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Review

Molecular biology of viroid–host interactions and disease control strategies

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ABSTRACT

Viroids are single-stranded, covalently closed, circular, highly structured noncoding RNAs that cause disease in several economically important crop plants. They replicate autonomously and move systemically in host plants with the aid of the host machinery. In addition to symptomatic infections, viroids also cause latent infections where there is no visual evidence of infection in the host; however, transfer to a susceptible host can result in devastating disease. While there are non-hosts for viroids, no naturally occurring durable resistance has been observed in most host species. Current effective control methods for viroid diseases include detection and eradication, and cultural controls. In addition, heat or cold therapy combined with meristem tip culture has been shown to be effective for elimination of viroids for some viroid–host combinations. An understanding of viroid–host interactions, host susceptibility, and non-host resistance could provide guidance for the design of viroid-resistant plants. Efforts to engineer viroid resistance into host species have been underway for several years, and include the use of antisense RNA, antisense RNA plus ribozymes, a dsRNase, and siRNAs, among others. The results of those efforts and the challenges associated with creating viroid resistant plants are summarized in this review.

Contents

1. Introduction

Viroids are the smallest known agents of infectious disease [1]. The first viroid to be identified and characterized was *Potato spindle tuber viroid* (PSTVd). Potato spindle tuber disease was described in the early 1920s in Irish Cobbler potato (*Solanum tuberosum* L.) in North America by Martin [2], who suggested that the disease might be caused by an infectious virus. Shultz and Folsom [3] investigated the disease and found that it was present in the tuber and could be spread mechanically in the field by leaf damage, tuber and stem grafts, with some evidence of insect transmission by aphids. Symptoms of the disease were characterized by stunting of the plants and elongated tubers; hence the disease was named 'spindle tuber'. Although the causal agent was initially described as the potato spindle tuber 'virus', it was later found not to be a conventional virus, with a nucleic acid encapsidated by a viral protein, but

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a small, naked RNA molecule [4–6]. Diener [6,7], credited with the discovery of this novel pathogen, advanced the concept of viroids and proposed the term 'viroid' to denote this new class of subviral pathogens. Similar observations of infectious, low-molecular weight nucleic acids were reported as the causal agent of citrus exocortis disease [8,9] and chrysanthemum stunt disease [10], and confirmed the viroid concept proposed by Diener.

Since the discovery of viroids, many plant diseases of considerable economic importance have been shown to be caused by viroids, for example PSTVd in potato, *Chrysanthemum stunt viroid* (CSVd) in chrysanthemum, *Citrus exocortis viroid* (CEVd) in citrus, *Coconut cadang-cadang viroid* (CCCVd) in coconut palm, and *Avocado sunblotch viroid* (ASBVd) in avocado, among others [11,12]. Viroids are restricted to higher plants and their hosts include monocots and dicots, herbaceous and woody plants, agronomic and ornamental plants. Viroids and viroid diseases are distributed globally, and their distribution may reflect exchange of infected germplasm and transmission through seeds [12]. Although diseases caused by viroids are thought to be a relatively recent occurrence, Bar-Joseph [13] questioned this assumption and provided a thoughtful perspective that viroids may have been associated with perennial crops, and were agents of older diseases, for hundreds of years, and their emergence in the last century may reflect changes in horticultural practices, the introduction of sensitive genotypes, and the development of sensitive diagnostic methods. With the recent emergence of viroid diseases in both horticultural and agricultural crops [14–20], control measures must be adopted that may include transgenic approaches in addition to traditional methods.

The molecular properties of viroids and interactions with their hosts have been extensively studied, and conventional as well as molecular approaches have been developed for their control. The strategies for introducing resistance to viroids rely on a basic knowledge of viroid structure and biology of infection, as well as the host response to viroid invasion. In this review, the properties of viroids and viroid–host interactions and points where molecular control mechanisms might be developed are summarized, in addition to current control measures and biotechnological approaches to viroid control. Other comprehensive resources on viroids and viroid diseases include Diener [11], Hadidi et al. [12], Ding [21], Riesner and Gross [22], Tsagris et al. [23], Cho et al. [24], Flores et al. [25], and Tabler and Tsagris [26], and on the development of strategies for control of viroid diseases can be found in the recent literature [27–31].

2. Molecular characteristics and biology of viroids

Mature viroids are composed of small covalently closed, circular single-stranded RNA molecules that range in size from 239 to 401 nucleotides, do not encode peptides or proteins, and use host proteins for replication, movement, and processing of replication intermediates, which distinguishes them from plant viruses. There are over 30 known viroid species (43 complete genomes, and greater than 4700 sequence variants described and assigned to eight genera) that are taxonomically divided into two families, the *Pospiviroidae* (the type species of which is PSTVd) and the *Avsunviroidae* (the type species of which is ASBVd) [12] and several proposed, unclassified viroids. Most known viroids are members of the *Pospiviroidae*; for an up-to-date list of known viroids and their sequences, see http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?opt=viroid&taxid=12884, http://www.ebi.ac.uk/genomes/viroid.html, and the Subviral RNA Database (http://subviral.med.uottowa.ca/cgi-bin/home.cgi). The application of a new homology-independent approach that combines deep sequencing of small RNAs with a computational algorithm may lead to the discovery of novel viroid pathogens in the future and expand the list of currently known viroids [32].

The origin of viroids remains unknown, although Diener speculated that viroids may be derived from host cellular RNAs, transposable elements, plasmids or introns and may be relics of pre-cellular RNA evolution [33–35]. It is also not known why viroids appear to be restricted to the plant kingdom. Several studies have noted the similarities between viroids and *Hepatitis delta virus* (HDV), a small satellite virus of *Hepatitis B virus* (HBV) [36,37]. HDV is a circular, single-stranded RNA that is replicated independently from its helper virus by DNA dependent RNA polymerases, and has partial structural similarity to viroids; however, the HDV RNA is larger and encodes an antigen that plays a critical role in the viral life cycle and HDV requires HBV for transmission [36,37].

Viroids are classified into the two families based primarily on three criteria – their mode and site of replication, the presence/absence of a hammerhead ribozyme, and structural properties. Members of the *Pospiviroidae* replicate and accumulate in the nucleus using a host DNA-dependent RNA polymerase II, normally involved in mRNA synthesis, *via* an asymmetric rolling circle mechanism that results in the synthesis of oligomeric, greater-than-unit length RNA replicative intermediates (plus and minus single strand and double-stranded RNA (dsRNA)) that are processed to unit length, and finally to mature viroid circles, by one or more host-encoded enzymes [38–40]. Members of the *Avsunviroidae* replicate and accumulate in the chloroplast using a symmetric pathway and host enzymes, and the oligomeric intermediates undergo self-cleavage to unit length and mature circles *via* an internal hammerhead ribozyme [41–44]. For a more detailed review of viroid replication, see Flores et al. [45].

