those involved in RNA interference in other organisms might be involved. This entailed purifying small RNA from silenced, non-silenced, and partially silenced transformants, resolving the RNA by polyacrylamide gel electrophoresis, and hybridizing using a probe for *inf1*.

The results indicated that partially silenced strains, but not those that are fully silenced or non-silenced, contain small RNAs

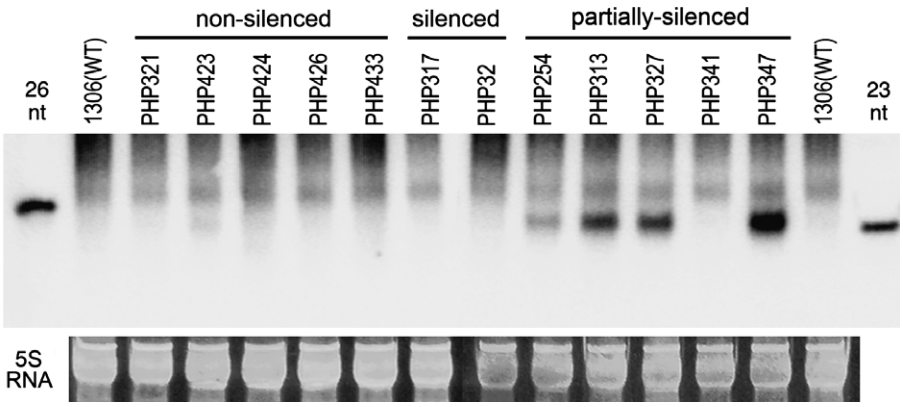

Fig. 6. Detection of small RNAs in protoplast-derived transformants containing pINFHP. The top panel shows an *inf1* probe hybridized to 10 μg of small RNA from mycelia of silenced, partially silenced and non-silenced transformants. Also shown is a lane containing small RNA from the non-transgenic strain 1306 as a control, and 23-nt and 26-nt DNA oligomers from the *inf1* as size markers. The bottom panel is an image of ethidium bromide-stained 5S rRNA as a loading control.

homologous to *inf1* (Fig. 6). For example, such a band is evident in partially silenced strains such as PHP254 and PHP347 after hybridization with a double-stranded *inf1* probe. The band has the same mobility as a 23-nt oligonucleotide derived from *inf1*, but since DNA migrates 10% faster than RNA markers of equal length, the major siRNA band is likely 21-nt (Hamilton et al., 2002). Other signals on the blots, including a weaker 26-nt band, were detected in all strains including the wild-type progenitor 1306 and are thus likely to represent non-specific hybridization unrelated to *inf1* silencing. Based on hybridization levels to the size standards, *inf1* siRNA is estimated to represent 0.002% of total RNA.

The level of silencing was not proportional to siRNA concentration in the partially silenced strains. For example, more siRNA signal was in transformant PHP347 than PHP254 even though these produce similar amounts of *inf1* mRNA (Fig. 2). This suggests that silencing may involve the integration of several mechanisms. Moreover, the PTGS stage could be transient, converting over time to TGS in some nuclei. However, such comparisons are difficult to make if cultures of silenced strains are chimeras of nuclei in various states of TGS or PTGS.

In other systems, the proportion of sense and antisense species within an siRNA population can vary, which reflects the specificity of the enzymes involved in silencing. For example, plant siRNAs contain roughly equal amounts of sense and antisense bands, while the antisense polarity predominates in several animal systems (Ronemus et al., 2006; Sijen et al., 2007). To address this issue in *P. infestans*, siRNA samples were hybridized against sense and antisense-specific RNA probes (Fig. 7). Based on phosphorimager analysis, sense and antisense molecules were present in approximately equal ratios within the small RNA population.

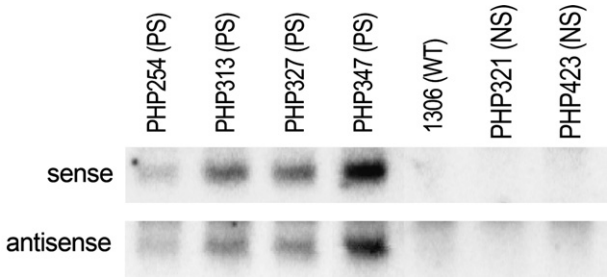

Fig. 7. Detection of sense and antisense *inf1* siRNAs in protoplast-derived transformants obtained using pINFHP. RNA from transformants that are partially silenced for *inf1* (PS), non-silenced transformants (NS), and the progenitor strain 1306 were hybridized with single-stranded RNA probes designed to detect *inf1* sequences of sense (top panel) or antisense (bottom panel) polarities.

