

SHORT COMMUNICATION

**SILENCING OF CRYPARIN, A CELL WALL HYDROPHOBIN,
IN *CRYPHONECTRIA PARASITICA***

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SUMMARY

Cryparin is a cell wall hydrophobin abundantly expressed in *Cryphonectria parasitica*, the ascomycete fungus causing chestnut blight. In this study we compare the cryparin-minus phenotype of a previously characterized knockout strain to that of silenced strains obtained by the transformation of the fungus with a strong elicitor of post-transcriptional gene silencing. We show that cryparin expression can be silenced very efficiently and that the knockout phenotype is similar to the phenotypes obtained through gene silencing. Silenced strains were also experimentally infected with *Chryphonectria hypovirus 1* (CHV-1) with no obvious effect on cryparin expression. We also tested the possibility that p29, a protease expressed by the CHV-1 genome, might act as a suppressor of gene silencing in plants.

Key words: Silencing, hydrophobins, chestnut blight, cryparin, *Cryphonectria parasitica*.

The ascomycete fungus *Cryphonectria parasitica* is the causal agent of chestnut blight, a disease that has severely impacted the American chestnut [*Castanea dentata* (Marsh) Bork.] (Anagnostakis, 1982) and is still causing serious damage in various parts of Europe (Milgroom and Cortesi, 2004). *C. parasitica* infects the host through wounds which then develop into cankers, after which the fungus emerges through the bark as stromal pustules that provide conidia for plant-to-plant dissemination. Recently, the epidemiological relevance of biocontrol through hypovirulent strains has been critically analysed (Milgroom and Cortesi, 2004). Hypovirulence in *C. parasitica* is caused by *Chryphonectria hypovirus 1* (CHV-1), genus *Hypovirus*, family *Hypoviridae* (McCabe *et al.*, 1999; Hillman *et al.*, 2000; Linder-Basso *et al.*, 2005).

Viral infection of *C. parasitica* causes down-regulation of a number of genes (Allen *et al.*, 2003). Important

and among the first to be characterized is the gene encoding cryparin (Zhang *et al.*, 1994), a cell wall hydrophobin. Previous studies characterized the properties of cryparin and its secretion pattern (Carpenter *et al.*, 1992; McCabe and Van Alfen, 1999).

Hydrophobins are a class of fungal proteins with various functions (Whiteford and Spanu, 2002). However in *C. parasitica*, cryparin appears to be involved in the formation of stromal pustules, and compared to the wildtype, cryparin knockout mutants produce stromal pustules that have difficulty erupting through the bark, although pathogenicity is not affected directly (Kazmierkzac *et al.*, 2005).

Reverse genetic approaches in filamentous fungi rely mainly on successful production and complementation of gene knockout mutants and other disruption techniques. However, these techniques are not always successful, especially when vital genes or genes involved in hyphal growth are involved. Additionally, some specific loci do not easily undergo the double recombination that is necessary for such techniques. Moreover, redundancy within the genome can make the functional knockout of multi-copy genes particularly difficult. These techniques are even less useful when the unmasking of gene dosage phenomena is needed. A valid alternative is the use of post-transcriptional gene silencing (PTGS), and this is particularly true for functional genomic studies (Kamath *et al.*, 2003; Boutros *et al.*, 2004).

PTGS is also known in filamentous fungi as “quelling” (Romano and Macino, 1992), and is thought to be a natural defence mechanism against viruses and transposons (Cogoni and Macino, 2000; Springer and Yanofsky, 2000). The mechanism is activated, among other stimuli, by the presence of “aberrant” RNA in the cytoplasm. Once activated, a complex of enzymatic reactions specifically digests all the mRNA carrying the aberrant sequence (Kuznetsov, 2003). PTGS can be artificially stimulated in filamentous fungi through transformation of the fungal strains with plasmid constructs able to express specific RNAs: in particular, hairpin constructs with minimal dsRNA structure are good elicitors of PTGS (Nakayashiki *et al.*, 2005).

No homologous recombination is required for the expression of such constructs, which greatly increases

the likelihood of success. RNA silencing is currently being used as a tool for exploring gene functions in various ascomycete and some basidiomycete fungi (Fitzgerald *et al.*, 2004; Nakayashiki *et al.*, 2005; Walti *et al.*, 2006). Our work is aimed at validating the possibility of using PTGS in *C. parasitica* as an alternative to gene knockout. We compare the traditionally prepared *cryparin* gene knockout mutants with strains in which *cryparin* expression has been silenced through PTGS.

