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Short communication

A sensitive and rapid RNA silencing suppressor activity assay based on alfalfa mosaic virus expression vector

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ABSTRACT

Plant viruses express RNA silencing suppressor (RSS) proteins to counteract plant defence mechanisms. Here, we describe a method to assess the RSS activity based on an alfalfa mosaic virus (AMV) RNA 3 expression vector and transgenic *Nicotiana tabacum* plants that express the P1 and P2 subunits of the AMV replicase (P12 plants). Inoculation of P12 plants with different AMV RNA 3 constructs expressing different HC-Pro mutants that differ in their RSS capabilities, revealed a perfect correlation between necrotic lesions on inoculated leaves and RSS activity. Protoplast analysis showed that the RSS activity correlated with the accumulation of the AMV RNAs. A direct comparison between three RSS activity assays and the AMV-P12 system revealed that the coat protein of carnation mottle virus displays RSS activity with the four assays and reduced the accumulation of the siRNAs.

RNA silencing acts as a basal defense mechanism against viruses and is one of the main plant immune responses against viral pathogens (Vaucheret, 2006; Ding and Voinnet, 2007). To block or attenuate plant host defense mechanisms, particularly RNA silencing, plant and animal viruses express RNA silencing suppressor (RSS) proteins acting at different steps of the silencing process. A large number of RSSs have been identified using different procedures (Li and Ding, 2006; Moissiard and Voinnet, 2004; Qu and Morris, 2005). The most common assay is the 'patch' technique (Voinnet et al., 1998) based on the infiltration of *Agrobacterium tumefaciens* cultures harboring the putative RSS and a reporter gene (usually the green fluorescent protein gene, GFP) in *Nicotiana benthamiana*, in which the natural silencing process of the overexpressed reported gene is delayed by the presence of a RSS. Another assay is based on viral vectors, such as potato virus X, in which the expression of a RSS is associated with a more aggressive viral infection (Voinnet et al., 1999). Other approaches for RSS screening are based on functional complementation of defective viral mutants (Chiba et al., 2006; Powers et al., 2008) or in viral vectors in which the RSS is correlated with symptoms appearance (Guilley et al., 2009). Here, we describe a simple experimental approach to screen for RSS activity based on a viral system derived from alfalfa mosaic virus (AMV).

AMV is the type member of the *Alfamovirus* genus within the family *Bromoviridae* (Pallas et al., 2013). Its genome consists of three ssRNAs with positive polarity. Monocistronic RNAs 1 and 2 encode P1 and P2 subunits of the RNA replicase complex, respectively. RNA 3 contains two open reading frames encoding the movement protein (MP) and coat protein (CP), which is expressed through a subgenomic RNA or RNA 4. The use of AMV to screen RSS was based on a modified RNA 3 that permits the expression of heterologous proteins by duplication of the subgenomic promoter of RNA4 (Sanchez-Navarro et al., 2001) and transgenic *Nicotiana tabacum* plants that express the P1 and P2 subunits of the AMV polymerase (P12 plants) (Taschner et al., 1991). AMV RNA3 wt transcripts or those coming from a modified RNA3 expressing the GFP cause asymptomatic infections in P12 plants. However, it was observed that the expression of a RSS induced a specific necrotic lesion. This test was used to assess the RSS activity of three well known RSSs and a viral protein for which no evidence of its potential RSS has been demonstrated. In addition, this new method was compared with the three most common RSS activity assays described in the literature.

To evaluate the capacity of this AMV vector to detect RSS we have expressed several mutants of tobacco etch virus HC-Pro affected in their silencing suppressor activities (Torres-Barceló et al., 2008; GenBank acc.: DQ986288) from null (mutant CLA10) to reduced (mutant AS20) or incremented (mutant PC22) activity (Fig. 1). To do this, a modified infectious cDNA 3 clone of AMV that expresses the green fluorescent protein (pGFP/MP/CP) (Sanchez-Navarro et al., 2001), was used to introduce all genes, previously amplified with the corresponding primers carrying the appropriate restriction sites (Table 1), by exchanging

