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Short communication

Cocksfoot mottle virus P1 suppresses RNA silencing in *Nicotiana benthamiana* and *Nicotiana tabacum*

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Abstract

The *Sobemovirus* genome consists of positive sense, single-stranded polycistronic RNA. The 5′-terminal ORF, encoding the protein P1, is its most variable region. Sobemoviral P1 has been described as dispensable for replication but indispensable for systemic infection. The P1 of *Rice yellow mottle virus*-Nigerian isolate (RYMV-N) is the only RNA silencing suppressor reported for sobemoviruses until now. Using an agrobacterium-mediated transient assay, we demonstrate here that P1 of *Cocksfoot mottle virus*-Norwegian isolate (CfMV-NO) suppresses RNA silencing in *Nicotiana benthamiana* and *Nicotiana tabacum*, two non-host plants. CfMV-NO P1 was able to suppress the initiation and maintenance of silencing. The suppression of systemic silencing was weaker with CfMV-NO P1 than in the case of RYMV-N P1. In the case of suppression at the local level, the reduction in the amount of 25-nucleotide small interfering RNAs (siRNAs) was less pronounced for CfMV-NO P1 than it was when RYMV-N P1 was used. At the same time, we show that CfMV-NO P1 did not bind siRNAs.
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Keywords: *Cocksfoot mottle virus*; *Sobemovirus*; P1; siRNA; RNA silencing; Suppressor

Plants have developed a number of defense mechanisms against pathogens. One of these is RNA silencing, a sequence-specific RNA degradation process conserved among eukaryotes (Voinnet, 2005). RNA silencing is induced by dsRNA, which is processed to 21–26-nt small interfering RNAs (siRNA) (Hamilton and Baulcombe, 1999) that mediate degradation of identical RNA molecules (Hammond et al., 2000). In plants, RNA silencing is initially activated at the single-cell level and a mobile silencing signal is generated. This signal moves systemically through plasmodesmata and also through the phloem reaching distant organs (systemic silencing; Palauqui et al., 1997).

The majority of plant viruses have genomes of (+)-ssRNA (Hull, 2002) and are thought to trigger RNA silencing by dsRNA replicative intermediates and by highly structured ds regions in genomic RNA (Molnar et al., 2005). However, viruses have evolved suppressor proteins to counteract RNA silencing (Voinnet, 2005). The identification of viral suppressors and the elucidation of their mode of action are important for understanding RNA silencing mechanisms.

The *Sobemovirus* genome consists of polycistronic (+)-ssRNA (Tamm and Truve, 2000b). ORF1 of *Cocksfoot mottle virus* (CfMV), which infects only monocots, encodes a protein (P1) of 12 kDa that is required for systemic infection but is dispensable for replication (Meier et al., 2006) as is also the case for *Rice yellow mottle virus* (RYMV) P1 and *Southern cowpea mosaic virus* P1 (Bonneau et al., 1998; Sivakumaran et al., 1998). Moreover, P1 of RYMV is reported to be a pathogenicity determinant (Bonneau et al., 1998) and in the case of the Nigerian isolate (RYMV-N) it has been also described as an RNA silencing suppressor (Voinnet et al., 1999). Surprisingly, however, there is no similarity between the amino acid sequences of sobemoviral P1s (Ngon A Yassi et al., 1994; Mäkinen et al., 1995; Othman and Hull, 1995). Since RYMV-N P1 acts as a silencing suppressor in *Nicotiana benthamiana*, a non-host species (Voinnet et al., 1999), we investigated the suppressor activity of P1 of CfMV-Norwegian isolate (CfMV-NO; Mäkinen et al., 1995) in *N. benthamiana* using the agrobacterium-mediated transient assay (Hamilton et al., 2002).

