Review

Resistance and Tolerance to Viroid Infection

Takashi Naoi[1] *, Shifang Li[2]*, Teruo Sano[3]

1. University of Tsukuba, Faculty of Environmental and Life Science, Tsukuba 305-8577, Japan.
2. State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China.
3. Hirosaki university, Faculty of Agriculture and Life Science, Hirosaki 036-8561, Japan.

* Correspondence: naoi.takashi.gn@u.tsukuba.ac.jp

Abstract: Viroids are known the smallest plant pathogens, and although their genome sequences do not encode proteins, they can cause disease in economically important crops. In order to control viroid diseases and mitigate their damage, genetic resources used for breeding of the viroid-resistant crop have been searched, but the practical resistant trait has not been found in almost all viroid-crop combinations, as well as the tolerant trait. Due to the difficulty in exploiting naturally occurring resistance or tolerance, various effective strategies have been devised to control viroid diseases using non-transforming or transforming techniques. Meanwhile, extensive findings related to viroid resistance and tolerance may lead to confer resistance or tolerance to viroid infection by combining with the recently emerged new technologies (e.g., spray-induced gene silencing and genome-editing technologies), which are believed to be more environmentally viable and acceptable to the general public than previously reported approaches. In particular, some genome-modified crops produced by the latter technology are already on the market. In this review, we comprehensively summarize the current status about investigation of naturally occurring genetic traits for viroid resistance and tolerance, accumulating knowledge about host factors involved in viroid pathogenicity, and various basic technologies developed to try to possible viroid disease control strategies. Furthermore, we discuss prospects and challenges for the achievement of more effective, practical, and sustainable disease control of viroid.

Keywords: viroid; resistance; tolerance; RNA silencing; plant hormone; spray-induced gene silencing; genome editing

1. Introduction

Viroids are known as the smallest plant pathogens, are single-stranded (ss) circular RNA molecules ranging from 246 to 434 nucleotides in length, and do not encode any proteins in their genome sequences (Sano, 2021). In spite of their non-coding nature, they autonomously replicate by using host factors, including an enzyme and a transcriptional factor (Mühlbach and Sänger, 1979; Warrilow and Symons, 1999; Navarro et al., 2000; Rodio et al., 2007; Wang et al., 2016; Gas et al., 2008; Nohales et al., 2012a; Nohales et al., 2012b). Viroids are classified into two families, *Pospiviroidae* and *Avsunviroidae*, according to their biological and structural characteristics such as replication mode and site, highly structure, and presence or absence of either of the central conserved region or the hammerhead ribozyme motif in its genome (Di Serio et al., 2014; Kovalskaya and Hammond, 2014).

Highly base-paired stem-loop structures and partially double-stranded (ds) replicative intermediates of viroid, formed transiently during replication, are potential inducers and targets of the host anti-virus/viroid RNA silencing mechanism which specifically cleaves foreign invaders such as viruses and transposons in a sequence-dependent manner (Kovalskaya and Hammond, 2014). In infected host cells, viroids are processed by an RNase III-like enzyme called Dicer-like (DCL) into 21–24 nucleotides (nt) small fragments (Itaya et al., 2001; Papaefthimiou et al., 2001; Martínez de Alba et al., 2002), called viroid-

 doi:10.20944/preprints202211.0169.v1

specific small RNA (vd-sRNA). Vd-sRNAs are recruited by RNA-induced silencing complex (RISC) including Argonaute (AGO) (Minoia et al., 2014), which is an RNase H-like enzyme, and RISC directs the cleavage of complementary target viroid RNA according to the incorporated vd-sRNA sequence. In general, RNA silencing is maintained and even amplified by RNA-dependent RNA polymerases (RDRs). RDRs convert RNAs with aberrant features into dsRNAs, and then, they are processed again by DCLs into small RNAs called secondary small interfering (si) RNAs. This process has not yet been clarified in the case of viroid infection.

Recently, it has become clear that RNA silencing is involved not only in defense responses against viroid but also in symptom expression. In several studies, it was indicated that vd-sRNAs processed by DCLs target host genes according to sequence homology and suppress their expression, resulting the development of disease symptoms (Navarro et al., 2012; Adkar-Purushothama et al., 2015; Adkar-Purushothama et al., 2017; Bao et al., 2019; Eamens et al., 2014). Post-transcriptional suppression of target genes by over-expression of endogenous micro (mi) RNAs in response to viroid infection, and consequent disruption of normal metabolic pathways, has also been implicated in symptom development (Suzuki et al., 2019; Fujibayashi et al., 2021).

In general, plant innate immune responses involve the fluctuation of several genes, such as mitogen-activated protein kinase, pathogenesis-related proteins, resistant proteins, and defense-related transcription factors, which is observed in viroid infection (Zheng et al., 2017; Góra-Sochacka et al., 2019; Thibaut and Bragard, 2018; Štajner et al., 2019; Wang et al., 2019; Xia et al., 2017; Więsyk et al., 2018; Navarro et al., 2021). These findings suggest that viroid can activate pathogen-associated molecular pattern (PAMP)–triggered immunity (PTI) and effector-triggered immunity (ETI). It is not clear how viroid is recognized as a genuine PAMP or effector in these plant immunity mechanisms, although it is possible to be associated with RNA silencing mechanism because distinct two plant defense pathways, dsRNA-mediated antiviral silencing and PTI, may be functionally linked (Niehl et al., 2016; Niehl and Heinlein, 2019). Meanwhile, plant hormones, such as salicylic acid, are thought to be involved in the plant innate immune responses as signaling substances, and the expression of plant hormone-related genes is significantly changed in viroid-infected host plants (Owens et al., 2012; Takino et al., 2019; Wang et al., 2019; Štajner et al., 2019; Olivier and Bragard, 2018; Góra-Sochacka et al., 2019; Zheng et al., 2017; Xia et al., 2017; Wang et al., 2011; Więsyk et al., 2018). At the same time, the fluctuation in plant hormone metabolism have been associated with symptom expression of viroid (Owens et al., 2012; Takino et al., 2019 Štajner et al., 2019; Bao et al., 2019; Hammond and Zhao, 2009; López-Gresa et al., 2016; Prol et al., 2020; Xin-xi et al., 2011; Wang et al., 2011).

Furthermore, one of the interactions between pathogens and host plants is the resistant responses (host resistance). “True resistance” is controlled by the resistant gene and characterized by containing the pathogen in the point of pathogen penetration through the hypersensitive cell-death responses (HR), and this is a part of defense responses in ETI (Muller and Haigh, 1953; Jones and Dangl, 2006; Balint-Kurti, 2019) On the other hand, in virus resistance, cell death is not necessarily a vital component of resistant mechanisms (Bendahmane et al., 1999; Komatsu et al., 2010), and the resistance, controlled by the resistant gene and characterized by a lack of symptoms and limited or lack of pathogen replication and pathogen spread, is called as “extreme resistance” (Bendahmane et al., 1999; de Ronde et al., 2014). Additionally, the presence of resistant components (eg, the plants latently infected with virus) either separately or in combination in a field may confer “field resistance” to a population of infectible individuals (Muller and Haigh, 1953; Cooper and Jones, 1982). Alternatively, the resistance shown by an entire plant species to all genetic variants of a pathogen is known as “non-host resistance” (Niks and Marcel, 2009), and is nearly synonymous with “immunity”. In some case, immunity may include strong host resistance. In the case of viroid, host resistance has not been classified in detail as described above, because viroid resistance has rarely been reported so far. Hence, in

 doi:10.20944/preprints202211.0169.v1

this review, "resistance" is broadly defined as the property of the host plant to inhibit systemic viroid infection unless otherwise specified. In contrast, "susceptible" refers to the property of the host plant to become systemically infected with viroid. Meanwhile, the property of host plants which exhibit the only mild symptoms or are symptomless even though the viroid systemically infects, is known as "tolerance" (so-called disease resistance). On the contrary, "sensitive" refers to the property of the host plant to develop disease symptoms during viroid infection. Tolerance is more general in viroid-host interactions and is the only genetic trait that can be used for viroid disease control in many crops, although the detailed mechanism of viroid tolerance remains unclear.

