Study of small RNA-mediated control of gene expression and viruses in plants: Roll of a viral suppressor of RNA silencing in viral survival

(Small RNA によって調節される植物体内の遺伝子発現と植物ウイルスに関する研究: ウイルス生存のために機能する RNA サイレンシングサプレッサーの役割)

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ABBREVIATIONS

AGO, Argonaute; amiRNA, artificial micro RNA; Avr, avirulence; CBB, Coomassie brilliant blue; CMV, *Cucumber mosaic virus*; DCL protein, Dicer-like protein; DFR, Dihydroflavonol 4-reductase; dsRNA, double-stranded RNA; *GAPDH, glyceraldehyde 3-phosphate dehydrogenase*; HC-Pro, helper component proteinase; hpRNA, hairpin RNA; HR, hypersensitive reaction; LYSV, *Leek yellow stripe virus*; miRNA, microRNA; mRNA, messenger RNA; MS medium, Murashige and Skoog medium; ORF, open reading frame; OYDV, *Onion yellow dwarf virus*; *PDS, Phytoene desaturase*; PDR, pathogen-drived resistance; PMMoV, *Pepper mild mottle virus*; Pol, RNA polymerase; PTGS, post transcriptional gene silencing; PVY, *Potato virus Y*; RdDM, RNA-derected DNA methylation; RDR, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; RSS, RNA silencing suppressor; siRNA, short-interference RNA; sRNA, small RNA; ssRNA, single-stranded RNA; SYSV, *Shallot yellow stripe virus*; ta-siRNA, trans-acting siRNA; TCV, *Turnip crinkle virus*; TEV, *Tobacco etch virus*; TMV, *Tobacco mosaic virus*; TRV, *Tobacco rattle virus*; VIGS, virus-induced gene silencing; vsiRNA, viral siRNA; ZYMV, *Zucchini yellow mosaic virus*

GENERAL INTRODUCTION

RNA silencing, or RNA interference, is an evolutionary conserved mechanism induced by small non-coding RNAs. The first description of RNA silencing-related phenomenon may have been published in a paper by Wingard et al. (1928). They reported a research result with several phenomena in tobacco ringspot virus-infected tobacco plants, in which the upper systemic leaves had somehow recovered and became resistant for secondary inoculation. Even though there was not enough knowledges to explain such a 'recovery' phenomenon at that time, consecutive researches have been elucidate the mechanism of systemic resistance conferred by viral infection. Since the concept of pathogen derived resistance (PDR) was proposed by Sanford and Johnston (1985), Lindbo and co-workers first suggested a model of sequence specific degradation of viral RNAs induced by expression of viral genes in plants (Lindbo et al., 1993). In present, it may be explained as RNA silencing mechanism against viral infection.

The RNA silencing triggers sequence-specific degradation of RNAs to regulate gene expressions for control of development, genome stabilization and responses to abiotic/biotic stresses in eukaryotic cells (Li and Wang, 2019; Llave, 2010). Over the past decades, the RNA silencing has been a main focus of plant biotechnology. Remarkable advancement in exploiting RNA silencing technologies have been developed for studies of functional genomics, crop development and pathogen controls (see the review by Frizzi and Huang, 2010). Among them, virus-induced gene silencing (VIGS) is one of the most frequently used tools for functional studies of plant genes. Because enormous viral small RNAs (sRNAs) are generated in host plants by viral infection, with recombinant viral vectors, silencing constructs can be easily designed for specific genes. Another application of RNA silencing to plants is a disease control

against various pathogens infecting crops. The RNA silencing-based technology for plants has been significantly progressed especially on protection of crop plants from various pathogens. RNA silencing in nematode and insect species can be induced by direct treatment of synthetic double-stranded RNA (dsRNAs) or intake of dsRNA from transgenic plants (Baum et al., 2007; Mao et al., 2007; Rosso et al., 2005; Tamilarasan and Rajam, 2013; Urwin et al., 2002; Wang et al., 2011a; Williamson and Kumar, 2006; Yu et al., 2013). Likewise, transgenic plants expressing fungi-derived dsRNA were significantly effective to induce antifungal resistance (Andrade et al., 2016; Chen et al., 2016; Cheng et al., 2015; Ghag et al., 2014; Hu et al., 2015; Koch et al., 2013), and simple spray-treatment of dsRNA targeting fungal gene also operates the fungal silencing machinery (Koch et al., 2016). Especially for viral resistance, numerous transgenic plants have been created with virus-derived sequences to induce antiviral RNA silencing against the target virus infection. In this text, I discuss about plant resistance based on RNA silencing technologies against viral infection, especially for exogenous dsRNA or short-interfering RNA (siRNA) treatment.

To survive from the antiviral RNA silencing, plant viruses express viral suppressor(s) of RNA silencing (RSSs), which interferes with almost all the stages of the plant RNA silencing pathway. Furthermore, some of RSSs have been identified to inhibit plant immune responses mediated by phytohormones such as salicylic acid and jasmonic acid (Mayers et al., 2005; Zhou et al., 2014; Love et al., 2012; Westwood et al., 2014). Therefore, RSSs generally play a role as a pathogenicity determinant of virus.

RNA silencing in plants

In plant cells, the RNA silencing is guided by sRNAs including microRNAs (miRNAs) and several classes of siRNAs, and it directly or indirectly targets gene expressions in a transcriptional or post-transcriptional manner (Rubio-Somoza et al., 2009). These sRNAs are originated from dsRNA or self-complementary hairpin RNA (hpRNA) (Wesley et al., 2001). The processing of endogenous dsRNAs, which are generated by plant RNA-dependent RNA polymerases (RDRs), or exogenous dsRNAs is performed by Dicer-like proteins (DCLs), RNase III enzymes, generating small 21–24 nt siRNAs (Carmell and Hannon, 2004), and the siRNAs are associated with Argonaute (AGO) proteins to form RNA-induced silencing complex (RISC) (Hammond et al., 2000; Pantaleo et al., 2007) and to guide them to their targets such as viral RNAs and messenger RNAs (mRNAs) in a sequence-specific manner. Baulcombe (2004) previously classified three pathways of RNA silencing in plants. The first pathway is cytoplasmic siRNA-induced RNA silencing. Complete dsRNAs or hairpin RNAs are processed into siRNAs by DCLs, and then induce post transcriptional gene silencing (PTGS). This pathway is important for virus-infected plant cells as a resistant mechanism. The second pathway is miRNA-mediated RNA silencing of endogenous mRNAs. In *Arabidopsis thaliana*, 21- to 24-nt mature miRNAs are generated by the DCL1-mediated cleavage of hairpin-structured pre-miRNA transcripts, which are transcribed from miRNA genes (*MIR*s) (Wang et al., 2012; Xie et al., 2005), and it is also involved in the DCL-mediated cleavage of endogenous *TAS* transcripts to generate trans-acting siRNAs (ta-siRNAs) (Allen et al., 2005; Peragine et al., 2004). The miRNAs and ta-siRNAs plays critical roles in regulating endogenous mRNAs to control plant development and responses against abiotic and biotic stresses (Li et al., 2017).

The third is related to RNA-directed DNA methylation (RdDM) and transcriptional gene silencing. The RdDM pathway is a conserved epigenetic modification in plant genome, which

is involved in transcriptional gene regulation, genomic imprinting, stress responses and transposon control (Matzke and Mosher, 2014; Yang et al., 2016). The DNA methylation occurs at cytosine residues in the context of the three different sequences (CG, CHG and CHH) by different DNA methyltransferase. It is not only induced by 24-nt siRNAs (canonical RdDM pathway) but also by 21–22-nt siRNAs (non-canonical RdDM pathway) (Matzke and Mosher, 2014).

Antiviral RNA silencing

The RNA silencing is a primary immune response of plants for viral infection (Li and Wang, 2019; Vaucheret, 2006). Enormous virus-derived siRNAs (vsiRNAs) are generated in virus-infected cells, in which dsRNAs could be a genome of dsRNA virus or replication intermediate of single stranded RNA (ssRNA) virus. For DNA viruses, bidirectional transcripts of viral genome could be the targets of antiviral RNA silencing. The viral dsRNAs are processed by DCLs, and then captured by AGOs for target either viral RNA degradation or viral genomic DNA methylation for DNA viruses (Wang et al., 2012).

Many studies have been conducted in *Arabidopsis* to identify the key factor proteins of antiviral RNA silencing. The genome of *Arabidopsis* encodes four DCL family proteins (DCL1–4 proteins), which contribute to plant resistance against viral infection (Guo et al., 2019). Among them, DCL4 plays a key role in antiviral RNA silencing by processing viral dsRNAs into 21-nt vsiRNAs (Garcia-Ruiz et al., 2010; Wang et al., 2010). DCL2 produce 22-nt vsiRNA to induce antiviral PTGS, but its effect is not as critical as 21-nt vsiRNAs generated by DCL4 (Wang et al., 2010; Wang et al., 2011b). The vsiRNAs are captured by either AGO1

or AGO2 forming RISC to guide it to target viral RNAs (Carbonell and Carrington, 2015). The cleaved viral RNAs become the targets of RDR6, being templates for endogenous viral dsRNA synthesis (Blevins et al., 2006). The endogenously synthesized viral dsRNAs are then processed by DCLs to generate secondary vsiRNAs, and these are dispersed through phloem to induce systemic antiviral resistance by RNA silencing. For DNA viruses, RdDM plays a role as a plant antiviral mechanism (Wang et al., 2012). Using viral genomic DNA as a template, plant DNA-dependent RNA polymerase IV (Pol IV) transcribes ssRNA complementary to the viral genome. The ssRNA is copied by RDR2 to generate dsRNA, and then it is processed into 24-nt vsiRNAs by DCL3 (Rodríguez-Negrete et al., 2009). The 24-nt siRNAs are captured by AGO4 forming RISC, which interacts with the transcript produced by RNA polymerase V (Pol V) to recruit methyltransferases such as DRM2 for *de novo* methylation of viral DNAs (Wierzbicki et al., 2008; Zhong et al., 2014).

Virus-induced gene silencing (VIGS)

The virus infection generates an enormous amount of vsiRNA to induce RNA silencing in its host cells. By exploiting the mechanism, VIGS has been widely used for functional genomics studies, mostly in *N. benthamiana* (Lu et al., 2003). Using the viral vector carrying a partial fragment of a gene sequence of interest, siRNAs against the target gene are generated from dsRNA of a recombinant viral vector (Burch-Smith et al., 2006). Instead of complete knock–out of gene expression, which takes generally a long time and much labor, the VIGS takes an advantage in that it is rapid and efficient in various host plants. Because some plant viruses have a broad host range (e.g. cucmber mosaic virus [CMV]), such a viral vector has an

advantage. Various viral vectors have been already constructed based on many plant RNA viruses, including barley stripe mosaic virus, cucumber mosaic virus (CMV), potato virus X, tobacco mosaic virus (TMV) and tobacco rattle virus (TRV) (Kumagai et al., 1995; Pacak et al., 2010; Ratcliff et al., 2001; Ruiz et al., 1998; Scofield and Nelson, 2009). Among them, The TRV vector has been most widely used for VIGS, especially in *N. benthamiana* plant (Ratcliff et al., 2001). In addition, several DNA viral vectors using geminiviruses have been also developed (Kjemtrup et al., 1998; Peele et al., 2001; Turnage et al., 2002; Tao and Zhou, 2004; Golenberg et al., 2009; Huang et al., 2009; Pandey et al., 2009; Chen et al., 2011), which have been applied to some model plants including *N. benthamiana* and *Arabidopsis*.

Application of antiviral RNA silencing for plant resistance against viral diseases

(1) Pathogen-derived resistance

Since Abel et al. (1986) demonstrated the TMV resistance in transgenic tobacco plants expressing the TMV CP gene, PDR became a key word for research in creating virus resistant plants. In the subsequent decade, many transgenic plants expressing viral genes have been generated for virus-resistant plants. Some of them has been led to practical applications for a commercial use; the production of transgenic papaya that is resistant against papaya ring spot virus in Hawaii is one of the most successful example (Ferreira et al. 2002). The main mechanism of the PDR-mediated viral resistance has been considered as RNA silencing even though some cases of PDR have been suggested to occur independently of RNA silencing (Baulcombe 1996).

(2) Small RNA (sRNA)-mediated resistance

Because PDR contributes to RNA silencing-based viral resistance, transgenic constructs which can efficiently induce antiviral RNA silencing have been extensively created. The common strategy is to generate antiviral sRNAs in plant cells. Various constructs have been devised to produce virus-resistant transgenic plants which express sRNAs of the target viruses, including hpRNAs, artificial micro RNAs (amiRNAs), synthetic ta-siRNA and their combinations.

Even simple hpRNAs generated from inverted-repeat constructs containing partial viral sequences have been identified to be effective enough to induce viral resistance against inoculated viruses (Duan et al. 2008; Gaba et al. 2010). For hpRNA-mediated viral resistance, high sequence similarity of siRNAs to the target virus, and a high concentration of siRNAs are required. Therefore, the resistance could be broken by the other virus strains generated in field. For instance, a transgenic plant containing the ~600-nt inverted-repeat construct for the potato virus Y (PVY) potato strain was infected by the PVY strains of tomato and pepper even though there is ~86% similarity in viral sequences among the three PVY strains (Gaba et al. 2010).

For efficient vsiRNA production in transgenic plants, amiRNAs have been devised (Liu et al. 2017). In this system, the original mature miRNA sequence in an endogenous pre-miRNA is replaced with a vsiRNA sequence of the target virus, which have been verified as efficiently generated by the target virus infection. Many miRNAs including miR159, miR167, miR168, miR169, miR171 and miR319 in a model plant, *Arabidopsis*, have been exploited as the backbone miRNAs for viral amiRNAs and evaluated to be effective for many viruses (Ai et al. 2011; Duan et al. 2008; Niu et al. 2006; Qu et al. 2007; Simón-Mateo and García 2006; Zhang

et al. 2011). Furthermore, miRNAs of *N. benthamiana* and several crop plants, such as rice, also have been successfully used for scaffold of amiRNAs to induce resistance to target virus infection (Ali et al. 2013; Fahim et al. 2012; Kis et al. 2016; Sun et al. 2016; Vu et al. 2013). However, amiRNA-induced viral resistance is not perfect. For example, Martínez et al. (2013) have demonstrated that mixed infections of turnip mosaic virus with other viruses can make the amiRNA-mediated resistance invalid. Furthermore, because amiRNA-mediated resistance relies on only a specific siRNA sequence, virus variants on the siRNA binding site or chimeric viruses, which may emerge in the field, can escape the RNA silencing due to mismatches between the miRNA and the target virus sequence (Simón-Mateo and García 2006). To overcome this weak point, synthetic ta-siRNA, which can generate several sets of antiviral siRNAs simultaneously when it is triggered by miRNA, were developed to control the infections of two viroid families (Carbonell and Daròs 2017). As another improvement of sRNA-mediated antiviral resistance, a combination strategy using both hpRNA and amiRNA, in which hpRNAs are expressed in the form of miRNAs, has been verified to efficiently express hpRNAs. Aslam et al. (2018) recently determined that an hpRNA fused in the miR168a backbone conferred RNA silencing-based resistance against sugarcane mosaic virus infection.

(3) Treatment with exogenous dsRNAs

As described above, transgenic plants producing viral dsRNAs and amiRNAs have been extensively generated and verified to be effective against various virus infections. The application of exogenously synthesized dsRNAs of viral sequences to plants has been also tested to prevent viral diseases through the antiviral RNA silencing (Figure 1A). In the early

researches based on this strategy, two research groups demonstrated that direct treatment of synthetic viral dsRNAs and *Agrobacterium*-mediated transient-overexpression of inverted repeat construct are effective to induce antiviral RNA silencing (Carbonell et al. 2008; Tenllado and Díaz-Ruíz 2001; Tenllado et al. 2004). Now, we know that exogenously synthesized viral dsRNAs are effective enough to induce antiviral resistance against the corresponding viruses or viroids after the plants are either pretreated with the viral/viroid's dsRNA fragments or simultaneously inoculated together with the corresponding viruses/viroids (Figure 1A). However, for practical application of these strategy, the biggest hurdle is the establishment of dsRNA production technique to obtain a large amount of dsRNAs in a low cost and short time. One approach to solve the problem is the production of dsRNAs in *E. coli* system. *E. coli* strain HT115 (DE3), an RNase III-deficient and T7 RNA polymerase-expressing cell, has been demonstrated to be a proper platform to produce dsRNAs on a massive scale (Gan et al. 2010; Shen et al. 2014; Tenllado et al. 2003; Yin et al. 2009). In addition, Huang et al. (2013) devised a system to produce vsiRNAs in *E. coli* by using a tombusvirus protein, p19, which is able to stabilize siRNAs generated by RNase III in *E. coli*. Considering that *in vitro*-synthesized siRNAs can also efficiently downregulate plant gene expressions through the RNA silencing (Dalakouras et al. 2016), siRNAs can be also used for viral protection.

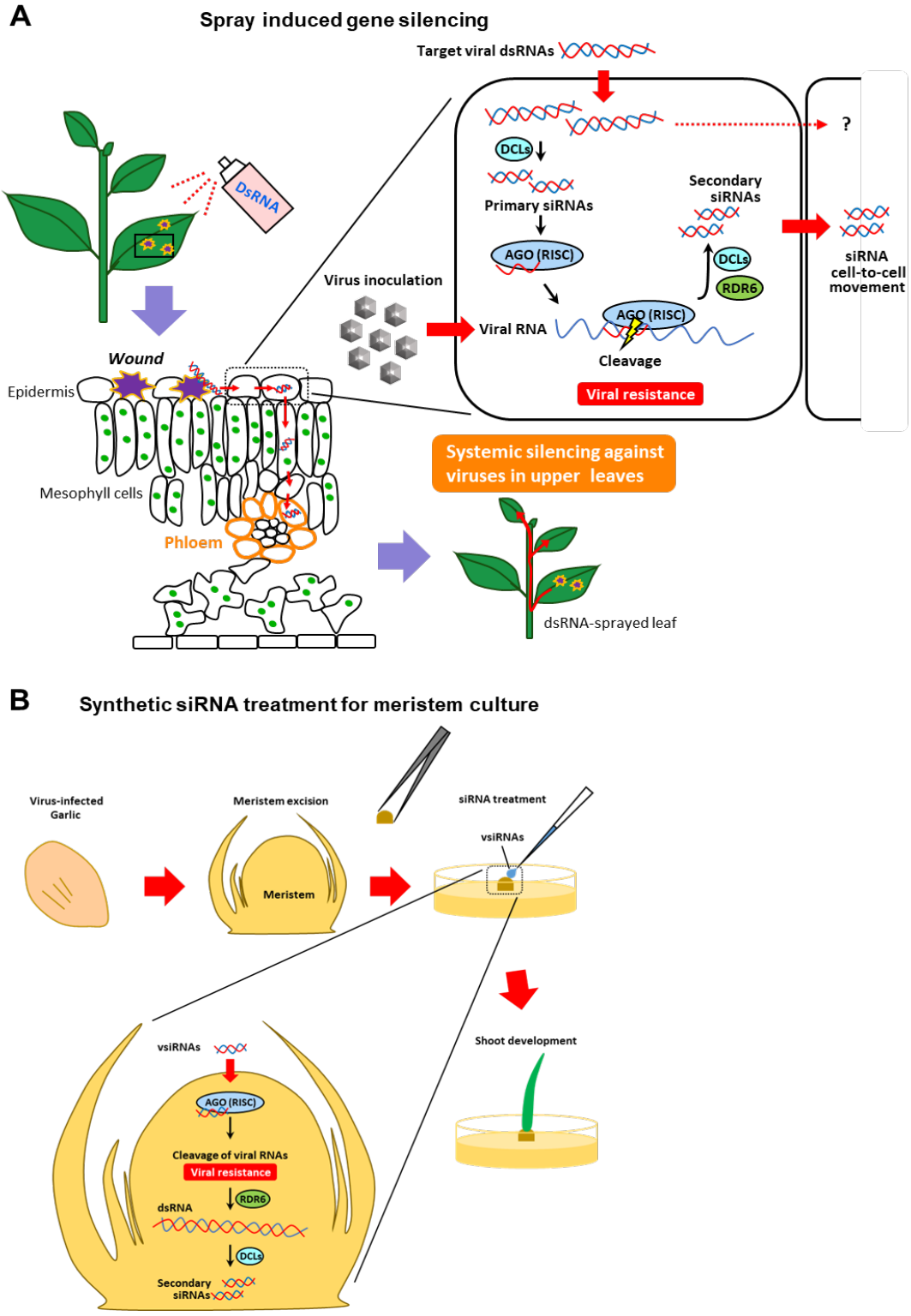

Figure 1 Application of exogenous viral dsRNAs on leaves to induce antiviral RNA silencing against target virus. (A) Synthetic dsRNAs are sprayed with high pressure, they will penetrate

cells through a wound and then processed into vsiRNAs by DCL proteins. The vsiRNA incorporates with AGO protein forming RISC, and guide it to target viral RNAs for cleavage. The secondary siRNAs are generated by RDR6 and DCL proteins and systemically spread through phloem to whole plant (Kim et al., 2019). (B) Production of virus free garlic plant using dsRNA and siRNAs of the target virus. The meristem of original garlic clove is grown on the MS medium. *In vitro* synthesized vsiRNA is treated on the meristem explant. The siRNAs are smeared into meristem and associated with AGO proteins to form RISC. The RISC is guided by vsiRNAs to their target viral RNAs to cleave them. The secondary siRNAs are produced by RDR6 and DCLs and spread over the meristem to eliminate viral RNAs. Shoot is developed from the meristem treated with siRNAs and grow up as a virus free plant.

Recently, Mitter et al. (2017) reported an exogenous dsRNA treatment technology by spraying layered double hydroxide clay nanosheets as a carrier of dsRNAs against pepper mild mottle virus (PMMoV), demonstrating that PMMoV infection has been inhibited even more than 20 days after treatment. This formulation of dsRNAs indicates that, depending on the carrier molecule or material of the dsRNAs (or siRNAs), viral protection can be sustainable for a long time and that sprayed dsRNAs can penetrate into plant cells and induce systemic RNA silencing (Figure 1). As to cost effectiveness, Zotti et al. (2018) discussed the practical production costs of dsRNAs in detail, suggesting that field application of dsRNAs might become feasible as a cost-effective and efficient formulation methods of dsRNAs (or siRNAs) are developed.