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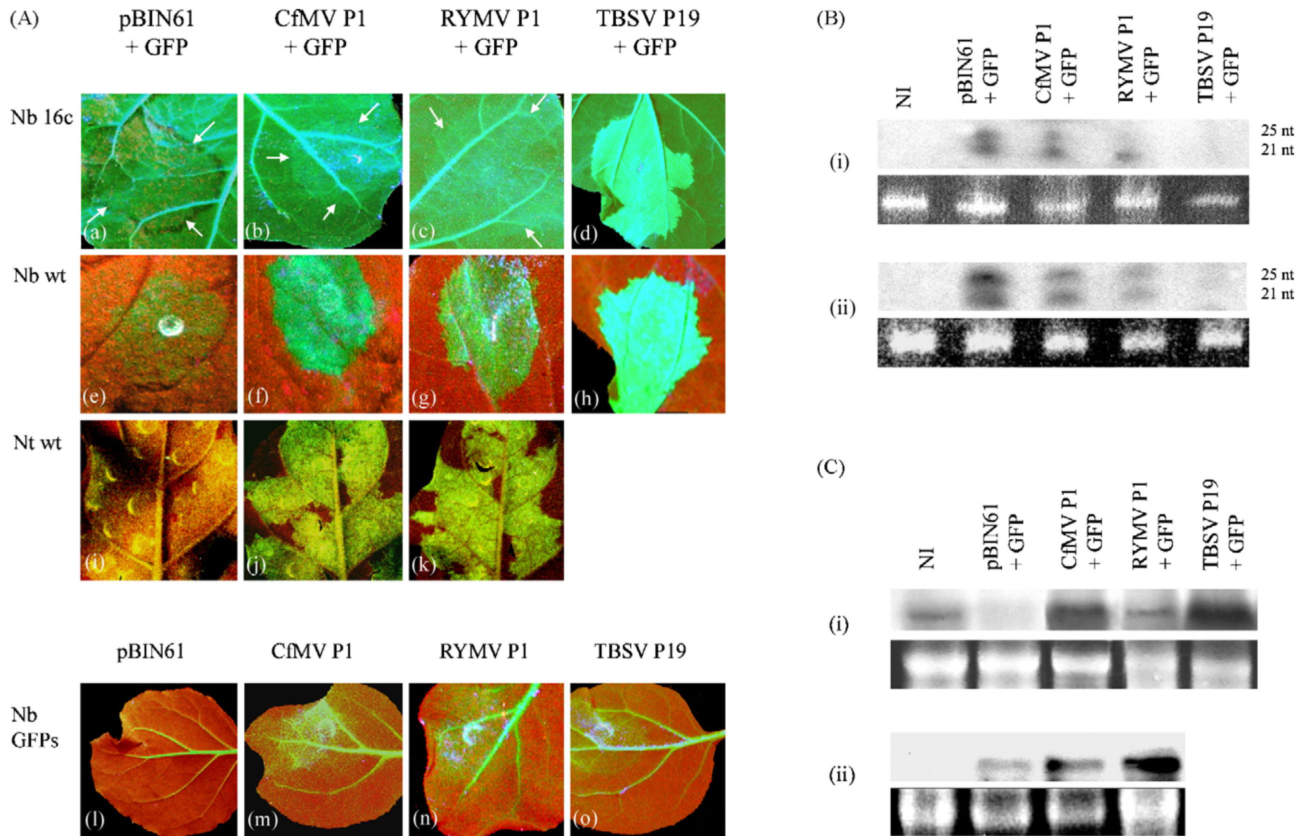

