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Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections

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Abstract

Background: Double-stranded RNA (dsRNA) is a potent initiator of gene silencing in a diverse group of organisms that includes plants, *Caenorhabditis elegans*, *Drosophila* and mammals. We have previously shown and patented that mechanical inoculation of *in vitro*-transcribed dsRNA derived from viral sequences specifically prevents virus infection in plants. The approach required the *in vitro* synthesis of large amounts of RNA involving high cost and considerable labour.

Results: We have developed an *in vivo* expression system to produce large amounts of virus-derived dsRNAs in bacteria, with a view to providing a practical control of virus diseases in plants. Partially purified bacterial dsRNAs promoted specific interference with the infection in plants by two viruses belonging to the tobamovirus and potyvirus groups. Furthermore, we have demonstrated that easy to obtain, crude extracts of bacterially expressed dsRNAs are equally effective protecting plants against virus infections when sprayed onto plant surfaces by a simple procedure. Virus infectivity was significantly abolished when plants were sprayed with French Press lysates several days before virus inoculation.

Conclusion: Our approach provides an alternative to genetic transformation of plant species with dsRNA-expressing constructs capable to interfere with plant viruses. The main advantage of this mode of dsRNA production is its simplicity and its extremely low cost compared with the requirements for regenerating transgenic plants. This approach provides a reliable and potential tool, not only for plant protection against virus diseases, but also for the study of gene silencing mechanisms in plant virus infections.

Background

Posttranscriptional gene silencing (PTGS) in plants is a homology-dependent RNA degradation system designed to act as a natural defence barrier against virus infections [1]. Mechanistically similar processes are known in fungi (quelling, [2]) and animals (RNA interference, [3]) and are considered a surveillance mechanism against mobilization of transposons [4,5]. Moreover, it has been widely conjectured that RNAi may also serve as an antiviral system in vertebrates [6,7]. It is even possible that these related processes are part of an ancient, RNA-based regulatory network aimed at regulating gene expression [8,9]. In addition, induction of RNA silencing in different organisms can be activated by exogenously supplied double-stranded RNA (dsRNA) [10,11]. In this sense, RNAi has become a powerful genetic tool for selectively silencing gene

expression in many eukaryotes [12]. In *Drosophila melanogaster*, the RNase III-like enzyme Dicer cleaves the dsRNA silencing trigger into RNA species of approximately 21 to 25 nucleotides. These small interfering RNAs (siRNAs) can act as guide sequences for specific cleavage of homologous RNAs [13,14]. The presence of siRNAs and core PTGS proteins in plants [15,16] and the existence of Dicer homologs in *Arabidopsis* [17] suggest that these mechanisms are conserved across kingdoms. Furthermore, wheat germ extracts have recently been shown to recapitulate *in vitro* many aspects of RNA silencing in animals [18]. Thus, the PTGS degradation machinery in plants would lead to the elimination of the viral RNA, as long as the dsRNA trigger contains sequences homologous to the invading virus.

We have previously shown that dsRNA derived from viral sequences can interfere with virus infection in a sequence-specific manner by directly delivering *in vitro*-transcribed dsRNA into leaf cells by mechanical inoculation [19]. Moreover, several characteristic hallmarks of PTGS were associated with viral interference mediated by transiently expressed hairpin RNA homologous to the virus [20]. These findings suggest that homologous dsRNA could serve as protective molecules against virus infections, provided that inexpensive and effective means of production and delivery of adequate interference products onto plants surfaces were previously developed. Obviously, interference with virus infection mediated by *in vitro*-transcription products or via *Agrobacterium*-mediated transient expression of dsRNA is limited to fundamental studies.

In *Caenorhabditis elegans*, RNAi can be achieved by feeding worms *Escherichia coli* expressing dsRNA corresponding to a specific gene [21]. *E. coli* strain BL21(DE3) was used in the initial development of bacteria-induced RNAi. However, the specific interference was limited in penetrance and expressivity and this was due to the production of variable amounts of partially degraded dsRNA [22]. Exhaustive cleavage of dsRNA by *E. coli* RNase III leads to duplex products averaging 12–15 bp in length [23]. These short dsRNA are unable to trigger an RNAi response in mammalian cells [24]. Afterwards, it was found that a bacterial strain, HT115(DE3), lacking the dsRNA-specific RNase III could produce high levels of specific dsRNA and that this strain could effectively trigger strong and gene-specific interference responses when fed to *C. elegans* [22]. These attributes make HT115(DE3) a promising strain for preparing massive amounts of viral dsRNA *in vivo*. Here we report the use of an RNase III-deficient strain of *E. coli* to produce virus-derived dsRNA and the application of a bacterial crude preparation that could interfere with virus infection in plants. In this study, we targeted two viruses, *Pepper mild mottle virus* (PMMoV) and *Plum pox virus* (PPV), that represent two of the main widespread groups of plant viruses in nature. We reasoned that, if successful, our studies would additionally contribute a reliable and simple technology for specific gene silencing in plant virus infections.

Results

Production of virus-derived dsRNA using an RNase-deficient E. coli *strain*

E. coli strain HT115(DE3) was chosen to take advantage of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase gene contained within a stable insertion of a modified lambda prophage λ DE3 [22]. This strain is deficient for RNase III, an enzyme that normally degrades a majority of dsRNAs in the bacterial cell, thereby improving the accumulation of extended dsRNA duplexes compared to BL21(DE3), an RNase III-expressing strain [22].