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the green fluorescent protein gene (Fig. 1A). The resultant constructs express the HC-Pro protein with the HA epitope (MSYPYDVPDYA) fused at their N-terminus. The results observed in the inoculated leaves at 3–4 days post inoculation (dpi) revealed a perfect correlation between the silencing suppressor activity and the necrotic lesion (Fig. 1A). Thus, the AMV RNA 3 construct carrying the CLA10 HC-Pro mutant with null RSS activity, generated an asymptomatic infection meanwhile the rest of constructs induced clear necrotic lesions, more evident in the PC22 mutant with an increased RSS activity. In the case of the mutant AS20 (reduced silencing activity) we also observed some chlorotic lesions (data not shown). No extra symptoms were observed with longer infection time. Similar necrotic response was observed using the AMV RNA 3 vector expressing the well-known RSS P19, 2b and P25 proteins from tomato bushy stunt virus (TBSV; GenBank acc.: AY579432.1), cucumber mosaic virus (CMV; GenBank acc.: NC_002035) and potato virus X (PVX; GenBank acc.: M72416.1), respectively (Fig. 1A). In the case of the AMV RNA 3 construct expressing the P25, some chlorotic lesions were also observed (data not shown). To confirm the expression and the stability of the corresponding protein in absence of any necrotic lesions, all constructs were transfected into P12 protoplasts and the protein accumulation was analyzed at 16 h post transfection by western blot analysis using a monoclonal mouse anti-HA antibody (Fig. 1B). All constructs accumulated the expected protein meanwhile no signal was detected in the water transfected protoplast. Also, we observed that the accumulation of the HC-Pro mutant with null RSS activity (mutant CLA10), was lower when compared with the rest of the HC-Pro variants with different RSS activity, suggesting a high replication and/or expression of AMV (see below). All together, these results showed that the expression of a silencing suppressor in the AMV context induced a clear necrotic lesion. In this sense, RSS can not only elicit hypersensitive response (HR) as effectors (Dodds and Rathjen, 2010; Jones and Dangl, 2006), but also induce host resistance (R) genes expression (Li et al., 2012; Shivaprasad et al., 2012; Boccara et al., 2014). In addition, the necrosis phenotype has been associated to the RSS activity by the interaction with either some cellular factors (Inaba et al., 2011; Masuta et al., 2012) or small RNAs (Vargason et al., 2003). In the case of wild type AMV, the lack of necrotic symptomatology associated to the infection could indicate that none of its viral proteins are RSS. In this sense, transient expression of the four AMV proteins (P1, P2, MP or CP) in *Nicotiana benthamiana* 16c plants did not show any RSS activity (data not shown). However, we cannot discern if the necrotic lesions associated to the expression of a RSS through the AMV RNA 3 in P12 plants is a direct consequence of the RSS *per se*, an indirect effect (e.g. over accumulation of any of MP or CP AMV proteins) or both. Alamillo et al., 2006; Boccara et al., 2014; Bueso et al., 2017; Campos et al., 2014; Chiba et al., 2006; Choi et al., 2004.

In the next step, the influence of the RSS in the viral RNA accumulation was analyzed in protoplasts (Fig. 2). For this purpose, all constructs analyzed in Fig. 1 together with a new AMV RNA 3 derivatives expressing the coat protein of carnation mottle virus (CarMV, genus *Alphacarmovirus*), which was assayed for its RSS activity in the final experiment, were transfected into P12 protoplasts. The results indicated that the presence of a RSS induces an increment of the AMV RNAs accumulation. Interestingly, the accumulation of the AMV RNAs correlated with the RSS activity described for the different HC-Pro mutants. Such effect could be explained by the inherent capacity of the RSS to block the silencing defense machinery preventing the viral RNA degradation, a process that could be modulated by the plant defense hormones. In this sense, there is evidence for cross-talk between phytohormone signaling and silencing defense pathways (Collum and Culver, 2016; Alamillo