More than 25 diseases induced by viroid infection have been reported in more than 15 crop species, including vegetables, fruits, trees, and flowers (Sano, 2021), which include economically important crops. Therefore, resistance to prevent viroid infection has been searched for, but so far, no resistance that can be used for cross-breeding have been reported for most crop-viroid combinations, as well as tolerance to suppress symptom expression. Little is known about the mechanism of viroid resistance, but it is probably due to inhibition of viroid replication or cell-to-cell/systemic movement, given the example of virus resistance. Due to the difficulty in exploiting natural resistance and tolerance, various strategies have been devised to control viroid diseases using non-transforming or transforming techniques. These strategies are diverse, and the most confer viroid resistance or tolerance into plants by inhibiting infection and accumulation, through degradation of viroid (Flores et al., 2017). In addition, deepening our understanding of the mechanisms, such as RNA silencing and changes in plant hormone metabolism, involved in viroid resistance and tolerance, and disease development is important because it may lead to the development of new control strategies for viroid diseases.

In this review, we comprehensively summarize about not only the viroid resistance and tolerance, and the various strategies that can confer resistance or tolerance to plants, but also the relevant findings possibly applied to disease control of viroid. Namely, we describe search and analysis of viroid-resistant and -tolerant genetic resources (chapter 2), findings on the mechanisms involved in viroid resistance/tolerance and disease development (chapter 3), and diverse strategies to induce viroid resistance (chapter 4). Furthermore, we discuss prospects and challenges for the achievement of more effective and practical control of viroid disease in chapter 5.

2. Current status of search and analysis of viroid-resistant and -tolerant genetic resources

In economically important crops, viroid-resistant and tolerant genetic resources that can be used for crop breeding have been explored, but have been found, scarcely. In this section, we describe the findings on resistance or tolerance to each viroid, reported so far**.**

2-1. Chrysanthemum stunt viroid

The only viroid resistance to render systemic viroid infection impossible, reported in cultivated crops is chrysanthemum stunt viroid (CSVd) resistance in chrysanthemums (*Chrysanthemum* × *morifolium*), and this is the only example of multiple analyzes of viroid resistance (Nabeshima et al., 2018). Screening for CSVd resistance in 67 self-pollinated progenies of the chrysanthemum cultivar 'Utage', whose CSVd concentration increased slowly, found three plants exhibiting strong CSVd resistance. In these plants, CSVd was detected locally in the youngest expanded leaves, or hardly detected in the whole body (Omori et al., 2009). An *in vitro* screening method, in which leaf primordia free shoot apical meristems (SAMs) were attached to the root tips of CSVd-infected chrysanthemum roots and analyzed for CSVd infection in newly developed leaves, showed that CSVd-resistant plants were classified into the two types, a CSVd-uninfected or slow titer-increasing type and a CSVd-disappearance type. Chrysanthemum cultivars 'Mari-Kazaguruma' or 'Seino-Issei', respectively classified into a slow titer-increasing type, or a CSVd-uninfected

 doi:10.20944/preprints202211.0169.v1

or slow titer-increasing type, were used for subsequent grafting assay. As a result, in the cultivar 'Seino-Issei', CSVd was detected temporarily after grafting and titers decreased in the newly expanded leaves, while CSVd was not detected in a shoot tip and detected in a young leaf at a low level. In addition, CSVd-infected 'Seino-Issei' plants did not exhibit symptoms. Namely, this cultivar has the characteristics of not only a CSVd-uninfected type but also a CSVd-disappearance type. On the other hand, in the cultivar 'Mari-Kazaguruma', CSVd was not detected in the youngest leaves at 2 months after-grafting, but CSVd accumulation had slightly increased by six months, accompanied by no symptoms. Namely, this cultivar has the characteristics of a slow titer-increasing type. These results suggest the existence of more than one type of CSVd resistance in chrysanthemums (Nabeshima et al., 2012). CSVd infection was not completely inhibited, CSVd accumulation gradually increased, and the "undetectable-area" characteristic of 'Seino-Issei' could not observed in 'Mari-Kazaguruma', suggesting that CSVd resistance in 'Mari-Kazaguruma' is a characteristic close to tolerance to viroid infection.

Further analyses by graft- and agro-inoculation revealed that systemic infection with CSVd was not observed in 'Mari-Kazaguruma' chrysanthemums even though a large amount of CSVd accumulated in inoculated leaves, suggesting that cell-to-cell movement but not replication of CSVd in leaves is associated with CSVd resistance (Nabeshima et al., 2017). In the CSVd-resistant cultivars 'Mari-Kazaguruma' and 'Seino-Issei', and the self-pollinated progeny exhibiting CSVd resistance of the cultivar 'Utage', absence of CSVd accumulation in shoot apex was observed even when CSVd was continuously provided from the CSVd-infected rootstock (Nabeshima et al., 2012; Omori et al., 2009). These findings suggest that the mechanisms related to suppression of viroid invasion into the SAMs, such as RNA silencing associated with RDR6 and callose deposition, reported previously (Di Serio et al., 2010; Naoi et al., 2020; Zhang et al., 2015), may be contribute to CSVd resistance. However, this possibility has not been validated, and further analyses are required in the future.

Regarding the genetic analysis of CSVd resistance, it is difficult to analyze because chrysanthemums are the high-order polyploid species, and many aspects remain to be elucidated. When two susceptible cultivars 'Sei Elza' and 'Anri' were crossed as the pollen parents with the resistant cultivar 'Okayama Heiwa', which was the female parent, only a portion of the first filial generation (F1) plants demonstrated resistance to CSVd infection (Matsushita et al., 2012; Nabeshima et al., 2018). In addition, F1 populations generated by mating different CSVd-resistant cultivars involved CSVd-resistant and -susceptible plants (Torata et al., 2018). On the other hand, it has been recently reported that some strains of *C. seticuspe*, a diploid wild chrysanthemum, have CSVd resistant properties (Matsushita and Osaka, 2019), expected the further progress in genetic analyses of CSVd resistance by using *C. seticuspe* instead of *C. morifolium*.

2-2. Citrus exocortis viroid, hop stunt viroid, and other citrus viroids

Partial resistance to viroid capable of infecting citrus was observed in *Eremocitrus glauca* and *Microcitrus australis*, one of the members of the subfamily *Aurantioideae* in the family *Rutaceae*. The investigation of accumulation and movement of six citrus viroids, citrus exocortis viroid (CEVd), hop stunt viroid (HSVd), citrus bent leaf viroid, citrus dwarfing viroid, citrus bark cracking viroid, and citrus viroid V, in *E. glauca* and *M. australis* revealed that these plants either do not replicate/accumulate viroids or are very inefficient viroid hosts. However, downward and upward movement of viroids to grafted citrus plants, in which replication and accumulation of viroid occurs efficiently, was not impaired (Hashemian et al., 2010).

Since apricots (*Prunus armeniaca*) are susceptible to viruses and viroids including HSVd, the resistance/susceptibility of 26 Mediterranean and North American apricot cultivars to HSVd infection was investigated in controlled greenhouse conditions (Rubio et al., 2015). However, no aplicot cultivars exhibiting resistance to HSVd infection were found.

