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Identification and characterization of two RNA silencing suppressors encoded by ophioviruses

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ABSTRACT

Citrus psorosis virus and *Mirafiori lettuce big-vein virus* are two members of the genus *Ophiovirus*, family *Ophioviridae*. So far, how these viruses can interfere in the antiviral RNA silencing pathway is not known. In this study, using a local GFP silencing assay on *Nicotiana benthamiana,* the 24K–25K and the movement protein (MP) of both viruses were identified as RNA silencing suppressor proteins. Upon their co-expression with GFP in *N. benthamiana* 16c plants, the proteins also showed to suppress systemic RNA (GFP) silencing. The MP^{CPsV} and $24K^{CPsV}$ proteins bind long (114 nucleotides) but not short-interfering (21 nt) dsRNA, and upon transgenic expression, plants showed developmental abnormalities that coincided with an altered miRNA accumulation pattern. Furthermore, both proteins were able to suppress miRNA-induced silencing of a GFP-sensor construct and the co-expression of MP^{CPsV} and $24K^{CPsV}$ exhibited a stronger effect, suggesting they act at different stages of the RNAi pathway.

1. Introduction

RNA silencing, also named RNA interference (or simply RNAi), is a highly conserved gene regulation mechanism present in almost all eukaryotes. This mechanism relies on the production of small (s)RNA molecules of 21–24 nt in size, that play an important role in plant development and genome integrity by regulation of endogenous mRNA expression. That regulation can be exerted post-transcriptionally via sequence specific mRNA cleavage or translational repression, or at a transcriptional level through histone methylation and heterochromatin formation (Brodersen and Voinnet 2006).

RNAi has also been recognized as an (adaptive) antiviral defence mechanism (Voinnet, 2001). Virus infections usually lead to the formation of dsRNA molecules resulting from viral replicative intermediates, secondary RNA folding structures in genomic or mRNA molecules or from the conversion of aberrant viral RNA molecules into dsRNA by the action of host RNA-dependent RNA polymerases (RDRs) (Wang et al., 2012; Baulcombe, 2004). Their processing into viral (v) siRNAs leads to subsequent activation of an antiviral RISC complex that is able to sense and degrade viral RNA target molecules. In plants, but not in mammals nor insects, the primary siRNA signal is being further amplified. Aberrant RNA molecules, *e.g.* those resulting from the first siRNA-guided cleavage, are converted into dsRNA by host RDRs, and further processed to generate a population of secondary siRNAs. Without this amplification, the antiviral RNAi response is generally weak, and plants highly susceptible to virus infection (Vaistij et al., 2002). Small RNAs can move systemically/long distance and thereby also trigger the non-cell autonomous systemic silencing pathway (Himber et al., 2003).

MicroRNAs (miRNAs) present another class of sRNAs that structurally resemble siRNAs but differ in their biogenesis. Plant miRNAs are encoded from host genes as primary miRNAs (pri-miRNA) (Lee et al., 2004; Xie et al., 2005) that fold into imperfect stem-loop structures. These are processed into miRNA precursors (pre-miRNAs) and ultimately into mature miRNA/miRNA* duplexes (Kurihara and Watanabe, 2004; Park et al., 2002). Several miRNA families are fundamental gene regulatory players to fine-tune correct plant development (Bartel, 2004; Jones-Rhoades et al., 2006; Schwab et al., 2005), but they also have important functions in response to biotic stress (Katiyar-Agarwal and Jin, 2010). Both plant and animal viruses can interfere with miRNA-mediated regulation in the host through different mechanisms exerted at transcriptional or posttranscriptional levels (Cazalla et al., 2010; Chapman et al., 2004; Chen et al., 2004; Kasschau et al., 2003; Pfeffer and Voinnet, 2006; Reyes et al., 2016; Silhavy and Burgyan, 2004),

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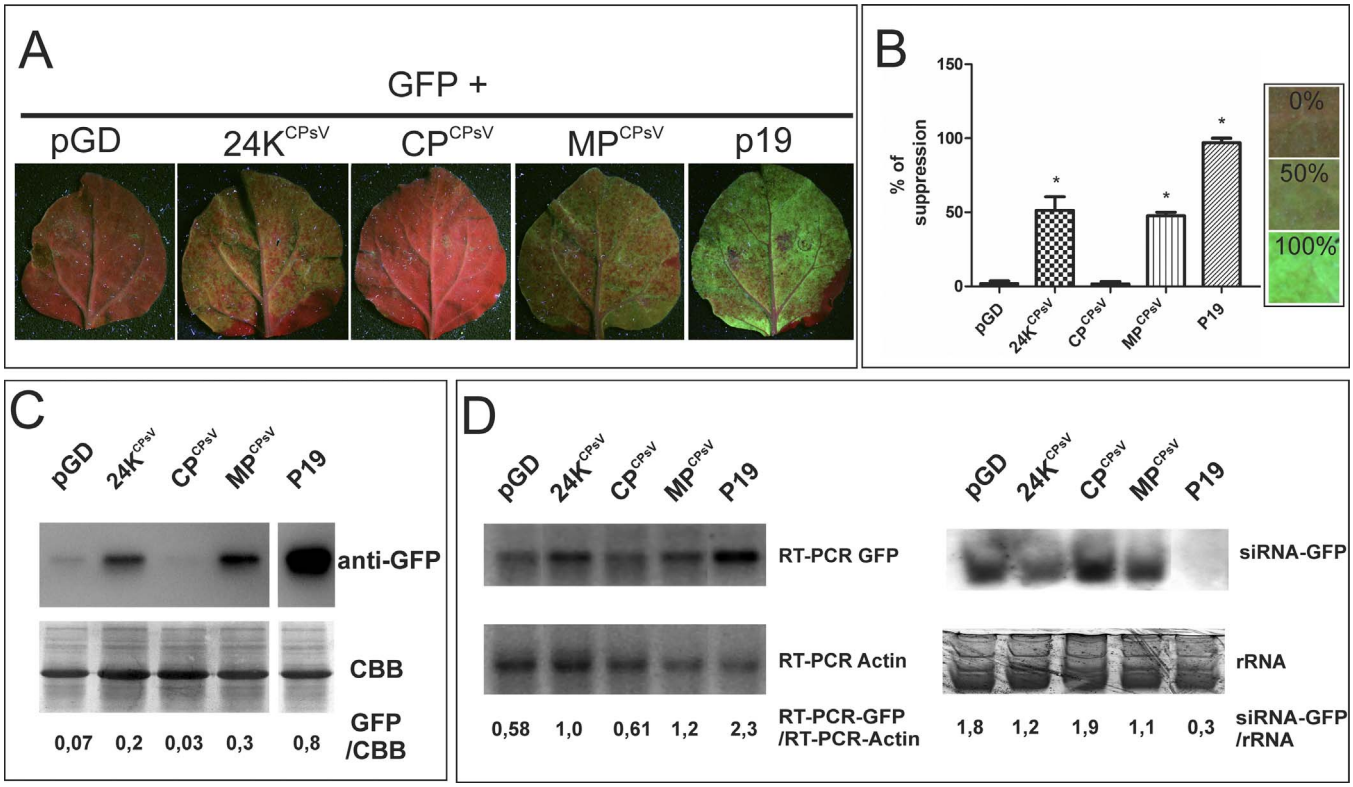