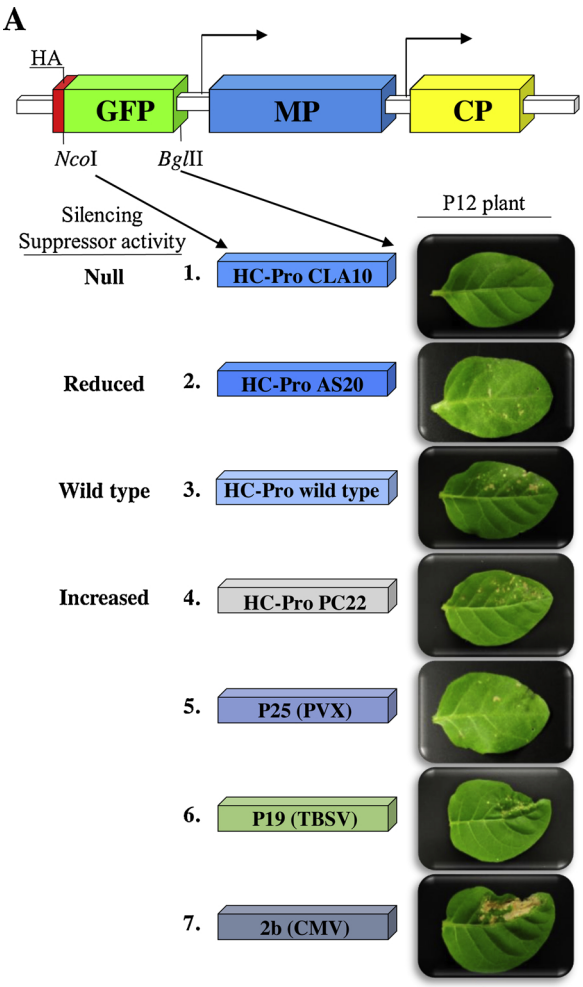

Fig. 1. A), Inoculation of P12 plants with the derivative AMV RNAs 3 expressing the tobacco etch virus HC-Pro, its mutants affected in the silencing suppressor activity (Torres-Barceló et al., 2008) or RSS P19, 2b and P25 proteins from tomato bushy stunt virus (TBSV), cucumber mosaic virus (CMV) and potato virus X (PVX), respectively. T7 Transcripts (5 μg) generated from *PstI* linearized plasmids and corresponding to all AMV RNA 3 derivatives, were inoculated into P12 plants, as described previously (Taschner et al., 1991), and the phenotypic changes were monitored during two weeks. Symptoms were observed at 4 days post inoculation. B) Western blot analysis of the accumulation in P12 protoplasts of the proteins carrying the HA epitope. Protoplasts were inoculated with RNA transcripts (5 μg) from constructs assayed in A, as described previously (Loesch-Fries et al., 1985). At 16 h post transfection, total proteins extracted from 40.000 protoplast were analyzed by western blot using an anti-HA antibody from mouse (Sigma-Aldrich, Steinheim, Germany) and a secondary anti-mouse antibody conjugated with peroxidase (Sigma-Aldrich, Steinheim, Germany) together with a chemiluminescence substrate (Amersham™ ECL™ Prime Western Blotting Detection Reagent). Schematic representation shows the derivative GFP/MP/CP AMV RNA 3 (Sanchez-Navarro et al., 2001). Open reading frames encoding the Human influenza hemagglutinin epitope (HA), green fluorescent protein (GFP), movement protein (MP) and coat protein (CP) are represented by red, green, red and yellow boxes, respectively. The *NcoI/BglII* restriction sites used to exchange the genes are indicated. The silencing suppressor activity of the HC-Pro mutants are relative to wild-type HC-Pro (Torres-Barceló et al., 2008). M, mock inoculated protoplast. The film was exposed 3 min.

Table 1
Primers used to amplify the different viral proteins.

Virus/protein		Sequence 5′ –3′[c]	Location in the viral RNA	Acc. Nº
TEV/ HC-Pro	S [a]	ACACCATGGGAAGCGACAAATCAATCTCT	1057-1074	DQ986288
	A[b]	ACAAGATCTTATCCAACATTGTAAGTTTTC	2433-2415	
PVX/P25	S	ACACCATGGATGGATATTCTCATCATTAG	4486-4505	M72416.1
	A	ACAAGATCTCTATGTCCCTGCGCGGAC	5149-5166	
TBSV/P19	S	ACACCATGGAACGAGCTATACAAG	3878-3896	AY579432.1
	A	ACAAGATCTTACTCGCTTTCTTCTTTG	4378-4396	
CMV/2b	S	ACACCATGGAATTGAACGTAGGTG	(RNA 2) 2419-2437	NC_002035
	A	ACAAGATCTCAGAAAGCACCTTCCGCC	(RNA 2) 2733-2751	
CarMV/CP	S	ACACCATGGATGGAAAATAAAGGAGAAAG	2668-2687	AJ304989
	A	ACAAGATCTTCACATCCTATAAACAACCC	3695-3714	

[a] Sense primer.
[b] Antisense primer with an STOP codon before the *Bgl*II restriction site.
[c] Underlined sequence indicated the primers used to introduce the corresponding gene into the PVX expression vector.

et al., 2006; Campos et al., 2014). Recently, it has been reported that the HC-Pro of turnip mosaic virus (TuMV) increments the virus accumulation via the repression of salicylic acid (SA)-mediated defense responses acting as a negative regulator of SA-binding protein SABP3 (Poque et al., 2017). Such hormone repression, derived from RSS, could be even more drastic in the AMV infection since this virus decreased jasmonic acid (JA) having no effects on the accumulation of abscisic acid (ABA), SA and auxins (IAA) (Bueso et al., 2017).