For comparison, HT115 and BL21 strains were transformed with a single plasmid, pGEM/IR 54, designed to express a hairpin RNA containing PMMoV replicase gene sequences (PMMoV IR 54) under the control of the T7 promoter. Upon induction with 0.4 mM IPTG, dsRNA produced in these bacteria was analyzed by preparation of total nucleic acid followed by treatment with RNase A to remove single-stranded RNA (Fig. 1). We found that the RNase III-deficient HT115 strain accumulated substantial levels of PMMoV IR 54, as judged by comparison with migration in gels of *in vitro*-transcribed PMMoV IR 54 and by hybridization analysis (see below). We estimated that the average yield of PMMoV IR 54 produced in cells was 4 μg per ml culture, based on ethidium bromide staining of an *in vitro*-synthesized RNA used as a mass marker. Accumulation of PMMoV IR 54 by the RNase III-expressing strain was not detectable by agarose gel or Northern blot analysis (data not shown).

To optimize the production of PMMoV IR 54 in HT115 strain, we assessed the effect of varying the concentration of IPTG (0.4 mM versus 1 mM), induction temperature (37°C versus 30°C), induction time (2 hr versus 4 hr) and induction media (LB versus Terrific). Beside some minor variations, there was no generalizable difference in the production of PMMoV IR 54 varying the parameters tested (data not shown). For subsequent production of dsRNA, IPTG was added to 0.4 mM, and the culture (LB media) was incubated for 2 h at 37°C.

Bacterial nucleic acid extracts inhibit PMMoV infection

To evaluate the capability of the PMMoV IR 54 produced in bacteria to interfere with PMMoV infection, *Nicotiana benthamiana* plants were inoculated with mixtures of PMMoV (5 μg/ml) and phenol-extracted, nucleic acid extracts prepared from HT115 harboring either pGEM/IR 54 (HT115/IR 54) or the empty vector (HT115). Total

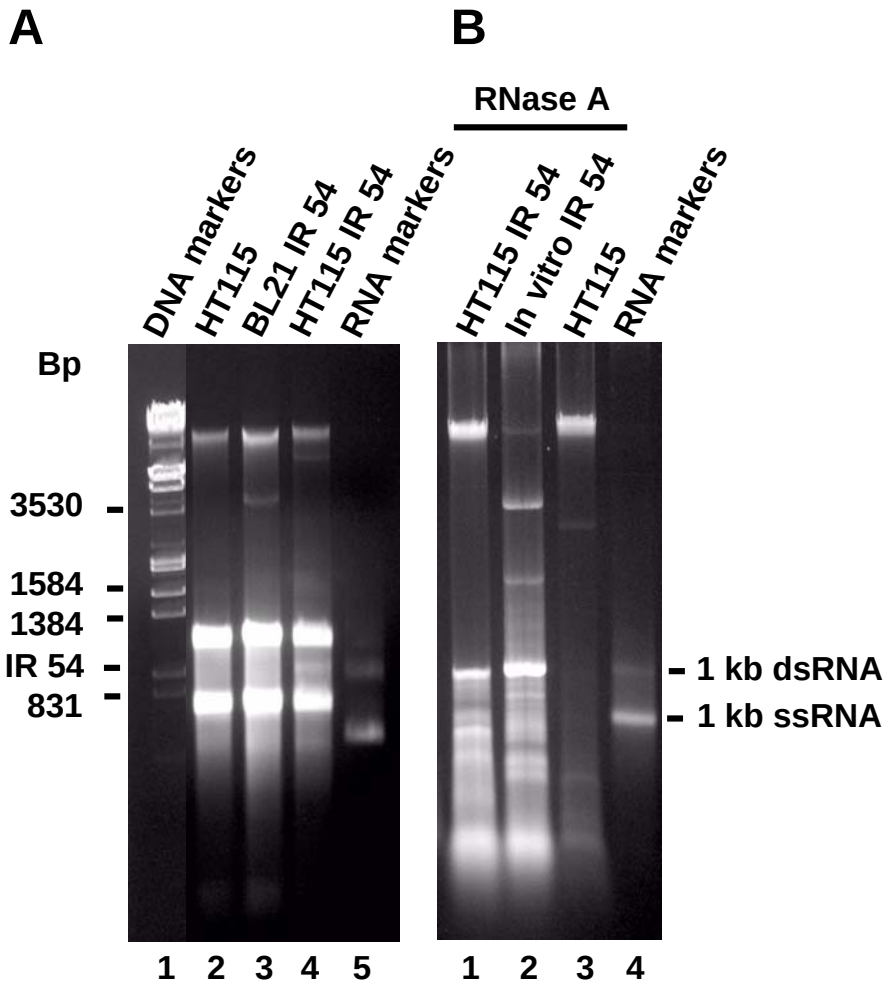

Figure 1

Production of PMMoV dsRNA in an *E. coli* strain deficient for RNase III. Bacteria of the indicated genotypes were transformed with either pGEM/IR 54, a plasmid designed to express a hairpin structure consisting of 977 bp of PMMoV RNA (IR 54), or with the empty plasmid (HT115). Bacterial cultures were induced with IPTG and processed for total nucleic acid. Samples were resolved by electrophoresis on 1% agarose gel before (A) or after treatment with RNase A (B), and nucleic acid was visualized by staining with ethidium bromide. DNA markers, λ*Eco*RI-*Hin*dIII molecular weight markers; RNA markers, 100 ng of *in vitro*-synthesized Neo poly(A) RNA. In vitro IR 54, T7 *in vitro*-transcription product of pGEM/IR 54. The position of bacterially expressed IR 54 is indicated.

