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ARTICLE

Deletion series in the P1 protein of the *Sweet potato mild mottle virus* identifies the shortest fully functional RNA silencing suppressor domain

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ABSTRACT RNA silencing is a part of the plant innate immune system that could effectively cope with intruders, like viruses. However, viruses evolved proteins that can suppress RNA silencing thus supporting virus spreading in the host. To counteract RNA silencing, suppressor proteins attack different players of RNA silencing pathway. The P1 protein of the *Sweet potato mild mottle* virus binds and inactivates small RNA loaded RISC complexes. Using a deletion series in the P1 protein we aimed to identify the possible smallest working version of P1. Our results revealed that the minimal RNA silencing suppressor domain of P1 is as small as 210 amino acids in size.

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RNA silencing is a post-transcriptional gene regulation mechanism conserved in almost all eukaryotes and involved in many essential biological processes, from development, physiological activity to the regulation of abiotic and biotic stress responses by micro RNAs (miRNA), genome defense by 24 nucleotide (nt) long small interfering RNAs (siRNAs) and in particular antiviral defense by 21 nt siRNAs. RNA silencing negatively regulates genes expression (Herr and Baulcombe 2004).

The trigger of RNA silencing can be pri-miRNAs for miRNAs or double stranded (ds) RNAs as replicative forms of plant viruses. Trigger RNAs are processed into si- and miRNAs by the RNAse III type enzymes Dicers, then small RNAs are loaded into the Argonaute (AGO) protein containing protein complexes called RNA induced silencing complex (RISC). This process is referred to as RISC assembly. In RISC, one strand of the small RNA is eliminated resulting in the single stranded (ss) small RNA containing RISC complex (active RISC). Active RISC complexes are able to hamper gene expression either by cleaving the target RNA or by inhibiting the translation of the target RNA (Hutvagner and Simard 2008). Thus, Argonaute (AGO) proteins play a key role in RNA silencing.

In the last few years, several AGO binding proteins were identified. Most of them contain GW/WG (Gly-Trp/Trp-Gly) domains, which mediate the interaction with AGO. This group of proteins was named GW/WG proteins after the founding member GW182 protein of human. GW182 in animals binds AGO and mediates the interaction with the polyA binding proteins to repress the translation of the target RNA (Pfaff et al. 2013). In *Schizosaccharomyces pombe*, transcriptional silencing involves AGO and TAS3 that shows high similarity to animal GW182 proteins (Pfaff et al. 2013). In plants, heterochromatin silencing exploits a couple of GW/WG proteins, such as the second largest subunit of the RNA polymerase V complex and SPT5 protein (Bies-Etheve et al. 2009; Herr et al. 2005). Moreover, the GW/WG protein SUO was identified in Arabidopsis thaliana to be involved in miRNA driven RNA silencing (Yang et al. 2012). Thus, these GW/WG proteins have a positive effect on RNA silencing and are absolutely required for the efficient carrying RNA silencing into execution.

Two viral proteins bearing WG/GW domains were found to have a negative effect on RNA silencing. The p38 protein of the *Turnip crinkle virus* (TCV) sequesters small RNA unloaded AGO1 (Azevedo et al. 2010). However, the P1 RNA silencing suppressor protein of the *Sweet potato mild mottle virus* (SPMMV) is markedly different from that of p38. SPMMV P1 binds AGO1 loaded with si- or miRNAs, thus inhibiting target cleavage of active RISC complexes. Mutational analysis revealed three WG/GW motifs, resembling the AGO binding platform conserved in plants and metazoans, at the N-terminal part of P1, which are required for AGO binding and silencing suppressor activity (Giner et al. 2010).