Fig. 1. Local silencing suppression activity of MP^{CPsV} and $24K^{CPsV}$ proteins.Panel A. GFP fluorescence of *N. benthamiana* wild type representative leaves agroinfiltrated with binary constructs coding for GFP and viral proteins as indicated, monitored at 5 days post infiltration. Leaves infiltrated with the empty vector (pGD) or the p19 construct, were used as negative and positive controls, respectively.Panel B. Percentage of suppression of CPsV proteins expressed as percentage of fluorescence for each construct. Fluorescent values were set as high (100%), moderate (50%) and low (0%), based on the scale shown at the right of the panel. The average percentage of five independent experiments is shown here. * Indicates significant differences with pGD (negative control) at $P < 0.05$ using a *t*-test.Panel C. Western immunoblot detection of GFP protein from infiltrated leaf patches (upper panel). Total protein was stained with Coomassie brilliant blue (CBB) and used to verify for protein loading (lower panel). The amount of GFP was calculated relative to the CBB signal and is indicated below the panel. (Although not contiguous, lanes correspond to the same gel).Panel D. Left; Accumulation of mRNA-GFP determined by RT-semiquantitative PCR relative to actin as reference gene. Representative results are shown. Empty vector and p19 were included as negative and positive controls, respectively. The relative amount of mRNA-GFP to mRNA-Actin, is shown at the bottom. Right; Northern blot detection of GFP-siRNAs. Ethidium bromide-stained rRNA and tRNA were included as loading controls. Empty vector and p19 were used as negative and positive controls, respectively. The relative amount of siRNA was calculated using rRNA as internal control, and is indicated below the panel.

involving, in some cases, miRNA processing alteration, accumulation and activity.

Plant viruses are able to counteract antiviral RNA silencing, and most are doing so by encoding for a protein, often referred to as viral suppressor of RNA silencing (VSR), that employs a myriad of diverse strategies to interfere at different phases of the RNA silencing pathway. Commonly, two main VSR strategies are distinguished: 1) binding of long dsRNA and/or sRNA to prevent their processing into sRNAs or loading into RISC respectively, and 2) activity inhibition of host protein components of the RNAi machinery by direct or indirect protein–protein interaction. Representatives of the first class of VSR proteins are tombusvirus p19, *Cucumber mosaic virus* (CMV) 2b and *Turnip crinkle virus* (TCV) p38 (Lakatos et al., 2006; Silhavy et al., 2002; Deleris et al., 2006; Goto et al., 2007; Merai et al., 2006). Examples of VSR proteins that employ the second strategy are the *Sweet potato mild mottle virus* (SPMMV) p1 protein, the polerovirus P0 protein, and also p38 which target the AGO1 component of RISC (Zhang et al., 2006; Azevedo et al., 2010; Csorba et al., 2010; Giner et al., 2010). Some VSR proteins even employ both strategies, like CMV 2b and TCV p38. VSR proteins that bind sRNAs also prevent them from moving long distance to activate the systemic silencing pathway (Dunoyer et al., 2010) but are often observed to interfere in the miRNA pathway as well, altering their processing, accumulation and activity (Csorba et al., 2015; Incarbone and Dunoyer, 2013; Csorba and Burgyan, 2016). Many studies have focused on posttranscriptional mechanisms where increased miRNA levels were associated with the presence of VSR silencing (Chapman et al., 2004; Chen et al., 2004; Dunoyer et al., 2004; Lakatos et al., 2006; Silhavy and Burgyan, 2004). Most viral suppressors have also been identified as pathogenicity determinants interacting with host factors (Anandalakshmi et al., 2000; Endres et al., 2010; de Ronde et al., 2013).

Psorosis is a graft-transmissible and widespread disease, affecting most citrus varieties worldwide and sources for commercial resistance breeding are lacking (Moreno et al., 2015). It is caused by *Citrus psorosis virus* (CPsV) the type member of the genus *Ophiovirus,* family *Ophioviridae* (Vaira et al., 2011). Another member of this family, *Mirafiori lettuce big-vein virus* (MiLBVV), is the causal agent of lettuce big vein disease and one of the most serious olpidium-transmitted viral diseases (Lot et al., 2002). The ophiovirus genome is divided into three (CPsV) or four (MiLBVV) ssRNA segments of negative polarity that code for the putative RNA-dependent RNA polymerase and a 24–25K protein in the RNA1, the 54–55K cell-to-cell movement protein (MP) in the RNA2, the coat protein (CP) in the RNA3, and the 37K protein in the RNA4 (van der Wilk et al., 2002; Hiraguri et al., 2013; Naum-Ongania et al., 2003; Peña et al., 2012; Robles Luna et al., 2013; Sanchez de la Torre et al., 2002, 1998). Although a transgenic, RNAi-mediated resistance approach has been developed against CPsV and MiLBVV (Reyes et al., 2011; Kawazu et al., 2010), so far no VSR protein of ophioviruses has been identified. Recently the processing and accumulation of miRNAs has been shown to become affected during a CPsV infection in *Citrus sinensis* plants. Furthermore, the 24K protein was observed to co-immunoprecipitate with miRNA precursors from *Nicotiana benthamiana* (Reyes et al., 2016), altogether suggesting a possible role of CPsV 24K as VSR. In this study, not only the 24–25K but also the MPs of CPsV and

MiLBVV have been identified as VSR. CPsV proteins have been shown to act by binding long dsRNA and have inhibitory effects in miRNA-induced silencing. *In vivo* and *in vitro* experiments designed to identify the different RNAi stages and silencing mechanisms targeted by these proteins are presented and discussed.

2. Material and methods

2.1. Local and systemic GFP RNA silencing assays

To test for local GFP silencing suppression, *Nicotiana benthamiana* plants were infiltrated in the abaxial side of the leaf with GV3101 *Agrobacterium tumefaciens* cultures bearing pBIN19-GFP5 at an OD_{600nm} = 0,4 and binary constructs coding for CPsV (Fig. 1) and MiLBVV (Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.virusres.2017.04.013) non tagged proteins as previously described (Peña et al., 2012; Robles et al., 2013). RNA-silencing suppressor constructs coding for tombusvirus p19, and CMV 2b (Q strain, Ding et al., 1995) were infiltrated in *N. benthamiana* plants at an OD_{600} = 0.2–0.3. To increase the level of viral protein expression, *A. tumefaciens* cultures bearing viral gene constructs were infiltrated 24hs prior to agroinfiltation with a binary GFP construct. The% of suppression was assigned for each leaf according to the scale showed in Fig. 1B based on the GFP fluorescence intensity. The% of suppression was calculated taken 4–6 leaves (one leaf per plant) for each protein, in each experiment. Five independent experiments were plotted in Fig. 1B and analyzed using the *t*-test (α = 0,05).

For the analysis of systemic GFP silencing suppression, *N. benthamiana* 16c plants (Ruiz et al., 1998), constitutively expressing GFP, were agroinfiltrated (locally) with binary constructs coding for GFP and CPsV proteins and monitored for GFP RNA silencing and suppression in systemic leaf tissues. The% of suppression was calculated as the number of suppressed plants (Fig. 2A, pictures iii and iv) divided by the total number of plants (10–15 plant per experiment) assayed for each protein evaluated. The% of suppression of five independent experiments for each protein were plotted as showed in Fig. 2B, and subject to statistical analysis (t-test; α = 0,05).

Northern blotting was performed as described in Reyes et al. (2011) and Western blotting as described in Peña et al. (2012).

2.2. MiRNA-induced GFP silencing assay

Transient miRNA-induced RNA silencing was performed using GFP sensor construct GFP171.1, containing a miR171 binding site downstream the stop codon of the GFP open reading frame (Parizotto et al., 2004). GFP sensor construct GFP171.2, containing a mutated miR171 target site, was included as negative control (Parizotto et al., 2004). (GFP171.1 and GFP171.2 constructs were kindly provided by Dr. Patrice Dunoyer). To test for suppression of miRNA-induced GFP silencing, GFP171.1 was co-infiltrated with binary constructs coding for CPsV proteins. As a positive control on VSR activity, the *Tomato spotted wilt virus* (TSWV) NSs gene was used (Schnettler et al., 2010). *N. benthamiana* were infiltrated with of *A. tumefaciens* strain GV3101, harboring GFP171.1 or GFP171.2, at OD_{600} = 0.2. CPsV proteins or TSWV NSs were co-infiltrated with the sensor constructs at OD_{600} = 0.4. When MP^{CPsV} and 24 K^{CPsV} were co-expressed half-dose of each culture was used. Infiltrated patches were used for total RNA and protein extraction, then analyzed by Northern blotting as described in Reyes et al. (2011) and by Western blotting as described in Peña et al. (2012).