The CarMV CP showed a significant increment of the AMV RNA 3 accumulation, suggesting that this protein could be a RSS. To confirm this observation, we analyzed the capacity of the protein to reduce the accumulation of the small RNAs derived from the transiently expressed GFP. To do that, wild type *N. benthamiana* plants were agroinfiltrated with a construct expressing the GFP together with different constructs expressing two well-characterized RSS (P25 and HC-Pro) and the CarMV CP protein. All proteins were expressed under the control of the CaMV 35S promoter and the PoPit terminator in the pMOG800 binary plasmid, introduced into C58C1 cells (Martinez-Gil et al., 2009). The

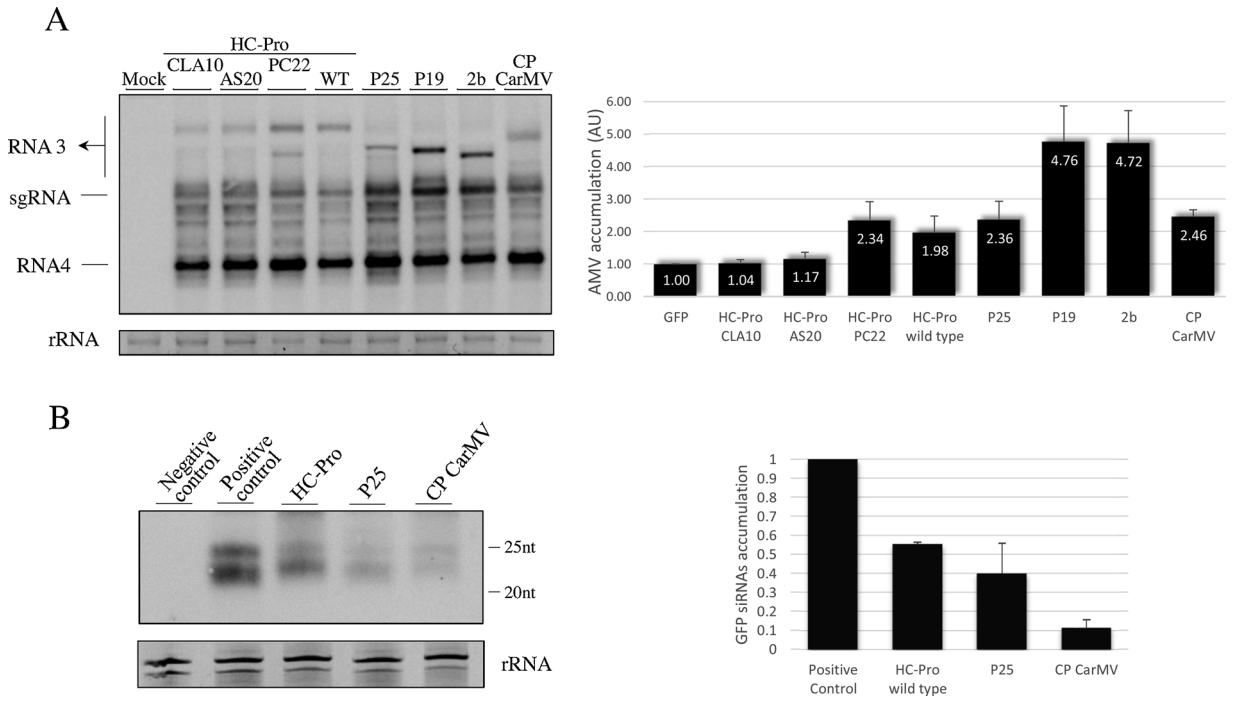

Fig. 2. Northern blot analysis of the accumulation of AMV RNAs in P12 protoplasts and GFP derived small RNAs in agroinfiltrated *N. benthamiana* plants. Transcripts corresponding to the constructs described in Fig. 1, plus a new AMV RNA 3 derivative carrying the coat protein (CP) of carnation mottle virus (CarMV), were transfected into P12 protoplast as described previously (Sanchez-Navarro et al., 2006). Total RNA was extracted at 16 h post inoculation using TRI Reagent (Sigma Steinheim, Germany), electrophoresed through formaldehyde-denatured gel and transferred to positively charged nylon membranes (Roche Mannheim, Germany). Hybridization and detection were conducted as previously described (Pallas et al., 1998) using a dig-riboprobe (Roche) complementary to the AMV 3´UTR. A) Northern blot showing accumulation of AMV RNAs. The localization of the RNA 3, 4 and the first subgenomic RNA (sgRNA) of AMV are indicated. The graph represents the relative accumulation of AMV RNAs, referred to the construct carrying the GFP, in four independent experiments. B) Northern blot analysis of the accumulation of GFP derived small RNAs in *N. benthamiana* plants agroinfiltrated with a construct expressing the GFP plus several constructs expressing the P25, CarMV CP, HC-Pro or an empty binary plasmid (Positive control). Negative control corresponds to total RNA extracted from non-infiltrated leaves. Eight µg of the total RNAs per sample extracted at 4 dpi of the infiltrated leaves (TRI Reagent; Sigma-Aldrich Steinheim, Germany), were electrophoresed through a 17% denaturing polyacrylamide gel and hybridized at 37 °C with a mix of three 50 nt riboprobes labelled with digoxigenin and complementary to nt 707–756, 761–810, 881–930 of the GFP gene (Genbank Accession: U76561). The film corresponds to an exposition of 20 min. Numbers on the right indicate the migration of two DNA primers of 20 nt and 25 nt. The graph represents the relative accumulation of GFP derived small RNAs in three different experiments. In both graphs, the bands were quantified using Image J 1.48c software (http://imagej.nih.gov/ij) and the error bars represent standard deviation.

