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Virus-induced silencing of a sequence coding for loricrin-like protein in *Phytophthora infestans* upon infection of a recombinant vector based on tobacco mosaic virus

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Abstract: *Phytophthora infestans* is the oomycete responsible for late blight disease of Solanaceae that causes both yield and economic losses. With the aim of reducing high management costs mainly due to wide fungicide applications, alternative eco-sustainable control strategies to avoid plant wilt are needed. RNA interference (RNAi) is a powerful tool for gene function studies that can be accomplished by constitutive transformation or transient expression like in virus-induced gene silencing (VIGS). VIGS makes use of viruses to deliver sequence homologous to a target gene fragment and trigger RNAi. Indeed, a *P. infestans* ortholog of plant loricrin-like protein (*LLP*), named *PiLLP*, has been silenced using the direct infection of a recombinant vector based on the plant virus, tobacco mosaic virus (TMV-*PiLLP*-1056), aiming to reduce the oomycete sexual reproduction. In TMV-GFP-1056 the gene coding for the green fluorescent protein has been replaced with an antisense construct obtained by fusion PCR from the 5'-UTR and 3'-UTR of the *PiLLP* sequence. Here we show that RNAi can be expressed in the A1 mating type of *P. infestans* 96.9.5.1 by VIGS using the direct infection of TMV-*PiLLP*-1056. We provide evidence that the recombinant vector can enter, replicate and persist in mycelia of *P. infestans* where it induces the partial downregulation of the *PiLLP* transcript. Compared with the wild type, the *PiLLP*-silenced mating type 96.9.5.1 had slower colony growth and a diminished virulence in detached tomato leaflets. This seems to be the first evidence for the downregulation of a constitutive gene of *P. infestans* using a recombinant vector based on a plus-sense RNA plant virus.

Keywords: Solanaceae; oomycetes; mating type; A1; A2; GFP; *P. infestans*; late blight; VIGS; recombinant.

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1. Introduction

Oomycetes form a lineage of eukaryotic organisms that resemble filamentous fungi, but phylogenetic studies demonstrated a more extensive relatedness with brown algae and diatoms than with fungi, thus leading to the inclusion of oomycetes in the stramenopiles ([1] and references cited therein). Similarly to true fungi, oomycetes include threatening plant pathogens like the heterotallic species *Phytophthora infestans* (Mont.) de Bary, the causal agent of potato late blight disease and other destructive diseases of Solanaceae [1]. Brownish black and water-soaked lesions covered by fungal sporulation are the main symptoms of late blight on leaves and stems, until plant wilt; similar symptoms are observed on other crops within the same family [2]. Late blight disease caused by *P. infestans* is well-known due to 1840s Irish potato famine, although it is considered a "re-emerging" disease [3] causing new severe epidemics that entail yield

and economic losses, e.g. high management costs mainly due to wide fungicide applications (up to ten sprays per season).

With the aim of reducing pesticide applications and favouring disease control by natural pathogen and host resources, recently proposed control strategies exploit ways for the identification and inhibition of pathogen's effectors [4,5]. Upon being secreted into the plant host cytoplasm, effector proteins may transit to the host nucleus, where they modulate plant signaling and the plant's immune responses [6,7]. Indeed, host immunity can be negatively regulated favouring pathogen penetration or fulfilling metabolic or structural needs of the pathogen during infection [8]. The plasticity of *P. infestans* genome and the abundance of predicted genes coding for disease-promoting effector proteins with high copy number variation, high substitution rates, and differential expression [9] pose continuous challenges to disease control. In *Phytophthora* spp., effectors drive the rapid sequence divergence of infection-related genes and have an exceptional high potential to adapt to new control strategies [10-12]. The situation was worsened by the recent emergence of more aggressive lineages of *P. infestans* such as the 13_A2 (Blue13) of the A2 mating type in Europe, which allowed the oomycete to exploit a sexual reproduction cycle. Until the late 1970s, only mating type A1 of *P. infestans* was distributed worldwide and reproduced asexually allowing some success in its management [13]. The emergence and worldwide distribution of aggressive A2 mating types, enabled sexual cycles, and induced genetic variations and gene transfer in populations of *P. infestans* producing isolates with increased virulence and creating new challenges for late blight management ([14] and references quoted therein). Integrated pest management (IPM) strategies could provide more durable and environmentally friendly crop protection against late blight [15]. In recent years, research have been focused on exploiting photosynthetic purple non-sulfur bacteria (PNSB) for bioremediation [16,17] and biosensing in hybrid devices [18,19]. Their versatile metabolism allows them to be used as a promising inoculant to promote plant growth while control pathogenic attacks, enhancing the immune response of solanaceous plants [20,21]. RNA interference (RNAi) (syn. RNA silencing) is a promising and sustainable strategy that has been used to target and silence specific pathogens' genes by means of the small RNAs (sRNAs) produced during the RNAi process. Basically, there are two ways for RNAi exploitation in *Phytophthora* spp.: constitutive and transient gene silencing that have been proven to be effective [22]. Constitutive gene silencing implies that transformation constructs must be integrated in pathogen's genome and expressed via a specific promoter. In the transient gene silencing, pathogen's protoplasts are electroporated with *in vitro*-synthesized silencing constructs, mainly hairpin-like RNA structures, and then regenerated into new colonies. The recent host-induced gene silencing (HIGS) approach seems more effective than other strategies but implies that sRNA molecules, generated in transgenic plants expressing double-stranded RNAs (dsRNAs) of oomycete sequences, are then translocated into the pathogen involved in plant attack [23]. Similarly, plants infected with viral vectors carrying effectors' sequences, can induce synthesis of sRNAs and then target effector proteins [24]; this technique is named virus-induced gene silencing (VIGS). In both HIGS and VIGS, it is expected that sRNAs generated by plant RNAi can move into pathogen during infection process to silence the homologous endogenous gene. Being a more direct way, VIGS induces silencing via a recombinant viral vector triggering pathogen rather than host.

