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Detection of Proteins with Viral Suppressor of RNA Interference Activities from Insect-transmitted Viruses Using *Drosophila* S2 Cells

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Abstract

Sap-sucking insects, such as aphids, leafhoppers, thrips, and whiteflies, are vectors for > 70% of plant viruses. Presently, the development of insecticide resistance is causing a problem in agriculture worldwide as control of these insect vectors and of virus transmission is increasingly difficult. Insects lack an adaptive immune response system but use RNA interference (RNAi) functions as antiviral defense systems. Nevertheless, some viruses that infect insects encode proteins termed as viral suppressors of RNA silencing (VSR) proteins that can act against the RNAi systems. VSR proteins enable viruses to keep and increase in number in the body of the insects. The present study aimed to construct a VSR detection system for use in the detection of VSRs of viruses transmitted by insect vectors. The activities of VSR proteins were measured using a dual-luciferase reporter assay in S2 *Drosophila* cells. The analyses showed that HC-pro protein from Zucchini yellow mosaic virus had the highest level of activity among the tested VSR proteins. This methodological approach enables the detection of VSR proteins and measurement of VSR activities of insect viruses as well as plant viruses transmitted by insect vectors.

Discipline: Biotechnology
Additional key words: drosophila cells, plant virus, reporter assay, VSR

Introduction

In agriculture, losses of crops caused by viral diseases are > 30 billion dollars every year (Sastry & Zitter 2014), and to control the diseases, plant breeding, cross-protection, control of insect vectors, or other procedures have been performed (Rubio et al. 2020). Among insect vectors, the sap-sucking insects are common pests in agriculture that act as vectors of plant viruses. These insect vectors are believed to be responsible for transmission of > 70% of plant viruses (Hogenhout et al. 2008). Recently, control of insects and virus transmission has become increasingly difficult because of the development of insecticide resistance. Thus, in this study, we searched for methods to control plant virus transmission without controlling insects using immunities of insects.

Insects lack an adaptive immune response system and use RNA interference (RNAi) as an antiviral defense mechanism instead (Han et al. 2011, Blair & Olson 2015). This defense mechanism functions to control viruses through the generation of small RNAs that regulate viral gene expression (Fire et al. 1998, Salas-Benito & Nova-Ocampo 2015). Nevertheless, many viruses can infect insects and increase their numbers in insect bodies. These viruses produce proteins that act as viral suppressors of RNAi (VSRs) that block the RNAi functions of the host insect (Ding & Voinnet 2007).

Sap-sucking insects including aphids, planthoppers, thrips, and whiteflies are vectors of plant viruses that cause plant diseases (Kanakala & Ghanim 2016). The viruses escape the immune systems of plants and insect vectors through VSR mechanisms. Thus, detection of VSR proteins and measurement of their activities will be valuable for the control of virus transmission by insect vectors. In the present study, viral proteins from insect vectors were expressed in S2 *Drosophila* cells and VSR activities were detected using a luciferase reporter assay.

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Materials and methods

1. Materials

Cauliflower mosaic virus (CaMV: MAFF104018) and Zucchini yellow mosaic virus (ZYMV: MAFF104048) were obtained from NARO Genebank. Pepper vein yellows virus (PeVYV: AB594828, Murakami et al. 2011) was obtained from sweet pepper in Japan. Rice dwarf virus (RDV: Kimura et al. 1987), rice grassy stunt virus (RGSV: Hibino & Kimura 1982), and rice ragged stunt virus (RRSV: Hibino et al. 1985) were maintained in rice plants and affected leaves were stored at −80°C. Reverse transcription PCR was used to produce the following cDNAs from infected leaves: P2 and P4 proteins from CaMV; P0 protein from PeVYV; P6, P9, P10, and P12 proteins from RDV; P1, P2, P3, P4, and P5 proteins from RGSV; P7, P9, and P10 proteins from RRSV; and HP-pro protein from ZYMV. A cDNA of the P2 protein from TSWV was provided by Dr. K. Ishibashi (Ishibashi et al. 2017).