Recently, we found that the first 383 amino acid (aa) region of the SPMMV P1 renders RNA silencing suppressor activity (Giner et al. 2010). Thus, our aim was to find the smallest region of the P1 protein bearing fully functional suppressor activity. Our results based on a series of truncation of

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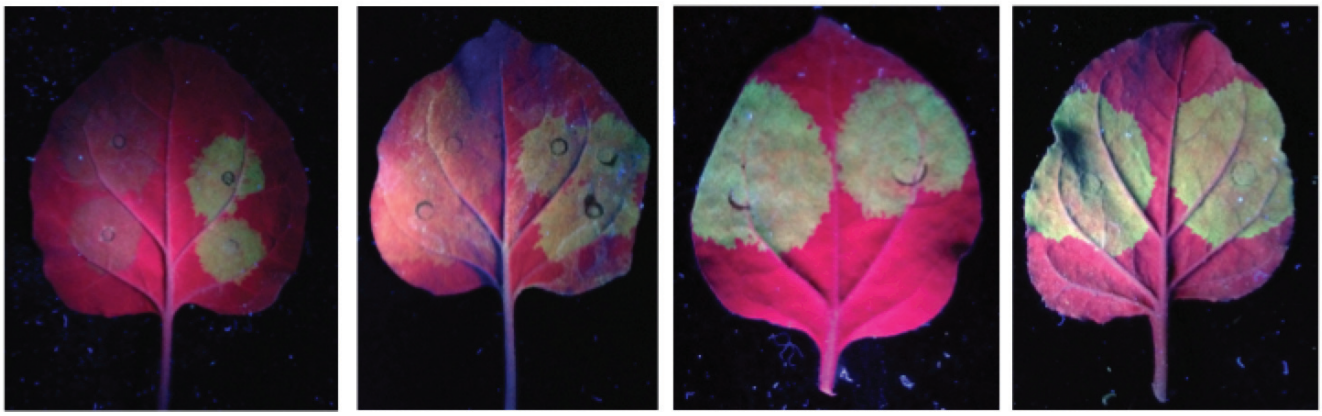

Figure 1. RNA silencing suppressor activity of the truncated mutants. The first panel shows empty vector infiltrated sample, used as negative control. The mutant of interest was infiltrated as shown in the second, third and fourth panel. On the right side of each panel $P1_{1-383}$ was infiltrated, as positive control.

the P1 protein from the C-terminal end showed that the 1-210, but not 1-120, amino acids region is fully functional.

Materials and Methods

Plant materials

Wild-type *Nicotiana benthamiana* plants grown in soil under normal growth conditions were used for, virus infection and agroinfiltration. Plants were grown in Phytotron (Versatile Environmental Test Chambers; Sanyo, Tokyo, Japan) under a 14-h light (50 mE m±2s±1) and 10-h dark regime at 23 °C.

Agroinfiltration

Agroinfiltration was essentially carried out as described earlier (Silhavy et al. 2002). Plasmid DNA was introduced into the *Agrobacterium tumefaciens* C58C1 strain by triparental mating (Wise et al. 2006). *Agrobacterium* strains harboring the expression plasmids were cultured at 28 °C in LB medium in the presence of 5 µg/ml tetracycline and 100 µg/ml kanamycin. Cultures were centrifuged at room temperature with 1000 g to pellet the bacteria, then resuspended in the induction solution containing 10 mM MES (pH 5.6), 10 mM $MgCl_2$ and 150 µM acetosyringone. Optical density of the cultures were determined and diluted to an OD_{600} of 0.3 for suppressor and 0.1 for reporter genes. Diluted and mixed *Agrobacterium* strains were incubated in the induction solution for 1-3 hours at room temperature before infiltration.

Primers used in this study

P1-5'-end: 5'GGGGATTCCCTAGAATGGGGAAATCCAAACTC 3'
$P1_{1-360}$-3'-end: 5' TGAATTCTCATGCCACCGTGATGGGACACACCATAGC 3'
$P1_{1-305}$-3'-end: 5' TGAATTCTCATGTCTTTGTAACCCACATAACTGC 3'
$P1_{1-210}$-3'-end: 5' TGAATTCTCAAATTGAGAAAACAGTTTCCTCAAAAACC 3'
$P1_{1-120}$-3'-end: 5' TGAATTCTCAGTTATCAAGTACATTGTCGTCGCGCTTGTT 3'

Western blot analysis

To the blot analysis, samples were collected in 2 cm diameter. The samples were extracted in 2x sodium dodecyl sulfate (SDS) loading puffer and boiled for 5 minutes, after sample preparation 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run. Blocking was carried out in 5% dry milk in phosphate buffered saline (PBS) at every turn. Anti-HA (anti-influenza hemagglutinin, 1:3000 in 5% dry milk) and anti-GFP (anti-green fluorescent protein, 1:5000) was used for HA and GFP tagged samples, respectively. Anti rabbit HRP (anti-rabbit IgG conjugated to horseradish peroxidase, 1:5000) was used as secondary antibody. At the co-immunoprecipitation anti-Ha and anti-myc antibody was used (1:5000 in 2% dry milk; secondary antibody was anti-rabbit HRP, 1:5000).

