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

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چکیده

در سال‌های اخیر نقش اتوفاژی در خاموشی آرانای القاء شده توسط ویروس از طریق فعالیت پروتئین‌های سرکوبگر خاموشی آرانای به اثبات رسیده است. به منظور درک بهتر نقش احتمالی سرکوبگر خاموشی HC-Pro ویروس ای سیب زمینی در فرآیند اتوفاژی (autophagy)، اثر این سرکوبگر ویروسی روی بیان تعدادی از ژن‌های مهم و دخیل در اتوفاژی مورد بررسی قرار گرفت. ژن *HC-Pro* ویروس ای سیب زمینی در ناقل بیان pGWB17 با برچسب myc در انتهای کربوکسیلی و پروموتور 35S در انتهای آمینی توسط تکنولوژی gatewayهمسانه‌سازی شد. کشت‌های اگروباکتریوم حاوی HC-Pro ویروس ای سیب زمینی با استفاده از سرنگ در ناحیه پشتی برگ‌های گیاه *Nicotiana benthamiana* تزریق شدند. بیان ژن‌های *ATG2*، *ATG7*، *ATG6* و*AGO1* در پاسخ به سرکوبگر خاموشی HC-Pro ویروس ای سیب زمینی، در فاصله زمانی پنج روز پس از تزریق، با استفاده از تکنیک ریل تایم پی‌سی‌آر (Real-Time PCR) اندازه‌گیری شدند. سرکوبگر خاموشی HC-Pro ویروس ای سیب زمینی میزان بیان ژن‌های *ATG6*، *ATG2* و *ATG7* را به میزان ۸۹/۵، ۳/۷ و ۶/۷ برابر نسبت به برگ‌های تزریق شده با سازه حاوی پلاسمید خالی pGWB افزایش داد. در حالی‌که میزان رونوشت *AGO1* به‌عنوان جزء کلیدی کمپلکس خاموشی القاء شده توسط آرانای، به میزان ۳۶/۱ برابر نسبت به میزان آن در برگ‌های فاقد HC-Pro کاهش نشان داد. نتایج این تحقیق حاکی از دخالت ژن‌های *ATG2*، *ATG7*، *ATG6* و *AGO1* در پاسخ‌های دفاعی گیاه *N .benthamiana* در مقابل سرکوبگر خاموشی HC-Pro ویروس ای سیب زمینی و امکان استفاده از آن در تدوین روش‌های جدید مدیریت ویروس‌های گیاهی می‌باشد.

کلیدواژه: اتوفاژی، سرکوبگر خاموشی، HC-Pro، *Nicotiana benthamiana*، خاموش سازی آرانای

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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). 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%.

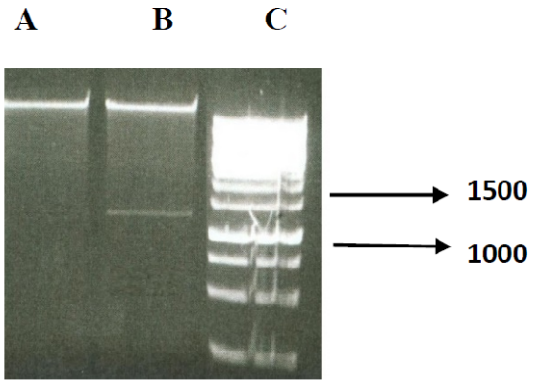

Figure 1. Digestion of pGWB EP (lane A) and pGWB17 PVA HC-Pro (lane B), colonies from HC-Pro and EP construct were inoculated into YEP broth culture in the presence of appropriate antibiotics, then incubated at 28°C for 20 h with shaking. From these Agrobacterium cultures, plasmids were purified using GeneJET Plasmid Miniprep kit (Thermo FisherScientific). Then purified plasmids were digested with *Xba*I and *Sac*I enzymes. Lane C represents the Gene Ruler 1 kb DNA Ladder mix.

Plasmid constructs

The cDNA of ORF PVA-HC-Pro Isolate U (1374 nt) was amplified with HC-Pro for, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT TGACC-3′; HC-Pro rev, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT ATCCAACCGGTAGTGCTACATT-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 35S 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 *Xba*I and *Sac*I 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.