In plants, suppressors of gene silencing are often used to restore gene function and demonstrate that a certain phenotype is associated with an apparently silenced gene and is indeed caused by the silenced gene itself. Currently there are no known suppressors of gene silencing that can serve this purpose in fungi, but we note that p29, a protein expressed by CHV-1 ORF A through autoproteolytic cleavage and recently shown to be located in *trans*-Golgi network vesicles (Jacob-Wilk *et al.*, 2006), is related to HC-Pro (potyvirus helper component-protein), a well characterized suppressor of gene silencing in plants (Koonin *et al.*, 1991).

We first compared the silencing efficiency of three different constructs:

(i) pCB1004, the negative control plasmid used for *C. parasitica* transformation expressing only the selection marker (Carrol *et al.*, 1994).

(ii) PCryGen, a plasmid expressing wild-type *cryparin* mRNA. A 3.6 kb *EcoRI* fragment was digested from a cosmid genomic library and inserted in the plasmid vector pBluescript SK⁻ (Stratagene, Cedar Creek, TX, USA). This intermediate construct, called pBS-Crygen, was then digested with *EcoRV*, dephosphorylated and ligated to a gene conferring resistance to hygromycin B derived from the plasmid pCB1004.

(iii) PCrySil, a plasmid expressing *cryparin* mRNA with an artificially inserted inverted repeat to create a hairpin secondary structure. Cloning began with the plasmid pBS-Crygen, which was digested with *Bam*HI, klenowed and re-ligated, in order to eliminate a *Bam*HI site at the end of the terminator of the gene. This plasmid was then used as template in a site-directed mutagenesis reaction performed by PCR to insert a new *Bam*HI site in position 710 (numbering correspond to the GenBank accession number L09559): we used EXPAND long template kit (Roche, Mannheim, Germany) following the suggested protocol and using the oligonucleotides Cry-Bam-710F (5'-AAAGGATCCGATTCCGAAGTCTGATAGCA-3') and Cry-Bam710R: (5'-AAAGGATCCCAGACCAACTGGGTCCC-3'). The 6 kb product was digested with *Bam*HI and re-ligated. The intermediate plasmid obtained was called pBS-Crygen+Bam710, with a newly inserted *Bam*HI site in position 710. We then inserted into the *Bam*HI site of this intermediate a DNA fragment encoding a 220 bp *cryparin* genomic segment obtained by PCR using as template pCrygen and as oligonucleotides Cry331R-BamHI

(5'-AAAGGATCCTCGCTGTACAGAGTGG-3') and Cry100F-BamHI (5'-AAAGGATCCTCTCAATTCCGTAACCTTGC-3'). Clones were selected which contained the inserted DNA in opposite orientation to the wild type: the expressed RNA included a 220 inverted repeat separated by a linear sequence. The putative secondary structure of this mRNA is a hairpin having a 220nt dsRNA stem and a 490nt loop; introns, are present in the *cryparin* gene, and were maintained in the transformation vector. We then inserted a gene conferring resistance to hygromycin into the *EcoRV* site as described in the previous paragraph.

