Short Communication

The helper component-proteinase of the *Zucchini yellow mosaic virus* inhibits the Hua Enhancer 1 methyltransferase activity *in vitro*

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The helper component-proteinase (HC-Pro) is a multifunctional protein found among potyviruses. With respect to its silencing suppressor function, small RNA binding appears to be the major activity of HC-Pro. HC-Pro could also exhibit other suppressor activities. HC-Pro may inhibit the Hua Enhancer 1 (HEN1) activity. There is indirect evidence showing that either transient or stable expression of HC-Pro in plants results in an increase of non-methylated small RNAs. Here, we demonstrated that recombinant *Zucchini yellow mosaic virus* (ZYMV) HC-Pro inhibited the methyltransferase activity of HEN1 *in vitro*. Moreover, we found that the HC-Pro^{FINK} mutant, which has lost small RNA-binding activity, inhibited HEN1 activity, while the truncated proteins and total soluble bacterial proteins did not. Using the ELISA-binding assay, we provided evidence that the HC-Pro^{FRNK} wild-type and HC-Pro^{FINK} both bound to HEN1, with HC-Pro^{FRNK} binding stronger than HC-Pro^{FINK}. Motif mapping analysis revealed that the amino acids located between positions 139 and 320 of ZYMV HC-Pro were associated with HEN1 interaction.

Suppression of plant RNA silencing is a mechanism which pathogens (e.g. viruses) use for survival. In general, viruses carry genes encoding silencing suppressor proteins which can either bind to small RNAs (sRNAs) (Silhavy *et al.*, 2002; Kasschau *et al.*, 2003), interfere with Argonaute proteins or Dicer-like and/or probably with Hua Enhancer 1 (HEN1) (Csorba *et al.*, 2010; Jin & Zhu, 2010; Wu *et al.*, 2010, Yu *et al.*, 2006). The helper component-proteinase (HC-Pro) is a multifunctional protein that plays multiple roles in the life cycle of potyviruses. It exhibits silencing suppressor activity by binding sRNA duplexes, e.g. small interfering RNA (siRNAs) and microRNAs (miRNA), rendering these RNAs inactive (Mérai *et al.*, 2006; Shiboleth *et al.*, 2007). In addition, using a yeast-two-hybrid system, binding of HC-Pro to a host protein, the Calmodulin-related protein, rgs-CaM from *Nicotiana tabacum*, was reported (Anandalakshmi *et al.*, 2000).

The RNA methyltransferase HEN1 is responsible for the 3′-terminal 2′-*O*-methylation of sRNAs in *Arabidopsis* (Yu *et al.* 2005), *Drosophila* (Horwich *et al.*, 2007; Saito *et al.*, 2007) and mouse (Kirino & Mourelatos, 2007). *Arabidopsis* HEN1 (AtHEN1) methylates double-stranded sRNAs (Yu *et al.* 2005; Yang *et al.* 2006), probably before they are loaded onto Argonaute proteins (Ramachandran & Chen, 2008a; Fang & Spector, 2007). This 2′-*O*-methylation protects sRNAs from exonuclease degradation (Ramachandran & Chen, 2008b). The loss of this modification causes a general reduction in the levels of sRNAs in plants (Boutet *et al.*, 2003; Li *et al.*, 2005). Although HEN1 was assumed to be exclusively localized in the nucleus (Xie *et al.*, 2004), it was also recently found in the cytoplasm (Csorba *et al.*, 2007).

Transgenic *Arabidopsis* plants expressing *Turnip mosaic virus* (TuMV) P1/HC-Pro differentially methylated miRNAs (Yu *et al.*, 2006). It was proposed that HC-Pro may prevent HEN1 from interacting with duplexes or block it from accessing the 2′-OH of the 3′-terminal nucleotide. Alternatively, HC-Pro may bind directly to HEN1 and inhibit its activity (Yu *et al.*, 2005). Here, we report for the first time that *Zucchini yellow mosaic virus* (ZYMV) HC-Pro inhibits the methylase activity of HEN1 *in vitro*.

The plasmid constructs MBP : HA-HC-Pro^{FRNK} and MBP : HA-HC-Pro^{FINK} used for protein expression were

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prepared as described previously (Fuellgrabe *et al.*, 2011). To identify the HEN1-binding domain in the HC-Pro protein, different N- and C-termini truncated (Δ) HC-Pro constructs were generated (Supplementary Fig. S1, available in JGV Online) (Laible & Boonrod, 2009).