2. Measurement of VSR activities

VSR activities were measured using a modification of the methods described by Cleef et al. (2011). The cDNAs of viral proteins were cloned into pAc5.1/V5-His A (*p*Ac) vectors (Invitrogen, Tokyo), and firefly luciferase from *p*SP-luc vector (Promega, Tokyo) and *Renilla* luciferase *p*RL-null vector (Promega) were separately cloned into *p*MT/V5-His A (*p*MT) vectors (Invitrogen).

For the preparation of *ds*RNAs, T7 promotor-flanked *Renilla* luciferase PCR products were amplified using KOD-Plus (TOYOBO, Osaka) from a *p*RL-null vector using the primers T7-Ruc-F (5′-TAATACGACTC ACTATAGGGAGATA-3′) and T7-Ruc-R (5′-TAATACG ACTCACTATAGGGAGATA-3′). T7 promotor-flanked EGFP PCR products were amplified using p EGFP vector (Clontech, Kusatsu) and the primers T7-GFP-F (5′-TAATACGACTCACTATAGGGAGAAGCTGACCC TGAAGTTCATCTG-3′) and T′-GFP-R (5′-TAATACGA CTCACTATAGGGAGAGGTGTTCTGCTGGTAGTG GTC-3′). RNAs were amplified with the RiboMAX Large Scale RNA Production System-T7 (Promega) and were annealed to prepare *ds*RNAs of *Renilla* luciferase.

Drosophila S2 cells (Thermo Fisher Scientific, Tokyo) were cultured in Schneider's insect medium (Thermo Fisher Scientific) with 50 U/mL of penicillin, 50 μg/mL of streptomycin, and 10% fetal bovine serum (FBS) at 25°C and kept at 40%-80% confluency in cell culture flasks. An aliquot of 7×10^4 cells was incubated in a well of a 96 well plate and the medium was replaced with a medium without FBS. The cells were transfected with 300 ng of *p*Ac vector cloned cDNAs of viral proteins, 2 ng of *p*MT vector cloned cDNAs of firefly luciferase, and 2 ng of *p*MT vector cDNA of *Renilla* luciferase using FuGENE HD Transfection Reagent (Promega). The cells were incubated for 24 h and the medium was replaced with a medium containing FBS. Then, 1.7 ng of *ds*RNA of *Renilla* luciferase was added to the culture of transfected cells, and 0.8 mM $CuSO_4$ was subsequently added after *ds*RNA feeding.

After 48 h of incubation, a reporter assay was performed using the Dual-Luciferase reporter assay system (Promega) following the manufacturer's instructions. After measurement of firefly luciferase luminescence, the activity of the firefly luciferase was blocked and luminescence of the *Renilla* luciferase was then measured with a luminometer (LUMI-COUNTER NU-700, Microtec, Funabashi).

3. Statistics

The experiments were repeated five times, and the ratio of *Renilla* luciferase luminescence to firefly luciferase luminescence (RF) was calculated as the VSR activity of each viral protein. Pairwise comparisons of RF values were performed using Bonferroni *t*-tests (R ver. 3.6.2).

Results

1. Detection of VSR proteins

Figure 1 illustrates the protocols for detecting VSR proteins and measuring their activities. Transfection of S2 cells with *p*MT plasmid carrying firefly luciferase and *p*MT plasmid carrying *Renilla* luciferase gave RF values in the range 0.16-0.18 (Fig. 2). When *p*Ac plasmid without viral protein was transfected into the cells, RF values increased. RF values fell when *ds*RNA and *Renilla* luciferase were also transfected. These results showed that the expression of *Renilla* luciferase was knocked down by *ds*RNA of *Renilla* luciferase.

P10 protein from RDV (Zhou et al. 2010), P2 protein from RGSV, and P6 and P9 proteins from RRSV (Nguyen et al. 2015) have been reported as suppressors in a transient expression analysis using *Nicotiana benthamiana* leaves. VSR proteins from RDV, RGSV, and RRSV suppressed the RNAi mechanisms of *Renilla* luciferase in S2 cells (Fig. 3). When the VSR proteins were transfected into the cells, the RF value increased. Thus, we concluded that the reporter assay using S2 cells was an effective method for the detection of VSR activity, and we used P2 protein from RGSV as a positive control in the following experiments.