RNA silencing suppressors (RSSs)

For successful infection, most plant viruses encode the RSS genes in their genome to overcome antiviral RNA silencing in host plants (Wu et al., 2010b). These RSSs are highly diverse among different viruses in their sequence or structure, suggesting that the RSSs may have evolved independently (Cheng and Wang, 2017a). The RSSs can suppress the antiviral RNA silencing pathway by interfering with almost all steps. Many RSSs, such as cucumovirus 2b, tombusvirus P19, tenuivirus NS3 and potyvirus HC-Pro, suppress RNA silencing in the initial stage of infection by sequestering viral dsRNAs or siRNAs (Molnar et al., 2010; Hemmes et al., 2007; Havelda et al., 2005; Lakatos et al., 2006). Some other RSSs also inhibit the RNA silencing pathway by directly or indirectly sequestering some key players in RNA silencing including DCLs, AGOs, HEN1 and RDRs. Cauliflower mosaic virus P6 has been

reported to interact with dsRNA binding protein 4 to suppress the function of DCL4 (Haas et al., 2008), and potyvirus VPg, polerovirus P0 and potexvirus P25 interferes with host RNA silencing by destabilizing AGO proteins or SGS/RDR6 (Cheng and Wang, 2017b; Chiu et al., 2010; Hendelman et al., 2013). Some of RSSs, such as cucumoviral 2b and carmoviral P38, plays a role as an all-round player in suppression of RNA silencing by targeting multiple steps in the RNA silencing pathway (Csorba et al., 2015).

Except for their function of suppression of RNA silencing, RSSs also serve as a helicase, protease, coat protein, replicase, symptom determinant, helper component for vector transmission and so on to play important roles in viral infection and replication cycle (Csorba et al., 2015; Yang and Li., 2018; Li et al., 2018; Valli et al., 2018). Therefore, RSSs are generally a disease determinant factor in virus control.

CHAPTER I. Application of cucumber mosaic virus to efficient induction and long-term maintenance of virus-induced gene silencing in spinach

INTRODUCTION

Spinach (*Spinacia oleracea*) belong to *Chenopodioideae*, a subfamily of the family *Amaranthaceae*. It is a very important crop containing plenty of nutrients such as vitamins, minerals and several plant pigments such as carotenoids and flavonoids (Gil et al., 1999; Hedges and Lister, 2007; Lester et al., 2013), and for these reasons, it is grown over 50 countries (Fuentes-Bazan et al. 2012). It is generally considered as dioecious plants, although certain cultivars can develop both staminate and pistillate flowers, as a monoecious plant (Janick and Stevenson 1955b; Onodera et al. 2008). The alternativeness of dioecism and monoecism in spinach are used for generating commercial seeds by hybridization, and hence the comprehension of the mechanisms for determining sex expression is crucial for breeding of spinach (Janick 1998; Onodera et al. 2011). Spinach has served as a model plant to study sex determination and expression of plant as well as flavonoid biosynthesis and function of chloroplast (Beerhues and Wiermann 1988; Beerhues et al. 1988; Chailakhyan 1979; Ellis 1981; Janick and Stevenson 1954; Janick and Stevenson 1955a; Janick and Stevenson 1955b; Janick and Stevenson 1955c; Sherry et al. 1993; Shimada et al. 2004; Yamamoto et al. 2014). Recently, the draft genome sequences and transcriptome data of spinach have been released (Okazaki et al. 2019; Xu et al. 2015; Xu et al. 2017), which are valuable to identify the loci responsible for important agronomic traits such as sex determination and disease resistance, enhancing the worth of spinach as a potential model plant. Despite of accumulating information on the genomic organization, rapid and efficient system for functional genomics studies are still not well built up for spinach; although spinach transformation methods have been developed, they are based on callus-mediated regeneration systems, requiring much time and labor to obtain transformants (Chin et al. 2009; Nguyen et al. 2013).

Virus-induced gene silencing (VIGS) is one of the most widely used technique for studies of functional genomics especially in plant species to which conventional transgenic methods are hardly applicable. So far, a VIGS system has been produced based on two viruses, beet curly top virus (BCTV) (Golenberg et al. 2009; Sather et al. 2010) and tobacco rattle virus (TRV) (Lee et al. 2017). Although both VIGS systems based on BCTV and TRV have been demonstrated to be efficient in silencing several spinach genes, there are some limitations. In the BCTV VIGS system, the virus could not systemically spread in spinach plants when homeotic transcription factor genes, *ribulose bisphosphate carboxylase small subunit* and *transketolase* were targeted (Golenberg et al. 2009). For the TRV VIGS system, when the *phytoene desaturase* (*PDS*) gene was targeted, the chlorosis phenotype gradually became disappeared one month later due to the decrease in viral accumulation (Lee et al. 2017). Therefore, it is better to establish an additional VIGS system in spinach with its full potential, because for several plants, such as legume and lily, systemic infection is absolutely required to efficiently induce VIGS (Pflieger et al. 2013; Tasaki et al. 2016). In addition, it is better to get more options of reverse genetics tool because the viral vectors may show differential behavior in their replication, cell to cell or long-distant movement and symptom development depending on spinach cultivars with diverse genotypes. Accordingly, I have developed the cucumber mosaic virus (CMV) vector as another option of convenient VIGS system for spinach.

CMV has a broad host range with 1200 plant species in over 100 families, and hence it has been exploited as a useful VIGS vector for many plant species including *Arabidopsis thaliana*, banana, lily, maize, *Nicotiana benthamiana*, pepper, soybean and tomato (Hong et al. 2012; Liu et al. 2010; Otagaki et al. 2006; Tzean et al. 2019; Wang et al. 2016). Recently, it has been demonstrated that CMV VIGS is able to silence even a gene of mycorrhizal fungi which

colonized the root of *N. benthamiana* (Kikuchi et al. 2016). In this study, I used an optimized pseudorecombinant for VIGS in spinach by mixing genomic RNAs of two CMV strains. Because CMV can systemically infect spinach, and the infection could be maintained until the flowering stage, I expect that our CMV VIGS system contributes to elucidate some spinach-specific traits such as plant's sex expression.

MATERIALS AND METHODS

Plant materials and viral inocula

The seeds of several spinach cultivars were purchased in the market: 'Akajiku-Salad' (Musashino-Shubyo, Tokyo, Japan), 'Akakuki-Minster' (Nakahara-Saishujyo, Fukuoka, Japan), 'All Right' (TAKII & CO., LTD., Kyoto, Japan), 'Kuroshio' (Kobayashi-Shubyo, Hyogo, Japan), 'Miyabi' (TOHOKU, Utsunomiya, Japan), 'Surprise' (TOHOKU SEED Co. LTD., Utsunomiya, Japan) and 'Wase-Salad-Akari' (TAKII, Kyoto, Japan). *N. benthamiana* plants were grown at 24 ℃ in a 16 h/8 h day and night period, and spinach plants were raised at 22 ℃ growth chamber in a condition of 12 h light and 12 h dark. Transcripts of CMV RNA 1, 3 and the vector RNA 2 were *in vitro* synthesized by using T7 RNA polymerase (Takara) with 5´ capping analogue. The *in vitro* transcripts of CMV RNA 1–3, which are infectious when co-inoculated, were first inoculated onto *N. benthamiana* plants for propagation of viral vectors, and then the infected *N. benthamiana* leaf sap was inoculated onto spinach plants.

Construction of VIGS vectors

Full-length cDNA clones of three genomic RNAs of two CMV strains (CMV-Y and CMV-L) have been constructed into the pUC plasmid vectors (Suzuki et al., 1991; Otagaki et al., 2006); the three genomic RNAs of CMV-Y and CMV-L were named as Y1–Y3 and L1–L3. The viral vector A1, which has the cloning site between StuI and MluI restriction enzyme sites was constructed based on RNA 2 of CMV-Y (Figure 2). For construction of the VIGS vector

for two spinach genes, *PDS* and *dihydroflavonol 4-reductase* (*DFR*), the partial sequences of the genes were amplified by RT-PCR. Total RNA was extracted from spinach leaf using Trizol (Roche) and its cDNA was synthesized by using Reverse Transcriptase XL (AMV) (Takara). The partial sequences of *PDS* and *DFR* genes were amplified by RT-PCR using Ex-Taq (Takara) with Spi-PDS-5-Ml2/Spi-PDS-3-St2 and Sp-DFR-5-2-Ml/Sp-DFR-3-2-St primer pairs, respectively (Table 1). The VIGS vectors were generated by directly inserting the PCR amplicons into the CMV-A1 vector, and the correct sequences of inserts were confirmed by sequencing.

Table 1. The list of primers used for construction of CMV VIGS clones and real-time RT-PCR.

Primer	Primer sequence (5´–3´)	Primer
Spi-PDS-5-Ml2	CGCACGCGTCTTATGACCATCTACTATTC	Construction of CMV-A1-PDS clone
Spi-PDS-3-St2	CGAGGCCTGATCCATTCTTCTGCAGGTGCA	
Sp-DFR-5-2-Ml	CGCACGCGTCAAGGACTTCGAAGAGGATATG	Construction of CMV-A1-DFR clone
Sp-DFR-3-2-St	CGAGGCCTGACCTTTGTTCTGCAAGTATCAAC	
Sp-PDS-5-100	CAAGCCTGGTGAATTTAGCC	Real-time RT-PCR for PDS
Sp-PDS-3-100	GCAGGCAAGAGACCAATAGC	
Sp-DFR-5-1	GGCTCATGGCTTGTCAAGAGG	Reat-time RT-PCR for DFR
Sp-DFR-3-1	CATAGTGTCAAGTGAGTGTTTG	
CMV-DET-5-340	GTTGACGTCGAGCACCAACGC	Real-time RT-PCR for CMV
CMV-DET-3-340	TGGTCTCCTTTTGGAGGCCC	
Sp-GAPDH-5	TGTGTGCGACATCCCCCTCGTGTCA	Real-time RT-PCR for reference
Sp-GAPDH-3	CCACCCGTTGGCTGTAACCCCACTCAT	
2b-5-up	GTACAGAGTTCAGGGTTGAGCG	RT-PCR for insert check
R2-2814-R2	AGCAATACTGCCAACTCAGCTCC	

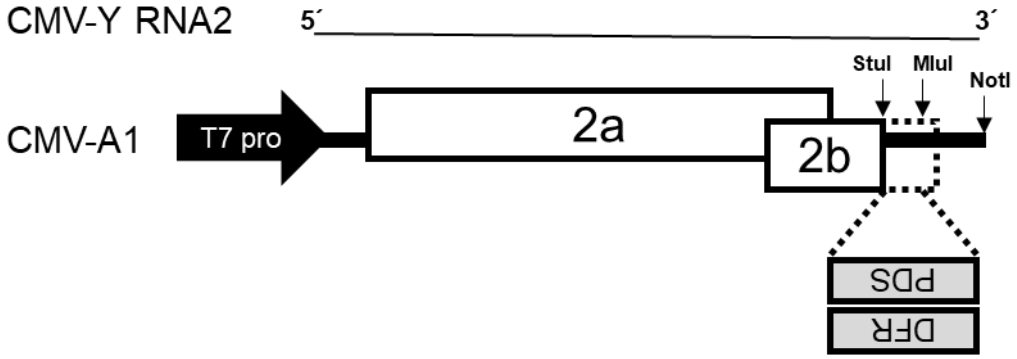

Figure 2 Construction of CMV VIGS vector. Simplified scheme of the CMV vector A1 (CMV-A1), whose backbone construct is CMV RNA 2 including the 2a and 2b open reading frames (ORFs). The partial sequences of spinach genes (*PDS* and *DFR*) were inserted into the A1 vector using StuI and MluI restriction enzyme sites in the antisense orientation.

Real-time RT-PCR

Total RNAs of leaves in virus-infected spinach plants were extracted using Trizol (Roche), and then DNase I (Takara) was treated for removal of DNA contaminants. cDNAs from the RNA extracts were synthesized by reverse transcription using Reverse Transcriptase XL (AMV) (Takara), and then these were used for real-time RT-PCRs with Powerup SYBR master mix (Applied Biosystems) and the StepOnePlus real-time PCR apparatus (Applied Biosystems). CMV-DET-5 and CMV-DET-3 primer pair was used to measure the CMV viral titer, and Sp-PDS-5-100/Sp-PDS-3-100 and Sp-DFR-5-1 and Sp-DFR-3-1 primer pairs were used for quantifying the mRNA levels of *PDS* and *DFR* genes, respectively (Table 1). Sp-GAPDH-5/Sp-GAPDH-3 primer pair was used as a reference. Statistical analysis was performed by Tukey's multiple test with significance $*P < 0.05$.

RESULTS

Construction of CMV VIGS vectors using a pseudorecombinant

As a preliminary experiment, I inoculated a combination of CMV Y1A1Y3 by inoculating Y1, A1 and Y3 transcripts together, and a pseudorecomninant, L1A1L3 with L1, A1 and L3 RNAs onto several spinach cultivars (Figure 3). At 10 to 14 days postinoculation (dpi), CMV Y1A1Y3-inoculated spinach plants showed severe mosaic symptoms although the symptom severity varied depending on the tested cultivars. Meanwhile, the spinach plants infected with a pseudorecombinant virus, CMV L1A1L3, induced relatively mild symptoms (Figure 3). Because severe symptoms can make the silencing phenotype hard to distinguish, the attenuated symptom induction may be advantageous for a VIGS vector.

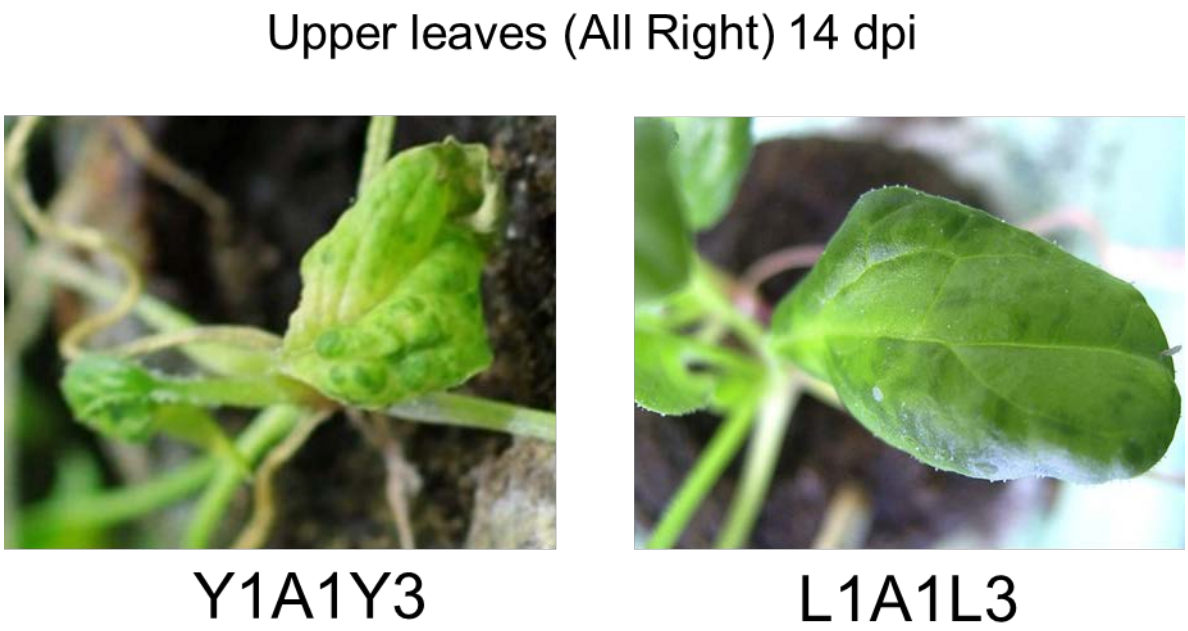

Figure 3 Construction of the pseudorecombinant CMV vector for VIGS. Spinach plants cultivar 'All Right' were inoculated with the CMV-Y vector derivatives Y1A1Y3 (left) and the pseudorecombinant vector L1A1L3 (right). The symptoms were compared on the upper systemically infected leaves at 14 dpi.

Silencing of spinach PDS using CMV VIGS

To validate CMV-based VIGS system in spinach, partial *PDS* gene sequence was cloned into the A1 vector in the reverse orientation to create A1-PDS (Figure 2). *In vitro* transcripts of CMV RNAs, L1, A1-PDS and L3, were inoculated onto *N. benthamiana*, and subsequently the sap of infected *N. benthamiana* leaves was mechanically inoculated onto two spinach cultivars (‘All Right’ and ‘Surprise’), which showed different susceptibility to L1A1L3. At 7 to 10 dpi, distinct chlorosis phenotype was observed along the veins of systemically infected leaves (Figure 4), and the photobleaching lasted over two months (Figure 5). To further confirm the phenotype in molecular level, the *PDS* mRNA levels were quantified by real-time RT-PCR. As expected, the *PDS* mRNA levels were significantly downregulated to less than 1/4 of the *PDS* expression of the healthy plants at 10 dpi (Figure 4). There was little difference in photobleaching phenotype between the two spinach cultivars when the vector contains insert sequence, suggesting that the introduction of an insert fragment into the vector may somehow affect the viral pathogenicity. However, the virus accumulation level in cultivar ‘Surprise’ was about 1/200 of the control, L1A1L3, while about 1/5 in cultivar ‘All Right’, suggesting that the viral titers are different in cultivars and that such a low level of virus accumulation can still efficiently induce VIGS against *PDS* (Figure 6).

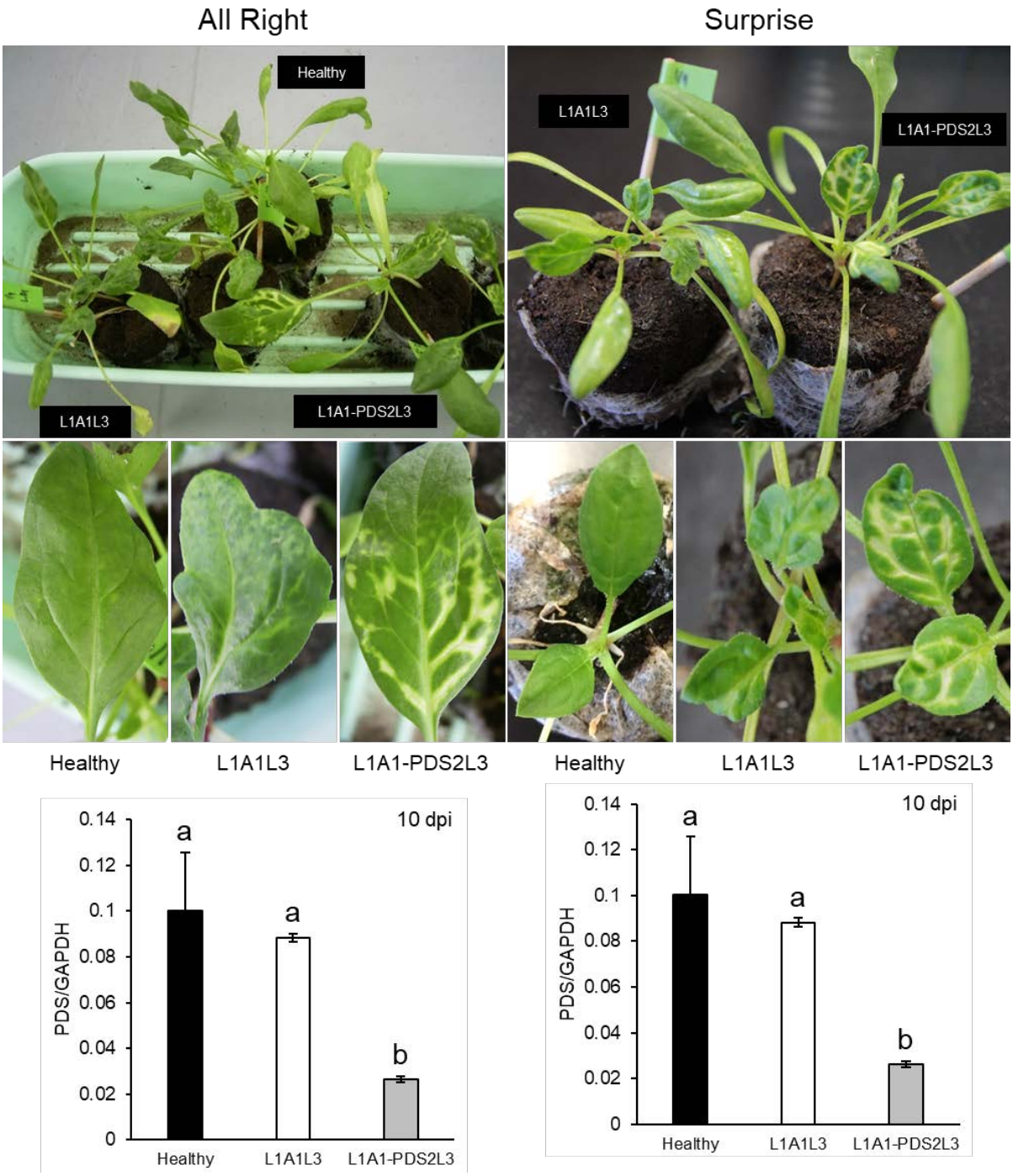

Figure 4 CMV VIGS for the *PDS* gene silencing in two spinach cultivars 'All Right' and 'Surprise'. Photobleaching phenotype was observed on the systemic leaves of L1A1-PDS2L3-infected plants at 10 dpi. *PDS* expression levels were compared by real-time RT-PCR (right-top) using upper systemic leaves at 10 dpi. The sequence *glyceraldehyde 3-phophate*

dehydrogenase (*GAPDH*) gene was amplified as a reference gene. Statistical analysis was conducted by Tukey's multiple comparison test (*P < 0.05); different letters above each bar indicates a significant difference among isolates.

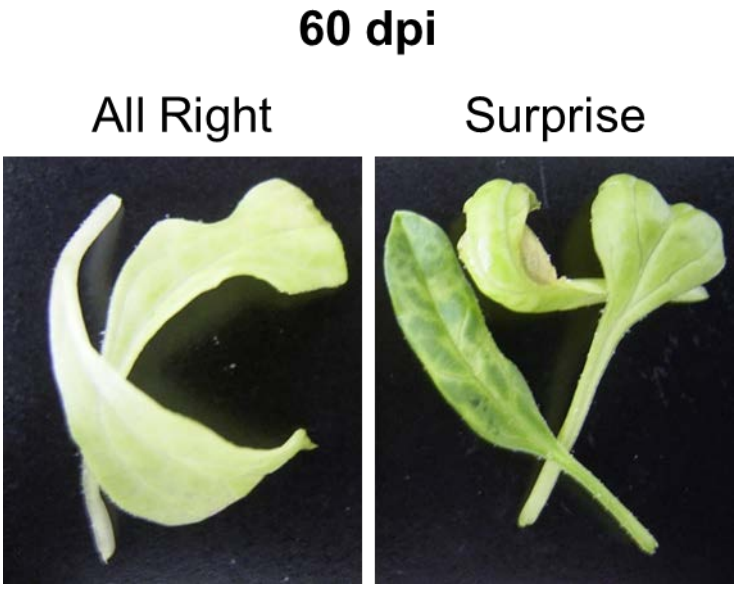

Figure 5 PDS-silenced photobleaching phenotype on the leaves of spinach at 60 dpi. The spinach cultivars ‘All Right’ and ‘Surprise’ were inoculated with CMV L1A1-PDS2L3. The picture of the systemic leaves was taken at 60 dpi.

