تغییر بیان زندهای مرتبط با انفوژه در پاسخ به سرکوبگر خاموشی HC-Pro ویروس ای سیب زمینی (Potato virus A) (PVX) در گیاه Nicotiana benthamiana

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چکیده

در سال‌های اخیر نقش انفوژی در خاموشی آرانی این اتفاق شده توسط ویروس از طریق فعالیت پروتئین‌های سرکوبگر خاموشی آرانی بی‌این انتظار می‌رود. به منظور دکر بهتر نقص احتمالی سرکوبگر خاموشی HC-Pro ویروس ای سیب زمینی در فرآیند انفوژه HC-Pro (autophagy) اثر انگشتی و ویروس روی بیان تعدادی از زندهای مهم و دیگر در انفوژی سورد پرستی قرار گرفت. زندهای این ویروس ای سیب زمینی در نوای گیاه pGWB17 با چرخه myc در انفوژه کروپسیلی و پرومتر 3S در انتهای آمیزه توسط تکنولوژی Gateway در پاسخ به سرکوبگر خاموشی HC-Pro ویروس HRG1 و HRG2 و ATG6، ATG7 و ATG10 در گیاه Nicotiana benthamiana گیاهی و اسیدهای سرکوبگر خاموشی (Real-Time PCR) در فاصله زمانی پنج روز پس از انفوژه با استفاده از تکنیک ریل تایم اسی آر سرکوبگر خاموشی HC-Pro ویروس ای سیب زمینی میزان بیان زندهای HRG6 و HRG7 و ATG6 و ATG7 با واژن 68 و 77 درصد و HRG1 با واژن 48 درصد در مطالعه کننده میزان رونوشت به گرده‌های تزیین شده با سازه‌های پلاسمید خالی می‌باشد. کمیل‌گردان خاموشی گیاه شده توسط آرانی، به نوبت به میزان در گرده‌های قادر کامل نشان داد. نتایج این تحقیق حاکی از دخله‌ی زندهای ATG6، ATG7 و ATG10 در پاسخ‌های دفاعی گیاه N. benthamiana و ویروس ای سیب زمینی و امکان استفاده از آن در تدوین روش‌های جدید مدیریت ویروس‌های گیاهی می‌باشد.

کلیدواژه‌ها: انفوژه، سرکوبگر خاموشی، نیکوتینیا بنتهامیانا، HC-Pro

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Altered expression of autophagy-related genes in Nicotiana benthamiana plants in response to Potato virus A HC-Pro silencing suppressor

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Abstract
Accumulating data have revealed the role of autophagy in virus-induced RNA silencing via viral RNA silencing suppressor activity. To extend our understanding for the possible role of Potato virus A-HC-Pro (PVA-HC-Pro) suppressor protein in autophagy process, we investigated the effect of PVA-HC-Pro on expression of some important genes involved in autophagy. The cDNA of ORF PVA HC-Pro was cloned in pGWB17 vector with a C-terminal myc tag and N-terminal 35S promoter using Gateway technology. Agrobacterium cultures harboring PVA-HC-Pro were infiltrated into the abaxial side of N. benthamiana leaves. The expression of ATG6, ATG2, ATG7 and AGO1 genes were measured at 5 days post infiltration in response to PVA-HC-Pro using qRT-PCR technique. PVA HC-Pro as a suppressor of RNA silencing increased the expression level of ATG6, ATG2 and ATG7 5.89, 7.3 and 7.6 fold, respectively, compared to corresponding values in leaves infiltrated with empty plasmid (EP) as a control. In contrast, the transcript level of Argonaute1 (AGO1), a key component of RNA-induced silencing complex (RISC), was decreased by 1.36-fold compared to the level of AGO1 in infiltrated leaves without PVA-HC-Pro. Results of this study indicated that PVA HC-Pro can alter the expression of autophagy related genes in N. benthamiana plant. These findings suggest the involvement of ATG6, ATG2, ATG7 and AGO1 in defense responses of N. benthamiana against PVA-HC-Pro, which might be considered in plant breeding programs as a novel approach to the control of plant viruses.

