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RESEARCH ARTICLE

Characterization and function of *Tomato yellow leaf curl virus*-derived small RNAs generated in tolerant and susceptible tomato varieties

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Abstract

Virus-tolerant plant, which allows the accumulation of virus and then generates virus-derived small RNAs (vsRNAs), is a valuable material to reveal the antiviral efficiency of vsRNAs. Here, a comparison of vsRNAs in *Tomato yellow leaf curl virus* tolerant and in susceptible tomato varieties showed the consistent trend of vsRNAs' distribution on virus genome, which is presented as an obvious characteristic. However, the expression level of vsRNA in tolerant variety is less than that in susceptible variety. Slicing targets of vsRNA-mediated viral transcripts were investigated using parallel analysis of RNA ends, and geminivirus DNA methylation was determined by bisulfite sequencing, which uncovered that not all vsRNAs participated in viral mRNA degradation and DNA methylation. Additionally, by comparing with the expression pattern of vsRNAs, viral DNA and mRNA, we proposed the quantity of vsRNAs is corresponding to the expression level of viral mRNA, while the virus-suppression of vsRNAs is not high-efficient.

Keywords: *Tomato yellow leaf curl virus*, virus-induced RNA silencing, virus-derived small RNA, degradome

1. Introduction

In the wild, plants may be infected by a variety of viruses. RNA silencing has been evolved as an immune strategy against virus infection, termed virus-induced RNA silencing or RNA-based antiviral immunity (Li and Ding 2006; Ding 2010). Indeed, virus-derived small RNAs (vsRNAs), which trigger RNA silencing, have been detected in many plants infected with viruses (Ding 2010; Llave 2010). Similar to the situation in host plants, the canonical pathway of virus-induced RNA silencing requires host proteins participating in three stages. First, Dicer-like (DCL) ribonucleases recognize viral precursors and slice them into 21–24 nt primary vsRNAs (Blevins *et al.* 2006; Bouche *et al.* 2006; Deleris *et al.* 2006). vsRNAs are then incorporated into the RNA-induced silencing complex, which contains a distinct active component, ARGONAUTE (AGO), and guides the target viral RNA degradation and/or translational inhibition in a sequence-specific manner (Morel *et al.* 2002; Jones *et al.* 2006). By contrast, interaction with target viral DNA molecules causes transcriptional repression through the methylation (Raja *et al.* 2008). Amplification of vsRNAs involves the activity of host RNA-dependent RNA

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polymerases (RDRs) that synthesize double-stranded small RNA (dsRNA) with single-stranded RNA (ssRNA) as template (Garcia-Ruiz *et al.* 2010; Wang *et al.* 2010), which serves as a substrate for the DCL-dependent formation of secondary vsRNAs. Secondary vsRNAs support the systemic silencing that spreads throughout the plant (Molnar *et al.* 2010). To counteract this host antiviral strategy, viruses have also evolved genes that encode viral RNA silencing suppressors (Wu *et al.* 2010).

Although virus-induced RNA silencing has been proven to exist widely in host plants, it obviously does not work efficiently in virus-susceptible plant varieties. A part from the RNA silencing suppressors encoded by the virus, this may be explained by insufficiency of vsRNAs. Using a transgenic strategy, *via* strong promoter-inducing abundant virus-homologous small interfering RNA (siRNA), susceptible plant varieties can acquire viral resistance (Waterhouse *et al.* 1998; Zhu *et al.* 2009). However, the mechanism of host resistance genes competing against virus molecules is seemingly different from that of virus-induced RNA silencing. *Tomato yellow leaf curl virus* (TYLCV, genus *Begomovirus*, family Geminiviridae), with an ssDNA circular genome, infects tomato plants with obvious symptoms (typical yellowing and curling of the leaves) and causes significant losses in tomato production (Czosnek 2007; Yadava *et al.* 2010). Five genes (loci), named *Ty-1* to *Ty-5*, with different levels of resistance to TYLCV, have been reported so far. *Ty-1*, the first documented locus, is a partially dominant gene that originated from *Solanum chilense* accession LA1969 and was mapped to the distal end of chromosome 6 (Michelson *et al.* 1994; Zamir *et al.* 1994). Recently, Bai Yuling's group found that *Ty-1* and *Ty-3* are allelic and represent a new class of resistance gene that encodes an RDR belonging to the RDRγ type (Verlaan *et al.* 2013). Nevertheless, whether the amplification of virus-induced RNA silencing by RDR activity results in tomato TYLCV resistance remains unknown.