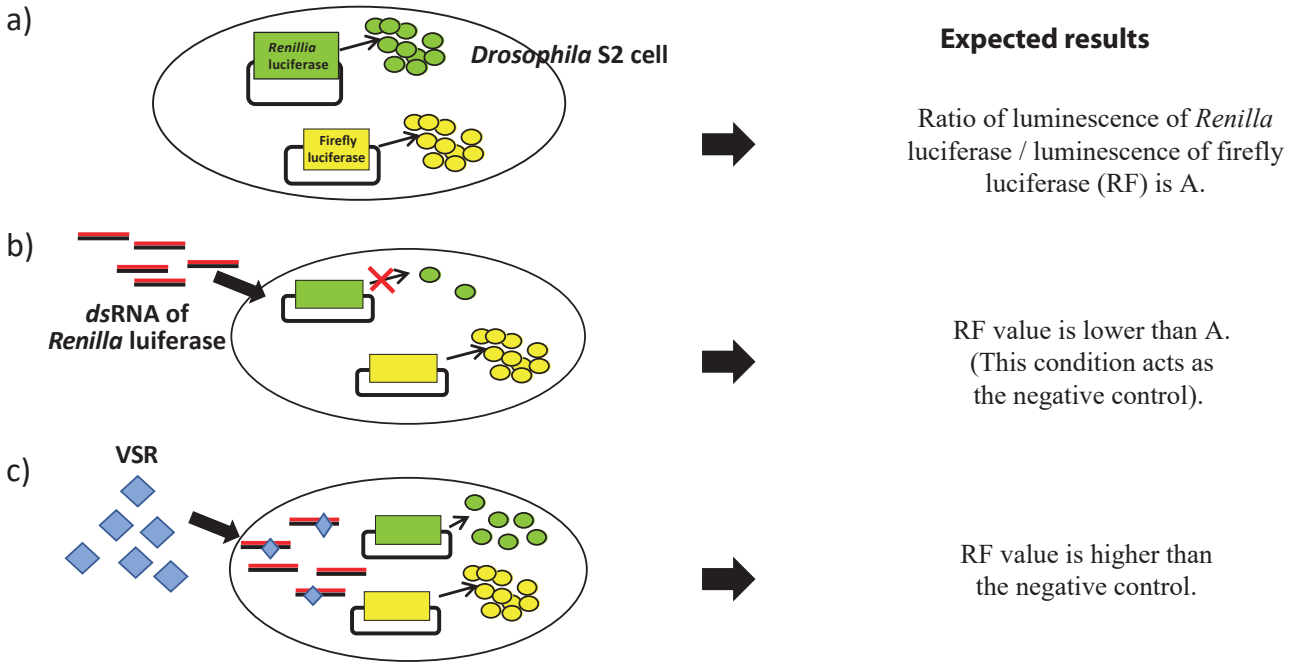

Fig. 1. Protocol for detection of VSR proteins and measurement of VSR activities

a) Plasmid with cDNA for *Renilla* luciferase and plasmid with cDNA for firefly luciferase are transfected into Drosophila cells. The ratio of the levels of *Renilla* luciferase luminescence to firefly luciferase luminescence (RF value) is A.

b) When double stranded RNA (dsRNA) of *Renilla* luciferase is transfected into the cells, the dsRNA interferes with translation of *Renilla* luciferase and the RF value is lower than A. This experimental condition acts as a negative control condition.

c) When viral suppressor of RNA silencing (VSR) protein is inoculated into negative control cells, it interferes with dsRNA of *Renilla* luciferase. The RF value is higher than that of the negative control when dsRNA for *Renilla* luciferase is added to negative control cells.