Recently, Cao et al. [25] demonstrated that nonpersistent acquisition of plant viruses by plant-associated fungi may commonly occur in nature, highlighting a possible role for fungi in the life cycle, spread, and evolution of plant viruses. Moreover, Mascia et al. [26,27] have shown that six taxonomically different plant viruses can replicate and persist in *P. infestans* and, as in plants, a tobacco mosaic virus-based recombinant vector (TMV) triggers and suppresses RNAi in *P. infestans*. Furthermore, Mascia et al. [28]

demonstrated that the same vector based on TMV could infect three fungal species within *Colletotrichum* genus, express a reporter gene ectopically and silence the related reporter transgene expressed in *Colletotrichum* spp. Based on the above-mentioned evidence it would not be surprising if an RNAi-based response could have the potential to be fruitfully exploited for specific gene silencing in *P. infestans*, using VIGS with a TMV recombinant vector. Recently, the CRISPR/Cas9 system-mediated gene knockout and *in situ* complementation of RNA-binding protein involved in oospore formation, have been applied to oomycetes; furthermore, this post-transcriptional regulator, i.e. PuM90, specifically binds to the 3′-UTR of a flavodoxin-like protein, repressing its mRNA level in *Pythium ultimum* [29]. In *P. infestans* regulatory mechanisms involved in sexual reproduction are scarcely known except for a loricrin-like protein (LLP) that is strongly over-expressed during oospore formation [30].

PiLLP, a *P. infestans* ortholog of the plant LLP, was silenced by protoplast transformation. Compared with the wild type, the *PiLLP*-silenced transformants were defective in oospore formation, had slower colony expansion rates, produced less sporangia with lower germination and zoospore-release rates, and also had severely diminished virulence levels [31]. We selected *PiLLP* sequence as target to silence via the direct infection of a TMV-based recombinant vector carrying the 3′UTR and 5′UTR of the *PiLLP* coding sequence.

2. Materials and Methods

2.1. *P. infestans* mating types and culture conditions

The mating types A1 (strain 96.9.5.1) and A2 (strain 13_A2) of *P. infestans* used in this study were kindly provided by Dr. David Cooke (The James Hutton Institute, Invergowrie, Dundee, Scotland). Both mating types were grown on pea agar (PA; [32]) medium at 18±1 °C in the dark. To test the mycelial growth rate, culture plugs (5 mm diameter), cut from the actively growing edge of each colony, were transferred on PA Petri dishes and the orthogonal colony diameters were measured after 14-day incubation. Sporangia of each mating type were aseptically collected by washing 20-day-old cultures with 7 mL of distilled water and gently rubbing mycelia with a glass rod. Zoospores were released by consecutively incubating the sporangial suspension at 4±1 °C for 2 h and then at room temperature for 1 h. For mating type confirmation, each mycelium was grown in liquid culture and harvested, then it was filtered and air-dried by sterile Whatman No.1 filter paper (Whatman, Maidstone, U.K.), freeze-dried at -20±1 °C, and finally homogenized by Tissue Lyser II (Qiagen, Hilden, Germany) prior to DNA extraction with DNeasy Plant Mini Kit (Qiagen), as manufacturer's recommendation. DNA preparations were subjected to PCR using primer pairs S1a/S1b [33] and PHYB-1/PHYB-2 [34] for mating type A1 and A2, respectively, and amplified by DreamTaq Hot Start DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.). All PCRs were performed in T100 Thermal Cycler (Bio-Rad, Bio-Rad Laboratories, Hercules, California, U.S.A.). Annealing temperatures were 50 °C and 59 °C for S1a/S1b and PHYB-1/PHYB-2 primer pair, respectively. For further confirmation, DNA preparations were amplified with W16-1/W16-2 primer pair [35] using 61 °C as primer annealing temperature; PCR products were digested with *HaeIII* (New England Biolabs, Ipswich, Massachusetts, U.S.A.). All PCR products were analyzed by 1.2 % agarose gel electrophoresis in 1X TBE (90 mM Tris, 90 mM boric acid, 1 mM EDTA) and stained with Gel Red (Biotium, Biotium Inc., Fremont, California, U.S.A.).

