Identification of an ARGONAUTE for Antiviral RNA Silencing in *Nicotiana benthamiana*[^1][^[C][W][OA]]

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ARGONAUTE proteins (AGOs) are known to be key components of the RNA silencing mechanism in eukaryotes that, among other functions, serves to protect against viral invaders. Higher plants encode at least 10 individual AGOs yet the role played by many in RNA silencing-related antiviral defense is largely unknown, except for reports that AGO1, AGO2, and AGO7 play an antiviral role in Arabidopsis (*Arabidopsis thaliana*). In the plant virus model host *Nicotiana benthamiana*, *Tomato bushy stunt virus* (TBSV) P19 suppressor mutants are very susceptible to RNA silencing. Here, we report that a *N. benthamiana* AGO (NbAGO2) with similarity to Arabidopsis AGO2, is involved in antiviral defense against TBSV. The activity of this NbAGO2 is shown to be directly associated with anti-TBSV RNA silencing, while its inactivation does not influence silencing of transiently expressed transgenes. Thus, the role of NbAGO2 might be primarily for antiviral defense.

Virus-induced gene silencing (VIGS) is a host RNA interference (RNAi) or RNA silencing response that specifically recognizes and degrades viral RNA (Baulcombe, 2004; Voinnet, 2005; Li and Ding, 2006). In turn, many viruses have evolved suppressors that block this RNA silencing defense (Roth et al., 2004; Qu and Morris, 2005) to prevent degradation of their genomic RNA or mRNAs (Lakatos et al., 2006; Mérai et al., 2006; Scholthof, 2006).

Tombusviruses like *Tomato bushy stunt virus* (TBSV) are well suited to study antiviral RNA silencing because they generate abundant substrates for DICER to yield high levels of duplex short-interfering RNAs (siRNAs; Molnár et al., 2005; Omarov et al., 2006) and they encode a 19-kD protein (P19) that is a potent suppressor of RNA silencing (Voinnet et al., 1999; Scholthof, 2006). P19 is used for RNA silencing research in many organisms because it universally blocks this process (Scholthof, 2006) by sequestering 21-bp siRNAs (Vargason et al., 2003; Ye et al., 2003). The proposed model in the context of TBSV infection is that the appropriation of virus-derived siRNAs by P19 prevents their subsequent incorporation into an antiviral RNA-induced silencing complex (vRISC; Silhavy and Burgyán, 2004; Scholthof, 2006). In support of this, we and others have provided direct evidence for a vRISC in *Tombusvirus*-infected plants, using TBSV P19 mutants and various biochemical approaches (Omarov et al., 2007; Fantale et al., 2007). More recently we isolated a sequence-specific vRISC from monocot and dicot plants infected with other viruses (Ciomperlik et al., 2011). Thus, plants have conserved the ability to mount an antiviral defense by activating a discrete and virus sequence-specific vRISC that can be isolated and analyzed in vitro.

The model for RNAi in eukaryotes implies that members of the ARGONAUTE protein (AGO) family members form key catalytic units of RISC to target RNAs for translational repression or cleavage (Baulcombe, 2004). Higher plants encode 10 more AGO genes, but other than a role for AGO4-like proteins in R-gene-induced virus resistance (Bhattarajee et al., 2009) and antiviral RNAi contributions by AGO1, AGO2, and AGO7 in Arabidopsis (*Arabidopsis thaliana*; Morel et al., 2002; Qu et al., 2008; Azavedo et al., 2010; Harvey et al., 2011; Wang et al., 2011; Jaubert et al., 2011), the contribution of AGOs in antiviral silencing is virtually unknown for other plant species (Alvarado and Scholthof, 2009). *Nicotiana benthamiana* is a well-established host for plant-virus research (Goodin et al., 2008) that mounts a biochemically tractable antiviral RNAi response (Omarov...
et al., 2007; Pantaleo et al., 2007), for which genomic information is rapidly accumulating, and it is susceptible to many more viruses than the genetic plant model Arabidopsis. For instance, Arabidopsis is not susceptible to TBSV although this virus has a vast host range spanning approximately 20 plant families and approximately 120 species (Yamamura and Scholthof, 2005); TBSV also replicates in yeast (Saccharomyces cerevisiae; Panavas and Nagy, 2003). Studies with TBSV and P19 in N. benthamiana have contributed significantly to our understanding of RNA silencing (Silhavy and Burgyán, 2004; Scholthof, 2006; Ding and Voinnet, 2007); therefore, results obtained with these model systems can be expected to yield novel results of use and guidance to systems beyond Arabidopsis.