2.3. Semiquantitative RT-PCR

Total RNA was extracted from *N. benthamiana* leaves using TriReagent® (Molecular Research Center, Inc. Cincinnati, OH, USA.). Semi quantitative RT-PCR was performed on RNA samples treated with DNAse (Promega, USA) according to the manufacturer's instructions. Reverse transcriptase reactions were performed with MMLV-RT (Promega, USA) and oligo-dT as reverse primer. PCR was performed with primers for GFP (GFP5_forw: 5′-dGTGCAGGAAAGGACCATCTTCT-3′; GFP5 rev: 5′-dGATTCCCTTAAGCTCGATCCTGTT-3′) and actin (ActinL: 5′-dGATGGACAAGTCATCACCATTG-3′ ActinR: 5′-dCTGAGGACAATGTTTCCGTACA-3′). RT-PCR conditions and resolution limits were evaluated using samples expressing pGD (empty vector) and p19 as negative and positive controls for VSR activity and thus lower and upper gfp detection limits were set. The PCR amplification was adjusted and stopped at cycle 20. Densitometric analysis was carried out with ImageJ software (https://imagej.nih.gov/ij/) normalized to actin as internal control.

2.4. Recombinant protein expression and purification

His-tagged MP^{CPsV} protein was expressed in *E. coli* BL21(DE3)-CodonPlus-RIL and His-tagged 24 K^{CPsV} protein was expressed in *Trichoplusia ni* Hi5 cells, using the Bac-to-Bac® Baculovirus Expression System. Both proteins were extracted from cells using 6 M guanidinium chloride and subsequently purified by affinity column chromatography using a Ni-NTA His·Bind® Resin (Novagen) under denaturing conditions. Protein samples were dialyzed against PBS buffer to remove final traces of urea. Protein fractions were flash frozen in aliquots in liquid nitrogen and stored at −80 °C until further use. Protein concentrations were determined by the Bio-Rad protein assay according to the manufacturer's protocol and the quality of protein purification analyzed by SDS-PAGE and subsequent staining with Coomassie brilliant blue (Fig. 3C).

2.5. In vitro synthesis of radio-labelled dsRNA

Long dsRNA molecules 114-nt in size were generated by T7 RNA polymerase (Promega) transcription on a gel-purified (High Pure PCR purification kit; Roche) eGFP template, containing T7 RNA polymerase promoter sequences at both ends, in the presence of [α-^{32}P]CTP (Perkin Elmer) essentially as described in Schnettler et al. (2010). Preparation of radio-labelled siRNAs was performed via end-labelling. To this end 100 pmol of double-straded siRNAs 21-nt long coming from *hygromycin* gene were dephosphorylated with Calf intestinal alkaline phosphatase (CIAP) (New England Biolabs) following the manufactureŕs recommendation. Next, dephosphorylated siRNAs were end-labelled with [γ-^{32}P]ATP (Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs) and then PAGE purified essentially as previously described (Schnettler et al., 2010).

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA procedures with purified his-tagged viral proteins were performed as described (Hemmes et al., 2007), in which each 15 ul binding reaction contained 100 pM radio-labelled dsRNA and purified MP^{CPsV}, 24 K^{CPsV} or *Groundnut ringspot tospovirus* (GRSV) NSs. Binding reactions were incubated for 10 min at 4 °C and then loaded on gel. Complexes of viral proteins with dsRNAs were resolved on a non-denaturing acrylamide gel (5% for long dsRNAs or 8% for siRNAs) in 0.5× TBE running buffer at 4 °C at 150 V for 1.5 h. After electrophoresis, gels were vacuum-dried at 80 °C for 30 min and exposed overnight to a phosphor image screen (Molecular Dynamics Typhoon Phosphorimager; Amersham Biosciences).

2.7. Production of transgenic $24K^{CPsV}$ N. benthamiana transgenic plants

Transgenic *N. benthamiana* plants were generated by infection of leaf disks with *A. tumefaciens* GV3101 carrying pBinGFP/24 (Zanek et al., 2008) as previously described by Peña et al. (2012). $24K^{CPsV}$ protein was detected with anti–$24K^{CPsV}$ antiserum followed by anti-rabbit-IgG conjugated to alkaline phosphatase (GibcoBRL, Life Tech-

