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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 plant wilt and high management costs mainly due to wide fungicide applications, alternative eco-sustainable control strategies are needed. RNA interference (RNAi) is a powerful tool for gene function studies that can be accomplished by constitutive transformation or transient expression such as virus-induced gene silencing (VIGS) experiments. VIGS makes use of viruses to deliver sequences 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. For this purpose, the gene coding for the green fluorescent protein (GFP) present in the TMV-GFP-1056 vector has been replaced with an antisense construct obtained by fusion PCR of the *PiLLP* 5′-UTR and 3′-UTR sequences. Here, we show that RNAi can be expressed in the A1 mating type of *P. infestans* strain 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 A1 mating type had slower colony growth and a diminished virulence in detached tomato leaflets. This seems to be the first evidence of a constitutive gene downregulation of *P. infestans* using a recombinant vector based on a plus-sense RNA plant virus.

Keywords: A1; A2; mating type; *P. infestans*; recombinant virus; VIGS

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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]. Similar to true fungi, oomycetes include threatening plant pathogens such as 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 and high management costs mainly due to up to ten sprays of fungicide applications per season [3].

With the aim of reducing pesticide applications and favoring disease control by natural pathogens 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 favoring pathogen penetration or fulfilling its metabolic or structural needs 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 the mating type A1 of *P. infestans* was distributed worldwide and reproduced asexually allowing for some success in its management [13]. The emergence and worldwide distribution of the aggressive A2 mating types, enabled sexual cycles, induced genetic variations and gene transfer in populations of *P. infestans*, produced isolates with increased virulence, and created new challenges for late blight management [14]. Integrated pest management (IPM) strategies could provide more durable and environment friendly crop protection against late blight [15]. In recent years, research has 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 controlling pathogenic attacks and 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 the nonpersistent acquisition of plant viruses by plant-associated fungi might 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. For example, the PuM90, a stage-specific RNA-binding protein, which regulates oospore formation in *Pythium*

ultimum, has been knocked out by the CRISPR/Cas9 approach. The resulting PuM90-knockout mutants were significantly defective in oospore formation [29]. In *P. infestans*, regulatory mechanisms involved in sexual reproduction are scarcely known except for a loricrin-like protein (LLP), which is strongly overexpressed during oospore formation [30].

PiLLP, a *P. infestans* ortholog of the plant LLP, was silenced by protoplast transformation. Compared with the wild-type (WT), 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. The TMV RNA genome 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 [32]. TMV-GFP-1056, one of the most used TMV-based vectors, 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 [33–35]. The TMV sgRNA promoter directs the expression of any gene inserted downstream between the *Pac*I and *Xho*I cloning sites. For the purpose of this study, the gene coding for GFP in the TMV-GFP-1056 vector has been replaced with an antisense construct obtained by fusion PCR of the *PiLLP* 5′-UTR and 3′-UTR sequences. The resulting TMV-*PiLLP*-1056 vector did enter, replicate, and persist in mycelia of *P. infestans* where it induced the partial downregulation of the *PiLLP* transcript. Compared with the WT, the *PiLLP*-silenced A1 mating type strain 96.9.5.1 had slower colony growth and a diminished virulence in detached tomato leaflets. This seems to be the first evidence of a constitutive gene downregulation of *P. infestans* using a recombinant vector based on a plus-sense RNA plant virus.

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 in a dark chamber on pea agar (PA) [36] medium at 18 ± 1 °C. 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 from 20-day-old cultures and washed with 7 mL of distilled water, and the mycelia were gently rubbed with a glass rod. Zoospores were released after incubation of the sporangia suspension at 4 ± 1 °C for 2 h and then at room temperature for 1 h. To distinguish between the two mating types, each mycelium was grown in liquid culture, and the harvested material was filtered and air-dried on a sterile Whatman No.1 filter paper (Whatman, Maidstone, UK), before being freeze-dried at −20 ± 1 °C. The DNA was extracted from the mycelia with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) after homogenization with Tissue Lyser II (Qiagen, Hilden, Germany), as manufacturer's recommendation. DNA preparations were subjected to PCR using primer pairs S1a/S1b [37] and PHYB-1/PHYB-2 [38] for mating type A1 and A2, respectively, and amplified with DreamTaq Hot Start DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). All PCRs were performed in a T100 Thermal Cycler (Bio-Rad, Bio-Rad Laboratories, Hercules, CA, USA). 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 [39,40] using 61 °C as primer annealing temperature. Then, PCR products were digested with *Hae*III (New England Biolabs, Ipswich, MA, USA). 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 GelRed® (Biotium, Biotium Inc., Fremont, CA, USA).