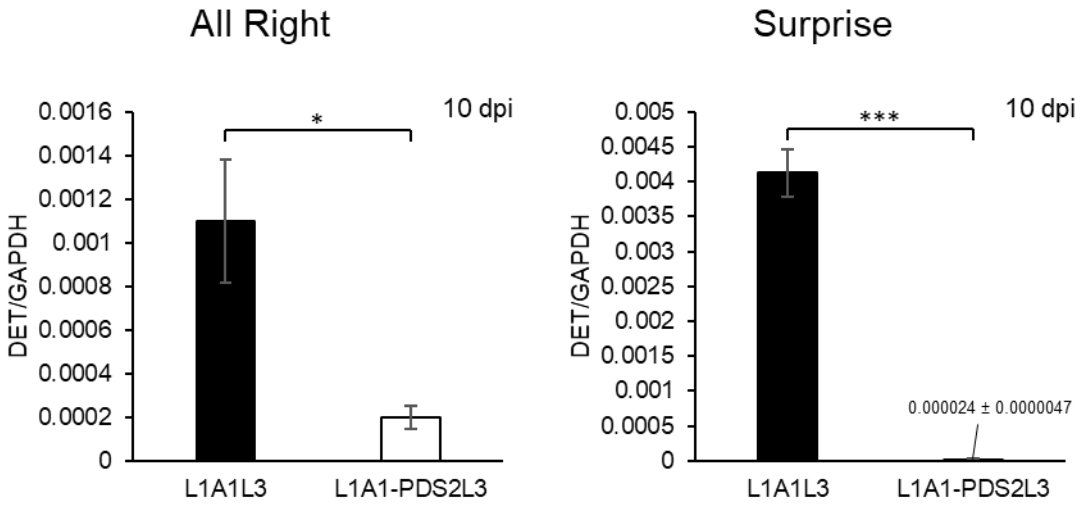

Figure 6 Real-time RT-PCR for quantification of the CMV viral accumulation levels. Significant difference were analyzed by Student's t-test (*$P < 0.05$, ***$P < 0.001$).

Silencing of spinach DFR using CMV VIGS

To further test the usefulness of CMV VIGS system using another spinach gene, I selected the *DFR* gene, which is a key gene for anthocyanin synthesis pathway (Shimada et al. 2004). Generally, green spinach cultivars synthesize betacyanins instead of anthocyanin, but it has been reported that several red-purple spinach cultivars produced anthocyanin in leaves and stem (Cai et al. 2018). I cloned the 130-nt of partial *DFR* gene sequence into the A1 vector in the reverse orientation to create A1-DFR, and induced VIGS by inoculating A1-DFR together with L1 and L3 onto several red-purple spinach cultivars. The inoculated plants did not show severe symptoms but very mild symptoms were detected in the systemically infected leaves (Figure 7). Although the red pigmentation was not diminished in most of the tested spinach cultivars by infection of A1-DFR VIGS, I observed decoloration in the stem of a spinach cultivar 'Kuroshio' (Figure 8), suggesting that the cultivar may produce anthocyanins, considering that anthycyanins and betacyanins are never synthesized together in same plant (Shimida et al., 2004), and that the *DFR* gene play a role as key gene for the anthocyanin synthesis pathway. In addition, *DFR* mRNA levels were measured to compare the *DFR* expression by real-time RT-PCR, and the A1-DFR-infected plants showed a half of the *DFR* expression in the healthy plants at 10 dpi, even though the empty vector (L1A1L3) infection strongly induced *DFR* expression (Figure 8). Real-time RT-PCR for CMV showed that the viral accumulation in L1A1-DFR2L3-infected plants were about 1/1000 of the empty vector control (L1A1L3), suggesting that the high level of CMV accumulation may promote *DFR* expression (Figures 8 and 9). Additionally, to determine whether L1A1-DFR2L3 loses the insert in systemically infected upper leaves, I checked the leaves just beneath the flowers of the infected plants at 60 dpi. The RT-PCR result showed that the virus (L1A1-DFR2L3) still retained the

complete insert fragment (Figure 10), and that the virus accumulation level was about 1/5 of the empty vector control (L1A1L3) (Figure 9), suggesting that the virus with the insert can maintain systemic infection even after 8 weeks postinoculation. These results also demonstrate that developed CMV VIGS system can be used for study of functional genomics in spinach without severe symptoms.

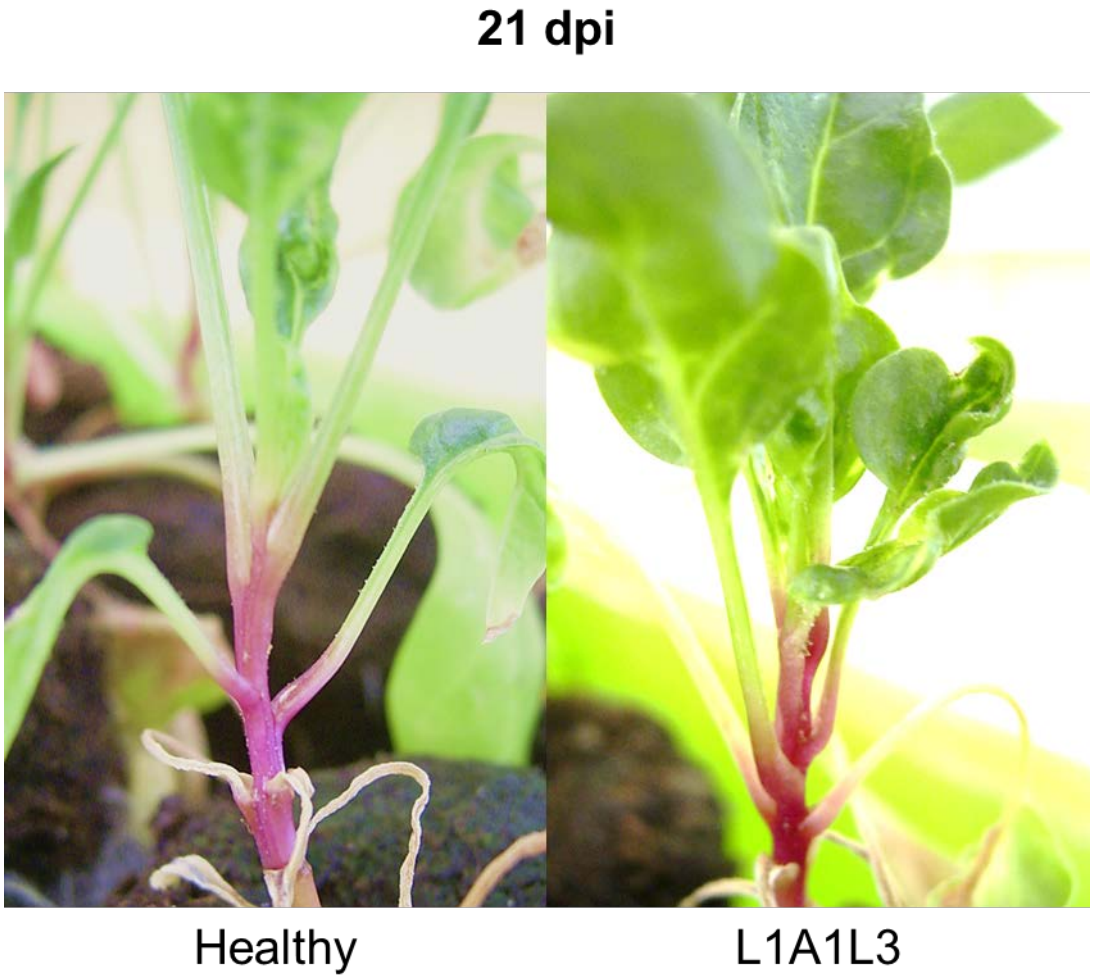

Figure 7 Symptom observation of spinach cultivar ‘Kuroshio’ inoculated the CMV VIGS vector L1A1L3 at 21 dpi. Distinct symptoms were little observed on the inoculated plants.

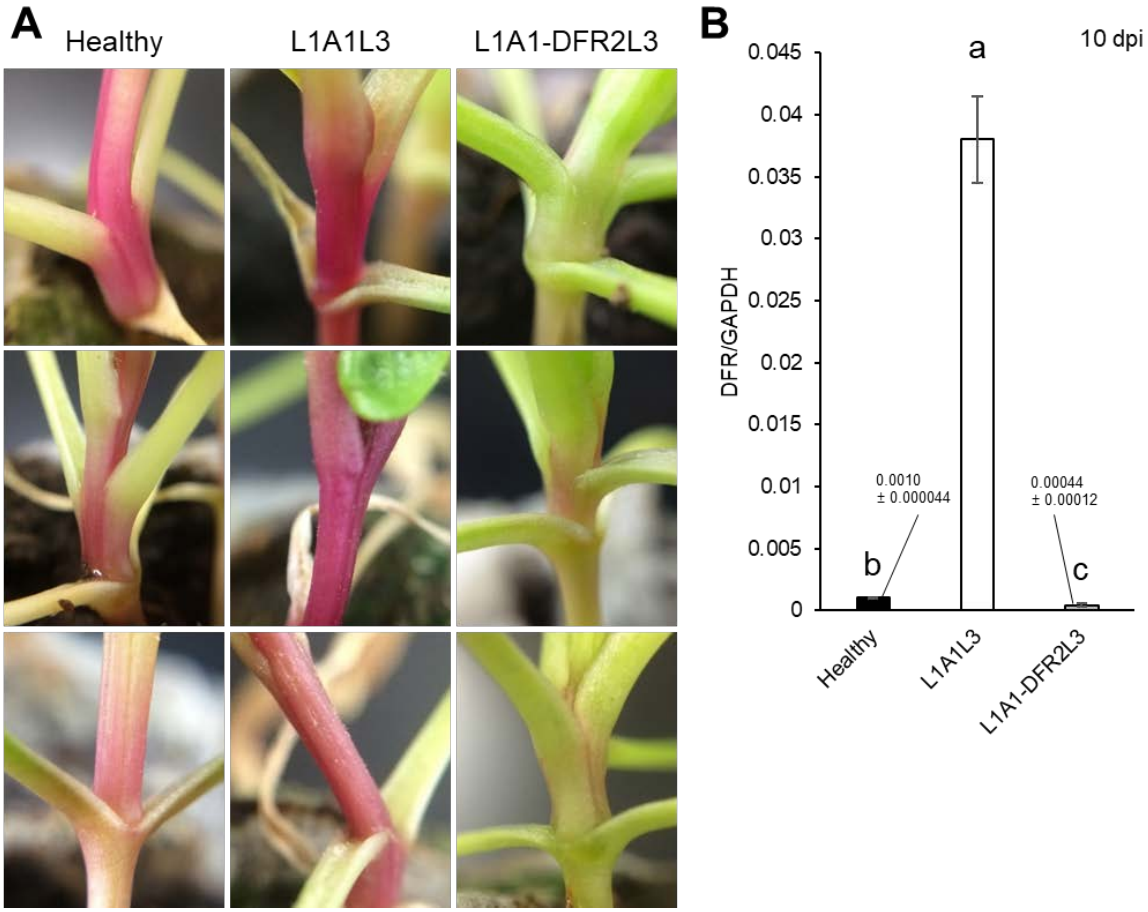

Figure 8 CMV VIGS against the *DFR* gene in the spinach cultivar 'Kuroshio'. (A) Decoloration phenotype was observed in the stem of spinach plants inoculated with the L1A1-DFR2L3 vector at 10 dpi. (B) Real-time RT-PCR was performed to quantify *DFR* mRNA levels in the upper systemically infected leaves at 10 dpi. The *GAPDH* gene was used for a reference gene. Mean values (±SE) among the healthy, L1A1L3-infected and L1A1-DFR2L3-infected plants were analyzed for significant differences by Tukey's multiple comparison test (*P < 0.05). The different letters above the bars represent a significant difference among the groups.

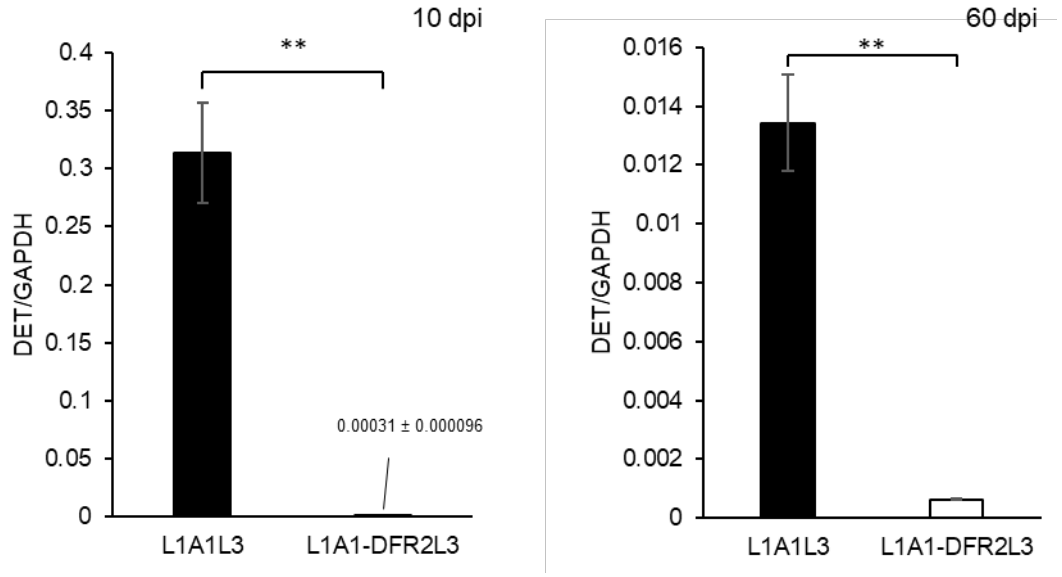

Figure 9 Real-time RT-PCR for quantification of the CMV accumulation levels. For real-time PCR at 10 dpi, the same leaves used for real-time PCR of the *DFR* gene were analyzed. At 60 dpi, leaves just beneath the bolted flowers were analyzed. Statistical analysis was performed by Student's t-test (**$P < 0.01$).

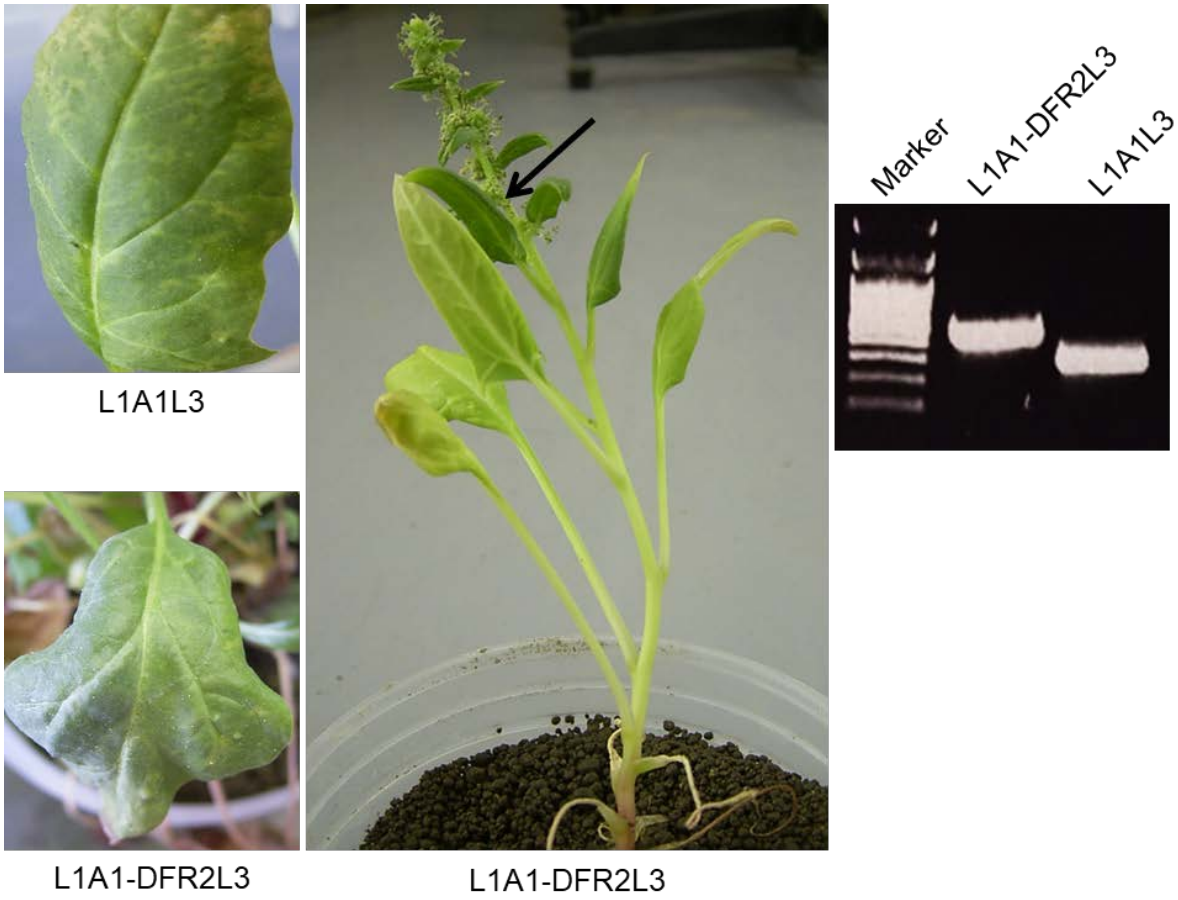

Figure 10 Virus detection in the leaves just beneath the flowers at 2 months postinoculation in the infected spinach plants. Symptoms on the systemically infected leaves just beneath the bolted flowers were little observed in L1A1L3- and L1A1-DFR2L3-infected plants. The arrow indicates the position of leaf used for RT-PCR to amplify the 2b gene (right-top). 100-bp ladder was used as a size marker.

DISCUSSIONS

Because CMV has extremely a wide host range, the CMV VIGS vector can be used for various plant species or cultivars for functional studies of the plant genes. In addition, various CMV strains adapting to many plant species have been already characterized (Roossinck, 2002). Moreover, by simply exchanging RNAs, CMV pseudorecombinant viruses are easily constructed. In this study, I developed a pseudorecombinant CMV VIGS system which is superior for some spinach cultivars in that it can systemically infect spinach plants with silencing phenotypes (*PDS* and *DFR*) and that its infection can be maintained more than 2 months for flowering stage. Therefore, such a pseudorecombinant virus may work even when the two BCTV and TRV VIGS systems are not available or do not provide clear silencing phenotypes because the successful CMV construct can systemically infect various spinach cultivars and efficiently accumulate in infected plants until the flowering stage. Because our laboratory keeps several CMV strains isolated directly from spinach, and moreover, many CMV genomic RNA sequences are available in GenBank, those viral RNAs can be used to adjust the viral pathogenicity or symptoms, and broaden host range. For example, even though the initial vector construct does not efficiently work on a cultivar of interest due to a resistant gene, other pseudorecombinant viruses using CMV spinach strains which can overcome the resistance will be useful.

CHAPTER II. Helper component proteinase of onion yellow dwarf virus isolated from Japanese garlic lost a long stretch of amino acids at the N-terminal region affecting RNA silencing suppressor activity

INTRODUCTION

Onion yellow dwarf virus (OYDV), a garlic infecting *Potyvirus*, is one of the most widely dispersed virus in *Allium* species such as garlic, leek and onion plants. OYDV-infected garlic plants show irregular yellow-striped and curled leaves with a dwarfed stature (Arya et al., 2006; Manglli et al., 2014). OYDV has occurred worldwide in many countries with high incidence (Dovas et al., 2001; Conci et al., 2003; El-Wahab et al., 2009; Fayad-André et al., 2011; Kumar et al., 2010; Mohammed et al., 2013). It has been reported that the virus is transmitted in a non-persistent manner by more than 50 aphid species (Drake et al., 1933). Several studies on OYDV-infected garlic or other *Allium* spp. showed that the virus had been frequently detected in mixed infections with other plant viruses of *Potyvirus*, *Carlavirus* and *Allexivirus* (Fajardo et al., 2001; Fayad-André et al., 2011; Bagi et al., 2012; Mohammed et al., 2013). Like other potyviruses, the genome of OYDV consists of a large genomic RNA (~ 10,000 nucleotides) encoding a large polyprotein, which is processed into ten viral proteins: P1, helper component protease (HC-Pro), P3, 6 K1, CI, 6 K2, VPg, NIa-Pro, NIb and CP (Verma et al., 2015). Additionally, PIPO, which is a small protein embedded in the P3-coding sequence, is translated from the second short open reading frame (Chung et al., 2008; Manglli et al., 2014).

The potyviral HC-Pro protein is a multifunctional protein which plays key roles in viral replication cycle such as autoproteolytic cleavage of viral polyprotein, aphid transmission, long-distance movement, genome replication and suppression of RNA silencing (Valli et al., 2018). Several loss-of-function studies have been conducted to map crucial domains or motifs for HC-Pro functions. The conserved KITC motif in the N-terminal region has been reported to be important for aphid transmission (Blanc et al., 1998). The FRNK motif, which is located in the central region, in zucchini yellow mosaic virus (ZYMV) HC-Pro has been found to be

required for sRNA binding and symptom development (Shibolath et al., 2007; Wu et al., 2010a). The IGN and CCC motifs on the central region of tobacco etch virus (TEV) HC-Pro are responsible for viral systemic movement (Cronin et al., 1995). The C-terminal region of ZYMV HC-Pro plays a role in protease and includes the PTK motif, which is also required for aphid transmission by providing an epitope for the interaction with the DAG motif of the coat protein (CP) (Peng et al., 1998).

In Japan, the Aomori prefecture is the main garlic-producing region, but several local varieties have also been cultivated in Hokkaido (Yoshida et al., 2012). To protect the garlic cultivation from viral infection, biological controls using attenuated viral strains have been proposed and attempted for cross protection. The group of Takaki et al. (2005) characterized the severe and attenuated strains of leek yellow stripe virus (LYSV) by complete nucleotide sequencing. This group also isolated a mild OYDV isolate G79, which lacks the N-terminal 92 amino acid in its HC-Pro (Takaki et al., 2006). The effect of N-terminal deletion on the functions of potyviral HC-Pro have been described in several literatures. The deletion of ~100 amino acids in the N-terminal region of lettuce mosaic virus HC-Pro affected its viral aphid transmissibility (Plisson et al., 2003). For TEV, the deletion of the N-terminal domain of HC-Pro showed a reduced level of viral accumulation, and the TEV containing the N-terminal truncated HC-Pro was not transmitted by aphids (Dolja et al., 1993). Similarly, the 75 amino acids deletion in the N-terminal region of tobacco vein mottle virus HC-Pro revealed totally impaired infection, and with point mutations on the KITC motif, its aphid transmission was abolished (Atreya and Pirone, 1993).