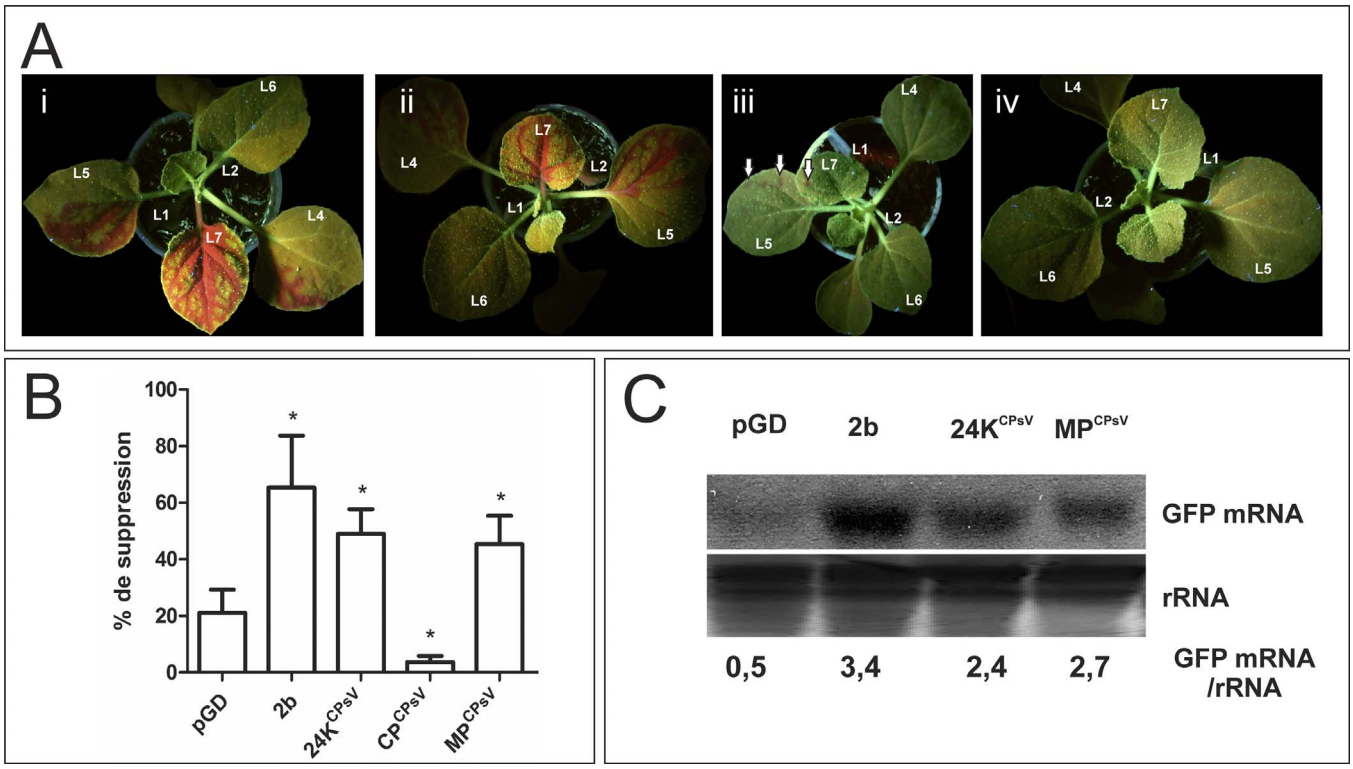

Fig. 2. Systemic silencing suppression activity of MP^{CPsV} and $24K^{CPsV}$ proteins.Panel A: *N. benthamiana* 16c plants were agroinfiltrated for the expression of GFP together with empty vector (pGD) or the viral genes in leaves L1 and L2. 14 days after infiltration plants were monitored under UV light to evaluate suppression of systemic GFP silencing on top leaves (L4, L5, L6 and L7). Representative plants with different degree of silencing suppression are shown in i-iv). i) and ii) were considered as not suppressed plants, the plants showed here were agroinfiltrated for the expression of GFP + empty vector (pGD). iii) and iv) were considered as suppressed plants, the plants showed here were agroinfiltrated for the expression of GFP + 2b. Arrows in iii) indicate small regions with GFP silencing. Panel B: The percentage (%) of systemic silencing suppression was calculated by counting the number suppressed plants over the total number of plants. The% of suppression of five independent experiments is shown here (15 plants per treatment).* indicates significant differences with pGD (negative control) at $P < 0.05$ using a *t*-test. Panel C: Northern blot detection of GFP-mRNA. Ethidium bromide-stained rRNAs is shown as loading control. The relative amount of GFP mRNA was calculated using the signal for rRNAs as internal control, and is indicated at the bottom.

nologies).

2.8. miRNA detection

For miRNA analysis by Northern blot, total RNA was extracted from *N. benthamiana* leaves using TriReagent® (Molecular Research Center, Inc. Cincinnati, OH, USA.). Total RNA was resolved on denaturing polyacrylamide gels, transferred to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA) using a Bio-Rad (Hercules CA, USA) transfer unit and chemically fixated according to Pall and Hamilton (2008). Northern blots were hybridized to ^{32}P-radiolabelled oligodeoxynucleotides complementary to the mature miRNA or to the U6 snRNA sequence as internal loading control. Hybridizations were performed at 50 °C overnight and signals were detected by autoradiography. The signals for miRNAs were quantified using ImageJ software normalized to U6.

3. Results

3.1. MPs and 24–25K proteins of CPsV and MiLBVV suppress local GFP RNA silencing

To test for VSR activity of CPsV or MiLBVV proteins, an agroinfiltration leaf patch assay (Li and Ding, 2006) was performed in wild type *N. benthamiana* plants. To this end, *Agrobacterium tumefaciens* carrying a binary GFP gene construct was co-infiltrated with *A. tumefaciens* containing binary constructs coding for 24K ($24K^{CPsV}$), 48K (CP^{CPsV}) or 54K (MP^{CPsV}) as described in Materials and Methods. As negative and positive controls for VSR activity, a binary vector without an insert (pGD) or containing the *Tomato bushy stunt* virus (TBSV) p19 gene respectively (Voinnet et al., 2003), were included. Leaf patches agroinfiltrated with a binary GFP construct in the presence of the empty binary vector (pGD), revealed a very weak GFP fluorescence 5 days post infiltration (pi) and indicating the effective induction of GFP RNA silencing (Fig. 1A, first panel). A similar result was obtained when GFP was expressed in the presence of a binary construct coding for CP^{CPsV} (Fig. 1A, third panel). In the presence of the $24K^{CPsV}$ and MP^{CPsV} proteins GFP signals were consistently stronger compared to the negative control (Fig. 1A, second and fourth panels) although not as strong as observed with the TBSV p19 positive control (Fig. 1A, fifth panel). The results clearly indicated that the $24K^{CPsV}$ and MP^{CPsV} proteins were both able to suppress local GFP RNA silencing. In order to quantify these observations, the level of suppression was measured using the fluorescence scale showed in Fig. 1B and the average for the total number of leaves calculated from the 5 independent experiments. The CP did not show suppressor activity (0%), while the $24K^{CPsV}$ and MP^{CPsV} proteins exhibit similar suppression activity (50% and 47,5% respectively). The results were further supported by Western blot detection of GFP at 5 days post agroinfiltration from leaf patches agro-infiltrated with GFP and $24K^{CPsV}$ or MP^{CPsV}, and the positive (p19) control, while hardly any GFP was detected during co-expression with CP^{CPsV}, similar to the negative control (Fig. 1, panel C). When the GFP protein signal was quantified relative to the Coomassie brilliant blue (CBB)-stained protein load, values for the ratio GFP/CBB from samples containing $24K^{CPsV}$ or MP^{CPsV} were in between those from the negative (0.07) and positive p19 (0.8) control (0.2 and 0.3 for $24K^{CPsV}$ and MP^{CPsV}, respectively). To rule out that the absence of any GFP silencing suppression with CP^{CPsV} was due to the absence of viral protein expression, CP^{CPsV} was detected by western blot, as well as all other viral proteins (data not shown). As further proof for the