 doi:10.20944/preprints202211.0169.v1

2-3. potato spindle tuber viroid

Potato spindle tuber viroid (PSTVd) is one of the most studied viroids for resistance and tolerance to viroid infection. In the early study to evaluate host reaction to PSTVd infection, local necrotic spots by the hypersensitive reaction, characteristically observed in virus-resistant plants, were observed in *Scopolia sinensis* (Singh, 1971); however, the response was observed only under the limited circumstances (Flores et al., 2017). PSTVd resistance to sap inoculation was observed in some strains of wild potato *Solanum berthaultii*, although these failed to be resistant after graft-inoculation (Singh and Slack, 1984; Singh, 1985). Moreover, *Solanum acaule* OCH 11603 was found to be resistant to mechanical inoculation with PSTVd, but was susceptible following agro-infection (Salazar et al., 1988; Kovalskaya and Hammond, 2014). These resistance to PSTVd observed in potato wild relatives was likely to be so-called "field resistance" rather than the resistance as plant immune responses (Salazar et al., 1988). PSTVd resistance of wild potato relatives is also reported in *S. guerreroense*, *S. etuberosum*, *S. sucrense*, and *S. chacoense*, and *S. stoloniferum* and several cultivars of potatoes (*S. tuberosum*) exhibited tolerance to PSTVd infection (Pfannenstiel et al., 1980; Palukaitis, 2012; Sofy et al., 2013). However, to date, these PSTVd resistance have not been introduced into susceptible cultivated potatoes (Palukaitis, 2014), as well as tolerance. Tomatoes (*S. lycopersicum*) are susceptible to pospiviroid infection and important crops for research on PSTVd and other pospiviroids. Most of tomato cultivars are sensitive to PSTVd infection, while some are tolerant, such as 'Moneymaker', 'Harzfeuer', 'Micro-Tom', and 'Tiny-Tim' (Wang et al., 2011; Owens et al., 2012; Matoušek et al., 2007; Naoi and Hataya, 2021). F1 hybrids generated by crossing between PSTVd-tolerant 'Harzfeuer' and -sensitive 'Rutgers' tomatoes exhibited severe symptoms, but their symptoms were weaker than those in 'Rutgers' tomatoes. This finding suggests that PSTVd-tolerance in the tomato cultivar 'Harzfeuer' may be an incompletely dominant trait or a quantitative trait controlled by multiple genes (Matoušek et al., 2007). PSTVd-resistance and -tolerance available for cross-breeding were investigated in wild tomato species. Although no host species exhibiting resistance to PSTVd were found, the wild tomato relatives *S. pimpinellifolium* LA0373 and *S. chmielewskii* LA1028 exhibited tolerance even to the lethal strain of PSTVd, showing few disease symptoms. In addition, PSTVd-tolerance was inherited to F1 hybrids produced by crossing a highly sensitive wild tomato (*S. lycopersicum* var. *cerasiforme* LA1286) with these wild species. Further analyses in PSTVd accumulation revealed that in these PSTVd-tolerant wild relatives and F1 hybrids, PSTVd accumulation was lower than in PSTVd-sensitive wild tomatoes, especially in the early stage of infection. The mechanism of PSTVd-tolerance and suppression of accumulation in these wild tomato relatives has not been analyzed in details, and further analysis is required in the future (Naoi and Hataya, 2021).

Taken together, tolerance is more general than resistance in the viroid-host interaction, but there are few examples in which the inheritance pattern has been clarified. Therefore, at present, CSVd resistance in chrysanthemums and PSTVd tolerance in wild tomato relatives are considered the only traits that can be used for generation of viroid-resistant or -tolerant crops by conventional cross-breeding, respectively.

3. Current state of research on mechanisms involved in viroid-resistance and -tolerance, and disease development.

Previous numerous studies on viroid resistance/tolerance and disease development suggest the existence of the complex mechanisms involving various pathways that respond or fluctuate during viroid infection. In this section, we focus on RNA silencing and alterations in plant hormone metabolism, as the mechanisms involved in viroid resistance/tolerance and disease development, especially.

3-1. Two different aspects of RNA silencing mechanism in viroid infection

 doi:10.20944/preprints202211.0169.v1

RNA silencing is one of the anti-virus/-viroid defense mechanisms, whereas it is involved in disease development of viroid. Tolerance to PSTVd infection in tomatoes may attributed to defense mechanisms including RNA silencing that inhibits PSTVd accumulation. PSTVd-tolerant 'Moneymaker' tomatoes, in which the key factors DCL2 and DCL4 in RNA silencing mechanism were down-regulated by RNA interference (i), exhibited lethal symptoms with systemic necrosis during PSTVd infection, other words, PSTVd-tolerance was impaired. In addition, over-expression of miR398 and miR398a-3p, suppression of the target *superoxide dismutase* (*SOD*) genes encoding reactive oxygen species (ROS) scavenging enzymes, and excessive ROS accumulation associated with the development of systemic necrosis were observed in these transgenic plants (Suzuki et al., 2019). Subsequent comparative analyses in the PSTVd-sensitive 'Rutgers' and -tolerant 'Moneymaker' tomatoes revealed that PSTVd accumulation in the pre-symptomatic early stage of infection was low in the PSTVd-tolerant cultivar, which is possibly associated with the tolerant cultivar-specific expression pattern of the *DCL* genes during PSTVd infection, especially up-regulation of *DCL4* mRNA. Additionally, over-expression of miR398 and miR398a-3p, suppression of the *SOD* genes, excessive ROS accumulation, and severe systemic necrosis were observed only in PSTVd-sensitive 'Rutgers' tomatoes during virulent PSTVd strain infection (Fujibayashi et al., 2021). These findings suggested that RNA silencing involving DCLs, which suppress PSTVd accumulation in the early stage of infection, and normal ROS scavenging mechanisms are important for induction of tolerance to PSTVd infection in tomatoes. RNA silencing may also contribute to CSVd resistance in chrysanthemums, because CSVd resistance is characterized by the absence of CSVd in SAM (Nabeshima et al., 2012; Omori et al., 2009), and, invasion of virus and viroid into the SAM is thought to be suppressed by the RNA silencing mechanism involving RDR1 or RDR6 (Schwach et al., 2005; Di Serio et al., 2010; Lee et al., 2016; Naoi et al., 2020). Meanwhile, viroid resistance strategies, which will be described later in Chapter 4, include some viroid-resistant strategies based on viroid-targeted RNA silencing to prevent viroid infection and accumulation.

Alternatively, vd-sRNAs, processed by DCLs, in RISC suppress the expression of host genes with complementary sequences to the vd-sRNAs, resulting disease development of viroid (Navarro et al., 2012; Adkar-Purushothama et al., 2015; Adkar-Purushothama et al., 2017; Bao et al., 2019; Eamens et al., 2014).

Taken together, RNA silencing has the two different aspects: it is important in suppressing viroid accumulation, symptom expression, and viroid invasion into SAM, whereas endogenous or exogenous sRNAs processed by DCLs can cause the silencing of host gene expression depending on their sequence homology, resulting viroid disease symptoms.

3-2. Fluctuation of plant hormone metabolism during viroid infection

Plant hormones regulate metabolic pathways associated with plant growth, development, and response to biotic and abiotic stress. Large-scale alterations in regulation of gene expression by plant defense responses induced during viroid infection disrupt normal plant hormone metabolism, which can be associated with viroid disease development (Owens et al., 2012; Takino et al., 2019 Štajner et al., 2019; Bao et al., 2019; Hammond and Zhao, 2009; López-Gresa et al., 2016; Prol et al., 2020; Xin-xi et al., 2011; Wang et al., 2011). In this section, we focus on plant hormones associated with anti-viroid defense responses and disease development, and discuss the possibility of suppressing disease development by altering hormone metabolism through hormone treatment, use of inhibitors, or regulating the expression of hormone metabolism-related genes.

3-2-1. Salicylic acid, Gentisic acid, Gibberellic acid, Brassinosteroid, and Auxin

Salicylic acid (SA) is known as one of the plant hormones which plays major roles in regulating plant defense responses against plant pathogens as well as abiotic stresses (Bari

 doi:10.20944/preprints202211.0169.v1

and Jones, 2009), and is crucial for plants to establish the resistance response in many plant-pathogen interactions (Delaney et al.,1994). Gentisic acid (GeA) is a metabolic derivative of SA and has been proposed as a signal molecule for plant defense responses in compatible non-necrotizing interactions (Bellés et al., 1999). Accumulation of SA and GeA were reported in CEVd-infected 'Rutgers' tomatoes (Bellés et al., 1999), and exogenous treatment of SA and GeA significantly delayed the disease development of CEVd in *Gynura aurantiaca*, resulting tolerance to CEVd infection (Campos et al., 2014). NahG tomato plants, expressing the *salicylate hydroxylase* gene from *Pseudomonas putida* and being unable to accumulate SA, exhibited severe symptoms during CEVd infection, and the hyper-sensitive phenotype of NahG plants against CEVd were rescued with the treatment of SA analogue benzothiadiazole, by reducing the titers, the disease development, and the severity at the beginning of CEVd infection (López-Gresa et al., 2016). SA or GeA treatment induced basal resistance of plants and gene expression of RNA silencing components, DCL2, RDR1, and RDR2 in tomatoes (López-Gresa et al., 2016; Campos et al., 2014), resulting delayed viroid accumulation and acquisition of tolerance to CEVd infection, probably. This hypothesis is also supported by the report that exogenous treatment with SA induced RDR1 expression, delayed PSTVd accumulation, and enhanced tolerance to PSTVd in 'Rutgers' tomatoes (Li et al., 2021).