4. Discussion

By systematically examining variables associated with silencing, the utility of this important procedure for oomycete functional genomics has been improved. Although most silencing studies performed in *Phytophthora* transformants report the use of sense or antisense constructs, our data unequivocally indicate that an inverted repeat transgene that generates a hairpin mRNA yields the highest rate of *inf1* silencing. However, silencing induced by hairpin as well as sense or antisense transgenes was not always absolute. Silencing was also found to be associated with the production of small-interfering RNAs (siRNA), which have not been previously detected in oomycetes.

Our observations concerning DNA-directed RNAi in *P. infestans* are consistent with current knowledge of the eukaryotic TGS and PTGS machinery, as hairpin transgenes would be most efficient at generating the dsRNA molecules that stimulate both pathways. siRNA derived from hairpin transcripts can either associate with the cytoplasmic RISC complex to trigger mRNA degradation, or with the nuclear RITS complex to block transcription (Martienssen et al., 2005; Matzke and Birchler, 2005; Pickford and Cogoni, 2003). Components of these pathways overlap, and may even be involved in gene inactivation processes thought previously to be distinct from RNA interference. For example, some genes subjected to RIP in *Neurospora* (a TGS process that occurs during sexual reproduction) are now recognized to be transcribed and processed into siRNA (Chicas et al., 2004). Cells may use cooperation between silencing pathways to ensure that potentially deleterious sequences are silenced, reflecting their evolution as an ancient antiviral defense system (Pickford and Cogoni, 2003).

A model for silencing in *Phytophthora* can now be proposed, which involves cooperation between PTGS and TGS processes. This is based on our observations that partially silenced transformants contain *inf1* siRNA, and that such strains can convert over time to more complete silencing. It is suggested that silencing usually involves a transient phase of PTGS during which siRNA is detectable, which then shifts to TGS. The variation seen between transformants in the timing of the conversion between PTGS and TGS may reflect differences in chromatin structure at the sites of transgene integration, transcription rate, or transgene copy number which was correlated positively with *inf1* silencing. Although there are exceptions, a positive correlation between transgene copy number and silencing is also reported in other species (Dorer and Henikoff, 1994; Henry et al., 2007; Jorgensen et al., 1996). It has been suggested that the tandem repeats that are typically present in high-copy transformants may themselves be targets for silenc-

ing, or that they aid silencing by enhancing the perpetuation of the siRNA pool. The latter would occur since sequences generated by RNA-dependent RNA polymerase (RdRP) will match sites both upstream and downstream of the RdRP initiation point in a tandem array (Martienssen, 2003). This would not be required in the case of a hairpin transgene, as its transcript would be an effective substrate for siRNA generation by itself. However, RdRP could still generate siRNAs from tandem repeats of the promoter and add to the effect of the hairpin transcript.

Genes encoding the key proteins needed for both PTGS and TGS can be found within the sequenced genome of *P. infestans*, which is can be accessed at the web site of the Broad Institute of Harvard and MIT. One RdRP gene is present, having a best match in Genbank against a *Nicotiana tabacum* RdRP (Blast $E = 10^{-102}$; gene model PITG_10457). Five Argonaute-encoding genes are detected (PITG_01400, PITG_01443, PITG_01444, PITG_04470, and PITG_4471); all have best Blast *E* values below 10^{-130}, with four being having the highest affinity to metazoan orthologs and one closest to AGO-1 of *Nicotiana benthamiana*. Two Dicer-like genes are present; one is predicted to be cytoplasmic and thus a likely participant in PTGS (PITG_09951; Blast *E* versus *Vitis vinifera* ortholog of 10^{-45}), while the other contains a nuclear localization signal and is probably responsible for TGS (PITG_09292; Blast *E* against *Arabidopsis thaliana* ortholog of 10^{-27}). However, *P. infestans* does not encode cytosine methyltransferases which are central to TGS in most non-oomycetes. This is consistent with the absence of methylated DNA around silenced loci in *P. infestans* (Judelson and Tani, 2007; van West et al., 1999) although other likely participants in silencing such as histone acetyltransferases and methyltransferases are present (Matzke and Birchler, 2005). Although this study did not test for a change in chromatin at silenced *inf1* loci, this likely has occurred since we demonstrated heterochromatinization at other loci in *P. infestans* upon silencing (Judelson and Tani, 2007).

The possibility that DNA–DNA recognition events might also mediate silencing in *P. infestans* was proposed in a study that reported that promoter-lacking constructs trigger silencing at rates similar to plasmids designed to generate sense or antisense transcripts (van West et al., 1999). However, we observe that the backbones of plasmids commonly used for oomycete transformation contain cryptic promoters that can generate transcripts spanning the cloning region (Ah Fong and Judelson, unpublished). These can result in dsRNA formation and hence silencing. Such unintended transcripts are also believed to participate in silencing in other systems, for example by generating dsRNA from sense plasmids (Jorgensen et al., 1999).