2.2. *TMV-PiLLP-1056 vector construction*

Total RNA was extracted from *P. infestans* mycelia with Eurogold RNA pure (Euroclone S.p.a., Pero, Milan, Italy) according to manufacturer's protocol. RNA was sus-

pended in RNase-free sterilized water and the concentration was adjusted to 1 µg/µL. Standard reverse-transcription reactions were carried out with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, Massachusetts, U.S.A.). The 5′ and 3′ untranslated regions (UTR) of *PiLLP* were amplified in two separate reactions with specific primers (5′UTR for/rev and 3′UTR for/rev) designed to obtain *XhoI* restriction site at 5′ and *PacI* restriction site at 3′ of the joined fragment using 54 °C and 59 °C as annealing temperatures respectively. Size of the two amplified PCR products was estimated by electrophoresis (2% low melting agarose gel, TBE buffer, Gel Red staining) and expected length of DNA fragments were 201 bp and 126 bp. Products were purified using Agarose Gel DNA Extraction Kit (Roche, Roche Diagnostics Corporation, Indianapolis, Indiana, U.S.A.) according to the manufacturer's instructions. Using 1:1 ratio of the amplified and excised fragments of 5 ′ UTR and 3 ′ UTR, fusion PCR was carried out using Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen™, Waltham, Massachusetts, U.S.A.) and the annealing temperature was set at 50 °C using 5′UTR for/3′UTR rev primers. The 327 bp amplified product was purified and eluted from agarose gel as described above. The fused fragment was cloned in pGem-T Easy Vector System (Promega, Promega Corporation, Madison, Wisconsin, U.S.A.), and inserted in anti-sense orientation into the *XhoI* and *PacI* sites of the vector pTMV-GFP-1056 [36]. The plasmid pTMV-GFP-1056 was used as backbone to construct recombinant plasmid pTMV-*PiLLP*-1056. The fused fragment and the vector were double digested with *XhoI* and *PacI* (New England Biolabs), loaded on 2% low melting agarose gel (TBE buffer, Gel Red staining), and eluted as described above. Ligation of the 5′UTR+3′UTR joined fragments to the viral vector pTMV-1056 without GFP was accomplished using T4 DNA ligase (Promega) followed by transformation of TOP10 *E. coli* competent cells (Invitrogen). The correct insertion of the construct in the pTMV-1056 plasmid was verified by DNA sequencing in both directions using GFP pro/CP pro primer pair. Vector map and cloning strategy are described in S1. Primers used in this work are listed in Table 1.

Table 1. Primer pairs used for amplifications of the genes in this study. From left to right, each column shows primer name and related sequences, amplicon size (bp), annealing temperature (Ta), use purpose, and bibliographical reference (Ref.).

Primer name	Sequence	Size (bp)	Ta	Purpose	Ref.
S1a S1b	5′-AGGATTTCAACAA-3′ 5′-TGCTTCCTAAGG-3′	1250	50	Mating type identification	[33]
PHYB-1 PHYB-2	5′GATCGGATTAGTCAGACGAG-3′ 5′-GCGTCTGCAAGGCGCATTTT-3′	347	59	Mating type identification	[34]
W16-1 W16-2	5′-AACACGCACAAGGCATATAAATCTA-3′ 5′-GCGTAATGTAGCGTAACAGCTCTC-3′	557	61	Mating type identification	[35]
5′UTR for rev	5'-TCTCCTCGAGTCCTCCTCTCTGCGT-3′ 5'-CGACAATATTCCATAACTGCTAAGGTGGGGCTGTAA-3'	201	54	5'UTR region of *PiLLP* cloning	This study
3′UTR for rev	5'-TATGGAATATTGTCGAGTACAGCAACGAGGCTTCGA-3' 5'-CCCCCTTAATTAAACGACACTTGAAACATCGTGAG-3'	126	59	3'UTR region of *PiLLP* cloning	
5′UTR for 3′UTR rev	5'-TCTCCTCGAGTCCTCCTCTCTGCGT-3′ 5'-CCCCCTTAATTAAACGACACTTGAAACATCGTGAG-3'	327	50	5'UTR+3′UTR of *PiLLP* fusion PCR	
GFP pro CP pro	5′-CGATGATGATTCGGAGGCTA-3′ 5′-ATCCAAGACACAACCCTTCG-3′	~500	55	pTMV-1056 plasmid sequencing	
REP for rev	5′-CAGTGCATATCGGCATTGTC-3′ 5′-CGTGACTCCTCTTCCGTCTC-3′	241	50	TMV replicase of *P. infestans* detection	
*Pi*EF for rev	5′-ACTCCAAGAACGACCCTGCTAAGGCAACC-3′ 5′-TCCGACGGCTCGAGGATGACCATGCAG-3′			Real Time PCR of ef1 of *P. infestans*	[31]
*Pi*ORF for rev	5′-CAGTCTCCCGTCACCAGTCC-3′ 5′-GGCAGTCGGCAATACCACAT-3′	~300	55	Real Time PCR of *PiLLP*	

