Identification of silencing suppressors of potato virus M

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Abstract
Gene silencing is an important regulatory and defense mechanism in plants. Plant viruses evolved some proteins that suppress cellular RNA interference mechanism. In this work identification of silencing suppressors of potato virus M (PVM) is reported. The cDNA sequences of different PVM open reading frames were cloned into the Gateway-compatible binary T-DNA destination vector pMDC32, harboring a double 35S promoter. Each of these constructs was electroporated into Agrobacterium tumefaciens C58C1 which then were agroinfiltrated into Nicotiana benthamiana 16C line. It was shown that viral coat protein (34K) as well as some other PVM proteins (12K, 11K) possessed suppressor properties of cellular silencing activity.

Keywords: Gene silencing, suppressors of silencing, potato virus M, agroinfiltration, Nicotiana benthamiana 16C line

Patates virüsü M’nin susturucu baskılayıcılarının tayini

Özet

Anahtar Sözcükler: Gen susturma, susturmanın baskılayıcıları, patates virüsü M, agroinfiltrasyon, Nicotiana benthamiana 16C hatti

Introduction
Recently a new defense system was detected that does function in plants, namely post-transcriptional gene silencing (PTGS) or RNA interference (RNAi). In response to virus infection, this “adaptive immune-system” is induced based on the recognition of double-stranded (ds) RNA or excessive quantities of RNA (Jorgensen, 2003). RNAi is an evolutionary conserved mechanism in many, if not all, eukaryotes, to target and degrade aberrant endogenous or exogenous RNA molecules (Sontheimer, 2005; Voinnet, 2005).

Due to this silencing defense system, plant viruses are thought to need a counter defense mechanism to successfully infect a plant. Depending on the virus, such PTGS suppression can be a secondary function of almost any type of viral protein. In plants, RNA silencing has been demonstrated to be one of the most important
antiviral mechanisms (Li and Ding, 2001; Ding et al., 2004). As a response to this highly efficient antiviral RNA silencing pathway, plant viruses have evolved specific suppressor proteins. Many of these proteins were previously described as virulence factors or pathogenicity determinants. It has been reported that many plant viruses encode suppressor proteins to combat against RNA silencing (Voinnet, Pinto and Baulcombe, 1999; Li and Ding, 2001). Identification of silencing suppressors of different viruses is a crucial step in direction of development of resistance against viral infection (Ding and Voinnet, 2007).

Silencing suppressors can interfere with several steps of the silencing pathway, i.e. dsRNA cleavage to produce siRNAs, destabilization of siRNAs, binding of siRNAs and siRNA usage as part of RISC, which is required for target RNA elimination. Silencing suppressors are usually multifunctional viral proteins and in different virus families different viral proteins gain suppressor function as an additional property. Interference with suppressor function would inhibit the viral counter-defense system.

The research and identification of silencing suppressors of PVM were carried out. Potato is one of the most important crops, and PVM, together with PVY, is responsible for 80% of all virus-caused losses of potato yields, especially in Kazakhstan (Sozinova et al., 2007). Thus the identification of PVM’s silencing suppressors points to the role of gene silencing as a natural antiviral defense system in plants.

Materials and methods

RNA isolation

A PVM isolate was maintained in *Nicotiana tabacum* plants. Leaves of *N. tabacum* were harvested and frozen in liquid nitrogen, ground to a fine powder and mixed with Tri-reagent (“Sigma”), extracted once with 1/5 volume of chloroform. RNA was precipitated with the addition of an equal volume of isopropanol and incubated at +4°C for 30 min. RNA was washed with 70% ethanol and dissolved in water.

Construction of PVM vectors

The last 5 of 6 protein sequences of PVM were obtained with using SuperScript II Reverse Transcriptase according to manufacture protocol. PVM cDNA fragments encoding the 25kDa, 12kDa, 7 kDa, coat protein (CP) and 11 kDa were PCR-amplified from cDNA and individually cloned using Gateway cloning kit (Invitrogen). First, the PCR products were cloned into pDONR207 vector using BP clonase, then they were re-cloned into pMDC32 destination vector harbouring double 35S promoter.

