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Temperature-dependent symptom recovery in *Nicotiana benthamiana* plants infected with tomato ringspot virus is associated with reduced translation of viral RNA2 and requires ARGONAUTE 1

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ABSTRACT

Symptom recovery in nepovirus-infected plants has been attributed to the induction of RNA silencing. However, recovery is not always accompanied with viral RNA clearance. In this study, we show that recovery of *Nicotiana benthamiana* plants infected with the tomato ringspot virus (ToRSV) is associated with a reduction of the steady-state levels of RNA2-encoded coat protein (CP) and movement protein but not of RNA2. *In vivo* labeling experiments revealed efficient synthesis of the CP early in infection, but reduced RNA2 translation later in infection. Silencing of *Argonaute*1-*like* (*Ago*1) genes prevented both symptom recovery and RNA2 translation repression. Similarly, growing the plants at lower temperature (21 °C rather than 27 °C) alleviated the recovery and the translation repression. Taken together, our results suggest that recovery of ToRSV-infected plants is associated with an *Ago*1-dependent mechanism that represses the translation of viral RNA2.

Introduction

Symptom recovery in virus-infected plants is characterized by the emergence of asymptomatic leaves after a systemic symptomatic phase of infection and has been linked with the induction of RNA silencing (Baulcombe, 2004). RNA silencing, a ubiquitous gene regulation mechanism, is a well-established antiviral defense response in higher eukaryotes, notably in plants (Burgyan and Havelda, 2011; Ding, 2010; Omarov and Scholthof, 2012; Pumplin and Voinnet, 2013; Wang et al., 2012). Small RNAs provide the sequence specificity of RNA silencing by guiding the RNA-induced silencing complex (RISC) to its target. In the case of RNA viruses, regions of the viral genome with extensive secondary structure or double-stranded RNA replication intermediates are cleaved by DICER-LIKE endoribonucleases to produce viral-derived small-interfering RNAs (vsiRNAs). ARGONAUTE (AGO) proteins are the main components of the RISC and the effectors of silencing. Plant AGO proteins interact with the vsiRNAs and have been shown to direct the degradation of the viral RNA genome (Carbonell et al., 2012; Ciomperlik et al., 2011; Omarov et al., 2007; Pantaleo et al., 2007). Consistent with the notion that symptom recovery is caused by the induction of RNA silencing, recovered leaves of plants infected by some wild-type viruses, notably nepoviruses, caulimoviruses and tobraviruses, have reduced levels of viral RNAs compared to symptomatic leaves and display sequence-specific resistance to secondary infection (Al-Kaff et al., 1998; Covey et al., 1997; Ratcliff et al., 1997; Ratcliff et al., 1999). Many plant viruses encode suppressors of RNA silencing (VSRs) that sequester vsiRNAs and prevent the loading of RISC or that target one or several RNA silencing enzymes, often AGO proteins (Burgyan and Havelda, 2011; Omarov and Scholthof, 2012). Viruses with strong VSRs usually cause severe systemic infections, while mutant derivatives that are defective in their silencing suppression activity display attenuated symptoms, sometimes leading to recovery. For example, *Nicotiana benthamiana* plants infected with mutant tombusviruses that lack the p19 suppressor of silencing recover from infection and recovered leaves show decreased levels of viral RNAs (Omarov et al., 2007; Szittya et al., 2002; Szittya et al., 2003). RISC complexes purified from these recovered leaves have RNA slicing activity directed to the tombusvirus sequences (Omarov et al., 2007; Pantaleo et al., 2007), reinforcing the idea that symptom recovery requires the induction of an RNA silencing mechanism that degrades the viral RNA. However, symptom recovery is not always linked with viral RNA clearance, although it is generally associated with the induction of an RNA silencing response (Jovel et al., 2007; Xin and Ding, 2003; Zhang et al., 2012). Recently,

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temperature-dependent symptom attenuation in *Arabidopsis thaliana* plants infected with turnip crinkle virus was linked to an RNA silencing mechanism that depends on DCL2, AGO2 and HEN1 (an RNA methyltransferase) but allows robust accumulation of viral RNAs (Zhang et al., 2012). The genetic determinants leading to the establishment of symptom recovery in plant infected with other viruses have not been studied. For example, it is not known whether deficiency in one or several AGO proteins can affect the outcome of these natural infections.

AGO1, AGO2 and AGO7 have been implicated in the antiviral response based on the increased susceptibility of plants deficient for these proteins (Alvarado and Scholthof, 2012; Carbonell et al., 2012; Harvey et al., 2011; Jaubert et al., 2011; Morel et al., 2002; Pumplin and Voinnet, 2013; Qu et al., 2008; Scholthof et al., 2011). VsiRNAs co-immunoprecipitate with AGO1 and AGO2 (Azevedo et al., 2010; Wang et al., 2011) and double *ago1ago2* mutants show synergistic effects compared to single mutants, suggesting that they have distinct functions in antiviral RNA defenses (Pumplin and Voinnet, 2013). The antiviral RNA slicing activity of AGO2 has been experimentally confirmed (Carbonell et al., 2012). However, the role of AGO1 in antiviral defenses remains to be further clarified. In addition to RNA slicing, AGO1 represses the translation of messenger RNAs (mRNAs) (Brodersen et al., 2008; Lanet et al., 2009; Yang et al., 2012). The translation repression is mediated by plant microRNAs (miRNAs) or siRNAs that share perfect or near-perfect complementarity with the target mRNA (Aukerman and Sakai, 2003; Brodersen et al., 2008; Chen, 2004; Gandikota et al., 2007). That translation repression functions as an antiviral mechanism in plants has been proposed (Dunoyer and Voinnet, 2005), but so far only a few lines of evidence support this suggestion. *A. thaliana* plants deficient for dsRNA-binding protein 4 (DRB4, a partner of DICER4) showed enhanced accumulation of the coat protein (CP) of turnip yellow mosaic virus without changes in viral RNA concentration (Jakubiec et al., 2012), suggesting the relief of a silencing mechanism involving translation repression of the viral genome. Translation repression was also implicated in the virus resistance induced by NB-LRR resistance genes (Bhattacharjee et al., 2009).

