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Transgenic down-regulation of ARGONAUTE2 expression in *Nicotiana benthamiana* interferes with several layers of antiviral defenses

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ABSTRACT

The present study aimed to analyze the contribution of *Nicotiana benthamiana* ARGONAUTE2 (NbAGO2) to its antiviral response against different viruses. For this purpose, dsRNA hairpin technology was used to reduce *NbAGO2* expression in transgenic plants as verified with RT-PCR. This reduction was specific because the expression of other *NbAGOs* was not affected, and did not cause obvious developmental defects under normal growth conditions. Inoculation of transgenic plants with an otherwise silencing-sensitive GFP-expressing *Tomato bushy stunt virus* (TBSV) variant resulted in high GFP accumulation because antiviral silencing was compromised. These transgenic plants also exhibited accelerated spread and/or enhanced susceptibility and symptoms for TBSV mutants defective for P19 or coat protein expression, other tombusviruses, *Tobacco mosaic virus*, and *Potato virus X*; but not noticeably for *Foxtail mosaic virus*. These findings support the notion that NbAGO2 in *N. benthamiana* can contribute to antiviral defense at different levels.

Introduction

Antiviral RNA silencing is a host RNA-mediated defense mechanism that specifically recognizes and degrades single-stranded viral RNA (Baulcombe, 2004). During replication of RNA viruses, double-stranded (ds) or highly structured single-stranded (ss) RNA accumulates and that triggers the host silencing cascade. DICER-LIKE proteins assisted by dsRNA binding proteins cleave these RNAs into short interfering RNAs (siRNAs) of 21–24 nucleotides. Upon their methylation (Yang et al., 2006) siRNAs are recognized by and programmed into an RNA induced silencing complex (RISC) which targets and specifically cleaves cognate mRNA (Alvarado and Scholthof, 2009). The proposed model for RNA silencing in eukaryotes suggests that members of the ARGONAUTE protein (AGO) family form key catalytic units of RISC, which target RNAs for cleavage or translational repression (Baulcombe, 2004).

In the dicotyledenous plant model *Arabidopsis thaliana*, the functions and developmental regulatory capabilities of its 10 known AGOs have been fairly well characterized (Morel et al., 2002; Vaucheret, 2008; Mallory and Vaucheret, 2010). For example, while AGO4, −6, and −9 carry out transcriptional RNA silencing involving 24-nucleotide small RNAs (Havecker et al., 2010; Zheng et al., 2007; Zilberman et al., 2003); AGOs 1 and −7 are known to be programmed with 21- to 22-nucleotide small RNAs such as miRNAs, ta-siRNAs, or exogenously derived siRNAs, such as those from viruses and transgenes (Baulcombe and Baumberger, 2005; Montgomery et al., 2008; Qi et al., 2005). AGOs 1 and −10 are also required for translational control of other miRNA targets and autoregulation (Brodersen et al., 2008; Mallory et al., 2009). AGO1 is a critical developmental regulator, and *ago1* mutants display multiple phenotypes, most notably tubular shaped leaves that resemble the tentacles of an argonaute squid (hence the name-argonaute) (Bohmert et al., 1998). Up-regulation of *AGO1* mRNA has been observed to be a general response to virus infection (Csorba et al., 2007; Havelda et al., 2008; Zhang et al., 2006), probably as an innate defense mechanism. Accordingly, *ago1* mutants exhibit extreme susceptibility to virus infections (Morel et al., 2002). This and other work (Harvey et al., 2011; Jaubert et al., 2011; Qu et al., 2008; Wang et al., 2011) (and as reviewed (Alvarado and Scholthof, 2009; Ding and Voinnet, 2007)) strongly suggest that even though in Arabidopsis AGO2 and AGO7 may contribute to antiviral silencing, AGO1 is key to establishing

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an antiviral response, presumably because once programmed with siRNAs it forms the slicer component of RISC to specifically target the homologous viral RNA for degradation.

The functional analysis of AGOs in Arabidopsis was possible in part through the availability of shared genetic resources, including specific gene knock-outs. However, for studying plant–virus interactions Arabidopsis has its limitations because of the relatively few viruses that infect this plant. Instead over many decades plant virologists have preferred to use *Nicotiana benthamiana* as the platform to study plant–virus interactions. However, even though the genome sequence (Bombarely et al., 2012) and transcriptome (Nakasugi et al., 2013) of *N. benthamiana* have recently been characterized there is not yet a library available with gene specific knock-outs. Instead gene knock-down studies on *N. benthamiana* are now routinely performed using *Tobacco rattle virus* (TRV) vectors to induce virus-induced gene silencing (VIGS) of specific host mRNAs (Burch-Smith et al., 2004). Even though the knock-down that is achieved yields incomplete loss-of-function (Orzaez et al., 2006; Pflieger et al., 2008), this may occasionally be advantageous, and importantly, sufficient to observe causal effects.

Despite the numerous advantages of the use of the VIGS approach, skeptics often point to possible limitations. For instance, an often-expressed concern is that VIGS necessitates the infection of a host with a virus (*e.g.*, TRV) that may perturb numerous host functions that in turn may mask or interfere with the manifestation of expected silencing phenotypes. This may become especially problematic when the (TRV)-infected plants are challenged with another virus to study its performance in a background where specific mRNAs are targeted by TRV-mediated VIGS. Even when including "empty-vector" TRV controls, this can lead to unexpected synergistic or antagonistic interactions that can influence observations and conclusions. Also, with VIGS experiments there is the potential influence of variation in experimental conditions, and plant-to-plant variation. When studying the antiviral silencing response there is also a paradoxical situation that one depends on an active VIGS to inactivate silencing components that are necessary for VIGS.