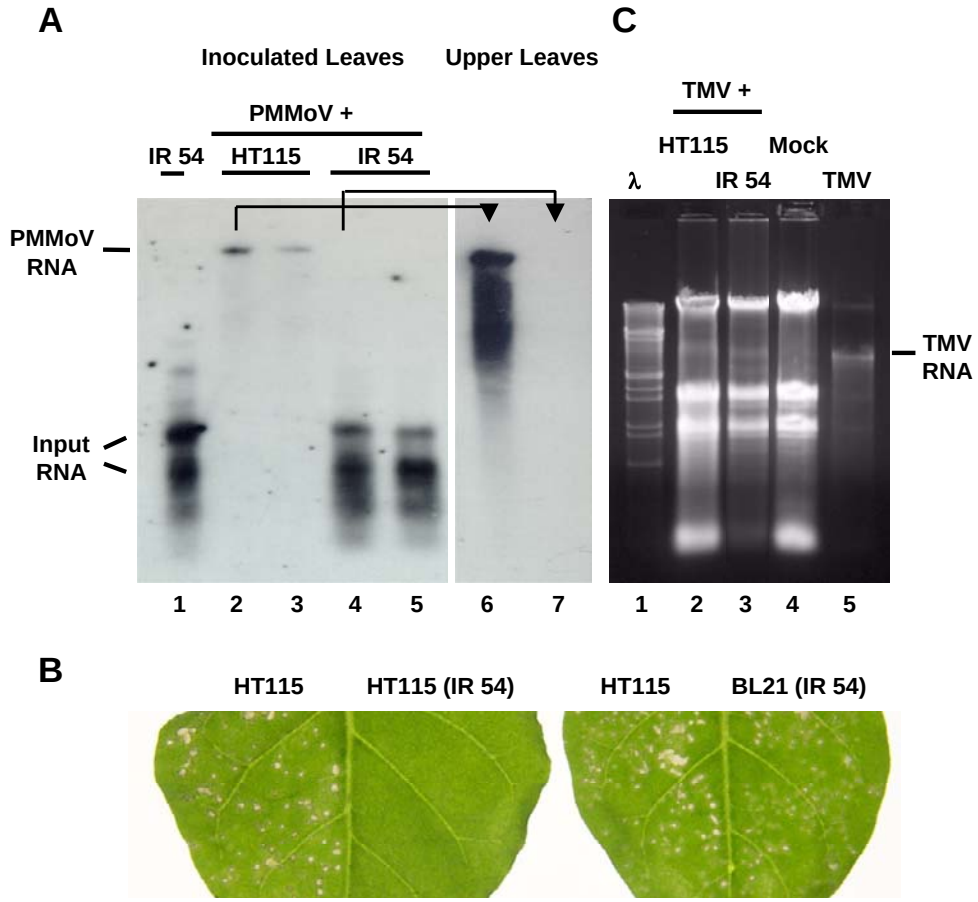

Figure 2

HT115-expressed PMMoV IR 54 specifically interferes with PMMoV infection. (A) Northern blot analysis of total RNA extracted from inoculated (lanes 2 to 5) or uppermost systemic leaves (lanes 6 and 7) of *N. benthamiana* at 7 dpi. Plants were inoculated with mixtures of PMMoV (5 μg/ml) plus nucleic acid extracts prepared from HT115 harboring either pGEM/IR 54 (IR 54) or the empty vector (HT115). Extract from HT115/IR 54 used in the inoculum was run on lane 1 for comparison. RNA samples (1 μg) were fractionated by 1% agarose gel electrophoresis, and a DIG-labeled PMMoV 54-kDa RNA was used as a probe. (B) *N. benthamiana* plants were initially inoculated with mixtures of PMMoV plus bacterial nucleic acid extracts as indicated above. In addition, mixtures of PMMoV plus nucleic acid extracts prepared from BL21 carrying pGEM/IR 54 (BL21/IR 54) were included. After 7 days, 1:1000 diluted extracts from systemic leaves were assessed on opposite half-leaves of *N. tabacum* cv. Xanthi nc as indicated. Similar numbers of local lesions were observed in both halves of the leaf shown at the right. No visible local response was observed in the half-leaf inoculated with plant extracts derived from PMMoV plus HT115/IR 54 shown at the left. (C) Agarose gel analysis of total RNA (3 μg) extracted from systemic leaves of *N. benthamiana* plants that were mock inoculated or were inoculated with mixtures of TMV (5 μg/ml) plus nucleic acid extracts prepared from HT115 harboring either pGEM/IR 54 (IR 54) or the empty vector (HT115), as indicated. M, λ*Eco*RI-*Hind*III molecular weight markers. TMV, purified TMV RNA (100 ng) was loaded as a control. The positions of PMMoV RNA, TMV RNA, and RNA species derived from partially denatured, input dsRNA are indicated.

nucleic acid extracted from BL21 carrying pGEM/IR 54 (BL21/IR 54) was used as control. Plants inoculated with PMMoV plus bacterial extracts derived from HT115 or BL21/IR 54 displayed typical disease symptoms in upper leaves at 6 days post inoculation (dpi). In contrast, all the plants (35 plants in 6 independent experiments) that were inoculated with PMMoV plus extracts derived from HT115/IR 54 were free of symptoms until they flower, typically after 10 weeks post-inoculation. The failure of nucleic acid extracts derived from BL21/IR 54 to interfere

with PMMoV infection precludes any effect concerning plasmid DNA homologous to the virus on the interference observed with extracts derived from HT115/IR 54. Northern blot analysis of total RNA showed that PMMoV RNA accumulated in both the inoculated and the upper leaf tissue of HT115- and BL21/IR 54-treated plants at 7 dpi (Fig. 2A and data not shown). In contrast, viral RNA levels were below the limit of Northern blot detection in plants coinoculated with the virus and the PMMoV IR 54-containing HT115 extract. Instead, two faster migrating signals that hybridised with the PMMoV-specific probe were consistently detected in the inoculated leaves of these plants. These hybridization bands have been previously reported in plants inoculated with *in vitro*-transcribed PMMoV 54 dsRNA [19] and in mosquitoes injected with dsRNA corresponding to the *Defensin* gene [25]. We interpret these bands as denatured and non-denatured input dsRNA, as they are also present in the bacterial extract used as inoculum (Fig. 2A, lane 1). A corollary is that the loop region present in the hairpin structure encoded by pGEM/IR 54, is probably cleaved by nucleases in the course of bacterial induction rendering non-covalently linked dsRNA.