Viroids have unique, thermodynamically stable structures that are composed of a series of helices and loops due to intramolecular base pairing, with the result that they are partially double-stranded and, although they are circular molecules, can assume rod-like or multibranched secondary structures. The intermediate strain of PSTVd, the type species of the family *Pospiviroidae*, was the first viroid to be sequenced [46] and the thermodynamically optimal native secondary structure of the mature, circular form of most pospiviroids, including PSTVd, was calculated to be rod-like and composed of an unbranched series of short helices and small loops that was established by biophysical methods [22], including electron microscopy [22,47–51]. Comparative sequence analysis suggests that PSTVd and other pospiviroids contain five structural domains – the terminal left (T_L) and terminal right (T_R) domains, the pathogenicity (P) and variable (V) domains, and the central conserved region (CCR) [52]. These domains may play a role in viroid evolution. Site-directed mutagenesis has revealed that sequence motifs within one or more of these domains play essential roles in replication, intracellular and cell-to-cell movement, induction of disease, and entry/exit of the viroid molecule from the vascular system [53]. The secondary structures of several members of the *Avsunviroidae*, the type species of which is ASBVd [54–56] are generally multibranched (excluding ASBVd), lack a CCR, and strands of both polarities contain hammerhead ribozymes which function to self-cleave the RNAs [42–44]. The significance of multibranched conformations *in vivo* is an area of active study. In addition to thermodynamically stable structures described above, viroids are also composed of essential, thermodynamically metastable structures, *e.g.*, secondary hairpins and loops (*e.g.*, hairpins I and II and Loop E) and pseudoknots, that have been shown to function in viroid replication, intra- and intercellular movement, and pathogenicity [57–59].

In infected plants, viroids are composed of complex populations of closely related, but not identical sequence variants which arise during replication and form what is known as quasispecies [60]. One or more of the variants may predominate in the infection and the variants are relevant as single nucleotide changes may have major effects on symptom expression. Passage through

a host or environmental factors may result in selection of variants and impact disease management strategies [61,62]. Viroids have the highest calculated per-base mutation rate yet measured for any disease-causing agent at 2.5×10^{-3} per base per round of replication, that is, one mutation per 400 nucleotides, and homologous and heterologous recombination can also generate diversity in these pathogens [63]. This variability may lead to host adaptation and/or escape from naturally occurring or introduced host resistance mechanisms [61].

Key steps in the colonization of a host plant by viroids are their ability to move within the cell to the site of replication [64,65], then cell-to-cell through plasmodesmata [66], and, finally, systemically through vascular tissues, particularly through phloem cells, that occurs parallel to the photosynthate from source to sink organs [67–70]. In contrast to viruses, which evolved specialized movement proteins to exploit endogenous host RNA and protein transport systems to move throughout the plant, viroids do not encode proteins and use other mechanisms for systemic infection that apparently involve plant developmental and cellular factors.

RNA-binding host proteins that may facilitate movement of viroids through the plant have been isolated using several approaches [68,71–77]. Characterization of host factors interacting with the viroid RNA may also contribute to the elucidation of RNA-related movement pathways of host plants. In addition, RNA signatures on viroid molecules that regulate the cell-to-cell and long distance movement of viroids in their hosts [66,69], the nuclear targeting of PSTVd [38,78–80] as well as movement of the avsunviroid, *Eggplant latent viroid* (ELVd) into and out of the nucleus and chloroplast have been identified [81,82].

Owens et al. [73] reported that the most abundant component of phloem exudate – phloem protein 2 (PP2; dimeric lectin) isolated from *Cucurbitaceae* – could interact with a variety of RNA molecules, specifically with highly structured viroid RNAs such as *Hop stunt viroid* (HSVd), and polyadenylated mRNAs *in vitro*, suggesting the facilitating role of PP2 in systemic movement of viroids and other RNAs *in vivo*. Martínez de Alba et al. [83] identified a bromodomain-containing protein (Virp1) with an atypical RNA binding domain and a nuclear localization signal naturally produced in tomato plants and capable of specifically interacting with PSTVd (+) RNA *in vitro* and *in vivo*. Gozmanova et al. [84] analyzed the specific binding of Virp1 protein to the terminal right domain of PSTVd, and found that two asymmetric internal loops within the PSTVd (+) RNA, each composed of the sequence elements 5′-ACAGG and CUCUUCC-5′, are responsible for the specific RNA–protein interaction. It was found that 5′-ACAGG/CUCUUCC-5′ motif, located close to the terminal right hairpin loop of the PSTVd secondary structure, has an approximately 5-fold stronger binding affinity than the more centrally located 5′-ACAGG/CUCUUCC-5′ motif. The individual inactivation of these motifs revealed that each motif could bind Virp1 without the need of the other. Maniataki et al. [77] reported on the specific *in vivo* interaction of Virp1 protein with full-length viroid RNAs (PSTVd and HSVd) and their sub-fragments in the yeast three-hybrid system [85]. HSVd did not possess as strong a Virp1-binding region as PSTVd, which may explain the low infectivity of HSVd in tomato plants. The authors proposed that the 5′-AGG/CCUUC-5′ motif bolsters recognition of the terminal right domain by Virp1 to achieve access of the viroid to pathways that propagate endogenous RNA systemic signals in plants. Kalantidis et al. [86] investigated the role of Virp1 in the viroid infection cycle by the use of transgenic lines of *Nicotiana tabacum* and *Nicotiana benthamiana* that either over expressed the tomato Virp1 RNA or suppressed the orthologous tobacco genes through RNA silencing. Virp1-suppressed lines were not infected by PSTVd or CEVd through mechanical inoculation, indicating a major role of Virp1 in viroid infection. On the other hand, over expression of Virp1 in both tobacco plants did not affect PSTVd infectivity or symptom appearance in these species. Transfection experiments with isolated protoplasts revealed that Virp1-suppressed cells were unable to sustain viroid replication, suggesting that resistance to viroid infection in Virp1-suppressed plants is likely the result of cell-autonomous events. In earlier studied preceding the identification of the Virp1 protein, mutagenesis of the right and left terminal hairpin loops of the PSTVd RNA rod-like structure revealed that the left terminal loop mutants were non-infectious whereas right terminal loop mutants were able to establish infection in tomato plants, though the character of mutant viroid distribution was different from the wild-type PSTVd [87]. The mutations in the right terminal loop may alter the interaction of PSTVd with the Virp1 host protein, thereby disrupting the normal pattern of intercellular transport of the viroid or limit its replication to a cell type.

In a separate study, Solovyev et al. [88] recently reported the possible role of the Nt-4/1 protein in systemic viroid transport. The *Arabidopsis thaliana* 4/1 protein has a highly α-helical structure that interacts with plant virus tubule-forming movement proteins and has the potential for self-interaction. Plants in which the expression of *N. tabacum* homolog, Nt-4/1 protein, was suppressed by virus-induced gene silencing resulted in alteration of viroid accumulation and movement.

