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Research paper

In silico prediction and validation of potential gene targets for pospiviroid-derived small RNAs during tomato infection

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ABSTRACT

Viroids are small, covalently closed, circular non-coding RNA pathogens of flowering plants. It is proposed that the symptoms of viroid pathogenesis result from a direct interaction between the viroid genomic RNA and unknown host plant factors. Using a comparative genomic approach we took advantage of the detailed annotation of the *Arabidopsis thaliana* (Arabidopsis) genome to identify sequence homologies between putative viroid-derived small RNAs (vd-sRNAs) and coding regions in the plant genome. A pool of sequence homologies among 29 species of the *Pospiviroidae* family and the Arabidopsis genome was analyzed. Using this strategy we identified putative host gene targets that may be involved in symptom expression in viroid-infected plants. In this communication, we report the in silico prediction and the experimental validation of pospiviroid-derived sRNAs conserved in the lower strand of the pathogenicity domain of seven viroid species infecting tomato; those vd-sRNAs targeted for cleavage of the host mRNA encoding a conserved tomato WD40-repeat protein (*SolWD40-repeat*; SGN_U563134). Analysis of *SolWD40-repeat* expression indicated that this gene is down-regulated in tomato plants infected with tomato planta macho viroid (TPMVd). Furthermore, 5′ RLM-RACE revealed that the *SolWD40-repeat* mRNA is cleaved at the predicted target site showing complementarity to a corresponding TPMVd-sRNA identified in silico. Our approach proved to be useful for the identification of natural host genes containing sequence homologies with segments of the *Pospiviroidae* genome. Using this strategy we identified a functionally conserved gene in Arabidopsis and tomato, whose expression was modified during viroid infection in the host genome; regulation of this gene expression could be guided by vd-sRNA:mRNA complementarity, suggesting that the comparison of the Arabidopsis genome to viroid sequences could lead to the identification of unexpected interactions between viroid RNAs and their host.

Abbreviations: Aa, amino acid; ABSVd, avocado sunblotch viroid; AFCVd, apple fruit crinkle viroid; AGO, argonaute; AGVd, Australian grapevine viroid; ASSVd, apple scar skin viroid; CBVd, *Coleus blumei viroid*; CBCVd, citrus bark cracking viroid; CBLVd, citrus bent leaf viroid; CChMVd, chrysanthemum chlorotic mottle viroid; CCR, central conserved region; cDNA, complementary DNA; CEVd, citrus exocortis viroid; CLVd, Columnea latent viroid; CSVd, chrysanthemum stunt viroid; CVd, citrus viroid; DCL, dicer-like; DNA, deoxyribonucleic acid; HLVd, hop latent viroid; HSVd, hop stunt viroid; GYSV, grapevine yellow speckle viroid; KH_2PO_4, potassium phosphate; miRNA, microRNA; MMLV, Moloney Murine Leukemia Virus; MPVd, Mexican papita viroid; mRNA, messenger RNA; nat siRNA, antisense interfering RNA; nt, nucleotide; P, pathogenicity; piRNA, piwi protein interactor RNA; PLMVd, peach latent mosaic viroid; PVd, persimmon viroid; ss, double-stranded; sRNA, small RNA; siRNA, small interfering RNA; RT, reverse transcription; PCR, polymerase chain reaction; PSTVd, potato spindle tuber viroid; RISC, RNA-induced silencing complex; RLM-RACE, RNA-ligase-mediated rapid amplification of cDNA ends; RNA, ribonucleic acid; ss, single-stranded; T, terminal; TAIR, The Arabidopsis Information Resource Database; ta-siRNA, trans-activating interfering RNA; TASVd, tomato apical stunt viroid; TCDVd, tomato chlorotic dwarf viroid; TPMVd, tomato planta macho viroid; UTR, untranslated region; V, variable; vd-sRNA, viroid small RNA; VMR, virulence modulating region.

