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Expression analysis of host defense responses against the 8K (KDa) cysteine-rich viral silencing suppressor protein in *Nicotiana benthamiana*

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Abstract

Potato mop-top virus (PMTV) encodes the 8K cysteine-rich viral suppressor of RNA silencing. To gain an insight into *N. benthamiana* defense mechanisms against 8K suppressor protein, we expressed two 8K suppressors from Peruvian isolates in *Nicotiana benthamiana* and assessed the expression of its defense genes involved in autophagy (*ATG6, ATG2* and *ATG7, AGO1*), salicylic acid (SA) (*ICS1, NPR1* and *PR1*) and jasmonic acid (JA) (*OPR3, COI1* and *PDF1.2*) pathways. To do this, the 8K cDNAs of two Peruvian PMTV isolates were cloned in pGWB17 vector with a C-terminal myc tag and N-terminal 35S promoter using Gateway technology. *Agrobacterium* cultures harboring PMTV 8K were syringe infiltrated into the abaxial side of *N. benthamiana* leaves. The expression levels of defense genes were examined in *N. benthamiana* leaves infiltrated with P1 8K, P11 8K and the control constructs at 2 and 5 days post infiltration in response to PMTV 8Ks using q-PCR technique. Our results showed that the expression levels of *ATG6, ATG2, ATG7, ICS1, OPR3, NPR1, PR1, COI1* and *PDF1.2* were increased in response to both 8K suppressors. However, the transcript level of *Argonaute1* (*AGO1*) was decreased in response to both 8K suppressors compared with the control. These results indicated that 8K suppressor proteins can alter the expression of autophagy, SA and JA signaling pathway genes in *N. benthamiana*. Taken together, it seems that despite the 8K role in virus pathogenicity, it can also induce host defense responses to modulate plant-virus interactions and fine-tune host-virus coexistence.

Keywords: Coevolution, Defense responses, Gene expression, 8K, Potato mop-top virus, RNA silencing suppressor. **Abbreviations**: JA_Jasmonic acid; PMTV _ Potato mop-top virus; SA_ Salicylic acid; VSR_Viral suppressor of RNA silencing.

Introduction

PMTV is a member of Pomovirus genus causing brown arcs and circles in potato tubers that make them unmarketable. The tripartite PMTV genome consists of three singlestranded RNAs with totally eight open reading frames (ORFs). RNA1 and RNA2 function in virus replication and encapsidation/transmission, respectively (Savenkov et al., 1999; Sandgren et al., 2001). RNA3 encodes a triple gene block (TGB) and a cysteine-rich protein, 8K (8kDa) (Savenkov, 2002). Three TGB proteins are responsible for viral cell-tocell movement in plants (Morozov and Solovyev, 2003; Zamyatnin et al., 2004). 8K protein increases the virulence of PMTV and also acts as a viral suppressor of RNA silencing (VSR) (Lukhovitskaya et al., 2005; Lukhovitskaya et al., 2013). VSRs can block host antiviral RNA silencing pathway at different levels (Pumplin and Voinnet, 2013). For example, VSRs degrade Argonaute1 (AGO1) protein through an autophagy based-system for the inactivation of the plant RNA silencing machinery (Derrien et al., 2012). AGO1 as a component of RISC (RNA-induced silencing complex) guides the sequence-specific degradation of the homologous viral RNAs (Baumberger and Baulcombe, 2005).

Autophagy can act as a plant defense system against VSRs by modulation of plant-pathogen interaction via a number of

plant defense pathways including SA, JA and RNA silencing (Zhou et al., 2014; Nakahara et al., 2012). The SA and JA phytohormones participate in antiviral plant defense responses and their levels change in response to virus infections (Alazem and Lin, 2015). On the other hand, VSRs are also capable of regulating plant defense pathways including SA and JA signaling (Westwood et al., 2014).

Since the origin of potato lies in Peru (Spooner et al., 2005), it is common to find the greatest variability of potato viruses there (Hooker, 1982). The Peruvian PMTV isolates showed the highest variability compared to other worldwide isolates and the susceptibility of potato commercial cultivars was also confirmed in response to Peruvian PMTV isolates (Tenorio et al., 2006). Consequently, the 8K protein variability was much greater among Peruvian isolates than that in worldwide isolates (Kalyandurg et al., 2017).

Although the role of 8K protein in PMTV-host interactions has been studied, little is known about host plant responses to this viral protein. Therefore, in this study we investigated plant host defense responses against 8K suppressors by expression of 8K from two Peruvian isolates in *N. benthamiana*. Understanding of networks involved in the virus-plant interactions could help in the dissection of the plant defense mechanisms against viruses.