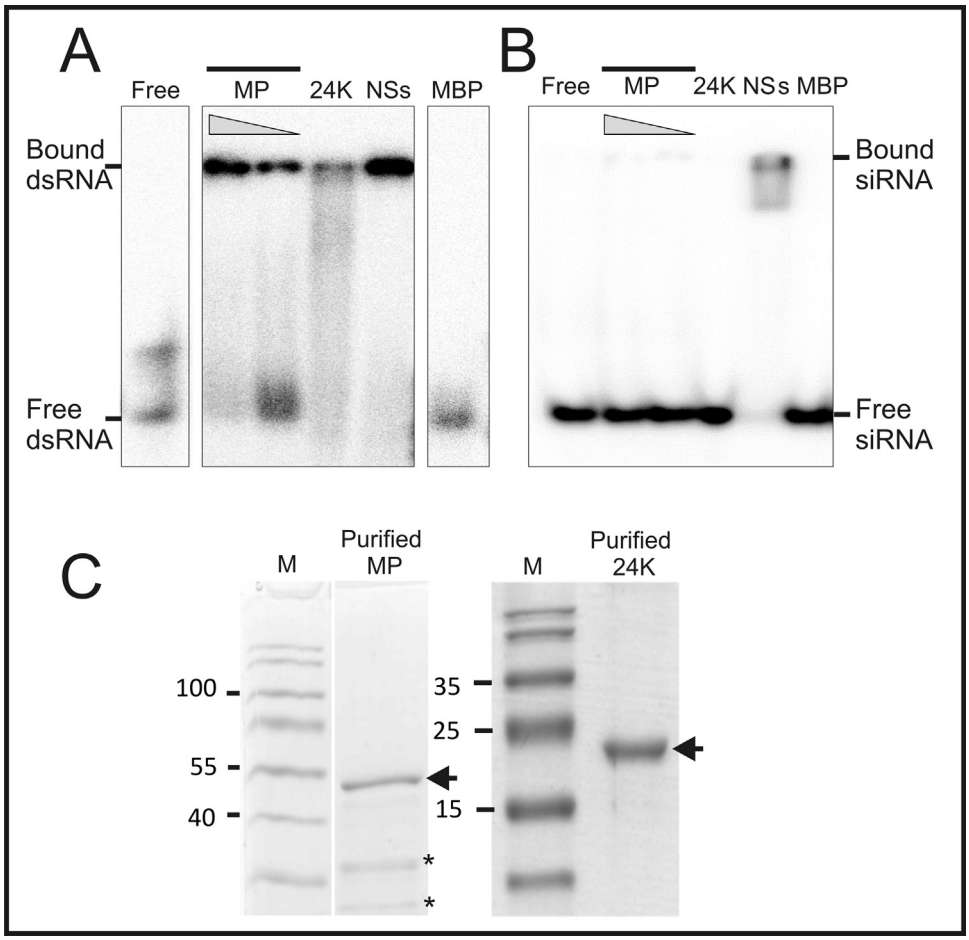

Fig. 3. Affinity of MP^{CPsV} and $24K^{CPsV}$ for 114-nt dsRNA. and 21-nt siRNAs.Electrophoretic mobility shift assays of long and small dsRNA in the presence of purified recombinant MP^{CPsV} protein at two concentrations (2.7 μM or 1.12 μM), or 24 K^{CPsV} at 1.12 μM. Proteins were incubated 20 min at room temperature, with 100 pmol of a synthetic radio-labelled dsRNA of 114 nt (panel A) or siRNA (panel B). The NSs protein of *Groundnut ringspot virus* (GRSV) was used as positive control. Two negative controls were analyzed in parallel: RNA incubated with buffer reaction, or with MBP expressed in *E. coli* and purified by chromatography. Position of Protein-dsRNA complexes and free dsRNA are indicated. Panel C: SDS-PAGE analysis of the purified MP^{CPsV} and 24 K^{CPsV} proteins. M: protein maker, * indicate MP^{CPsV} cleavage products (Robles Luna unpublished results).

occurrence of GFP silencing and suppression by $24K^{CPsV}$ and MP^{CPsV}, the accumulation of GFP-mRNA and derived siRNA was assessed by semiquantitative RT-PCR and Northern blot, respectively. While the relative level of GFP mRNA normalized to actin (internal control) for samples containing CP^{CPsV} was about 0.6 and similar to the negative control, those in samples containing $24K^{CPsV}$ or MP^{CPsV} were significantly higher (1.0–1.2), although less than the positive p19 control (2.3) (Fig. 1D, left panel). Concomitantly, the accumulation of GFP-derived siRNAs in the samples containing $24K^{CPsV}$ or MP^{CPsV} was consistently lower than in the samples containing the negative (empty pGD vector) control or CP^{CPsV} (Fig. 1D, right panel). In the presence of the strong VSR p19, hardly any siRNAs were visualized (Fig. 1D, right panel). Altogether, these results indicated that $24K^{CPsV}$ and MP^{CPsV} proteins, but not CP^{CPsV}, were able to suppress local RNA silencing.

To further strengthen these observations and simultaneously verify whether this behaviour was generic for other ophiovirus, the homologous $25K^{MiLBVV}$ and MP^{MiLBVV} proteins of MiLBVV were likewise tested on VSR activity during local GFP silencing assays. The results obtained (Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.virusres.2017.04.013) indicated that similar to CPsV, the $25K^{MiLBVV}$ and MP^{MiLBVV} proteins, but not CP^{MiLBVV}, were able to suppress local RNA silencing (Supplementary Fig. S1A and B in the online version at DOI: http://dx.doi.org/10.1016/j.virusres.2017.04.013). Results from GFP Western blot analysis (Supplementary Fig. S1C in the online version at DOI: http://dx.doi.org/10.1016/j.virusres.2017.04.013) and semiquantitative RT-PCR on GFP mRNA were in agreement with this finding (Supplementary Fig. S1D in the online version at DOI: http://dx.doi.org/10.1016/j.virusres.2017.04.013).

3.2. MP^{CPsV} and $24K^{CPsV}$ proteins suppress systemic GFP RNA silencing in *N. benthamiana* 16c plants

To determine whether the CPsV proteins also were able to impede the spread of the systemic silencing signal, cultures bearing binary constructs coding for CP^{CPsV}, $24K^{CPsV}$, and MP^{CPsV} were co-infiltrated with cultures bearing a GFP construct on leaves of *N. benthamiana* 16c. After 14 days pi, the plants were observed under UV light (Fig. 2A) and percentage of suppression was calculated (Fig. 2B), as described in Material and Methods. Results from 5 independent experiments showed a 49% and 45% systemic silencing suppression for $24K^{CPsV}$ and MP^{CPsV} respectively. Significant differences in systemic GFP silencing between CP and the negative control was observed (Fig. 2B), suggesting that CP^{CPsV} somehow enhance systemic RNA silencing. To further support the occurrence of GFP silencing suppression, the production and relative levels of GFP-mRNA were analyzed in the systemic leaves (Fig. 2C). Hardly any GFP mRNA was detectable by Northern blot in the negative control, indicative of strong systemic GFP silencing. However, in the presence of $24K^{CPsV}$ or MP^{CPsV} higher levels of GFP-mRNA were accumulating in systemic leaves. Concomitant with these, a decrease in the levels GFP-siRNA was observed on these leaves (data not shown). Systemic leaves from the positive CMV 2b control, as expected, contained high levels of GFP mRNA and concomitant low levels of

GFP-siRNAs. These data altogether indicate that $24K^{CPsV}$ and MP^{CPsV}, but not CP^{CPsV}, also exert suppression of systemic RNA silencing.

3.3. MP^{CPsV} and $24K^{CPsV}$ proteins exhibit affinity for long but not short dsRNAs

Many viral proteins exert VSR activity through binding of long and/or small dsRNA to prevent their processing into siRNAs and/or loading into RISC and systemic spread (Deleris et al., 2006; Goto et al., 2007; Merai et al., 2005). To analyse whether the two viral proteins showing suppression activity (MP^{CPsV} and $24K^{CPsV}$) were also able to bind short and/or long dsRNA molecules, electrophoretic mobility shift assays (EMSAs) were performed. To this end, MP^{CPsV} and $24K^{CPsV}$ were expressed in *E. coli* or insect cells respectively, and purified by affinity chromatography via a His-tagged fusion. After purification, proteins were incubated with radiolabeled 114-nt dsRNA or 21-nt siRNA molecules and subsequently analyzed by EMSA on native acrylamide gels (Materials and Methods). The results revealed a shift for the 114-nt dsRNA in the presence of MP^{CPsV} and $24K^{CPsV}$ (Fig. 3A) but not for 21 nt siRNAs (Fig. 3B) indicating that both MP^{CPsV} and $24K^{CPsV}$ were able to bind only long dsRNA. Very faint shifted bands were observed for siRNAs and MP^{CPsV}, but such a poor binding at that high protein concentrations (2700 nM or 1120 nM) reflects very low or no significant siRNA binding affinity. As a positive control the *Groundnut ringspot virus* (GRSV) NSs protein showed a shift with siRNAs and dsRNAs, as previously reported (Schnettler et al., 2010) at concentrations as low as 35 nM and long dsRNA at 140 nM (Hedil and Kormelink, 2016). Purified N-terminally His-tagged maltose binding protein (MBP), used as negative control, did not reveal any shift with dsRNAs or siRNAs, even at the highest concentrations tested (Fig. 3A and B).