To further confirm the complete interference with PMMoV infection, sap homogenates from the inoculated and upper leaves of *N. benthamiana* plants that had been coinoculated with PMMoV and the bacterial extracts seven days before, were used to back-inoculate the local lesion host, *N. tabacum* cv. Xanthi nc. No virus was present in any kind of leaves from three plants coinoculated with the virus and the PMMoV IR 54 containing HT115 extract, as judged by the absence of lesions in the hypersensitive host. In contrast, plant sap derived from HT115- or BL21/IR 54-treated plants elicited a high number of lesions in the hypersensitive host (Fig. 2B).

The interfering activity on PMMoV infection exhibited by nucleic acid extracts prepared from HT115 expressing PMMoV IR 54 could reflect any kind of unspecific, inhibitory effect of this particular strain on virus replication. However, coinoculation of bacterial extract derived from HT115/IR 54 together with *Tobacco mosaic virus* strain U1 (TMV-U1) (5 μg/ml) in *N. benthamiana*, had no effect on symptom expression and TMV RNA accumulated in upper leaves as in HT115-treated plants (Fig. 2C). TMV-U1 shares 73% sequence identity with PMMoV in the region of the 54-kDa gene [26], and only a few runs of identity of 17 nucleotides were observed. Thus, interference with virus infection exhibited by nucleic acid extracts derived from HT115/IR 54 was dependent on a high level of sequence identity with the target, viral RNA.

Bacterial crude preparations inhibit infection by plant viruses

Extraction of bacterial nucleic acid used above included a phenol-chloroform step prior to application on plants. To avoid the use of such chemicals in large-scale production and delivery of interference products onto plant surfaces, bacterial crude preparations derived from HT115/IR 54 were made by lysing cell pellets with the French Press. Partially degraded RNA was obtained from bacteria, as the internal markers 16S and 23S rRNA gave smeared bands on agarose gels, thus precluding the detection of dsRNA products (data not shown). However, Northern blot analysis of French Press lysates using a PMMoV-specific probe showed a specific signal in preparations derived from bacteria expressing PMMoV IR 54 but not in HT115-control lysates (Fig. 3A, lanes 1 and 2).

To test the interfering activity on PMMoV infection by the French Press preparation and, further, to assess the relationship between dose and inhibition of virus infection, a series of dilutions (1/2 to 1/20) were made from the PMMoV IR 54 preparation (3 μg of total nucleic acid/μl) to which a fixed concentration of PMMoV was added. PMMoV accumulation in the inoculated leaves was completely inhibited by the PMMoV IR 54 preparation diluted 10-fold or less, as assessed by Northern blot analysis at 7 dpi (Fig. 3A). A low level of PMMoV RNA, however, accumulated in leaves of plants coinoculated with PMMoV and the 1/20-diluted PMMoV IR 54 preparation, as compared to plants coinoculated with the virus and French Press preparations expressing non-homologous, PPV HC dsRNA (see below) or the empty vector (HT115) used as controls (Fig. 3A and data not shown). Plants coinoculated with the virus and the PMMoV IR 54 preparation diluted 1/10 or less did not display disease symptoms upon completion of their life cycles (Fig. 3B). In contrast, symptom expression in plants coinoculated with PMMoV and the 1/20 dilution was delayed by only 2 days compared to that of plants inoculated with the mixture PMMoV plus either HT115 or PPV HC dsRNA preparation.

To test whether extracts derived from bacteria expressing viral dsRNA could prevent infection by plant viruses other than PMMoV, gene sequences corresponding to PPV were introduced into HT115 strain. For these experiments, we cloned cDNA fragments containing either the helper component (HC) or the coat protein (CP) genes of PPV into L4440, a plasmid vector which has two convergent T7 promoters flanking the multiple cloning site [21]. Upon IPTG induction, prominent bands corresponding in size to full-length HC (1492 bp) and CP (1081 bp) dsRNAs were detected in bacterial nucleic acid extracts by agarose gel analysis and confirmed by resistance to RNase A (Fig. 4A).