Protein expression and purification were carried out according to Fuellgrabe *et al.* (2011). In order to produce high amounts of the MBP : HA-HC-Pro^{FRNK}, MBP : HA-HC-Pro^{FINK} and truncated MBP : HA-HC-Pro^{FRNK} proteins, the corresponding plasmids were transformed into BL21(DE3) codon$^+$ bacteria cells. After induction of the cells with IPTG, bacteria were cultured at 14 °C overnight. Cells were harvested and lysed to extract the total soluble protein fraction. After affinity purification using amylose magnetic beads (NEB), the eluate was analysed by 9 % SDS-PAGE. Protein staining with Coomassie blue revealed that MBP : HA-HC-Pro^{FRNK}, MBP : HA-HC-Pro^{FINK} and the truncated MBP : HC-Pro^{FRNK} proteins were clearly expressed and purified (for protein purification see Supplementary Fig. S2, available in JGV Online). In addition to the full-length protein (99 kDa), a lower molecular mass fragment of about 55 kDa was detectable in all preparations. The 55 kDa protein was probably a bacterial protein co-purified with the HC-Pro proteins (Fuellgrabe *et al.*, 2011).

An ELISA was carried out in order to check the *in vitro* binding of HC-Pro$^{FRNK/FINK}$ with AtHEN1, and to determine the binding site of HC-Pro^{FRNK} with AtHEN1. A microtitre plate was coated with 50 U AtHEN1 RNA methyltransferase (cloned from *Arabidopsis* plants with a physical purity of 95 %; NEB) diluted in 100 µl 3 % BSA in PBS buffer. The plate was incubated at 4 °C overnight. Non-specific binding was blocked with 3 % BSA in PBS buffer for 1 h. The plate was then washed three times with PBS buffer, then equal amounts (~0.5 µg) of MBP : HA-HC-Pro$^{FRNK/FINK}$ and all ΔN/ΔC-MBP : HA-HC-Pro^{FRNK} deletion mutant proteins diluted in 100 µl of 3 % BSA in PBS buffer were added. MBP was used as a negative control for HC-Pro, while the total bacterial soluble proteins [non-induced BL21(DE3) codon$^+$] were used as negative control for AtHEN1. The plate was incubated at room temperature (RT) for 2 h. Unbound protein was washed out four times with 0.001 % Tween–PBS buffer and once with PBS buffer. Binding of AtHEN1 to HC-Pro was detected by ELISA with 1 : 10 000 of anti-MBP HRP conjugate (NEB) specific for the MBP and 1 : 2000 of anti-mouse antibody conjugated with alkaline phosphatase (AP; Sigma-Aldrich). Finally, fresh substrate was added to the plates followed by incubation at 37 °C for 15 min. The absorbance was then measured using a plate reader (Multiscan Ascent) at 405 nm for the detection of protein–protein interaction.

In order to demonstrate direct binding of HC-Pro to AtHEN1, a microtitre plate was coated either with AtHEN1 (50 U) or total bacterial soluble proteins [1 µg of non-induced BL21(DE3) codon$^+$] as a negative control. Two micrograms of purified MBP : HA-HC-Pro^{FRNK} and MBP : HA-HC-Pro^{FINK} were added, respectively. Successful binding of HC-Pro(s) to AtHEN1 or to total bacterial soluble proteins was shown using anti-MPB, followed by anti-mouse AP detection. Our results indicated that MBP : HA-HC-Pro$^{FRNK/FINK}$ bound AtHEN1. However, MBP : HA-HC-Pro^{FRNK} appeared to bind stronger than MBP : HA-HC-Pro^{FINK} (Fig. 1). The lower binding of MBP : HA-HC-Pro^{FINK} implied that the arginine in the FRNK box may play a role not only in binding to siRNA (Shiboleth *et al.*, 2007; Fuellgrabe *et al.*, 2011), but also to AtHEN1. The lower binding between HC-Pro and total bacterial proteins (about threefold) indicated the specificity of binding between HC-Pro and AtHEN1.