3.4. Transgenic expression of $24K^{CPsV}$ and MP^{CPsV} proteins in N. benthamiana produces developmental defects and alters miRNA accumulation

Viral proteins exhibiting VSRs have been expressed as transgenes in different host plants to study their role. Some of these transgenes severely disturb plant phenotypes through alteration of miRNA accumulation (Lewsey et al., 2009; Soitamo et al., 2011, 2012; Siddiqui et al., 2008). To analyse whether the CPsV proteins indeed also interfere in the miRNA pathway and cause for abnormal plant phenotypes, *N. benthamiana* plants expressing CP^{CPsV}, MP^{CPsV} (Peña et al., 2012) or $24K^{CPsV}$ (generated in this work) were analyzed. Independent F0 transgenic lines were self-pollinated to obtain seeds of the next generations (F1 and F2). Seedlings from those were tested for viral protein expression and they were detected in all the selected transgenic plants. Lines CP:02 and 54:06 showed the highest expression levels for CP^{CPsV} and $54K^{CPsV}$ respectively (Peña et al., 2012). $24K^{CPsV}$ lines had similar protein accumulation (Supplementary Fig. S2 in the online version at DOI: http://dx.doi.org/10.1016/j.virusres.2017.04.013). Two lines corresponding to each construct were selected and were phenotypically examined and categorized according to Siddiqui et al. (2008). Whereas lines expressing CP^{CPsV} (CP:02 and CP:04) did not show any developmental alterations comparing to wild type plants (data not shown), those expressing MP^{CPsV} (54:01 and 54:06) exhibited clear changes in phenotype. Plants from line 54:01 showed mild stunting, rolling of the leaf blades into a mild cup-shaped form and blistered leaf epidermis (Fig. 4A and B). Plants of line 54:06 also showed mild stunting and cup shaped leaves (Fig. 4C and D). Phenotypic alterations caused by the expression of $24K^{CPsV}$ (lines 24:46 and 24:52) included mild stunting and multiple branching as well as small and malformed flowers (Fig. 4E and F) and scarce seed production.

To analyse whether the transgenic expression of MP^{CPsV} and $24K^{CPsV}$ indeed interfere in the miRNA pathway altering their processing, accumulation and activity as recently described in citrus (Reyes et al., 2016), leaf samples were collected from the transgenic *N. benthamiana* lines (CP, 54 and 24) and total RNA prepared. Northern blots analysis were performed using probes against two conserved and well-characterized miRNAs, i.e. miR156 and miR167. The level of miRNA expression in transgenic lines was quantified and normalized to the accumulation in non-transgenic control lines (Fig. 5). Whereas CP-transgenic lines did not show significant differences with the non-transgenic control, the expression levels of miR156 and miR167 were clearly up-regulated in the transgenic plants expressing $24K^{CPsV}$. In the lines expressing the MP^{CPsV} only miR156 was up-regulated (Fig. 5). Compared to non-transgenic control, miR156 increased two folds in lines 24 and three folds in lines 54. In lines 24 a two folds up-regulation of miR167 was observed. Considering that transgenic lines 24 showed floral abnormalities and miR172 was earlier reported to regulate the floral homeotic gene Apetala2 (Mlotshwa et al., 2006), lines 24 were also assessed for accumulation of miR172 (Fig. 5). Relative to the non-transgenic control plants, an up-regulation of miR172 was observed in line 24:52 (2.0 folds).

3.5. Co-expression of $24K^{CPsV}$ and MP^{CPsV} exerts a collaborative inhibition on miRNA-induced RNA silencing

Viral proteins might compromise either the biogenesis or a later stage of the miRNA pathway like the RISC assembly and/or the final effector function. To further substantiate the findings that $24K^{CPsV}$ and MP^{CPsV} interfere in the miRNA-induced RNA silencing pathway, transient silencing assays were performed with sensor constructs (Parizotto et al., 2004). To this end, leaves of *N. benthamiana* were agroinfiltrated for the expression of GFP171.1 and GFP171.2 target constructs with or without the viral suppressors candidates $24K^{CPsV}$ and/or MP^{CPsV}. In these sensors a full complementary miR171 binding site was placed downstream of the STOP codon of GFP ORF allowing miR171-mediated silencing of the GFP171.1 mRNA, while GFP171.2 possesses a mutant miR171 target site (negative control) and does not drive miR171-guided RNA silencing (Parizotto et al., 2004). As a positive control for suppression of miRNA-induced RNA silencing, the NSs of *Tomato spotted wilt virus* (TSWV) was included (Schnettler et al., 2010). At five days pi GFP fluorescence was monitored under UV light (Fig. 6A), and the infiltrated leaf patches collected for RNA and protein isolation. As expected, endogenous miR171-driven RNA silencing downregulated GFP171.1, but not the negative control GFP171.2 (Fig. 6A, upper panel), that coincided with reduced levels of the GFP mRNA and protein (Fig. 6B). When $24K^{CPsV}$ or MP^{CPsV} were expressed, miR171-induced GFP silencing was clearly suppressed (Fig. 6A, second and third panel), and even stronger when both VSRs were combined (Fig. 6A, fourth panel). These results were consistent in three independent assays., Besides, ratios of GFP mRNA and protein levels for GFP171.1 relative to GFP171.2 detected in samples infiltrated with VSRs was higher compared with the control. GFP mRNA GFP171.1/GFP171.2 ratios were very low for the control (silenced) sample with a value of 0.1. Ratios from samples additionally infiltrated with $24K^{CPsV}$ or MP^{CPsV} were substantially higher (0.54 and 0.5, respectively) and more in the range found with TSWV NSs (0.7) (Fig. 6B). When $24K^{CPsV}$ and MP^{CPsV} were combined at half-dose, the relative level went even up to 1.2 (Fig. 6B), indicating a collaborative ability to suppress miRNA-induced RNA silencing. Western blots results were consistent with those of Northern blots.

4. Discussion

Nowadays virtually all plant viruses analyzed encode at least one silencing suppressor. The enormous diversity in sequence and structure of VSRs indicate that they likely have evolved independently (Csorba and Burgyan, 2016). Here, we have identified the cell movement protein (54K/55K) and the 24/25K proteins from two different ophioviruses, *in casu* CPsV and MiLBVV, as VSR. They were not only