Here, we report that down-regulating expression of an N. benthamiana AGO with similarity to Arabidopsis AGO2 plays a key and specific role in anti-TBSV RNA silencing.

RESULTS

A Role of NbAGO2 in Susceptibility of N. benthamiana to Suppressor-Defective TBSV

To examine a possible role of an AGO2-like candidate in anti-TBSV silencing in N. benthamiana, we identified a tobacco (Nicotiana tabacum) AGO2 homolog by searching the publicly available tobacco sequences for similarity with the 10 and 18 AGOs from Arabidopsis and rice (Oryza sativa), respectively. This identified a gene that is relatively well conserved in solanaceous plants based on comparisons with tomato (Solanum lycopersicum) and tobacco (Fig. 1A). Phylogenetic analysis suggests that like Arabidopsis AGO2, the solanaceous homolog falls within a clade with AGO3 and AGO7 (Fig. 1B).

Using primers based on the identified sequences an approximately 0.6-kb AGO2 (NbAGO2-1, accession JF815524) cDNA fragment was amplified from N. benthamiana. Sequencing showed it to be 96% similar to the tobacco sequence, and approximately 65% nucleotide and 50% amino acid identity with Arabidopsis AGO2 (Fig. 1A), the closest match. At the nucleotide level no other meaningful similarities were evident when NtAGO2 and NbAGO2 were directly compared with other AGO genes identified from solanaceous species or when used in nucleotide sequence BLAST queries, minimizing the potential for cross-silencing genes other than NbAGO2-1, in experiments described below.

To facilitate the use of Tobacco rattle virus (TRV)-mediated VIGS, the fragment of NbAGO2-1 cDNA, referred to hereafter as NbAGO2, was cloned into TV-00, which is an effective silencing vector for AGO genes in N. benthamiana (Jones et al., 2006; Bhattacharjee et al., 2009), to yield TV-NbAGO2 that was used for VIGS. Four weeks post TV-RNA1 + TV-NbAGO2 infection, reverse transcription (RT)-PCR tests using primers specifically designed to only amplify endogenous NbAGO2

Figure 1. NbAGO2. A, Schematic representation and alignment of the AGO2 proteins from Arabidopsis, tomato, tobacco, and N. benthamiana. The predicted amino acid sequence encoded by the amplified approximately 0.6-kb NbAGO2 cDNA fragment is shown in red. The underlined region highlights the Piwi-AGO-Zwille (PAZ) domain. Multiple alignment was performed using ClustalW. B, Phylogenetic tree of the PAZ domain sequences from NbAGO2, NtAGO2, SlAGO2, and AtAGO1 to AtAGO10. Bootstrapping was performed using 1,000 bootstrap replicates, and percentage of bootstrap support is shown by values at the branch nodes of the tree. The NbAGO2 nucleotide sequence is approximately 96% identical to the homologous gene of tobacco (NtAGO2). A comparison of NbAGO2 and NtAGO2 with Arabidopsis (AtAGO2) and tomato (SlAGO2) is provided. Multiple alignment was performed using ClustalW. B, Phylogenetic tree of the PAZ domain sequences from NbAGO2, NtAGO2, SlAGO2, and AtAGO1 to AtAGO10. Bootstrapping was performed using 1,000 bootstrap replicates, and percentage of bootstrap support is shown by values at the branch nodes of the tree. The NbAGO2 amino acid sequence is 50% identical to AtAGO2 (65% at the nucleotide level) and the predicted molecular mass for NbAGO2 is approximately 95 KD.
The NbAGO2-silenced plants were then tested for susceptibility to P19 suppressor-defective TBSV mutants that can initially infect *N. benthamiana* but are subsequently effectively silenced, resulting in recovery of the plants (Chu et al., 2000; Omarov et al., 2006). The reasoning was that compromised silencing of a crucial antiviral NbAGO would prevent recovery and yield symptoms reminiscent of those observed upon infection with wild-type TBSV. Inoculation of the NbAGO2-silenced plants with wild-type TBSV (T) resulted in a normal progression of a systemic infection and severe symptoms (Fig. 2C). As expected, p19-defective TBSV (TdP19; Scholthof, 2006) was unable to establish a severe systemic infection in various control plants, i.e. those not silenced or silenced with TRV containing other NbAGO inserts or no insert (Supplemental Fig. S1). In contrast, in NbAGO2-silenced plants the TdP19 mutant induced very severe systemic symptoms reminiscent of those caused by the wild-type virus and the plants did not recover (Fig. 2C). These findings strongly suggest that NbAGO2 silencing compensates for the lack of suppressor in the TdP19 mutant virus, indicating that silencing is suppressed even in the absence of P19.