In order to identify the binding domain of HC-Pro to AtHEN1, deletions in the N and C termini of HC-Pro^{FRNK} were generated as described above (Supplementary Fig. S1). Two micrograms of each protein fraction were used for a HEN1-binding assay using ELISA. With one exception the deletion of either the N or the C terminus of HC-Pro strongly reduces AtHEN1 binding (Fig. 2). Interestingly ΔC7, in which most of the HC-Pro C terminus was deleted, showed strong binding to AtHEN1. This strong binding could be due to folding of the HC-Pro ΔC7 that would promote an efficient exposition of the HC-Pro domain interacting with AtHEN1. This result suggests that the HC-Pro–AtHEN1 interaction requires proper folding more than the structure of the protein for the strong binding (Atreya & Pirone, 1993). Our data did not enable us to identify the HC-Pro-binding domain at the amino acid level. Due to the observation that all N- and, with the exception of HC-Pro ΔC7, all other C-terminal HC-Pro deletions led to a loss of interaction with AtHEN1, we assume that the putative-binding domain of HC-Pro is

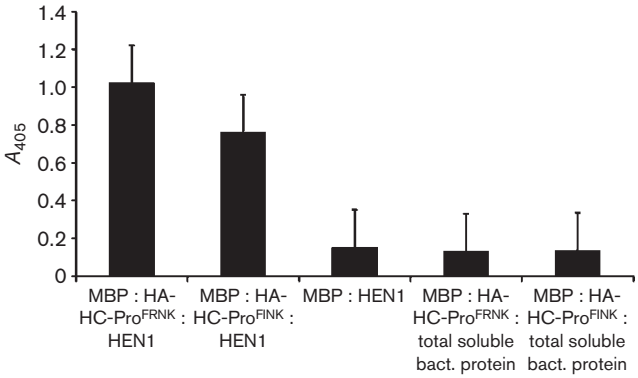

Fig. 1. Binding of HC-Pro to HEN1 *in vitro*. AtHEN1 or total soluble bacterial proteins [non-induced BL21(DE3) codon$^+$] were incubated with HC-Pro in a microtitre plate. Bound HC-Pro proteins were detected after incubation with anti-MBP followed by anti-mouse IgG-conjugated AP-mediated reaction. The signal was measured at A_{405}. Values are the mean of triplicate determination with SD represented by error bars reduced by the mean value of the negative control (MBP).

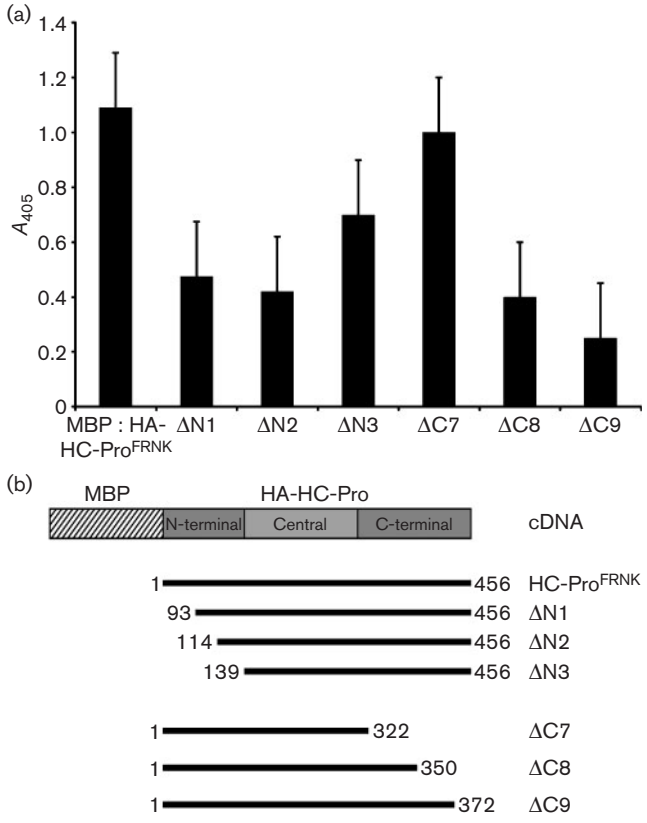

Fig. 2. Rough mapping of the domains of HC-Pro interacting with HEN1 using ELISA. (a) Analysis of the binding activities between AtHEN1 and the N-terminal deletion mutants (ΔN1–ΔN3) and the C-terminal deletion mutants (ΔC7–ΔC9) of MBP : HA-HC-Pro^{FRNK}. Bars in Fig. 2(a) represent the mean of triplicate determinations deducted by SD of the mean value of the negative control (MBP). (b) Schematic maps of MBP : HA-HC-Pro^{FRNK} and its mutants. The cDNA of MBP : HA-HC-Pro^{FRNK} is presented by the open box. The N-, C-terminal and the central domains of the HC-Pro^{FRNK} are indicated. HC-Pro^{FRNK} and its mutants are shown as solid lines.