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 suspended 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, MA, USA). 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) annealed at 54 °C and 59 °C, respectively. Primers were designed to introduce a restriction site at 5′ for the *XhoI* enzyme and a restriction site at 3′ for the *PacI* enzyme to join the two PCR fragments. The size of 201 bp and 126 bp expected for the two amplified PCR products was estimated by electrophoresis in 2% low melting agarose gel in TBE buffer after GelRed® staining. Products were purified using Agarose Gel DNA Extraction Kit (Roche, Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. Fusion PCR was carried out using Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen™, Waltham, MA, USA) using 1:1 ratio and 5′UTR for/3′UTR rev primers annealed at 50 °C. 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, WI, USA) and inserted in anti-sense orientation into the *XhoI* and *PacI* sites of the pTMV-GFP-1056 vector [41]. To obtain the recombinant plasmid pTMV-*PiLLP*-1056, the plasmid pTMV-GFP-1056 was used as backbone. Briefly, both the fused fragment and the vector were double digested with *XhoI* and *PacI* (New England Biolabs, Ipswich, MA, USA), loaded on 2% low melting agarose gel in TBE buffer, stained with GelRed®, and eluted as described above. Ligation of the 5′UTR + 3′UTR joined fragments to the viral vector pTMV-1056 deprived of GFP was accomplished using T4 DNA ligase (Promega, Madison, WI, USA) followed by transformation of TOP10 *E. coli* competent cells (Invitrogen™, Waltham, MA, USA). 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 Figure S1. All primers used in this work are listed in Table 1.

2.3. Plant Inoculation

Inocula of TMV-*PiLLP* were prepared in tobacco (*Nicotiana tabacum* L.) cv. Samsun by rub-inoculation of a biologically active transcript synthesized from the *KpnI*-linearized pTMV-*PiLLP*-1056 plasmids with T7 RNA polymerase and the mMessage mMachine kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA), following the protocol of the manufacturer. To perform the assays, young plants were transplanted at cotyledon stage and used for the mechanical inoculation after two weeks. The second and third 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 controls, whereas tobacco plants cv. Samsun inoculated with 100 mM (Na_2-K) phosphate buffer, pH 7.2 served as mock-inoculated controls. All the plants were grown at 22 ± 2 °C with a 16-hour light/8-hour dark photoperiod and daily monitored for symptom appearance. To ensure virus maintenance, systemically infected leaves of tobacco were collected at 10 days post inoculation (dpi), and the plant sap was extracted in 100 mM (Na_2-K) phosphate buffer, pH 7.2 for rub-inoculation onto a new stock of plants. Viral particles of TMV-*PiLLP*-1056 were purified from systemically infected tobacco leaves collected at 10 dpi as described by Lot et al. [42] 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 assess the correct insertion of the 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). Two µg of total RNA preparations were spotted onto 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 GelRed®, 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.

Table 1. Primer pairs used for amplifications of the genes in this study.

Primer Name	Sequence	Size (bp)	Ta	Purpose	Ref.
S1a S1b	5′-AGGATTTCAACAA-3′ 5′-TGCTTCCTAAGG-3′	1250	50	Mating type identification	[37]
PHYB-1 PHYB-2	5′GATCGGATTAGTCAGACGAG-3′ 5′-GCGTCTGCAAGGCGCATTTT-3′	347	59	Mating type identification	[38]
W16-1 W16-2	5′-AACACGCACAAGGCATATAAATCTA-3′ 5′-GCGTAATGTAGCGTAACAGCTCTC-3′	557	61	Mating type identification	[39,40]
5′UTR for 5′UTR rev	5′-TCTCCTCGAGTCCTCCTCTCTGCGT-3′ 5′-CGACAATATTCCATAACTGCTAA GGTGGGGCTGTAA-3′	201	54	5′UTR region of *PiLLP* cloning	This study
3′UTR for 3′UTR rev	5′-TATGGAATATTGTCGAGT ACAGCAACGAGGCTTCGA-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 REP rev	5′-CAGTGCATATCGGCATTGTC-3′ 5′-CGTGACTCCTCTTCCGTCTC-3′	241	50	TMV replicase of *P. infestans* detection	
*Pi*EF for *Pi*EF rev	5′-ACTCCAAGAACGACCCTGCTAAGGCAACC-3′ 5′-TCCGACGGCTCGAGGATGACCATGCAG-3′	239	>60	Real Time PCR of ef1 of *P. infestans*	[31]
*Pi*ORF for *Pi*ORF rev	5′-CAGTCTCCCGTCACCAGTCC-3′ 5′-GGCAGTCGGCAATACCACAT-3′	~300	>60	Real Time PCR of *PiLLP*	

From left to right, each column shows primer name and related sequence, amplicon size (bp), annealing temperature (Ta), use purpose, and bibliographical reference (Ref.).