Keywords: Autophagy, HC-Pro, N. benthamiana, RNA silencing, Suppressor of RNA Silencing

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Introduction

Autophagy is considered as an important intracellular process in eukaryotes for the removal and recycling of cytoplasmic components including damaged proteins and organelles (Boya et al. 2013, Klionsky 2005). More than 30 autophagy-related (ATG) genes have been identified in Arabidopsis and other plants including tobacco, rice and maize (Bassham et al. 2006, Liu & Bassham 2012). Many ATG genes involved in the core process of autophagy have been functionally analyzed in plants (Chung et al. 2009, Liu et al. 2005, Shin et al. 2009, Su et al. 2006). ATG6, ATG2 and ATG7 genes have been shown to play roles in antiviral defense (Carbonell & Carrington 2015, Garcia-Ruiz et al. 2015). Activated autophagy can be induced in response to a variety of stresses including viruses (Boya et al. 2013). Autophagy is considered as an important intracellular process for the removal and recycling of cytoplasmic components including damaged proteins and organelles (Boya et al. 2013, Klionsky 2005). More than 30 autophagy-related (ATG) genes have been identified in Arabidopsis and other plants including tobacco, rice and maize (Bassham et al. 2006, Liu & Bassham 2012). Many ATG genes involved in the core process of autophagy have been functionally analyzed in plants (Chung et al. 2009, Liu et al. 2005, Shin et al. 2009, Su et al. 2006). ATG6, ATG2 and ATG7 genes have been shown to play roles in antiviral defense (Carbonell & Carrington 2015, Garcia-Ruiz et al. 2015). Activated autophagy can be induced in response to a variety of stresses including viruses (Boya et al. 2013). Autophagy can be induced in response to a variety of stresses including viruses (Boya et al. 2013). Activated autophagy can promote antiviral immunity through the transfer of viruses from the cytoplasm to the lysosomes for degradation or the transfer of viral components to specific subcellular compartments for the activation of innate or adaptive antiviral immunity (Shoji-Kawata & Levine 2009). Mutations of autophagy genes increased host susceptibility to bacterial, viral and protozoan pathogens (Deretic 2012, Levine et al. 2011, Shoji-Kawata & Levine 2009). On the other hand, pathogens have evolved mechanisms to avoid death by autophagy pathways through the evasion of autophagic recognition, inhibition of autophagy, modulation of autophagosome maturation and modification of the overall state of autophagy. It demonstrates the key role of autophagy in host innate immunity in response to pathogens (Deretic 2012, Levine et al. 2011, Shoji-Kawata & Levine 2009). There is an emerging knowledge on the role of autophagy in virus-induced RNA silencing. Autophagy can act either as an antiviral collaborator for targeted degradation of viral RNA silencing suppressors (VSR) or as a counter-defense mechanism against invading viruses for inactivating plant RNA silencing machinery (Derrien et al. 2012, Nakahara et al. 2012, Tadamura et al. 2012). This process is involved in the degradation of plant and viral proteins associated with RNA silencing (Agius et al. 2012). RNA silencing is important for the regulation of development in animals and plants, but also plays an antiviral role in plants. Plant antiviral RNA silencing involves the processing of dsRNA by the enzyme Dicer into small RNAs, 21 to 25 nucleotides in length (Voinnet 2009). One of the two RNA strands is then incorporated into a protein complex called RNA-induced silencing complex (RISC) that contains AGO protein (Vaucheret 2008). AGO1 as the key constituent of the major antiviral RISC recruits virus-specific siRNAs (Zhang et al. 2006). Furthermore, AGO1 was indicated as one of the major antiviral AGOs. In addition to AGO1, other AGO proteins can be involved in RNA silencing-based defense responses (Alvarado & Scholthof 2011, Harvey et al. 2011, Jaubert et al. 2011, Scholthof et al. 2011, Wang et al. 2011). Also, it has been shown that ago1 mutants are more susceptible to viral infections (Morel et al. 2002, Qu et al. 2008). The incorporated small RNA then guides the complex to partially or fully silence complementary RNA. In virus infection, viral-derived small RNAs are incorporated into the RISC complex to guide degradation of the corresponding viral RNA (Ding 2010). As a counter defense, viruses have evolved VSRs that suppress the antiviral PTGS defense response (Ding 2010). VSRs counter host defense by different strategies to inactivate RNA silencing machinery. A large number of VSR proteins with diverse structures and functions have been identified in many plant viruses and some animal viruses (Brigneti et al. 1998, Kasschau & Carrington 1998, Li & Ding 2006, Voinnet 2005).

