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Short communication

Improved silencing suppression and enhanced heterologous protein expression are achieved using an engineered viral helper component proteinase

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ABSTRACT

RNA silencing limits transient expression of heterologous proteins in plants. Co-expression of viral silencing suppressor proteins can increase and prolong protein expression, but highly efficient silencing suppressors may stress plant tissue and be detrimental to protein yields. Little is known whether silencing suppression could be improved without harm to plant tissues. This study reports development of enhanced silencing suppressors by engineering the helper component proteinase (HCpro) of *Potato virus A* (PVA). Mutations were introduced to a short region of HCpro (positions 330–335 in PVA HCpro), which is hypervariable among potyviruses. Three out of the four HCpro mutants suppressed RNA silencing more efficiently and sustained expression of co-expressed jellyfish green fluorescent protein for a longer time than wild-type HCpro in agroinfiltrated leaves of *Nicotiana benthamiana*. Leaf tissues remained healthy-looking without any visible signs of stress.

Production of heterologous proteins in plants using transgene or transient expression based systems is used commonly for research, pharmaceutical and other purposes (Desai et al., 2010). However, protein yields are often lower than expected (Daniell et al., 2001), because expression of genes under a strong promoter may trigger RNA silencing, a cytoplasmic surveillance mechanism that targets the over-expressed or aberrant mRNAs to degradation (Dougherty and Parks, 1995; Sijen et al., 1996). This mechanism is induced by double-stranded RNA (dsRNA) produced by the cellular RNA-dependent RNA polymerase using the single-stranded RNA (ssRNA) transcripts of the transgene as a template, and can reduce mRNA quickly to undetectable levels (Baulcombe, 2007; Sijen et al., 1996, 2001). RNA silencing is also an antiviral defence system in plants and other cellular organisms (Wang et al., 2012). Viruses, in turn, have evolved to obstruct RNA silencing with the suppressor proteins they produce (Burgyán and Havelda, 2011).

Transient protein expression in leaves by agro-infiltration (Kapila et al., 1997) is used as an alternative to transgenic plants. *Nicotiana benthamiana* is a well-suited species for production of foreign proteins in leaves, because the leaves of this species are easy to agro-infiltrate and high yields of the protein can be obtained (Nausch et al., 2012; Sheludko et al., 2007). Viral RNA silencing suppressor proteins can be used to enhance transient expression of co-expressed genes (Dhillon et al., 2009). The helper component proteinase (HCpro) of potyviruses (genus *Potyvirus*; *Potyviridae*) is an efficient suppressor of RNA silencing. It was first shown to enhance transient expression of the jellyfish (*Aequorea victoria*) green fluorescent protein (GFP) in agroinfiltrated leaves of *N. benthamiana* (Johansen and Carrington, 2001) and later many other proteins (*e.g.*, Chiera et al., 2008; Chiu et al., 2010; Wan et al., 2012). Recent studies show that amino acid (aa) substitution introduced to the HCpro of *Tobacco etch virus* (TEV) can enhance or impair silencing suppression by HCpro, indicated by changes in the levels of co-expressed GFP mRNA in agroinfiltrated leaves of *N. benthamiana*, however, the amounts of GFP and HCpro proteins accumulating in the leaves were not studied (Torres-Barceló et al., 2008).

There is little information as to whether or not the efficiency of heterologous protein expression could be enhanced by engineering viral silencing suppressor proteins. The aim of this study was to compare the wild-type HCpro (wtHCpro) of *Potato virus A* (PVA) with four PVA HCpro mutants for their capacity to sustain transient protein expression in agroinfiltrated leaves of *N. benthamiana*. The tested HCpro mutants carry aa substitutions in a recently described, highly variable region (HVR; aa 330–335 in PVA HCpro), whose aa sequence varies greatly among potyviruses (Haikonen et al., 2013a). Prediction of protein structures with the latest protein modelling tools suggests that mutations in HVR alter the structure of HCpro (Haikonen et al., 2013a). The results presented here indicate that accumulation of HCpro and the co-expressed heterologous proteins can be enhanced significantly by mutation of HVR in HCpro.