Nepoviruses have a bipartite positive-strand RNA genome (Sanfacon, 2008; Sanfacon et al., 2006). The two RNAs are translated into two large polyproteins, which are cleaved by the viral protease. Replication proteins and the protease are encoded by RNA1, while the CP and movement protein (MP) are encoded by RNA2. Symptom recovery in two plant–nepovirus interactions (tomato black ring virus-infected *Nicotiana clevelandii* plants and tobacco ringspot virus-infected *N. benthamiana* plants) results in reduced levels of viral RNAs in the recovered leaves (Ratcliff et al., 1997; Siddiqui et al., 2008). Consistent with the idea that RNA silencing directs viral RNA clearance, recovery from tobacco ringspot virus infection is prevented in transgenic lines expressing various suppressors of silencing and this is concomitant with increased viral RNA accumulation (Siddiqui et al., 2008). In contrast, recovery of tomato ringspot virus (ToRSV, another nepovirus)-infected *N. benthamiana* plants is not associated with viral RNA clearance in spite of active RNA silencing triggered against viral sequences (Jovel et al., 2007). Thus, the molecular mechanisms that lead to symptom recovery are not well-established for the ToRSV-*N. benthamiana* interaction. In this report, we show that recovery of ToRSV-infected plants is associated with a reduction in the steady-state levels of viral proteins and decreased translation of the corresponding viral RNA. We also show that the recovery does not occur in plants silenced for *Ago1* or in wild-type plants grown at lower temperature and that translation of the viral RNA remains active under these conditions. We discuss a model in which symptom recovery is associated with an *Ago1*-dependent mechanism that represses the translation of the viral genome.

Results

Temperature-dependent symptom recovery in ToRSV-infected N. benthamiana plants is associated with reduced viral CP accumulation and increased vsiRNAs levels

RNA silencing is highly regulated and environmental conditions such as elevated temperature have been shown to enhance silencing efficiency against viruses, often resulting in decreased symptomatology or enhanced recovery (Chellappan et al., 2005; Szittya et al., 2003; Velazquez et al., 2010; Zhang et al., 2012). Symptom recovery was previously described in ToRSV-infected *N. benthamiana* plants grown under greenhouse conditions (Jovel et al., 2007). In this study, we re-examined the kinetics of viral infection in controlled environmental growth chambers at two different temperatures (21 °C and 27 °C). At 27 °C, ringspot symptoms developed on the inoculated leaves 2–3 days post-inoculation (dpi, Fig. 1(A)-(1)). At 4 dpi, young leaves above the inoculated leaf developed vein clearing symptoms (Fig. 1(A)-(2)). By 8 dpi, newly emerging leaves were asymptomatic and the plant recovered from infection (Fig. 1(A)-(3) and (7)). In contrast, symptom recovery was not observed at 21 °C. At this temperature, the infection progressed slower and the first symptoms appeared at 4 dpi. Ringspot symptoms developed on inoculated leaves but were less prominent than those observed in plants grown at 27 °C (Fig. 1(A)-(4)). Symptoms developed on upper non-inoculated leaves at 5 dpi and progressed to severe necrosis (Fig. 1(A)-(5–7)). At late stages of infection, although some young leaves remained non-necrotic, they were highly symptomatic (Fig. 1(A)-(7)). Thus, the outcome of ToRSV infection was influenced by the temperature.

Accumulation of viral RNA and proteins was evaluated during the course of infection. We focussed our attention on the accumulation of the MP and CP, for which high affinity specific antibodies were available, and of RNA2, which encodes these proteins. Pools of 18–25 leaves were taken from 9–10 plants at each time point. Leaves were collected from different plants from the same batch for each time point to ensure that collection of leaves at an earlier time point did not affect the progress of infection. Experiments were repeated 3–5 times with consistent results. Accumulation of RNA2 increased rapidly at 27 °C (Fig. 1(B)). As previously shown (Jovel et al., 2007), the level of RNA2 was high in symptomatic and in recovered leaves. At 5 dpi, MP and CP also accumulated to high levels in young systemically-infected symptomatic leaves (Fig. 1(B) and (C)). The steady-state levels of these proteins declined later in infection and were very low in recovered leaves (Fig. 1(B) and(C)). At 21 °C, RNA2 accumulation progressed slower (compare levels of RNA2 in systemically infected leaves at 5 dpi at 21 °C and 27 °C) but attained a high steady-state level by 8 dpi. The accumulation of CP and MP progressively increased during the course of infection at 21 °C. These results indicate that symptoms are associated with accumulation of viral proteins while symptom recovery is concomitant with a reduction in viral protein concentration.

We next examined the induction of host defense responses. At 27 °C, vsiRNAs accumulated earlier in infection and reached higher levels when compared to their accumulation at 21 °C (Fig. 1(D)), suggesting that the antiviral RNA silencing machinery was less active at lower temperature, possibly in part due to the slower accumulation of viral RNAs. Temperature-dependent activation of RNA silencing has been described by others (Szittya et al., 2003; Zhang et al., 2012). The results also confirm our previous observation that