Potato virus A (PVA) belongs to the genus Potyvirus, family Potyviridae, the largest virus family infecting plants (Urcuqui-Inchima et al. 2001). PVA genome encodes a large polyprotein subsequently cleaved by three virus-encoded proteinases to yield up to 10 mature proteins (Rajamäki et al. 2004; Urcuqui-Inchima et al. 2001). Helper component proteinase (HC-Pro) is a multifunctional protein involved in potyvirus replication, cell-to-cell movement and long-distance movement (Rojas et al. 1997, Saenz et al. 2002), aphid transmission (Pirone & Blanc 1996), symptom development (Redondo et al. 2001), viral synergism (Wang et al. 2002), inhibition of the endonuclease activity (Ballut et al. 2005), the protease activity (Sahana et al. 2012) of the 20S proteasome and suppression of RNA silencing–based antiviral defense in plants (Rajamäki et al. 2004). Recent studies revealed that autophagy is involved in the degradation of VSRs, which plays...
a critical role in plant antiviral defense (Agius et al. 2012). A tobacco calmodulin-like protein (rgs-CaM) functions as a secondary antiviral mechanism in antiviral defense. Rgs-CaM binds to different VSRs including HC-Pro and 2b to reduce the suppressor activity and promote their degradation by autophagy (Nakahara et al. 2012). The protein levels of rgs-CaM and interacting VSRs increased in plant cells after treatment with an inhibitor of autophagy or by silencing of tobacco ATG6 (Nakahara et al. 2012). In addition, accumulated rgs-CaM and interacting VSRs co-localized with LysoTracker-stained autophagosomes, suggesting that they are recruited into autophagosomes for degradation after complex formation (Nakahara et al. 2012). Over-expression of rgs-CaM in transgenic plants enhanced resistance, whereas silencing lead to more susceptible to viral infection (Nakahara et al. 2012). P0, another VSR from polerovirus, targets the autophagic degradation of AGO1 (Derrien et al. 2012). Thus, P0 hijacks a host cellular ubiquitin E3 ligase to ubiquitinate AGO1, and the ubiquitinated AGO1 is targeted for degradation before the RISC assembly via the selective autophagy (Zhou et al. 2014). Viral genome-linked protein (VPg) as a VSR of Turnip mosaic virus interacts with suppressor of gene silencing 3 (SGS3), a key component of the RNA silencing pathway that functions in double-stranded RNA synthesis for virus-derived siRNA production. Expression of VPg alone is sufficient to induce the degradation of SGS3 and its intimate partner, RNA-dependent RNA polymerase 6. Moreover, VPg-mediated degradation of SGS3 is via both the 20S ubiquitin-proteasome and autophagy pathways (Cheng & Wang 2016). Little is known about the interaction of VSRs with autophagy genes. Hence, the main objective of this study was to demonstrate changes in the expression of autophagy related genes with antiviral function, including AGO1, ATG6, ATG2 and ATG7, against PVA HC-Pro as a strong VSR.

**Materials and Methods**

**Plant materials**

*N. benthamiana* plants were grown under long day photoperiod (16/8 hr) at 18±4°C, with a relative humidity of 80%.