We also screened NbAGO1-silenced and NbAGO4-silenced plants (Jones et al., 2006) as above. However, in contrast to the observations for NbAGO2-silenced plants, the results failed to show any effect of silencing NbAGO1 or NbAGO4 on symptoms or ability to exhibit recovery associated with infection by the P19-defective TBSV (Supplemental Fig. S1).

### NbAGO2 Mediates RNA Silencing against TBSV

To determine whether the strong stimulatory effect of NbAGO2 silencing on invasion by the TdP19 mutants is exclusively associated with RNA silencing, and not due to some unspecified effect on the ability of the mutants to systemically invade the plants, we monitored early infection events in inoculated leaves. For this, we developed a versatile, modified TBSV-GFP coat protein replacement vector (TG) that yields high levels of GFP expression in inoculated leaves. This vector is available in two constructs: the first allows for the generation of in vitro produced infectious transcripts that can be rub inoculated onto plants and the second can be agroinfiltrated to launch the infection (Supplemental Fig. S2A). For both systems the vector is also available as a version that is defective for P19 expression (TGDp19) and this leads to dramatic attenuation of GFP expression (Supplemental Fig. S2A). For both transcript inoculation and agroinfiltration, the absence of P19 can be compensated for by agroinfiltration with T-DNA constructs expressing P19 or by restoring P19 expression from the virus (TG; Supplemental Fig. S2A). Furthermore, the defect can also be complemented by coexpression of the potyvirus HC-Pro or hordeivirus yb suppressors of RNA silencing (Ciomperlik, 2008; Supplemental Fig. S2B). Therefore, the attenuation of GFP expression associated with TdP19 infection is due to the absence of suppressor activity and thus strictly caused by RNA silencing.

Inoculation of the control construct TG (encoding P19), by transcript inoculation or agroinfiltration, leads to rapid (visible in 1–2 d) and high levels of GFP expression in inoculated leaves of control plants as well as in NbAGO2-silenced plants (Fig. 3). In comparison, even though inoculation of TdP19 (not expressing P19) onto control plants may lead to some early GFP accumulation, GFP expression is rapidly silenced (Fig. 3). However, in NbAGO2-silenced plants high levels of GFP were observed, both visually and

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**Figure 2.** Effect of NbAGO2 silencing on infection with p19-defective TBSV. A, Four weeks postinfiltration with the TRV VIGS constructs to silence either NbAGO1 (A1) or NbAGO2 (A2). RNA from plants was subjected to RT-PCR with primers specific for Actin or NbAGO2. B, Healthy (H) plants or those silenced either with the empty TRV vector (00) or with TV-NbAGO2 (A2) were subjected to total protein extraction for SDS-PAGE, followed by Coomassie staining (left) or by western-blot analysis for detection of NbAGO2 (right). The positions of the marker proteins are indicated in kD, and NbAGO2 predicted to be approximately 95 kD is visible slightly below the 100-kD marker (*). The significance of the approximately 70-kD NbAGO2 antibody recognized band is presently unknown. C, Plants were silenced for NbAGO2 and 1 month after initiation of VIGS, silenced plants were either not inoculated (-) or inoculated with TBSV (T) or the p19-defective mutant (TdP19). Images were taken 3 weeks after inoculation. The plants shown are from the same experiment as those shown in Supplemental Figure S1, which serve as control comparisons. [See online article for color version of this figure.]
by western blotting, in leaves infected with the TGdP19 variant, whether inoculated as transcript or launched by agroinfiltration (Fig. 3). To further confirm these results, silencing assays were performed with different treatments on the same leaves (Supplemental Fig. S3). The results showed that TG yields bright fluorescence on 00-silenced plants as well as on NbAGO2-silenced plants whereas TGdP19-expressed GFP accumulated to high levels only in the latter, as also verified with plants whereas TGdP19-expressed GFP accumulated to high levels only in the latter as also verified with plants whereas TGdP19-expressed GFP accumulated to high levels only in the latter, as also verified with plants whereas TGdP19-expressed GFP accumulated to high levels only in the latter, as also verified with plants on 00-silenced plants as well as on NbAGO2-silenced. Plants were agroinfiltrated (ag) with cultures expressing TG (lanes 1 and 4) or TGdP19 (lanes 2 and 5), or rub inoculated with TGdP19 transcripts (lanes 3 and 6; text). Note: TG infections will not yield systemic GFP expression due to rapid accumulation of recombinants (Qiu and Scholthof, 2007). Comparative protein loading of the samples is shown in the Coomassie-stained gel on the bottom.