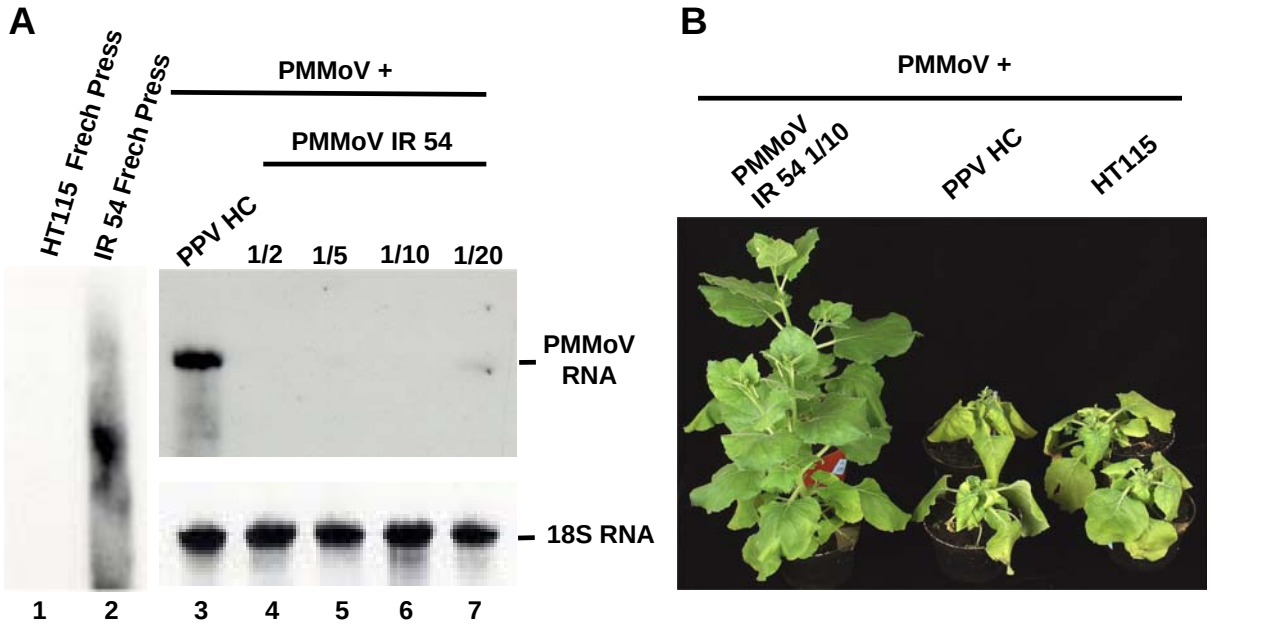

Figure 3

Dose-dependent interference with PMMoV infection by bacterial crude preparations expressing PMMoV IR 54. (A) Northern blot analysis of total RNA extracted from inoculated leaves (lanes 3 to 7) of *N. benthamiana* at 7 dpi. Plants were inoculated with mixtures of PMMoV (5 μg/ml) plus either a series of dilutions (1/2 to 1/20) of the PMMoV IR 54 preparation, or the PPV HC dsRNA preparation diluted 1/2, as indicated. French Press preparations derived from bacteria expressing the empty vector (HT115) or PMMoV IR 54 used in the inoculum were run in lanes 1 and 2, respectively. RNA samples (1 μg) were fractionated by 1% agarose gel electrophoresis and the filter was hybridised with a DIG-labeled PMMoV 54-kDa RNA probe. Equal loading of RNA samples was assessed using a DIG-labeled RNA probe complementary to the 18S ribosomal RNA. The positions of PMMoV RNA and 18S RNA are indicated in the margin. (B) Response of *N. benthamiana* to a combination of PMMoV plus French Press preparations derived from either PMMoV IR 54 (left), PPV HC dsRNA (middle) or HT115 (right). Plants displaying disease symptoms (one-half-diluted, PPV HC dsRNA and HT115 preparations) or showing protection to virus infection (1/10-diluted PMMoV IR 54 preparation) were photographed at 30 dpi.

Other minor bands present in the extracts likely represent partial RNA duplexes.

One-half diluted French Press preparations derived from bacteria expressing either PPV HC dsRNA, PPV CP dsRNA, PMMoV IR 54 or the empty vector (HT115) were mixed with PPV (0.3 μg/ml), and used to inoculate *N. benthamiana*. In three independent experiments, PPV was detected in upper leaves of all the plants coinoculated with PPV and either PMMoV IR 54 or HT115 preparations by double antibody sandwich indirect (DASI)-ELISA at 12 dpi. In contrast, a high proportion of plants inoculated with combinations of PPV plus HC (82%) or CP (73%) dsRNA-expressing preparations showed no symptoms and DASI-ELISA tests failed to detect any virus (Table 1). Furthermore, no traces of PPV replication were detected by reverse transcriptase (RT)-PCR in upper leaves from these