Protoplasts obtained from *C. parasitica* strain Ep67

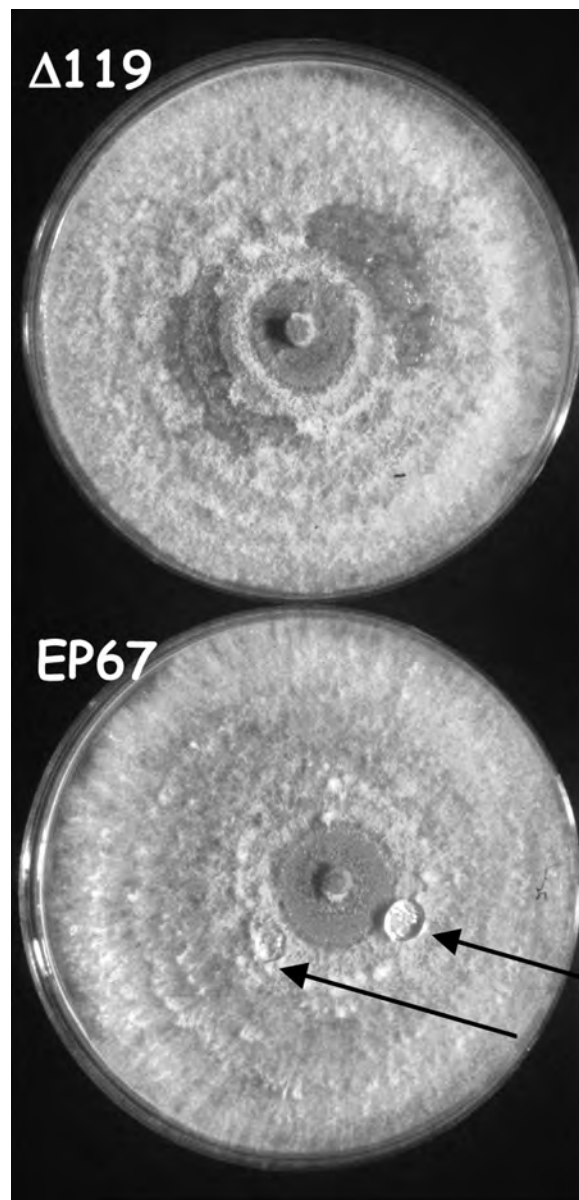


Fig. 1. PD Amb plates inoculated with a wild type strain (EP67) and a *cryparin* knockout strain Δ 119. Fungal colonies were grown for 1 week. A 30 μ l drop of water (arrow) deposited on aerial mycelia allows estimation of the hydrophobicity of the fungal thallus.

were transformed with the aforementioned plasmids as previously described (Turina *et al.*, 2003). Mitotically stable transformants were obtained from single spores and the hydrophobicity of their aerial mycelium was assessed as this was shown to be a good indicator of the presence or absence of cryparin (Kazmierczac *et al.*, 2005). Each selected transformant was grown on PDAMB (Turina *et al.*, 2003) medium for seven days at which point a 30 μ l drop of water was placed on the aerial mycelium. Fig. 1 shows the shape maintained by the water droplet on a wild-type mycelium vs the mycelium of a Cryparin knockout strain (Δ 119). For each of the three constructs (pCrySil, pCryGen, and pCB1004), the hydrophobicity of at least 100 transformants was assayed.

Transformation with pCrySil resulted in 93 hydrophilic colonies out of 142 transformants; the two control plasmids pCryGen and pCB1004 gave 17 out of 124 and 6 out of 119 hydrophilic colonies, respectively. The transformation vector expressing the hairpin construct (pCrySil) was thus much better at triggering gene silencing (65% of the normally growing transformants) than the two controls used in the same experiment: pCB1004 only occasionally produced strains that lack hydrophobicity (0.5%), possibly because of random insertion in loci important for cryparin expression, transport or regulation, and pCryGen, which only occasionally (13%) expressed aberrant cryparin mRNA depending on the number of copies and locus of insertion, or other factors not completely elucidated. Since the hydrophobicity of