Results and Discussion

Transcript analysis of autophagy-related genes

The transcript levels of ATG6, ATG2 and ATG7 were increased in P1 8K treatment by 7.6, 2.5, 5.2-fold and in P11 8K treatment by 1.1, 12.2, 4.8-fold, respectively, when compared with their counterparts in infiltrated leaves with control constructs (Fig. 2). It has been shown that PMTV 8K is associated with endoplasmic reticulum (ER)-membranes and its expression induces rearrangements of the ER membranes in plant cells (Lukhovitskaya et al., 2005). Autophagosomes can arise from extending membranes of several sources including the ER (Hamasaki et al., 2013). Autophagy might act as a defense system against VSRs (Nakahara et al., 2012). The enhancement of ATG6, ATG2 and ATG7 expressions might be as a result of inducing plant antiviral autophagy process in response to 8K suppressors. Our results were in agreement with other studies which showed that autophagy genes were up-regulated in response to plant viruses and VSRs (Tahmasebi et al., 2017; Liu et al., 2005; Liu et al., 2014; Miozzi et al., 2014; Ascencio-Ibáñez et al., 2008). The induced autophagy can limit virus replication and/or spread either through degradation of viruses or via its effects on other antiviral defense pathways (Agius et al., 2012).

The transcript level of *AGO1*, a key component of RISC was decreased by 39.9- and 30.6-fold lower than that of *AGO1* in infiltrated leaves without P1 8K and P11 8K, respectively (Fig. 2). This result was in accordance with other studies which showed that VSRs repressed the AGO1 (Derrien et al., 2012; Azevedo et al., 2010; Giner et al., 2010; Zhang et al., 2006; Chiu et al., 2010; Várallyay et al., 2010). VSR-mediated control of AGO1 can be a mechanism to alleviate the action of RNA silencing-based defenses through a reduction in RISC formation.

Quantitative measurement of relative expression of SA and JA-related genes

The expression levels of the SA and JA upstream genes, *ICS1* and *OPR3* were increased by 6.2 and 2.6-fold in P1 8K and 13.2 and 4.9-fold, in P11 8K, respectively, in comparison to the level of genes in the control infiltrated leaves (Figs. 3 and 4). The transcript levels of SA downstream genes (*NPR1* and *PR1*) were also found to be upregulated by 5.92- and 1.55-fold in P1 8K and 2.3-and 1.1-fold in P11 8K, respectively (Fig. 3). Moreover, JA downstream genes (*COI1* and *PDF1.2*) were also upregulated by 1.72 and 4.86-fold in P1 8K and 1.63- and 1.92-fold in P11 8K, respectively (Fig. 4).

In addition to defense against VSRs, autophagy can also affect other plant defense pathways including hypersensitive cell death, SA- and JA-based defense (Nakahara et al., 2012). We showed that expression of 8K enhanced transcript level of SA-related genes (*ICS1, NPR1* and *PR1*). Similar to our results, it has been shown that the plants expressing 2b transgenes induce SA accumulation (Lewsey et al., 2010). SA accumulation can cause increased reactive oxygen species production and necrosis in *N. benthamiana*. TMV (PMTV-8K) caused necrosis on the inoculated *N. benthamiana* leaves

(Lukhovitskaya et al., 2005). On the other hand, systemic necrosis can increase SA biosynthesis (Jovel et al., 2011). Therefore, it can be hypothesized that SA accumulation in response to 8k protein might be associated with HR suppression and necrosis induction in *N. benthamiana* in response to 8K. We also found that JA-related genes (*OPR3, COI1* and *PDF1.2*) were up-regulated in response to both 8K suppressors. These results are in line with other reports which showed that expression of HC-Pro from Tobacco etch virus and the P6 from Cauliflower mosaic virus enhanced JA responses in *Arabidopsis thaliana* (Endres et al., 2010; Love et al., 2012). Enhanced expression of SA- and JA-related genes can be as a result of induction of autophagy pathway. The results indicate that 8K may itself be the target of host defense responses.

Materials and Methods

Plant and growth conditions

N. benthamiana plants were grown in a greenhouse under long day (16 hr light/8 hr dark) photoperiodic conditions at $18^{\circ}C \pm 4^{\circ}C$ and 80% relative humidity.