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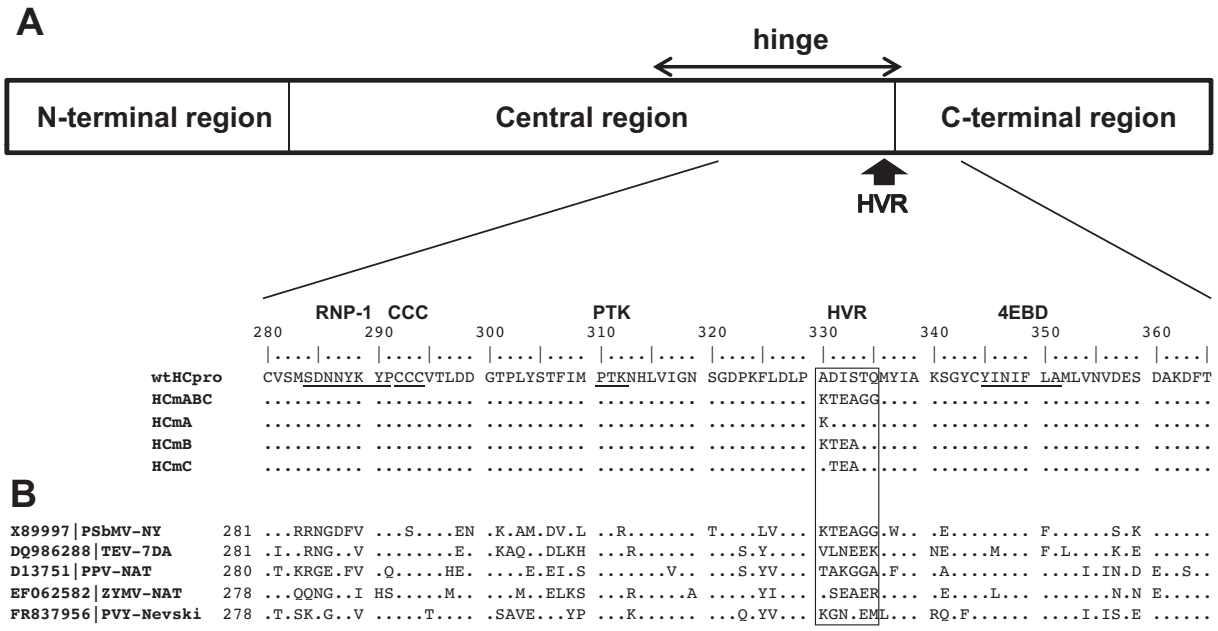

Fig. 1. The hypervariable region (HVR) in helper component proteinase (HCpro). (A) Schematic presentation of HCpro and alignment of HCpro sequences of *Potato virus A* (wtHCpro) and four mutants (HCmABC, HCmA, HCmB, and HCmC). Amino acid substitutions in HVR (boxed) are indicated in mutants and correspond to the residues in HVR of *Pea seed-borne mosaic virus* (PSbMV). Numbers above the alignment indicate the amino acid positions in PVA HCpro. RNP-1, CCC, PTK and 4EDB are conserved amino acid motifs (underlined). (B) A partial amino acid alignment of the HCpro sequences of PSbMV, *Tobacco etch virus* (TEV), *Plum pox virus* (PPV), *Zucchini yellow mosaic virus* (ZYMV) and *Potato virus Y* (PVY). Genebank sequence accession numbers are indicated. Numbers in the beginning of the sequences refer to the position in HCpro of the first residue shown.

Cloning and mutagenesis of the HCpro-encoding sequence of PVA (NCBI database accession no. AJ296311) have been described (Haikonen et al., 2013a). In brief, aa residues were substituted in the HVR of PVA HCpro to make one, three, four, or all six of them identical to the HVR of *Pea seed-borne mosaic virus* (Fig. 1), in which all HVR aa residues differ from PVA. The mutated HCpro clones (HCmA, HCmB, HCmC and HCmABC) (Fig. 1) were inserted into the binary expression vector pLH-YN under the *Cauliflower mosaic virus* 35S promoter as described for wtHCpro (Ala-Poikela et al., 2011). The binary vectors were used to transform *Agrobacterium tumefaciens* (C58C1, Ti-plasmid pGV2260) and the proteins were expressed by agroinfiltration in leaves of *N. benthamiana*. GFP and ß-glucuronidase (GUS) were expressed from the binary vectors pBIN-mGFP4 (Haseloff et al., 1997) and pA-GUS (Vancanneyt et al., 1990), respectively, as described (Kreuze et al., 2005).