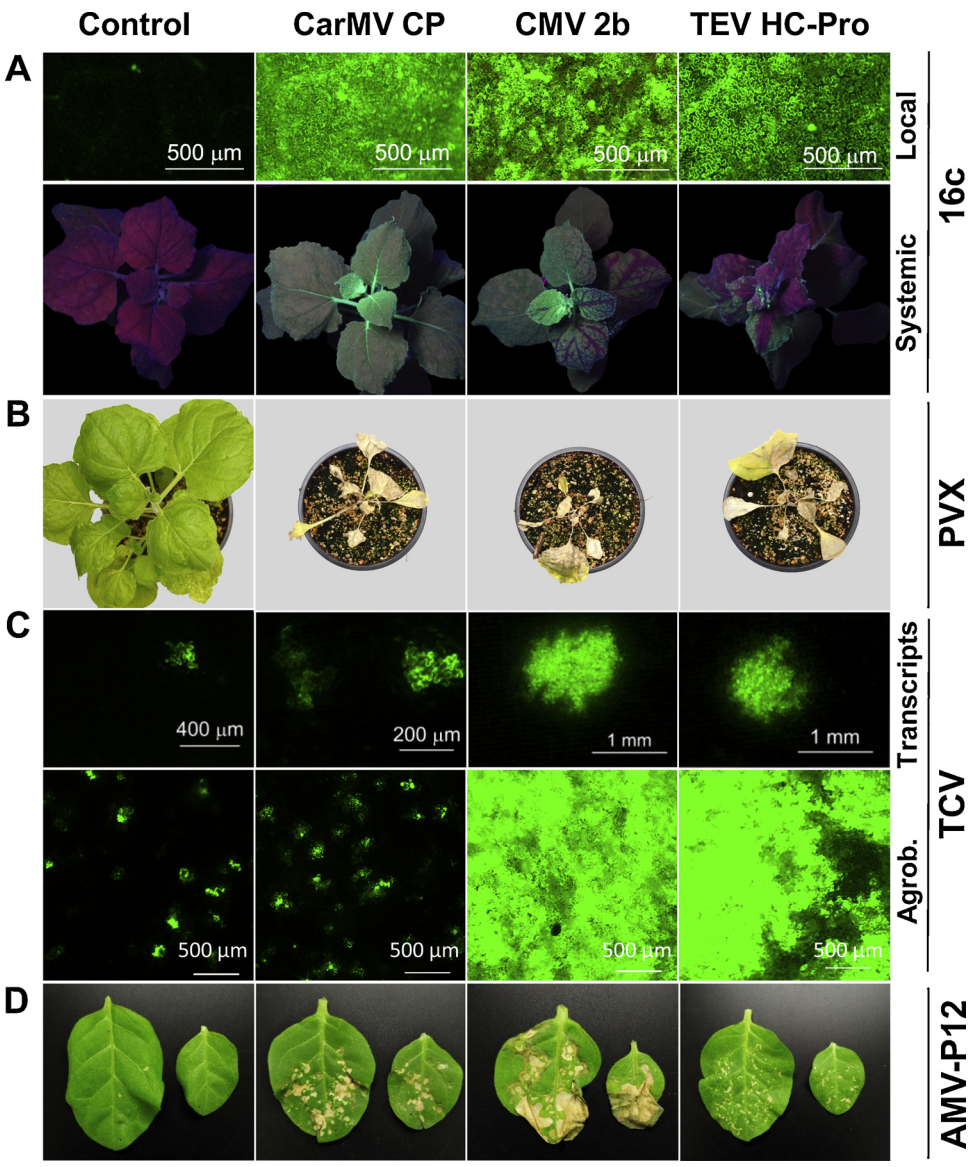

Fig. 3. Comparison of RNA silencing activity of the CarMV CP and two well-known viral suppressors, CMV 2b and TEV HC-Pro, using three different standard assays: *N. benthamiana* line 16c (Hamilton et al., 2002), PVX and TCV vectors plus the novel AMV-P12 system. A) Fluorescence visualization at local (7 dpi) or systemic (21 dpi) level of GFP in transgenic *N. benthamiana* line 16c plants after transient expression of the viral proteins together with the GFP. The ORFs were cloned between the CaMV 35S promoter and potato proteinase inhibitor terminator (PoPit) in the pMOG800 binary plasmid, introduced into C58C1 cells and co-infiltrated with equal volumes (OD600 of 0.5) of an *A. tumefaciens* culture containing pMOG(GFP), which express the eGFP. Control plants were infiltrated with a mixture consisting of equal volumes of *A. tumefaciens* cultures carrying pMOG(GFP) or empty pMOG800. Growing conditions included a recurring two-step cycle of 10 h of darkness at 18 °C and 14 h of light at 20 °C. Pictures of infiltrated and systemic leaves were taken at 6 dpi using a Leica MZ16 F fluorescence stereomicroscope or at 30 dpi under long-wavelength UV light (UVGL-58 Handheld UV lamp; UV Products) by using a tripod and a Nikon D3000 digital camera at F11 aperture value and 1/10 s shutter speed (Yaegashi et al., 2012), respectively. Five independent experiments were performed, each one included the infiltration of three plants per construct. B) Infectivity assay using pGR107 (Lu et al., 2003), a potato virus X (PVX) vector expressing each heterologous viral protein in *N. benthamiana* plants. PVX-derivatives either containing the CarMV CP, CMV 2b, or TEV HC-Pro were constructed by cloning each ORF into the SmaI site of pGR107 downstream of the duplicated PVX CP