**Plasmid constructs**

The cDNA of ORF PVA-HC-Pro Isolate U (1374 nt) was amplified with HC-Pro for, 5′-GGGGACAAAGTTTGTACAAAAAAGCAGGCTTGACC-3′; HC-Pro rev, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTATCCAACCGGTAGTGCTACATT-3′, then cloned into a pDONRTM/Zeo vector (Invitrogen). Clones were checked by sequencing, then recombined into the Gateway (Invitrogen) destination vector pGWB17 (Nakagawa et al. 2007) with a C-terminal myc tag and N-terminal 3S promoter for agro-infiltration tests. Also, Agrobacterium tumefaciens strain C58C1 carrying empty plasmid, pGWB (EP) was prepared (Savenkov & Valkonen 2001) and used as a negative control. Subsequently, these clones were checked by restriction enzyme analysis with XbaI and SacI enzymes (Figure 1). Each of these plasmids was introduced into A. tumefaciens C58C1 by a freeze-thaw method (Weigel & Glazebrook 2006). The A. tumefaciens cells were transformed with a binary vector p35-GFP for expression of the GFP (Germundsson et al. 2007).
Table 1. Details of specific primers used in this study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer name</th>
<th>Sequence from 5′ to 3′</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG01</td>
<td>AG01-F</td>
<td>GCCATGGGCGACCTTCTG</td>
<td>132</td>
<td>Gursinsky et al. 2015</td>
</tr>
<tr>
<td></td>
<td>AG01-R</td>
<td>GAGACGAGAACCAGCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG6</td>
<td>ATG6-F</td>
<td>ACCTGCGTAAGGAGTCTG</td>
<td>164</td>
<td>Wang et al. 2013</td>
</tr>
<tr>
<td></td>
<td>ATG6-R</td>
<td>AGAGCTTGTGAACCTTTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG2</td>
<td>ATG2-F</td>
<td>GCAATGGCGTGAGTTG</td>
<td>159</td>
<td>Wang et al. 2013</td>
</tr>
<tr>
<td></td>
<td>ATG2-R</td>
<td>CCGTGCGGCTGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG7</td>
<td>ATG7-F</td>
<td>CCGAGCTGGAGGAGTCTT</td>
<td>166</td>
<td>Wang et al. 2013</td>
</tr>
<tr>
<td></td>
<td>ATG7-R</td>
<td>GCCACCCATTTCCGTATCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>GFP-F</td>
<td>TCCATGCGCAACACTTGCA</td>
<td>102</td>
<td>Han et al. 2016</td>
</tr>
<tr>
<td></td>
<td>GFP-R</td>
<td>GGCATGGCGACTCTTAAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIF1-α</td>
<td>EIF1-α-F</td>
<td>AGCTTTACCTCCCAAGTCATC</td>
<td>116</td>
<td>Lukhovitskaya et al. 2014</td>
</tr>
<tr>
<td></td>
<td>EIF1-α-R</td>
<td>AGAAGCCTGGTTAATCTGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F-Forward strand, R-Reverse strand

Infiltration of Agrobacterium cultures

Agrobacterium cultures harboring PVA-HC-Pro, EP and GFP constructs were prepared for infiltration. Individual colonies from PVA-HC-Pro and EP constructs were inoculated into 3-ml yeast extract peptone (YEP) broth in the presence of appropriate antibiotics and incubated at 28°C for 20 h with shaking. One ml from each culture was used to inoculate 50-ml YEP broth with the antibiotics supplemented with acetosyringone (20 µM final concentration) and 0.5 M M2-(N-morpholino) ethane sulfonic acid (MES), pH 5.6. These cultures were similarly incubated at 28°C for 20 h with gentle shaking. When the cultures reached an OD_{600} of 0.5, they were centrifuged at 3500g for 5 min and the pellet was re-suspended in induction buffer containing 10 mM MgCl₂, 10 mM MES, pH 5.6, and 150 µM acetosyringone and incubated for at least 2 h at room temperature with constant agitation. Liquid Agrobacterium cultures of OD_{600}=0.5 were diluted in induction buffer containing the GFP plus PVA-HC-Pro or EP constructs (final OD_{600}= 0.5) and were immediately mixed and syringe-infiltrated into the abaxial side of 6-8 week-old N. benthamiana leaves (Lukhovitskaya et al. 2013).