2.3. Infection in plants

Inocula of TMV-*PiLLP* were prepared in tobacco (*Nicotiana tabacum* L.) cv. Samsum by rub-inoculation of a biologically active transcript synthesized from the *KpnI*-linearized plasmids pTMV-*PiLLP*-1056 with T7 RNA polymerase and the mMessage mMachine kit (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.), following the protocol of the manufacturer. Two true leaves of four tobacco plants were inoculated with 30 μL of the transcription mixture obtained from pTMV-*PiLLP*-1056. Inocula of TMV-GFP-1056 were used as control. All the plants were grown at 22±2 °C with a 16-hours photoperiod and daily monitored for symptom appearance. To ensure both virus maintenance and purification, systemically infected leaves of *N. tabacum* cv. Samsum were collected at 10 days post inoculation (dpi) extracting plant sap in 100 mM (Na_2-K) phosphate buffer, pH 7.2. Viral particles of TMV-*PiLLP*-1056 were purified from tobacco leaves as described by Lot et al. [37] and stored at -20±1 °C in 50 mM NaCl, 30% glycerol solution until use. Viral RNA was extracted using phenol-chloroform protocol. Reverse-transcription polymerase chain reactions (RT-PCR) and DNA sequencing were used to detect the correct insertion of 327 bp fused fragment into pTMV-1056 using GFPpro/CPpro primer pair. Furthermore, total RNA from infected tobacco leaves was extracted using RNeasy Mini Kit (Qiagen). RNA preparations were spotted on positively-charged nylon membranes (Roche) and preliminary tested for hybridization with digoxigenin (DIG)-labeled DNA probes specific for: i) tomato mild green mosaic virus (TMGMV) coat protein (CP) present in TMV-1056 vector; ii) GFP present in TMV-GFP-1056 vector and iii) 3'UTR+5'UTR *PiLLP* fragment present in TMV-*PiLLP*-1056 vector. RNA preparations were separated by electrophoresis through 1.2% agarose gel in TBE buffer, stained with Gel Red, and blotted by capillarity onto positively-charged nylon membranes. Northern blots were subjected to hybridization with digoxigenin (DIG)-labeled DNA probe specific for TMGMV CP.

2.4. Infection in *P. infestans*

To inoculate *P. infestans* A1 and A2 mating types, 50 μL of purified preparations of TMV-*PiLLP* (1 μg/mL) were added to sporangia growing in 100 mL of PB supplemented with ampicillin (100 mg/mL; Sigma-Aldrich, Merck KGaA, St. Louis, Missouri, U.S.A.). Fungal cultures inoculated with 50 μL of 50 mM NaCl and 30% glycerol solution served as wild-type (WT) negative control. All cultures were prepared in triplicates and incubated at 18±1 °C under continuous shaking at 50 rpm. Total RNA, extracted from freeze-dried mycelia at 10 dpi was reversely transcribed as described above. RT-PCR was used throughout to detect the TMV-*PiLLP* in mycelia of *P. infestans*. Reactions were carried out with primer pairs REP for/rev mapping to TMV-1056 sequences that code for replicase using 50 °C as annealing temperature. Purity and size of the amplified products was estimated as above. Quantitative real-time PCR (qPCR) was used to evaluate the abundance of *PiLLP* transcripts in the two mating types of *P. infestans* exposed to TMV-*PiLLP*-1056 inoculum. Reactions were carried out in Applied Biosystems StepOne™ Real-Time PCR System with Fast SYBR®Green Master Mix (Applied Biosystems). Samples were run in triplicate and reactions were set up in a total volume of 10 μL using 50 ng of cDNA and primers PiEF for/rev and PiORF for/rev (250 nM each). Primer pairs were designed to target the constitutive elongation factor 1 (*ef1*) gene of *P. infestans*, as an internal control, and the *PiLLP* ORF [31]. The cycling conditions were: 95 °C for 20 sec; 40 cycles at 95 °C for 3 sec and 60 °C for 30 sec. Specificity of the qPCR products was confirmed by analysis of the dissociation curves. Results were evaluated using the StepOne™ Software v2.3. Means and standard deviations were calculated among three replicates. The whole experiment was repeated twice with a different set of biological samples.

2.5. Pathogenicity test

Three-weeks-old *P. infestans* WT and TMV-*PiLLP*-challenged mycelia cultured on PA in optimal growth conditions were used to cause plant infection: fungal plates were flooded with 5 mL Pea Broth (PB) and scraped with a glass rod to release sporangia. Each resulting solution was collected and used for inoculation of detached leaflets of tomato cv UC82. Leaflets were previously surface-sterilized with sodium hypochlorite solution (2% vol/vol), rinsed in sterile distilled water, aseptically air-dried, and transferred on sterile moist filter paper placed in Petri dishes (90 mm Ø). Each leaflet was inoculated on abaxial side with 30 µL of sporangia suspension ($1x10^5$ sporangia/mL) near the main vein and kept in growth chambers (16±1 °C, 16 h light/8 h dark photoperiod). Each replicate was made of three leaflets placed in the same Petri dish, and two replicates were arranged for each tested inoculum. Disease severity was estimated by measuring the area of the lesions developed using ImageLab software (Bio-Rad).

3. Results and Discussion

3.1. *P. infestans* mating types identification

The mating type of the two *P. infestans* isolates was confirmed by several tests. According to S1a/S1b PCR assay, isolate 96.9.5.1 yielded a product of about 1.25 kbp confirming that it was the A1 mating type, whereas no amplification was observed from 13_A2 (Blue 13) isolate. Amplification with PHYB-1/PHYB-2 primer pair aimed to target PHYB marker, resulted in 347 bp fragment obtained only for isolate 13_A2; no amplification product was obtained for 96.9.5.1 isolate. (Figure 1) From both *P. infestans* isolates, amplification with primer pair W16–1/W16–2 produced amplicons of 557 bp. After digestion with *Hae*III endonuclease, three fragments of approx. 557 bp, 457 bp, and 100 bp and two fragments of approx. 457 and 100 bp were observed, respectively, for the A1 mating type and A2 mating type (not shown).