Plant material and *A. tumefaciens* infiltration

GFP transgenic 16C line of *Nicotiana benthamiana* plants were grown as described previously (Marano and Baulcombe, 1998; Voinnet et al. 1998). Recombinant *Agrobacterium tumefaciens* strain C58C1, carrying different constructs, were grown at 28°C in 2 ml of LB medium with 2,5 μg/ml tetracycline and 50 μg/ml kanamycin for two days, then transferred to 18 ml of fresh LB medium with appropriate antibiotics and were grown overnight at 28°C. The bacteria were centrifuged at 4000 g for 10 min. The pellet was resuspended in agroinfiltration solution (10mM MgCl₂, 10 mM MES and 100 μM acetosyringone) up to desired optical density (OD₆₅₀). The bacteria solution was left at room temperature for overnight before infiltration. Infiltration carried on underside leaves of 5-7-week-old *N. benthamiana* plants. For co-infiltration, equal volumes of indicated *A. tumefaciens* cultures (OD₆₅₀=1) were mixed before infiltration. After 5-10 days post infiltration, plants were observed for silencing effect.

GFP Imaging

Visual detection of GFP fluorescence in whole plant was performed using a 100W hand-held long-wave ultraviolet lamp. Plants were photographed with a yellow filter.

Results

Potato virus M belongs to the genus *Carlavirus* of the *Flexiviridae* family. The PVM genome is a single positive-strand RNA of 8534 nucleotides. The sequence contains six open reading frames (ORFs) (Figure 1), and non-coding regions consisting of 75 nucleotides at the 5'-end and 70 nucleotides followed by a poly(A) tail at the 3'-end (Zavriev et al., 1991).
The ORF beginning at the first initiation codon at nucleotide 74 encodes a polypeptide of 223K, virus RNA replicase (Table 1). The next coding block consists of three ORFs encoding polypeptides of 25K, 12K and 7K, which is responsible for viral cell-to-cell movement. The third block consists of two ORFs encoding polypeptides of 34K (coat protein) and 11K. The 11K polypeptide contains a pattern resembling the consensus for a metal-binding nucleic acid-binding zinc-finger motif.

Table 1. Proteins of PVM and their sizes

<table>
<thead>
<tr>
<th>Symbol of protein</th>
<th>Protein</th>
<th>Start (nt)</th>
<th>End (nt)</th>
<th>Length (bp)</th>
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<td>ORF 1</td>
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<tr>
<td>ORF 6</td>
<td>11kDa protein</td>
<td>8136</td>
<td>8462</td>
<td>108</td>
</tr>
</tbody>
</table>

According to articles for last years (Chiba et al., 2006), the protein 11K is most putative silencing suppressor. The aim of our investigation was the check of suppressor activity of all PVM proteins except first one, largest polyprotein due to its size.

The primers for cloning were designed using the GeneRunner programme version 3.0 (ORF2sense 5'-gggacactttgtacaaaagcagctatgtgaaatgt-3' and ORF2antisense 5'-ggggacagttagaagaatgggtcatttacctattatacgtaatctgt-3' for ORF2; ORF3sense 5'-gggacactttgtacaaaagcagctatggagctaaacacgagc-3' and ORF3antisense 5'-gggacactttgtacaaaagcagctatgcacttacccgc-3' for ORF3; ORF4sense 5'-gggacactttgtacaaaagcagctatgtgaaatgttgaatc-3' and ORF4antisense 5'-gggacactttgtacaaaagcagctatgtgaaatgttgaatc-3' for ORF4; ORF5sense 5'-gggacactttgtacaaaagcagctatgcacttacccgc-3' and ORF5antisense 5'-gggacactttgtacaaaagcagctatgcacttacccgc-3' for ORF5; ORF6sense 5'-gggacactttgtacaaaagcagctatgcacttacccgc-3' and ORF6antisense 5'-gggacactttgtacaaaagcagctatgcacttacccgc-3' for ORF6). The Gateway-kit (Invitrogen) was used for cloning. The DNA of the different PVM ORFs were obtained by RT-PCR, and cloned into the Gateway-compatible binary T-DNA destination vector pMDC32. Each of these constructs were verified by nucleotide sequencing.

The *Nicotiana benthamiana* plant, constitutively expressing GFP transgene (line 16C; a gift from David Baulcombe), and the *Agrobacterium tumefaciens* C58C1 strain according to the manufacturer’s instructions. All constructs described above were verified by nucleotide sequencing.

The *Nicotiana benthamiana* plant, constitutively expressing GFP transgene (line 16C; a gift from David Baulcombe), and the *Agrobacterium tumefaciens* infiltration operation have been described previously (Hamilton et al., 2002). The *N. benthamiana* line 16C were cultured in growth chambers at 22 to 24°C before and after infiltration. For coinfiltration, equal volumes of individual *Agrobacterium* cultures (OD_{600}= 1) were mixed.
prior to infiltration. HC-Pro of potato virus Y and P19 of tombusviruses were used as a positive control. GFP fluorescence was observed under long-wavelength UV light and photographed using a digital camera with a yellow filter.