**DISCUSSION**

Inactivation or down-regulation of AGO2 does not induce a readily identifiable morphological phenotype in Arabidopsis (Vaucheret, 2008; Jaubert et al., 2011) or in *N. benthamiana* (Fig. 2). Likewise, NbAGO2 appears to be dispensable for transgene silencing and miRNA-mediated translational repression (Fig. 4), or is at least required at much lower threshold than is necessary for antiviral activities. These observations suggest a role that is distinct from other characterized AGO proteins.

Collectively, these results demonstrate that NbAGO2 is not involved in transgene- or miRNA-induced silencing but instead has a specific role in antiviral silencing.
protein we have identified is an analog of AtAGO2 (Fig. 1). This is further supported by a conserved role of AtAGO2 in antiviral resistance (Harvey et al., 2011; Wang et al., 2011; Jaubert et al., 2011).

Although hypomorphic Arabidopsis ago1 (Morel et al., 2002; Qu et al., 2008) and null ago7 mutants (Qu et al., 2008) are reported to be compromised for antiviral silencing, our experiments with TBSV and N. benthamiana failed to reveal such an obvious or primary role for NbAGO1. Experiments for NbAGO7 are ongoing. At this point it is not clear whether the failure to detect a role of NbAGO1 in anti-TBSV silencing is related to perhaps incomplete silencing using VIGS or if this is associated with properties characteristic of the TBSV-N. benthamiana system used in this study. However, we point out that the developmental phenotypes induced by VIGS of NbAGO1 are at least as severe as those observed in ago1 hypomorphic alleles used to study the role of AGO1 in Arabidopsis (Bhattacharjee et al., 2009). Alternatively, AGO1-like proteins such as AGO10 might have broader expression patterns in N. benthamiana allowing them to compensate for AGO1 activity, as has been shown for certain functions in Arabidopsis (Mallory et al., 2009; Mallory and Vaucheret, 2010).

Even though it cannot be ruled out that NbAGO2 is functionally equivalent to AtAGO1 or AtAGO7, the present evidence suggests that the role of NbAGO2 in N. benthamiana is comparable to that of Arabidopsis AGO2 shown to be active against Turnip crinkle virus and Cucumber mosaic virus (Harvey et al., 2011; Wang et al., 2011) or Potato virus X (Jaubert et al., 2011). However, direct comparisons must take into account the fact that the latter evidence was obtained with different, often wild-type viruses, whereas our experiments were conducted in N. benthamiana with TBSV in absence of P19 and therefore results directly relate to the effect of RNA silencing against this tombusvirus.

The fact that TRV-VIGS can be used to induce silencing of an antiviral AGO (NbAGO2) in N. benthamiana may at first seem counterintuitive. This paradox can be extended to our observations that apparently neither NbAGO1 nor NbAGO4 are required for TRV to induce and maintain VIGS, as was also found by others for NbAGO1 and NbAGO4 (Jones et al., 2006) and AtAGO4 (Dunoyer et al., 2004). However, these observations may in fact underscore the functional specialization of AGO proteins. That is, whereas certain AGO proteins, such as AGO2, may be required for the targeting of viral RNAs, nuclear-encoded mRNAs clearly seem to be directly targeted by AGO1 and/or AGO10 (Brodersen et al., 2008; Mallory and Vaucheret, 2010). Thus, although silencing of AGO2 might result in a loss of targeting of viral RNAs, NbAGO2 does not appear to

Figure 4. Virus-specific silencing by NbAGO2. A, Expression of 35S:GFP and 35S:GFP + P19 in N. benthamiana leaves at 2 and 7 d postinfiltration (dpi). B, VIGS was performed with either TV-00 (00) or TV-NbAGO2 (NbAGO2), and 3 weeks later plants were agroinfiltrated with either 35S:GFP or TGdP19. Images were taken 9 d later. C, VIGS was performed with either TV-00 (00) or TV-NbAGO2 (NbAGO2), and 3 weeks later plants were agroinfiltrated with either 35S:GFP or 35S:GFP171.1. In addition, 35S:GFP171.1 was expressed in non-TRV-infected N. benthamiana leaves together with either empty vector (EV) or P19 to demonstrate its ability to express GFP when RNA silencing has been compromised. Images were taken 3 and 6 d later. Note: Some images show a grayish color at the site of infiltration but that is distinct from green fluorescence.
affect nuclear-transcribed genes (Fig. 4), and thus a lack of NbAGO2 would not affect targeting of mRNAs by NbAGO1 or other AGOs.