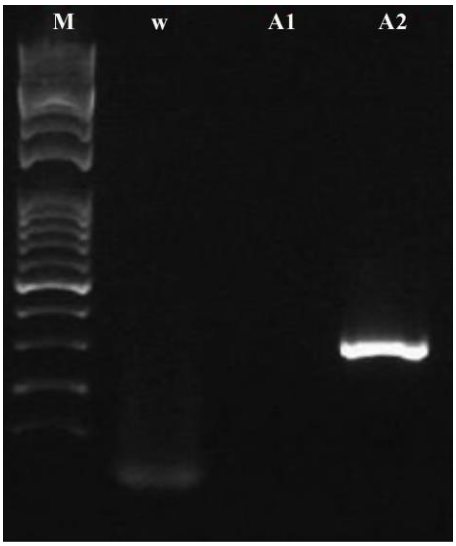


Figure 1. Gel electrophoresis of A1= DNA extracted from mating type A1 amplified with PHYB-1/PHYB-2; A2= DNA extracted from mating type A2 amplified with PHYB-1/PHYB-2. The arrow shows the 347 bp fragment obtained for isolate 13_A2. M= HyperLadder™ 100bp (Bioline, Meridian Bioscience Inc., Cincinnati, Ohio, U.S.A.); w= negative control (no DNA).

3.2. *Infectivity in tobacco plants*

To investigate whether *PiLLP* could be silenced by VIGS, the pTMV-*PiLLP*-1056 vector was assembled to express the 5' and 3' untranslated regions of *PiLLP* under the subgenomic RNA (sgRNA) promoter of TMV CP. The 201 bp amplified fragments of 5' UTR and 126 bp of 3'UTR were joined by fusion PCR and the 327 bp resulting fragment was inserted in anti-sense orientation between the *XhoI* and *PacI* sites of pTMV-1056 in replacement of GFP. The correct insertion and orientation of the 5'UTR+ 3'UTR joined fragment was confirmed by sequencing the region of interest in both directions (data not shown). The recombinant transcript of TMV-*PiLLP*-1056 was synthesized *in vitro* and its infectivity tested in tobacco healthy plants. A transcript of TMV-GFP-1056 synthesized from the original pTMV-GFP-1056 was used as control. Disease symptoms appeared on tobacco leaves at 7 dpi and consisted in pale yellow discolorations of main veins. (Figure 2a) These symptoms appeared also in newly formed leaves and persisted up to 30 dpi. Absence of possible contaminants in which the original GFP sequence was not removed was excluded by the absence of fluorescence when plants were irradiated with an UV lamp (UVP, Upland, CA, U.S.A) (Figure 2b) suggesting complete replacement of GFP with 3'-5'UTR *PiLLP* in TMV-*PiLLP*-1056 vector. GFP fluorescence was clearly observed in plants rub-inoculated with the recombinant transcript TMV-GFP-1056. (Figure 2c)

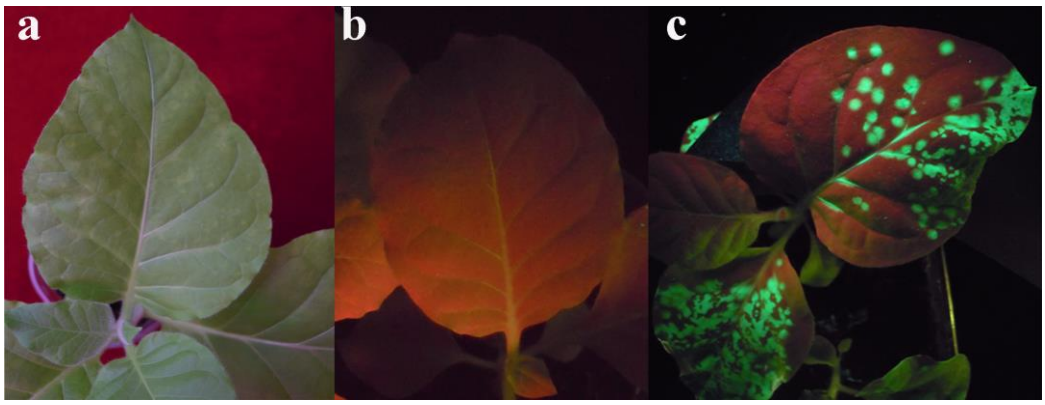


Figure 2. a) Expression of TMV-*PiLLP*-1056 on *N. tabacum* leaves; b) No GFP fluorescence of tobacco leaf infected by TMV-*PiLLP*-1056 when exposed to UV light; c) GFP fluorescence in tobacco plant inoculated with TMV-GFP-1056 used as control.

DNA sequencing of the reversely transcribed TMV-*PiLLP*-1056 RNA extracted from infected plants confirmed the stable and correct insertion of the 327 bp fused fragment in the TMV-*PiLLP*-1056 after replication in tobacco plants (data not shown). The TMV RNA genome indeed encodes four proteins of approx.126, 183, 30 and 17.5 kDa. The 183 kDa polypeptide is synthesized by readthrough of the leaking termination codon of the gene for the 126 kDa polypeptide. Both the 126 and 183 kDa polypeptides, namely replicase (REP), are required for viral RNA replication. The 30 kDa polypeptide, also called movement protein (MP) is required for viral cell-to-cell movement whereas the 17.5 kDa polypeptide is the viral coat protein (CP) subunit. MP and CP are synthetized via a subgenomic RNA (sgRNA) strategy [38]. TMV-GFP-1056, one of the most used TMV-based vector, drives the expression of the reporter green fluorescent protein (GFP) isolated from jellyfish, for functional studies in plants. It contains a duplicated sgRNA promoter for the CP synthesis from the heterologous tobamovirus tobacco mild green mosaic virus (TMGMV) to prevent homologous recombination between duplicated TMV CP sgRNA promoters and the CP sequence of the same virus [39-41]. The TMV sgRNA promoter

directs the expression of any gene inserted downstream between the *Pac*I and *Xho*I cloning sites.