The critical interaction with host proteins for movement suggests that one strategy for introducing resistance to viroids may be to interfere with these interactions, and therefore, limit viroid movement in the plant.

3. Host range and transmission

The host range of viroids includes monocots and dicots, vegetable crops, ornamentals, and woody perennials, depending upon the viroid species [12]. The experimental host range of PSTVd, *e.g.*, includes several plant families, most of which were determined to be symptomless carriers of the viroid [89]; members of the *Solanaceae* generally produce visible symptoms, the severity of which depends on the viroid strain.

Field and greenhouse studies have demonstrated that pospiviroids are easily transmitted mechanically through contact with contaminated pruning tools and farming implements, by human hands, and by contact between plants [17]. They can also be spread vegetatively by graft inoculation, cuttings, micro-plants and tuber propagation. Pospiviroids are also transmitted through infected seed, pollen [12,90], and insects (described in more detail below). Interestingly, developmental activation of pollen nucleases eliminates the pospiviroid *Hop latent viroid* (HLVd) naturally [91]. The principal mode of transmission of the avsunviroids ASBVd and *Peach latent mosaic viroid* (PLMVd) is primarily through grafting and budding during propagation [92,93].

The green peach aphid (*Myzus persicae*) transmits PSTVd from plants co-infected with the luteovirus, potato leafroll luteovirus (PLRV) to potato, *Physalis floridana*, and *Datura stramonium* [94–96], although it was found to be an insignificant vector of PSTVd alone [96,97]. It was observed that the coat protein of PLRV transencapsidates PSTVd, allowing co-transmission of both pathogens by the insect vector [95,96]. The co-transmission of PLRV and PSTVd by aphid vectors has important implications for epidemiology, transmission and control of PSTVd in potato fields. Additional studies revealed that PSTVd is encapsidated *in vivo* at a low frequency by *Velvet tobacco mottle virus* [98], and that *Tomato planta macho viroid* (TPMVd) could be transmitted at a high efficiency by *M. persicae* to *Solanum nigrescens* and *Physalis foetens* [99], however it is not known if this was mechanical transmission by the insects or feeding-associated transmission. There is conflicting evidence for the transmission of viroids by pollinating insects, including bees, in greenhouse-grown crops [100–102].

4. Host responses to viroid infection

4.1. Symptoms

Viroid pathogenicity is a complex phenomenon that is influenced by both the viroid and host genomes, *e.g.*, infection of different viroid strains on the same host can result in latent (asymptomatic) infections [103], or mild to severe symptoms [21,70,104,105]. Macroscopic symptoms of viroid infection are similar to those associated with many plant virus infections, and include stunting, epinasty, vein discoloration and clearing, leaf distortion and mottling, chlorotic or necrotic spots, cankers, scaling and cracking of bark, malformation of tubers, flowers, and fruits, and rarely, death of the plant.

Cytopathic effects of viroid infections were first reported in *Gynura aurnatiaca* infected with CEVd by Semancik and Vanderwoude [106] who identified paramural bodies – invaginations of the plasmalemma – termed plasmalemmasomes (PSs) which appeared to be associated with leaf epinasty and blistering. In addition, there was distortion and irregular thickness of cell walls. In a later report, Wahn et al. [107] presented evidence that PSs were found in both healthy and CEVd-infected tissues although viroid infection caused a change in the morphology of the PSs. Despite these reports, the role of PSs in viroid pathogenesis remains unanswered.

Abnormal development of chloroplasts by both pospi- and avsunviroids has been observed [108,109], but it is unknown if these effects are specific to viroid infection or are a general response to biotic stress. Di Serio et al. [110] summarized the evidence linking viroid-induced cytopathic effects with macroscopic symptoms and the potential biochemical pathways underlying the observed effects and concluded that, although a tentative model of modified gene expression and its role in cytopathic effects has been put forward, additional multidisciplinary studies are needed to integrate molecular data with ultrastructural studies.

4.2. Molecular biology of viroid–host interactions

As early as the first description of viroid RNAs, it was proposed that they may function as an abnormal regulatory RNA [6]. Since their discovery, much has been learned about their biochemical nature, while their mechanism of pathogenesis remains elusive. As viroids replicate and exert pathogenic effects without encoding proteins, the implications are that viroid functions are mediated through sequence and structural signals. Sequence analysis of viroid variants and reverse genetics on infectious cDNA copies of viroids with the introduction of mutations has revealed that there are complex relationships between viroid sequence/structure and function [53,105]. Although single nucleotide changes in the P domain of PSTVd and CEVd can lead to pathogenicity, and naturally occurring sequence variants, or introduced mutations in that region, lead from mild to severe symptoms [111,112], Sano et al. [113] demonstrated that multiple structural regions may be responsible for pathogenicity of pospiviroids. In addition, single or multiple nucleotides affect host range and replication levels [114,115]. Although variants of the avsunviroid PLMVd possess high sequence variability, the molecular determinant for the peach calico disease caused by PLMVd, was mapped to a 12–14 nucleotide insertion in loop A of a hammerhead arm [116,117]. Insertions can be acquired and lost during infection [116,117] resulting in the emergence of symptomatic variants; the molecular mechanism generating the variants is unknown but has important consequences to host pathology.

In spite of the relative simplicity of viroid genomes, they can trigger complex host responses. PSTVd and CEVd infections alter host metabolism and markedly change the levels of various host proteins [118,119]. A comprehensive analysis of the differential gene expression patterns of tomato plants at various stages of infection by mild and severe strains of PSTVd revealed that both of these strains altered expression of genes encoding products involved in defense/stress response, cell wall structure, chloroplast function, protein metabolism, hormone signaling, and other diverse functions [120,121]. Proteomic analysis of viroid–host interactions of CEVd in tomato, performed using two-dimensional PAGE and mass spectroscopy, resulted in the identification of differentially expressed proteins, such as defense-related proteins, translation elongation factors, and translation initiation factors, and provided evidence that pathogenicity may involve gentisic acid (GA) signaling and interaction of CEVd with eukaryotic elongation factor 1a (eEF1a) [122,123].

PSTVd infection of tomato plants has been shown to selectively alter the phosphorylation state of the host-encoded protein p68 that led to activation of its dsRNA-dependent protein kinase activity [124]. Immunological assays revealed that this phosphoprotein was related to a dsRNA-dependent protein kinase from virus-infected, interferon-treated human cells. Nucleotide photoaffinity labeling experiments indicated that p68 contained an ATP binding site with characteristics consistent with protein kinase activity. Similar results were obtained when the influence of *Tobacco mosaic virus* (TMV) infection on nucleotide binding and phosphorylation of host-encoded p68 protein was examined [125]. Diener et al. [126] demonstrated the differential activation of the mammalian p68 by intermediate and mild strains of PSTVd, and suggested that activation of a plant enzyme homologous to mammalian p68 protein kinase may represent the triggering event in viroid pathogenesis. Hammond and Zhao [127] identified a specific protein kinase gene (*pkv*) that is transcriptionally activated in plants infected with PSTVd. The encoded PKV protein is a novel member of the AGC VIIIa group of signal-transducing protein kinases. Expression of *pkv* antisense RNA in tomato plants using a viral-based vector resulted in marked suppression of viroid symptoms (Zhao and Hammond, unpublished data).