Transient expression assay in N. benthamiana

The cDNAs encoding the 8K protein of two PMTV isolates from Peru (P1, KU955501 and P11, KU955498 accession numbers) were PCR amplified (Fig. 1) using full-length clones (provided by Eugene Savenkov) and specific primers (Table 1) followed by cloning into a pDONRTM/Zeo vector (Invitrogen) (Earley et al., 2006). Following confirmation of the clones' identity by sequencing, they were recombined into the Gateway (Invitrogen) destination vector pGWB17 (Nakagawa et al., 2007), with a C-terminal myc tag and Nterminal 35S promoter to generate pBin8K for agroinfiltration test. Wild-type N. benthamiana plants were used for agro-infiltration tests (Ruiz et al., 1998). Leaves of N. benthamiana which were divided into two equal parts were infiltrated with A. tumefaciens cells harboring pGWB17 8K and opposite side of leaf was infiltrated with the pGWB plasmid. pGWB plasmid construct was used as a negative control in gene expression analysis.

RNA extraction and cDNA synthesis

Total RNAs from infiltrated leaves with *A. tumefaciens* cells harboring pGWB17 8K or pGWB constructs were extracted with the total RNA kit (S-1010-1, Dena Zist Asia, Iran) with DNase I (Invitrogen) according to the manufacturer's instruction. 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 the iScript reverse transcriptase first-strand cDNA synthesis kit (MBI, Fermentas). cDNA was synthesized using 1 μ I of oligo (dT) primer (0.5 μ g/ μ I), 4 μ I of 5x reaction buffer, 1 μ I of Revert AidTM M-MuLV reverse transcriptase (Invitrogen) and 9 μ I of DEPC- treated water and 2 μ I of 10mM dNTP mix. The mixture was incubated at 42°C for 30 min, and then 5 min at 80°C.

Template		Primer Sequence (5' to 3') PC	CR product Size (bp)		
8 K P1	K P1 F GGGGACAAGTTTGTACAAAAAGCAGGCTTGATGGAA				
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTAACGGCAT			
8 K P11	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGAA			
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTAACGGCAT			
		a M P1 P11 C			
	265 bp				
		b P1 P11 C			
		12.7 KDa			

Table 1. Nucleotide sequences of specific primer pairs which were used in this study for amplification of DNA fragment of 8K gene of PMTV isolates (product sizes are shown).

Fig 1. Gel electrophoresis pattern of PCR products for amplification of *8K* gene of PMTV isolates (**a**) using specific primer pair (Table 1). Lane M represents the Gene Ruler 1 kb plus DNA Ladder. Western blot detection of the 8K proteins in the *N. benthamiana* leaves infiltrated with Agrobacterium constructs containing 8K P1, 8K P11 and C, control) (**b**). A total of 30 µg extracts for each sample was prepared and subjected to immunoblot analysis with myc tag-specific antibody.

Table 2. Features of specific primers used in expression of genes involved in autophagy process (ATG6, ATG, ATG7 and AGO1), SA
(ICS1, NPR1 and PR1) and JA (OPR3, COI1 and PDF1.2) signaling pathways.

Gene name	Primer	Sequence from 5' to 3'	PCR product Size (bp)	Accession number
	name			
AG01	AG01-F	GCCATGGGGCACCTTCTG	132	KR942296.1
	AG01-R	GAGACGAGGAACCAGCCTC		
ATG6	ATG6-F	ACCTGCGTAAAGGAGTTTGCTGAC	164	AY701316.1
	ATG6-R	AGAGCTTTGGTCCAACTTTCCTGC		
ATG2	ATG2-F	GCAATTGGGCTTGGAGTGCATTTG	159	KR336559.1
	ATG2-R	CCTGTCGGGCATCTCTAGGTTGAT		
ATG7	ATG7-F	CCAGCAGTGGAAGCAGAAGGTCTT	166	KX369398.1
	ATG7-R	GCCACCGACTTTCCCGTGTATCA		
COI1	COI1-F	CACTTGATAATGGTGT	172	AY547493.1
	COI1-R	AGGCCTTCATCGGATTCC		
NPR1	NPR1-F	TGCATGTTGCAGCTATGAGGA	156	KY402167.1
	NPR1-R	CTTCCTCCGGAGACTTGCTG		
PR1	PR1-F	CGACCAGGTAGCAGCCTATG	140	JN247448.1
	PR1-R	TCTCAACAGCCTTAGCAGCC		
OPR3	OPR3-F	GTGGAGCTGCACCAATATCT	149	NM_001325344.1
	OPR3-R	TGCCTTGCGATAATCTTCAACC		
ICS1	ICS1-F	TTCAGTCTGGAGTCTCCCCC	102	LC222288.1
	ICS1-R	GCTGTGTGTTCCAGCGTTTTA		
PDF1.2	PDF1.2-F	GACCAACGACAATTGCAGAGG	100	KP017278.1
	PDF1.2-R	GTCAAACAGACGGTGGCACA		
<i>EF1-α</i>	EF1-α-F	AGCTTTACCTCCCAAGTCATC	135	NM_001326165.1
	EF1-α-R	AGAACGCCTGTCAATCTTGG		