promoter (Jones et al., 1999). Each of the recombinant construct was propagated in *A. tumefaciens* strain C58C1 containing the helper plasmid pSoup, grown to OD600 of 0.5 and infiltrated into *N. benthamiana* plants. The empty pGR107 was used as control. Three weeks later, entire plants were photographed. C) TCV assay based on the complementation in trans of the movement-deficiency phenotype of a TCV CP deletion mutant that expresses GFP (TCV-sGFP) (Transcripts) or in the increment of the fluorescent when TCV-sGFP construct was delivered by *Agrobacterium* (Agrob.). For the virus movement complementation assay, three *N. benthamiana* leaves per plant were infiltrated with *A. tumefaciens* cultures containing the empty pMOG800, as control, or the pMOG800 constructs expressing CarMV CP, CMV 2b, or TEV HC-Pro at an OD600 = 1. One day postinfiltration, TCV-sGFP infectious RNA transcripts were mechanically inoculated onto the abaxial surfaces of the infiltrated leaves. Cell-to-cell movement was evaluated 3 days after inoculation, with a Leica MZ16 F fluorescence stereomicroscope. Each assay was repeated three times. For the second approach, the *A. tumefaciens* cultures described above, were co-infiltrated with an *Agrobacterium* culture carrying the TCV-sGFP construct at an OD600 = 0.0025. The fluorescent signal of the infiltrated leaves was monitored at 5 dpi with a Leica MZ16 F fluorescence stereomicroscope. D) In the novel AMV-P12 system, the AMV RNA 3 harboring each heterologous viral protein was inoculated in transgenic *N. tabacum* P12 plants. An early increase in the severity of local symptoms was observed in all viral proteins tested indicating that all of them can suppress in some extension the RNA silencing. Negative control corresponds to CLA10 HC-Pro mutant with null RSS activity.