RNA extraction and Real-time quantitative RT-PCR

Total RNAs from co-infiltrated leaves were extracted with the spectrum plant total RNA kit (Sigma-Aldrich) with DNase 1 (Sigma-Aldrich) according to the manufacturer’s instructions. Total RNA concentrations were measured with a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, U.S.A.), and one-microgram aliquots of total RNA samples were used for cDNA synthesis with iScript reverse transcriptase first-strand cDNA synthesis kit (MBI Fermentas). Primers amplifying the GFP, autophagy related genes (ATG) and elongation factor-1 alpha (EIF-1α), were used as target and control for equal cDNA amounts (Table 1). The cDNA was quantified with the qPCR green master kit (Jena Bioscience, Germany) using a Bio-Rad IQ5 iycler apparatus according to the manufacturer’s recommendations. PCR was run in 96-well optical reaction plates. The PCR conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. At the end of the PCR, dissociation kinetics analysis was performed to check the specificity of annealing. Three biological and technical replicates were used. Accordingly, the fold expression of target mRNAs over the reference values were calculated by the equation 2^{-DDCT} (Livak & Schmittgen 2001), where DCT was determined by subtracting the CT value of internal control from the specific CT of the target, and DDCT was obtained by subtracting the DCT of each experimental sample from that of the control sample.

Western blot of HC-Pro protein

Leaf samples (100 mg fresh weight) from
infiltrated *N. benthamiana* leaves at 4 dpi were ground and homogenized in 300 µl of Laemmli protein buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 10% 2 -mercaptoethanol; 20% glycerol; 0.004% bromophenol blue), heated at 95°C for 5 min, and centrifuged for 10 min at 13000 rpm to remove insoluble material. The supernatant was run on a 12% SDS-PAGE and after electrophoresis, proteins were transferred onto a PVDF membrane as described (Sambrook & Russel 2001) and blocked with 5% (w/v) nonfat milk powder in phosphate-buffered saline containing 0.05% (v/v) Tween 20. Detection was carried out using an anti HC-Pro monoclonal antibody, Sigma Aldrich (dilution 1:1000 in PBS-T plus 2.5% milk) and secondary anti mouse polyclonal antibody (dilution 1:5000 in PBS-T plus 2.5% milk) and signals were detected using the ECL Prime kit (Amersham, GE Healthcare) with the enhanced Chemilluminescence plus protein detection system (Amersham). They were imaged in a LAS-3000 Luminescent Image Analyzer (Fujifilm, Fuji Photo Film, Kleve, Germany).

**Statistical analysis**

The experimental data for gene expression were statistically analyzed using analysis of variance (ANOVA) with subsequent Duncan’s multiple range test (Duncan 1951) using SAS 9.4 software. The differences at the 95 % confidence level were considered to be significant. In all cases, a P value of < 0.05 was considered significant. The error bars denote SD.