Gene name	Primer name	Sequence from 5′ to 3′	Product size (bp)	Reference
AG01	AG01-F AG01-R	GCCATGGGGCACCTTCTG GAGACGAGGAACCAGCCTC	132	Gursinsky *et al.* 2015
ATG6	ATG6-F ATG6-R	ACCTGCGTAAAGGAGTTT GCTGAC AGAGCTTTGGTCCAACTTTCCTGC	164	Wang *et al.* 2013
ATG2	ATG2-F ATG2-R	GCAATTGGGCTTGGAGTGCATTTG CCTGTCGGGCATCTCTAGGTTGAT	159	Wang *et al.* 2013
ATG7	ATG7-F ATG7-R	CCAGCAGTGGAAGCAGAAGGTCTT GCCACCGACTTTCCCGTGTATCA	166	Wang *et al.* 2013
GFP	GFP-F GFP-R	TCCATGGCCAACACTTGTCA GGCATGGCACTCTTGAAAAAG	102	Han *et al.* 2016
EIF1-α	EIF1-α-F EIF1-α-R	AGCTTTACCTCCCAAGTCATC AGAACGCCTGTCAATCTTGG	116	Lukhovitskaya *et al.* 2014

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 $MgCl_2$, 10 mM MES, pH 5.6, and 150 µM acetosyringone and incubated for at least 2h 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 I (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* (*ElF-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 icycler 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

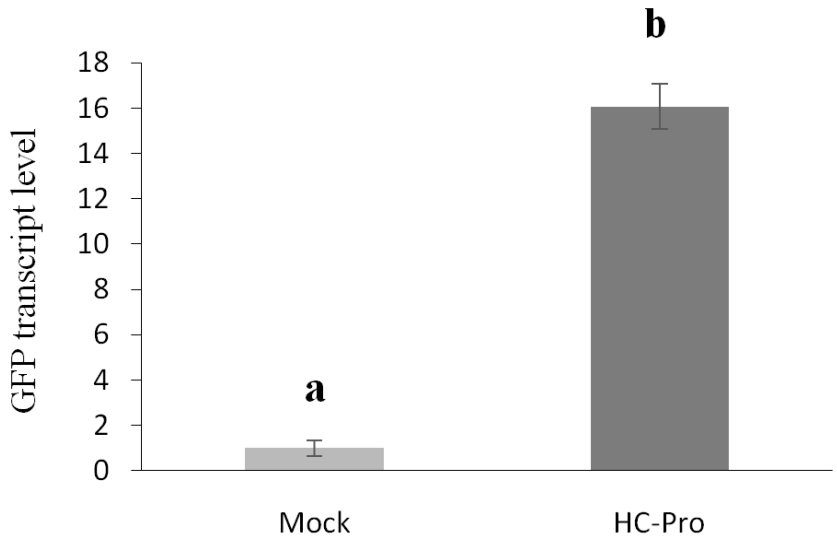

Figure 2. GFP RNA accumulation in co-infiltrated tissues measured by quantitative RT-PCR. Leaves were co-infiltrated with agrobacterium cultures containing GFP and HC-Pro. Values represent means from three biological and technical replications. Error bars denote SD; different letters indicate statistically different values (P,0.05, Duncan test).