Replication of TMV-*PiLLP*-1056 in plants was confirmed by the detection of three sgRNAs corresponding to sequences replicated by the TMV MP promoter, TMV CP promoter and TMGMV CP promoter, respectively. (Figure 3) sgRNA 1 has been synthesized by the TMV MP promoter, has an estimated size of 2.4 kbp and contains sequences of TMV-MP, 3'-5' *PiLLP* and TMGMV CP; sgRNA 2 has been synthesized by TMV CP promoter, has an estimated size of 1.6 kbp and contains sequences of 3'-5' *PiLLP* and TMGMV CP; sgRNA3 has been synthesized by the TMGMV CP promoter, has an estimated size 0.48 kbp and contains the sequence of TMGMV CP. sgRNA 1 seems also present in the RNA preparation extracted from TMV-*PiLLP*-1056 particles and probably accounts for the shorter viral particles observed in purified preparations (Figure 5).

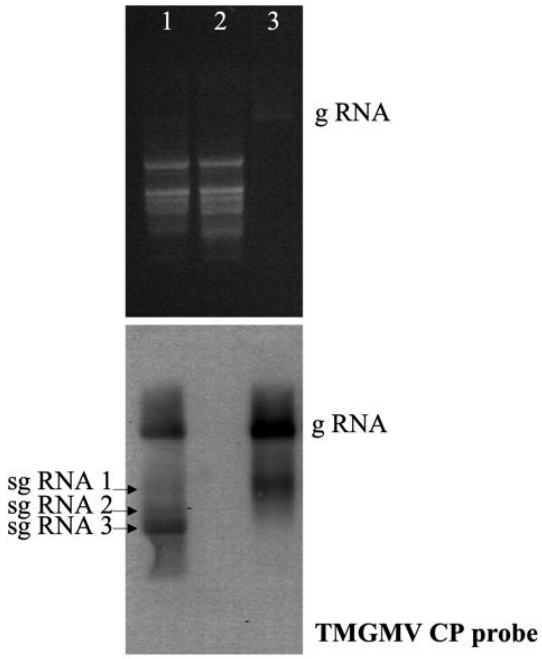


Figure 3. Electrophoresis of RNA preparations extracted from: mock-inoculated tobacco plant (track 1); tobacco plant infected by TMV-*PiLLP*-1056 at 14 dpi (track 2); purified preparation of TMV-*PiLLP*-1056 (track 3). Northern blot hybridization with a DIG-labelled DNA probe for TMGMV CP.

Further confirmation of the presence of TMV-*PiLLP*-1056 and absence of any transcript expressing GFP in plants was obtained by dot blot hybridization of sap extracted from infected leaves of *N. tabacum* collected at 14 dpi and hybridized with DIG-labeled DNA probes specific for TMGMV CP, GFP and 3'-5' *PiLLP* fragment. (Figure 4) Specific hybridization signals are present in leaf infected with TMV-*PiLLP*-1056 and back-inoculated with sap of a TMV-*PiLLP*-1056 infected leaf using TMGMV CP probe whereas the GFP expression is totally absent using GFP probe. Hybridization signals are visible in the same samples using 3'-5' *PiLLP* probe, confirming the presence of the recombinant viral vector.

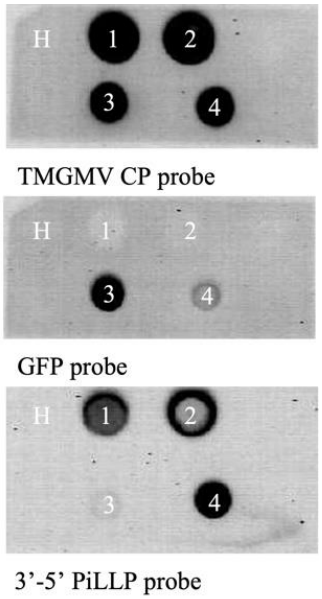


Figure 4. Dot blot hybridization with DIG-labeled DNA probes specific for TMGMV CP, GFP and 3'-5' *PiLLP* fragment on sap extracted from cv Samsun tobacco plants at 14 dpi with TMV-*PiLLP*-1056. H = sap from healthy tobacco plant; 1 = sap extracted from leaf infected with TMV-*PiLLP*-1056; 2 = sap extracted from leaf back-inoculated with sap of a TMV-*PiLLP*-1056 infected leaf; 3 = 2 ng of pTMV-GFP-1056; 4 = 2 ng of pTMV-*PiLLP*-1056.

TMV-*PiLLP*-1056 infection was transferred to a larger number of tobacco plants by rub-inoculation with sap extracted from plants at 14 dpi with the recombinant transcript and purified from systemically infected leaves collected at 21 dpi. Virus preparations were observed under the transmission Philips-Morgagni 282D electron microscope and compared to standard TMV particles that appeared swollen at one end, probably as consequence of the TMGMV CP that replaced the authentic TMV CP in the recombinant vector. Particles shorter than the standard TMV modal length were also present (Figure 5).