Extremely resistant material can suppress virus replication in the initial infection period, and identify vsRNAs in infected single cell is difficult; therefore, most virus-induced RNA silencing studies have been based on susceptible host varieties, not on resistant varieties. Upon infection with TYLCV, tomato plants containing the extreme resistance gene *Ty-2* do not express any vsRNAs which was assessed using deep sequencing. The reaction of the *Ty-1* locus-carrying lines to TYLCV isolates has been described as 'tolerance' because the plants became infected (with detectable levels of viral DNA) but displayed attenuated symptoms (Barbieri *et al.* 2010). The study of virus-induced RNA silencing in this tolerant material and comparison with susceptible varieties will help us to understand the relationship between the two anti-virus strategies (resistance genes and vsRNA), and will permit the evaluation of vsRNA efficiency.

2. Materials and methods

2.1. Plant materials and viral inoculations

Tomato (*Solanum lycopersicum*) *cv*. Moneymaker (TYLCV susceptible strain) and FL505 (containing *Ty-1* gene, Asian Vegetable Research and Development Center, Taiwan of China) at six to eight leaf stages (five weeks old) were agroinoculated with infectious clones of pBinPLUS-SH2-1.4A that contained TYLCV-[CN:SH2] (AM282874), as described previously (Zhang *et al*. 2009). The two samples were termed 'MMS' and 'TY1S', respectively. Mock inoculations was performed by inoculating plants with the *Agrobacterium tumefaciens* strain GV3101 containing pBinPLUS, these samples were called MMC and TY1C, respectively. Inoculated plants and controls were kept in an insect-free chamber at 25–27°C with 16 h of light per day. PCR was used to check for the presence of TYLCV in MMS and TY1S. Systemically infected plant leaves were harvested at 21 and 30 days post inoculation (dpi), at the same stage. Healthy leaves from mock-inoculated plants were used as a control. Symptoms and phenotype before and after virus inoculation are shown in Appendix A. Each leaf sample was divided into several parts, each part was used, separately, for small RNA (sRNA) and degradome library construction by deep sequencing, RNA extractions to identify viral RNA by quantitative RT-PCR (qRT-PCR), for total DNA extractions by quantitative PCR (qPCR) and for bisulfite sequencing PCR (BSP) for the detection of viral DNA methylation (Appendix B). Three biological replicates for each treatment were harvested for RNA isolation (for deep sequencing, all the replicates were combined into one sample).

2.2. Total DNA and RNA extractions

Total DNA from infected and mock-inoculated tomato leaves was isolated. RNA was removed with RNase I (Invitrogen, Carlsbad, CA, USA) treatment and then used for viral DNA qPCR analyses. Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's protocol and DNA was removed with DNase I (TaKaRa, Japan) treatment. First-strand cDNA was synthesized from 1 μg total RNA using an oligo(dT) primer and SuperScript™ III cDNA Synthesis Kit (Invitrogen), according to the manufacturer's instructions.

2.3. Quantitative analyses of viral RNA and DNA

Quantitative PCR was performed in 20-μL reactions including 20 ng of cDNA synthesized from viral RNA (or viral DNA), 0.2 mmol L^{-1} primer (primers used are listed in Appendix C, and 10 μL of SYBR Premix *ExTaq* (TaKaRa). PCR was