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1. Introduction

Viroids are plant-restricted parasites that represent a remarkable model system to analyze many aspects of host–pathogen interactions at the genomic level (Ding and Itaya, 2007; Flores et al., 2005; Navarro et al., 2012a; Tabler and Tsagris, 2004). As the smallest known agents of infectious disease (246–401 nucleotides, nt), viroids are characterized by a highly structured, single-stranded (ss), closed circular, unencapsidated non-coding RNA genome (Flores et al., 2003; Semancik, 2003). Despite their lack of mRNA polypeptide-coding capacity, they are still able to replicate autonomously in natural hosts using host enzymes, causing diseases in several susceptible plant species (Diener, 1987). Systemic viroid infection is commonly associated with the expression of severe symptoms, including stunting, leaf epinasty, leaf distortion, veinal chlorosis, reduction of flower size, flower abortion, and reduced size and number of fruits (Ding, 2009; Flores et al., 2005; Hadidi et al., 2003). How these parasites – with a minimal genome lacking coding capacity – cause changes that result in drastic alterations in plant development remains one of the most intriguing questions in viroid research.

Based on comparative analyses of the primary and highly conserved secondary structures, the International Committee on Taxonomy of Viruses classification scheme for viroids released in 2013 (http://ictvonline.org/virusTaxonomy.asp) has classified thirty-two viroid species into two families (reviewed by Di Serio et al., 2014). These families have significant differences in their predicted structures, replication mechanisms, and location of subcellullar accumulation. Members of the family *Avsunviroidae* possess hammerhead ribozyme activity and replicate via double-stranded RNA (dsRNA) intermediates in the chloroplast (Delgado et al., 2005; Navarro et al., 2000), while members of the family *Pospiviroidae* (most viroids) replicate via dsRNA intermediates in the nucleus and adopt a quasi-rod like secondary structure in which five structural domains can be distinguished (Branch and Robertson, 1984; Keese and Symons, 1985). These domains are the central conserved region (CCR), which contains conserved sites among species from the same genus and is associated with viroid replication; the terminal left and right domains (T_L, T_R) related to duplication and movement of the viroid; the variable domain (V) that is the most different among viroid species from the same genus; and the pathogenicity domain (P) containing structural elements that contribute substantially to the regulation of symptom expression (Hammond and Owens, 1987; Henco et al., 1979; Owens et al., 1996; Visvader and Symons, 1985). Functional analysis of the role of specific RNA sequences and structural motifs residing in the viroid genome has revealed specific elements, such as the virulence modulating region (VMR), RY motifs (structural elements located near the T_R which are involved in host protein binding for systemic viroid movement) (Gozmanova et al., 2003; Maniataki et al., 2003), temporally metastable structures known as Hairpin I and Hairpin II, and the E-Loop motif involved in viroid processing during replication (Baumstark et al., 1997; Eiras et al., 2007); for a recent detailed review see Flores et al. (2012).

It has been proposed that the mechanism of viroid pathogenesis is mediated directly by the viroid genome itself, or by viroid genome-derived ss- or dsRNAs, and that expression of symptoms as a result of systemic infection may be an outcome of direct interactions of viroid-derived RNAs with unknown host factors (protein or nucleic acid), either in the organelle where the viroid replicates or in the cytoplasm where they accumulate during its movement (Flores et al., 2005). Three general pathways account for this mechanism: mature viroid RNA–host factor interactions (via induction or activation of proteins; mainly pathogenesis-related protein kinases), or blocked protein functions; hormone-mediated responses (altered plant endogenous miRNA and siRNA pathways); and viroid dsRNAs processed by specific Dicer-like (DCL) enzymes into small RNAs which guide the RNA-induced silencing complex (RISC) (host mRNA cleavage/host DNA methylation and transcriptional silencing); all of these influencing host gene expression (for reviews see Gómez et al., 2009; Hammann and Steger, 2012; Sano et al., 2010).