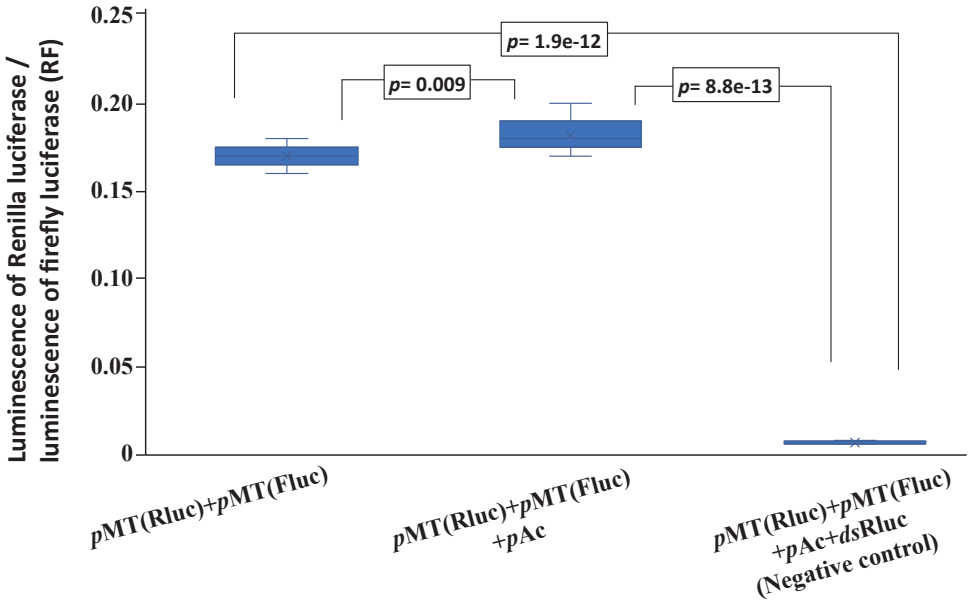

Fig. 2. Luminescence of vectors using reporter assay in S2 *Drosophila* cells

When pAc5.1/V5-His A vector (pAc) was added to pMT/V5-His A vector (pMT) carrying cDNA of *Renilla* luciferase and pMT carrying cDNA of firefly luciferase, luminescence of *Renilla* luciferase/luminescence of firefly luciferase (RF) increased. The RF value decreased when dsRNA of *Renilla* luciferase was added to the cells (Negative control).