2.4. Infection in P. infestans

The infection of the *P. infestans* A1 and A2 mating types was performed with 50 µL of purified preparations of TMV-*PiLLP* (1 µg/mL) added to the sporangia growing in 100 mL of Pea Broth (PB) supplemented with 100 mg/mL ampicillin (Sigma-Aldrich, Merck KgaA, St. Louis, MO, USA). Fungal cultures inoculated only with 50 µL of 50 mM NaCl, 30% glycerol solution were used as 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 reported above. RT-PCR was used to detect the TMV-*PiLLP* in mycelia of *P. infestans*. Reactions were carried out with REP for/rev primer pair using 50 °C as annealing temperature to amplify the replicase gene of TMV-1056. Purity and size of the amplified products was estimated as above. The comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method corrected for PCR efficiencies (qPCR) was used to evaluate the relative abundance of *PiLLP* transcripts in the two mating types of *P. infestans* exposed to TMV-PiLLP-1056 inoculum. Reactions were carried out

in an Applied Biosystems StepOne™ Real-Time PCR System apparatus using the Fast SYBR®Green Master Mix (Applied Biosystems, Waltham, MA, USA). Samples were run in triplicate, and reactions were set up in a total volume of 10 µL using 50 ng of cDNA and 250 nM each of for and rev primers pair. The constitutive elongation factor 1 (*ef1*) gene of *P. infestans* (PiEF), amplified with the PiEF for/rev primers pair, was used as housekeeping gene for *PiLLP* ORF gene transcripts normalization amplified by using the PiORF for/rev primer pair [31]. The cycling conditions were: 95 °C for 20 s, 40 cycles at 95 °C for 3 s, and 60 °C for 30 s. Specificity of the qPCR products was confirmed by analysis of the melting curves, which showed a single Tm peak obtained for each primer combination. Results were evaluated using the StepOne™ Software v2.3. PCR efficiency for each amplified fragment was derived from the slope of the regression line obtained by interpolating values from triplicates of five serial 1:2 dilutions of input cDNA amount and the relative Ct values using StepOne software (Applied Biosystems, Waltham, MA, USA). 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-week-old *P. infestans* WT and TMV-*PiLLP*-1056 challenged mycelia cultured on PA in optimal growth conditions were used as inoculum for tomato leaf infection. For this purpose, fungal plates were flooded with 5 mL Pea Broth and scraped with a glass rod to release sporangia. Each resulting suspension was collected and used for the inoculation of detached leaflets of four-week-old tomato plants cv UC82. Leaflets were previously surface-sterilized with sodium hypochlorite solution (2% vol/vol), rinsed in sterile distilled water, aseptically air-dried, and transferred to sterile moist filter paper placed in Petri dishes (90 mm Ø). Each leaflet was inoculated on abaxial side with 30 µL of sporangia suspension (1×10^5 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 6.1 (Bio-Rad, Hercules, CA, USA).

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, A1 mating type was confirmed by the amplification of a product of about 1.25 kbp, whereas no amplification was observed for the A2 mating type with the same primer pair (Figure 1a). Amplification with PHYB-1/PHYB-2 primer pair aimed to target PHYB marker and resulted in a 347 bp fragment obtained only for the A2 mating type whereas no amplification product was obtained for the A1 mating type (Figure 1b). From both the *P. infestans* isolates, amplification with primer pair W16–1/W16–2 produced amplified products of 557 bp [39,40]. After digestion with *Hae*III endonuclease, two fragments of approx. 557 bp and 457 bp and one fragment of approx. 457 bp were observed, for the A1 and A2 mating types, respectively [39,40].

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 5′UTR fragment and the 126 bp of 3′UTR fragment 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 the ORF for GFP expression (Figure S1). The correct insertion and orientation of the 5′UTR + 3′UTR joined fragment were confirmed by sequencing the region of interest in both directions. Tobacco cv Samsun was used to biologically assess the successful transformation of the original TMV-GFP-1056 into TMV-*PiLLP*-1056 by the GFP screening, sequencing,

and recombinant vector purification to validate the insertion of the *PiLLP* fragment and to prepare the inoculum, respectively. Thus, the recombinant transcript of TMV-*PiLLP*-1056 was synthesized in vitro, and its infectivity was tested in healthy plants of tobacco cv. Samsun. A transcript of TMV-GFP-1056 synthesized from the original pTMV-GFP-1056 was used as control, whereas tobacco plants cv. Samsun inoculated with 100 mM (Na_2-K) phosphate buffer, pH 7.2 served as mock-inoculated controls. Disease symptoms consisting in pale yellow discolorations along the main veins (Figure 2a) were observed on tobacco leaves at 7 dpi. These symptoms appeared also in the newly formed leaves and persisted up to 30 dpi. Possible contaminants of the original GFP sequence still present in the recombinant plasmid were excluded by the absence of fluorescence when plants were irradiated with a UV lamp (UVP, Upland, CA, USA) (Figure 2b), confirming the complete replacement of GFP with the 5′UTR + 3′UTR *PiLLP* fragment in the TMV-*PiLLP*-1056 vector. GFP fluorescence was clearly observed in plants rub-inoculated with the recombinant transcript TMV-GFP-1056 (Figure 2c), while mock-inoculated leaves did not show disease symptoms (Figure 2d) or GFP fluorescence when exposed to UV light (Figure 2e).