In this study, I sequenced the HC-Pro genes from several Japanese OYDV isolates, and found that all of the tested HC-Pro genes from the Japanese OYDV isolates lack ~100 amino acids in

their N-terminal region. Furthermore, by a novel assay for the suppression of GFP silencing using onion epidermis, I examined the suppression of RNA silencing in onion, which is a natural host plant for OYDV. Finally, I also analyzed the structures of HC-Pro transcripts and proteins for the possible explanation of the evolutionary history of OYDV HC-Pro. These findings propose a model of template-switching for the deletion of the N-terminal region of OYDV HC-Pro.

MATERIALS AND METHODS

Plant materials

Garlic samples (leaves or bulbs) were collected from the fields of Hokkaido and other Japanese local regions, and garlics from America, China and Spain were purchased at the market. *Nicotiana benthamiana* plants were grown at 25 °C in a condition of 16 h/8 h day and night period, and used for agroinfiltration. Onion bulbs from Hokkaido were also purchased at the market.

Total RNA extraction and RT-PCR

Garlic bulbs and leaves were ground in the RNA extraction buffer (25 mM KCl, 25 mM $MgCl_2$, 1% SDS and 25 mM Tris-HCl pH 7.5), and the total RNAs were isolated by the conventional phenol–chloroform method. The total RNA extracts were used as a template for cDNA synthesis using AMV reverse transcriptase (Nippon gene), and RT-PCR was conducted using Ex Taq (Takara) and OYDV-HC5-450/OYDV-HC3-1400 primer pair to amplify the HC-Pro genes (Table 2). The PCR products were separated by electrophoresis in a 1% agarose gel.

Table 2. List of primers used for amplification of HC-Pro genes and real-time RT-PCR

Primer	Primer sequence (5´–3´)	PCRs
OYDV-HC5-450	TAAATCCYGACAAYATAATYGG	RT-PCR amplification of HC-Pro genes
OYDV-HC3-1400	ACCTACATTRTAGTYTTTCATTTC	
S65T-5-168	TCACGGCAGACAAACAAAAG	Real-time RT-PCR for GFP
S65T-3-168	AAAGGGCAGATTGTGTGGAC	
Nb-L23-5-110	AAGGATGCCGTGAAGAAGATGT	Real-time RT-PCR for L23
Nb-L23-3-110	GCATCGTAGTCAGGAGTCAACC	

DNA sequencing for multiple alignment and phylogenetic analysis

The amplified PCR products of OYDV HC-Pros were purified by using QIAEX II gel extraction kit (Qiagen), and then cloned into the pGEM-T easy vector plasmid (Promega) according to the manufacturer's instruction. The plasmids containing the HC-Pro genes were sequenced in the two directions with M13-F and M13-R primers. The sequencing data and the HC-Pro sequences registered in GenBank were used for multiple-alignment by the MEGA v7 program; some places were adjusted manually if necessary. Phylogenetic analysis was conducted by the maximum likelihood method based on the amino acid or nucleotide sequence of HC-Pro with 1,000 bootstraps using the MAGA v7 program.

Analysis of RNA silencing suppression

The RSS activity of the HC-Pro proteins was examined in both *N. benthamiana* and onion plants. The expression vectors of the GFP and HC-Pro genes were constructed under the 35S promoter by cloning into the pBE2113 binary vector between the SacI and XbaI restriction enzyme sites. *Agrobacterium tumefaciens* strain KYRT1 was transformed with the recombinant expression vectors by the conventional freeze-thaw method. The *Agrobacterium* transformants were cultured in the medium (YEP, 1% yeast extract, 1% peptone and 0.5% NaCl) as a seed culture, and then the cells were re-cultured in a new YEP medium containing 10 μM of acetosyringone. The harvested cells were then resuspended in the buffer (10 mM $MgCl_2$, 10 mM MES and 0.2 mM acetosyringone), and the concentration of the resuspension was adjusted to $O.D_{600}$ of 1.0 for *N. benthamiana* and 0.2 for onion. The inocula were prepared by mixing 35S:GFP and 35S:HC-Pro in the 1:2 ratio for *N. benthamiana* and the 1:1 ratio for onion, and

then incubated for ~2 hour in the room temperature. The inocula were infiltrated into *N. benthamiana* leaves using 1-ml syringe without a needle. For infiltration into onion bulbs, 1-ml syringes with a needle were used. The GFP signals were observed at 5 days postinfiltration (dpi) for *N. benthamiana*. For the onion bulbs, the GFP was observed using epifluorescence microscopy (Leica DMI 6000B, Leica) at 3 dpi. To quantify the GFP expression in onion cells, the intensity of GFP signals were measured using the LAS AF program.

Real-time RT-PCR

Total RNAs were isolated from the infiltration patches of *N. benthamiana* leaves at 5 dpi, and then DNase I was treated to remove DNA contamination. Reverse transcription for the cDNA synthesis was performed using the PrimeScript RT reagent kit (Takara), and then real-time RT-PCR was conducted with Powerup SYBR green master mix (Applied Biosystems). S65T-5-168/S65T-3-168 primer pair was used for quantifying mRNA of GFP, and the primer pair of Nb-L23-5/Nb-L23-3 was used for a reference (Table 2). Statistical analysis was performed by Tukey's multiple test with $P < 0.05$ level.

Western blot analysis

Total proteins were extracted with the PBS-T buffer from the infiltration patches in *N. benthamiana* leaves at 5 dpi, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 14% polyacrylamide gel. The GFP was detected by western blotting using anti-GFP antibodies. The expression levels of GFP were estimated with the Multi Gauge

program.

Structure prediction of the HC-Pro transcripts and peptides

To predict the two-dimensional structures of the deleted region of the HC-Pro RNAs, the partial RNA sequences of HC-Pro genes were analyzed by mfold (http://unafold.rna.albany.edu/?q=mfold). The *ab initio* 3D structures of HC-Pros were predicted using the I-TASSER server v5.0 (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The protein data bank files were provided from the I-TASSER server, and the 3D structures were visualized with the UCSF Chimera v1.9 program.

Production of virus-free garlic plants

Original garlic bulbs were purchased at the market. The meristems of garlic cloves were excised and cultured on the Murashige and Skoog medium (MS medium). Viral dsRNAs were synthesized from the PCR products containing a viral sequence with the T7 promoter sequence at both ends by using T7 RNA polymerase, and then processed with RNase III (Promega) to produce siRNAs according to the manufacturer's protocol. One μg of the purified siRNAs was added to the excised garlic meristems, kept for 10 min, and the siRNA-treated meristem tissues were transferred to new MS medium plate. The tissues were kept in a 24 ℃ growth chamber with a 16 hrs/8 hrs day and night period. Overall steps are explained in Figure 1.

RESULTS

N-terminal deletion of HC-Pro in newly isolated Japanese OYDV strains

The study conducted by Takaki et al. (2006) reported that one of the OYDV isolates identified in Aomori Prefecture lacked the N-terminal region of HC-Pro, and that such an isolate was a spontaneous mutant and a rare case. In this study, I initially performed RT-PCRs with the primer pair to detect the HC-Pro full-length ORF, but failed to amplify any PCR products. On the other hand, relatively shorter bands than expected was successfully amplified using another 5' end primer, which hybridizes the upstream region of the HC-Pro gene (Figure 11), suggesting that the HC-Pro genes of all the tested Japanese OYDV isolates lacked the 5' end region. Furthermore, the HC-Pro PCR products from China and all the Japanese garlic samples were ~300-nt smaller than those from America and Spain samples. To confirm the results of RT-PCR and compare the HC-Pro sequences of OYDV isolates from different geographic regions, I performed a sequencing analysis of the HC-Pro genes. All the ten PCR products from the six Japanese local regions, China, America and Spain garlic plants were sequenced, and all the HC-Pro sequences, which are registered in Genbank (AB219833, AB219834, AJ510223, NC005029, JX429964, HQ258894, KJ451436, KF632714, JX433019 and JX433020), were multiple-aligned. Multiple alignment showed that the HC-Pros of China and all the seven Japanese OYDV isolates lacked the initial ~300-nt sequence (~100 amino acids) in the N-terminal region (Figures 12 and 13). In particular, the amino acid sequences of the HC-Pro proteins from Iwate, Aomori1 and Tokoro had several unique amino acid residues (Figure 12, residue 3–9, 189 and 313).

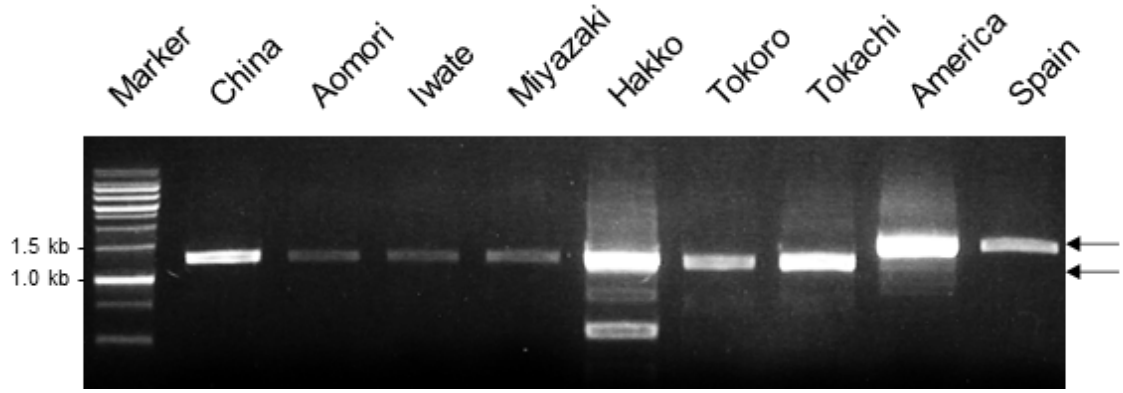

Figure 11 N-terminal deletion of HC-Pros of OYDV Japanese isolates. RT-PCR was conducted to compare the OYDV HC-Pro genes from different regions. OYDV-HC5-450/OYDV-HC3-1400 primer pair was used for detection. Arrows on the right side represent the amplified HC-Pro products.

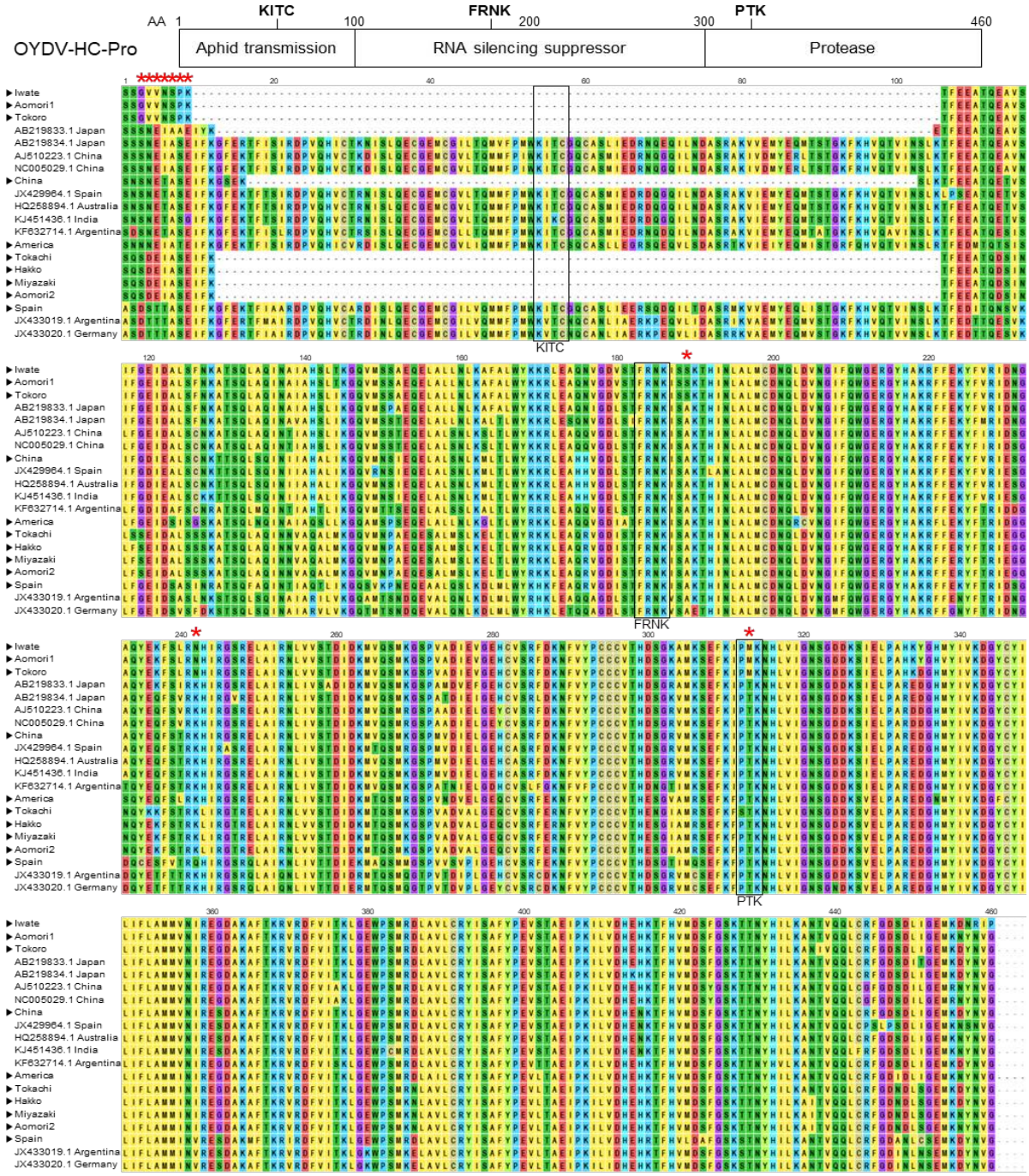

Figure 12 Multiple alignment of OYDV HC-Pro peptide sequences. Schematic structure of OYDV HC-Pro represents the putative functional motifs of amino acids for aphid transmission (KITC), suppression of RNA silencing (FRNK) and coat protein binding (PTK). The alignment was performed using the MEGA v7.0 program. Arrowheads on the left side indicate the OYDV

HC-Pro sequences analyzed in this study. OYDV HC-Pro sequences of GenBank used in this study are as follows: HQ258894, JX429964, KJ451436, AJ510223, KF632714, NC005029, JX433019, JX433020, AB219834 and AB219833. Red asterisks indicate the residues unique in the Iwate, Aomori1 and Tokoro isolates.

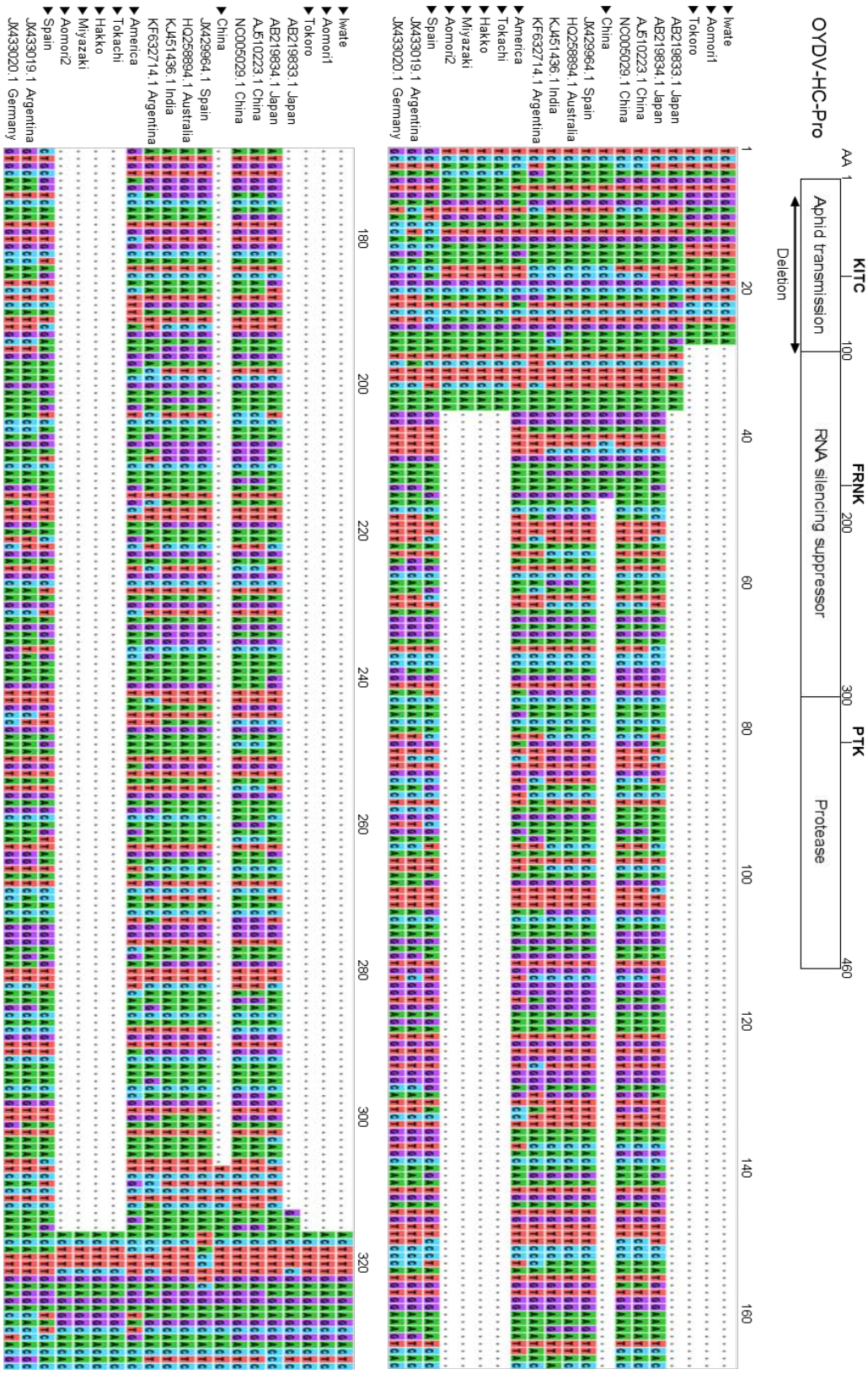

Figure 13 Multiple alignment of the N-terminal region of the OYDV HC-Pro nucleotide

sequences. The alignment was created using the MEGA v7.0. Arrowheads on the left indicate the OYDV HC-Pro genes sequenced in this study. All the ten OYDV HC-Pro sequences in the NCBI database were included in the analysis.

Phylogenetic analysis of OYDV isolates based on HC-Pro

For comprehension of the phylogenetic relationship of the obtained short-type OYDV HC-Pros to the previously reported sequences, a phylogenetic analysis of OYDV-HC-Pro was conducted based on the amino acid sequence (Figure 14). Ten HC-Pro sequences obtained in this study and another ten sequences from GenBank were used. As an outgroup control, HC-Pro of shallot yellow stripe virus (SYSV, GenBank: AJ865076) was included in the phylogenetic analysis. The phylogenetic tree divided the OYDV isolates into three major groups (Group I–III). The HC-Pros with the deletion in their N-terminal region seem to have been originated from a single ancestor containing a long type HC-Pro, even though at least two ancestors for each of Group I and II are assumed in this tree, implying that all the short type isolates may not necessarily share a common ancestor. For the clade of Group II, four Japanese HC-Pros shared a common ancestor, America, a long type HC-Pro. Considering that Aomori1 and Aomori2 isolated from the same garlic bulb are divided into two different groups, the topology of the phylogenetic tree reflects neither the geographic divergence in the origin of virus nor host cultivar difference.

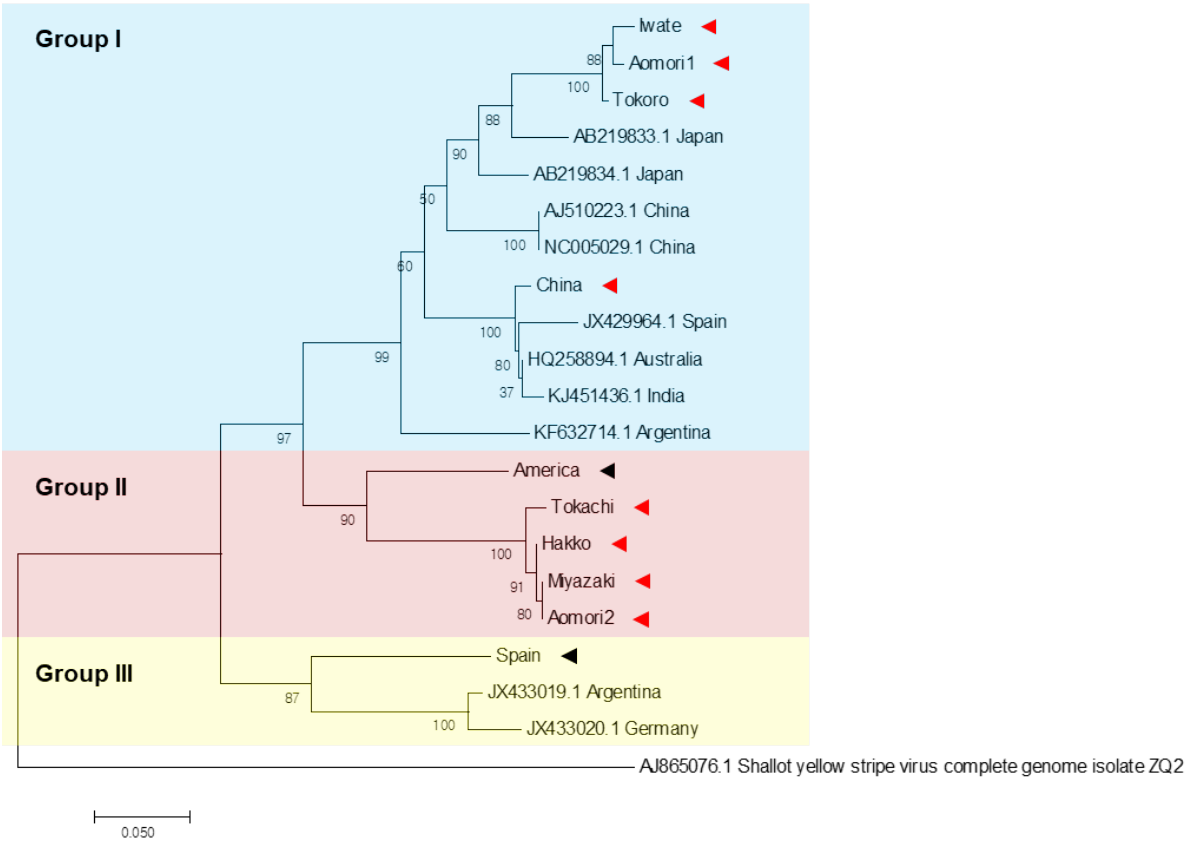

Figure 14 Phylogenetic analysis of OYDV HC-Pro at the level of amino acid sequences. The phylogenetic tree was constructed by using the maximum likelihood method of peptide sequences based on the Jones-Taylor-Thornton model. The values of bootstrap (%) from 1000 replicates are indicated as each node. The OYDV HC-Pro sequences, which analyzed in the multiple alignment in Figure 12, are used; black and red arrowheads indicate long and short type HC-Pros, respectively. The SYSV (AJ865076) HC-Pro sequence was included as an outgroup control. The length of branch is scaled by the number of amino acid substitution per site.