Early evidence that post transcriptional gene silencing (PTGS) occurs during viroid replication was reported by Wassenegger et al. [128], where over expression of viroid RNAs resulted in methylation of PSTVd genes incorporated into the tobacco genome. Although replication occurs in different subcellular compartments, several groups have subsequently reported the accumulation of short RNA fragments of 21–24 nucleotides representing different regions of the viroid genome in both pospi- and avsunviroid-infected plants [121,129–140]. These fragments are characteristic of PTGS, or RNA silencing, which provides a multilayer defense system that protects plants from invasion by RNA replicons, including viruses and viroids. For example, Di Serio et al. [138] demonstrated that RNA-dependent RNA polymerase 6 (RDR6), which catalyzes the amplification producing the double-stranded precursors of secondary silencing RNAs (siRNAs), plays a role in the restriction of PSTVd spread into floral and vegetative meristems.

Several lines of evidence suggest that most of these short fragments may originate from restricted regions of the viroid molecule, including the pathogenicity and CCR domains of the plus strand genomic RNA of PSTVd, and the potential binding sites to host genes have been predicted [137,141]. In PLMVd, hotspots of siRNA accumulation were generated during infection; however, the 12–14 nucleotide insertion associated with peach calico disease was underrepresented, leading the authors to suggest that it is unlikely that symptoms may result from the accidental targeting of host mRNAs by viroid-derived small RNAs derived from this region [140]. An alternative proposal is that viroid genomic RNAs, or the replication process, could be the effectors of pathogenesis and may impair the normal function of the RNA silencing machinery in the host.

The presence of small viroid-specific RNAs in the cytoplasm of viroid-infected plants indicates that viroids can trigger RNA silencing in a host and are substrates for dicer-like cleavage to produce short interfering RNAs (siRNAs) [135]; however, mature, monomeric, partially double-stranded, highly structured viroid molecules can overcome this RNA interference (RNAi)-mediated machinery [142,143]. The reasons behind the failure of the RNA-induced silencing complex (RISC) to target viroid RNA may include but are not restricted to: (1) the highly ordered secondary structure of the mature viroid RNA; (2) viroid RNA can be closely associated with host factors that protect it against RNA silencing; (3) differences in the subcellular localization of viroid RNA that replicates in the nucleus, or chloroplast, and the RISC-siRNA complex, which is found in the cytoplasm, or (4) possible activation of a novel silencing suppressor [136]. The question of how viroid RNA can avoid RNA-silencing is still a matter of investigation.

5. Host resistance *versus* non-host resistance

Although there is no known resistance to PSTVd in potato and tomato cultivars, Singh and Slack [144] and Singh [145] reported the identification of clones of *Solanum berthaultii* with resistance to PSTVd by sap inoculation, but failed to be resistant after graft inoculation. Singh [146] reported the identification of a local lesion host, *Scopolia sinensis* Hemsl., for 'potato spindle tuber virus'. The local lesions were produced on the leaves of *S. sinensis* after mechanical inoculation with crude sap from tomato leaves infected with PSTVd. *Solanum acaule* OCH 11603 was found to be resistant to mechanical inoculation with PSTVd, but was susceptible following agroinfection with PSTVd-containing cDNAs indicating that the observed resistance was likely to be to mechanical inoculation ('field resistance'), rather than immunity to infection [147]. Harris et al. [148] and Pfannenstiel and Slack [149] reported tolerance of some potato cultivars to PSTVd infection.

Attempts to breed CSVd-resistant chrysanthemum plants were reported by Omori et al. [150] and Matushita et al. [151]. Thirty-five chrysanthemum lines and cultivars, wild chrysanthemum species, and interspecific hybrids were screened for resistance to CSVd following grafting of scions of screened cultivars onto CSVd-infected chrysanthemum roots [151]. CSVd could not be detected in the scion of one cultivar "Okayamaheiwa" 210 days post-grafting. Investigation of the heritability of viroid resistance in chrysanthemum plants showed that interspecific hybrids that were obtained by crossing of "Okayamaheiwa" with susceptible cultivars also expressed resistance to CSVd in the first hybrid generation. Although the pattern of inheritance of resistance and the mechanism of resistance remain unclear, this approach for developing chrysanthemum cultivars with resistance to the devastating CSVd infections seems to be promising.

Non-host resistance describes the resistance that is observed when 'all members of particular plant species exhibit resistance to all members of a given pathogen species' [152–155]. No viroids have been reported to naturally infect *A. thaliana* or other members of the *Brassicaceae* family and efforts to infect *Arabidopsis* ecotypes with pospiviroids have been unsuccessful, suggesting that Arabidopsis is a non-host for these viroid species. Transgenic introduction of dimeric minus strands and dimeric plus strands of pospi-and avsunviroids resulted in the generation of viroid RNA replicative intermediates and processing to circular plus strand monomers of HSVd and CEVd, suggesting that the RNAs can serve as templates for synthesis of complementary strands, and that RNA processing could occur [156]. However, the replication efficiency was very low and the viroids could not spread systemically, suggesting that these are limiting steps of viroid infection in plants [156].

6. Control of viroid diseases

6.1. Non-transgenic methods of control

To date, several different non-transgenic strategies have been developed to control plant viroid diseases. They include: detection and eradication of viroid-infected plants, chemotherapy, thermotherapy alone or combined with tissue culture methods, grafting technology, viroid cross-protection, electrotherapy, and different combinations of the approaches mentioned above. These approaches are summarized in the present section.

Detection, prevention, and eradication: The most effective means of viroid disease control are the prevention of the introduction of infected plant material into the field or greenhouse, strict hygiene procedures, and monitoring of crops for unusual symptoms. This includes the use of seed and germplasm certified to be viroid-free and the maintenance of sanitary growing conditions (disinfection and cultural controls). Seed certification programs and quarantine enforcement by the European and Mediterranean Plant Protection Organization (EPPO) and the North American Plant Protection Organization (NAPPO) for viroids of quarantine and certification importance have resulted in effective control of several diseases caused by viroids and rely on sensitive diagnostic and detection methods [157].