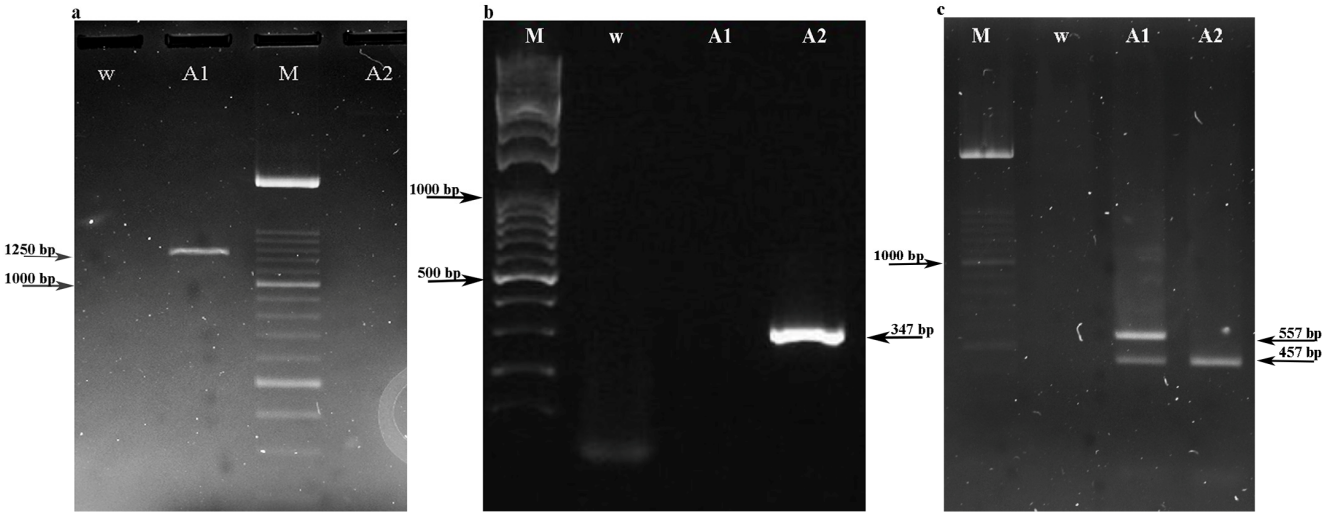

Figure 1. Detection of PCR products of *P. infestans* A1 and A2 mating type obtained with mating-type-specific primers. (**a**) product of 1250 bp (pointed by higher arrow) obtained from the A1 mating type with S1a/S1b primers; (**b**) product of 347 bp (pointed by lower arrow) obtained from the A2 mating type with PHYB-1/PHYB-2 primers; (**c**) products of 557 bp and 457 bp (pointed by two lower arrows) obtained from the A1 and A2 mating types with W16-1/W16-2 primers, followed by *Hae*III digestion. A1 = DNA extracted from WT A1 mating type; A2 = DNA extracted from WT A2 mating type; M = HyperLadder™ 100 bp (Bioline, Meridian Bioscience Inc., Cincinnati, OH, USA.); w = negative control (no DNA).

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). According to the TMV expression strategy and the pTMV-*PiLLP*-1056 map shown in Figure S1, sgRNA 1 has been synthesized by the TMV MP promoter; it has an estimated size of 2.4 kb and contains sequences of TMV-MP, 3′-5′ *PiLLP*, and TMGMV CP; sgRNA 2 has been synthesized by TMV CP promoter; it has an estimated size of 1.6 kb and contains sequences of 3′-5′ *PiLLP* and TMGMV CP; sgRNA3 has been synthesized by the TMGMV CP promoter; it has an estimated size 0.48 kb and contains the sequence of TMGMV CP. All sgRNAs share the sequence coding for the CP of TMGMV, which thus can be revealed by the 643 bp-long TMGMV CP probe. However, the three sequences are under the control of different promoters and the relative abundance of each sgRNA may vary according to the efficiency of its transcription, which was not evaluated.