RSS ability of OYDV HC-Pros

To determine whether the lack of N-terminal region of HC-Pro affects the function as an RSS, I first examined local suppression of GFP silencing in *Nicotiana benthamiana* leaves by the conventional agroinfiltration assay. The full-length of HC-Pros from America (long type), China (short type) and three Japanese OYDV isolates (all short types; Tokoro, Hakko and Tokachi) were cloned under the 35S promoter to generate the 35S:HC-Pro constructs. Leaves of *N. benthamiana* plants were coinfiltrated with *Agrobacterium* carrying the expression vectors of the GFP and HC-Pro genes. At five days postinfiltration, the co-infiltration of 35S:GFP with 35S:TEV HC-Pro strongly suppressed the local silencing of GFP compared to 35S:GUS, a control (Figures 15 and 16). For the OYDV HC-Pro proteins, only China HC-Pro showed a slight RSS activity in *N. benthamiana* while those of America and three Japanese isolates (Hakko, Tokachi and Tokoro) had none. Because in previous experiments, the viral RSS activity could differ depending on the assay host plants, I then examined whether HC-Pro can suppress silencing of GFP using onion, which is a natural host of OYDV (Figures 17 and 18). Onion epidermis tissues were infiltrated with the same *Agrobacterium* constructs as used for *N. benthamiana*. In the onion assay, I unexpectedly found that the TEV HC-Pro could not suppress local silencing in spite of the fact that it is a very strong suppressor in *N. benthamiana* (Figures 18). In contrast, America and China HC-Pros showed strong suppression of RNA silencing, and also significantly higher value of GFP fluorescence intensity compared to GUS. However, three Japanese HC-Pro did not show suppression of GFP silencing in onion epidermis cells. Because the China HC-Pro has been considered as an exception as discussed later, the N-terminal deletion of Japanese OYDV HC-Pro seems to have caused a reduction of suppression of RNA silencing in *Allium* species.

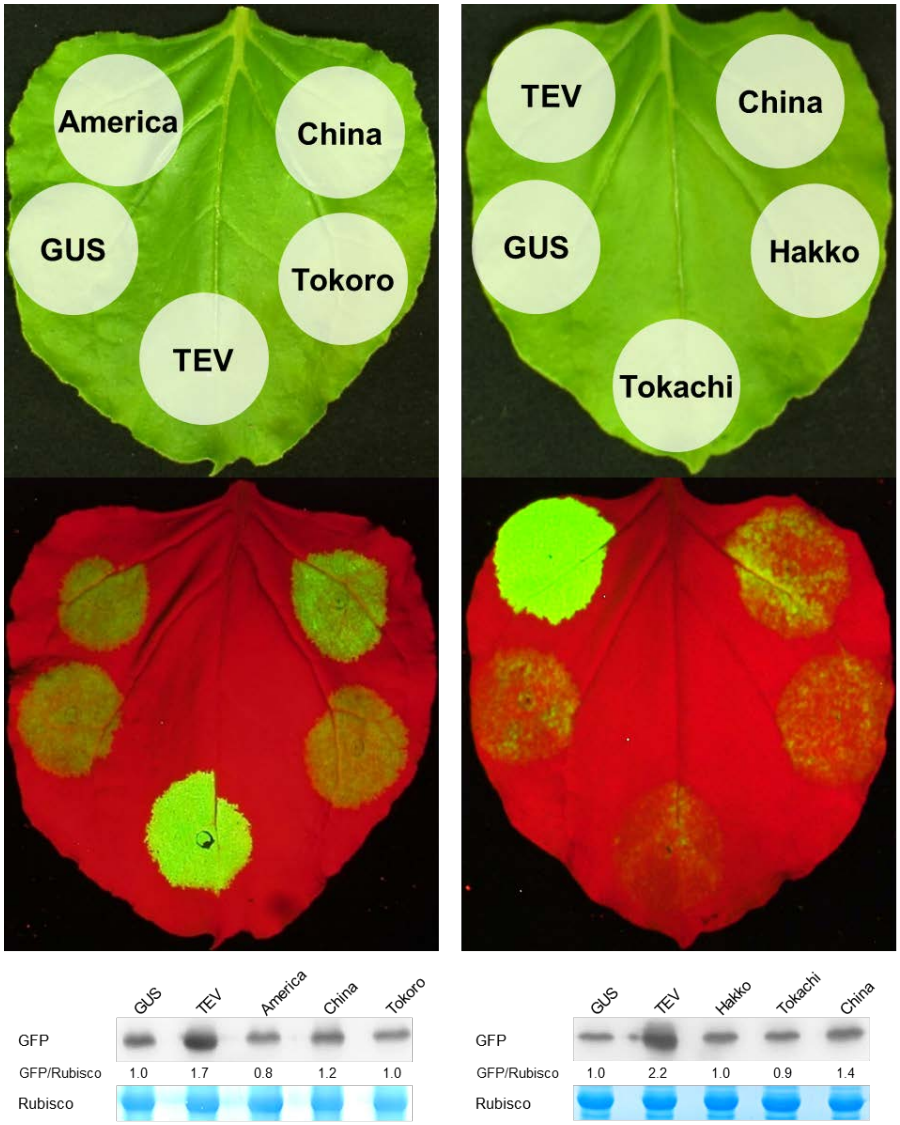

Figure 15 RSS activity of OYDV HC-Pros in *N. benthamiana* leaves. *Agrobacterium* carrying the 35S:GFP construct was coinfiltrated with those with 35S:HC-Pro. The 35S:GUS and 35S:TEV HC-Pro constructs were included as negative and positive controls, respectively. The GFP signals were observed under UV light at 5 days postinfiltration. Western blots were conducted to compare GFP expressions. Total proteins were extracted using the PBS-T buffer and separated in a 14% polyacrylamide gel. The GFP was detected using anti-GFP antibodies. The same gels were stained by Coomassie brilliant blue (CBB). The numbers between the western blot and CBB staining images indicate the relative GFP/Rubisco ratios.

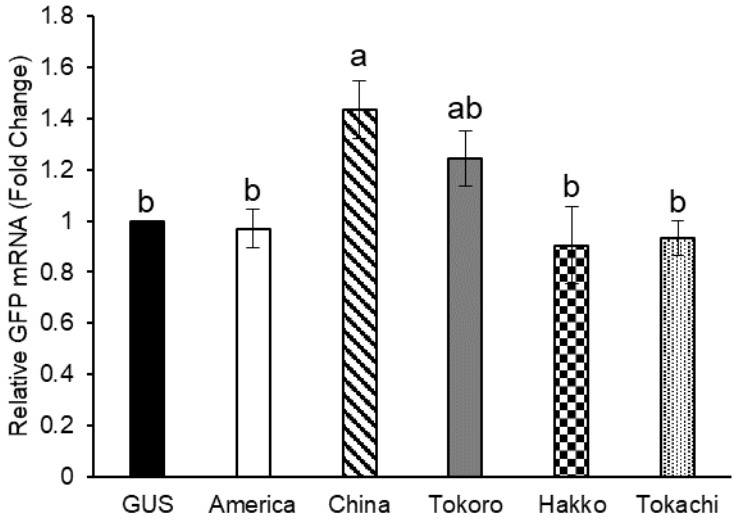

Figure 16 Real-time RT-PCR for comparison of GFP mRNA levels. Total RNAs were extracted from the agroinfiltration patches expressing GFP and HC-Pro together. The mRNA of *N. benthamiana* 60S ribosomal protein L23 was amplified as a control. Means (±SE) among the isolates were analyzed for significant differences by Tukey's multiple comparison test (*P < 0.05). Different letters above each bar represent a significant difference among the isolates.

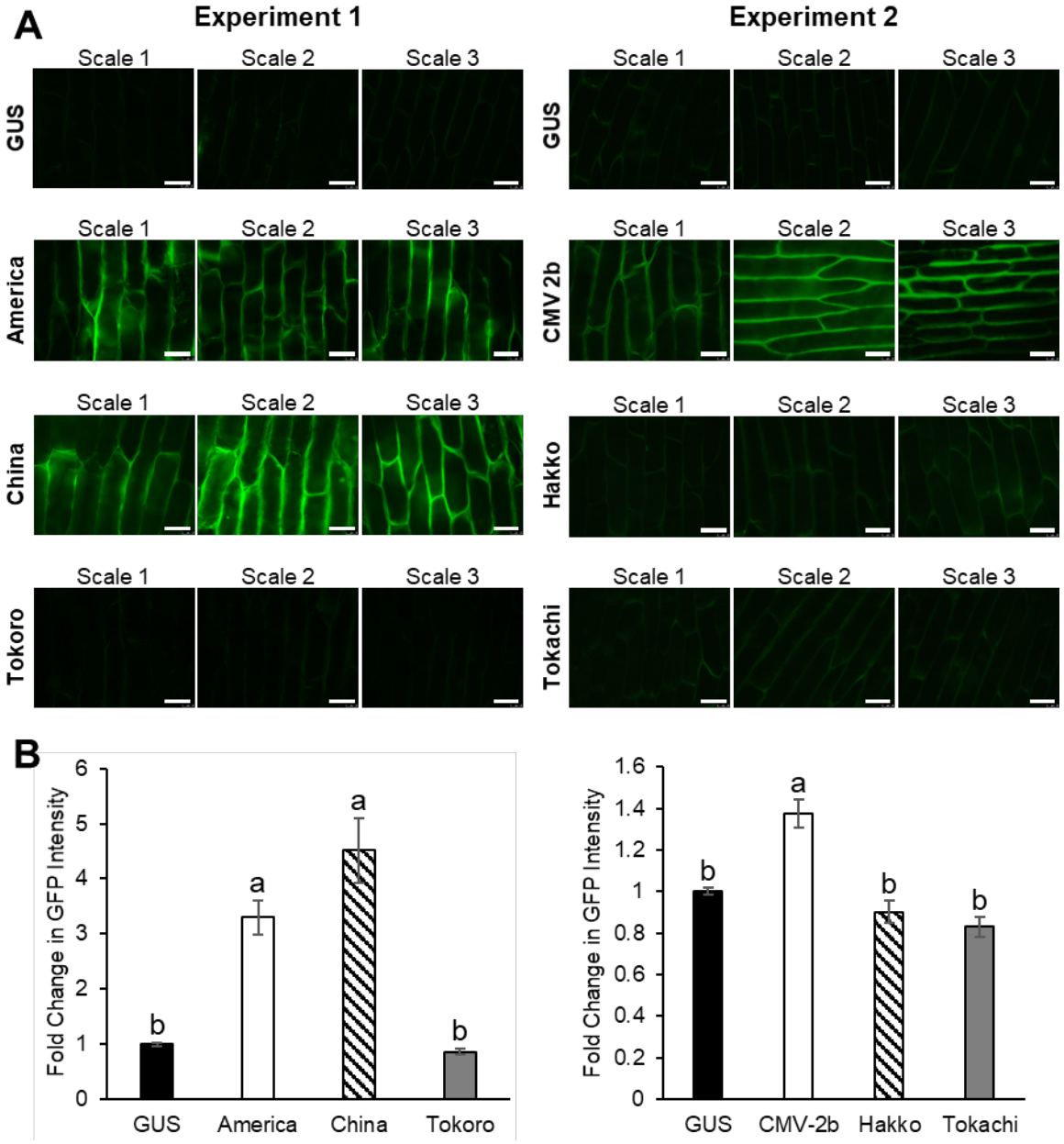

Figure 17 Examination of local RSS activity of OYDV HC-Pro in onion epidermis. (A) *Agrobacterium* carrying the 35S:GFP construct was coinfiltrated with 35S:HC-Pro in the 1:1 ratio. 35S:GUS and 35S:CMV-2b were used as negative and positive controls, respectively. GFP images were taken by using epifluorescence microscope (Leica DMI 6000B). (B) The GFP fluorescence intensities were calculated in LAS AF program, and the values were converted to fold change based on the level of negative control, GUS. Statistical analysis was

conducted by Tukey’s multiple comparison test (*P < 0.05); different letters above the bars indicate a significant difference among isolates.

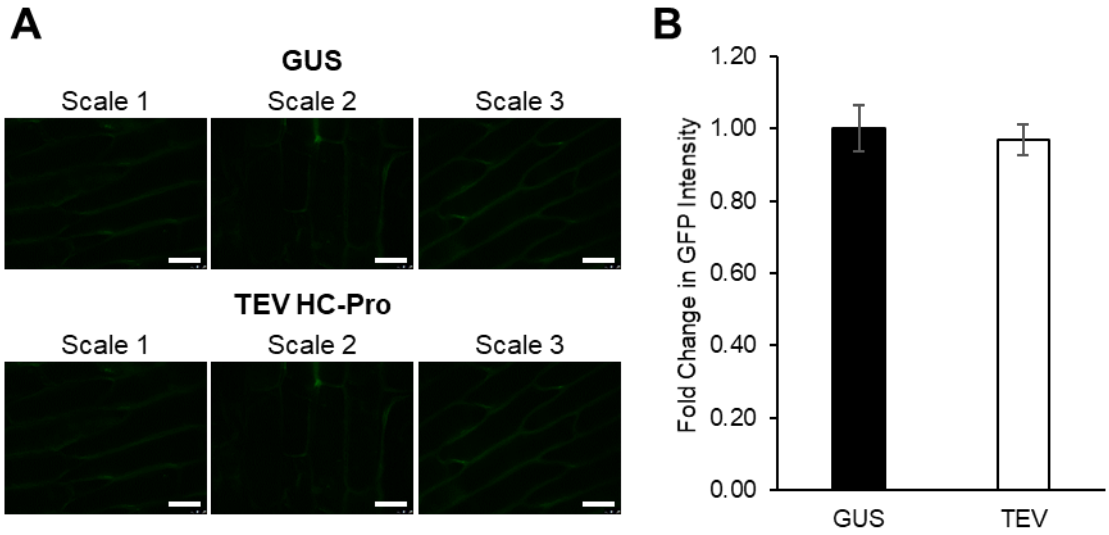

Figure 18 Examination of local RSS activity of TEV HC-Pro in onion epidermis. (A) *Agrobacterium* carrying the 35S:GFP construct was coinfiltrated with 35S:HC-Pro in the 1:1 ratio. The GFP images were taken by using epifluorescence microscope (Leica DMI 6000B). (B) The GFP fluorescence intensities were calculated in the LAS AF program, and the values were converted to fold change based on the level of negative control, GUS. Statistical analysis was conducted by Student't test (*$P < 0.05$).

Structural analysis of OYDV HC-Pro transcripts

To obtain a clue for the evolutionary history of the short type OYDV HC-Pro, I analyzed the secondary structure of the deletion region in the HC-Pro transcripts by using the m-fold program. RNA sequences of OYDV HC-Pros from 15-nt upstream to 15-nt downstream of the deletion were analyzed to generate secondary structure prediction models (Figure 19). Interestingly, the prediction models showed that the HC-Pro transcripts can be classified by their secondary structures; a type of which start and end positions of the N-terminal deletion are close to each other (Close S-E) and start and end positions of deletion are isolated each other (Distant S-E).

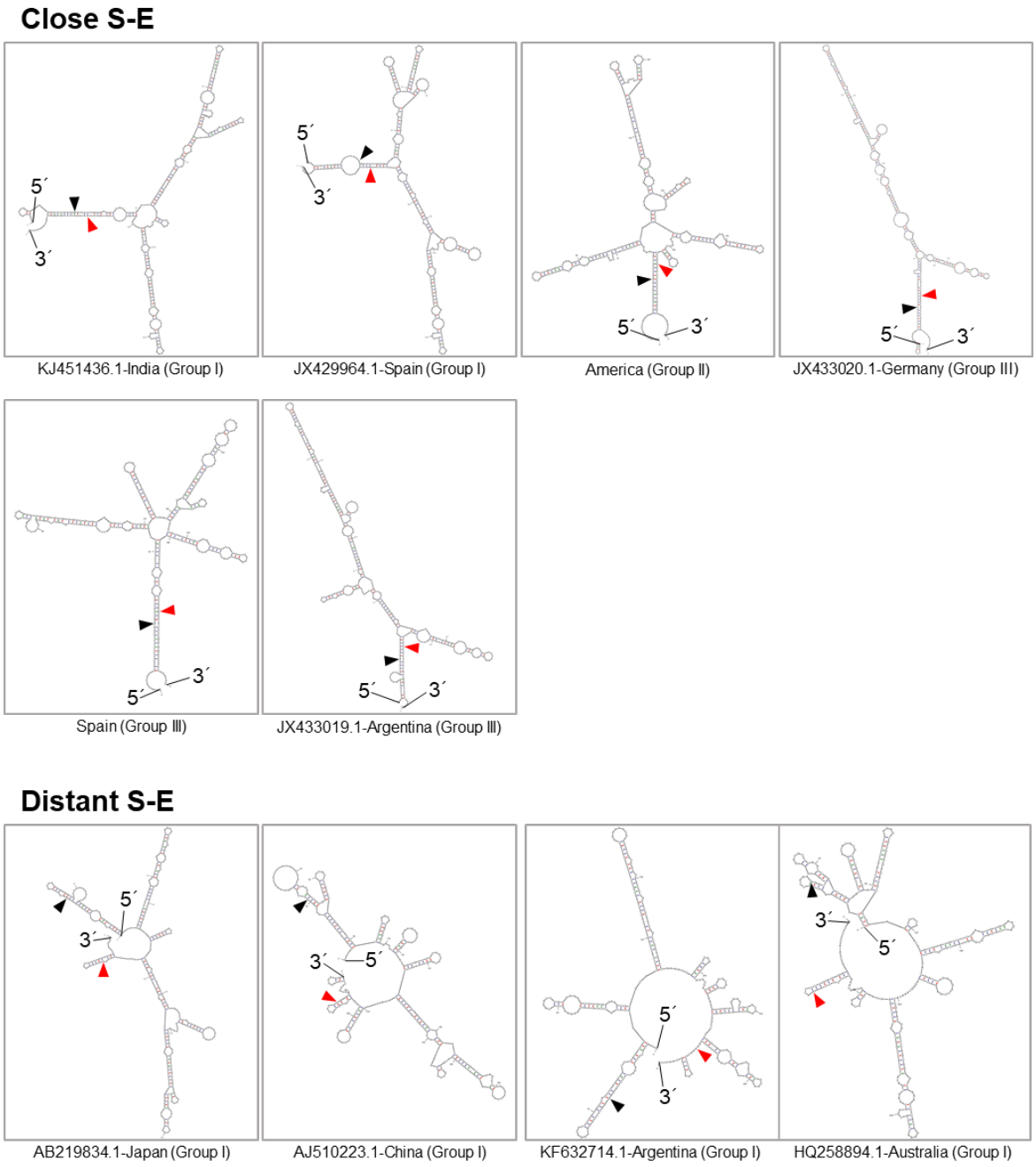

Figure 19 Secondary structure prediction of OYDV HC-Pro RNAs. RNA sequences including the N-terminal deletion were analyzed by the mfold web server v2.3. Black and red arrowheads indicate the start and end sites of N-terminal deletion, respectively. The predicted OYDV RNA structures were classified into two groups: (1) start and end sites were close to each other (Close S-E) and (2) start and end sites were distant (Distant S-E).

Construction of virus-free garlic plants

Because garlic plants are produced by vegetative propagation, they are usually infected with a mixture of some plant viruses; hence it is difficult to produce virus-free garlic plant. Intensive attempts to generate virus-free garlic plants have been encouraged and conducted, but unfortunately, the virus-free garlics were easily re-infected by insect vectors or contaminated through farm implements (Takaki et al., 2005).

To examine aphid transmissibility of Japanese OYDV isolates containing short type HC-Pros in future, I developed a technique to produce virus-free garlic plants by synthetic siRNA treatment. To obtain OYDV-infected garlic, LYSV was targeted by directly treating the meristem tissues with exogenously synthesized LYSV siRNA. In the result, LYSV has totally disappeared when confirmed by RT-PCR using LYSV specific primers (Figure 20). Now, the OYDV-infected plants can be used for an aphid transmission test to examine whether the OYDV with the short type HC-Pro can function as a helper compornent for aphid transmission. Considering the short type HC-Pro lack the crucial motif for aphid transmission KITC, I expect that the Japanese OYDV alone will not be aphid-transmissible.

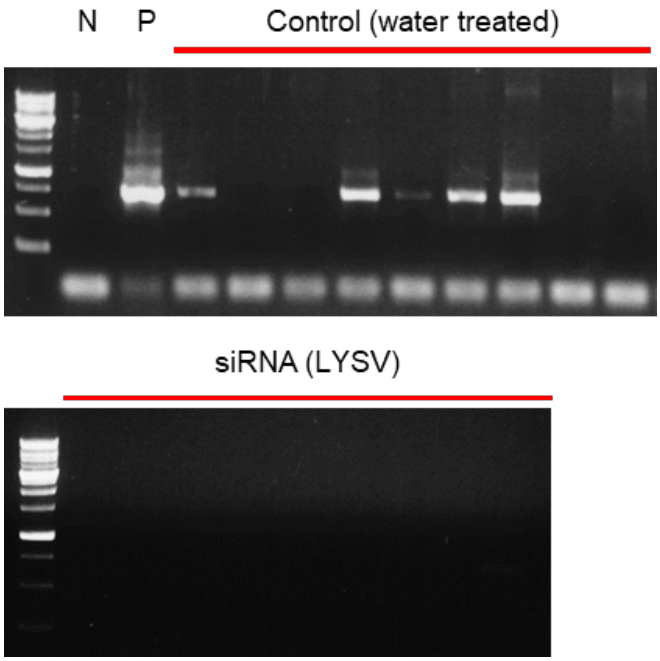

Figure 20 Detection of LYSV in viral dsRNA- or siRNA-treated garlic meristem tissues. siRNA targeting LYSV was directly added to the meristem tissues. RT-PCRs were conducted with primer pair for detection of LYSV at 14 days after treatment. N and P represent the negative control and positive control for RT-PCR, respectively. A 1kb ladder was used as a size marker.