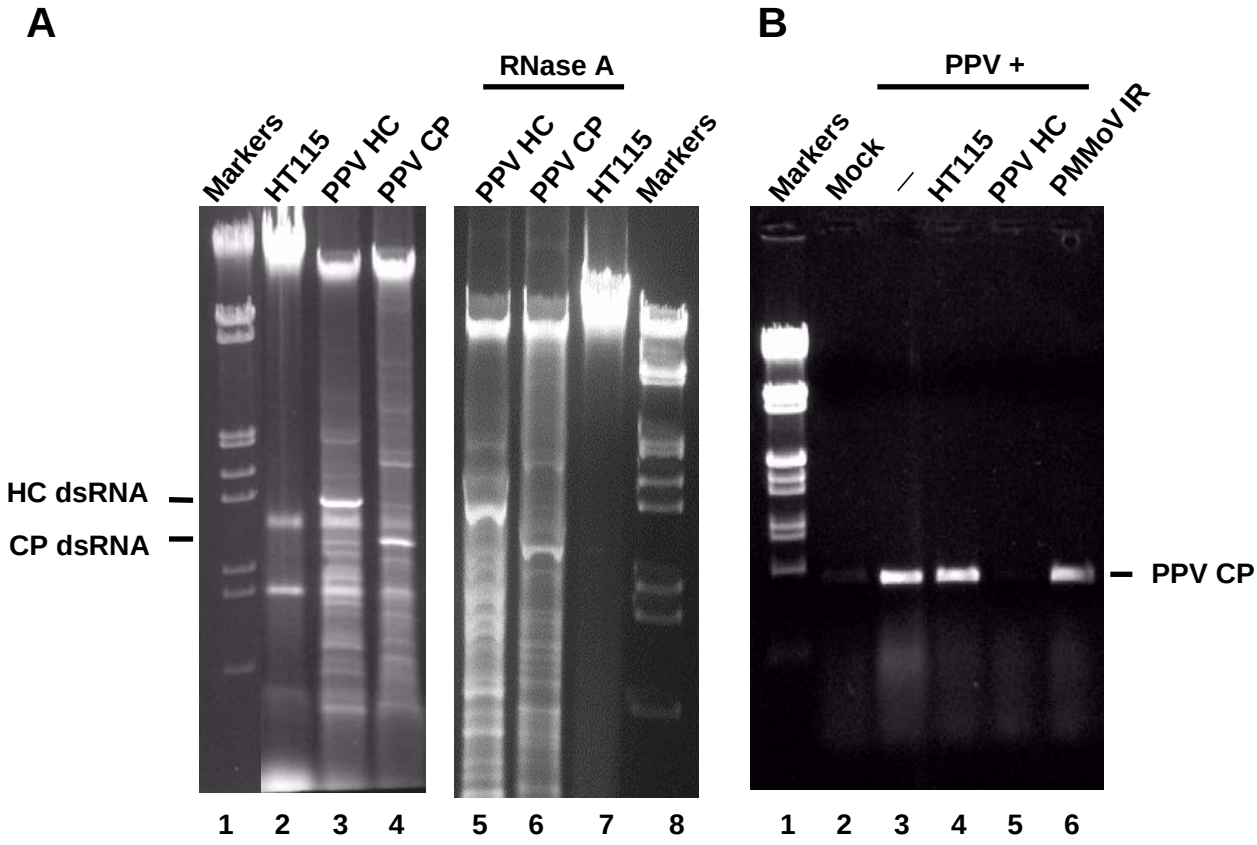

Figure 4

Bacterially expressed dsRNA interferes with PPV infection. (A) Production of PPV dsRNAs in *E. coli*. HT115 cells were separately transformed with the L4440 double-T7 vector containing either the HC or the CP genes of PPV. Bacterial cultures were induced with IPTG and processed for total nucleic acid. Samples were resolved by electrophoresis on 1% agarose gel before (lanes 1 to 4) or after treatment with RNase A (lanes 5 to 8), and nucleic acid was visualized by staining with ethidium bromide. Markers, λ*Eco*RI-*Hin*dIII molecular weight markers. The positions of bacterially expressed 1492-bp HC and 1081-bp CP dsRNAs are indicated in the margin. (B) Detection of PPV in total RNA extracted from systemic leaves of *N. benthamiana* by RT-PCR at 14 dpi. Plants were mock inoculated or were inoculated with PPV (0.3 μg/ml) alone (-), or with mixtures of PPV plus French Press preparations derived from HT115 harboring either PPV HC dsRNA, PMMoV IR 54 or the empty vector, as indicated. Markers, λ*Eco*RI-*Hin*dIII molecular weight markers. RT-PCR was performed with 1 μg of total RNA using primers corresponding to the CP coding sequence of PPV. The position of the 510-bp amplified fragment is indicated in the margin.

asymptomatic plants at 14 dpi (Fig. 4B and data not shown).

Sprayed-bacterial crude preparations inhibit viral infections

From a practical standpoint, we tested a simple spray technique for the delivery of interfering products onto the surface of plant leaves. Furthermore, to evaluate the protective effect against viral infection at different time points after delivery of French Press lysates on plants, PMMoV was inoculated on plants simultaneously or one, three, five or seven days after spraying with either PMMoV IR 54 or HT115 preparations in the same leaves. In these experiments, with at least five plants per treatment, one-half diluted preparations were used. All HT115-sprayed plants displayed systemic symptoms at 6 dpi. Interestingly, a delay of up to 5 days between spraying with PMMoV IR 54 preparation and virus inoculation was able to protect plants until their life cycles were completed. Analysis of the upper leaves from PMMoV IR 54-treated plants by

Table 1: Specific interference with virus infection by bacterial crude preparations

Bacterial preparation	Phenotype	Virus titer
Coinoculation (PPV)*		
HT115	S 3/3	1.221 ± 0.105
PPV HC dsRNA	R 14/17	0.007 ± 0.004
	S 3/17	1.197 ± 0.060
PPV CP dsRNA	R 8/11	0.017 ± 0.017
	S 3/11	1.310 ± 0.103
PMMoV IR 54	S 7/7	1.149 ± 0.078
Non-inoculated controls		0.012 ± 0.007
Spraying (PMMoV)‡		
PMMoV IR 54 (0 day)	R 5/5	0.004 ± 0.002
PMMoV IR 54 (1 day)	R 5/5	0.009 ± 0.003
PMMoV IR 54 (3 days)	R 5/5	0.011 ± 0.010
PMMoV IR 54 (5 days)	R 18/18	0.015 ± 0.012
PMMoV IR 54 (7 days)	R 1/5	0.012
	S 4/5	1.360 ± 0.141
HT115 (5 days)	S 10/10	1.420 ± 0.121
Non-inoculated controls		0.010 ± 0.009
Spraying (PPV)‡		
HT115	S 7/7	1.042 ± 0.260
PPV HC dsRNA	R 11/12	0.004 ± 0.008
	S 1/12	0.278
Non-inoculated controls		0.008 ± 0.003

*Plants were inoculated with mixtures of PPV plus French Press preparations derived from HT115 harboring either PPV HC dsRNA, PPV CP dsRNA, PMMoV IR 54 or the empty vector (HT115). The number of plants showing resistant (R) or susceptible (S) phenotypes to viral infection is indicated. Virus titer corresponds to the mean ELISA values ± SE at 12 dpi. †PMMoV was inoculated on plants simultaneously or one, three, five or seven days after spraying with either PMMoV IR 54 or HT115 preparations in the same leaves. ‡PPV was inoculated on plants five days after spraying with either PPV HC dsRNA or HT115 preparations in the same leaves.

DASI-ELISA at 12 dpi showed the absence of PMMoV CP compared to the upper leaves from HT115-sprayed plants (Table 1). In contrast, inoculation with PMMoV seven days after spraying with PMMoV IR 54 was unable to protect plants against viral infection. Four out of five plants showed systemic disease symptoms. However, symptom expression in these plants was delayed by three days as compared with controls, indicating a partial interference with virus infection. Viral replication limited to the newly developed, non-sprayed tissue of the inoculated leaves could reduce the rate of systemic spread in these plants.

Similar strong inhibition of virus infection was observed in plants inoculated with PPV five days after spraying with one-half diluted, PPV HC dsRNA preparation in the same leaves. Most of the plants, 11 out of 12, exhibited protection against virus infection in upper leaves according to DASI-ELISA test at 12 dpi. PPV was detected in only one PPV HC dsRNA-sprayed plant, albeit virus titer was reduced compared to that of plants sprayed with HT115 preparation (Table 1). The DASI-ELISA-negative plants were assayed again for PMMoV or PPV infection 35 days after inoculation and remained noninfected (data not shown).