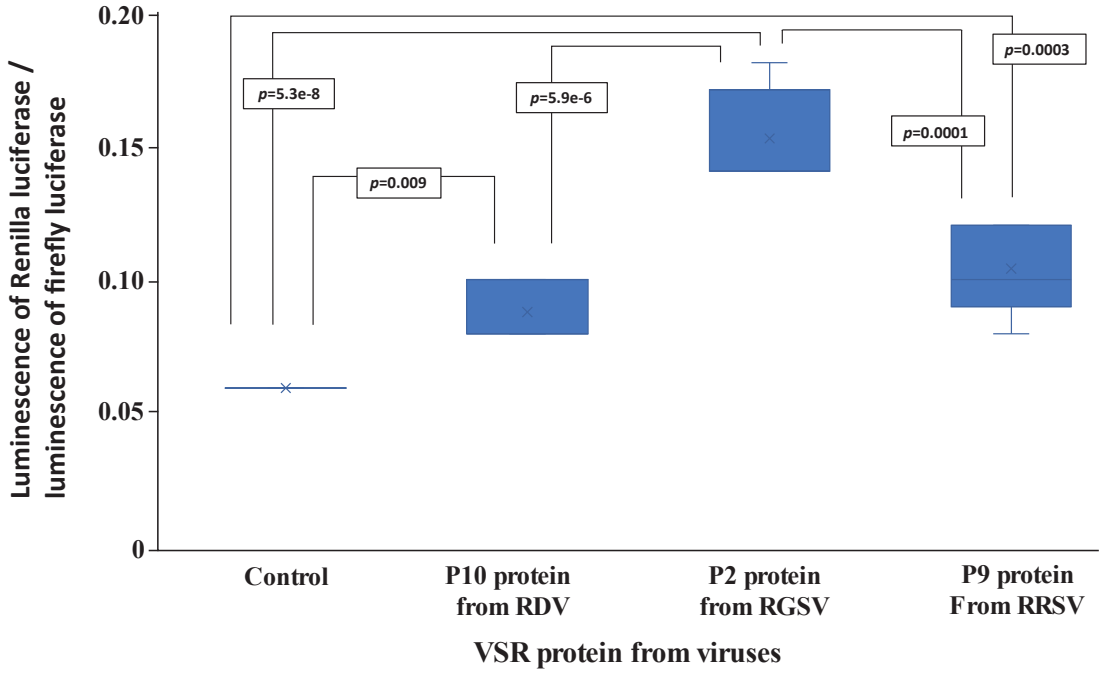

Fig. 3. Reporter assay system using *Drosophila* S2 cells
cDNAs encoding viral suppressor RNA interference (VSR) proteins were cloned into pAc5.1/V5- His A vectors and transfected into S2 cells, the RF value for *Renilla* luciferase luminescence/firefly luciferase luminescence increased because RNAi of *Renilla* luciferase was induced by the VSR protein.
RDV: Rice dwarf virus, RGSV: Rice grassy stunt virus, RRSV: Rice ragged stunt virus (Positive control)

2. Detection of VSR

Figure 4 shows the RF values of proteins from various viruses that are transmitted by insects. There were significant differences in RF values among the control and many of the proteins, and HC-pro protein from ZYMV had the greatest activity among the tested proteins. P2 protein from RGSV, the positive control, had the second-highest activity. However, there were no significant differences among the RF values of P4 protein from CaMV and P6 and P9 proteins from RDV and that of the control. Therefore, we judged that P4 protein from CaMV and P6 and P9 proteins from RDV might be not VSR protein.

Discussion

In Figure 4, positive control (P2 from RGSV) showed higher VSR activities than negative control. Thus, we judged that this system is useful for the detection of VSR proteins and measuring VSR activity of the tested proteins. The analysis showed that RF values of P4 protein from CaMV and P6 and P9 proteins from RDV and the negative control did not differ significantly (Fig. 4). Thus, P4 protein from CaMV and P6 and P9 proteins from RDV were judged that they might have not VSR activity. P4 protein from CaMV is a coat protein (Bak et al. 2013), and P6 and P9 proteins from RDV have low molecular weights; however, their functions are unknown. The results of coat protein from CaMV and P6 and P9 unknown proteins from RDV had not been in conflict with those reported litertures. By contrast, HC-pro protein showed the highest activity among the proteins tested in S2 cells. Further, HP-pro protein from ZYMV has been reported to function as a suppressor in host plants (Shiboleth et al. 2007). P2 protein from TSWV is also a suppressor in host plants (Eifan et al. 2013), and this protein showed VSR activity in the present study. The function of P0 protein from PeVYV (*Polerovirus*) is unknown, but *Polerovirus* P0 protein has been reported as a suppressor in host plants (Bortolamiol et al. 2007, Nicolas et al. 2007). Here, P0 protein from PeVYV showed VSR activity in S2 cells. Thus, the results from S2 cells are consistent with those obtained from host plants.

P2 protein from CaMV; P12 protein from RDV; P1, P3, P4, and P5 proteins from RGSV; and P7 and P10 proteins were observed to show VSR activities. P2 protein from CaMV is an aphid transmission factor (Bak et al. 2013). P12 protein from RDV; P1, P3, P4, and P5 proteins from RGSV; and P7 and P10 proteins from RRSV are low molecular weight proteins of unknown function. Viral proteins can be multifunctional, and these