Fig. 1. Effect of CfMV-NO P1 on local RNA silencing in *N. benthamiana* and *N. tabacum*. (A) Infiltrated patches (5 dpi). *Agrobacterium* transformed with constructs indicated on the top were infiltrated into: *N. benthamiana* 16c (Nb 16c, a–d), wild-type *N. benthamiana* (Nb wt, e–h), wild-type *N. tabacum* (Nt wt, i–k), GFP-silenced *N. benthamiana* (Nb GFPs, l–o). Infiltration was carried out as previously described (Hamilton et al., 2002). Plants were photographed with Olympus CAMEDIA digital camera and pictures were processed using Adobe Photoshop 6.0. (B and C) Northern-blot analysis showing GFP siRNAs (B (i) at 5 dpi, B (ii) at 7 dpi) and GFP mRNA (C) extracted from *N. benthamiana* 16c (for B and C (i)) or *N. tabacum* (for C (ii)) patches infiltrated with the indicated strains. siRNA detection with ^{32}P-labeled in vitro transcript corresponding to the anti-sense strand of GFP was carried out as described (Sarmiento et al., 2006). DIG-labeled GFP-specific PCR fragment was used for mRNA detection at 5 dpi as described (Bucher et al., 2004). Ethidium bromide-stained rRNA is shown as loading control. NI: non-infiltrated leaf. Arrows indicate edges of infiltrated patches.

CfMV-NO ORF1 was amplified with primers 5′-CCTAGATCTAGCTTAGATGTGCGAACCTCC-3′ and 5′-GAGCTGCAGAACAACCCATTCTTGGTCACCCT-3′ and inserted into pTZ57R/T (Fermentas) to generate pTZ:CfMVORF1. CfMV-NO ORF1 (nt 62–441) was excised with *Xba*I and *Bam*HI from pTZ:CfMVORF1 and cloned into pBIN61 between the 35S promoter and Nos terminator to give pBIN61-P1. 35S-C_P1 refers to *Agrobacterium tumefaciens* (C58C1) containing pBIN61-P1.

To test if CfMV-NO P1 acts as a suppressor of RNA silencing, we first infiltrated leaves of GFP-transgenic *N. benthamiana* line 16c (Ruiz et al., 1998) with both 35S-C_P1 and 35S-GFP (*A. tumefaciens* carrying the GFP gene). In parallel, we co-infiltrated the 16c line with 35S-GFP together with pBIN61 (*A. tumefaciens* containing the empty pBIN61) or 35S-P19 (*A. tumefaciens* containing the strong suppressor P19 of TBSV). *A. tumefaciens* carrying P1 of RYMV-N (called 35S-R_P1) was also co-infiltrated with 35S-GFP to compare the effect of both sobemoviral proteins. At 5 days post-infiltration (dpi), GFP silencing has just been established and could be visualized by the appearance of a red ring at the border of the patch infiltrated with 35S-GFP plus pBIN61 and the weak red fluorescence inside this patch (Himber et al., 2003; Fig. 1A a). In contrast, the patches infiltrated with 35S-GFP and 35S-C_P1 or 35S-R_P1 still showed GFP expression (green fluorescence) at 5 dpi (Fig. 1A (b and c)). The patch infiltrated with 35S-GFP and 35S-P19 emitted at this time a very strong green fluorescence (Fig. 1A (d)). At 7 dpi, the patch infiltrated with 35S-GFP and 35S-C_P1, as well as the one co-infiltrated with 35S-R_P1, turned red (also a red ring appeared at the border of the patch) and by 11 dpi it was as red as the patch infiltrated with 35S-GFP and pBIN61 (data not shown). As expected, the area infiltrated with 35S-GFP and 35S-P19 remained intensely green after 11 dpi, due to the strong suppressor effect of P19 (Voinnet et al., 2003). The difference in the fluorescence of the patches indicates that CfMV-NO P1 suppressed GFP silencing in *N. benthamiana* 16c. At the local level, C_P1 was similar to R_P1, whose suppression in the same system has been partially described (Himber et al., 2003). To confirm these results we determined the levels of GFP siRNAs in the infiltrated patches. At 5 dpi, 21 and 25-nt GFP siRNAs were abundant in the pBIN61 control patch, whereas in the presence of any suppressor the levels changed: with P19 no siRNA was detected, as expected (Hamilton et al., 2002), with R_P1 only the shortest class of siRNAs were above the detection limit,

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