The earliest method developed for viroid detection was biological indexing, or bioassay, before the physical/chemical nature of viroids was known, and it is still an important step in the detection and identification of viroid infections [158]; however, the number of hosts and host plants required for the assay, the time required to perform the assay, and the potential lack of symptoms in host plants are disadvantages for the use of biological indexing as the only means of detection. Detection of viroids by polyacrylamide gel electrophoresis (PAGE) played a key role in viroid research [5]. The development of a simplified purification scheme for low-molecular weight nucleic acids and two-dimensional, non-denaturing/denaturing PAGE [159] provides a powerful method for viroid detection as it can detect small circular RNAs, which is due to the fact that they migrate more slowly than linear RNAs in the denaturing phase of electrophoresis. The circular RNAs can then be visualized and recovered from the gels for further cDNA cloning and characterization [160].

With the development of rapid and sensitive detection methods for viroids that include nucleic acid hybridization, first demonstrated by Owens and Diener [161], reverse transcription-polymerase chain reaction and real-time PCR assays [12,14,162,163], successful management and eradication of PSTVd in the USA and Canada has been achieved [164,165]. DNA microarrays and next-generation sequencing technologies may also have applications for detection of viroid infections [32,166,167].

To prevent viroid transmission under field conditions, different chemical substances such as 1–5% sodium hypochlorite, 6% hydrogen peroxide, 2% sodium hydroxide with 2% formaldehyde are used for disinfection of cutting surfaces of agricultural tools such as knives, pruning, grafting and other appliances to eliminate viroid spread through contaminated equipment [54,168–170].

Insecticidal and antiviral sprays have also been used to prevent spread of viroid infection from infected plants. Application of 1 and 2% piperonyl butoxide (insecticide) before challenge inoculation with PSTVd prevented infection of potato and *S. sinensis* plants, whereas attempts to protect tomato plants with various piperonyl butoxide concentrations as well as with sesame oil, corn oil, paraffin oil and mineral oil failed [171]. Application of the antiviral agent ribavirin, at a concentration 300 mg/L, on *Gynura aurantiaca* DC plants infected with CEVd led to almost complete remission of symptoms on newly developed leaves and prevented

the establishment of viroid infections when applied three days prior to CEVd inoculation [172].

In the event that preventative measures are not effective in controlling viroid spread, or if the viroid is already present in valuable germplasm, alternative approaches to protect plants and eliminate viroids are used.

Thermotherapy and tissue culture methods: The application of either heat- or cold-therapy for viroid elimination in host plants has had mixed results [173]. Chung et al. [174] showed that, although the titer of CSVd was reduced in chrysanthemum grown for 2 months at low temperatures (10 °C or 20 °C), 8 weeks after the plants were moved to a normal temperature (30 °C) the CSVd concentration increased to that of control (untreated) plants. El-Dougdoug et al. [175] demonstrated that cold- and heat-therapy can have opposite effects on certain viroid–plant systems, *i.e.*, heat-therapy (37 °C for 3 weeks) *in vitro* was not an efficient method for obtaining HSVd-free peach and pear plants, whereas cold-therapy (4 °C for 3 weeks) led to HSVd elimination from the same plants by 18% for both cases. Matousek et al. [176] found a negative correlation between HLVd titer and nuclease activity during the thermotherapy of hop plants. Incubation of *in vitro*-grown hop plants for 2 weeks at 35 °C led to a dramatic decrease (70–90%) in the HLVd titer, and at the same time, nuclease activity capable of cleaving HLVd and fully double-stranded RNA (dsRNA) increased significantly in hop tissues during thermotherapy cycles, or after the heat shock. Strong tissue-specific gradients of viroid concentrations (the lowest level in stem apex and the highest level in roots) were observed in young plants, showing a negative correlation with the dsRNase activity.

The analysis of the trafficking pattern of PSTVd in *N. benthamiana* and tomato (*Solanum lycopersicum*) revealed the absence of PSTVd RNA in the shoot apical meristem and lateral shoot meristems of mechanically inoculated plants [65]. Mahfouze et al. [177] obtained 83.3% recovery of PSTVd-free potato plants after application of meristem-tip culture alone.

The combination of thermotherapy with meristem-tip culture resulted in more efficient viroid elimination from infected plants [178,179]. The severe strain of PSTVd was successfully eliminated from a potato clone by a combination of low temperature (5–8 °C) and low light treatments of viroid-infected plants and subsequent meristem culture. Seven of 13 plants (~54%), which developed from meristems of plantlets grown *in vitro* for 6 months at 5–6 °C, were found to be PSTVd-free. From plantlets derived from infected tubers and grown at 8 °C for 4 months, 17 excised meristems grew to plants and 5 of them (~29%) were free of PSTVd [179]. In another study, viroid-free chrysanthemum plants were obtained from meristem-tips cut from chrysanthemum infected with CSVd, *Chrysanthemum chlorotic mottle viroid* (CChMVd) or *Cucumber pale fruit viroid* (CPFVd) after 6 months of cold-therapy (5 °C) [180]. In the same study, PSTVd-free plants were obtained from meristem-tips cut from sprouts grown from potato tubers infected with severe or mild strains of PSTVd after 6 months cold-therapy at 6–7 °C in the dark. The efficiency of 6 months therapy in viroid elimination was dependent on the viroid and plant species and was from 18.5% to 80.0% [180]. Postman and Hadidi [181] reported elimination of *Apple scar skin viroid* (ASSVd) from infected pear plants with 85 and 86% recovery of ASSVd-free plants for heat- and cold-treated meristems, respectively. Adams et al. [182] demonstrated 36% recovery of HLVd-free hop plants after storage of infected plants at low temperature (2–4 °C in the dark) for several months followed by meristem culture using small explants. Mahfouze et al. [177] treated the potato tubers infected with PSTVd with low temperature (21 °C) and cold-therapy (4, 5 and 8 °C) for 4 months along with sprout excision or meristem-tip excision from the sprouts. It was shown that the maximum PSTVd elimination, in case of sprout excision, was 71.4% for temperatures 4 and 5 °C and, in case of sprout meristem-tip excision, recovery of PSTVd-free plants reached 100% after tuber incubation at 4, 5 and 8 °C. Jeon et al. [183] showed that efficiency of CSVd elimination from chrysanthemum was influenced by the size of meristem used for plant regeneration, namely, there was a negative correlation between excised shoot tips and percentage of viroid-free plantlets obtained. The small-sized meristems with 1 or 2 leaf primordia regenerated into the highest number of CSVd-free plantlets showing 28.6% and 22.2% recovery, respectively, although they demonstrated a lower survival ratio than larger meristems. In the same work, the prolonged plant heat treatment (37 °C) led to damage of leaves or shoots of *in vitro* explants leading to decrease of survival percentage from 37.8% to 18.5% according to the increase of the period of heat treatment. The percentage of CSVd-free plants recovered from heat treatment along with meristem-tip culture varied from 29.4% to 16.7% depending on percentage of survival plants. Savitri et al. [184] showed that the combination of low temperature (4 °C) treatment of CSVd-infected chrysanthemum for 2 months followed by meristem-tip culture increased CSVd elimination rate up to 42.8%.