Gibberellic acid (GA) is the plant hormone which affects stem elongation, germination, elimination of dormancy, flowering, sex expression, enzyme induction, and leaf and fruit senescence (Rodrigues et al., 2012). Relationships between GA metabolism and viroid infection in host plants have been studied in details primarily in combination with tomatoes or potatoes and PSTVd. A combination of microarray and large-scale RNA sequence analyses revealed that the expression of *GA20ox1* and *GA3β* genes, contribute to GA biosynthesis, was down-regulated in both PSTVd-infected 'Rutgers' and 'Moneymaker' tomatoes (Owens et al., 2012). In addition, expression analyses of the potential target genes for PSTVd-sRNAs in tomatoes revealed that expression of the *Gibberellin β-hydroxylase* gene, which is involved in GA biosynthesis, was reduced in PSTVd-infected 'Rutgers' tomatoes (Wang et al. 2011). Expression of the genes involved in GA biosynthesis and signaling was also suppressed in 'Rutgers' tomatoes infected with tomato planta macho viroid or Mexican papita viroid and in *Etrog citron* infected with CEVd, in which disease symptoms including stunting were observed (Aviña-Padilla et al., 2018; Vidal et al., 2003). On the other hand, in contrast, it is reported that expression of some genes involved in GA biosynthesis, including *GA20ox1*, was up-regulated in PSTVd-infected 'Rutgers' tomatoes (Więsyk et al., 2018). This contradiction suggests the existence of the complex feedback or feedforward regulation of biologically active GA levels, in which GA biosynthetic and inactivating enzymes are related, in viroid-infected plants (Yamaguchi, 2008; Więsyk et al., 2018). At least in potatoes, it has been clarified that PSTVd-sRNAs post-transcriptionally suppress the expression of TCP23 belonging to a group of transcription factor, which plays an important role in regulating plant growth and development, and disrupts GA metabolism, resulting the induction of specific disease symptoms observed in PSTVd infection. In addition, it was shown that exogenous treatment of GA recovered the disease symptom-like spindle tuber phenotype observed in transgenic potatoes in which the PSTVd-sRNA thought to target the sequence of *TCP23* mRNA was expressing as artificial-mi (ami) RNAs (Bao et al., 2019). Recovery of plants by means of exogenous GA treatment was also observed in the transgenic tobacco exhibiting PSTVd-induced symptom-like dwarf phenotype, in which the PKV (protein kinase viroid-induced) protein is over-expressing and the expression of *GA20x1* and *GA3β* genes was up-regulated (Hammond and Zhao, 2009).

Brassinosteroid (BR) is the plant hormone which regulates numerous developmental processes including root and shoot growth, vascular differentiation, fertility, flowering, and seed germination, as well as in responding to environmental stresses (Bajguz et al., 2020). Additionally, the crosstalk between BR and GA signaling pathways is supported

 doi:10.20944/preprints202211.0169.v1

by some evidences (Li and He, 2013). The *DWARF4* gene encoding cytochrome P450 protein, catalyzing the rate-limiting step of BR biosynthesis, were down-regulated in 'Rutgers' and 'Moneymaker' tomatoes during PSTVd infection. In 'Micro-Tom' tomatoes showing the dwarf phenotype, containing mutations affecting both BR biosynthesis and GA signaling (Martí et al. 2006) and exhibiting tolerance to PSTVd infection (Naoi and Hataya, 2021), exogenous BR treatment did not induce disease symptoms observed in PSTVd-infected 'Rutgers' tomatoes. Meanwhile, the treatment up-regulated several genes involved in responses to stress and other stimuli in PSTVd-infected 'Micro-Tom' tomatoes, but not non-hormone, GA, or BR+GA (Owens et al., 2012). In PSTVd-infected potatoes in which the suppression of TCP23 expression resulted in inhibition of GA metabolism and induction of disease symptoms, expression of the *DWARF1* and *Gibberellin 7-oxidase* genes was down-regulated in leaves and tubers (Bao et al., 2019; Katsarou et al., 2016). As described, although changes in the expression of BR metabolism-related genes during PSTVd infection have been reported, it is unclear whether the BR metabolism or the crosstalk between BR and GA signaling is associated with the induction or suppression of viroid symptoms. Thus, the effects of exogenous treatment of BR or inhibition of BR metabolism in tomatoes and potatoes, sensitive to PSTVd infection, on the development of PSTVd symptoms are currently unknown.

Auxin (AUX) is a key plant hormone to regulate nearly all aspects of plant growth and development, from embryogenesis to senescence (Li et al., 2016). AUX signaling contributes to promote virulence and cause disease in various pathogens, including tobacco mosaic virus (TMV) (Culver and Padmanabhan 2007). Flat-top symptoms observed in 'Rutgers' tomatoes infected with mutant IntU257A of PSTVd were shown to be correlated with suppressed cell proliferation associated with down-regulation of the *expansin (EXP) 2* gene (Qi and Ding, 2003). Expression of several *EXP* genes is down-regulated in 'Rutgers' tomatoes infected with severe strain of PSTVd, along with expression of most genes involved in AUX biosynthesis and signaling, for example, *AUX/IAA* (auxin-responsive protein IAA), *GH3* (auxin-responsive GH3 gene family), and *SAUR* (small auxin up-regulated RNA) (Więsyk et al., 2018). In rice, it has been reported that GH3 inhibits plant growth by suppressing genes, involved in AUX biosynthesis and signaling, and *EXP* genes (Ding et al., 2008; Domingo et al., 2009). These findings suggest the existence of a relationship between symptom expression and AUX metabolism during PSTVd infection in tomatoes. In addition, pathogenicity analyses in 'Rutgers' tomatoes using mutants between mild and intermediate strains of PSTVd indicated that the expression levels of all six *EXP* genes, including *EXP2*, were significantly reduced by PSTVd infection regardless of the severity of disease symptoms, except for that of the *EXP5* gene in mild strain infection (Kitabayashi et al., 2020). At present, the possibility that exogenous treatment of AUX can reduce disease symptoms such as flat-top in PSTVd-infected tomatoes has not been verified.

Taken together, these findings suggest that exogenous treatment of SA, GeA, or GA has the effect of alleviating the symptoms of viroid disease, that is, inducing tolerance to viroid infection, depending on the combination with the viroid and host plant.

3-2-2. Ethylene

Ethylene (ET) is a plant hormone which governs the development of leaves, flowers, and fruits, may also promote, inhibit, or induce senescence depending upon the ET levels, and plays major roles in regulating plant defense responses against plant pathogens, as well as abiotic stress (Konings and Jackson, 1979; Khan, 2005; Pierik et al., 2006, Iqbal et al., 2017; Bari and Jones, 2009). It has reported that ET production in tomato leaves, in which exhibit systemic symptoms during CEVd infection, is activated at the level of the production of ET precursor, 1-aminocyclopropane-l-carboxylic acid (Bellés et al., 1989). Furthermore, NahG tomatoes, SA accumulation is defective, exhibited hyper-sensitivity to CEVd and excessive accumulation of ET during CEVd infection, indicating a positive correlation between the accumulation of ET and the induction of CEVd symptoms (López-

 doi:10.20944/preprints202211.0169.v1

Gresa et al., 2016). In inoculation assays of CEVd, ethylene-insensitive *Never ripe* (*Nr*) tomato mutant exhibited more severe symptoms (hyper-sensitive to CEVd) and the higher level of ET emission than in wild-type. This result indicates that the expression of symptoms during CEVd infection may be caused by ET accumulation even in the absence of ET signaling (Prol et al., 2020). Meanwhile, it has been suggested that low-concentration ET rather than high-concentration may prevent viroid infection (Conejero and Granell, 1986). Based on these findings, a dual role of ET is proposed in defense responses to CEVd infection; low ET levels could delay disease development, while high ET levels may contribute to the severity of CEVd symptoms (Prol et al., 2020). It has also been suggested that ET plays a partial role in the disease development of tomato chlorotic dwarf viroid (TCDVd). The expression of disease symptoms was slightly suppressed in the *Nr* mutant compared with the wild-type, and the partial suppression was also observed in wild-type by means of the treatment with silver thiosulfate, an inhibitor of the reaction to ET (Xinxi et al., 2011). In PSTVd infection, the induction of ET-related genes has been reported in tomatoes (Wang et al., 2011; Owens et al., 2012), but a relationship with symptom expression has not been studied in details.