located close to the centre of the protein between positions 139 and 320 of ZYMV HC-Pro. Alternatively, one may speculate that an N- and a C-terminal domain of HC-Pro are both required for AtHEN1 binding. However, the observation that HC-Pro ΔC7 strongly bound to AtHEN1 argues against this hypothesis.

A methyltransferase inhibition assay was carried out to analyse the effect of the ZYMV HC-Pro on HEN1 activity. The inhibition assay was performed according to Yu *et al.* (2005) with some modifications. The reaction was carried out with 100 µl of total reaction mixture that contained 1× NEB 2 buffer (NEB), 1 µg miR173 (Sigma-Aldrich), 2 µl Ribo Lock RNase-Inhibitor (40 U µl^{-1}; Fermentas), 2 µl AtHEN1 RNA methyltransferase, 4 µl *S*-adenosyl-L-[methyl-^{14}C] methionine (58.0 mCi mmol^{-1}; GE Healthcare) and 5 µg protein (MBP : HA-HC-Pro$^{FRNK/FINK}$). As negative controls MBP, MBP buffer or total bacterial soluble proteins were added instead of MBP : HA-HC-Pro$^{FRNK/FINK}$. After 1 h of incubation at 37 °C, the reaction was stopped by the addition of 100 µl stopping solution [100 mM Tris/HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl, 2 % SDS and 0.4 mg proteinase K ml^{-1}] and incubation for 15 min at 65 °C. The reaction was extracted with phenol/chloroform and the RNA was precipitated by adding 3 µl glycogen and 300 µl 2-propanol, 10 M ammonium acetate (10 : 2) followed by incubation for 1 h at −20 °C for RNA precipitation. The reaction mixture was washed with 70 % ethanol (−20 °C) and the pellet was dried for 10 min at RT before it was resuspended in 10 µl RNase-free water. RNA samples were electrophoresed at 80 V in 1× TBE buffer using a 20 % TBE-polyacrylamide gel (www.anamed-gel.com). The gel was dried at 65 °C for 1 h, exposed to FujiFilm Imaging Plates (Fujifilm) and scanned using PharosFX Plus PhosphorImager (Bio-Rad).

HEN1 has an *S*-adenosyl methionine (SAM)-binding pocket and can methylate the 3′-terminal 2′-*O*-methylation of sRNAs (Yu *et al.*, 2005). It was clear from our ELISA results that MBP : HA-HC-Pro$^{FRNK/FINK}$ can bind to AtHEN1. To test whether HC-Pro can inhibit the methlytransferase activity of HEN1 *in vitro*, a methyltransferase inhibition assay was performed as described above using adenosyl-^{14}C. In such an assay, the incorporation of labelled methyl-^{14}C in the 3′ end of RNA templates can be detected (Kurth & Mochizuki, 2009). Purified MBP : HA-HC-Pro$^{FRNK/FINK}$ and the truncated proteins ΔN1 and ΔC9 were incubated with AtHEN1. To exclude that the possible impurities in the protein preparation, including the co-purified 55 kDa protein (Supplementary Fig. S2), could inhibit the AtHEN1 activity the total soluble bacterial proteins [non-induced BL21(DE3) codon^{+}] were included in the assay. Subsequently, a synthetic miRNA duplex of 22 bp whose 3′ ends carried a 2′-hydroxyl and *S*-adenosyl-L[methyl-^{14}C] methionine, which served as a methyl group donor, were added. The methyltransferase inhibition assay showed that HC-Pro$^{FRNK/FINK}$ clearly inhibited AtHEN1 activity *in vitro* (Fig. 3). It is worth noting that the total bacterial extracts did not inhibit the AtHEN1 activity. This indicated that no interaction between bacterial proteins and AtHEN1 occurred. This result also argued against the binding of putative protein contaminants (Fig. 1) to AtHEN1.