Using TRV-mediated VIGS to reduce expression of individual AGOs in *N. benthamiana*, we recently reported that instead of AGO1 (as in Arabidopsis), the antiviral response in *N. benthamiana* against *Tomato bushy stunt virus* (TBSV) is controlled by an AGO2 (Scholthof et al., 2011) analog (NbAGO2). Partly because of the aforementioned reasons relating to possible issues with VIGS, but importantly also to: (i) address the question whether NbAGO2 is specifically used against viral RNA or also for silencing of endogenous (ds)RNA; (ii) to create a stable platform of plants with the inheritable *NbAGO2*-silenced trait; and, (iii) to permit studies on the involvement of NbAGO2 in developmental processes and antiviral defense, we aimed in the present study to transgenically silence *NbAGO2* in *N. benthamiana* without resorting to VIGS. For this purpose a dsRNA-hairpin approach was employed to effectively trigger transient or transgenic gene silencing of *NbAGO2* in *N. benthamiana*. The results showed that the dsRNA approach effectively and specifically down-regulated *NbAGO2* mRNA expression indicating that NbAGO2 is not involved in dsRNA-mediated silencing. Furthermore, the silencing of *NbAGO2* did not affect development under normal growing conditions, but it prevented an effective silencing response against TBSV-GFP not expressing both CP and P19, and it enhanced the susceptibility towards individual TBSV CP or P19 mutants, and selected other viruses.

Results

Transient silencing of NbAGO2

Agrobacterium cultures that harbor the *NbAGO2* dsRNA hairpin-expressing plasmid (*NbAGO2hp*) were infiltrated into *N. benthamiana* leaves. Ten days post-agroinfiltration with cultures expressing *NbAGO2hp*, the silencing-sensor construct TGdP19 (TBSV-GFP neither expressing CP nor P19 (Shamekova et al., 2013)) was also agroinfiltrated onto the putative *NbAGO2*-silenced leaf as well as on an adjacent leaf to monitor the possible movement of the silencing signal. GFP accumulation was monitored under UV light every 5 days (Fig. 1). Five days post-infiltration with the TGdP19 cultures, clear GFP signal was visible on both *NbAGO2hp* and empty vector infiltrated leaves. In the adjacent leaves that had not been infiltrated with *NbAGO2hp* but only with TGdP19, GFP intensity was comparable. This suggested that at this early time-point antiviral silencing of TGdP19 had not yet manifested itself.

However, at 10 days post-agroinfiltration with TGdP19, no GFP signal was visible on the empty-vector agroinfiltrated plants indicating that antiviral silencing had been activated against the

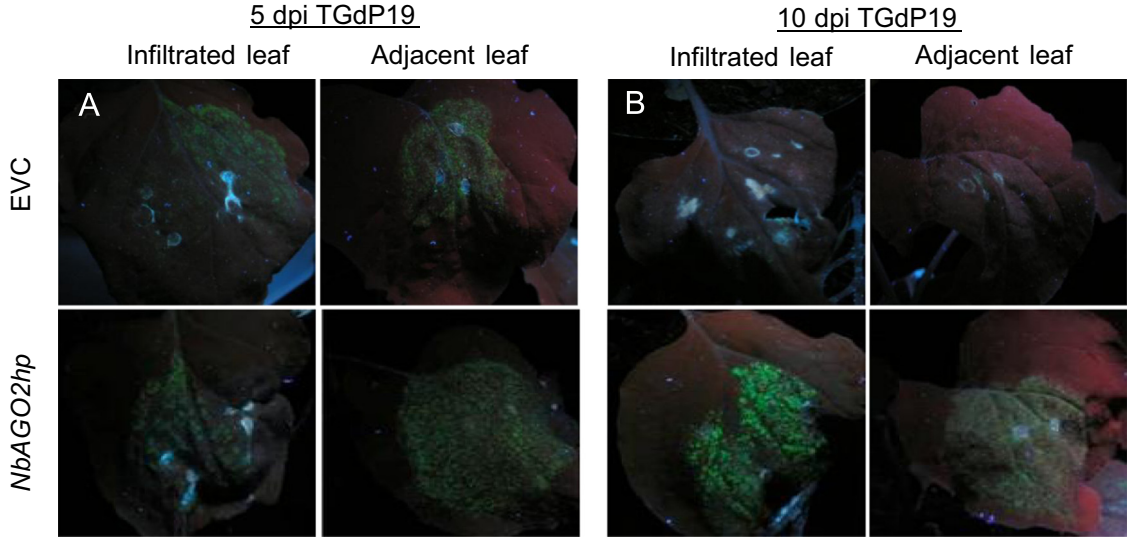

Fig. 1. Transient silencing of *NbAGO2* using the hairpin vector (*NbAGO2hp*). The empty vector control (EVC) or *NbAGO2hp* agroinfiltrated, or non-infiltrated adjacent leaves, were subsequently infected with TGdP19 ten days later, and GFP accumulation was monitored over a 10 day period. The panels show leaves at: A) 5 days post-infiltration (dpi) with TGdP19; B) 10 dpi with TGdP19.