Taken together, it is suggested that the use of transformation techniques or inhibitors to suppress ET biosynthesis or signaling may suppress the disease development of CEVd or TCDVd in tomatoes.

4. Current status of various resistant strategies to viroid infection

Due to the difficulty in using viroid-resistant and -tolerant genetic resources in many economically important crops, various resistance strategies have been devised so far. In this section, we describe the findings about various resistance strategies to viroid infection and their mechanisms.

4-1. Cross-protection

Cross-protection is a phenomenon in which prior infection with attenuated virus/viroid strains inhibits an infection with subsequent virulent strains of the same or closely related species, and the detailed mechanism has not been elucidated. However, it is thought to be a phenomenon that follows nucleotide sequence homology, possibly involving RNA silencing mechanism (Flores et al., 2005, Kovalskaya and Hammond, 2014; Flores et al., 2017). This phenomenon has been reported between several viroid species or strains, in some different host plants (Fernow, 1967; Niblett et al., 1978; Branch et al., 1988; Khoury et al., 1988; Pallás and Flores, 1989; Singh and Boucher, 1990). Pre-inoculation with CSVd or mild or severe strains of PSTVd in chrysanthemums or with mild strain of PSTVd in tomatoes, suppressed the disease development of CEVd in chrysanthemums, or attenuated disease symptoms of CEVd and severe PSTVd strain in tomatoes. This effect was not observed in the case of pre-inoculation with chrysanthemum chlorotic mottle viroid (CChMVd), a member of the family *Absunviroidae*. No symptoms of CEVd and severe PSTVd strain inoculated in pre-protected tomatoes was observed during the test period (65 days), whereas symptoms of CEVd inoculated in pre-protected chrysanthemums began to appear at 50-60 days post-inoculation of CEVd (Niblett et al., 1978). Cross-protection between PSTVd strains or PSTVd and CEVd has also been reported in other subsequent studies. Pre-inoculation of mild PSTVd strain in tomatoes delayed the accumulation and disease development of severe PSTVd strain subsequently inoculated (Khoury et al., 1988). In addition, when a severe strain of PSTVd was inoculated into *G. aurantiaca* in advance and then inoculated with a severe strain of CEVd, some plants showed CEVd-characteristic severe symptoms, while the others exhibited only mild symptoms induced by PSTVd. In the former plants, both PSTVd and CEVd were detected, but only PSTVd was detected in the latter plants (Pallás and Flores, 1989). Differences in the effects of cross-protection between crop cultivars are shown in studies with PSTVd and potatoes. In PSTVd-sensitive 'Russet Burbank' potatoes, pre-inoculated with mild strain of PSTVd,

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severe strain of PSTVd subsequently inoculated was not detected. On the other hand, in PSTVd-tolerant 'BelRus' potatoes, the subsequent inoculated severe strain of PSTVd was detected in several plants, indicating that cross-protection by pre-inoculation with mild PSTVd strain against severe strain subsequently inoculated was not completely functional in this cultivar (Singh and Boucher, 1990). Thus, it seems necessary to examine the optimal combination of viroid species or strains for each plant species or cultivar, although cross-protection is effective in conferring resistance or tolerance to viroid infection in viroid-sensitive host plants. However, it has not been used in the field as a general means of controlling viroid disease due to the risk of biological contamination of the environment (Flores et al., 2017).

4-2. Virus-induced gene silencing and agro-infiltration.

Down-regulation and over-expression of host factors, important for viroid replication and movement, may inhibit accumulation or infection of viroid. Transient suppression or over-expression of the *DNA ligase 1* gene by means of virus-induced gene silencing (VIGS) or agro-infiltration resulted the decrease or increase of PSTVd accumulation in *Nicotiana benthamiana* (Nohales et al., 2012a). In addition, ectopic over-expression of ribosomal protein L5 (RPL5), the negative regulator of alternative splicing to generate the transcriptional factor (TF) IIIA-7ZF, by agro-infiltration induced the reduction of PSTVd concentration in PSTVd-infected leaves of *N. benthamiana*, which is correlate with the reduction of TFIIIA-7ZF protein (Jiang et al., 2018). These methods use viruses or agrobacteriums to suppress or over-express a target gene and the effects are transient, so it is not practical for controlling viroid disease. However, the findings obtained from these experiments may be applied to generate viroid-resistant or -tolerant crops by transformation technologies.

4-3. Transgenic plant

To date, practical viroid resistance has not been reported in almost all viroid-crop combinations. Transformation of plants can be used not only for reverse-genetical analysis to presume the role of target gene through RNAi-mediated suppression but also for induction of resistance or tolerance to virus and viroid infection. Therefore, the use of transformation techniques aimed to confer resistance or tolerance to viroid infection into various plants has been considered as one of the most effective methods for controlling viroid diseases. Many viroid-resistant or -tolerant transgenic plants targeting various viroids have been generated so far, and they can be classified into the following three types: the first is to directly degrade target viroid, the second is to indirectly induce the degradation of viroid through the RNA silencing mechanism, and the third is to inhibit viroid infection through down-regulation or over-expression of host factors, involved in viroid replication or movement.

4-3-1. Direct degradation of viroid.

4-3-1-1. Ribonuclease

The dsRNA-specific ribonuclease pac1 protein is found in yeast *Schizosaccharomyces pombe* and is characterized structural similarity to RNase III derived from *Escherichia coli* (Iino et al., 1991). It has been reported that expression of pac1 in *N. tabacum* confer resistance to several RNA viruses (Watanabe et al., 1995). In the transgenic potato lines expressing pac1 protein, infection and accumulation of PSTVd were prevented and no disease symptom was observed at different study temperatures: 25–32°C (favorable for viroid replication and accumulation) and 20–28°C (resembling actual field conditions). In addition, carryover of PSTVd through seed potatoes was reduced in transgenic potato lines at 25–32°C (Sano et al., 1997). These results were supported by the subsequent study that pac1-expressing transgenic potatoes inoculated with PSTVd showed no disease symptoms, low accumulation of PSTVd (compared with wild-type) was detected only in 1/2 to

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1/3 of them, and viroid-free tubers were produced from transgenic plants in which PSTVd was not detected in leaves (Ishida et al., 2002). These findings indicate that the expression of the *pac1* gene confers resistance to PSTVd infection into potatoes. Transgenic chrysanthemums expressing pac1 were also generated and tested for resistance to CSVd infection. In transgenic plants, stunting observed in CSVd-infected wild-type plants was not observed, and CSVd was detected only in 20% of the transgenic lines efficiently expressing pac1 protein (Ishida et al., 2002). Similarly, three selected transgenic lines, stably expressing pac1 after CSVd inoculation, showed infection at the low frequency and low accumulation of viroid, and attenuated growth retardation compared to controls (Ogawa et al., 2005).

Taken together, these reports indicate that it is possible to confer resistance or tolerance to viroid infection on viroid-susceptible or sensitive plants by expressing pac1 protein by means of transformation technique, for the purpose of direct degradation of viroid.