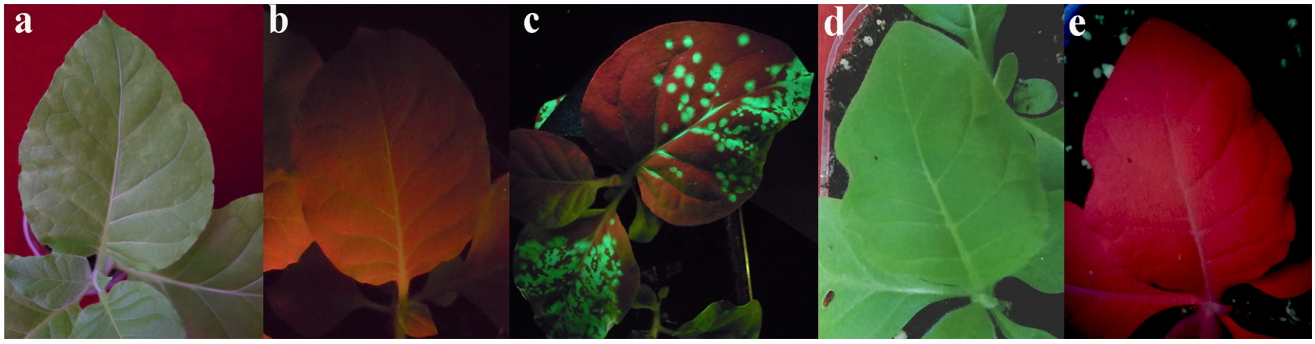

Figure 2. Exposure of tobacco plants to UV lamp to check the replacement of GFP with 5′UTR + 3′UTR *P. infestans* ortholog of plant loricrin-like protein (*PiLLP*) in TMV-*PiLLP*-1056 vector. (**a**) Disease symptoms consisting in pale yellow discolorations along the main veins in a tobacco leaf systemically infected with TMV-*PiLLP*-1056 not exposed to UV light. (**b**) No residual 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, (**d**) tobacco leaf of mock-inoculated plant used as negative control not exposed, and (**e**) exposed to UV light.

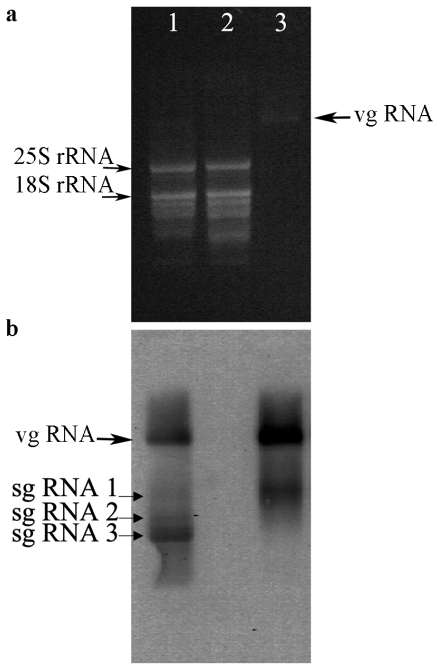

Figure 3. Detection of TMV-*PiLLP*-1056 RNA by northern blot hybridization with a 643-bp DIG-labeled DNA probe for TMGMV CP. (**a**) RNA gel electrophoresis and (**b**) northern blot hybridization of: lane 1 = 2 µg of total RNA preparation extracted from tobacco plant infected by TMV-*PiLLP*-1056 at 14 dpi; lane 2 = 2 µg of total RNA preparation extracted from mock-inoculated tobacco plant used as negative control; lane 3 = 0.2 µg of RNA preparation extracted from purified preparation of TMV-*PiLLP*-1056 used as control. Arrows indicate the positions of tobacco leaf 25S and 18S rRNAs of 3.7 and 1.9 kb, respectively, (**a**) and TMV genomic RNA (vgRNA) and subgenomic RNA1, RNA2, RNA3 (sgRNAs) RNAs (**b**).

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 tobacco leaves collected at 14 dpi and hybridized with DIG-labeled DNA probes specific for the TMGMV CP, GFP, and 3′-5′ *PiLLP* fragment (Figure 4). Specific hybridization signals were observed in leaf infected with TMV-*PiLLP*-1056 and in a leaf back-inoculated with sap of a TMV-*PiLLP*-1056 infected leaf using TMGMV CP probe whereas the GFP

expression was absent using GFP probe. Hybridization signals were observed in the same samples using 3′-5′ *PiLLP* probe, confirming the presence of the recombinant viral vector.