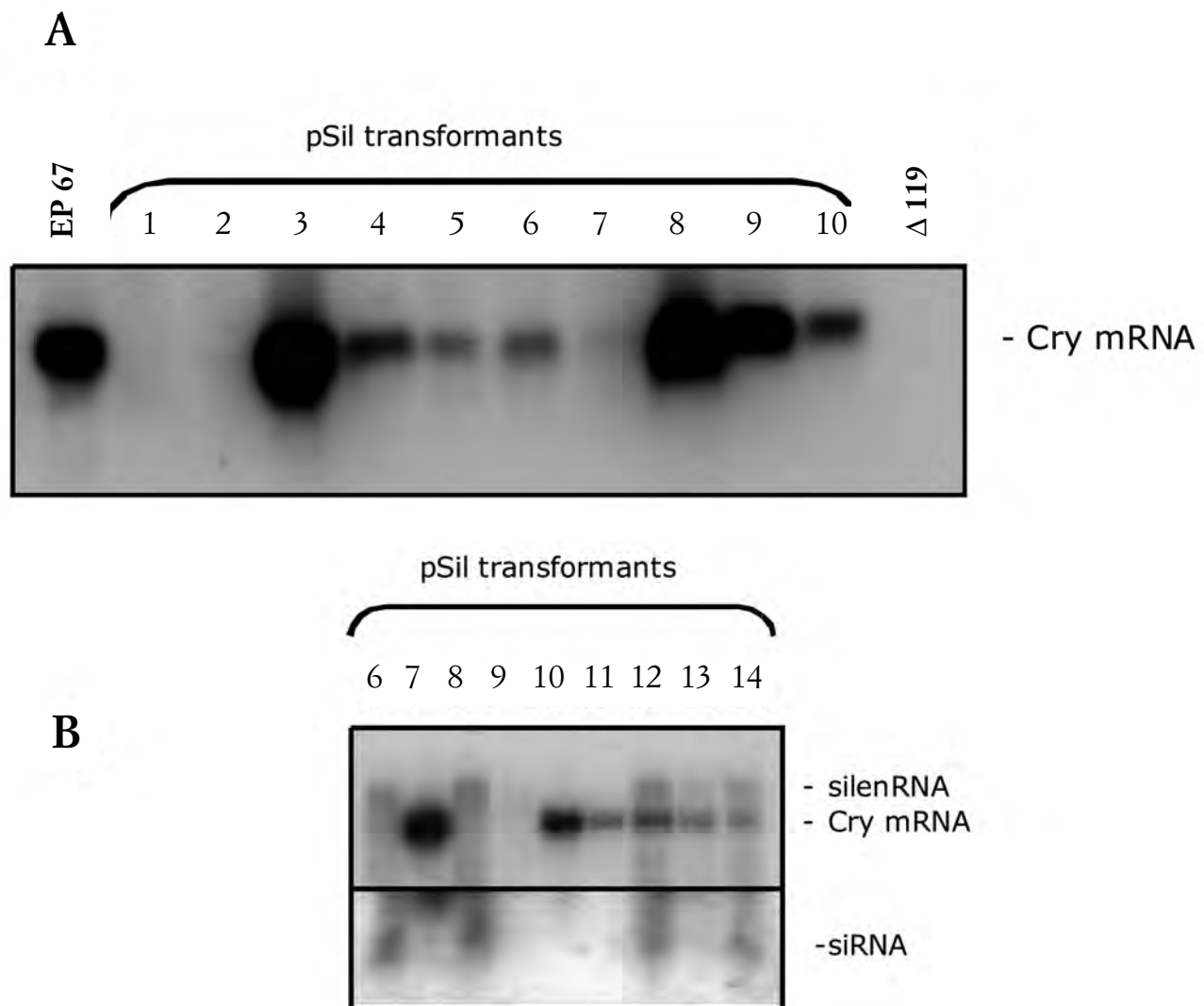


Fig. 2. **A**) Northern blot analysis of total RNA from a number of mycelial extracts from *C. parasitica* transformants (liquid cultures). The plasmid used for transformation was pCrySil. A cryparin probe was labelled as previously described (Turina *et al.*, 2003). A wild type strain (EP67) and a cryparin knockout strain (Δ 119) are also present. **B**) Northern blot analysis of a number of *C. parasitica* single spore colonies from the previous experiment. The position of cryparin mRNA (Cry mRNA), the mRNA derived from transcription of the inserted transformation vector (silenRNA), and the accumulation of small interfering RNAs (siRNAs) is shown. Each lane was loaded with the same amount of total RNA, and equal ribosomal RNA loadings were estimated by ethidium bromide staining.