It was shown that the FRNK box in the central domain of HC-Pro, which is highly conserved among all sequenced members of the genus *Potyvirus*, is required for binding of double-stranded sRNA duplexes and that replacement of arginine at position 180 (R180) by leucine (I) within this sequence impaired sRNA binding and led to the attenuation of the ZYMV infection symptoms (Shiboleth *et al.*, 2007). There is evidence suggesting that HC-Pro affected HEN1 activity *in vivo* resulting in the suppression of RNA-mediated silencing. However, the mechanism of HEN1 interference by HC-Pro has not yet been demonstrated. The finding that mutating the FRNK box of HC-Pro results in its loss of sRNA-binding activity, even though silencing

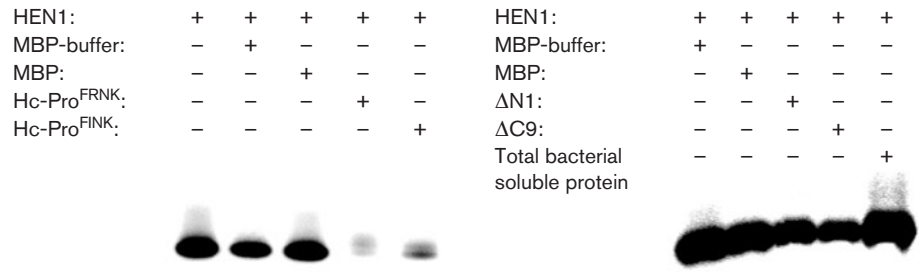

Fig. 3. Methyltransferase inhibition assay. The methyltransferase inhibition assay was carried out to analyse whether the ZYMV HC-Pro interferes with the activity of AtHEN1. Hc-Pro proteins (MBP : HA-HC-Pro^{FRNK} and MBP : HC-Pro^{FINK}), ΔN1 (N-terminal deletion mutant MBP : HA-HC-Pro^{FRNK}), ΔC9 (C-terminal deletion mutant MBP : HA-HC-Pro^{FRNK}) or MBP and total soluble bacterial proteins [non-induced BL21(DE3) codon^{+}] as negative controls were mixed with miR173 duplex, HEN1 (AtHEN1) and *S*-adenosyl-L-[methyl-^{14}C] methionine and incubated for 1 h at 37 °C. RNA samples were separated using 20 % TBE-polyacrylamide gels. The gels were dried and scanned using PharosFX Plus PhosphorImager.

suppression remains (Shiboleth *et al.*, 2007) prompted us to further investigate the silencing suppression function of HC-Pro.

Our results show that HC-Pro$^{FRNK/FINK}$, but not the truncated proteins (ΔN1 and ΔC9) displaying decreased *in vitro* affinity for AtHEN1 binding, have inhibited the methyltransferase activity of AtHEN1 *in vitro*. It was proposed that HC-Pro could bind to sRNA and subsequently protect HEN1 from sRNA templates (Yu *et al.*, 2006). Other work (Lózsa *et al.*, 2008) has shown varying effects of some RNA silencing suppressors on siRNA 3′ modification in *Nicotiana benthamiana* plants infected with viruses expressing RNA silencing suppressors, namely the p19 protein of *Carnation Italian ringspot virus* (CIRV) and the HC-Pro of *Tobacco etch virus* (TEV). The effects ranged from slight modification by CIRV to significant inhibition of si/miRNA modifications by TEV. It was proposed that HC-Pro covers the 3′ overhangs of sRNAs, resulting in inhibition of 3′ modifications by blocking HEN1 accessibility to sRNA. Alternatively, it was speculated that HC-Pro competes with HEN1 (Lózsa *et al.*, 2008). However, it was shown that HC-Pro^{FINK} lost sRNA-binding activity *in vitro* and *in vivo* but retained its silencing suppressor activity (Fuellgrabe *et al.*, 2011; Shiboleth *et al.* 2007). Thus, our results strongly indicate that inhibition of AtHEN1 activity by HC-Pro$^{FRNK/FINK}$ is probably due to direct interaction between both proteins. It can therefore be concluded that HEN1 inhibition and sRNA-binding activities of HC-Pro are independent of each other.

However, further work is needed to identify the binding domain of HC-Pro with HEN1 at the amino acid level and also to confirm the interaction in living plant cells.

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