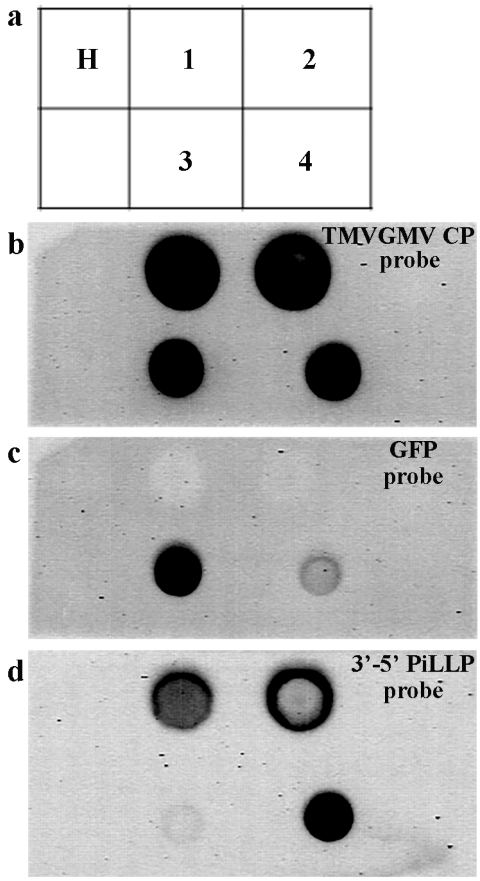

Figure 4. Detection of TMV-*PiLLP*-1056 presence in sap extracted from cv Samsun tobacco plants with DIG-labeled DNA specific probes. Dot blot hybridization scheme (**a**): H = sap extracted from healthy tobacco plant; 1 = sap extracted from leaf infected with TMV-*PiLLP*-1056 at 14 dpi; 2 = sap extracted from leaf back-inoculated with sap of a TMV-*PiLLP*-1056 infected leaf; 3 = 2 ng of recombinant plasmid pTMV-GFP-1056 served as positive control; 4 = 2 ng of recombinant plasmid pTMV-*PiLLP*-1056 used as positive control. Results of dot blot hybridization with (**b**) TMGMV CP, (**c**) GFP, and (**d**) 3′-5′ *PiLLP* probes. Non-specific hybridization signals are visible in spot 4 hybridized with GFP probe and in spot 3 hybridized with 3′-5′ *PiLLP* probe.

TMV-*PiLLP*-1056 infection was transferred to a larger number of tobacco plants by rub-inoculation with sap extracted from plants infected with the recombinant transcript at 14 dpi. TMV-*PiLLP*-1056 was purified from systemically infected leaves collected at 21 dpi. Virus preparations were viewed under the transmission Philips-Morgagni 282D electron microscope and compared to standard TMV particles; TMV-*PiLLP*-1056 particles 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).

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* A1 and A2 mating types. 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 (see pTMV-*PiLLP*-1056 map in Figure S1). The PCR yielded the expected amplified product of 241 bp that was observed in both *P. infestans* mating types exposed to the inoculation of the TMV-*PiLLP*-1056,

while no bands were amplified in the mock-inoculated controls (Figure 6a). RT-qPCR with the primer pair targeting the *PiLLP* ORF showed a 3.5-fold and a seven-fold reduced expression of *PiLLP* ORF transcript in mating types A1 and A2, respectively, infected with TMV-*PiLLP*-1056 at 10 dpi (Figure 6b) compared to A1 and A2 mock-inoculated controls, used as reference sample for gene expression normalization. Elongation factor 1 alpha (*EF-1a*) was used as housekeeping gene for target genes normalization. Melting curve of the housekeeping gene *ef1* and *PiLLP* ORF after RT-qPCR showed a single peak, suggesting the absence of non-specific 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*.

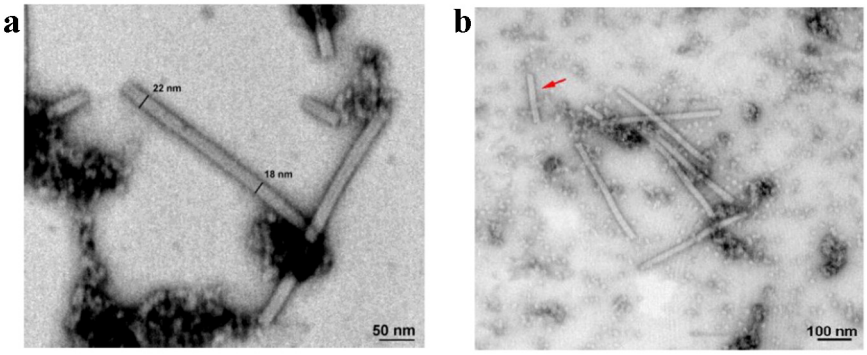

Figure 5. Electron microscope pictures of TMV-*PiLLP*-1056 and TMV virus preparations. TMV-*PiLLP*-1056 particles purified from Samsun tobacco plants showing an increase in particle diameter from 18 to 22 nm (**a**). In (**b**), arrow points particles shorter than the standard TMV modal length (Courtesy Dr. Angelo De Stradis IPSP CNR, Bari).