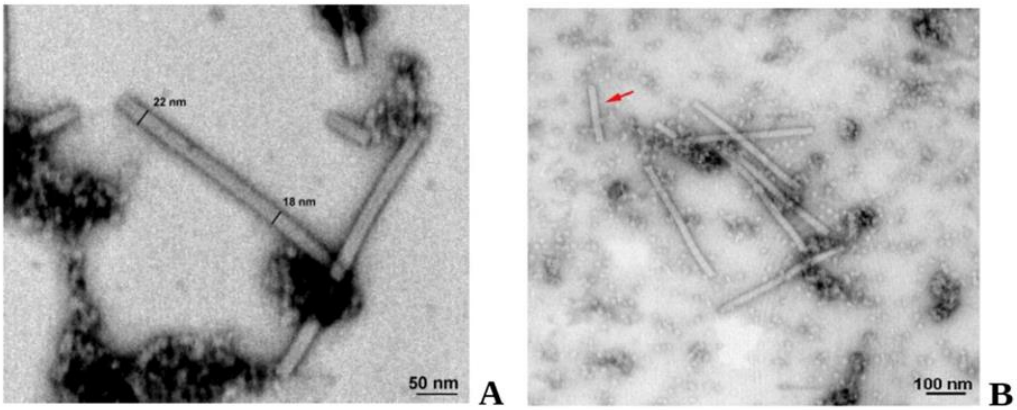


Figure 5. TMV-*PiLLP*-1056 particles purified from cv. Samsun tobacco plants showing an increase of particle diameter from 18 to 22 nm (panel A). In panel B, arrow points particles shorter than the standard TMV modal length (Courtesy Dr. Angelo De Stradis IPSP-CNR, Bari).

3.3. *Infectivity in* P. infestans

A purified preparation of the recombinant virus TMV-*PiLLP*-1056 was used for the inoculation of liquid cultures of *P. infestans* 96.9.5.1 (mating type A1) and 13_A2 (mating type A2). The presence of the recombinant virus in mycelia of *P. infestans* collected at 10 dpi was confirmed by RT-PCR targeting TMV-*PiLLP*-1056 replicase, which was not present in sgRNAs. The PCR yielded the expected amplified product of 241 bp which was not present in the two *P. infestans* mating types not exposed to inoculation with TMV-*PiLLP*-1056. Real-time qPCR with the primer pair targeting the *PiLLP* ORF showed a 3.5-fold and a 7-fold reduced expression of *PiLLP* ORF transcript in mating types A1 and A2, respectively, in samples collected at 10 dpi with TMV-*PiLLP*-1056 (Figure 6) compared to mock-inoculated controls. Melting curve of the housekeeping gene *ef1* and *PiLLP* ORF after qPCR showed a single peak suggesting the absence of aspecific amplified products. Overall, these results suggest that the recombinant vector TMV-*PiLLP*-1056 was able to enter mycelia of both the mating types of *P. infestans* and induce silencing of the *PiLLP* gene due to sequence identity between the *PiLLP* UTRs regions in the recombinant vector and the constitutive gene transcribed in *P. infestans*.

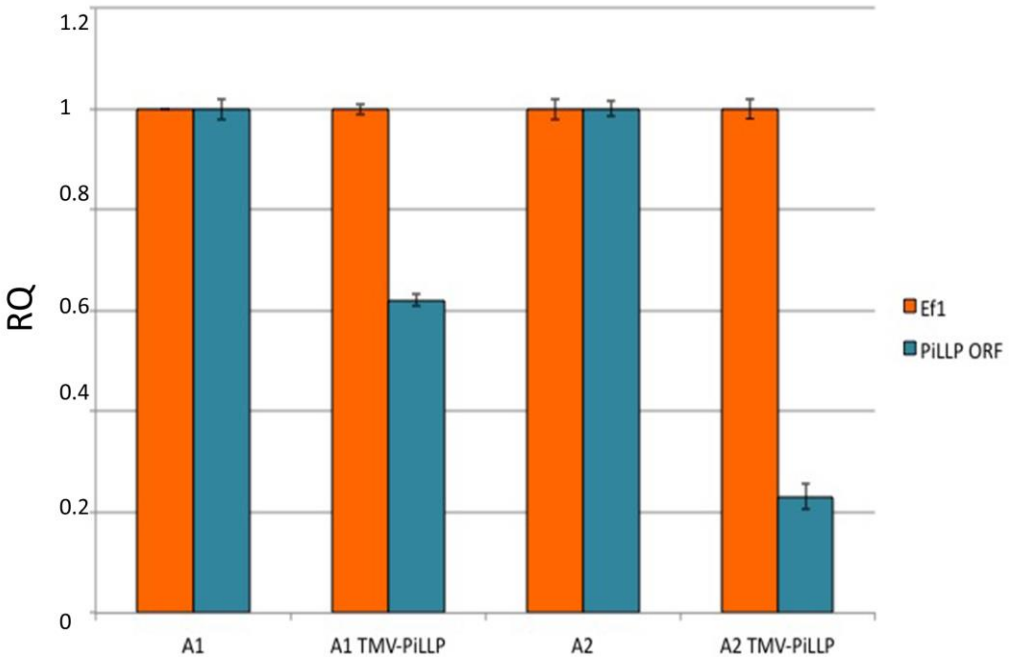


Figure 6. Relative abundance of *PiLLP* ORF transcripts (blue bar) compared with that of the housekeeping gene *Ef1* (orange bar) and mock-inoculated controls in samples of *P. infestans* A1 and A2 mating types at 10 dpi with TMV-*PiLLP*-1056.