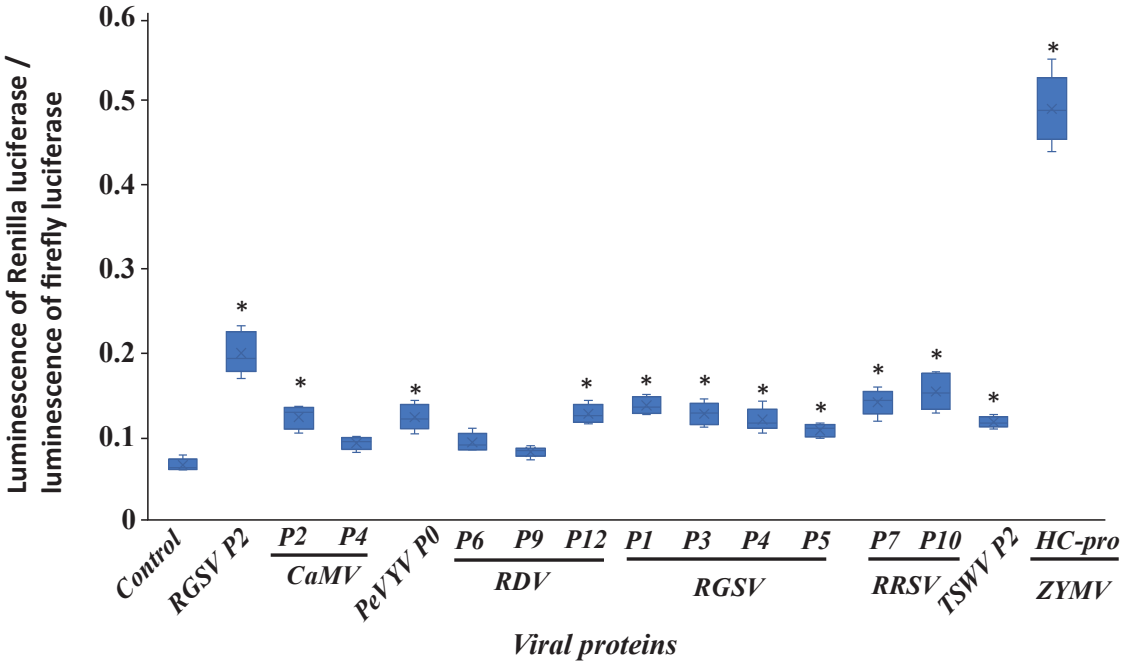

Fig. 4. VSR activities of viral proteins in the S2 *Drosophila* cell reporter assay
Control: negative control, RGSV P2: positive control, CaMV: Cauliflower mosaic virus, PeVYV: Pepper vein yellows virus, RDV: Rice dwarf virus, RGSV: Rice grassy stunt virus, RRSV: Rice ragged stunt virus, TSWV: Tomato spotted wilt virus, ZYMV: Zucchini yellow mosaic virus. *: significant difference between negative control and viral proteins, $p < 0.05$

proteins also showed weak VSR activities. More studies analyzing the VSR activities function of these proteins are warranted.

CaMV, ZYMV, and PeVYV are transmitted by aphids (Antignus et al. 1989, Yonaha et al. 1995, Martiniere et al. 2009). RDV, RGSV, and RRSV are transmitted by planthoppers and can accumulate in planthopper bodies (Rivera et al. 1966, Hibino & Kimura 1982, Kimura et al. 1987). TSWV is transmitted by thrips and can replicate in these insects (Ullman et al. 1992). Plant viruses transmitted by insect vectors are categorized into three types based on their viral transmission system: nonpersistent, which do not enter the insect cells; semipersistent, which enter the cells but are not replicated; and persistent, which enter the cells and are replicated. CaMV and ZYMV are nonpersistent viruses; PeVYV is a semipersistent virus; and RDV, RGSV, RRSV, and TSWV are persistent viruses. The present study used S2 cells identified VSR activities in proteins from all three virus transmission types.

In the present analysis, the levels of VSR activity varied among proteins. VSR activity determines viral pathogenicity, and VSR proteins have different levels of activity depending on their suppression activities in insects (Nayak et al. 2010, Nguyen et al. 2015). Thus, the results obtained here do not contradict previously reported results. Analyses using host plant leaves have also shown that VSR activities may determine viral pathogenicity (Desbiez et al. 2010).

Thus far, investigations of suppressor activity by viral proteins have been carried out using transient expression on *Nicotiana benthamiana* or host plant leaves (Zhou et al. 2010, Nicolas et al. 2007, Shiboleth et al. 2007, Nguyen et al. 2015). HC-pro protein from Tobacco etch virus has been shown to have VSR activity using RNAi suppression of RNAi of β-galactosidase activity in S2 cells, and the proportion of cells showing reporter activity indicated the relative strength of VSR activities (Reavy et al. 2004). Nevertheless, β-galactosidase activity was unstable because of the cell system or the efficiency of expression vector plasmid in the cells, but the luciferase reporter assay is not affected by these constraints. Therefore, the method used here should be of value for detecting VSR proteins and measuring their activities not only for insect viruses but also for plant viruses transmitted by insect vectors.

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