4-3-1-2. Nucleic acid antibody

The 3D8 single-chain variable fragment (3D8 scFv) is a catalytic nucleic acid antibody with anti-viral activity against a broad spectrum of viruses that can bind and hydrolyze nucleic acid in a sequence-independent manner (Lee et al., 2015; Kim et al., 2012). In addition, this antibody is superior to accumulate at low levels and to induce no harmful effects on plant development in spite of its sequence-independent activity (Lee et al., 2013a). It has been reported that expression of 3D8 scFv confers resistance to DNA or RNA viruses in tobaccos and Chinese cabbages (*Brassica rapa*) (Lee et al., 2013a; Lee et al., 2013b; Jung et al., 2009). When CSVd was inoculated into transgenic chrysanthemums expressing the conventional 3D8 scFv or codon-optimized for chrysanthemum, no disease symptoms were observed in either case, and all transgenic lines showed no disease symptoms. Additionally, CSVd was detected by RT-PCR in the inoculated leaves in all the lines produced, but was hardly in the newly developed upper leaves in almost all lines (Tran et al., 2016). This indicates that 3D8 scFv expression in plants can confer resistance not only to a wide range of viruses but also to viroids.

4-3-1-3. Ribozyme

A ribozyme is an RNA molecule with the ability to catalyze a biochemical reaction, commonly carried out by proteins. Synthetic RNA transcribed from ribozyme gene, containing three hammerhead ribozyme catalytic domains derived from tobacco ringspot virus satellite RNA sequences, cleaved the target plus- and minus-strand CEVd RNA in vitro, whereas no significant change in CEVd accumulation was observed in transgenic tomatoes transformed with the ribozyme gene (Atkins et al., 1995). Subsequently, transgenic potatoes, expressing a hammerhead ribozyme with a shorter antisense recognition sequence (9–11 nt) than that used in previous studies targeting the minus-strand PSTVd RNA, were generated and inoculated with PSTVd. As a result, 23 out of 34 lines (approximately 68%) exhibited high resistance to PSTVd infection, in which PSTVd was not detected by Northern blot analysis of total RNA. The others exhibited weak resistance (in other words, tolerance), accompanied by the low-level accumulation of PSTVd. On the other hands, transgenic tomato expressing the same ribozyme did not exhibit resistance to PSTVd, because of efficient replication of PSTVd in tomato than in its natural host, potato, probably (Yang et al., 1997). An extended trans-cleaving peach latent mosaic viroid (PLMVd)-derived hammerhead ribozyme with natural tertiary stabilizing motifs (TSMs), critical for the catalytic activity of hammerheads (Saksmerprome et al., 2004; Weinberg and Rossi, 2005), indicated high efficiency in cleavage of short RNA substrates and highly structured long RNA containing the complete sequence of PSTVd, at the low Mg^{2+} concentration existing *in vivo*. Moreover, the hammerhead ribozyme derived from PLMVd prevented systemic PSTVd infection in *N. benthamiana* when transiently co-expressed on leaves with infectious dimeric minus strand PSTVd RNA (Carbonell, et al., 2011). These

 doi:10.20944/preprints202211.0169.v1

observations suggest that trans-acting modified hammerhead ribozymes with TSMs may confer viroid resistance. No other ribozymes, apart from hammerhead structures, have been examined against viroid RNAs (Flores et al., 2017)

4-3-2. Indirect induction of viroid degradation through the RNA silencing mechanism

4-3-2-1. Sense and Antisense RNA

In general, introduction of a transgene that express sense or antisense RNAs to the target gene-derived transcripts can induce post-transcriptional suppression of the target gene expression in plants. Therefore, it may be possible to induce degradation of viroid by expressing these RNAs against the plus- or minus-strand viroid RNA in host plants. Transgenic potatoes expressing antisense RNA, which correspond to plus- or minus-strand PSTVd replication intermediates and formed complexes with them in vitro, were generated and inoculated with PSTVd. In those transgenic plants, PSTVd accumulation was significantly inhibited, while severe symptoms were observed at 6–8 weeks after inoculation (Matoušek et al., 1994). The similar study was conducted against CEVd in tomatoes, and transgenic plants, expressing an antisense construct targeting the plus- or minus-strand of CEVd RNA, were inoculated with CEVd. As a result, a moderate decrease in accumulation of CEVd RNA in transgenic tomatoes expressing an antisense construct targeting the minus-strand of CEVd RNA, whereas, interestingly, CEVd accumulation was increased in the case of expressing an antisense construct targeting the plus-strand CEVd RNA, compared with control wild-type plants. (Atkins et al., 1995). Subsequently, transgenic chrysanthemums were generated using four different constructs containing 75–82 nt sense- or antisense CSVd-specific RNAs corresponding to the plus-strand of CSVd. Infection analyses by RT-PCR revealed that CSVd was detected in inoculated leaves at a low level, while hardly detect in upper leaves of almost all transgenic lines. Among them, the transgenic lines expressing the AS3 construct (82 nt, derived from variable and terminal right region of CSVd) were shown to be the best by RT-PCR results. These results indicate that expressing sense or antisense RNA confer CSVd resistance, and suggests that antisense RNA may be more effective than sense RNA in suppressing replication of viroid RNA in transgenic plants (Jo et al., 2015).

4-3-2-2. hairpin RNA

Hairpin RNA is transcribed from an inverted repeat (IR) construct and efficiently processed into siRNAs by dicer-like proteins (Dunoyer et al., 2005; Fusaro et al., 2006; Wesley et al., 2001), and RISCs loading siRNAs induces the cleavage of target viroid RNAs, resulting resistance to viroid, probably. PSTVd resistance conferred by hpRNA derived from nearly full-length PSTVd was observed in two lines of transgenic tomatoes accumulating high-level hpPSTVd and vd-sRNA, and the severity of symptoms was correlated with PSTVd accumulation (Schwind et al., 2009). Prevention of PSTVd infection by hpRNA was also tested in another study. Seven partial or truncated versions of PSTVd sequences selected according to the hotspots of both PSTVd-sRNAs and functional domains of the PSTVd were used for generation of 21 transgenic lines of *N. benthamiana*. In five of these lines, decrease of PSTVd accumulation was observed, which correlated with the accumulation of PSTVd-sRNA derived from hpRNA expressed at high levels by regulation of the 35S promoter derived from cauliflower mosaic virus. And then, the short IR sequence of 26-49 nt used to express hpRNA in this study were shown to allow both efficient expression of PSTVd-sRNA and inhibition of PSTVd infection (Adkar-Purushothama et al., 2015). Induction of PSTVd-sRNA accumulation via expression of hpRNA was combined with grafting-mediated siRNA transport technology (Kasai et al., 2011). Wild-type tobacco scions were grafted on top of a transgenic *N. benthamiana* expressing the PSTVd hairpin RNA (hpRNA) under the control of a strong companion cell-specific promoter. Inoculation analyses of PSTVd into scions revealed that PSTVd accu-

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mulation was clearly attenuated in scions with highly accumulating PSTVd-sRNA compared with control grafted plants. These results demonstrate that transgenic rootstocks expressing vd-sRNA can attenuate viroid accumulation in non-transgenic scions grafted onto the rootstocks (Kasai et al., 2013).

4-3-2-3. Artificial micro RNA and synthetic trans-acting small interfering RNA

AmiRNAs and synthetic trans-acting siRNAs (syn-tasiRNAs) are artificial sRNAs engineered to silence specific transcripts in plants, post-transcriptionally. These are produced in plants by the expression of a functional miRNA or tasiRNA precursor including modified double-stranded miRNA or tasiRNA sequences, respectively, and have been used to selectively inactivate endogenous and reporter genes (Alvarez et al., 2006; de la Luz Gutierrez-Nava et al., 2008; Schwab et al., 2006). Moreover, transgenic plants expressing these sRNAs exhibited anti-viral resistance (Niu et al., 2006; Qu et al., 2007; Tien et al., 2013; Singh et al., 2015). The use of these sRNAs to prevent viroid infection was tested in *N. benthamiana* against PSTVd. Using a high-throughput platform for time- and cost-effective design and synthesis of amiRNAs and syn-tasiRNAs, multiple sRNAs to target sites in plus- and minus-strands of PSTVd were effectively screened. Most of amiRNAs were highly active in agroinfiltrated leaves of *N. benthamiana* when co-expressed with infectious PSTVd transcripts, and syn-tasiRNAs derived from a construct including the five most effective amiRNA sequences were also the same. The effects of the most effective amiRNA and of the syn-tasiRNAs were observed in both agroinfiltrated and upper non-agroinoculated leaves, resulting the significant delay of systemic PSTVd accumulation. These results suggest that these artificial sRNAs are possible to control viroid infection, efficiently, when expressed in transgenic plants, stably (Carbonell and Daròs, 2017).