DISCUSSION

Japanese OYDV may have lost aphid transmissibility

The potyviral HC-Pro has been identified as a key factor for aphid transmission by joining viral coat protein and aphid stylet as a 'bridge' (Pirone and Blanc, 1996). Several studies supporting the bridge hypothesis have been reported. Loss-of-function studies of the HC-Pro proteins in TEV and tobacco vein mottling virus have revealed that the conserved KITC motif of the N-terminal region of HC-Pro is essential for aphid transmission, especially for the binding to aphid stylet (Atreya and Pirone, 1993; Blanc et al.,1998). In addition, Peng et al. (1998) identified that the conserved C-terminal PTK motif is required for the interaction with viral capsid. In Japan, a short type of OYDV HC-Pro has been detected from OYDV isolate G79, an attenuated and rare isolate, implying that the N-terminal deletion in HC-Pro may affect the OYDV's pathogenicity and aphid transmission (Takaki et al., 2006). In this study, I analyzed seven OYDV isolates from six Japanese local regions and detected that all the seven HC-Pros lacked ~100 amino acids in the N-terminal region (Figures 12 and 13). Because the missing regions include the KITC motif, which is necessary for aphid transmission, I assume that the aphid transmissibility of short type HC-Pros may be restricted or totally impaired. Interestingly, I noticed that the infection of OYDV always occurred together with another potyvirus, LYSV, and that it has been never detected alone (Table 3). Therefore, only when co-infected with LYSV, Japanese OYDV strains may be transmitted by aphids, similar to the case of potato virus Y, which partially complement the aphid transmissibility of a TEV variant containing a truncated HC-Pro (Dolja et al., 1993).

Table 3. Detection of garlic-infecting viruses in Japanese garlic samples obtained from a market

Region	Detected viruses		
	LYSV	OYDV	Allexivirus
Hokkaido			
Tokoro-a	+	-	-
Tokoro-b	+	-	+
Furano-a	+	-	-
Furano-b	+	-	+
Furano-c	+	-	-
Hokkaido	+	+	-
Unknown	+	-	-
Kiyosato	+	-	-
Touyako	+	-	-
Nakasatsunai	+	+	-
Asahikawa	+	-	-
Obihiro	+	-	-
Makubetsu	+	+	-
Shinshinotsu	+	+	-
Hokudai-a	+	+	-
Hokudai-b	+	+	+
Other regions in Japan			
Aomori-a	-	-	-
Aomori-b	+	-	+
Miyagi	+	-	+
Fukuoka	+	+	+
Okinawa1	+	+	+
Okinawa2	+	+	+

Host specific RSS activity of OYDV HC-Pro in the viral natural host, onion

In this study, the function of OYDV HC-Pro as a RNA silencing suppressor was evaluated in two plants, a conventional model plant, *N. benthamiana* (Figures 15 and 16), and the natural host of OYDV, onion (Figures 17 and 18). Using the transient expression system by agroinfiltration in onion epidermis (Xu et al., 2014), I performed the GFP silencing-suppression assay to compare the RSS activity of the OYDV HC-Pro. America HC-Pro and China HC-Pro successfully suppressed the GFP silencing in onion cells but only China HC-Pro showed slight RSS activity in *N. benthamiana*. In contrast, TEV HC-Pro efficiently suppressed the local silencing of GFP in *N. benthamiana* but not in onion tissue, implying that the HC-Pro proteins of OYDV and TEV suppress the RNA silencing better in its host cells. There are not many reports which describe that viral RSS activity was differentially detected depending on the assay plants. For instance, the 2b protein of a CMV soybean strain (CMV-Sj) showed strong suppression against *CHS* silencing but the 2b of CMV-Y strain, which cannot infect soybean, did not (Senda et al., 2004). In addition, p19 of tomato bushy stunt virus suppressed RNA silencing in *Arabidopsis thaliana*, but not in potato plants, suggesting that p19 discriminates plants for its RSS activity (Ahn et al., 2011). It is conceivable that viral RSSs can work better in their natural host plants than in artificially prepared plants.

Effect of N-terminal deletion of HC-Pro on RSS activity

I also found a loss of the RSS activity of the OYDV HC-Pro (short type) in the three Japanese OYDV isolates (Tokoro, Hakko and Tokachi) in both *N. benthamiana* and onion (Figures 15-17). Potyviral HC-Pro protein interferes with multiple steps in the antiviral RNA silencing

pathway of host plants. The HC-Pro proteins of TEV and ZYMV have been reported to sequester vsiRNAs from the RISC (AGO)-mediated disruption of viral RNAs; the central region of potyviral HC-Pro including the conserved FRNK box was required for the symptom development of ZYMV and its binding to sRNAs (Lakatos et al., 2006; Gal-On, 2000; Shiboleth et al., 2007). Inhibition of vsiRNA 3' methylation by disturbing S-adenosyl methionine cycle or the direct interaction with HEN1 has been also reported (Ivanov et al., 2016; Soitamo et al., 2011; Jamous et al., 2011), and the interference of AGO1 (or AGO1-RISC) by HC-Pro was described in TEV and potato virus A (Varallyay and Havelda, 2013; Ivanov et al., 2016). Because the short HC-Pro found in this study had the conserved FRNK motif, which is responsible for siRNA binding, the lack of the N-terminal region may affect the RSS activity in a different way such as its interactions with AGO1 and HEN1. On the other hand, the China HC-Pro, another short type of OYDV HC-Pro, showed strong suppression of GFP silencing in both *N. benthamiana* and onion plants (Figures 15-17). One explanation may be given by computational analysis of the 3D structure prediction. As shown in Figure 21, the 3D structure of China HC-Pro (China) is totally different from the other short type of HC-Pros (Tokoro and Miyazaki). Unlike the other HC-Pros, the prediction of China HC-Pro structure revealed an exceptionally long helix structure (the residues 11 to 58). This result is in agreement with phylogenetic analysis; the China HC-Pro showed a rather unique evolutionary history among the other short type HC-Pros in Figure 14.

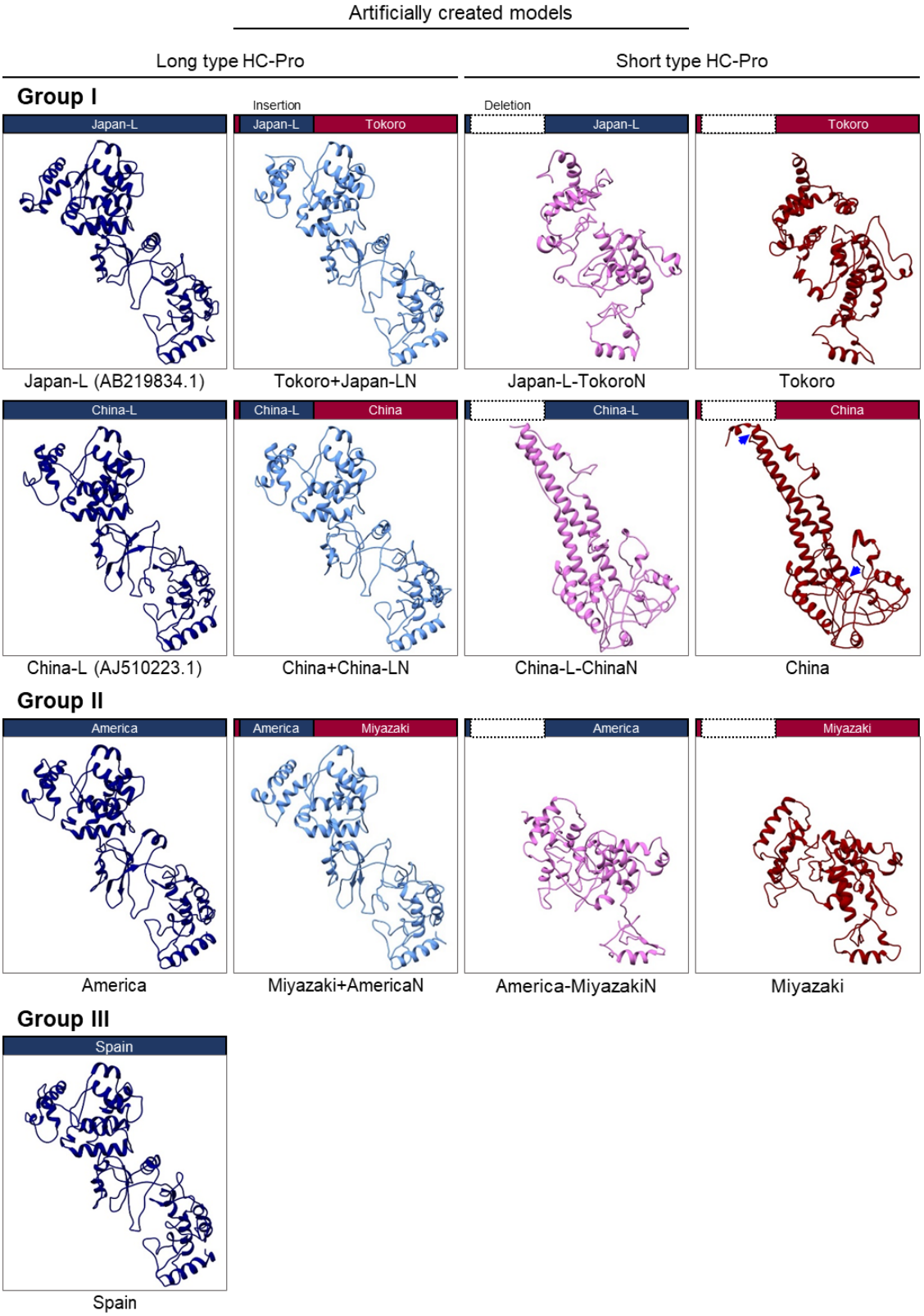

Figure 21 3D structure prediction of OYDV HC-Pros. *Ab initio* structure prediction models of

OYDV HC-Pros were generated by I-TASSER v5.1 server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The predicted models were provided in protein data bank files and visualized in the UCSF chimera program v1.9. The two blue arrowheads indicate the unique long α-helix structure in China HC-Pro. Several artificially created models by inserting or deleting the N-terminal region were also analyzed.

Effect of the N-terminal deletion on HC-Pro tertiary structures and HC-Pro-mediated aphid transmission

Phylogenetic analysis of OYDV HC-Pro based on the sequence similarity divided the short type of Japanese HC-Pros in two groups (Group I and II) (Figure 14). The evolution patterns of Group I including Iwate, Tokoro and Aomori1 and Group II including Tokachi, Hakko, Miyazaki and Aomori2 suggest that the short type HC-Pros from Japanese OYDV isolates may have been generated from the long type HC-Pro. As shown in Figure 12, sequencing and multiple alignment reveal that only the three isolates (Iwate, Tokoro and Aomori 1) contains several unique residues (indicated by asterisks). The PTK motif in the C-terminal region of HC-Pro has been reported to interact with CP in ZYMV, and it is also crucial for aphid transmission (Peng et al., 1998; Gal-On, 2007). The study conducted by Peng et al. (1998) showed that the point mutation of PTK to PVK or PSK resulted in lower transmission efficiency compared to wild-type and the HC-Pro with PSK weakly bind to CP *in vitro*. Therefore, the point mutation of PTK to PMK in those three isolates may affect the interaction between OYDV CP and HC-Pro and thus aphid transmission.

To understand how the insertion of the ~100 aa of the N-terminal region into the short HC-Pro or the deletion of the N-terminal in the long type HC-Pro affect the HC-Pro tertiary structures, I artificially created long type HC-Pros (Tokoro+Japan-LN, China+China-LN and Miyazaki+AmericaN) and short type HC-Pros (Japan-L-TokoroN, China-L-ChinaN and America-MiyazakiN). Interestingly, the predicted structures of Tokoro+Japan-LN, China+China-LN and Miyazaki+AmericaN were almost identical to the authentic type of Japan-L, China-L and America, respectively. Furthermore, the prediction models of Japan-L-TokoroN, China-L-ChinaN and America-MiyazakiN were very similar to those of the short

HC-Pros, Tokoro, China and Miyazaki, respectively. Together with the phylogenetic studies, the computational analysis of the protein structure supports a hypothesis that the short type HC-Pros were generated from long type of HC-Pros.

How did the short OYDV HC-Pros emerge in Japanese garlic fields?

Over the centuries, the virus-infected garlics in the field have been cultivated and selected by human. Because the infected plants with strong viral symptoms are easily distinguished and excluded by growers, and the selection by human may have acted as a selective force for garlic viruses. Therefore, the viruses with weak symptoms are advantageous for their survival in that they are not easily detectable. Considering that the potyviral HC-Pro is responsible for the OYDV symptom development, and that the OYDV isolate with a short HC-Pro induced mild symptoms (Takaki et al., 2006), the Japanese OYDV strains seems to conceal their infection through the N-terminal deletion of HC-Pro. In Japan, virus-free bulbs generated by meristem culture have been widely distributed. The farmers try to aggressively eliminate symptomatic plants. I assume that this Japanese cultivation form has created the short type HC-Pros.

Possible molecular mechanism for emergence of short type HC-Pros

Analysis on the secondary structure of HC-Pro transcript pattern gave me some clue to explain the mechanism for the deletion. The m-fold models of the partial sequences corresponding to the deletion of HC-Pro generated two patterns of folding by which the start and end positions of the N-terminal deletion became close to each other (Close S-E) or very

distant (Distant S-E) as shown in Figure 19. Six of ten long type OYDV HC-Pro RNAs showed that the start and end of the deletion region became close to each other, implying a possible template-switching mechanism; a hairpin-structure provides a recombination junction by template-switching with RNA-dependent RNA polymerase (Figure 22). RNA recombination by template-switching during replication due to a hairpin-structure has been proposed in cucumoviruses, Turnip crinkle virus (TCV) genomic and satellite RNAs (Carpenter et al., 1995; Cascone et al., 1990; Cascone et al., 1993). White and Morris (1995) has identified the preferred junction sites for RNA recombination at a stem-loop structure. Furthermore, the *in vitro* studies conducted by the group of Nagy revealed that some hotspots of TCV and cucumber necrosis virus RNA recombination was localized on the junction of a hairpin structure (Nagy et al., 1998; Cheng and Nagy, 2003). However, whether a short type HC-Pro of OYDV can be generated from a long type HC-Pro with a distant start-end structure remains to be solved.

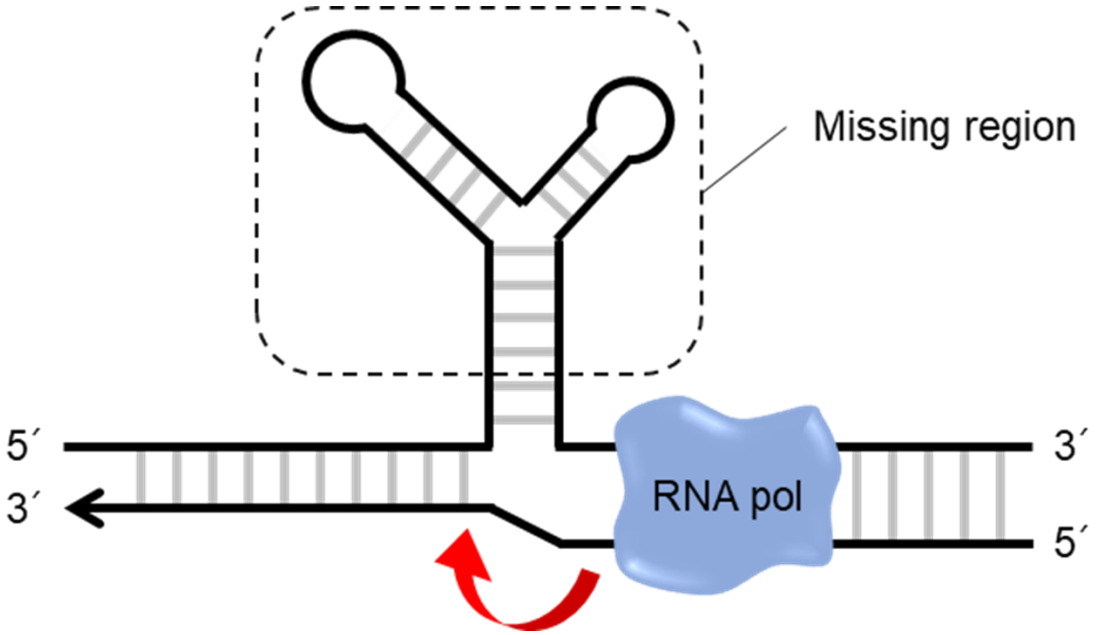

Figure 22 Possible template-switching model for the N-terminal deletion of the HC-Pro genes. A hairpin-structure may provide a recombination junction for template-switching by RNA-dependent RNA polymerase. The RNA polymerase may skip the secondary structure producing a short type HC-Pro transcript.

GENERAL DISCUSSIONS

Even though many studies have demonstrated that the RNA silencing-mediated plant immunity is effective to inhibit various virus infections, the plant-virus interaction is a very complex mechanism in which various host factors and viral genes are involved. In terms of plant immunity, the RNA silencing mechanism is considered to be most important. As described previously, the RSSs interfere with antiviral RNA silencing in all steps in the RNA silencing pathway; knocking-out of a RSS in a viral genome usually results in a decrease (even loss) of pathogenicity of the virus (Cheng and Wang, 2017). Meanwhile, several reports identified that some RSSs of plant viruses act as avirulence (Avr) factors by inducing plant antiviral immune response such as hypersensitive response (HR) in the counter-counter defense of plants. There are several examples on the molecular interactions between RSS and R-proteins. The tomato bushy stunt virus P19 was captured by an R-protein in *Nicotiana* species to induce HR and the NSs protein, the RSS of tomato spotted wilt virus acts as an Avr factor in pepper (Angel et al., 2011; de Ronde et al., 2013). In addition, the HC-Pro and P3 cistrons of soybean mosaic virus are recognized by the *Rsv1* locus in soybean plants (Wen et al., 2013), and cauliflower mosaic virus P6 elicits HR in *Nicotiana* species (Palanichelvam et al., 2000). Therefore, plants infected with a plant virus is indeed a battle field of armed race between host resistance and pathogenic factors of virus and the RSSs play a critical role in infection, directing viral evolution and thus viral survival.

In the same context as above, present study on OYDV HC-Pro in Japanese garlic plants gives some consideration on some aspects of RSSs: viral evolution, viral pathogenicity and survival. As previously described, I believe that with the HC-Pro protein containing a strong RSS activity, OYDV induces severe symptoms in garlic plants, which can be easily detected in the field.

Identification of the short type OYDV HC-Pros from most of Japanese garlic cultivars tested provide some implications on how the short type HC-Pros are generated and how the OYDV strains containing short type HC-Pros can survive. Because viral RSS activity is thought to be crucial for viral infection and symptom development, the RSS activity of Japanese OYDV strains must have eventually played an important role in the viral evolution and survival. In this study, I showed that the short-type HC-Pros from Japanese OYDV isolates lost their RSS activity but successfully survived in Japanese garlic plants, and that OYDV was detected only in the presence of LYSV. These results thus suggest that OYDV would depend on LYSV for aphid transmission and even strong RSS activity. In spite of the fact that OYDV lost its RSS activity, the virus found the way for survival.

On the other hand, VIGS vectors may need a strong RSS activity because a lot of siRNAs derived from the VIGS vector must be generated to efficiently induce RNA silencing against a certain plant gene. However, when the RSS has a strong activity, it must interfere with the RNA silencing against the target gene. I thus consider that there is some discrepancy between an efficient induction of RNA silencing and a degree of RSS activity. Therefore, it may be able to search for an optimizing RSS activity for successful VIGS. Taking this observation as a requirement for VIGS, the artificially designed RSSs inserted in a VIGS vector may be constructed. For CMV 2b, by loss-of-function studies, the domains or motifs crucial for the subcellular localization, suppression of RNA silencing, symptom development and pathogenicity have been well demonstrated. Using such information, I believe that fine-tuning for the RSS activity of 2b can be certainly conducted.

Taken together, I here demonstrated two techniques, applying RNA silencing to the practical field: (1) CMV VIGS system for functional gene analysis of spinach; (2) Exogenous siRNA

treatment to generate virus-free plants for an aphid transmission experiment of OYDV. I developed pseudorecombinant CMV-based VIGS system to optimize VIGS on spinach plants, and a novel technique to generate virus-free garlic plants. In both systems, elaborately tailored small RNAs or dsRNA may be a crucial factor to induce efficient RNA silencing for endogenous genes or viral RNAs. Even though I did not predict or analyze small RNAs induced by our VIGS system or antiviral RNA silencing in virus free garlic plants, I may detect hot-spot of siRNA synthesis region in plant or viral genomes by small RNA sequencing in whole genome level and generate better VIGS vectors or vsiRNAs to improve the efficiency to induce RNA silencing.

In addition, RSSs play a key role in modulating viral pathogenicity and viral survival. Additional studies about the RSSs of plant viruses including CMV are necessary for better understanding of the plant-virus interactions and application of RNA silencing to the practical fields.

SUMMARY

Plants have evoloved various RNA silencing pathways operated by several classes of small RNAs including miRNAs and siRNAs, and these pathways play vital roles in regulating development and growth, genome stabilization from transposable elements and responses against abiotic or biotic stresses. Numerous number of researches have been conducted to exploit the plant RNA silencing pathway as a tool of plant biotechnology, and remarkable advancements in RNA silencing-based techniques have been achieved especially for study of reverse genetics, crop development and pathogen control in plant. In this study, I developed two biotechnological tools based on RNA silencing in plant; (1) CMV VIGS system for study of functional genomics in spinach plants and (2) exogenious small RNA treatment to generate virus-free garlic plants. Furthermore, I analyzed a RSS gene, HC-Pro, of OYDV, which counteract host antiviral RNA silencing mechanism.

In Chapter I, I introduced a CMV VIGS system optimized in spinach plants. I first generated a CMV pseudorecombinant vector (L1A1L3), which has attenuated symptom in infected spinach cultivars, by exchanging genomic RNAs between two CMV strains, CMV-Y and CMV-L. Using the viral vector, I examined VIGS against two spinach genes, *PDS* and *DFR* in three spinach cultivars. In *PDS*-silenced plants, clear chlorosis was observed on the systemically infected leaves with significantly decreased level of *PDS* mRNA. For DFR silencing, decoloration of red pigment was observed in the stem of infected plants with decreased *DFR* gene expression. Furthermore, unlike other VIGS system based on BCTV or TRV, our CMV VIGS construct was still retained after two month postinoculation with silencing phenotype, suggesting that the CMV VIGS is sustainable until flowering stage, and hence can be applicable for some unique properties of spinach plants such as plasticity in sex

expression.