Fig 2. Accumulation level of autophagy-related genes (*ATG6*, *ATG2*, *ATG7* and *AGO1*) in *N. benthamiana* leaves infiltrated with P1 8K, P11 8K and control constructs at 2 and 5 days post infiltration (dpi), measured by qPCR. Mean was obtained from three biological replicates. Error bars are representative of the standard error (Mean \pm SD, n = 3). Different letters indicate statistically different values (*P*, 0.05, Duncan test).



Fig 3. Accumulation level of SA-related genes (*ICS1*, *NPR1* and *PR1*) in *N. benthamiana* leaves infiltrated with P1 8K, P11 8K and control constructs at 2 and 5 days post infiltration (dpi), measured by qPCR. Mean was obtained from three biological replicates. Error bars are representative of the standard error (Mean \pm SD, n = 3). Different letters indicate statistically different values (*P*, 0.05, Duncan test).



Fig 4. Accumulation level of JA-related genes (*OPR3, COI1* and *PDF1.2*) in *N. benthamiana* leaves infiltrated with P1 8K, P11 8K and control constructs at 2 and 5 days post infiltration (dpi), measured by qPCR. Mean was obtained from three biological replicates. Error bars are representative of the standard error (Mean \pm SD, n = 3). Different letters indicate statistically different values (*P*, 0.05, Duncan test).

Quantitative Real Time PCR (qPCR)

The expression level of ten genes involved in defense signaling pathways (Table 2) was analyzed at 2 and 5 days post infiltration (dpi) in response to P1 8K and P11 8K by qPCR using a lineGeneK thermal cycler (Exicycler TM96) according apparatus the manufacturer's to recommendations. The qPCR primers were designed for these genes (Table 2). The qPCR test was performed in a 20 μl volume reaction mixture containing cDNA (250 ng) template, 10 µM of each primer (0.6 µl), gPCR SYBR[®] Green master with low ROX (Jena Bioscience, Germany) (10 µl) and sterile water (fill up to 20 µl). The genes were analyzed using the following profile: 95°C for 2 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were expressed as quantification cycle (Cq). Three biological replicates were used, and three technical replicates were performed for each biological replicate. Accordingly, The fold expression of target mRNAs was calculated using the equation; $2^{\text{-}\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). EIF1- α was used as an internal reference.

Western blot analysis of 8K protein

One-hundred milligrams fresh weight of infiltrated *N.* benthamiana leaves were ground and homogenized in 300 μ l of laemmli buffer (0.125 M Tris–HCl, pH, 6.8; 4% Sodium dodecyl sulfate (SDS); 10% 2 mercaptoethanol; 20% glycerol; 0.004% bromophenol blue), centrifuged for 10 min at 13,000 rpm. The supernatant was run on a 16% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Then proteins were transferred onto a PVDF membrane (Sambrook and Russell, 2001). Detection was conducted with the primary mouse myc-specific monoclonal antibody (Roche) (diluted 1:1,000 in PBS-T plus 2.5% milk) followed by incubation with a secondary anti-mouse polyclonal antibody (diluted 1:5000 in PBS-T plus 2.5% milk). Signals were detected using the ECL Prime kit (Amersham, GE Healthcare). The membrane was imaged using a LAS-3000 Luminescent Image Analyzer (Fujifilm, Fuji Photo Film, Kleve, Germany) (Fig. 1).

Statistical analysis

The data were statistically analyzed using analysis of variance (ANOVA) with subsequent Duncan's multiple range test with SAS 9.4 software and those with p-values less than 0.05 were considered significant. All experiments reported in this study were repeated in three biological and technical replicates.

Conclusion

The results reported herein showed that autophagy, SA and JA-related genes are induced as defense systems in *N. benthamiana* against 8K suppressor protein. Besides the 8K role in virus pathogenicity, it can also induce host defense responses making it an important multifunctional viral protein that plays a pivotal role in the virus-plant interaction. Identification of defense signaling pathways involved in VSR-plant host interaction might be considered in plant genetic engineering as a novel approach to the control of plant viruses.

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