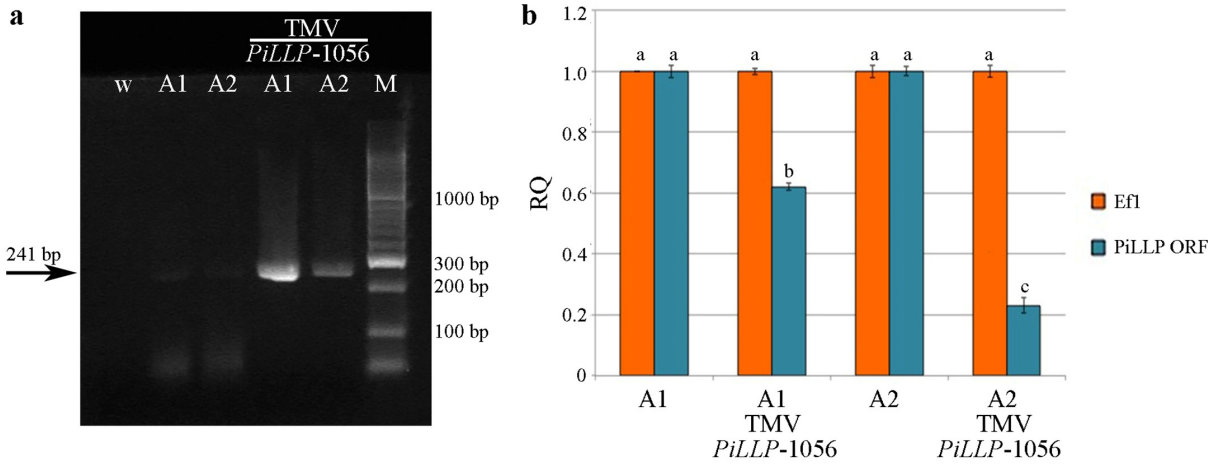

Figure 6. Amplification of a fragment of TMV-*PiLLP*-1056 replicase and *PiLLP* ORF in mycelia of *P. infestans* A1 and A2 mating types at 10 dpi with of TMV-*PiLLP*-1056. (**a**) PCR assay of TMV-*PiLLP*-1056 replicase yielded the expected amplified product of 241 bp (pointed by arrow) in infected mycelia compared to mock-inoculated controls; M = HyperLadder™ 100 bp (Bioline, Meridian Bioscience Inc., Cincinnati, OH, USA); w = negative control (no DNA); (**b**) Relative abundance of *PiLLP* ORF transcripts (blue bar) compared with that of the housekeeping gene *Ef1* (orange bar) in infected mycelia compared to mock-inoculated controls. RQ values ± St.dev. were calculated as mean of three biological replicates obtained from RT-qPCR. Letters indicate statistically significant differences ($p \leq 0.05$, Tukey post hoc test), for each genotype. The mock-inoculated condition was used as reference mycelia for gene expression normalization. Elongation factor 1 (*Ef1*) was used as housekeeping gene for target genes normalization.

Mycelia from liquid cultures of the two mating types exposed to TMV-*PiLLP*-1056 infection (denoted A1 TMV-*PiLLP* and A2 TMV-*PiLLP*), and 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 mock-inoculated control, whereas A2 TMV-*PiLLP* did not grow. The experiment was repeated twice with identical results.

Due to unavailability of the A2 TMV-*PiLLP*-1056, the variation in pathogenicity resulting from the partial downregulation of *PiLLP* transcript was estimated only for the A1 TMV-*PiLLP*. Infectivity/pathogenicity of A1 TMV-*PiLLP* was tested using detached tomato leaves instead of tobacco, since tomato is one of the most economically important hosts of *P. infestans*. Thus, detached tomato leaflets were inoculated in Petri dishes with equivalent amounts of germinated sporangia of infected A1 TMV-*PiLLP* and mock-inoculated A1 and A2 mating types as control. At 7 dpi, typical water-soaked lesions developed on leaflets inoculated with A1 and A2 mock controls, where the lesions observed in the A2 mating type were larger than in A1. Moreover, lesions induced by A1 TMV-*PiLLP* were smaller in size than those produced by the A1 mock controls and with poor aerial mycelium (Figure 7a,b). The colony diameter of A1 TMV-*PiLLP* cultures plated on PA was also smaller than that of the A1 WT.