An interesting and potentially useful observation from this study is that a significant fraction of transformants exhibited only a partial knock-down. This is much more than described in an earlier study of *inf1* where silencing was typically absolute (van West et al., 1999). Such differences might be explained by variation in the strains used, in the time between transformation and expression assays, or other factors. However, incompletely silenced transformants were also reported in a study of the CBEL gene from *P. nicotianae* (Gaulin et al., 2002). The ability of the silencing machinery to maintain a partially silenced state can aid studies of genes essential for growth, as complete silencing would be lethal. For example, in analyses of a gene encoding a Gβ-like protein from *P. infestans*, we have only been able to obtain partially silenced strains which suggests it plays a critical role (K. Kim and H. Judelson, unpublished). The bombardment method for gene transfer could also be used to assess such genes, since this generates heterokaryons of silenced and non-silenced nuclei; that a gene has an essential role would be suggested if partially silenced strains are detected among the heterokaryotic primary transformants, yet fully silenced homokaryons cannot be purified.

Why the frequency of *inf1* silencing in strains generated by electroporation or bombardment was lower than in protoplast-derived transformants can be explained by several factors. For bombardment, this is likely due to the fact that most transformants are heterokaryons in which the phenotype of silenced nuclei are masked. This appears to contradict a report that silencing spreads efficiently between silenced and non-silenced nuclei in heterokaryons made from fused zoospores, but the establishment of silencing could be slower in the bombardment-derived strains since only a minority of nuclei are typically transformed (Cvitanich and Judelson, 2003). The lower rate of silencing in strains generated by zoospore electroporation may reflect the fact that zoospores are mitotically dormant; in contrast, protoplasts are made from young hyphae in which DNA replication is active. There is strong evidence to support a linkage between TGS and DNA replication, which is probably connected to the histone repositioning and chromatin modification that occurs during mitosis (Elmayan et al., 2005; Kapoor et al., 2005). Another explanation for the variation in silencing displayed by the different gene transfer methods is that aberrant transcripts that generate dsRNA from the sense or antisense constructs, or the enzymes that process dsRNA, are not produced equally in all tissues.

While the protoplast method appears to be best for silencing genes in stable transformants of *P. infestans*, oomycetes not amenable to protoplasting will benefit from the fact that a fair degree of silencing also occurs when hairpin constructs are used with electroporation or bombardment. Either protocol might be useful with species that grow poorly (or not at all) in artificial media, and procedural modifications might exist that would enhance silencing. Even with the protoplast method other variables could be tested that would influence silencing, such as the size of the hairpin transgene or intron. These were not assessed here since data from other species suggest that such factors are typically gene-specific (Heilersig et al., 2006; Hirai et al., 2007; Wesley et al., 2001). Also, our prior test of a 21-nt hairpin indicated that it was 50 times less effective at causing silencing than a 1-kb hairpin (Judelson and Tani, 2007), possibly since longer hairpin mRNAs can be processed to multiple siRNAs each of which may trigger silencing. Our data with the *NIFC1* gene of *P. infestans* also suggests that hairpin plasmids are best in general for silencing genes in general, and not just for *inf1*. With *NIFC1*, hairpin constructs triggered silencing in four of thirteen transformants, sense constructs in only one of ten transformants, and antisense in zero of five transformants (S. Tani, H. Judelson, unpublished). Whether this trend holds for all *Phytophthora* genes remains to be proved.

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References

Bernstein, E., Allis, C.D., 2005. RNA meets chromatin. Genes Dev. 19, 1635–1655.
Blanco, F.A., Judelson, H.S., 2005. A bZIP transcription factor from *Phytophthora* interacts with a protein kinase and is required for zoospore motility and plant infection. Mol. Microbiol. 56, 638–648.
Chicas, A., Cogoni, C., Macino, G., 2004. RNAi-dependent and RNAi-independent mechanisms contribute to the silencing of RIPed sequences in *Neurospora crassa*. Nucleic Acids Res. 32, 4237–4243.
Chuang, C.-F., Meyerowitz Elliot, M., 2000. Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 97, 4985–4990.
Cvitanich, C., Judelson, H., 2003. Stable transformation of the oomycete, *Phytophthora infestans*, using microprojectile bombardment. Curr. Genet. 42, 228–235.
Dorer, D.R., Henikoff, S., 1994. Expansions of transgene repeats cause heterochromatin formation and gene silencing in *Drosophila*. Cell 77, 993–1002.

Elmayan, T., Proux, F., Vaucheret, H., 2005. *Arabidopsis* RPA2: a genetic link among transcriptional gene silencing, DNA repair, and DNA replication. Curr. Biol. 15, 1919–1925.