HCpro mutants were tested for suppression of sense-mediated RNA silencing (co-suppression) using the "silencing on the spot" assay described by Johansen and Carrington (2001). The cultures of agrobacteria transformed with the binary vectors were collected by centrifugation, diluted with infiltration medium (10 mM $MgCl_2$ and 20 mM acetosyringone) to final optical density at 600 nm (OD600) of 0.5, incubated at room temperature for 3 h, and combined in equal ratios for agroinfiltration of leaves of the transgenic *N. benthamiana* line 16c constitutively expressing GFP (Brigneti et al., 1998; seeds kindly provided by D.C. Baulcombe, University of Cambridge, UK). GFP was coexpressed with wtHCpro, HCmA, HCmB, HCmC, HCmABC, or GUS (negative control) and leaves were observed for GFP fluorescence under ultraviolet-A (UV-A) light (360–370 nm) at 3, 5, 7 and 9 days post-infiltration (dpi). The experiment was carried out three times.

At 3 dpi, all agroinfiltrated areas of the leaf expressed more or less similar levels of GFP fluorescence, which was higher than background fluorescence of the *gfp*-transgenic leaves of *N. benthamiana* line 16c (Fig. 2A). However, at 5 dpi, only background levels of GFP fluorescence were observed in the leaf tissue agroinfiltrated for co-expression of GFP and GUS or HCmABC (Fig. 2A, spots 6 and 2, respectively). Green fluorescence in the tissue co-expressing GFP and wtHCpro was above the background level (Fig. 2A, spot 1), and the fluorescence in the tissue co-expressing GFP and HCmA, HCmB or HCmC was brighter than with wtHCpro (Fig. 2A). At 7 dpi, no GFP fluorescence above the background level was observed in tissues co-expressing GFP and wtHCpro or HCmABC (Fig. 2A, spots 3–5, respectively). In contrast, the tissues co-expressing GFP and HCmA, HCmB or HCmC showed brighter GFP fluorescence than before (Fig. 2A) and continued displaying enhanced GFP fluorescence 9 dpi, after which the fluorescence declined (data not shown). The leaf tissue co-expressing GFP and GUS showed more pronounced red autofluorescence of chlorophyll than the surrounding tissues at 7 dpi, indicating that sense-mediated silencing of the GFP transgene expression had occurred in the absence of silencing suppressors (Fig. 2A, spot 6).

Correlation of GFP fluorescence with accumulation of HCpro and GFP in leaves was tested by immunoblotting using antibodies to PVA HCpro and GFP, as described (Ala-Poikela et al., 2011). In brief, total protein was extracted from 20 mg of leaf tissue, homogenized in liquid nitrogen, resuspended in 80 μl of 2× extraction buffer [0.1 M Tris-HCl, 20% glycerol, 10% (v/v) β-mercaptoethanol, 4% (w/v) sodium dodecyl sulfate (SDS), 0.2% bromophenol blue], boiled for 5 min and clarified by centrifugation. Aliquots of 25 μl were loaded on a 12% SDS polyacrylamide gel, separated by electrophoresis and electroblotted onto a polyvinylidene fluoride membrane (GE Healthcare, Buckinghamshire, UK). Proteins remaining in the gel were stained with Coomassie blue (Thermo Fisher Scientific, Waltham, MA) to document loading. The membranes were probed with PVA HCpro-specific or GFP-specific polyclonal rabbit antiserum and incubated with horse radish peroxidase-conjugated anti-rabbit donkey antibodies (GE Healthcare). Signals were developed on an X-ray film using an enhanced Super Signal West Femto chemiluminescent substrate (Thermo Scientific, Rockford, IL).

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