4-3-2-4. Over-expression of RNA silencing component

The roles of the key factors of RNA silencing, DCLs, AGOs, and RDRs, in the defense responses to viroid infection was reverse-genetically analyzed by evaluating the effects of down-regulation or over-expression of each gene on viroid infection. The roles of DCLs in the defense responses to viroid infection has been analyzed in *N. benthamiana*, tomatoes, and *N. tabacum*. Analyses of the effect of single or multiple suppression of DCL, by the combination of RNAi and crossing, on PSTVd infection in *N. benthamiana* revealed that simultaneous suppression of DCL2 and DCL3 especially increased the PSTVd accumulation and exacerbated viroid symptoms. In addition, suppression of DCL4 decreased PSTVd accumulation, likely because it is primarily targeted and processed by the DCL4 pathway instead of the most effective pathway by DCL2 and DCL3, normally (Katsarou et al., 2016). Furthermore, RNAi-mediated simultaneous suppression of DCL2 and DCL4 expression in PSTVd-tolerant tomatoes increased accumulation of PSTVd and exacerbated disease symptoms during the early stage of infection (Suzuki et al., 2019). Subsequent comparative analyses in PSTVd-sensitive 'Rutgers' and -tolerant 'Moneymaker' tomatoes revealed that PSTVd accumulation in 'Moneymaker' tomatoes was lower than in 'Rutgers' tomatoes in the early infection, and at the timing, DCL4 expression was specifically elevated in PSTVd-infected 'Moneymaker' tomatoes, suggesting the possible involvement of DCL4 in exerting PSTVd tolerance (Fujibayashi et al., 2021). On the other hand, RNAi-mediated suppression of DCL1 was suggested to reduce the efficiency of PSTVd infection (Dadami et al., 2013; Katsarou et al., 2016), and the function of DCL1 in the defense responses to viroid infection was further analyzed in association with the miRNA pathway. As a result, in *N. benthamiana* and *N. tabacum*, RNAi-mediated suppression of SERRATE and DCL1, which are important components of the miRNA biogenesis pathway, decreased the accumulation of PSTVd. This suggests that these factors have the positive effects to viroid infection (PSTVd and HSVd). However, the mechanism behind this effect remains unclear (Kryovrysanaki et al., 2019). These findings indicate that DCL2,

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DCL3, and DCL4, but not DCL1, play important roles in anti-viroid defense. The involvement of AGOs in anti-viroid defense responses is supported by the report that PSTVd-derived sRNAs are loaded into AGO1, AGO2, AGO4, and AGO5. Furthermore, transient expression of these four AGOs in leaves of *N. benthamiana* reduced the accumulation of PSTVd (Minoia et al., 2014). The role of RDRs in defense responses to viroid infection was analyzed in *N. benthamiana* and tomatoes. When scions of *N. benthamiana*, in which RDR6 expression was suppressed by RNAi, were grafted onto *N. benthamiana* rootstocks expressing dimeric forms of HSVd, HSVd-induced disease symptoms were suppressed in the RDR6-suppressed scions. Meanwhile, HSVd accumulation in RDR6-suppressed scions was similar to that in symptomatic wild-type scions or HSVd-expressing rootstocks (Go´mez et al., 2008). In the case of PSTVd, RNAi-mediated suppression of RDR6 in *N. benthamiana* increased the accumulation of PSTVd, and allowed PSTVd to completely enter the SAM (Di Serio et al., 2010). Similarly, VIGS-mediated suppression of RDR6 in *N. benthamiana* increased the PSTVd accumulation (Adkar-Purushothama and Perreault, 2019). On the other hand, RNAi-mediated suppression of RDR6 in tomatoes reduced the accumulation of PSTVd early in the infection and allowed PSTVd to enter the SAM, but it is only into the basal part. It is suggested that the difference in the effect of RDR6 suppression between these host plant species is probably due to RDR1, because *N. benthamiana* is a natural loss-of-function mutant of RDR1 (Naoi et al., 2020; Yang et al., 2004). RDR1 expression was enhanced in 'Rutgers' tomatoes or 'Suyo' cucumbers infected with PSTVd or HSVd (Schiebel et al., 1998; Xia et al., 2017), suggesting the involvement of RDR1 in the anti-viroid defense. The subsequent study showed that down-regulation of tomato RDR1 or over-expression of N. tabacum RDR1 by VIGS or agro-infiltration increased or decreased the accumulation of PSTVd in tomatoes or *N. benthamiana*, respectively (Li et al., 2021). These findings indicate that not only DCLs (DCL2, DCL3, and DCL4) but also AGOs (AGO1, AGO2, AGO4, and AGO5) and RDRs (RDR1 and RDR6) play important roles in the defense responses to viroid infection.

As described, it is assumed that over-expression of RNA silencing components can suppresses viroid accumulation, since many DCLs, AGOs, and RDRs have been found to favor viroid accumulation and infection when their expression was suppressed. In fact, transient over-expression of some AGOs and RDR1 suppressed viroid accumulation (Minoia et al., 2014; Li et al., 2021). Therefore, it may be possible to confer resistance or tolerance to viroid infection into plants by over-expression of RNA silencing-related factors, using transformation technology.

4-3-3. Inhibition of viroid infection through down-regulation or over-expression of host factors involved in viroid infection events

It may be possible to induce resistance or tolerance to viroid infection by modifying the gene expression of host factors that contribute to infection events such as replication and movement of viroid. The viroid RNA-binding protein 1 (VirP1), which was identified in tomatoes as a protein that specifically interacts with the terminal right region of PSTVd, had been suggested to be important for systemic movement of PSTVd (Maniataki et al., 2003; Martínez de Alba et al., 2003). RNAi-mediated suppression of VirP1 in *N. benthamiana* and *N. tabacum* inhibited infection with PSTVd and CEVd, and induced resistance to these pospiviroids. Further analysis by transfection of PSTVd into protoplasts derived from VirP1-suppressed *N. benthamiana* plants suggested that VirP1 is important for replication of PSTVd at the cellular level. On the other hand, over expression of VirP1 did not affect PSTVd infectivity or symptom development in both tobacco species (Kalantidis et al., 2007). Alternatively, suppression of VirP1 in tomatoes attenuated PSTVd symptoms (Kovalskaya and Hammond, 2015; Zhao and Hammond, unpublished data) Other targets for expression regulation include DNA ligase 1 and RPL5. When the expression of these genes was transiently regulated VIGS or agro-infiltration in *N. benthamiana*, PSTVd accumulation was reduced (Nohales et al., 2012a; Jiang et al., 2018, refer to sub-section 4-2). Several other host factors that interact with viroid have been reported (e.g., TFIII-7ZF or

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cucumber phloem protein 2 interacted with PSTVd or HSVd, respectively); however, the effect of down-regulation or over-expression of their host factors on viroid infection has not been examined. Additionally, it should be noted that modifications in the expression of host factors involved in viroid replication may adversely affect the normal plant development, because viroid autonomously replicate depending on the transcriptional machinery of host plants.

5. Future Perspectives

Due to the difficulty in using viroid-resistant genetic resources, various resistance strategies have been devised so far. However, although most are efficient, many are difficult to put into practical use in the field due to concern about the risk of adverse effects on the environment and the human body. In this section, we will discuss the possibilities and challenges of establishing a more practical viroid control method using two technologies, spray-induced gene silencing and genome editing, recently reported.