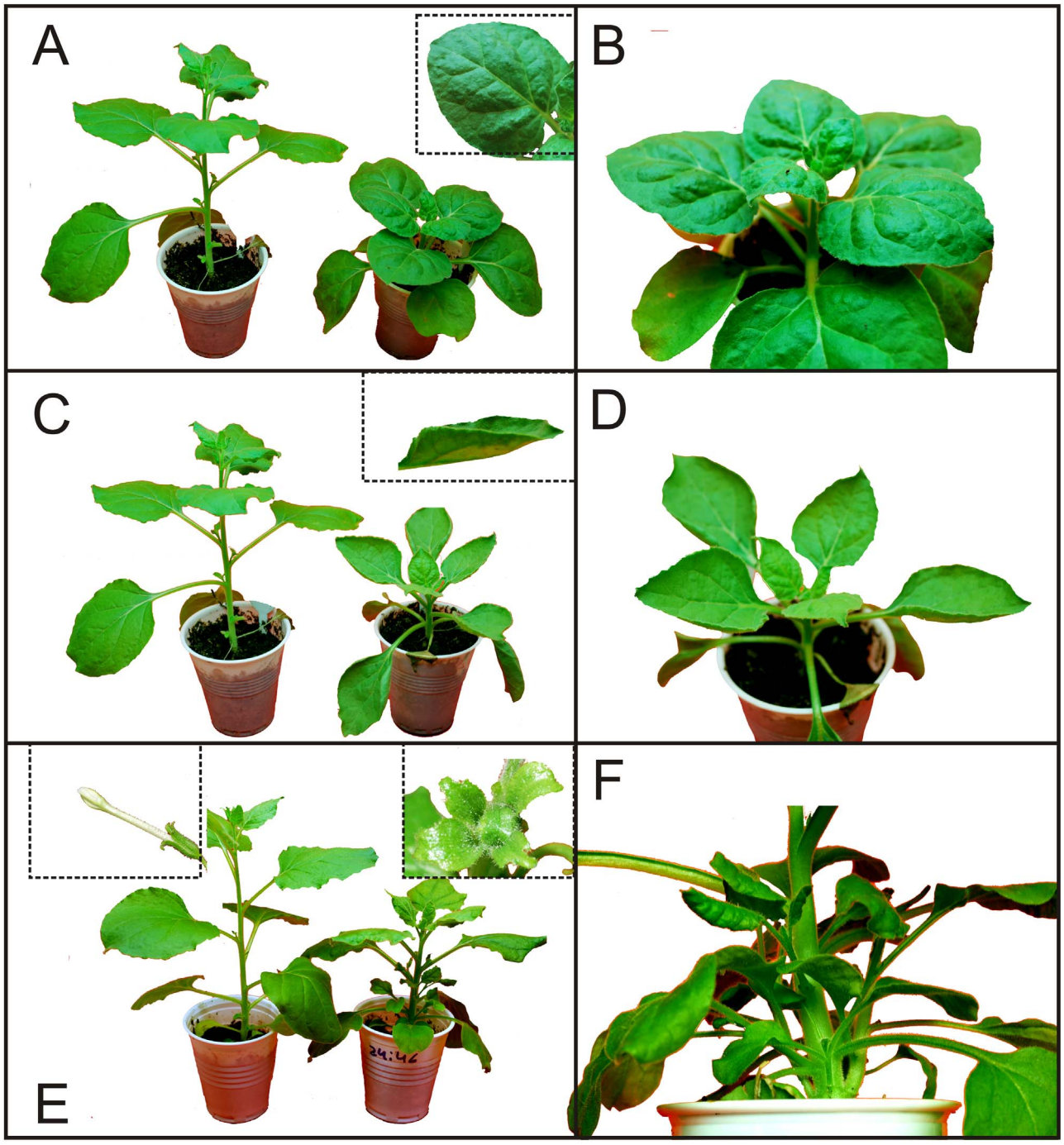

Fig. 4. Ectopic expression of MP^{CPSV} and $24K^{CPSV}$ proteins in *N. benthamiana* plants.Panel A: Alterations in the transgenic line 54:01 (right) expressing the MP^{CPsV} including mild stunting, rolling of the leaf blades into mild cup-shaped form and blistered leaf epidermis (see right inset) compared to wild type plants (left). Panel B: detail of the phenotype of a representative plant of line 54:01. Panel C: alterations in the transgenic line 54:06 (right) expressing MP^{CPsV} including mild stunting, cup-shaped form (see right inset) compared to wild type plants (left). Panel D: detail of the phenotype of a representative plant of line 54:06. Panel E: alterations in the transgenic line 24:46 (right) expressing 24 K^{CPsV} including mild stunting, branched, and small, malformed flowers (right inset) compared to wild type plants (left, left inset). Panel F: detail of the phenotype of a representative plant of line 24:46.

shown to suppress GFP silencing during transient *A. tumefaciens* co-infiltration assays in *N. benthamiana* plants, but also able to impede the spread of a systemic silencing signal, although in both situations less strong than TBSV p19 and CMV 2b. The silencing enhancement (instead of suppression) observed by CP^{CPsV} in the systemic assays, could indicate a mechanism by which CP modulate CPsV accumulation or/ and alter cellular environment to a more suitable for viral proliferation, maintaining the equilibrium between efficient virus multiplication and preservation of the host integrity. Mechanisms by which this effect is carried out could be diverse including targeting host defence factors by viral siRNAs and virus-induced RNA silencing (Miozzi et al., 2013; Qi et al., 2009; Shimura et al., 2011) or through enhancing cell-to-cell or systemic spread of RNA silencing (Zhou et al., 2008; Lacombe et al., 2010).

VSRs have been demonstrated to inhibit practically all steps of the RNA silencing machinery to establish a successful infection. Those include the silencing initiation-, effector- and, amplification phase during post transcriptional gene silencing (PTGS) of RNA viruses, but also chromatin modification and modulation of host gene transcription during transcriptional gene silencing (TGS) by DNA viruses (Csorba and

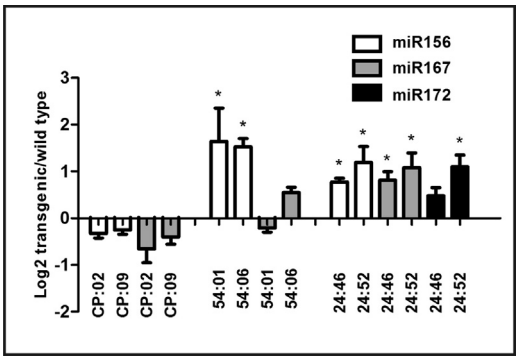

Fig. 5. MicroRNA accumulation in transgenic *N. benthamiana* lines expressing CP (lines CP), MP^{CPsV} (lines 54) and $24K^{CPsV}$ (lines 24).The average level of miRNAs, and standard error, are calculated from three independent samples. The graphs were plotted as log2 of the ratio transgenic/wild type; * Indicates significant differences with non transgenic control samples at $P < 0,05$ using a two tailed paired *t*-test.

Burgyan, 2016). Sequestration of siRNAs is one of the most widely used strategies of VSRs (p19, Hc-Pro, P21, NS3, NSs) (Hemmes et al., 2007; Lakatos et al., 2006; Merai et al., 2006; Silhavy et al., 2002; Hedil and Kormelink, 2016). The MP^{CPsV} and $24K^{CPsV}$ proteins, in contrast, only exhibit affinity for long dsRNAs and not significant or very low affinity (for the case of MP^{CPsV}) for siRNAs *in vitro* (Fig. 3B) and, GFP-derived siRNAs are still observed to accumulate in local silencing assays (Fig. 1D). The binding of long dsRNA by MP^{CPsV} and $24K^{CPsV}$ could lead to inhibition of the initiation silencing phase by preventing dsRNA dicing. Earlier, TCV p38 and CMV 2b have been shown to bind long dsRNA and thereby block the biogenesis of vsiRNAs (Deleris et al., 2006; Goto et al., 2007). Considering that siRNAs are still accumulating at detectable levels during GFP RNA silencing assays in the presence of MP^{CPsV} and $24K^{CPsV}$, we can infer that the affinity of these proteins for long dsRNA is not strong enough to prevent all dicing events. Binding long dsRNA by MP^{CPsV} may also be related to the cell-to-cell movement function of this protein (Robles Luna et al., 2013). However, other functions related to the long dsRNA binding capacity of MP^{CPsV} and $24K^{CPsV}$ cannot be ruled out. In agreement with the affinity for long dsRNA is the earlier observed *in vivo* association of $24K^{CPsV}$ protein fused to GFP to pre-miRNA precursors in *N. benthamiana* plants, causing their misprocessing and altered miRNA biogenesis profile (Reyes et al., 2016).

Interference at the RDR-mediated amplification cycle by VSRs is another strategy being used by viruses. Blocking this step leads to inhibition of the cell-autonomous silencing amplification, required to mount a strong local antiviral RNAi response, and to activate systemic silencing, the non-cell autonomous branch of the RNAi pathway in distant tissues (Csorba and Burgyan, 2016; Ren et al., 2010; Schwach et al., 2005). Since $24K^{CPsV}$ and MP^{CPsV} proteins do not bind siRNAs, and therefore do not prevent their movement to activate systemic silencing, the observation that these proteins are still able to suppress systemic silencing tempts us to speculate on a possible interference during the RNAi amplification cycle as well. An example for this is V2 from *Tomato yellow leaf curl virus* (TYLCV) that may compete with SGS3 (the RDR6 cofactor) for dsRNA binding. Long dsRNAs are RDR6/SGS3 substrates or intermediates during vsiRNA amplification (Fukunaga and Doudna 2009; Kumakura et al., 2009). Whether this applies to the CPsV VSRs as well, in light of their long dsRNA binding ability remains to be investigated.