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

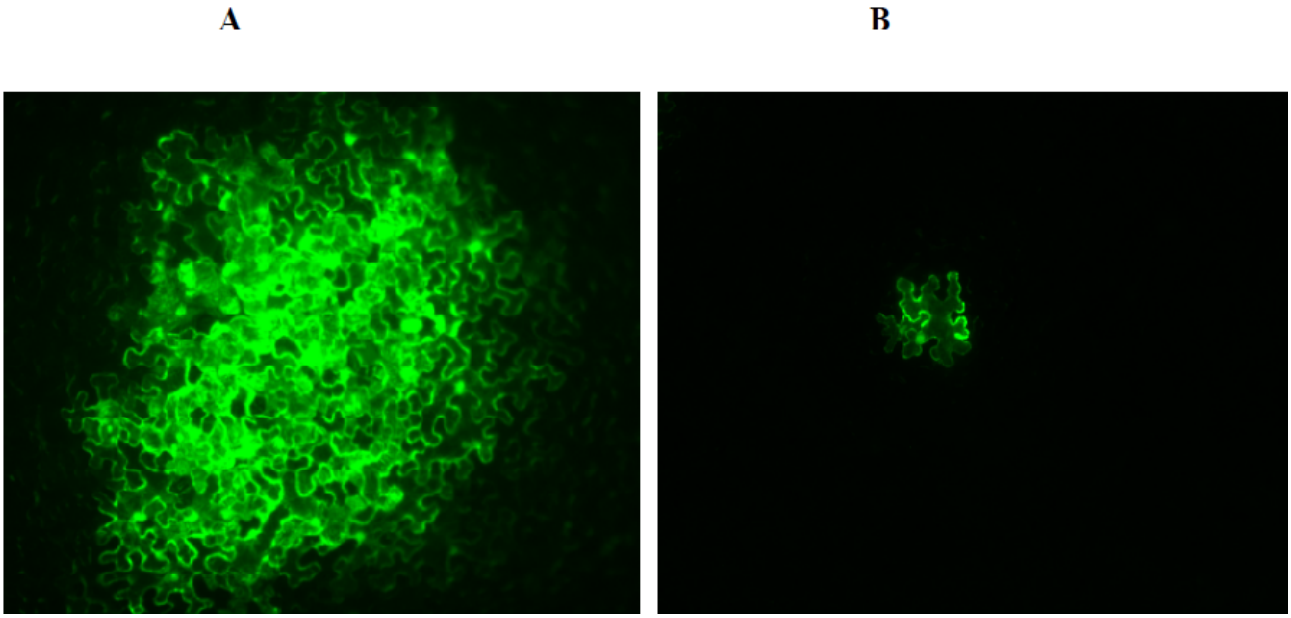

Figure 3. Green-color imaging of the GFP in the co-infiltrated leaves with PVA HC-Pro (panel A) or EP (panel B) in *Nicotiana benthamiana* at 5 dpi, with a Leica MZIII fluorescence dissecting microscope equipped with a GFP filter.

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

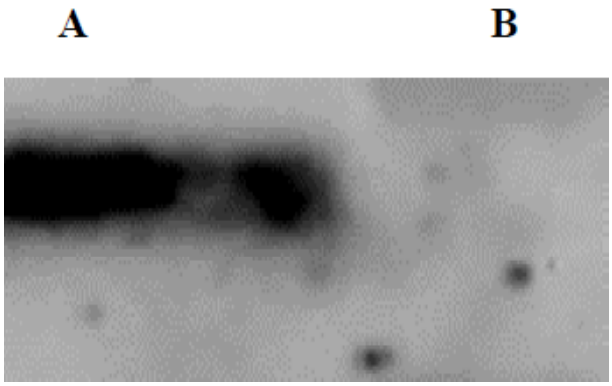

Figure 4. Western blot analysis of infiltrated *Nicotiana benthamiana* plants with PVA HC-Pro (lane A) and EP (lane B) at 5 dpi.

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*

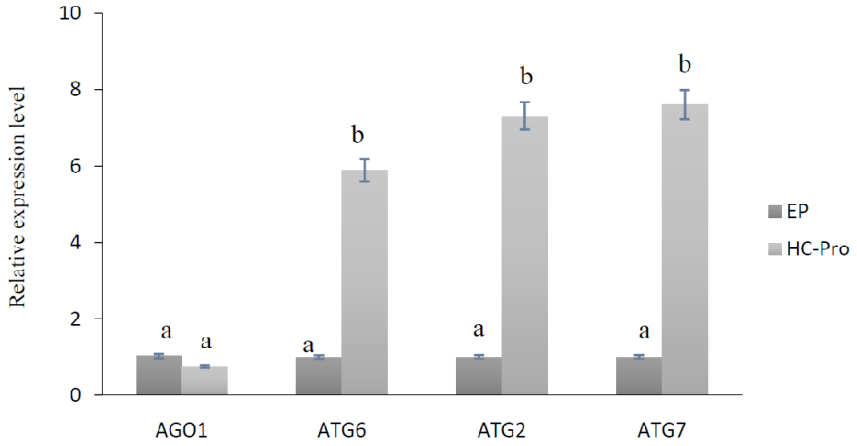

Figure 5. Accumulation level of *AGO1*, *ATG6*, *ATG2* and *ATG7*in infiltrated leaves with a PVA HC-Pro and EP constructs, measured by qRT-PCR. Mean and standard deviation (SD) were obtained from three biological replicates. Vertical bars represent SD. Means are statistically different at $p < 0.05$, when they share no common letter. The comparisons were made using the Duncan test.