**Results and discussion**

GFP is widely used as a quantitative and visual marker in plants (Stephan et al. 2011). Co-infiltration of separate *A. tumefaciens* cultures harboring the VSR and GFP reporter gene onto *Nicotiana benthamiana* leaves can quantitatively assess suppressor protein activity (Chiera et al. 2007). In this study, transient GFP-expression assay using *N. benthamiana*, addition of the PVA HC-Pro as a plant viral suppressor was shown to enhance fluorescent protein expression 16.1-fold at 5 dpi than patches without suppressor activity (Figure 2). In the absence of a functional VSR, GFP expression from the Ti-plasmid is recognized as exogenous by the host and is silenced (Johansen & Carrington 2001). RNA silencing has been shown to be involved in the decrease in transient expression of genes introduced into *N. benthamiana* plants by Agrobacterium (Johansen & Carrington 2001). Our results were in accordance with other transient expression studies which showed that the silencing effect is reduced by co-expressed plant viral silencing suppressors (Voinnet et al. 2003, Chiera et al. 2008), which
interfere with the RNA silencing machinery and lead to an increase in transiently expressed proteins (Johansen & Carrington 2001). PVA HC-Pro was shown to be useful to enhance and extend GFP-expression in N. benthamiana. It might be co-agroinfiltrated with any target protein construct to allow high protein yields. Strong suppressor activity of PVA HC-Pro was verified by the enhancement of transiently expressed GFP marker (Figure 3), while, GFP expression was silenced in co-infiltrated leaves with GFP and EP construct. These results confirm the strong silencing suppressor activity of PVA-HC-Pro. Meanwhile, HC-Pro transient expression using A. tumefaciens infiltration confirmed the expression of HC-Pro protein by western blot analysis, with an apparent molecular weight of 53 kDa, in infiltrated leaves with agrobacterium culture harboring PVA-HC-Pro. In contrast, no protein was detected in the extracts prepared from plants infiltrated with agrobacterium carrying an EP (Figure 4).

To investigate the effect of PVA-HC-Pro suppressor protein on autophagy process, we used transient expression of PVA-HC-Pro gene under constitutive expression of CaMV35S promoter in N. benthamiana leaves and the expression levels of ATG6, ATG2, ATG7 and AGO1 genes involved in autophagy process were evaluated.

This study showed that the expression level of ATG6, ATG2 and ATG7 in N. benthamiana plants infiltrated with PVA-HC-Pro was upregulated 5.89, 7.3 and 7.6-folds, respectively, compared to those of infiltrated leaves with EP control. However, the accumulation level of AGO1, was down-regulated 1.36-fold lower than the level of AGO1 in infiltrated leaves without PVA-HC-Pro (Figure 5).

Our result was in accordance with other studies which showed that VSRs (Turnip crinkle virus CP, Cucumber mosaic virus 2b, Tomato bushy stunt virus P19, Potato virus X P25, Polerovirus P0 and P1 of Sweet potato mild mottle virus) repressed the function of AGO1 (Derrien et al. 2012, Azevedo et al. 2010, Giner et al. 2010, Zhang et al. 2006, Chiu et al. 2010, Varallyay et
Figure 5. Accumulation level of AGO1, ATG6, ATG2 and ATG7 in infiltrated leaves with a PVA HC-Pro and EP constructs, measured by qRT-PCR. Mean and standard deviation (SD) were obtained from three biological replicates. Vertical bars represent SD. Means are statistically different at p < 0.05, when they share no common letter. The comparisons were made using the Duncan test.

Also, it was shown that under normal conditions, basal autophagy functions as a housekeeping process to clear damaged or unwanted cytoplasmic contents, whereas under certain stresses, autophagy is upregulated (Inoue et al. 2006, Slavikova et al. 2005, Xiong et al. 2007). These results indicate that autophagy can play a positive role in response to plant viruses. The induced autophagy can limit virus replication and/or movement either directly through degradation of viruses in vacuoles or indirectly through effects on other antiviral defense (Agius et al. 2012). In general, autophagy shapes the outcomes of plant-virus interaction through interfaces with a number of important pathways of plant innate immunity, including pathogen-induced hypersensitive cell death, SA- and JA-regulated defense and virus-induced RNA silencing through the modulation or reprogramming of globally important metabolic and signaling pathways (Zhou et al. 2014).

Thus, autophagy deployed as a defense system against viral RNA silencing suppressors. However, efforts to elucidate the complex roles of autophagy in plant innate immune responses will require the identification and characterization of additional defense mechanisms to develop a comprehensive understanding of the network of complex functional interactions between autophagy and plant defense responses. It will also be interesting to investigate the effect of PVA-HC-Pro on siRNAs and miRNAs profile and their regulation pathways.
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