Cryotherapy of shoot tips is a relatively new method for pathogen eradication based on a cryopreservation technique that was successfully applied in potato, sweet potato, grapevine, banana, raspberry and prunes to eliminate pathogens such as viruses, phytoplasmas, and bacteria [185]. During cryotherapy, the cells containing pathogens do not survive the exposure to liquid nitrogen (−196 °C). The size of meristem excised after cryotreatment is larger than the size of meristem used in traditional shoot tip grafting technique, and therefore, makes it easier to perform and increases the survival rate of plantlets (http://www.ars.usda.gov/research/projects/projects.htm?accn_no=424246). This approach is an especially promising strategy aimed at pathogen elimination for citrus orchards and requires further investigation of efficiency to combat viroid diseases.

Grafting technology: Over the years grafting became an important tool in agricultural management that has many applications including pathogen eradication from the host–plant [186–188]. Despite the fact that this approach requires a high level of expertise due to difficulties of technical performance, the shoot-tip grafting technology was successfully applied for elimination of *Citrus cachexia viroid* (CCaVd) and CEVd in citrus trees, showing 9.5% recovery of pathogen-free plants [189]. Hosokawa et al. [190] established a new method of chrysanthemum regeneration by attaching a leaf primordium-free apical meristem of a CSVd-infected chrysanthemum plant to a root tip (0.5 mm) of a CSVd-free chrysanthemum or a cabbage (*Brassica oleracea*). Using this technique, CSVd-free chrysanthemum plants were generated on the chrysanthemum and cabbage root tips with CSVd elimination rates 14 and 3%, respectively [191]. The same group obtained CChMVd-free chrysanthemum plants of different cultivars by attaching leaf primordium-free apical meristems of CChMVd-infected chrysanthemum plants to cabbage root tips free of viroid infection [192].

Different combinations of various approaches (chemotherapy, tissue culture technique, thermotherapy, electrotherapy) were applied to protect plants against viroid infection. Antiviral chemicals such as virazol (ribavirin), phosphonoacetic acid and amantadine were tested on CSVd-infected chrysanthemum shoot apices in tissue culture. Amantadine (50–100 mg/L) incorporated into a tissue culture medium for chrysanthemums was effective in obtaining chrysanthemum plantlets free of CSVd (10%) and did not reveal phytotoxicity at the concentrations used. Phytotoxicity was detected for phosphonoacetic acid even at the lowest concentrations (10 mg/L). Although no phytotoxicity was observed from virazol treatments, no plants were found to be free from CSVd [193]. Growing of the potato plantlets on medium containing the antiviroid agent ribavirin, acetyl salicylic acid or 2-thiouracil at a concentration of 50 mg/L led to 87.5%, 83.3% and 85.7% PSTVd

elimination, respectively [177]. El-Dougdoug et al. [175] used ribavirin and thiouracil at different concentrations in order to determine the efficiency for HSVd elimination in peach and pear fruit trees. It was revealed that ribavirin (30 mg/L) incorporated into culture medium had a better effect on viroid elimination in peach and pear plantlets (41% and 40% recovery, respectively) than thiouracil at the same concentration (18% and 30% recovery of HSVd-free peach and pear plantlets, respectively). The combination of cold therapy (4 °C for 1 month) and chemotherapy (ribavirin at concentration 20 mg/L) gave 40% elimination of HSVd from the tested plantlets. Savitri et al. [184] investigated the effect of low temperature (4 °C), antiviral chemicals (ribavirin and amantadine) and a combination of these treatments on CSVd elimination by meristem tip cultures using plantlets that originated from CSVd-infected chrysanthemum. The most effective results were obtained from a combination of low temperature for 3 months at 4 °C followed by meristem tip culture on media containing 50 and 100 mg/L ribavirin.

Mahfouze et al. [177] applied electricity to eliminate PSTVd from potato tubers by exposure of the tubers to an electrical current of 5, 10 and 15 mA for 5 and 10 min followed by excision of the tuber sprouts with subsequent shoot-tip culture. It was found that treatment with electrical currents of 10 and 15 mA for 10 min and 15 mA for 5 min were the most effective (100%) for PSTVd elimination. Earlier, electrotherapy along with shoot-tip culture was successfully used for virus elimination from potato plants [194,195].

Viroid cross-protection: This approach is based on the observation that infection of plants with a mild virus/viroid strain can protect the plant against severe virus/viroid strain. After McKinney [196] discovered viral cross-protection for TMV, Fernow [197] described this phenomenon for mild and severe PSTVd strains on tomato plants. Niblett et al. [198] showed that CSVd, and mild and severe strains of PSTVd, protected chrysanthemum against CEVd, whereas the mild strain of PSTVd protected tomato plants against expression of the symptoms caused by CEVd and the PSTVd severe strain. In both cases, the replication of protecting and challenging viroids was detected in plants. Branch et al. [199] reported the interference between PSTVd and HSVd in tomato plants. Tomato plants inoculated with dual transcripts, containing two copies of PSTVd linked to two copies of HSVd, developed characteristic symptoms of PSTVd infection. Dot blot hybridization revealed the presence of only PSTVd in plant tissues. In the same work, simultaneous inoculation of tomato plants with mild and severe PSTVd isolates led to a predomination of severe symptoms on 75% of plants even when the inoculum contained 100-fold excess of the mild isolate. No additive effects were observed on PSTVd co-inoculated plants. Singh et al. [200] showed that in the highly susceptible potato cultivar Russet Burbank pre-infected with mild PSTVd strain and challenged with severe PSTVd strain, the latter was not detected in all experimental plants, whereas in case of tolerant BelRus potato cultivar pre-infected with the mild PSTVd strain, cross-protection was not complete, *i.e.*, severe PSTVd strain was detected in 2 out of 10 tested plants. In the same study, second generation potato plants grown from tubers pre-infected with mild/severe PSTVd strain in previous generation were completely protected against challenging infection with severe/mild PSTVd strain, respectively.