al. 2010). Also, the p19 VSR of *Cymbidium ring spot virus* in *N. benthamiana* and crucifer-infecting Tobamovirus (crTMV) infection in *A. thaliana*, induced the over accumulation of miR168 and mediated the subsequent control of AGO1 protein (Varallyay *et al.* 2010). In crTMV-infected *N. benthamiana*, *AGO1* mRNA was down-regulated (Havelda *et al.* 2008). *AGO1* down regulation might be due to induction of miRNA pathway in response to PVA-HC-Pro. VSR-mediated control of AGO1 can be an additional mechanism to alleviate the action of RNA silencing, thereby facilitating efficient viral replication by reducing the effect of antiviral RISC in plant–virus interactions. Subsequently, a reduction in RISC formation can counteract host RNA silencing-based defenses. The enhancement in *ATG6*, *ATG2* and *ATG7* genes can be a result of inducing autophagy process in response to PVA-HC-Pro to play a significant role in plant antiviral immunity. Likewise, in tobacco *ATG6*-, *ATG3*- and *ATG7*-silenced plants, *Tobacco mosaic virus* accumulation was increased (Liu *et al.* 2005). In addition, several autophagy genes were up regulated in response to C2 suppressor of *Spinach curly top virus* (Liu *et al.* 2014). Also, autophagy-related genes were up-regulated during the *Tomato yellow leaf curl sardinia virus* /tomato compatible interaction. All these transcripts are related to the formation of autophagosomes (Miozzi *et al.* 2014). Interestingly, activation of *ATG8* gene was also observed in Arabidopsis infected by *Cabbage leaf curl virus* (Ascencio-Ibanez *et al.* 2008).

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.

References

Agius C., Eamens A. L., Millar A. A., Watson J. M. and Wang M. B. 2012. RNA silencing and antiviral defense in plants. Antiviral Resistance in Plants. Methods and Protocols 894:17-38.

Alvarado V. Y. and Scholthof H. B. 2012. AGO2: a new Argonaute compromising plant virus accumulation. Frontiers in Plant Science 2:112.

Ascencio-Ibáñez J. T., Sozzani R., Lee T.J., Chu T. M., Wolfinger R. D., Cella R. and Hanley-Bowdoin L. 2008. Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant Physiology 148(1):436-454.

Azevedo J., Garcia D., Pontier D., Ohnesorge S., Yu A., Garcia S. and Voinnet O. 2010. Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes and Development 24(9):904-915.

Ballut L., Drucker M., Pugniere M., Cambon F., Blanc S., Roquet F. and Badaoui S. 2005. HC-Pro, a multifunctional protein encoded by a plant RNA virus, targets the 20S proteasome and affects its enzymic activities. Journal of General Virology 86:2595-2603.

Bassham D. C., Laporte M., Marty F., Moriyasu Y., Ohsumi Y., Olsen L. J. and Yoshimoto K. 2006. Autophagy in development and stress responses of plants. Autophagy 2:2-11.

Boya P., Reggiori F. and Codogno P. 2013. Emerging regulation and functions of autophagy. Nature cell biology 15:713-720.

Brigneti G., Voinnet O., Li W. X., Ji L. H., Ding S. W. and Baulcombe D. C. 1998. Retracted: Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. The EMBO journal 17:6739-6746.

Carbonell A. and Carrington J. C. 2015. Antiviral roles of plant ARGONAUTES. Current Opinion in Plant Biology 27:111-117.

Cheng X. and Wang A. 2017. The potyvirus silencing suppressor protein VPg mediates degradation of SGS3 via ubiquitination and autophagy pathways. Journal of Virology 91:e01478-16.