Results and Discussion

It was previously demonstrated that the N-terminal 383 amino acid region of SPMMV P1 possessed the silencing suppressor domain (Giner et al. 2010). If the N-terminally HA-tagged full length SPMMV P1 was infiltrated into *N. benthamiana* plants and immunoprecipitated, the minority of the HA-tagged protein was proven full length (Lakatos, unpublished). This result might not be surprising, since it is already known that

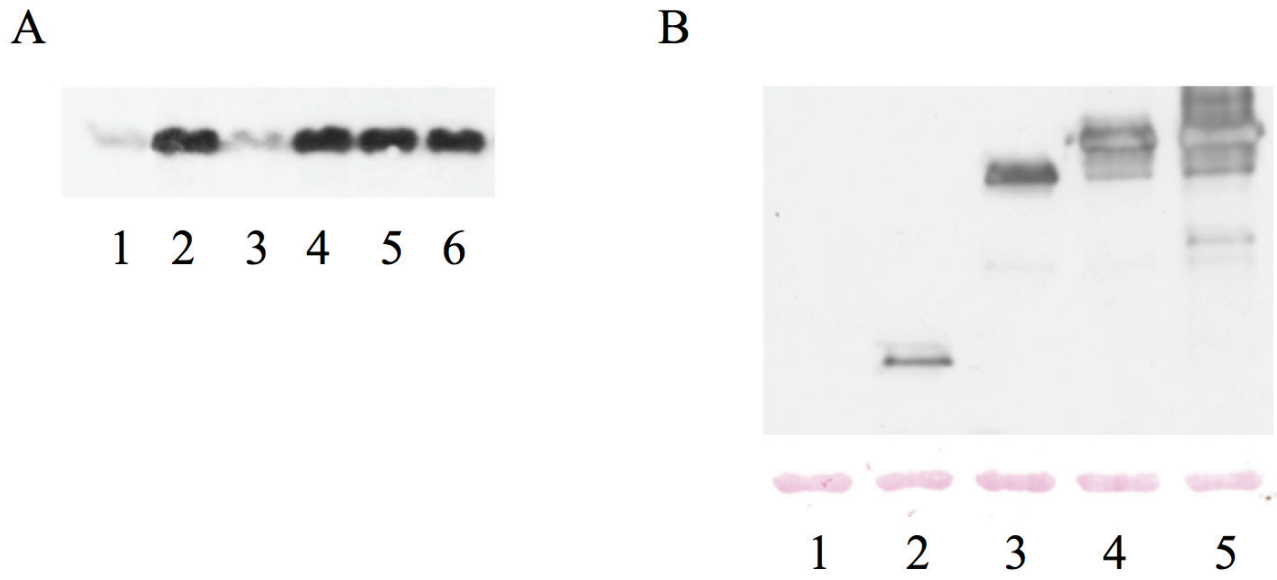

Figure 2. Expression analysis of GFP and the truncated suppressor mutants. Panel A, GFP detected by anti-GFP polyclonal antibody; lanes: 1, empty vector+GFP171; 2, GFP-171+ $P1_{1-383}$; 3, GFP-171+ $P1_{1-120}$; 4, GFP-171+ $P1_{1-210}$, 4 GFP-171+ $P1_{1-305}$, 5, GFP-171+ $P1_{1-360}$; GFP-171+ $P1_{1-383}$. Panel B, suppressor mutants detected by anti-HA antibody; lanes: 1, GFP-171+ $P1_{1-120}$; 2 GFP-171+ $P1_{1-210}$; 3 GFP-171+ $P1_{1-305}$; 4 GFP-171+ $P1_{1-360}$; GFP-171+ $P1_{1-383}$; lower panel represents the loading control.

maturation of the huge potyviral polypeptide translated occurs via the intrinsic protease activities of the polypeptide. Even P1 has a protease domain in its C-terminal, which is required for liberation of P1 from the primary translation product (Valli et al. 2007). Thus, the C-terminal truncation of P1 can be a consequence of its own protease activity, but the action of the plant proteases could not be excluded.