In spite of the relative efficiency of this approach against virus/viroid diseases it cannot be applied as a general practice due to danger of biological contamination of the environment [201]. Though the mechanism of cross-protection remains unclear it was proposed that cross-protection is mediated by various defense mechanisms such as protein-mediated or RNA-mediated resistance [202,203], where plant physiological status also plays an important role [204]. Ratcliff et al. [205] postulated that RNA-mediated cross-protection is functionally equivalent to PTGS. It is proposed that the mild ("protecting") strain serves as a 'primer' for PTGS initiation [201]; in this regard it is essential that the sequences of mild and severe ("challenge") strains must be similar. It is consistent with the results obtained by Niblett et al. [198] showing that CChMVd could not protect chrysanthemum plants against PSTVd, CEVd and CSVd infections, and PSTVd failed to protect these plants against CChMVd. On the other hand, CSVd and PSTVd were able to protect chrysanthemum plants against CEVd infection. The failing of cross-protection between members of *Pospiviroidae* (PSTVd, CEVd and CSVd) and *Avsunviroidae* (CChMVd) could be explained by quite distinct genome sequences between these two families. To investigate the cross-protection mechanism, Zhou and Zhou [206] developed a rapid micro-extraction method for the preparation of total nucleic acid that can be combined with other molecular methods. This method allows one to monitor the interaction of virus strains at short time intervals in young plants. Though the effectiveness of this method was demonstrated for *Citrus tristeza virus* (CTV) [207], according to this report, it can be applied for investigation of viroid cross-protection mechanisms as well.

6.2. Strategies to introduce resistance by transgenic methods

There are a number of natural plant protective mechanisms against pathogen invasion, such as production of resistance proteins (R-gene products) [208–210], phytoalexins [211], reactive oxygen species causing hypersensitive response [212,213], and RNA silencing [214] among others. However, in most cases the natural activation of such mechanisms is not enough to resist pathogenic microorganisms. Plant viruses overcome resistance mechanisms in plant cells by encoding multifunctional RNA silencing suppressor proteins. Viroids, on the other hand, do not encode any proteins and the viroid RNA molecule itself cannot function as an RNA silencing suppressor [136].

Based on molecular analysis of viroid replication mechanisms, plant–viroid interactions and host–pathogen relationships, various strategies have been developed to attenuate/prevent viroid infection using molecular transformation of the host plant. The first reported study of plant protection against viroid infection was the use of an *antisense RNA* strategy performed by Matousek et al. [215]. It was shown that antisense RNA directed against either plus- or minus-strand sequences (corresponding to the left half of the rod-like secondary structure of minus-strand replication intermediate, or to the upper central conserved region of plus-strand viroid replication intermediate, respectively) of PSTVd formed complexes with the corresponding target RNA *in vitro*. The same antisense RNA integrated and expressed in potato plants led to significant inhibition to PSTVd accumulation although severely infected plants were observed in all transgenic lines 6 to 8 weeks post challenge inoculation. Atkins et al. [216] demonstrated that inoculation of transgenic tomato seedlings expressing antisense constructs targeting the negative-strand of the CEVd RNA molecule with CEVd resulted in a moderate reduction in the accumulation of CEVd RNA in plant tissues. In contrast, similarly inoculated transgenic plants expressing constructs targeting the positive-strand CEVd RNA molecule resulted in an increase in the rate of CEVd RNA accumulation when compare with control (non-transformed) plants. Incorporation of ribozyme motifs to the antisense genes did not enhance their efficiency in the suppression of viroid replication in plants, in spite of the presence of catalytic activity of the ribozyme constructs *in vitro*.

Yang et al. [217] expressed a *hammerhead ribozyme* targeting the minus-strand RNA of PSTVd in potato plants and demonstrated that 23 out of 34 transgenic plant lines (about 68%) possessed high level resistance to viroid infection after plants were challenge inoculated with PSTVd. Northern blot analysis of total RNA isolated from transgenic potato lines revealed no PSTVd RNA in plants containing the ribozyme construct. The remaining lines showed a weaker

level of resistance to PSTVd with low levels of PSTVd accumulation. The resistance against PSTVd replication was stably inherited to the vegetative progenies. Carbonell et al. [218,219] tested ability of the *trans*-cleaving extended hammerhead ribozyme (HHe) derived from PLMVd [220] to control PSTVd infection in *N. benthamiana*. During viroid replication of avsunviroids, hammerhead ribozymes act in *cis* (self-cleaving the RNA in which they are embedded through a single turnover mechanism) [42]. However, the ribozyme can be manipulated to act in *trans* by splitting it into the ribozyme itself and the substrate [221]. As a result, hammerhead ribozymes can target specific RNAs for degradation and one molecule of ribozyme can act on several molecules of substrate, through a multiple turnover mechanism, thus increasing the catalytic efficiency. Previously, it was shown that tertiary stabilizing motifs (TSMs; particularly interactions between peripheral loops) appeared critical for the catalytic activity of hammerheads [222,223]. A natural TSM was incorporated into a *trans*-cleaving PLMVd-derived hammerhead giving rise to an extended format of this ribozyme (HHe-PLMVd). For experiments *in vivo*, two cultures of *Agrobacterium tumefaciens* transformed with constructs expressing the HHe-PLMVd and the substrate dPSTVd (−), which generates a head-to-tail dimeric PSTVd (−) RNA that triggers replication through the asymmetric variant of the rolling-circle mechanism were co-infiltrated in tobacco leaves. The experiment showed that HHe-PLMVd interfered with systemic PSTVd infection when co-expressed with the infectious dPSTVd (−) RNA, indicating that it may target the primary dimeric transcript and perhaps also the oligomeric (−) replicative intermediates. Constitutive expression in transgenic plants of a modified ribozyme like HHe-PLMVd may control PSTVd more efficiently [219].

Schwind et al. [143] demonstrated that two out of three transgenic tomato lines expressing a *hairpin RNA* (hpRNA) construct, derived from PSTVd sequences, exhibited resistance to PSTVd infection. This resistance was correlated with high-level accumulation of hpRNA-derived siRNAs in the plant tissues. Although small RNAs produced by the infecting viroid did not silence viroid RNAs efficiently to prevent their replication, the results of this work showed that hpRNA-derived siRNAs (hp-siRNAs) effectively targeted the mature PSTVd RNA.

Di Serio et al. [138] reported that silencing of the gene encoding the RNA-dependent RNA polymerase (RDR6), that catalyzes the amplification circuit producing double-stranded precursors of secondary silencing RNAs (siRNAs), led to the accumulation of increased amounts of PSTVd genomic RNAs compared to non-silenced plants. Gómez et al. [224], using a symptomatic, transgenic line of *N. benthamiana* that expresses and processes dimeric forms of HSVd, demonstrated that symptom expression is independent of HSVd accumulation levels but dependent on an active state of the viroid-specific silencing pathway. The scion of tobacco plants, in which the RDR6 is constitutively silenced, remained asymptomatic when grafted onto symptomatic plants, despite an accumulation of a high level of mature forms of HSVd, indicating the requirement of RDR6 for viroid-induced symptom production. These results indicate the involvement of the viroid-specific RNA silencing pathway in the symptom expression associated with viroid pathogenesis. The demonstration that siRNAs transported through grafts from rootstocks to scions induce silencing of an endogenous gene in the scion [225] led Kasai et al. [226] to explore the ability of PSTVd siRNAs, generated in transgenic *N. benthamiana* rootstocks, to reduce accumulation of viroid RNAs in challenge-inoculated scions. A truncated, near full-length PSTVd hp RNA sequence expressed from a strong companion cell-specific transcriptional promoter, to increase the potential siRNAs in phloem tissue, resulted in suppression of viroid accumulation in the early stages of infection of transgenic plants, although all plants were infected at a later stage. When wild-type scions were grafted onto the transgenic rootstocks, there was evidence of attenuation of PSTVd accumulation in the early stage of infection (12 dpi), but all plants became infected at a later stage. Although the approach is theoretically promising, and there is merit in the development of genetically modified rootstocks, further improvements are needed to increase its efficacy in controlling viroid infection.