5-1. Spray-induce gene silencing

Recently, fungal pathogens *Botrytis cinerea* and *Fusarium graminearum* have been shown to efficiently take up environmental dsRNAs, which are then processed into sRNAs that induce silencing of pathogen genes with complementary sequences. Based on these findings, a crop protection strategy, spray-induced gene silencing (SIGS), was developed. SIGS inhibits pathogen infection by locally applying dsRNA or sRNA molecules to plants to silence pathogen virulence-associated genes. Topical application of virus-specific dsRNAs to protect plants against plant viruses has attenuated viral infections of the respective host plants (Mitter et al. 2017b, Dalakouras et al. 2020, Das and Sherif 2020). To date, resistance to virus infection has been achieved in maize by spraying of crude extract from *Escherichia coli* HT115 (DE3) expressing dsRNA targeting the coat protein of sugarcane mosaic virus (Gan et al., 2010) and in *N. benthamiana* by spraying of RNA extracts from *Pseudomonas syringae* LM2691 expressing dsRNAs targeting the region of replicase and movement protein of TMV (Niehl et al., 2018). Furthermore, to increase dsRNA stability and prolong antiviral protection, dsRNA was bounded to the layered double hydroxide clay nanosheets BioClay, with average particle sizes of 80–300 nm. The dsRNA of 330 bp bounded to BioClay was detectable as early as 30 days post-application, and a single application to *N. tabacum* provided the protection to cucumber mosaic virus for at least 20 days (Mitter et al., 2017a).

No attempts have been made to suppress viroid infection by spraying viroid-derived dsRNA or sRNA, while co-inoculation of CEVd and CEVd-dsRNA or CChMVd and CChMVd-dsRNA into tomatoes and *G. aurantiaca* or chrysanthemums prevented viroid infection. Half of the plants did not indicate disease symptoms, and viroid accumulation was not detected in those plants. Furthermore, inoculation of PSTVd and PSTVd-dsRNA in tomatoes reduced the PSTVd accumulation, and the disease development was delayed and less severe compared with those of the control plants (Carbonell et al., 2008). This suggests that, similar to viruses and other pathogens, spraying solution containing dsRNA may induce resistance or tolerance to viroid, without using transgenic technologies. Furthermore, recent studies have indicated that miRNAs, produced by a plant, work as signaling molecules and influence the gene expression in other nearby plants (Betti et al., 2021). This suggests the possibility that uptake of sRNA derived from the viroid sequence from plant roots may lead to suppress viroid accumulation.

Taken together, these recent findings about disease control methods and relevant reports may lead to the development of new control strategies for viroid diseases, not relying on genetic modification techniques.

5-2. Genome editing

 doi:10.20944/preprints202211.0169.v1

The genome editing technology have enabled crops to be improved with great precision and speed, and have been used to achieve important agronomic and quality attributes of several crops. Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated protein 9 (CRISPR-Cas9) has emerged as a powerful genome-editing tool suitable for next-generation plant breeding including virus resistance (Saurabh, 2021; Tussipkan and Manabayeva, 2021; Zhang et al., 2020; Khatodia et al., 2016; Tiwari et al., 2022). CRISPR-Cas genome-editing tools consist of a guide RNA molecule and an enzyme that cleaves DNA, called Cas protein. The protein cleaves specifically at sites supported by guide RNA sequences, and then, the cleaved site is repaired by the cell's natural DNA repair machinery. Globally, genome-modified crops are classified into three Site-Directed Nuclease (SDN) categories based on their editing behavior (mutation type/donor DNA) and their respective repair mechanisms (Podevin et al., 2013; Kumari et al., 2022): SDN-1, DNA editing consisting of the insertion or deletion of a few DNA nucleotides resulting from incomplete repair, so-called non-homologous end joining (NHEJ). SDN-2, DNA editing consisting of the modification of several nucleotides in the DNA (insertion, deletion, or substitution of a few nucleotides) as a result of repair using a template to direct the repair, so-called homology-directed DNA repair (HDR). SDN-3, DNA editing is based on the same mechanism as SDN-2, but the used template results in the insertion of a larger DNA piece which constitutes an additional gene. The Cas9 expression cassette introduced for plant genome editing can be removed from progeny by genetic segregation. The genetic alterations introduced into the SDN-1 and SDN-2 type plants are no different than those occurred naturally or resulting from conventional breeding. Currently, two genome-edited crops are on the market, one is soybeans with high oleic acid in the United States (Demorest et al., 2016; Haun et al., 2014; Nagamine and Ezura, 2022) and the other is tomatoes with high φ-aminobutyric acid levels in Japan (Nonaka et al., 2017; Nagamine and Ezura, 2022). Both genome-edited crops are categorized into SDN-1.

The genome-editing technology have been used to create some resistant plants against various DNA/RNA viruses. For example, they include genome-edited tomatoes that exhibit resistance to potyvirus and cucumovirus by modifying the translation initiation factor *eIF4EI* gene, which is important for viral replication, and to tomato yellow leaf curl virus (TYLCV) by modifying the *Ty-5* gene, whose recessive allele is reported as the TYLCV-resistant gene *ty-5* (Atarashi et al., 2020; Yoon et al., 2020; Pramanik et al., 2021). The class 2 type VI CRISPR/Cas effector Cas13a is an RNA-targeting CRISPR effector that provides protection against RNA phages through the cleavage of single-stranded RNA (Abudayyeh et al., 2016). Transgenic potato lines expressing Cas13a/sgRNA (small guide RNA) constructs targeting genome RNA of potato virus Y (PVY) exhibited high-level resistance to PVY (Zhan et al., 2019).

To date, viroid-resistant crops produced by using genome-editing technology have not been reported, but there are several host factors that suppress viroid infection and accumulation when down-regulated (e.g., VirP1). It may be possible to create viroid-resistant or -tolerant plants by knocking out these host factors by genome editing, and these genome-edited plants are categorized into the SDN-1 type that do not correspond to genetically modified organisms. Further genome editing targets for the creation of viroid-resistant plants are host factors that suppress viroid infection and accumulation when over-expressed (for example, RPL5, AGOs, and RDR1). Modification of the regulatory region to up-regulate gene expression may confer resistance or tolerance to viroid infection. In addition, the genes contributing to CSVd resistance in chrysanthemums (Omori et al., 2009; Nabeshima et al., 2012) and PSTVd tolerance in wild tomato relatives (Naoi and Hataya, 2021), which can be used for conventional cross-breeding, have not yet been identified, but if identified, they could be potential targets for genome editing in the future. Plant hormone metabolism-related genes associated with the expression of viroid disease symptoms may also be candidate editing targets for creating viroid-tolerance crops. Alternatively, introduction of the Cas13a/sgRNA construct, directly targeting viroid RNAs, into plants may also enable conferring viroid-resistance or -tolerance to plants.

 doi:10.20944/preprints202211.0169.v1

Thus, the genome-editing technology is considered useful in producing crops exhibiting resistance or tolerance to viroid infection. In particular, the SDN-1 type of genome-edited plants, which contain modifications that are no different from mutations naturally occurring or caused by conventional breeding, and some have already been on the market, are considered to be highly practical. Therefore, further researches and developments are required in the future.

6. Conclusion

In this review, we comprehensively summarized the current status about investigation of naturally occurring genetic traits for viroid resistance and tolerance, accumulating knowledge about host factors involved in viroid pathogenicity, and various basic technologies developed to try to possible viroid disease control strategies. Many non-transgenic or transgenic approaches that can induce resistance or tolerance to viroid infection, reported so far, have not been used in the field as a general means of controlling viroid disease, due to concerns about the risk of biological contamination of the environment and possible adverse effects on the human body. On the other hand, as discussed in this review, viroid-resistance and -tolerance and related extensive findings may lead to confer resistance or tolerance to viroid infection by combining with the recently emerged new technologies (e.g., SIGS and genome-editing technologies), which are believed to be more environmentally viable and acceptable to the general public than previously reported approaches. In particular, some genome-modified crops produced by the latter technology are already on the market. Therefore, extensive further research on viroid-resistance and -tolerance is important for development of agriculturally more practical new approaches and following achievement of more effective and sustainable disease control of viroid.

Author Contributions: Conceptualization, T.N.; investigation, T.N.; writing—original draft preparation, T.N.; writing—review and editing, T.N., S.L., and T.S.; supervision, T.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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