In Chapter II, I analyzed an RSS of a potyvirus, OYDV. When I investigated viral infection of three garlic infecting virus, LYSV, OYDV and allexiviruses, I found that two potyviruses, OYDV and LYSV, always coinfected Japanese garlic plants. Because potyviral HC-Pro is a key player for aphid transmission of potyvirus, I focused the HC-Pro genes of OYDV. Interestingly, our RT-PCR result and sequencing data showed that all the tested HC-Pros of Japanese OYDV isolates lacked ~100 amino acids, which includes a crucial motif for aphid transmission (KITC), suggesting that the HC-Pros Japanese OYDV strains may not be able to mediate aphid transmission. Phylogenetic analysis based on the amino acid sequences revealed three distinct groups and the short type HC-Pros were divided into two groups, implying their different evolutionary history. However, at least a group may be explained by a possible template-switching mechanism during viral replication. To determine whether the lack of N-terminal affects the HC-Pro function as an RSS, I examined their RSS activity in a model plants, *N. benthamiana*, and its natural host, onion. The OYDV HC-Pros showed more efficient RSS activities in onion, and except the HC-Pro of China OYDV isolate, all the Japanese OYDV HC-Pros lost their RSS activity in both *N. benthamiana* and onion plants. To experimently examine whether the N-terminal deletion of HC-Pros affect their aphid transmissibility, I tried to generate virus-free garlic plants for aphid transmission assay. Using antiviral RNA silencing mechanism, a novel technique was developed to generate virus-free garlic plants by treating exogenous dsRNA to meristem tissues. Using the OYDV-infected garlic, I will conduct the aphid transmission assay. Together, this study provide a clue of OYDV viral evolution in Japanese garlic field and the roll of HC-Pro gene for control of pathogenicity and viral survival.

In this research, I used the virus-induced RNA silencing to target and eliminate viruses or to

apply it as a tool, VIGS, for functional genomics studies. In both systems, RSSs are key factor in counteracting antiviral RNA silencing or modulating viral pathogenicity. Much more detailed studies of RSSs may be required to enhance the efficiency of VIGS, antiviral RNA silencing techniques and more over every plant RNA silencing-based technologies.

REFERENCES

Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science.* 232, 738-743.

Ahn, J. W., Lee, J. S., Davarpanah, S. J., Jeon, J. H., Park, Y. I., Liu, J. R., and Jeong, W. J. (2011). Host-dependent suppression of RNA silencing mediated by the viral suppressor p19 in potato. *Planta.* 234, 1065-1072.

Ai, T., Zhang, L., Gao, Z., Zhu, C. X., and Guo, X. (2011). Highly efficient virus resistance mediated by artificial microRNAs that target the suppressor of PVX and PVY in plants. *Plant Biol.* 13, 304-316.

Ali, I., Amin, I., Briddon, R. W., and Mansoor, S. (2013). Artificial microRNA-mediated resistance against the monopartite begomovirus *Cotton leaf curl Burewala virus. Virol J.* 10, 231.

Allen, E., Xie, Z., Gustafson, A. M. and Carrington, J. C. (2005). microRNA directed phasing during trans-acting siRNA biogenesis in plants. *Cell.* 121, 207-221.

Andrade, C. M., Tinoco, M. L. P., Rieth, A. F., Maia, F. C. O., and Aragão, F. J. L. (2016). Host-induced gene silencing in the necrotrophic fungal pathogen *Sclerotinia sclerotiorum. Plant Pathol.* 65, 626-632.

Angel, C. A., Hsieh, Y. C., and Schoez, J. E. (2011). Comparative analysis of the capacity of tombusvirus P22 and P19 proteins to function as avirulence determinants in *Nicotiana* species. *Mol Plant Microbe Interact.* 24, 91-99.

Arya, M., Baranwal, V. K., Ahlawat, Y. S., and Singh, L. (2006). RT-PCR detection and molecular characterization of *Onion yellow dwarf virus* associated with garlic and onion. *Curr Sci.* 91, 1230-1234.

Aslam, U., Tabassum, B., Nasir, I. A., Khan, A., and Husnain, T. (2018). A virus-derived short hairpin RNA confers resistance against sugarcane mosaic virus in transgenic sugarcane. *Transgenic Res.* 27, 203-210.

Atreya, C. D., and Pirone, T. P. (1993). Mutational analysis of the helper component-proteinase gene of a potyvirus: effects of amino acid substitutions, deletions, and gene replacement on virulence and aphid transmissibility. *Proc Natl Acad Sci U S A.* 90, 11919-11923.

Bagi, F., Stojšin, V., Budakov, D., El Swaeh, S. M. A., and Gvozdanović-Varga, J. (2012). Effect of onion yellow dwarf virus (OYDV) on yield components of fall garlic (*Allium sativum* L.) in Serbia. *Afr J Agric Res.* 7, 2386-2390.

Baulcombe, D. (2004). RNA silencing in plants. *Nature.* 431, 356-363.

Baulcombe, D. C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell.* 8, 1833-1844.

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T., and Roberts, J. (2007). Control of coleopteran insect pests through RNA interference. *Nat Biotechnol.* 25, 1322-1326.

Beerhues, L., and Wiermann, R. (1988). Chalcone synthases from spinach (*Spinacia oleracea* L.): I. Purification, peptide patterns, and immunological properties of different forms. *Planta.* 173, 532-543.

Beerhues, L., Robenek, H., and Wiermann, R. (1988). Chalcone synthases from spinach (*Spinacia oleracea* L.): II. Immunofluorescence and immunogold localization. *Planta.* 173, 544-553.

Blanc, S., Ammar, E. D., Garcia-Lampasona, S., Dolja, V. V., Llave, C., Baker, J., and Pirone, T. P. (1998). Mutations in the potyvirus helper component protein: effects on interactions with virions and aphid stylets. *J Gen Virol.* 79, 3119-3122.

Blevins, T., Rajeswaran, R., Shivaprasad, P. V., Beknazariants, D., Si-Ammour, A., Park, H. S., Vazquez, F., Robertson, D., Meins, F., Hohn, T., and Pooggin, M. M. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids, Res.* 34, 6233-6246.

Burch-Smith, T. M., Schiff, M., Liu, Y., and Dinesh-Kumar, S. P. (2006). Efficient virus-induced gene silencing in *Arabidopsis*. *Plant Physiol.* 142, 21-27.

Cai, X., Lin, L., Wang, X., Xu, C., and Wang, Q. (2018). Higher anthocyanin accumulation associated with higher transcription levels of anthocyanin biosynthesis genes in spinach. *Genome.* 61, 487-496.

Carbonell, A., and Carrington, J. C. (2015). Antiviral roles of plant ARGONAUTES. *Curr Opin Plant Biol.* 27, 111-117.

Carbonell, A., and Daròs, J. A. (2017). Artificial microRNAs and synthetic trans-acting small interfering RNAs interfere with viroid infection. *Mol Plant Pathol.* 18, 746-753.

Carbonell, A., de Alba, Á. E. M., Flores, R., and Gago, S. (2008). Double-stranded RNA interferes in a sequence-specific manner with the infection of representative members of the two viroid families. *Virology.* 371, 44-53.

Carmell, M. A., and Hannon, G. J. (2004). RNase III enzymes and the initiation of gene silencing. *Nat Struct Mol Biol.* 11, 214-218.

Carpenter, C. D., Oh, J. W., Zhang, C., and Simon, A. E. (1995). Involvement of a stem-loop structure in the location of junction sites in viral RNA recombination. *J Mol Biol.* 245, 608-622.

Cascone, P. J., Carpenter, C. D., Li, X. H., and Simon, A. E. (1990). Recombination between satellite RNAs of turnip crinkle virus. *EMBO J.* 9, 1709-1715.

Cascone, P. J., Haydar, T. F., and Simon, A. E. (1993). Sequences and structures required for recombination between virus-associated RNAs. *Science.* 260, 801-805.

Chailakhyan, M. K., and Timiriazev, K. A. (1979). Genetic and hormonal regulation of growth, flowering, and sex expression in plants. *Am J Bot.* 66, 717-736.

Chen, L. F., Vivoda, E., and Gilbertson, R. L. (2011). Genetic diversity in curtoviruses: a highly divergent strain of *Beet mild curly top virus* associated with an outbreak of curly top disease in pepper in Mexico. *Arch Virol.* 156, 547-555.

Chen, W., Kastner, C., Nowara, D., Oliveira-Garcia, E., Rutten, T., Zhao, Y., Deising, H. B., Kumlehn, J., and Schweizer, P. (2016). Host-induced silencing of *Fusarium culmorum* genes protects wheat from infection. *J Exp Bot.* 67, 4979-4991.

Cheng, C. P., and Nagy, P. D. (2003). Mechanism of RNA recombination in carmo-and tombusviruses: evidence

for template switching by the RNA-dependent RNA polymerase *in vitro*. *J Virol.* 77, 12033-12047.

Cheng, W., Song, X. S., Li, H. P., Cao, L. H., Sun, K., Qiu, X. L., Xu, Y. B., Yang, P., Huang, T., Zhang, J. B., Qu, B., and Liao, Y. C. (2015). Host-induced gene silencing of an essential chitin synthase gene confers durable resistance to *Fusarium* head blight and seedling blight in wheat. *Plant Biotechnol J.* 13, 1335-1345.

Cheng, X., and Wang, A. (2017a). Multifaceted defense and counter-defense in co-evolutionary arms race between plants and viruses. *Commun Integr Biol.* 10, e1341025.

Cheng, X., and Wang, A. (2017b). The potyvirus silencing suppressor protein VPg mediates degradation of SGS3 via ubiquitination and autophagy pathways. *J Virol.* 91, e01478-16.

Chin, D. P., Bao, J. H., and Mii, M. (2009). Transgenic spinach plants produced by *Agrobacterium*-mediated method based on the low temperature-dependent high plant regeneration ability of leaf explants. *Plant Biotechnol.* 26, 243-248.

Chiu, M. H., Chen, I. H., 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. *Mol Plant Pathol.* 11, 641-649.

Chung, B. Y. W., Miller, W. A., Atkins, J. F., and Firth, A. E. (2008). An overlapping essential gene in the *Potyviridae*. *Proc Natl Acad Sci U S A.* 105, 5897-5902.

Conci, V. C., Canavelli, A., Lunello, P., Di Rienzo, J., Nome, S. F., Zumelzu, G., and Italia, R. (2003). Yield losses associated with virus-infected garlic plants during five successive years. *Plant Dis.* 87, 1411-1415.

Cronin, S., Verchot, J., Haldeman-Cahill, R., Schaad, M. C., and Carrington, J. C. (1995). Long-distance movement factor: a transport function of the potyvirus helper component proteinase. *Plant Cell.* 7, 549-559.

Csorba, T., Kontra, L., and Burgyán, J. (2015). Viral silencing suppressors: tools forged to fine-tune host-pathogen coexistence. *Virology.* 479, 85-103.

Dalakouras, A., Wassenegger, M., McMillan, J. N., Cardoza, V., Maegele, I., Dadami, E., Runne, M., Krczal, G.,

and Wassenegger, M. (2016). Induction of silencing in plants by high-pressure spraying of *in vitro*-synthesized small RNAs. *Front Plant Sci.* 7, 1327.

De Ronde, D., Butterbach, P., Lohuis, D., Hedil, M., Van Lent, J. W., and Kormelink, R. (2013). *Tsw* gene-based resistance is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*. *Mol Plant Pathol.* 14, 405-415.

Dolja, V. V., Herndon, K. L., Pirone, T. P., and Carrington, J. C. (1993). Spontaneous mutagenesis of a plant potyvirus genome after insertion of a foreign gene. *J Virol.* 67, 5968-5975.

Dovas, C. I., Hatziloukas, E., Salomon, R., Barg, E., Shiboleth, Y., and Katis, N. I. (2001). Incidence of viruses infecting *Allium* spp. in Greece. *Eur J Plant Pathol.* 107, 677-684.

Drake, C. J., Tate, H. D, and Harris, H. M. (1933). The relationship of aphids to the transmission of yellow dwarf of onions. *J Econ Entomol.* 26, 841-846.

Duan, C., Wang, C., and Guo, H. (2008). Delayed resistance to cucumber mosaic virus mediated by 3' UTR-derived hairpin RNA. *Chin Sci Bull.* 53, 3301-3310.

Ellis, R. J. (1981). Chloroplast proteins: synthesis, transport, and assembly. *Annu Rev Plant Physiol.* 32, 111-137.

El-Wahab, A. S. A. (2009). Aphid-transmission efficiency of two main viruses on garlic in Egypt, *Onion yellow dwarf virus* (OYDV-G) and *Leek yellow stripe virus* (LYSV-G). *Academic J Entomol.* 2, 40-42.

Fahim, M., Millar, A. A., Wood, C. C., and Larkin, P. J. (2012). Resistance to Wheat streak mosaic virus generated by expression of an artificial polycistronic microRNA in wheat. *Plant Biotechnol J.* 10, 150-163.

Fajardo, T. V. M., Nishijima, M., Buso, J. A., Torres, A. C., Ávila, A. C., and Resende, R. O. (2001). Garlic viral complex: identification of potyviruses and carlavirus in central Brazil. *Fitopatol Bras.* 26, 619-626.

Fayad-André, M. D. S., Dusi, A. N., and Resende, R. O. (2011). Spread of viruses in garlic fields cultivated under different agricultural production systems in Brazil. *Trop Plant Pathol.* 36, 341-349.

Ferreira, S. A., Pitz, K. Y., Manshardt, R., Zee, F., Fitch, M., and Gonsalves, D. (2002). Virus coat protein

transgenic papaya provides practical control of papaya ringspot virus in Hawaii. *Plant Dis.* 86, 101-105.

Frizzi, A., and Huang, S. (2010). Tapping RNA silencing pathways for plant biotechnology. *Plant Biotechnol J.* 8, 655-677.

Fuentes-Bazan, S., Uotila, P., and Borsch, T. (2012). A novel phylogeny-based generic classification for *Chenopodium* sensu lato, and a tribal rearrangement of *Chenopodioideae (Chenopodiaceae)*. *Willdenowia.* 42, 5-24.

Gaba, V., Rosner, A., Maslenin, L., Leibman, D., Singer, S., Kukurt, E., Shiboleth, Y. M., and Gal-On, A. (2010). Hairpin-based virus resistance depends on the sequence similarity between challenge virus and discrete, highly accumulating siRNA species. *Eur J Plant Pathol.* 128, 153-164.

Gal-On, A. (2000). A point mutation in the FRNK motif of the potyvirus helper component-protease gene alters symptom expression in cucurbits and elicits protection against the severe homologous virus. *Phytopathology.* 90, 467-473.

Gal-On, A. (2007). *Zucchini yellow mosaic virus*: insect transmission and pathogenicity—the tails of two proteins. *Mol Plant Pathol.* 8, 139-150.

Gan, D., Zhang, J., Jiang, H., Jiang, T., Zhu, S., and Cheng, B. (2010). Bacterially expressed dsRNA protects maize against SCMV infection. *Plant Cell Rep.* 29, 1261-1268.

Garcia-Ruiz, H., Takeda, A., Chapman, E. J., Sullivan, C. M., Fahlgren, N., Brempelis, K. J., and Carrington, J. C. (2010). *Arabidopsis* RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and small interfering RNA biogenesis during *Turnip Mosaic Virus* infection. *Plant Cell.* 22, 481-496.

Ghag, S. B., Shekhawat, U. K., and Ganapathi, T. R. (2014). Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against *Fusarium* wilt in banana. *Plant Biotechnol J.* 12, 541-553.

Gil, M. I., Ferreres, F., and Tomás-Barberán, F. A. (1999). Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. *J Agr Food Chem.* 47, 2213-2217.

Golenberg, E. M., Sather, D. N., Hancock, L. C., Buckley, K. J., Villafranco, N. M., and Bisaro, D. M. (2009). Development of a gene silencing DNA vector derived from a broad host range geminivirus. *Plant Methods.* 5: 9.

Guo, Z., Li, Y., and Ding, S. W. (2019). Small RNA-based antimicrobial immunity. *Nat Rev Immunol.* 19, 31-44.

Haas, G., Azevedo, J., Moissiard, G., Geldreich, A., Himber, C., Bureau, M., Fukuhara, T., Keller, M., and Voinnet, O. (2008). Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4. *EMBO J.* 27, 2102-2112.

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature.* 404, 293-296.

Havelda, Z., Hornyik, C., Válóczi, A., and Burgyán, J. (2005). Defective interfering RNA hinders the activity of a tombusvirus-encoded posttranscriptional gene silencing suppressor. *J Virol.* 79, 450-457.

Hedges, L. J., and Lister, C. E. (2007). Nutritional attributes of spinach, silver beet and eggplant. *Crop Food Res Confidential Rep.* 1928.

Hemmes, H., Lakatos, L., Goldbach, R., Burgyán, J., and Prins, M. (2007). The NS3 protein of *Rice hoja blanca tenuivirus* suppresses RNA silencing in plant and insect hosts by efficiently binding both siRNAs and miRNAs. *RNA*, 13, 1079-1089.

Hendelman, A., Kravchik, M., Stav, R., Zik, M., Lugassi, N., and Arazi, T. (2013). The developmental outcomes of P0-mediated ARGONAUTE destabilization in tomato. *Planta.* 237, 363-377.

Hong, J. S., Rhee, S. J., Kim, E. J., Kim, T. S., Ryu, K. H., Masuta, C., and Lee, G. P. (2012). Application of a reassortant cucumber mosaic virus vector for gene silencing in tomato and chili pepper plants. *Plant Pathol J.* 28, 81-86.

Hu, Z., Parekh, U., Maruta, N., Trusov, Y., and Botella, J. R. (2015). Down-regulation of *Fusarium oxysporum* endogenous genes by host-delivered RNA interference enhances disease resistance. *Front. Chem.* 3, 1.

Huang, C., Xie, Y., and Zhou, X. (2009). Efficient virus-induced gene silencing in plants using a modified

geminivirus DNA1 component. *Plant Biotechnol J.* 7, 254-265.

Huang, L., Jin, J., Deighan, P., Kiner, E., McReynolds, L., and Lieberman, J. (2013). Efficient and specific gene knockdown by small interfering RNAs produced in bacteria. *Nat Biotechnol.* 31, 350-356.

Ivanov, K. I., Eskelin, K., Bašić, M., De, S., Lõhmus, A., Varjosalo, M., and Mäkinen, K. (2016). Molecular insights into the function of the viral RNA silencing suppressor HCPro. *Plant J.* 85, 30-45.

Jamous, R. M., Boonrod, K., Fuellgrabe, M. W., Ali-Shtayeh, M. S., Krczal, G., and Wassenegger, M. (2011). The helper component-proteinase of the *Zucchini yellow mosaic virus* inhibits the Hua Enhancer 1 methyltransferase activity *in vitro*. *J Gen Virol.* 92, 2222-2226.

Janick, J. (1998). Hybrids in horticultural crops. In: Lamkey, K. R., and Staub, J. E. (eds) Concepts and breeding of heterosis in crop plants. Crop Scinece Society of America, pp 45-56.

Janick, J., and Stevenson, E. C. (1954). A genetic study of the heterogametic nature of the staminate plant in spinach. *Proc Am Soc Hort Sci.* 63, 444-446.

Janick, J., and Stevenson, E. C. (1955a). Environmental influences on sex expression in monoecious lines of spinach. *Proc Am Soc Hort Sci.* 65, 416-422.

Janick, J., and Stevenson, E. C. (1955b). Genetics of the monoecious character in spinach. *Genetics.* 40, 429-437.

Janick, J., and Stevenson, E. C. (1955c). The effects of polyploidy on sex expression in spinach. *J Hered.* 46, 150-156.

Kikuchi, Y., Hijikata, N., Ohtomo, R., Handa, Y., Kawaguchi, M., Saito, K., Masuta, C., and Ezawa, T. (2016). Aquaporin-mediated long-distance polyphosphate translocation directed towards the host in arbuscular mycorrhizal symbiosis: application of virus-induced gene silencing. *New Phytol.* 211, 1202-1208.

Kim, H., Shimura, H., and Masuta, C. (2019). Advancing toward commercial application of RNA silencing-based strategies to protect plants from viral diseases. *J Gen Plant Pathol.* 85, 321-328.

Kis, A., Tholt, G., Ivanics, M., Várallyay, É., Jenes, B., and Havelda, Z. (2016). Polycistronic artificial miRNA-

mediated resistance to *Wheat dwarf virus* in barley is highly efficient at low temperature. *Mol Plant Pathol.* 17, 427-437.

Kjemtrup, S., Sampson, K. S., Peele, C. G., Nguyen, L. V., Conkling, M. A., Thompson, W. F., and Robertson, D. (1998). Gene silencing from plant DNA carried by a geminivirus. *Plant J.* 14, 91-100.

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., Linicus, L., Johannsmeier, J., Jelonek, L., Goesmann, A., Cardoza, V., McMillan, J., Mentzel, T., and Kogel, K. H. (2016). An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *PLoS Pathog.* 12, e1005901.

Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J., and Kogel, K. H. (2013). Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase–encoding genes confers strong resistance to *Fusarium* species. *Proc Natl Acad Sci U S A.* 110, 19324-19329.

Kumagai, M. H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., and Grill, L. K. (1995). Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc Natl Acad Sci U S A.* 92, 1679-1683.

Kumar, S., Baranwal, V. K., Joshi, S., Arya, M., and Majumder, S. (2010). Simultaneous detection of mixed infection of *Onion yellow dwarf virus* and an *Allexivirus* in RT-PCR for ensuring virus free onion bulbs. *Indian J Virol.* 21, 64-68.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E. J., Carrington, J. C., Liu, Y. P., Dolja, V. V., Calvino, L. F., López-Moya, J. J., and Burgyán, J. (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO J.* 25, 2768-2780.

Lee, J., Cao, D. V., Kim, J., Pamplona, R. S., Ahn, J., Cho, S. K., Yang, S. W., Riu, K. Z., and Boo, K. H. (2017). Development of a virus-induced gene silencing (VIGS) system for *Spinacia oleracea* L. *In Vitro Cell Dev Biol Plant.* 53, 97-103.

Lester, G. E., Makus, D. J., Hodges, D. M., and Jifon, J. L. (2013). Summer (Subarctic) versus winter (Subtropic) production affects spinach (*Spinacia oleracea* L.) leaf bionutrients: Vitamins (C, E, Folate, K1, provitamin A),

lutein, phenolics, and antioxidants. *J Agr Food Chem.* 61, 7019-7027.

Li, F., and Wang, A. (2019). RNA-targeted antiviral immunity: more than just RNA silencing. *Trends Microbiol.* 9, 792-805.

Li, F., Yang, X., Bisaro, D. M., and Zhou, X. (2018). The βC1 protein of geminivirus–betasatellite complexes: a target and repressor of host defenses. *Mol Plant.* 11, 1424-1426.

Li, S., Castillo-González, C., Yu, B., and Zhang, X. (2017). The functions of plant small RNAs in development and in stress responses. *Plant J.* 90, 654-670.

Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M., and Dougherty, W. G. (1993). Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *Plant Cell.* 5, 1749-1759.

Liu, B., Watanabe, S., Uchimiya, T., Kong, F., Kanazawa, A., Xia, Z., Nagamatsu, A., Arai, M., Yamada, T., Kitamura, K., Masuta, C., and Harada, K. and Abe, J. (2010). Soybean stem growth habit gene *Dt1* is an orthologue of *Arabidopsis TERMINAL FLOWER1*. *Plant Physiol.* 153, 198-210.