Discussion

Virus resistance in plants containing virus-derived transgenes, usually by activation of a sequence-specific RNA degradation process, has been widely reported [1,27]. Our approach differs from strategies based on transgenic expression of RNAs but still relies on PTGS as a means to achieve pathogen-derived resistance in plants [20]. We have previously shown that dsRNA derived from viral sequences can specifically interfere with plant virus infection through a PTGS mechanism by directly delivering dsRNA to leaf cells through either mechanical inoculation or via an *Agrobacterium*-mediated transient expression assay [19,20]. The first approach required the *in vitro* synthesis of large amounts of RNA involving high cost and considerable labour. The transient RNA silencing system is limited by the failure of *Agrobacterium* to efficiently move for long distances in plants, precluding its use as a biocontrol agent.

Here we present an efficient production of dsRNA derived from viral sequences using a bacteria deficient for RNase III, with a view to providing a practical control of virus diseases. The obtained dsRNAs promoted specific interference with the infection of plants by two viruses belonging to the tobamovirus and potyvirus groups. Therefore, we

suggest that the effect mediated by dsRNA in plant virus infection reflects the phenomenon of RNAi reported in *C. elegans* by feeding worms *E. coli* expressing dsRNA corresponding to a specific gene [22]. Further, a method was developed to deliver interference products by a combination of lysing cells with the French Press and spraying bacterial crude preparations onto plant surfaces. The protocol is simple and quick, avoiding the use of long-lasting protocols to purify nucleic acids from bacteria. It was shown to be highly effective in interfering with virus infection without detrimental effect on plant surfaces. The results obtained in a dilution series of French Press preparations suggest that a 10-fold dilution of interfering products produced in bacteria is the threshold required for a complete interference with PMMoV multiplication. Furthermore, virus infectivity was significantly abolished when plants were sprayed with French Press lysates up to 5 days before virus inoculation. This is not surprising because a previous analysis of the stability of dsRNA molecules after delivery into leaves supported that most of the input dsRNA was relatively stable and persisted in the leaf several days after inoculation, even after exuberant watering [19]. This delivery method further contributes to making RNAi technology widely available and applicable for deployment in the field. One possible limitation of the procedure described here is inactivation of dsRNA by ultraviolet light crosslinking under field conditions, yet the crosslinking reaction is not efficient enough to create multiple crosslinking in a single given dsRNA [28]. Moreover, crosslinks within the nucleic acid helix can be reversed by photoreactivation which happens under visible light irradiation. Our approach provides an alternative to genetic transformation of plant species with dsRNA-expressing constructs capable to interfere with plant virus [1,27]. The main advantage of this mode of dsRNA production is its simplicity and its extremely low cost compared with the requirements for regenerating transgenic plants.

Since most plant viruses are transmitted by aphids in nature, we have been especially interested in using this procedure to extend the resistance against the inoculation by aphids. In the case of the potyvirus, experiments are currently being in progress trying to facilitate the penetration of bacterially expressed dsRNA into plant tissues other than epidermal cells, in order to protect plants against this natural means of infection.

Beyond the obvious application for plant protection, our approach provides a potential tool to study the onset of PTGS in viral infection, in contrast to dsRNA-transgenic plants which primarily allow to study PTGS maintenance. It is thought that the presence of the transgene encoded dsRNA switches on the host's PTGS response prior to the invasion of the virus, which in turn leads to the RNA degradation of the invading virus [27,1]. In our case, dsRNA penetrates in the cell in combination with virus particles, that is, in cells where a PTGS status is not established. Therefore, we propose that the bacterially expressed dsRNA activates the PTGS machinery in cells where replication of the virus genome is simultaneously initiated. The combination of both a bacterially expressed dsRNA and suppressors of PTGS transiently expressed by *Agrobacterium* could give valuable information about the balance between silencing and suppression of PTGS in the context of a viral infection.

Conclusions

In this report, we have established a simple, fast, safe and inexpensive procedure to produce large amounts of dsRNA derived from viral sequences using a bacteria deficient for RNase III, with a view to providing a practical control of plant virus diseases. When applied on plants, the dsRNAs caused specific degradation of the viral RNA and resultant protection against virus infections. The demonstration that dsRNA also works in interfering with virus infection when bacterial crude preparations are sprayed onto plant surfaces, further contributes to making this technology widely available and applicable. The infectivity of virtually any RNA virus in plants can now be inhibited with the corresponding dsRNA. This approach represents a dramatic improvement compared with the requirements for regenerating transgenic plants capable to interfere with plant virus infections. In addition, the strategy described here provides a reliable and potential tool for the study of gene silencing mechanisms in plant virus infections.

Methods

Plasmid constructs

A cDNA construct (pGEM/IR 54) with the potential to originate a hairpin RNA corresponding to part of the 54-kDa replicase gene of PMMoV (PMMoV IR 54) under the control of the T7 RNA polymerase promoter was described earlier [20]. This construct contains a non-homologous spacer sequence flanked by the antisense and sense orientations of 977 bp of the PMMoV 54-kDa gene (nucleotides 3411 to 4388 in PMMoV RNA, [26]). A cDNA fragment containing the HC gene and flanking regions of PPV 5.15 [29]. was amplified by RT-PCR using PPV RNA as template and cloned into *Hind III* and *ClaI* of L4440. The upstream primer was 5'TATAGCTCGAGGAAAACACAACTC3' (nucleotides 986 to 1009 of PPV sequence, [30]). The downstream primer was 5'TTTTGAATTCGTCACACTTATC3' (nucleotides complementary to 2457 to 2478). The complete CP coding sequence and flanking regions of PPV 5.15 were amplified by RT-PCR using PPV RNA as template and cloned into *Hind*III and *Cla*I of L4440. The upstream primer was 5'CGATATCTTGAAGCTTTTTAC3' (nucleotides 8511 to 8531). The downstream primer was

5'GTTTATCGATGATACCGAGACCA3' (nucleotides complementary to 9570 to 9592). L4440 (pPD129.36) contains two convergent T7 polymerase promoters in opposite orientation separated by a multicloning site [21].