Our aim was to find out, whether these shorter versions of P1 still have RNA silencing suppressor activity. To answer this question, we systematically performed C-terminal truncations on the SPMMV P1 protein. To this end, we amplified the P1 coding region with P1-5'-end primer (annealing to the translational start codon) and $P1_{1-360}$-, $P1_{1-305}$-, $P1_{1-210}$- and $P1_{1-120}$-3'-end primers resulting in 360-, 305-, 210- and 120-aa versions of P1. PCR products were cloned into the TOPO II vector (Invitrogen) and sequenced from both sides. These inserts were cloned into pSanyi plant expression vector (Kertesz et al. 2006) to express them as fusion protein with a HA-tag at their N-terminal end. *Agrobacterium* strains harboring these constructs were coinfiltrated with reporter construct GFP-171.1 (Parizotto et al. 2004) to check for silencing suppressor activity in *N. benthamiana* (Giner et al. 2010). SPMMV $P1_{1-383}$ was used as a control, because this truncated version was known to have silencing suppressor activity. Forty eight hours post-infiltration, visual inspection of the infiltrated leaves was carried out under UV light. We found that the SPMMV $P1_{1-383,}$ $P1_{1-360}$, $P1_{1-305}$, and $P1_{1-210}$ infiltrated leaves showed bright green fluorescence under UV light, indicating RNA silencing suppressor activity. However, the $P1_{1-120}$ infiltrated leaves were rather red, which demonstrated lack of silencing suppressor activity (Fig. 1). Then, Western blotting was carried out to detect GFP protein in the infiltrated leaves. Consistently with our visual inspection, strong GFP expression was found in the protein extracts of $P1_{1-383,}$ $P1_{1-360}$, $P1_{1-305}$, and $P1_{1-210}$ infiltrated leaves, while significantly less GFP was detected, when GFP-171 or GFP-171 and $P1_{1-120}$ were infiltrated (Fig. 2A). These experiments clearly revealed that silencing suppressor function was lost in the case of the $P1_{1-120}$ mutant. We showed earlier that SPMMV P1 contains three WG/GW motifs at positions 15, 101 and 131 (Giner et al. 2010). Although, we found that changing W131 to A (alanine) did not significantly affect the silencing suppressor function, but in the case of the $P1_{1-120}$ mutant, excluding W131 and ten more amino acids might be detrimental for silencing suppressor activity (Giner et al. 2010). Finally, the expression of the N-terminally HA-tagged truncated silencing suppressor proteins were examined by western blotting. Our results showed that extracts of leaves infiltrated with $P1_{1-210,}$ $P1_{1-305}$, $P1_{1-360}$, and $P1_{1-383}$ showed high suppressor expression in increasing protein size (Fig 2B). On the other hand, leaves infiltrated with $P1_{1-120}$ did not express the corresponding protein. Of note, that extracts used in this experiment contained nearly the same amount of protein, as shown in the protein loading panel (Figure 2B). However, the SPMMV $P1_{1-120}$ protein, which did not show suppressor activity, could be detected only when they coinfiltrated in plants with *Tobacco etch virus* HC-Pro (data not shown) (Azevedo et al. 2010; Szabo et al. 2012).

The prototype RNA silencing suppressor inhibiting preassembled RISC is the SPMMV P1 protein, which consists of 759 amino acids. As it was shown before, this large protein in size contains the RNA silencing suppressor domain at the N-terminal part (Giner et al. 2010) and a protease domain conserved in the *Potyvirideae* family (Valli et al. 2007). Shorter versions of P1 were found in unrelated experiments implicated the functionality of these proteins. Thus, we narrowed the silencing suppressor domain to the N-terminal 210 amino acids of SPMMV P1. This truncated protein has full silencing suppressor activity, compared to longer versions.

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