Fry, W.E., Goodwin, S.B., 1997. Resurgence of the Irish potato famine fungus. Bioscience 47, 363–371.

Gaulin, E., Jauneau, A., Villalba, F., Rickauer, M., Esquerre-Tugaye, M.T., Bottin, A., 2002. The CBEL glycoprotein of *Phytophthora parasitica* var. *nicotianae* is involved in cell wall deposition and adhesion to cellulosic substrates. J. Cell Sci. 115, 4565–4575.

Hamilton, A., Voinnet, O., Chappell, L., Baulcombe, D., 2002. Two classes of short interfering RNA in RNA silencing. EMBO J. 21, 4671–4679.

Heilersig, H.J., Loonen, A., Bergervoet, M., Wolters, A.M., Visser, R.G., 2006. Post-transcriptional gene silencing of GBSSI in potato: effects of size and sequence of the inverted repeats. Plant Mol. Biol. 60, 647–662.

Henry, C., Mouyna, I., Latge, J.P., 2007. Testing the efficacy of RNA interference constructs in *Aspergillus fumigatus*. Curr. Genet. 51, 277–284.

Hirai, S., Oka, S., Adachi, E., Kodama, H., 2007. The effects of spacer sequences on silencing efficiency of plant RNAi vectors. Plant Cell Rep. 26, 651–659.

Jorgensen, R.A., Cluster Paul, D., English, J., Que, Q., Napoli Carolyn, A., 1996. Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs antisense constructs and single-copy vs complex T-DNA sequences. Plant Mol. Biol. 31, 957–973.

Jorgensen, R.A., Que, Q., Stam, M., 1999. Do unintended antisense transcripts contribute to sense cosuppression in plants? Trends Genet. 15, 11–12.

Judelson, H.S., 1993. Intermolecular ligation mediates efficient cotransformation in *Phytophthora infestans*. Mol. Gen. Genet. 239, 241–250.

Judelson, H.S., Tani, S., 2007. Transgene-induced silencing of the zoosporogenesis-specific PiNIFC gene cluster of *Phytophthora infestans* involves chromatin alterations. Eukaryot. Cell 6, 1200–1209.

Kadotani, N., Nakayashiki, H., Tosa, Y., Mayama, S., 2003. RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. Mol. Plant–Microbe Interact. 16, 769–776.

Kamath, R.S. et al., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 421, 231–237.

Kapoor, A., Agarwal, M., Andreucci, A., Zheng, X., Gong, Z., Hasegawa, P.M., Bressan, R.A., Zhu, J.K., 2005. Mutations in a conserved replication protein suppress transcriptional gene silencing in a DNA-methylation-independent manner in *Arabidopsis*. Curr. Biol. 15, 1912–1918.

Latijnhouwers, M., Govers, F., 2003. A *Phytophthora infestans* G-Protein β subunit is involved in sporangium formation. Eukaryot. Cell 2, 971–977.

Latijnhouwers, M., Ligterink, W., Vleeshouwers, V.G.A.A., van West, P.F.G., 2004. A G-alpha subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. Mol. Microbiol. 51, 925–936.

Martienssen, R.A., 2003. Maintenance of heterochromatin by RNA interference of tandem repeats. Nat. Genet. 35, 213–214.

Martienssen, R.A., Zaratiegui, M., Goto, D.B., 2005. RNA interference and heterochromatin in the fission yeast *Schizosaccharomyces pombe*. Trends Genet. 21, 450–456.

Matzke, M.A., Birchler, J.A., 2005. RNAi-mediated pathways in the nucleus. Nat. Rev. Genet. 6, 24–35.

Pickford, A.S., Cogoni, C., 2003. RNA-mediated gene silencing. Cell. Mol. Life Sci. 60, 871–882.

Randall, T.A. et al., 2005. Large-scale gene discovery in the oomycete *Phytophthora infestans* reveals likely components of phytopathogenicity shared with true fungi. Mol. Plant–Microbe Inter. 18, 229–243.

Ronemus, M., Vaughn, M.W., Martienssen, R.A., 2006. MicroRNA-targetted and small interfering RNA-mediated mRNA degradation is regulated by argonaute, dicer, and RNA-dependent RNA polymerase in *Arabidopsis*. Plant Cell 18, 1559–1574.

Sambrook, J., Russell, D., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Woodbury, NY.

Sijen, T., Steiner, F.A., Thijssen, K.L., Plasterk, R.H., 2007. Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science 315, 244–247.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G., Waterhouse, P.M., 2000. Total silencing by intron-spliced hairpin RNAs. Nature 407, 319–320.

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

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

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

Tyler, B.M. et al., 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science 313, 1261–1266.

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

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

Wesley, S.V. et al., 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 27, 581–590.

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