An increasing body of evidence has revealed that RNA-mediated gene regulation, primarily via viroid-induced RNA silencing, might play an important role in disease development. As may be expected from their highly base-paired structures and the presence of dsRNA replication intermediates, viroid genomes can be processed by the DCL enzymes and loaded into the RISC by Argonaute (AGO) proteins (Minola et al., 2014). Structurally similar to endogenous small RNAs in plants, viroid-derived small RNAs (vd-sRNAs) range from 21–24 nt in length. These small RNAs have been detected, and in some instances characterized, in infected tissues from the following viroid–host combinations of both viroid families: *Pospiviroidae:* genus *Pospiviroid*: potato spindle tuber viroid (PSTVd)-tomato (*Solanum lycopersicum*, susceptible and tolerant cultivars), PSTVd-*Nicotiana benthamiana*, PSTVd-*Arabidopsis thaliana*, PSTVd-*Phelipanche ramosa*, citrus exocortis viroid (CEVd)-tomato, CEVd-*N. benthamiana*; genus *Hostuviroid* : hop stunt viroid (HSVd)-grapevine and HSVd-cucumber; genus *Apscaviroid*: grapevine yellow speckle viroid (GYSVd)1-grapevine; genus *Cocadviroid*: hop latent viroid (HLVd)-hops; mixtures: HLVd + apple fruit crinkle viroid (AFCVd)-hops, HLVd + HSVd-hops (Diermann et al., 2010; Itaya et al., 2007; Ivanova et al., 2014; Machida et al., 2007; Markarian et al., 2010; Martin et al., 2007; Matousek et al., 2007; Navarro et al., 2009; Owens et al., 2012; Papaefthimiou et al., 2001; Wang et al., 2011), and *Avsunviroidae*: genus *Avsunviroid*: avocado sunblotch viroid (ABSVd)-avocado, and genus *Pelamoviroid*: peach latent mosaic viroid (PLMVd)-peach, and chrysanthemum chlorotic mottle viroid (CChMVd)-chrysanthemum (Di Serio et al., 2009; Martínez de Alba et al., 2002; Navarro et al., 2012b; St-Pierre et al., 2009).

The two major classes of plant sRNAs are miRNAs (21–22 nt) and siRNAs (21–22 and 24 nt), which act by triggering an amplification cascade mediated by host RNA-dependent RNA polymerases (RDRs) and DCLs resulting in the generation of secondary 21-nt siRNAs that promote RNA silencing in a non-cell autonomous systemic manner (Voinnet, 2008). This system has been widely implicated as an antiviral defense in plants and animals, providing protection to plants from invasion by exogenous RNA replicons such as viruses and viroids (Akbergenov et al., 2006; Baulcombe, 2004; Darós et al., 2006; Ding, 2010; Fusaro et al., 2006; Garcia-Ruiz et al., 2010; Vance and Vaucheret, 2001). If this mechanism of gene regulation is also involved in viroid pathogenicity (the hypothesis that vd-sRNAs are involved in symptom expression in infected plants), vd-sRNAs would be complementary to host mRNAs and promote cleavage or repression of the target genes. In the post-genomic era, different strategies have been developed to identify potential host gene targets of pathogenic organisms. Comparative genomic approaches allow the systematic classification of gene regulatory regions and the identification of small RNAs; however a major challenge is the identification of host gene sequences that are potential targets of specific sRNAs.

Previous bioinformatic approaches have compared viroid genomes to the genome of tomato or *A. thaliana* (Diermann et al., 2010) yielding many sequences representing potential vd-sRNAs and their targets; however, these studies were not validated at the molecular level, and no evidence for specific functional interactions leading to the regulation of plant gene expression has been reported. An initial search for potential vd-sRNAs and their gene targets in transgenic tomato lines expressing a non-infectious PSTVd hairpin RNA yielded several 19–20 nt sequences corresponding to the A–G rich PSTVd VMR (nucleotides 45–68) and to identical sequences present in the genome of several plant species (Wang et al., 2004). These results suggested the possibility that vd-sRNAs derived from the VMR of the P domain of PSTVd could silence host regulatory genes involved in previously unknown pathways (Hammond and Zhao, 2000; Wang et al., 2004). Similar comparative approaches were used to search the grapevine genome for possible targets of sequenced vd-sRNAs and, although only one 21 nt sequence that completely matched the HSVd species genome was identified, several

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