Chiera J. M., Bouchard R. A., Dorsey S. L., Park E., Buenrostro-Nava M. T., Ling P. P. and Finer J. J. 2007. Isolation of two highly active soybean (*Glycine max* (L.) Merr.) promoters and their characterization using a new automated image collection and analysis system. Plant Cell Reports 26:1501-1509.

Chiera J. M., Lindbo J. A. and Finer J. J. 2008. Quantification and extension of transient GFP expression by the co-introduction of a suppressor of silencing. Transgenic Research 17:1143-1154.

Chiu M. H., Chen I. Baulcombe D. C. and Tsai C. H. 2010. The silencing suppressor P25 of *Potato virus X* interacts with Argonaute1 and mediates its degradation through the proteasome pathway. Molecular plant pathology 11:641-649.

Chung T., Suttangkakul A. and Vierstra R. D. 2009. The ATG autophagic conjugation system in maize: ATG transcripts and abundance of the ATG8-lipid adduct are regulated by development and nutrient availability. Plant physiology 149:220-234.

Deretic V. 2012. Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors. Current Opinion in Immunology 24:21-31.

Derrien B., Baumberger N., Schepetilnikov M., Viotti C., De Cillia J., Ziegler-Graff V. and Genschik P. 2012. Degradation of the antiviral component *ARGONAUTE1* by the autophagy pathway. Proceedings of the National Academy of Sciences 109:15942-15946.

Ding S. W. 2010. RNA-based antiviral immunity. Nature Reviews Immunology 10:632-644.

Duncan D. B. 1951. A significance test for differences between ranked treatments in an analysis of variance. Virginia journal of science 2:171–189.

Garcia-Ruiz H., Carbonell A., Hoyer J. S., Fahlgren N., Gilbert K. B., Takeda A. and Baladejo M. T. M. 2015. Roles and programming of Arabidopsis ARGONAUTE proteins during Turnip mosaic virus infection. PLoS Pathogens 11:e1004755.

Germundsson A., Savenkov E. I., Ala-Poikela M. and Valkonen J. P. 2007. VPg of *Potato virus A* alone does not suppress RNA silencing but affects virulence of a heterologous virus. Virus Genes 34:387-399.

Giner A., Lakatos L., García-Chapa M., López-Moya J. J. and Burgyán J. 2010. Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. PLoS Pathogens 6:e1000996.

Gursinsky T., Pirovano W., Gambino G., Friedrich S., Behrens S. E. and Pantaleo V. 2015. Homeologs of the *Nicotiana benthamiana* antiviral ARGONAUTE1 show different susceptibilities to microRNA168-mediated control. Plant Physiology 168:938-952.

Han T., Chen Q. and Liu H. 2016. Engineered photoactivatable genetic switches based on the bacterium phage T7 RNA polymerase. ACS Synthetic Biology.

Harvey J. J., Lewsey M. G. Patel K. Westwood J. Heimstädt S. Carr J. P. and Baulcombe D. C. 2011. An antiviral defense role of AGO2 in plants. PloS one 6:e14639.

Harvey J. J., Lewsey M. G., Patel K., Westwood J., Heimstadt S., Carr J. P. and Baulcombe D. C. 2011. An antiviral defense role of AGO2 in plants. PLoS ONE 6:e14639.

Havelda Z., Várallyay É., Válóczi A. and Burgyán J. 2008. Plant virus infection-induced persistent host gene downregulation in systemically infected leaves. The Plant Journal 55:278-288.

Inoue Y., Suzuki T., Hattori M., Yoshimoto K., Ohsumi Y. and Moriyasu Y. 2006. At ATG genes, homologs of yeast autophagy genes, are involved in constitutive autophagy in Arabidopsis root tip cells. Plant and Cell Physiology 47:1641-1652.

Jauber M., Bhattacharjee S., Mello A. F., Perry K. L. and Moffett P. 2011. ARGONAUTE2 mediates RNA-silencing antiviral defenses against *Potato virus X* in Arabidopsis. Plant Physiology 156:1556-1564.

Johansen L. K. and Carrington J. C. 2001. Silencing on the spot. induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. Plant Physiology 126:930-938.

Kasschau K. D. and Carrington J. C. 1998. A counter defensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell 95:461-470.

Klionsky D. J. 2005. The molecular machinery of autophagy: unanswered questions. Journal of Cell Science 118:7-18.