Another way in which VSR proteins may interfere during the effector stage of silencing is to block the maturation of a functional RISC assembly or inhibit the activity of an activated si/miRNA-loaded RISC complex (Csorba and Burgyan, 2016). P1 from *Sweet potato mild mottle virus* (SPMMV) interacts directly with siRNA and/or miRNA-loaded AGO1 present in the holo-RISC but not minimal-RISC through GW/WG-motifs (AGO-hook) and thereby inhibits RISC activity (Csorba and Burgyan, 2016; Giner et al., 2010; Szabo et al., 2012). The NSs protein from TSWV also contains WG residues and is postulated to be involved in AGO-interaction since mutation of this motif abrogated its VSR activity in a local GFP silencing assay (de Ronde et al., 2014; Hedil et al., 2015). GW motifs are lately described as implicated in nucleolar localization and also sRNA binding capability (Pérez-Cañamás and Hernández, 2015). Interestingly, the $24K^{CPsV}$ protein similarly contains

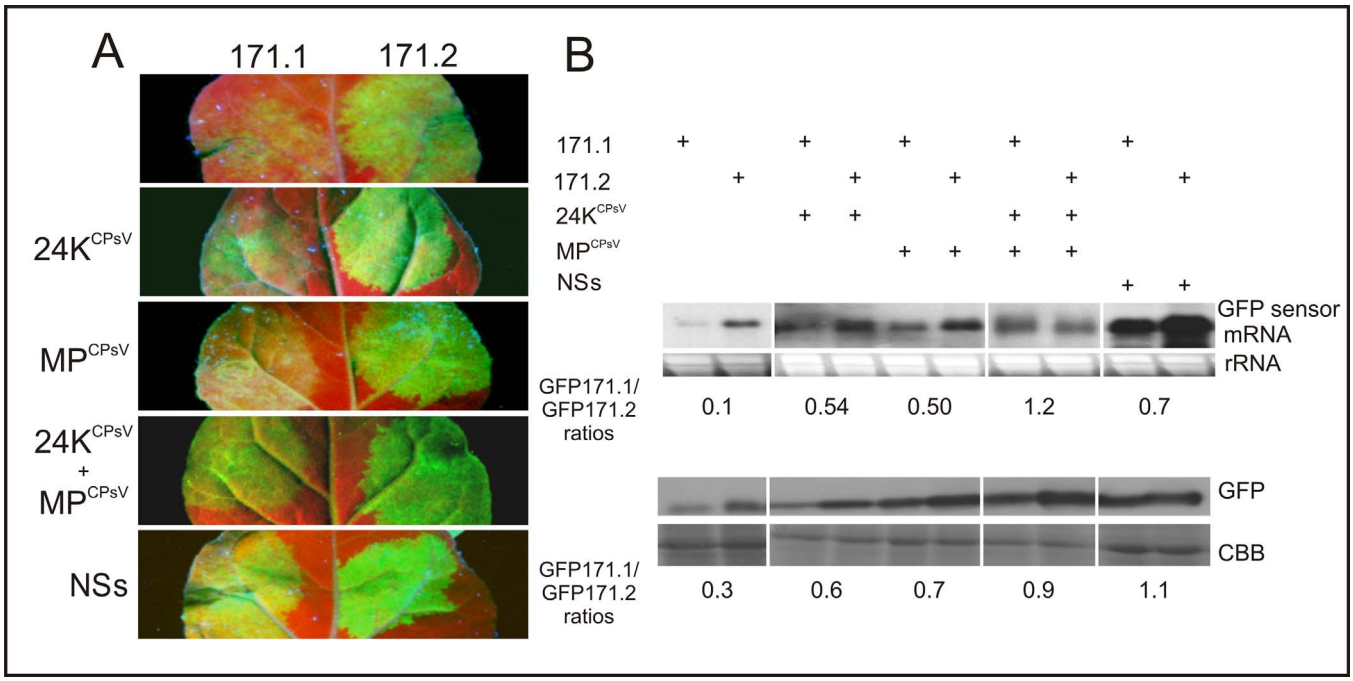

Fig. 6. MP^{CPSV} and $24K^{CPSV}$ interference on the miRNA pathway in *N. benthamiana* plants as visualized by a GFP-miRNA sensor assay.Constructs expressing MP^{CPSV} and 24 K^{CPSV} proteins were agroinfiltrated with GFP-171.1 (left side of the leaf) or GFP-171.2 (right side of the leaf) sensor constructs and monitored under UV light after 5 days post-infiltration (Panel A). Northern blot detection of GFP mRNA from leaf samples shown in A, and quantified relative to rRNA as internal control (B, upper panel). Western immunoblot detection of GFP from the infiltrated leaf patches. Protein samples were CBB stained to verify for protein load (B, lower panel). GFP-171.1/GFP-171.2 band density ratios is shown under both panels.

a GW motif, but whether this would mediate binding and subsequent destabilization of AGO1 or any other effect in RNAi is not known yet.

We show that $24K^{CPsV}$ and MP^{CPsV} also exhibit inhibition on miRNA-induced RNA silencing as revealed through the sensor assay. The effect on miRNA effector function exerted by $24K^{CPsV}$ could be explained by inhibition of miRNA171 maturation as already reported (Reyes et al., 2016). Although in this study MP^{CPsV} has been observed to bind long dsRNA, this protein was earlier not observed to interact with pre-miRNA171 (Reyes et al., 2016). Whether the interference in the miRNA pathway by MP^{CPsV} is thus a consequence of an inhibitory effect along the effector phase of RNA silencing, such as AGO1 degradation/inhibition, remains to be investigated. Although speculative, considering that a collaborative effect in suppression of miRNA-induced RNA silencing was manifested when both CPsV proteins were present, it supports the possible idea that each protein may target different steps of the RNAi machinery and would render accumulating suppression during a co-expression. It could also be the case that an interaction between $24K^{CPsV}$ and MP^{CPsV} is needed to act in miRNA-induced RNA silencing.

In agreement with the interference of MP^{CPsV} and $24K^{CPsV}$ on the miRNA pathway, is the observation that their constitutive expression in *N. benthamiana* plants leads to abnormal plant phenotypes associated to alteration in miRNA accumulation. Transgenic expression of VRSs can cause developmental abnormalities resembling disease symptoms (Kasschau et al., 2003; Dunoyer et al., 2004; Siddiqui et al., 2008). Tang et al. (2010) reported early leaf-curl phenotype in *Arabidopsis thaliana* plants overexpressing miR156b, coinciding with the phenotype observed for transgenic lines 54. In the case of lines 24 a significant up-regulation of miR167, related to the floral abnormalities, was observed. Many reports in these cases have shown an increment on miRNA levels after expression of VSRs (HC-Pro from potyviruses, NSs from tospoviruses), explained by stabilization of the miRNA duplexes in protein viral complexes that may include RISC reducing miRNA turnover (Kasschau et al., 2003; Schnettler et al., 2010). This apparent discrepancy occurs between two different expression systems: transgenic *N. benthamiana*, a symptomless herbaceous host expressing only one viral protein, and the natural host systemically infected, where all viral components are present. Changes in temporal and/or subcellular localization, interactions with host component together with the complete viral machinery may contribute to the observed differences.

In conclusion, VSRs from ophioviruses, as shown for CPsV and MiLBVV, would be capable to modulate the antiviral RNAi defence at various points, by their 24/25K and cell-to-cell movement protein (MP). In light of the nuclear localization of the 24K protein, long dsRNA binding would mostly comprise pri/pre-miRNA binding and misprocessing (Reyes et al., 2016). MP^{CPsV} protein in contrast, would involve dsRNA binding to inhibit the siRNA pathway and possibly competing for substrates during the RNAi amplification cycle in the cytoplasm. Whether, in light of their subcellular localization, both VSRs act strictly separate on different branches of the RNAi pathway, and are able to interact at a certain stage in the cytoplasm to exert a combined stronger RNAi suppression, will be challenging questions for the future.

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