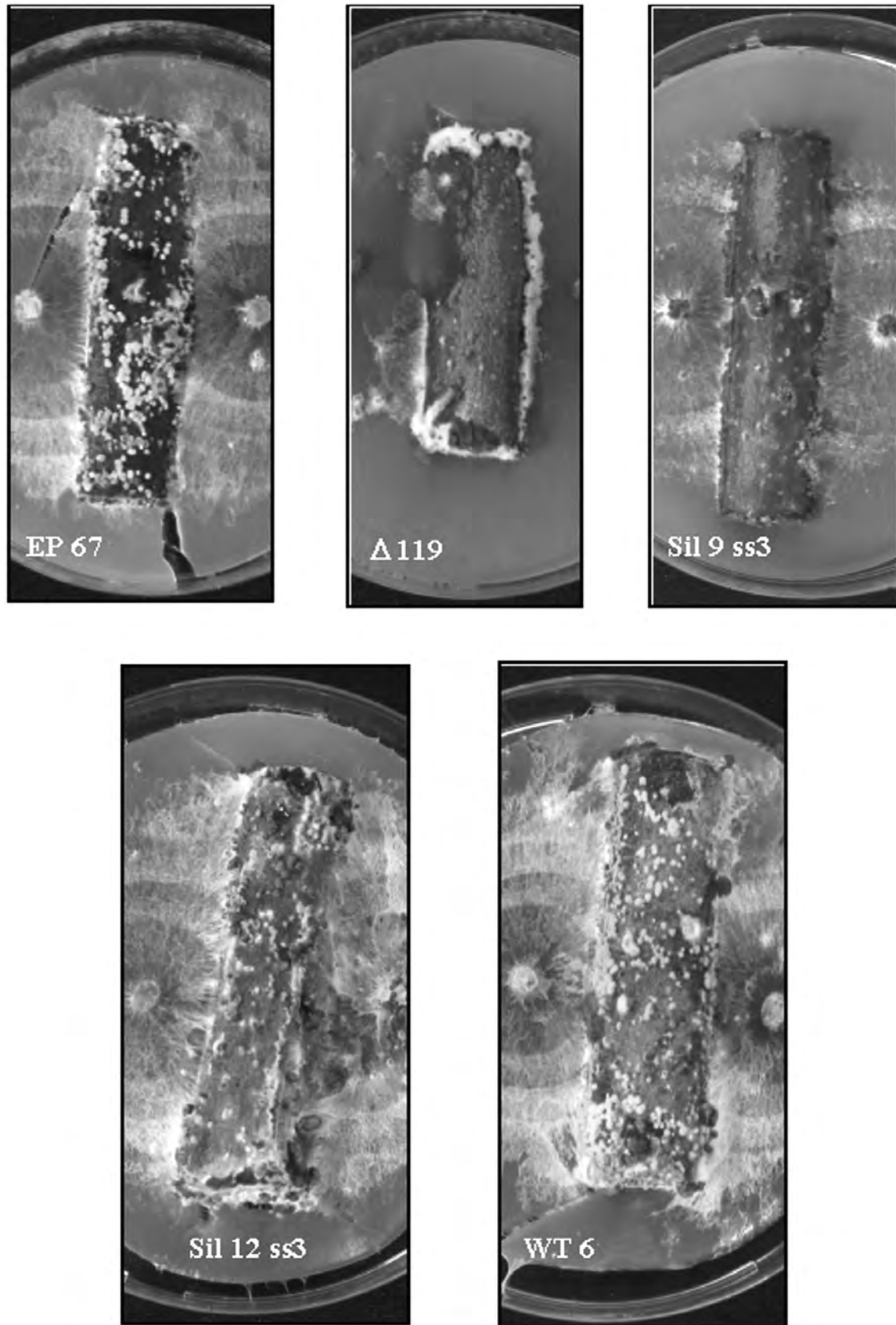


Fig. 3. Stromal pustule eruption on chestnut wood: Petri dishes contain stem pieces prepared as in Kazmierczak *et al.* (2005) at 45 days post-inoculation with various *C. parasitica* strains. EP67 and Δ 119 are the positive and negative controls; WT6 is a replacement strain where a functional genomic fragment is re-introduced in a Δ 119 background; pSil9ss3 and pSil12ss2 are two single-spore isolates of transformants number 9 and 12 from Fig. 2, showing different levels of silencing.

aerial mycelia might not exclusively depend on cryparin gene expression, we wanted to check that it was indeed correlated to cryparin expression. Northern blots were done on a number of transformants according to methods previously described (Turina *et al.*, 2003). Fig. 2 shows the cryparin expression level of some of the transformants together with a wild-type and a knockout cryparin strain as controls; the RNA expression level varied among the transformants, with some strains increasing the level and others where the level was depressed below the threshold of detection. Cryparin down-regulation was also checked through Western blot analysis (Kazmierczak *et al.*, 2005) which showed that different levels of accumulation for the various strains correlated with presence of the mRNA (data not shown). Presence of small RNA molecules hybridizing with a Cry mRNA probe were also detected indicating that down-regulation of cryparin is indeed due to PTGS (Fig. 2b), and their presence was strictly correlated with expression of the aberrant cryparin mRNA from the transformation vector.