Carbonell et al. [227] studied the effect of viroid-derived dsRNAs and small RNAs (vd-sRNAs) on viroid infectivity. Experiments were conducted on tomato plants (with PSTVd and CEVd), gynura (with CEVd) and chrysanthemum (with CChMVd) using an excess of the homologous dsRNA or vd-sRNAs in the inocula. The sequence specific effect was observed for all biological assays. The CEVd-gynura/tomato (CEVd + CEVd-dsRNA) and CChMVd-chrysanthemum (CChMVd + CChMVd-dsRNA) systems reduced the infectivity, showing that half of the tested plants did not express symptoms and Northern blot analysis failed to detect the viroid in these plants. In PSTVd-tomato system (PSTVd + PSTVd-dsRNA), co-inoculation of PSTVd-dsRNA and PSTVd was not as effective because all plants eventually became infected, although symptom appearance was delayed and less severe. Experiments with vd-sRNAs obtained by *in vitro* digestion of dsRNA and co-inoculated with their homologous viroid showed that the CEVd-gynura/tomato system (CEVd + CEVd-sRNAs) decreased infectivity, whereas PSTVd-tomato (PSTVd + PSTVd-sRNAs) and CChMVd-chrysanthemum (CChMVd + CChMVd-sRNAs) systems did not reveal any observable effects on viroid infection.

Another approach involved expression of the dsRNA-specific ribonuclease *pac*1 gene naturally found in yeast *Schizosaccharomyces pombe* [228] and encoding a protein structurally similar to RNase III from *Escherichia coli.* Pac1 protein is highly active and digests long dsRNAs into short oligonucleotides and also cleaves a small hpRNA substrate [228]. Expression of Pac I protein in transgenic plants led to resistance to several single-stranded plant RNA viruses [229–231]. Sano et al. [232] showed that five potato lines expressing *pac*1 and challenge-inoculated with PSTVd exhibited resistance to PSTVd infection and reduced viroid carryover through seed potatoes. Resistance assays were conducted at different temperatures 25–32 °C (more favorable for viroid replication and accumulation) and 20–28 °C (resembling actual field conditions). In both cases, none of the viroid-challenged Pac1 plants developed disease symptoms.

Ogawa et al. [231] obtained three transgenic lines of chrysanthemum plants stably producing Pac1 protein and tested them against viroid and virus infections. After challenge inoculation with CSVd, the tolerance assay showed that infection frequency for two of three transgenic lines was less than 50% in contrast to that in control plants (100% or 78%). One out of nine plants (11%) and two out of nine plants (22%) from the different transgenic lines revealed CSVd infection after 60 days post viroid inoculation (dpi), whereas approximately 70% of control plants were infected in 30 dpi. The infection frequency in control plants reached 100% in 40 dpi, while only about 50% of transgenic plants were infected at the same time. In another viroid-tolerance assay, transgenic and control plants were grafted on CSVd-infected plants. The transgenic plants showed better growth, and one line exhibited the least growth retardation. Analysis of CSVd accumulation in transgenic lanes by micro-plate hybridization, performed after 52 dpi, revealed that about half of the transgenic plants did not exhibit CSVd infection. The remaining plants were infected, but the accumulation of CSVd in these infected plants was suppressed compared with that in control plants. All three transgenic lines displayed significantly lower infection frequencies compared to control when chrysanthemum plants were challenge inoculated with *Tomato spotted wilt virus* (TSWV). Ishida et al. [230] reported the efficiency of the Pac1 protein to protect potato and chrysanthemum plants against PSTVd and CSVd infections, respectively. Pac1-transgenic potato plants

inoculated with PSTVd did not develop disease symptoms, where as non-transgenic control plants showed symptoms at 30–40 dpi. In transgenic potato lines, viroid RNA was detected in 1/3 to 1/2 of the plants, and where viroid was not detected in leaves following inoculation with PSTVd, viroid-free tubers were produced. *Pac1* transgenic chrysanthemum plants did not produce disease symptoms after challenge infection with CSVd, and CSVd was detected only in 20% of the highest Pac1 expressing plants. In addition, the chrysanthemum transgenic line displaying the highest expression of Pac1 was tested in an isolated field, and the transgenic chrysanthemums (cv. Reagan) produced a small amount of pollen that might cross-hybridize with, and be transmitted from, the transgenic trait to surrounding plants. In all of these reports, healthy transgenic plants grew without abnormal phenotypes, suggesting that there was no adverse effect of Pac1 over expression on the physiology of the plant.

7. Conclusions and outlook

Extensive research conducted since the first viroid was described [5] has resulted in the development of a variety of different strategies to protect plants against viroid invasion and transmission of viroid RNA from infected to healthy plants. Unfortunately, to date, efforts to produce transgenic plants with durable resistance to viroids using various biotechnological approaches that target viroid replicative intermediates have resulted in variable levels of resistance. A combination of factors, such as the resistance of secondary structures of mature viroid molecules to degradation, subcellular compartmentation, and association with host proteins may help viroids elude the host RNA silencing machinery, thereby reducing the efficacy of RNA silencing-based methods of control. There is no unique recipe that can be applied to protect any host-plant against any viroid species. Development of approaches that could avoid species-specificity is a promising strategy that can be applied to any pathogen–host system. In this regard, expression of the yeast dsRNA-specific ribonuclease *pac1* gene in host-plants seems an efficient method to combat viroid/virus infection. Non-hosts have been shown to have extremely low levels of viroid replication and the viroid cannot move from the initial site of infection. New approaches for molecular control of viroid diseases might include limitation of pathogen spread, *i.e.*, interference with cell-to-cell and long distance movement by disrupting viroid interaction with host proteins such as Virp1 through implementation of gene silencing mechanisms, or suppressing the synthesis of the proteins presumed to be involved in movement. To prevent viroid transmission from already infected plants, the classic means of control such as disinfection of cutting tool surfaces, control of aerial vector populations and the use of planting material that is free of viroids are the best ways to restrict spread of viroid diseases under field conditions. Although the mechanisms through which viroids interact with their hosts are beginning to be dissected, the key triggering events and molecular mechanisms underlying viroid pathogenesis are largely unknown. Further investigation of the molecular basis of viroid–host interactions will contribute to the development of new approaches to develop novel strategies to combat viroid diseases.

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