Plasmids were transformed into BL21(DE3) [31] or HT115(DE3) using standard $CaCl_2$ transformation protocols. HT115(DE3) is an RNAase III-deficient *E. coli* strain, which was modified to express T7 RNA polymerase from an IPTG-inducible promoter [22]. The RNase III gene is disrupted by a Tn10 transposon carrying a tetracycline-resistance marker [22].

Bacterial induction

Single colonies of HT115(DE3) or BL21(DE3) bacteria containing pGEM/IR 54 or cloned L4440 plasmids were grown for 16 h in LB with 100 μg/ml ampicillin at 37°C. For HT115(DE3) strain, tetracycline was used at 12.5 μg/ml. The culture was diluted 100-fold in 20 ml of the same medium and allowed to grow to OD_{595} = 0.5. T7 polymerase was induced by the addition of 0.4 mM IPTG, and the culture was incubated with shaking for 2 h at 37°C. Total nucleic acid was extracted from bacteria as previously described [22], except that DNAse I and RNase digestions were omitted. The extraction procedure included a phenol-chloroform step prior to ethanol precipitation. The nucleic acid pellet was resuspended at 1.5 μg/μl in 10 mM Tris, 1 mM EDTA, pH 7.5, and use for plant protection against virus infection. Accumulation of dsRNA in bacterial extracts was confirmed by resistance to RNase A under high salt conditions (0.3 M NaCl, 0.030 M sodium citrate). An *in vitro* synthesized Neo poly(A) RNA (DIG RNA labeling kit, Roche Molecular Biochemicals) was used as a ssRNA/dsRNA marker (1 kb) and to estimate the amount of PMMoV IR 54 produced in bacteria. It is well known that in vitro transcription with phage RNA polymerases generally produces a low-to-moderate level of dsRNA [10].

For large-scale preparations, IPTG-induced HT115 cells (800 ml) were pelleted by centrifugation and resuspended in 16 ml of 50 mM Tris, 10 mM EDTA, pH 7.5. The cell suspension was passed through the French Press twice at 12,000 psi, then centrifuged at 9,000 rpm for 20 min. The supernatant was tested for its interfering activity on viral infection. Concentration of total nucleic acid in different preparations was estimated to be 2.5–3 μg/μl by spectrophotometry using E_{260} = 40.

Virus inoculation

The origin of TMV-U1, PMMoV, and PPV 5.15 has been reported previously [29,32]. Viruses were purified from *N. benthamiana* plants as described [29,33]. In the experiments with PMMoV and TMV-U1, standard inoculum was 10 μg/ml of purified virus. For PPV 5.15, the inoculum was 3 μg of purified virus per ml. To test interference with virus infection, inoculation mixtures were made by adding 10 μl of each bacterial extract (nucleic acid extracts or French Press preparations) to either an equal volume of PMMoV or TMV-U1 or to 1 μl of PPV 5.15. French Press preparations were sprayed onto two leaves using an atomizer, then inoculated with PMMoV or PPV. Inoculation of plants was done on two fully expanded leaves of at least two plants per assay by gently rubbing the leaf surface with the inoculum using carborundum as an abrasive. To assess virus level in plants, sap from the inoculated and upper leaves of *N. benthamiana* plants was diluted 1:1000 in 0.02 M sodium phosphate buffer, pH 7.0, and was used to back-inoculate *N. tabacum* cv. Xanthi nc. The inoculated plants were kept in growth chambers with a 16 h light and 8 h dark cycle at 25°C, and the development of viral infection symptoms was monitored for as long as their life cycles (10 weeks post-inoculation). For the local lesion host, inoculated leaves were photographed 5 days after inoculation.

Analysis of virus in plants

Total RNA was extracted from inoculated leaves at 7 dpi and from upper leaves 7 to 14 dpi as described [34]. PMMoV RNA was detected by Northern blot analysis. RNA samples (approximately 1 μg) were separated on 1% agarose formaldehyde gels and transferred to Hybond-N membranes (Roche Molecular Biochemicals) by a low-pressure vacuum system (VacuGene XL, Pharmacia). PMMoV RNA was detected with a digoxigenin (DIG)-labeled riboprobe (Roche Molecular Biochemicals) complementary to PMMoV nucleotides 3411–4388 that was transcribed from pT3T7/54 kDa [35]. To confirm equal loading of RNA in each sample, blots were hybridised with a DIG-labeled riboprobe complementary to the 18S ribosomal RNA [36]. PPV RNA was detected by RT-PCR using primers corresponding to the CP gene. The upstream primer was 5'TTGGGTTCTTGAACAAGC3' (nucleotides 8390 to 8407 of PPV sequence, [30]. The downstream primer was 5'TGGCACTGTAAAAGTTCC3' (nucleotides complementary to 8883 to 8900 of PPV sequence).

Assessment of virus level in plants sprayed with French Press preparations was done in upper leaves by double antibody sandwich indirect (DASI)-ELISA at 12 and 35 dpi. Leaf samples were homogenized in ELISA sample buffer at 1 g/ 50 ml, and aliquots of sap (100 μl) transferred to two wells of a microtiter plate coated with polyclonal antibodies against PPV (REALISA-PPV, Durviz) or PMMoV [32]. Each microtiter plate had both the negative control, which was uninoculated *N. benthamiana* plant extract, and the positive control, a twofold-dilution series of quantitated purified virion.

Authors' contributions

FT carried out the molecular analysis and drafted the manuscript. BM-G prepared the PPV antiserum and assisted with the PPV constructs. MV performed the PPV constructs and assisted with the experiments illustrated in figures 1 and 4. JRD-R is the principal investigator and participated in this study's design and coordination.

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