Although the above scenario possibly explains the differential targeting of mRNAs versus viral RNAs, it still remains curious that TRV-mediated VIGS is not critically influenced by the compromised expression of aforementioned AGOs, including NbAGO2. It is possible that functional redundancies or imbalances in epistatic relations can lead to a dynamic virus-dependent switch in the primary AGO being recruited for vRISC, similar to what was suggested for the requirement of AGO1 or AGO2 in Arabidopsis (Harvey et al., 2011). At the same time, viruses with different properties (e.g., replication strategy or accumulation level of viral RNA and/or siRNAs) perhaps trigger the primary participation of different AGO effectors, or might not require the effective NbAGO to be expressed at high levels.

Another intriguing possibility relates to the involvement of primary versus secondary viral siRNAs by the AGO molecules forming the catalytic core of vRISCs. Primary siRNAs are DICER products directly derived from viral replicative intermediates or highly structured plus-sense RNA. Secondary siRNAs are amplified by plant-encoded RNA-dependent RNA polymerase (RDR) through mechanisms that may involve interactions with AGOs (Alvarado and Scholthof, 2009). It appears that RDR6 is involved in generating viral secondary siRNAs and depending on the virus this can affect silencing (Qu et al., 2005; Schwach et al., 2005; Vaistij and Jones, 2009; Szczyta et al., 2010; Wang et al., 2010). However, secondary siRNAs are suggested to play a minor role in silencing against tombusviruses like TBSV (Szczyta et al., 2010). This favors the hypothesis that if NbAGO2 is associated with siRNAs (Takeda et al., 2008) these will be predominantly primary siRNAs whereas other NbAGOs may preferentially recruit secondary siRNAs.

It is also noteworthy that the symptoms induced by TdP19 on NbAGO2-silenced plants resemble those observed upon infection of plants with wild-type TBSV that expresses P19. It has generally been the notion that P19 expression is directly or indirectly associated with more severe symptoms (Scholthof, 2006), but our present results suggest that at least some TBSV-mediated symptom effects can be mimicked even in absence of P19, when silencing is compromised.

The most straightforward interpretation of our results is that NbAGO2 is the core catalytic unit of the vRISC that is activated or assembled upon infection of N. benthamiana with TBSV (Omarov et al., 2007). However, we cannot rule out the possibility that NbAGO2 might have upstream or downstream regulatory roles that influence RNA silencing through a process not directly related to vRISC activity or composition. To address the mechanistic role it will be necessary to determine if and/or how virus infection influences vRISC properties. Regardless of the importance of such future studies, at present we can conclude that a newly identified AGO (NbAGO2) is required for antiviral RNA silencing against TBSV in N. benthamiana.

MATERIALS AND METHODS

Bioinformatic Analysis

A tobacco (Nicotiana tabacum) AGO homolog was identified by searching the tobacco genomic sequence downloaded from the Tobacco Genome Initiative (at North Carolina State University, http://www.pnpg.org/tgi/), and the entire EST and Unigenes databases from tobacco and Nicotiana benthamiana downloaded from the Sol Genomics Network (SGN, http://solgenomics.net/). A BLAST-based search was performed using the Arabidopsis (Arabidopsis thaliana) sequences of AGO2 (NP_174413) and AGO7 (NP_177103) from GenBank. Stand-alone BLAST software was obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.gov). Alignment of tobacco, tomato (Solanum lycopersicum), N. benthamiana, and Arabidopsis AGO sequences (accession nos: NbAGO2, SGN-U458085; NbAGO2, SGN-U65783; NbAGO1, NP_175274.1; ATAGO2, NP_174413.2; ATAGO3, NP_174414.1; ATAGO4, NP_565633.1; ATAGO5, NP_850110.1; ATAGO6, NP_180583.2; ATAGO7, NP_177103.1; ATAGO8, NP_197602.2; ATAGO9, NP_197613.2; ATAGO10, NP_199194.1) was performed using ClustalW (Thompson et al., 1994). Phylogenetic analysis was performed using the neighbor-joining method with 1,000 bootstrap replicates.