Levine B., Mizushima N. and Virgin H. W. 2011. Autophagy in immunity and inflammation. Nature 469:323-335.

Li F. and Ding S. W. 2006. Virus counter defense: diverse strategies for evading the RNA-silencing immunity. Annual Review of Microbiology 60:503.

Liu Y. and Bassham D. C. 2012. Autophagy: pathways for self-eating in plant cells. Annual Review of Plant Biology 63:215-237.

Liu L., Chung H. Y., Lacatus G., Baliji S., Ruan J. and Sunter G. 2014. Altered expression of Arabidopsis genes in response to a multifunctional geminivirus pathogenicity protein. BMC Plant Biology 14:302.

Liu Y., Schiff M., Czymmek K., Tallóczy Z., Levine B. and Dinesh-Kumar S. P. 2005. Autophagy regulates programmed cell death during the plant innate immune response. Cell 121:567-577.

Livak K. J. and Schmittgen T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402-408.

Lukhovitskaya N. I., Solovieva A. D., Boddeti S. K., Thaduri S., Solovyev A. G. and Savenkov E. I. 2013. An RNA virus-encoded zinc-finger protein acts as a plant transcription factor and induces a regulator of cell size and proliferation in two tobacco species. The Plant Cell 25:960-973.

Lukhovitskaya N. I., Vetukuri R. R., Sama I., Thaduri S., Solovyev A. G. and Savenkov E. I. 2014. A viral transcription factor exhibits antiviral RNA silencing suppression activity independent of its nuclear localization. Journal of General Virology 95:2831-2837.

Miozzi L., Napoli C., Sardo L. and Accotto G. P. 2014. Transcriptomics of the interaction between the monopartite phloem-limited geminivirus tomato yellow leaf curl Sardinia virus and *Solanum lycopersicum* highlights a role for plant hormones, autophagy and plant immune system fine tuning during infection. PloS one 9; e89951.

Morel J. B., Godon C., Mourrain P., Béclin C., Boutet S., Feuerbach F. and Vaucheret H. 2002. Fertile hypomorphic *ARGONAUTE* (*ago1*) mutants impaired in post-transcriptional gene silencing and virus resistance. The Plant Cell 14:629-639.

Nakagawa T., Kurose T., Hino T., Tanaka K., Kawamukai M., Niwa Y., Toyooka K., Matsuoka K. and Jinbo T. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction

of fusion genes for plant transformation. Journal of Bioscience and Bioengineering 104:34–41.
Nakahara K. S., Masuta C., Yamada S., Shimura H., Kashihara Y., Wada T. S. and Sekiguchi T. 2012. Tobacco calmodulin-like protein provides secondary defense by binding to and directing degradation of virus RNA silencing suppressors. Proceedings of the National Academy of Sciences 109:10113-10118.
Pirone T. P. and Blanc S. 1996. Helper-dependent vector transmission of plant viruses. Annual Review of Phytopathology 34:227-247.
Qu F., Ye X. and Morris T. J. 2008. Arabidopsis DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. Proceedings of the National Academy of Sciences 105:14732-14737.
Rajamaki M. L., Maki-Valkama T., Makinen K. and Valkonen J. P. 2004. Infection with potyviruses. In Plant-Pathogen Interactions. N. Talbot, ed (Oxford, UK: Blackwell Publishing), pp. 68–91.
Redondo E., Krause-Sakate R., Yang S. J., Lot H., Le Gall O. and Candresse T. 2001. *Lettuce mosaic virus* pathogenicity determinants in susceptible and tolerant lettuce cultivars map to different regions of the viral genome. Molecular Plant-Microbe Interactions 14:804-810.
Rojas M. R., Zerbini F. M., Allison R. F., Gilbertson R. L., Lucas Sáenz P., Salvador B., Simón-Mateo C., Kasschau K. D., Carrington J. C. and García J. A. 2002. Host-specific involvement of the HC protein in the long-distance movement of potyviruses. Journal of Virology 76:1922-1931.
Sahana N., Kaur H., Tena F., Jain R. K., Palukaitis P., Canto T. and Praveen S. 2012. Inhibition of the host proteasome facilitates papaya ringspot virus accumulation and proteosomal catalytic activity is modulated by viral factor HC-Pro. PLoS One 7:e52546.
Scholthof K. B. G., Adkins S., Czosnek H., Palukaitis P., Jacquot E., Hohn T. and Hemenway C. 2011. Top 10 plant viruses in molecular plant pathology. Molecular Plant Pathology 12:938-954.
Shin J. H., Yoshimoto K., Ohsumi Y., Jeon J. S. and An G. 2009. OsATG10b, an autophagosome component, is needed for cell survival against oxidative stresses in rice. Molecules and Cells 27:67-74.
Shoji-Kawata S. and Levine B. 2009. Autophagy, antiviral immunity, and viral countermeasures. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1793:1478-1484.
Sláviková S., Shy G., Yao Y., Glozman R., Levanony H., Pietrokovski S. and Galili G. 2005. The autophagy-associated Atg8 gene family operates both under favourable growth conditions and under starvation stresses in Arabidopsis plants. Journal of Experimental Botany 56:2839-2849.
Stephan D., Slabber C., George G., Ninov V., Francis K. P. and Burger J. T. 2011. Visualization of plant viral suppressor silencing activity in intact leaf lamina by quantitative fluorescent imaging. Plant Methods 7:25.
Su W., Ma H., Liu C., Wu J. and Yang J. 2006. Identification and characterization of two rice autophagy associated genes, OsAtg8 and OsAtg4. Molecular Biology Reports 33:273-278.
Tadamura K., Nakahara K. S., Masut C. and Uyeda I. 2012 Wound-induced rgs-CaM gets ready for counterresponse to an early stage of viral infection. Plant Signal Behav 7:1548–1551.
Urcuqui-Inchima S., Haenni A. L. and Bernardi F. 2001. Potyvirus proteins: a wealth of functions. Virus Research 74:157-175.
Várallyay É., Válóczi A., Ágyi Á., Burgyán J. and Havelda Z. 2010. Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation. The EMBO journal 29:3507-3519.
Vaucheret H. 2008. Plant argonautes. Trends in Plant Science 13:350-358.
Voinnet O. 2005. Induction and suppression of RNA silencing: insights from viral infections. Nature Reviews Genetics 6:206-220.
Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNAs. Cell 136:669-687.
Voinnet O., Rivas S., Mestre P. and Baulcombe D. 2003. Retracted: an enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. The Plant Journal 33:949-956.
Wang X. B., Jovel J., Udomporn P., Wang Y., Wu Q., Li W. X. and Ding S. W. 2011. The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative argonautes in Arabidopsis thaliana. The Plant Cell 23:1625-1638.

Wang Y., Gaba V., Yang J., Palukaitis P. and Gal-On A. 2002. Characterization of synergy between Cucumber mosaic virus and potyviruses in cucurbit hosts. Phytopathology 92:51-58.

Wang Y., Yu B., Zhao J., Guo J., Li Y., Han S., Huang L., Du Y., Hong Y., Tang D. and Liu Y. 2013. Autophagy contributes to leaf starch degradation. The Plant Cell 25:1383-1399.

Weigel D. and Glazebrook J. 2006. Transformation of agrobacterium using the freeze-thaw method. Cold Spring Harb Protoc doi/10.1101/pdb.prot4665.

Xiong Y., Contento A. L., Nguyen P. Q. and Bassham D. C. 2007. Degradation of oxidized proteins by autophagy during oxidative stress in Arabidopsis. Plant Physiology 143:291-299.

Zhang X., Yuan Y. R., Pei Y., Lin S. S., Tuschl T., Patel D. J. and Chua N. H. 2006. *Cucumber mosaic virus*-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. Genes & Development 20:3255-3268.

Zhang Y. H. P., Himmel M. E. and Mielenz J. R. 2006. Outlook for cellulase improvement: screening and selection strategies. Biotechnology Advances 24:452-481.

Zhou T., Murphy A. M., Lewsey M. G., Westwood J. H., Zhang H. M., Gonzalez I. and Carr J. P. 2014. Domains of the cucumber mosaic virus 2b silencing suppressor protein affecting inhibition of salicylic acid-induced resistance and priming of salicylic acid accumulation during infection. Journal of General Virology 95:1408-1413.