GFP expression reached the highest level in all leaves infiltrated with GFP, as well as GFP plus other genes at 5 to 7 days postinfiltration (dpi), as shown by the enhanced green fluorescence in the infiltrated patches. The green fluorescence intensity remained strong in the patches coinfiltrated with ORF 3 and ORF 5 during the 7-9 dpi. Coinfiltration with ORF 6 also resulted in GFP fluorescence over a similar period of observations, but not as strong and clear as above described samples (Figure 2). On the other hand, GFP was silenced upon inoculation with ORF 2 and ORF 4.

Figure 2. Effects of different ORFs of PVM on gene silencing in GFP transformants of Nicotiana benthamiana. Infiltrated leaves are shown. Silencing of GFP is manifested in red fluorescent. Green spots indicate suppression of silencing. Photographs were taken of UV-illuminated plants. 1 and 2: HC-Pro and P19, respectively, are shown as control; 3: ORF2, 25K protein; 4: ORF3, 12K protein; 5: ORF4, 7K protein; 6: ORF5, coat protein; 7: ORF6, 11K protein

According to these data the most promising candidates as silencing suppressors are ORF 3 (12K protein; Table 1), ORF 5 (coat protein; Table 1) and, in lesser extent, ORF 6 (11K protein; Table 1), while ORF 2 and ORF 4 revealed no suppressor activity. We have repeated this experiment three times which yielded the same results.

Discussion

Most of the viruses have some sort of silencing suppressor system as an evolutionary counter-response to the antiviral activity of the plant gene silencing defence. Gene silencing suppressors can inhibit silencing of transgenes or endogenous genes. Test for silencing suppression was based on a previously described experimental system (Brigneti et al., 1998). When leaves of N. benthamiana line 16C plants were agroinfiltrated with an A. tumefaciens C58C1 strain carrying the construct 35S GFP, the strong green fluorescent signal disappeared under UV light due to GFP silencing. However, if 35S-ds GFP and any strong silencing suppressor are co-agroinfiltrated, the fluorescent signal does not disappear and the area does not look dark under UV light. This is due to the inhibition of gene silencing caused by that suppressor.

Thus, the result of experiments shows that ORF3 (12K protein; part of triple block, cell-to-cell movement), ORF5 (coat protein) and ORF6 (11K protein) of potato virus M has a suppressor activity. It corresponds to the results of recent articles about suppression activity of virus proteins responsible for long-distance movement (Voinnet, 2001), viral coat proteins (Roth, Pruss and Vance, 2004) and 10K-16K proteins with a conserved amino acid sequence motif encoded by diverse filamentous viruses (Chiba et al., 2006).

The 12K protein encoded by the second ORF of PVM triple gene block (TGB) which involved in the cell-to-cell and long-distance movement of virus. This protein possesses conserved hydrophobic domain and plays significant role in TGB functions (Seppänen et al., 1997). TGB-containing viruses have evolved special mechanisms to suppress RNA silencing (Voinnet et al., 2000; Morozov and Solovyev, 2003).

The potex-like Carlavirus PVM requires the coat protein for virus cell-to-cell movement (Morozov and Solovyev, 2003; Verchot-Lubicz, 2005). In RNA silencing assays, plant viral suppressors differ by their ability to suppress intracellular and/or intercellular silencing (Li and Ding, 2001; Roth, Pruss and Vance, 2004). Spreading of silencing in plants occurs through plasmodesmata and results from a cell-to-cell movement of a short-range silencing signal, most probably 21-nt short interfering RNAs (siRNAs), that are produced by one of the plant Dicer enzymes (Kalantidis et al., 2008).

11K protein had been shown to be a non-specific nucleic acid-binding protein (Gramstat, Courtpozanis and Rohde, 1990), similar to p10 which is silencing suppressor of Grapevine virus A (Zhou et al., 2006). Interestingly, 11K is supposed to be translated from the CP subgenomic RNA by a (-1) frameshift within the A-rich region
AAUAGAAAAUGA preceding the 12K AUG translational start codon (underlined) and representing the 3' terminus of the CP coding region (UGA stop codon in bold letters) (Gramstat, Courtpozanis and Rohde, 1990).

This article describes the identification of viral suppressors of RNA silencing encoded by a natural pathogen. An exact role for any of the identified PVM suppressors in host infection remains to be established.

The potential of using viral suppressors to help understand the mechanism of RNA silencing in plants is largely unexplored, and these studies promise to be a fertile area of research. Viral suppressors of silencing also provide unique tools to understand the mechanism of RNA silencing as well as interaction between virus and their hosts. Moreover, it became an extremely useful technique for molecular biology and very powerful biotechnological tool.

Acknowledgement

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introduction of ectopic promoterless DNA. 