We then compared the phenotype of the silenced strains, with the knockout and wild-type strain, and noticed that the number of stromal pustules was proportional to cryparin expression level (Fig. 3). This shows that *C. parasitica* genes can be silenced, and that the silenced strains resemble the knockout strain in phenotype, confirming the role of cryparin in stromal pustule eruption through the bark (Kazmierczak *et al.*, 2005). One argument against using silencing for a reverse genetics approach in filamentous fungi is the impossibility of demonstrating the link between the observed phenotype and a certain silenced gene, thus excluding the pos-

sibility of a certain phenotype being due to a random mutation in the genome. In a knockout strain, such proof is generally obtained through replacement of the original gene with an ectopic expression vector. This approach is generally mimicked in plants by the availability of gene silencing suppressors, which are often used to reverse the silencing of a certain gene, showing phenotype recovery. Such a tool is not yet available for filamentous fungi, and the virus CHV-1 with its p29 protein being similar to potyvirus HC-pro seems to be a good candidate for a silencing suppressor to be used in filamentous fungi. A first step in looking for a suppressor of gene silencing in CHV-1, was the infection of silenced cryparin strains (pSil9) with CHV-1: virus was transmitted to several silenced strains with strain EP802 as previously described (Van Alfen *et al.*, 1975). We expected some silencing reversion if CHV-1 encodes for a silencing suppressor, and this would be shown by accumulation of cryparin. On the contrary, our results indicated lack of obvious strong suppressor activity (Fig. 4). This result should be taken cautiously though, since a further complexity is that CHV-1 infection was consistently shown to down-regulate cryparin, even if at the mRNA level, accumulation is still fairly abundant. The possibility of using different target genes to test for silencing suppressors will be evaluated in the near future. We also tested the possibility that p29 is a silencing suppressor in plants: after cloning p29 in a transient expression vector for agroinfiltration (pBin61), we agroinfiltrated p29 into leaves of C16 *N. benthamiana* together with GFP as previously described (Bendahmane *et al.*, 1999; Bendahmane *et al.*, 2002). We were able to detect