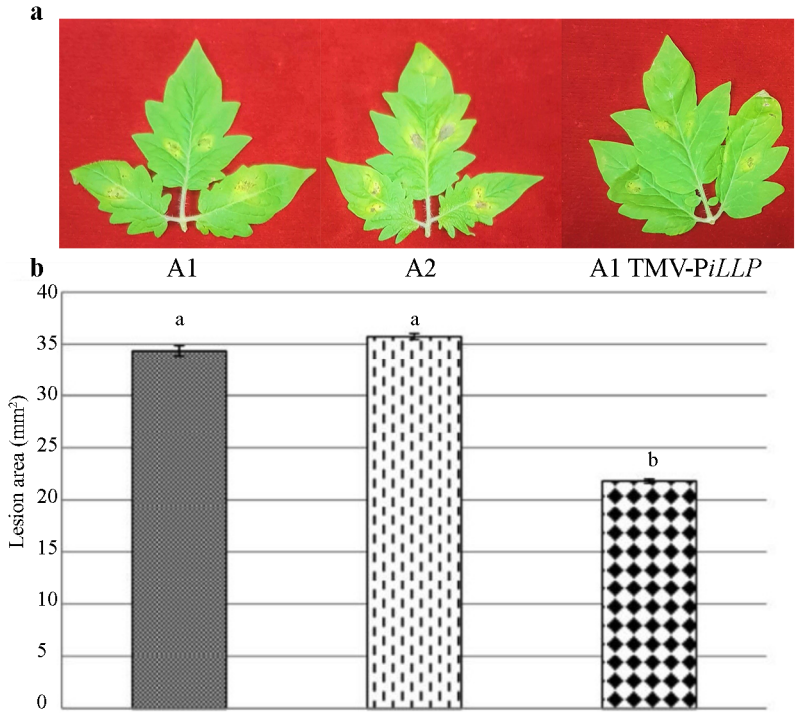

Figure 7. Lesions developed in detached leaflets of tomato cv UC82 inoculated with 30 µL sporangia suspension (1×10^5 sporangia/mL) of different inocula near the abaxial main vein. Lesions developed in detached leaflets of tomato cv UC82 inoculated with A1, A2 and A1 TMV-*PiLLP* (**a**). Sizes of lesions in mm^2 induced by A1 (grey bar), A2 (dotted bar) and A1 TMV-*PiLLP* (checkered bar) inocula (**b**). Vertical bars ± St.dev represent the mean of three biological replicates. Letters indicate statistically significant differences ($p \leq 0.05$, Tukey post hoc test), for each genotype. A1 = lesion area induced by germinated sporangia of the A1 mating type; A2 = lesion area induced by germinated sporangia of the A2 mating type; A1 TMV-*PiLLP* = lesion area induced by germinated sporangia of the A1 mating type infected with TMV-*PiLLP*-1056.

The same approach was used to evaluate the *PiLLP* downregulation effect on oospore development. WT isolates, infected 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 [43,44]. With α1 hormone, sporangia and asexual spores were produced and otherwise are suppressed [43,44]. This mechanism, shared by *Phytophthora* heterothallic species as *P. nicotianae* [45], *P. capsici* [46], *P. cambivora* [47], and *P. infestans* [27], demonstrates that hormones are the universal mating factors among *Phytophthora* species [44]. Unfortunately, according to Tomura et al. [44], certain strains did not produce hormones, and the production time of A1 and A2 mating types were not precisely synchronous [44]. Generally, the *P. infestans* life cycle is suited to its dissemination. Although numerous infections can be caused by asexual sporangia, these are short-lived such as hyphae; instead, oospores can persist for years in soil, surviving freezing and fungicides entailing as an important source of inoculum season by season. In addition, since during biotrophic stages, *P. infestans* can only feed from living cells of the host, often disease diagnosis is delayed. Bypassing plant-based resistance mechanisms, systemic chemicals fungicides remain a key-tool against late blight but are not economically and environmentally sustainable due to the high number of yearly needed applications. In this scenario, aimed at controlling sexual cycle of *P. infestans*, this molecular approach could be a long-term and effective solution to face late blight blocking its sexual reproduction over the years and obtaining positive feedback from farmers and stakeholders [48].

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 such as *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 as apparently inhibited its growth on PA plates. Although results of this study were not further corroborated by the expected effect on oospore formation, the full 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. If successful, this approach would benefit of the demonstration by Cao et al. [25] that the nonpersistent acquisition of plant viruses by fungi may commonly occur in nature for its exploitation in the IPM of *P. infestans*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9030360/s1, Figure S1: Construction of pTMV-PiLLP-1056 vector to express the 5′UTR + 3′UTR of PiLLP in antisense orientation, under the TMV CP sgRNA promoter.

Author Contributions: Conceptualization, R.L. and T.M.; methodology, R.L. and T.M.; validation, R.L., A.M., R.S. and T.M.; formal analysis, R.L., A.M. and R.S.; investigation, R.L. and T.M.; data curation, R.L.; writing—original draft preparation, R.L. and T.M.; writing—review and editing, R.L., A.M., R.S. and T.M.; All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available in the article or Supplementary Material.

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