accumulation of GFP derived siRNAs was analyzed by northern blot through a 17% denaturing polyacrylamide gel (Fig. 2B). The results revealed that all analyzed proteins reduced the accumulation levels of the small RNAs meanwhile a clear signal was observed in the leaves infiltrated with an empty pMOG800 binary vector. No hybridization signal was observed in the non-infiltrated leaves.

Finally, we performed a direct comparison between the different assays for the screening of RSS (*N. benthamiana* 16c plants, PVX or turnip crinkle virus –TCV- vectors), and the new AMV-P12 plants system. To do this, we selected two well- characterized RSS (2b and HC-Pro) and the CP of CarMV (Navarro and Pallas, 2017). In the *N. benthamiana* 16c plants assay the ability of the viral protein to prevent the systemic spread of the GFP transgene silencing signal is determined by the preservation of the green fluorescence in agroinfiltrated or non-agroinfiltrated upper leaves. In the case of the PVX system, an increase in the pathological effects of the PVX infection suggests the presence of an additional gene with silencing suppression activity. Finally, the turnip crinkle virus (TCV) assay was used in two different ways either as an infectious RNA transcript or via agroinfiltration (Powers et al., 2008). In the former, the system is based in the complementation in trans of the movement-deficiency phenotype of a TCV CP deletion mutant that expresses GFP (TCV-sGFP). In the second approach, the TCV-sGFP constructs was delivered by *Agrobacterium* and the presence of a RSS is associated with an increase of the GFP signal in the whole leaf. First, it was observed that the two previously described RSS (2b and HC-Pro) were clearly detected using the four assays. In the case of

HC-Pro, it was also observed that RSS activity was only detected at the infiltrated leaves of *N. benthamiana* 16c plants, being unable to block the systemic silencing pathway (Fig. 3, 16c Systemic). For the TCV assay, both RSS proteins were clearly detected in the two approaches, either by allowing the cell-to-cell transport of the movement defective TCV-sGFP transcripts (Fig. 3C, Transcripts) or by increasing the fluorescent signal when the TCV-sGFP construct was delivered by *Agrobacterium* (Fig. 3C, Agrob.). In the case of the CP of CarMV, the RSS activity was clearly detected in the line 16c (local and systemic), PVX and AMV-P12 assays. However, by using the TCV system, we detected the RSS capacity of the CarMV CP only with the approach based on the complementation in trans of the cell-to-cell defective TCV-sGFP construct (Fig. 3C, Transcripts). In addition, we observed foci of a reduced number of infected cells (3 to 6 cells), completely different than the foci observed for 2b and HC-Pro proteins, in spite of the fact that the three proteins (CarMV CP, 2b and HC-Pro) rendered the same strong RSS activity in the other three assays. Apparently, the TCV system does not correlate the RSS activity with the size of the infection foci or with the increment of the fluorescent signal, which leads to conclude that other factors rather than RSS activity could affect the TCV assay. The classification of the CarMV CP as a RSS correlates with other CPs from members of *Carmovirus* genus (Genoves et al., 2006; Meng et al., 2006; Martínez-Turiño and Hernández, 2009; Choi et al., 2004).

In summary, we present a new and very sensitive RSS activity assay, reliable and short time consuming (3–4 days), which allows for RSS activity quantification in protoplasts. In addition, the observation that the P12 plants over accumulated the AMV RNA 3 (Wu et al., 2017) permits hypothesize that the AMV-P12 plant system could be a very sensitive assay to detect RSS activity. Finally, we present a new RSSs corresponding to the CarMV CP which also blocked the accumulation of GFP-derived small RNAs in agroinfiltrated plants.

Conflict of interest

The authors have no conflict of interest to declare.

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