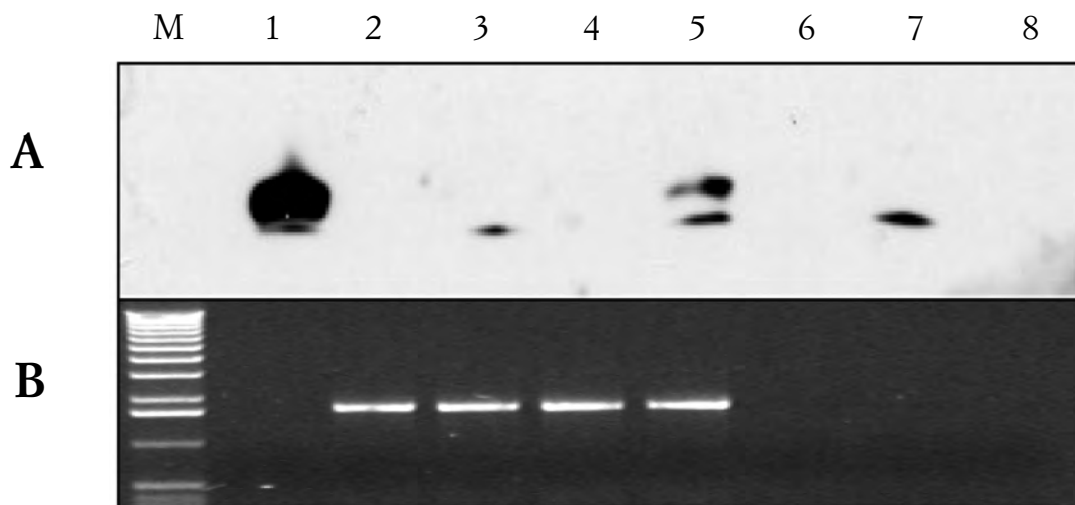


Fig. 4. **A)** Western blot analysis of whole cell lysate from cryparin silenced strains, with or without the presence of CHV-1 in their cytoplasm. EP67 (lane 1) and $\Delta 119$ (lane 8) are respectively the positive and negative controls. Lanes 6 and 7 correspond to two different single-spore isolates of strain pSil9, a strain displaying strong cryparin silencing. Lanes 2, 3 and 4 show the same single spore isolates after acquisition of CHV-1 through hyphal anastomosis. Lane 5 = strain EP67 after CHV-1 acquisition through hyphal anastomosis. **B)** RT-PCR product analysis (ethidium staining of 1% agarose gel in TBE) for the presence of CHV-1 in the viral genome. M = 1 kb ladder (Gibco-Life Technologies). The size of the expected band is 1700 bp, and the oligonucleotides used in the RT-PCR reaction designed on the CHV-1 sequence were CHV-1-8685F (5'-GCCACTTCGTGGGACAATAC-3') and CHV-1 10387R (5'-GGGAAGAAAGTGGACTTACC-3').

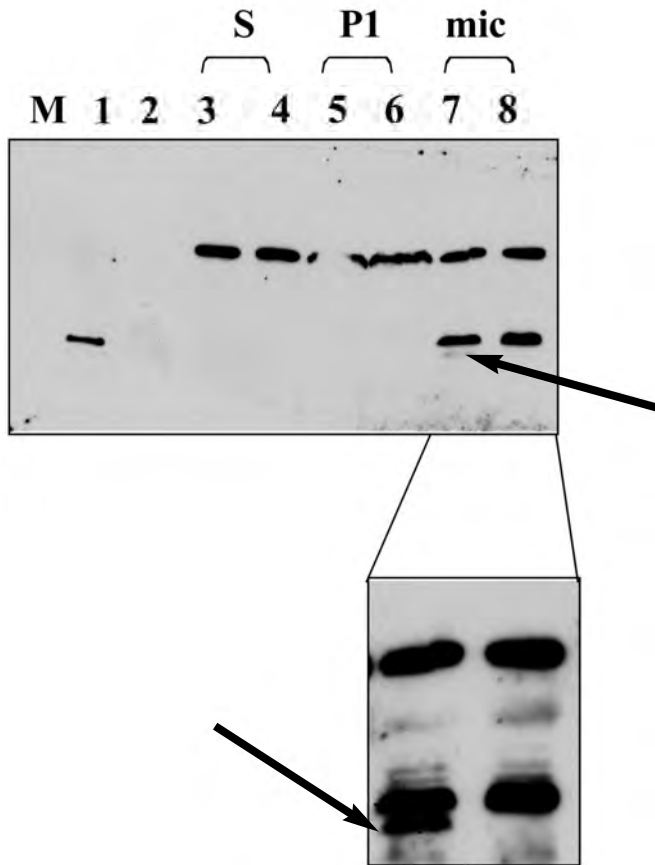


Fig. 5. Western blot analysis for the expression of p29 protein in *N. benthamiana* fractionated sub-cellular extracts agroinfiltrated with plasmid pBinp29 (lanes 3, 5 and 7) and with the negative control plasmid pBin61 (lanes 4, 6 and 8). S= soluble fraction; P1= low speed centrifugation pellet; mic= microsomal fraction. As positive and negative controls, we show virus-infected EP802 cell extracts (lane 1), and wild-type EP67 (lane 2) cell extracts. The arrows point to the p29 expression band in the microsomal fraction of *N. benthamiana* agroinfiltrated leaves.

p29 through western blot analysis in agroinfiltrated leaves (Fig. 5), but no obvious local reversion of silencing (displayed by the positive control HC-pro) was detected (data not shown). Recently, p29 silencing suppression activity was indeed demonstrated in newly emerging leaves (Segers *et al.*, 2006), showing suppression of systemic spread of silencing in GFP-expressing transgenic *N. benthamiana* line 16c. In summary, our data show that indeed PTGS can be used in *C. parasitica* for a reverse genetic approach where other techniques have failed. Previously this phenomenon was used for reverse genetics in *C. parasitica*, when the terms co-suppression or quelling were current, before the PTGS phenomenon was completely understood (Choi *et al.*, 1995). The hair-pin vectors we have used can be further improved with the use of inducible promoters, which would help understanding functions of housekeeping genes.

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