Liu, S. R., Zhou, J. J., Hu, C. G., Wei, C. L., and Zhang, J. Z. (2017). MicroRNA-mediated gene silencing in plant defense and viral counter-defense. *Front Microbiol*. 8, 1801.

Llave, C. (2010). Virus-derived small interfering RNAs at the core of plant–virus interactions. *Trends Plant Sci.* 15, 701-707.

Love A. J., Geri C., Laird J., Carr C., Yun B. W., Loake G. J., Tada Y., Sadanandom A., and Milner J. J. (2012). *Cauliflower mosaic virus* protein P6 inhibits signaling responses to salicylic acid and regulates innate immunity. *PLoS One.* 7, e47535.

Lu, R., Martin-Hernandez, A. M., Peart, J. R., Malcuit, I., and Baulcombe, D. C. (2003). Virus-induced gene silencing in plants. *Methods.* 30, 296-303.

Manglli, A., Mohammed, H. S., El Hussein, A. A., Agosteo, G. E., Albanese, G., and Tomassoli, L. (2014).

Molecular analysis of the 3' terminal region of *Onion yellow dwarf virus* from onion in southern Italy. *Phytopathol Mediterr.* 53, 438-450.

Mao, Y. B., Cai, W. J., Wang, J. W., Hong, G. J., Tao, X. Y., Wang, L. J., Huang, Y. P. and Chen, X. Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat Biotechnol.* 25, 1307-1313.

Martínez, F., Elena, S. F., and Daròs, J. A. (2013). Fate of artificial microRNA-mediated resistance to plant viruses in mixed infections. *Phytopathology.* 103, 870-876.

Matzke, M. A., and Mosher, R. A. (2014). RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nat Rev Genet.* 15, 394-408.

Mayers, C. N., Lee, K. C., Moore, C. A., Wong, S. M., and Carr, J. P. (2005) Salicylic acid-induced resistance to *Cucumber mosaic virus* in squash and *Arabidopsis thaliana*: contrasting mechanisms of induction and antiviral action. *Mol Plant Microbe Interact.* 18, 428-434.

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., Fletcher, S. J., Carroll, B. J., Lu, G. Q., and Xu, Z. P. (2017) Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat Plants.* 3, 16207.

Mohammed, H. S., Zicca, S., Manglli, A., Mohamed, M. E., El Siddig, M. A. R., El Hussein, A. A., and Tomassoli, L. (2013). Occurrence and phylogenetic analysis of potyviruses, carlaviruses and allexiviruses in garlic in Sudan. *J Phytopathol.* 161, 642-650.

Molnar, A., Melnyk, C. W., Bassett, A., Hardcastle, T. J., Dunn, R., and Baulcombe, D. C. (2010). Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science.* 328, 872-875.

Nagy, P. D., Zhang, C., and Simon, A. E. (1998). Dissecting RNA recombination *in vitro*: role of RNA sequences and the viral replicase. *EMBO J.* 17, 2392-2403.

Nguyen, Q. V., Boo, K. H., Sun, H. J., Cao, D. V., Lee, D., Ko, S. H., Kang, S., Yoon, S., Kim, S. C., Park, S. P., Riu, K. Z., and Lee, D. S. (2013). Evaluation of factors influencing *Agrobacterium*-mediated spinach

transformation and transformant selection by EGFP fluorescence under low-selective pressure. *In Vitro Cell Dev Biol Plant*. 49, 498-509.

Niu, Q. W., Lin, S. S., Reyes, J. L., Chen, K. C., Wu, H. W., Yeh, S. D., and Chua, N. H. (2006). Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat Biotechnol*. 24, 1420-1428.

Okazaki, Y., Takahata, S., Hirakawa, H., Suzuki, Y., and Onodera, Y. (2019). Molecular evidence for recent divergence of X- and Y-linked gene pairs in *Spinacia oleracea* L. *PLoS One*. 14, e0214949.

Onodera, Y., Yonaha, I., Masumo, H., Tanaka, A., Niikura, S., Yamazaki, S., and Mikami, T. (2011). Mapping of the genes for dioecism and monoecism in *Spinacia oleracea* L.: evidence that both genes are closely linked. *Plant Cell Rep*. 30, 965-971.

Onodera, Y., Yonaha, I., Niikura, S., Yamazaki, S., and Mikami, T. (2008). Monoecy and gynomonoecy in *Spinacia oleracea* L.: Morphological and genetic analyses. *Sci Hortic*. 118, 266-269.

Otagaki, S., Arai, M., Takahashi, A., Goto, K., Hong, J. S., Masuta, C. and Kanazawa, A. (2006). Rapid induction of transcriptional and post-transcriptional gene silencing using a novel *Cucumber mosaic virus* vector. *Plant Biotechnol*. 23, 259-265.

Pacak, A., Geisler, K., Jørgensen, B., Barciszewska-Pacak, M., Nilsson, L., Nielsen, T. H., Johansen, E., Grønlund, M., Jakobsen, I., and Albrechtsen, M. (2010). Investigations of barley stripe mosaic virus as a gene silencing vector in barley roots and in Brachypodium distachyon and oat. *Plant methods*. 6, 26.

Palanichelvam, K., Cole, A. B., Shababi, M., and Schoelz, J. E. (2000). Agroinfiltration of *Cauliflower mosaic virus* gene VI elicits hypersensitive response in *Nicotiana* species. *Mol Plant Microbe Interact*. 13, 1275-1279.

Pandey, P., Choudhury, N. R., and Mukherjee, S. K. (2009). A geminiviral amplicon (VA) derived from *Tomato leaf curl virus* (ToLCV) can replicate in a wide variety of plant species and also acts as a VIGS vector. *Virol J*. 6, 152.

Pantaleo, V., Szittya, G., and Burgyán, J. (2007). Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. *J Virol*. 81, 3797-3806.

Peele, C., Jordan, C. V., Muangsan, N., Turnage, M., Egelkrout, E., Eagle, P., Hanley-Bowdoin, L., and Robertson, D. (2001). Silencing of a meristematic gene using geminivirus-derived vectors. *Plant J.* 27, 357-366.

Peng, Y., Kadoury, D., Gal-On, A., Huet, H., Wang, Y., and Raccah, B. (1998). Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions. *J Gen Virol.* 79, 897-904.

Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L., and Poethig, R. S. (2004). SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis*. *Genes Dev.* 18, 2368-2379.

Pflieger, S., Richard, M. M. S., Blanchet, S., Meziadi, C., and Geffroy, V. (2013). VIGS technology: An attractive tool for functional genomics studies in legumes. *Funct Plant Biol.* 40, 1234-1248.

Pirone, T. P., and Blanc, S. (1996). Helper-dependent vector transmission of plant viruses. *Annu Rev Phytopathol.* 34, 227-247.

Plisson, C., Drucker, M., Blanc, S., German-Retana, S., Le Gall, O., Thomas, D., and Bron, P. (2003). Structural characterization of HC-Pro, a plant virus multifunctional protein. *J Biol Chem.* 278, 23753-23761.

Qu, J., Ye, J., and Fang, R. (2007). Artificial microRNA-mediated virus resistance in plants. *J Virol.* 81, 6690-6699.

Ratcliff, F., Martin-Hernandez, A. M., and Baulcombe, D. C. (2001). *Tobacco rattle virus* as a vector for analysis of gene function by silencing. *Plant J.* 25, 237-245.

Rodríguez-Negrete, E. A., Carrillo-Tripp, J., and Rivera-Bustamante, R. F. (2009). RNA silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. *J Virol.* 83, 1332-1340.

Roossinck, M. J. (2002). Evolutionary history of *Cucumber mosaic virus* deduced by phylogenetic analyses. *J Virol.* 76, 3382-3387.

Rosso, M. N., Dubrana, M. P., Cimbolini, N., Jaubert, S., and Abad, P. (2005). Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. *Mol Plant Microbe Interact.* 18, 615-620.

Rubio-Somoza, I., Cuperus, J. T., Weigel, D., and Carrington, J. C. (2009). Regulation and functional specialization of small RNA–target nodes during plant development. *Curr Opin Plant Biol.* 12, 622-627.

Ruiz, M. T., Voinnet, O., and Baulcombe, D. C. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell.* 10, 937-946.

Sanford, J. C., and Johnston, S. A. (1985). The concept of parasite-derived resistance—deriving resistance genes from the parasite's own genome. *J Theor Biol.* 113, 395-405.

Sather, D. N., Jovanovic, M., and Golenberg, E. M. (2010). Functional analysis of B and C class floral organ genes in spinach demonstrates their role in sexual dimorphism. *BMC Plant Biol.* 10, 46.

Scofield, S. R., and Nelson, R. S. (2009). Resources for virus-induced gene silencing in the grasses. *Plant Physiol.* 149, 152-157.

Senda, M., Masuta, C., Ohnishi, S., Goto, K., Kasai, A., Sano, T., Hong, J. S., and MacFarlane, S. (2004). Patterning of virus-infected *Glycine max* seed coat is associated with suppression of endogenous silencing of chalcone synthase genes. *Plant Cell.* 16, 807-818.

Shen, W., Yang, G., Chen, Y., Yan, P., Tuo, D., Li, X., and Zhou, P. (2014). Resistance of non-transgenic papaya plants to papaya ringspot virus (PRSV) mediated by intron-containing hairpin dsRNAs expressed in bacteria. *Acta Virol.* 58, 261-266.

Sherry, R. A., Eckard, K. J., and Lord, E. M. (1993). Flower development in dioecious *Spinacia oleracea* (*Chenopodiaceae*). *Am J Bot.* 80, 283-291.

Shiboleth, Y. M., Haronsky, E., Leibman, D., Arazi, T., Wassenegger, M., Whitham, S. A., Gaba, V., and Gal-On, A. (2007). The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. *J Virol.* 81, 13135-13148.

Shimada, S., Takahashi, K., Sato, Y., and Sakuta, M. (2004). Dihydroflavonol 4-reductase cDNA from non-anthocyanin-producing species in the Caryophyllales. *Plant Cell Physiol.* 45, 1290-1298.

Simón-Mateo, C., and García, J. A. (2006). MicroRNA-guided processing impairs *Plum pox virus* replication, but the virus readily evolves to escape this silencing mechanism. *J Virol.* 80, 2429-2436.

Soitamo, A. J., Jada, B., and Lehto, K. (2011). HC-Pro silencing suppressor significantly alters the gene expression profile in tobacco leaves and flowers. *BMC Plant Biol.* 11, 68.

Sun, L., Lin, C., Du, J., Song, Y., Jiang, M., Liu, H., Zhou, S., Wen, F., and Zhu, C. (2016). Dimeric artificial microRNAs mediate high resistance to RSV and RBSDV in transgenic rice plants. *Plant Cell Tissue Organ Cult.* 126, 127-139.

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N., and Takanami, Y. (1991). Functional analysis of deletion mutants of cucumber mosaic virus RNA 3 unsing an in vitro transcription system. *Virology.* 183, 106-113.

Takaki, F., Sano, T., and Yamashita, K. (2006). The complete nucleotide sequence of attenuated onion yellow dwarf virus: a natural poyvirus deletion mutant lacking the N-terminal 92 amino acids of HC-Pro. *Arch Virol.* 151, 1439-1445.

Takaki, F., Sano, T., Yamashita, K., Fujita, T., Ueda, K., and Kato, T. (2005). Complete nucleotide sequences of attenuated and severe isolates of *Leek yellow stripe virus* from garlic in northern Japan: Identification of three distinct virus types in garlic and leek world-wide. *Arch Virol.* 150, 1135-1149.

Tamilarasan, S., and Rajam, M. V. (2013). Engineering crop plants for nematode resistance through host-derived RNA interference. *Cell Dev Biol.* 2, 114.

Tao, X., and Zhou, X. (2004). A modified viral satellite DNA that suppresses gene expression in plants. Plant J. 38, 850-860.

Tasaki, K., Terada, H., Masuta, C., and Yamagishi, M. (2016). Virus induced gene silencing (VIGS) in *Lilium leichtlinii* using the *Cucumber mosaic virus* vector. *Plant Biotechnol.* 33, 373-381.

Tenllado, F., and Díaz-Ruíz, J. R. (2001). Double-stranded RNA-mediated interference with plant virus infection. *J Virol.* 75, 12288-12297.

Tenllado, F., Llave, C., and Díaz-Ruíz, J. R. (2004). RNA interference as a new biotechnological tool for the control of virus diseases in plants. *Virus Res.* 102, 85-96.

Tenllado, F., Martínez-García, B., Vargas, M., and Díaz-Ruíz, J. R. (2003). Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. *BMC Biotechnol.* 3, 3.

Turnage, M. A., Muangsan, N., Peele, C. G., and Robertson, D. (2002). Geminivirus-based vectors for gene silencing in Arabidopsis. *Plant J.* 30, 107-114.

Tzean, Y., Lee, M. C., Jan, H. H., Chiu, Y. S., Tu, T. C., Hou, B. H., Chen, H. M., Chou, C. N., and Yeh, H. H. (2019). *Cucumber mosaic virus*-induced gene silencing in banana. *Sci Rep.* 9, 11553.

Urwin, P. E., Lilley, C. J., and Atkinson, H. J. (2002). Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. *Mol Plant Microbe Interact.* 15, 747-752.

Valli, A. A., Gallo, A., Rodamilans, B., López-Moya, J. J., and García, J. A. (2018). The HCPro from the *Potyviridae* family: an enviable multitasking Helper Component that every virus would like to have. *Mol Plant Pathol.* 19, 744-763.

Várallyay, É., and Havelda, Z. (2013). Unrelated viral suppressors of RNA silencing mediate the control of ARGONAUTE1 level. *Mol Plant Pathol.* 14, 567-575.

Vaucheret, H. (2006). Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev.* 20, 759-771.

Verma, R. K., Mishra, R., Petrov, N. M., Stoyanova, M., Stoev, A., Bakardjieva, N., and Gaur, R. K. (2015). Molecular characterization and recombination analysis of an Indian isolate of *Onion yellow dwarf virus*. *Eur J Plant Pathol.* 143, 437-445.

Vu, T. V., Choudhury, N. R., and Mukherjee, S. K. (2013). Transgenic tomato plants expressing artificial

microRNAs for silencing the pre-coat and coat proteins of a begomovirus, *Tomato leaf curl New Delhi virus*, show tolerance to virus infection. *Virus Res.* 172, 35-45.

Wang, M. B., Masuta, C., Smith, N. A., and Shimura, H. (2012). RNA silencing and plant viral diseases. *Mol Plant Microbe Interact.* 25, 1275-1285.

Wang, R., Yang, X., Wang, N., Liu, X., Nelson, R. S., Li, W., Fan, Z., and Zhou, T. (2016). An efficient virus-induced gene silencing vector for maize functional genomics research. *Plant J.* 86, 102-115.

Wang, X. B., Jovel, J., Udomporn, P., Wang, Y., Wu, Q., Li, W. X., Gasciolli, V., Vaucheret, H., and Ding, S. W. (2011b). The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative argonautes in *Arabidopsis thaliana*. *Plant Cell.* 23, 1625-1638.

Wang, X. B., Wu, Q., Ito, T., Cillo, F., Li, W. X., Chen, X., Yu, J. L. and Ding, S. W. (2010). RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A.* 107, 484-489.

Wang, Y., Zhang, H., Li, H., and Miao, X. (2011a). Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. *PLoS One.* 6, e18644.

Wen, R. H., Khatabi, B., Ashfield, T., Saghai Maroof, M. A., and Hajimorad, M. R. (2013). The HC-Pro and P3 cistrons of an avirulent *Soybean mosaic virus* are recognized by different resistance genes at the complex *Rsv1* locus. *Mol Plant Microbe Interact.* 26, 203-215.

Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M., Rouse, D. T., Liu, Q., Gooding, P. S., Singh, S. P., Abbott, D. Stoutjesdijk, P. A., Robinson, S. P., Gleave, A. P., Green, A. G., and Waterhouse, P. M. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant Journal.* 27, 581-590.

Westwood, J. H., Lewsey, M. G., Murphy, A. M., Tungadi, T., Bates, A., Gilligan, C. A., and Carr, J. P. (2014). Interference with jasmonic acid-regulated gene expression is a general property of viral suppressors of RNA silencing but only partly explains virus-induced changes in plant–aphid interactions. *J Gen Virol.* 95, 733-739.

White, K. A., and Morris, T. J. (1995). RNA determinants of junction site selection in RNA virus recombinants

and defective interfering RNAs. *RNA.* 1, 1029-1040.

Wierzbicki, A. T., Haag, J. R., and Pikaard, C. S. (2008). Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. *Cell.* 135, 635-648.

Williamson, V. M., and Kumar, A. (2006). Nematode resistance in plants: the battle underground. *Trends Genet.* 22, 396-403.

Wingard, S. A. (1928). Hosts and symptoms of ring spot, a virus disease of plants. *J Agric Res.* 37(3), 127-153.

Wu, H. W., Lin, S. S., Chen, K. C., Yeh, S. D., and Chua, N. H. (2010a). Discriminating mutations of HC-Pro of *Zucchini yellow mosaic virus* with differential effects on small RNA pathways involved in viral pathogenicity and symptom development. *Mol Plant Microbe Interact.* 23, 17-28.

Wu, Q., Wang, X., and Ding, S. W. (2010b). Viral suppressors of RNA-based viral immunity: host targets. *Cell Host Microbe.* 8, 12-15.

Xie, Z., Allen, E., Fahlgren, N., Calamar, A., Givan, S. A., and Carrington, J. C. (2005). Expression of *Arabidopsis MIRNA* genes. *Plant Physiol.* 138, 2145-2154.

Xu, C., Jiao, C., Sun, H., Cai, X., Wang, X., Ge, C., Zheng, Y., Liu, W., Sun, X., Xu, Y., Deng, J., Zhang, Z., Huang, S., Dai, S., Mou, B., Wang, Q., Fei, Z., and Wang, Q. (2017). Draft genome of spinach and transcriptome diversity of 120 *Spinacia* accessions. *Nat Commun.* 8, 15275.

Xu, C., Jiao, C., Zheng, Y., Sun, H., Liu, W., Cai, X., Wang, X., Liu, S., Xu, Y., Mou, B., Dai, S., Fei, Z., and Wang, Q. (2015). *De novo* and comparative transcriptome analysis of cultivated and wild spinach. *Sci Rep.* 5, 17706.

Xu, K., Huang, X., Wu, M., Wang, Y., Chang, Y., Liu, K., Zhang, J., Zhang, Y., Zhang, F., Yi, L., Li, T., Wang, R., Tan, G., and Li, C. (2014). A rapid, highly efficient and economical method of *Agrobacterium*-mediated *in planta* transient transformation in living onion epidermis. *PLoS One.* 9, e83556.

Yamamoto, K., Oda, Y., Haseda, A., Fujito, S., Mikami, T., and Onodera, Y. (2014). Molecular evidence that the

genes for dioecism and monoecism in *Spinacia oleracea* L. are located at different loci in a chromosomal region. *Heredity.* 112, 317-324.

Yang, D. L., Zhang, G., Tang, K., Li, J., Yang, L., Huang, H., Zhang, H., and Zhu, J. K. (2016). Dicer-independent RNA-directed DNA methylation in *Arabidopsis*. *Cell Res.* 26, 66-82.

Yang, Z., and Li, Y. (2018). Dissection of RNAi-based antiviral immunity in plants. *Curr Opin Virol.* 32, 88-99.

Yin, G., Sun, Z., Liu, N., Zhang, L., Song, Y., Zhu, C., and Wen, F. (2009). Production of double-stranded RNA for interference with TMV infection utilizing a bacterial prokaryotic expression system. *Appl Microbiol Biotechnol*. 84, 323-333.

Yoshida, N., Shimura, H., Yamashita, K., Suzuki, M., and Masuta, C. (2012). Variability in the P1 gene helps to refine phylogenetic relationships among leek yellow stripe virus isolates from garlic. *Arch Virol.* 157, 147-153.

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H., and Smagghe, G. (2013). Delivery of dsRNA for RNAi in insects: an overview and future directions. *Insect Sci.* 20, 4-14.

Zhang, X., Li, H., Zhang, J., Zhang, C., Gong, P., Ziaf, K., Xiao, F., and Ye, Z. (2011). Expression of artificial microRNAs in tomato confers efficient and stable virus resistance in a cell-autonomous manner. *Transgenic Res.* 20, 569-581.

Zhong, X., Du, J., Hale, C. J., Gallego-Bartolome, J., Feng, S., Vashisht, A. A., Chory, J., Wohlschlegel, J. A., Patel, D. J., and Jacobsen, S. E. (2014). Molecular mechanism of action of plant DRM *de novo* DNA methyltransferases. *Cell*. 157, 1050-1060.

Zhou, T., Murphy, A. M., Lewsey, M. G., Westwood, J. H., Zhang, H. M., Gonzalez, I., Canto, T., 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. *J Gen Virol*. 95, 1408-1413.

Zotti, M., dos Santos, E. A., Cagliari, D., Christiaens, O., Taning, C. N. T., and Smagghe, G. (2018). RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. *Pest Manag Sci.* 74, 1239-1250.

ACKNOWLEDGMENT

It is a great honor for me to finally complete my Ph. D course in Graduate School of Agriculture, in Hokkaido University. My long life as a student becomes over. However, I always cherish my mental attitude as a student and a prospective young scientist pursuiting research on plant virus with a unchanged passion.

First, I want to express profound gratitude to my supervisor, Professor Chikara Masuta in Pathogen-Plant Interaction Laboratory for his unstinted support on my researches past 3 and a half years. His infinite passions and great ideas on research of plant virus always provide me motivations with vision. He always waited my visit in his office and discussed every my result of experiments with constructive criticisms. I am greatly indebted and never forget his favor.

I also truely grateful to Professor Takeshi Matsumura in National Institute of Advanced Industrial Science and Technology (AIST) for his detailed review on my thesis with constructive criticisms. His expert advices give me chance to learn how experiments should be designed and conducted. I would like to thank Lecturer Hanako Shimura from Laboratory of Crop Physiology for her close review on my thesis and for guiding several experiments with kind advices which helpful my research.

I speicially thank Lecturer Tsuyoshi Inukai from Laboratory of Cell Biology and Manipulation for teaching me several important experiments required for my research and helping me to prepare experimental equipments. I would like to extend my sincere thanks to Lecturer Tatsuji Hataya and Kenji Nakahara for giving me support on experimental equipments and constructive advices on plant virology. I always appreciate my previous advisor, Professor Kook-Hyung Kim from Plant Virology Laboratory in Seoul National University for his advice

on my vision as a prospective researcher of plant virology. I also thank all my lab members, Dr. Matsunaga, Wikum, Kawano, Takahashi, Kuribara, Kuroiwa and Mori, and I have been pleasure to work with them. I also appreciate my friends Dong-Geun Lee, Hyuksung Yoon and Kyoung-Min Lee.

I always thank my parents for supporting everything in my life and cheering me, and want to my deepest gratitude to my wife and my precious two babies.