Mycelia from liquid cultures of two mating types exposed to TMV-*PiLLP*-1056 infection (denoted A1 TMV-*PiLLP* and A2 TMV-*PiLLP*) and of the relative mock–inoculated controls were transferred to PA plates and grown under standard conditions. Compared with mock-inoculated control the growth of A1 TMV-*PiLLP* was slower than A1 WT whereas A2 TMV-*PiLLP* did not grow. The experiment was repeated twice with identical results.

We can speculate that due to unavailability of the A2 strain infected by TMV-*PiLLP*, variation in pathogenicity resulting from the partial downregulation of *PiLLP* transcript was estimated only for the A1 mating type infected by TMV-*PiLLP*. Detached tomato leaflets were inoculated *in vitro* with equivalent amounts of germinated sporangia of A1 TMV-*PiLLP* and WT A1 and A2 mating types as control. At 7 dpi, typical water-soaked

lesions developed on leaflets inoculated with A1 and A2 WT but those induced by A2 were larger. Lesions induced by A1 TMV-*PiLLP* were smaller in size than those produced by the A1 WT and with poor aerial mycelium (Figure 7). The colony diameter of A1 TMV-*PiLLP* cultures plated on PA was also smaller than that of the A1 WT (not shown).

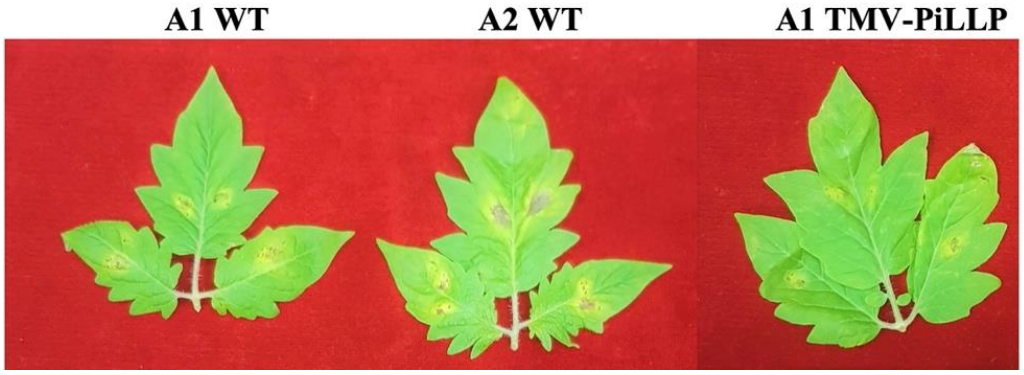


Figure 7. Lesions developed in detached leaflets of tomato cv UC82 inoculated with 30 µL sporangia suspension (1x10^5 sporangia/mL) of different inocula near the abaxial main vein.

The same approach was used to evaluate the *PiLLP* downregulation effect on oospore development. Wild type isolates and A1 TMV-*PiLLP* and A2 TMV-*PiLLP* isolates were mated on tomato leaflets with equivalent amounts of germinated sporangia for each isolate. Although the experiment was repeated twice, no oospore development was observed. Probably, this might be due to scarce α1 hormone produced by A1 mating type that inhibits A2 induction of oospore formation which generally secretes dose-dependently both α1 and α2 hormones [42,43]. Without α1 hormone, sporangia and asexual spores were produced otherwise are suppressed [42,43]. This mechanism, shared by *Phytophthora* heterothallic species as *P. nicotianae* [44], *P. capsici* [45], *P. cambivora* [46], and *P. infestans* [27], demonstrates that hormones are the universal mating factors among *Phytophthora* species [43]. Unfortunately, according to Tomura [43], certain strains did not produce hormones and the production time of A1 and A2 mating types were not precisely synchronous [43]. Although, if successful, knowledge reached by this study could offer new opportunities for the biological control of *P. infestans* as part of an integrated pest management strategy since Cao et al. [25] demonstrated that fungi could effectively be the living sources or reservoirs of plant viruses in nature as plant viruses can be maintained in fungal populations in nature. Based on these results, plant viruses can be naturally acquired in fungi during fungal colonialization of the plants [25], so our approach could represent a novel and green pest management technique.

4. Conclusions

The results of this work provide the first evidence for the downregulation of a constitutive gene of *P. infestans* using a recombinant vector based on a plus-sense RNA plant virus suggesting a therapeutic use of the RNAi in the control of threatening pathogens like *P. infestans*. Furthermore, interference with the sexual reproductive cycle by inhibiting or limiting the production of oospores represents a promising strategy. A partial downregulation of the transcription of *PiLLP* gene involved in oospore formation has been obtained for both tested mating types. The reasons for the partial success need to be addressed. Interestingly, such downregulation was significantly more evident in the A2 mating type and apparently inhibits its growth on PA plates. Although results of this study were not further corroborated by the expected effect on oospore formation, the fully exploitation of the approach and future research should primarily focus on the transmissibility of the *PiLLP*-downregulation and fully silenced phenotype to WT isolates during the sexual reproduction.

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