Constructs

Previous reports have described the properties of the Agrobacterium binary vectors for expressing HC-Pro and yb (Bragg and Jackson, 2004), 35S:GFP (Ruiz et al., 1998; Voinnet et al., 1998), or TV-NbAGO1 and TV-NbAGO4 (Jones et al., 2006). The tobacco AGO2-like sequence was used to identify homologous sequences from available N. benthamiana ESTs that yielded an AGO2-like gene of N. benthamiana (NbAGO2). To amplify an NbAGO2 cDNA fragment from N. benthamiana, total RNA was isolated and subjected to RT-PCR using forward (5′-GGATATTCTGCCTGCGATTAC-3′) and reverse (5′-TCTTCAGCCCGTACCATTTC-3′) primers. The resulting NbAGO2 products were cloned into pGEM-T Easy (Promega) and sequenced. Inserts were removed with Apai and PstI, and cloned in the minus-sense orientation into the RNA2 cloning vector pTV00 cleaved with the same enzymes. TV-00 clones containing the NbAGO2-1 insert (verified by sequencing) were electroporated into Agrobacterium strain GV3101 plus pSoup and cells were grown on KanR selective medium. To measure gene expression, RT-PCR experiments were conducted for NbAGO2 using forward (5′-GGATATTCTGCCTGCGATTAC-3′) and reverse (5′-TCTTCATCAACTCCATTTC-3′) to yield a 677-bp fragment. Actin gene expression was used as a comparative control using NtAct-forward (5′-CGCATGAGTACAGCGCGTTC-3′) and NtAct-reverse (5′-ATGGCAAGGCTGACGATAC-3′) primers.

Agroinfiltration to Launch TRV Infection

Agrobacterium cultures containing the TRV-RNA1 (in CSB1) and TRV-RNA2 cassettes were grown overnight (16–22 h) at 25°C to 28°C in 5 ml Luria-Bertani medium in presence of KanR. Cells were collected by centrifugation at 3K (Sorvall) for 15 min, and the pelleted cells resuspended in 10 ml 10 mM MgCl2. The optical density was measured and subsequently adjusted with 10 mM MgCl2 to 0.3. Five milliliters of these TRV-RNA2 expressing cells were mixed with 1 ml of the TRV-RNA1 cultures, and the mix was infiltrated with a needleless syringe into the abaxial side of two N. benthamiana leaves on young plants at the five-leaf stage. Infiltrated plants were grown in the greenhouse or in the laboratory at 23°C to 25°C daytime temperatures with 14 to 16 h daylight. Plants were grown for 4 weeks prior to infection with TBSV variants.

Constructs and procedures for co-infiltration of 35S:GFP and P19 expressing constructs were described recently (Savena et al., 2010).
mutants (Omarov et al., 2006, 2007), or for TBSV-GFP (TG) constructs with in vitro generated transcripts or by agroinfiltration. Construction and detailed properties of the newly generated TG constructs will be described separately (H.B. Scholthof, unpublished data). In essence, the TG variants are based on an infectious clone in which the coat protein open reading frame was largely removed and replaced with GFP. TBSV-GFP-dP19 (TGdP19) contains a p19 start codon mutation and two downstream stop codons. Both TG and TGdP19 were used to replace analogous TBSV backbone inserts of pJL54TG (H.B. Scholthof, unpublished data) to obtain T-DNA constructs expressing infectious viral RNAs, for agroinfiltration.

Protein Analysis

NbAGO2 Antibody
An NbAGO2 affinity-purified rabbit polyclonal antibody against a specific synthetic NbAGO2 peptide (CLEDPEKGDPPRDVF) was obtained from GenScript. The antibody was resuspended in water to a concentration of 1 mg/mL and a dilution of 1:3,000 was determined to be best for use in western blots.

SDS-PAGE and Western Blotting
Briefly, 80 mg of N. benthamiana leaf tissue was ground in 400 mL of extraction buffer containing: 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl, and 10 mM β-mercaptoethanol, mixed with 80 mL of 6X loading buffer containing dithiothreitol, and boiled for 5 min. Protein samples from total leaf tissues were separated by SDS-PAGE on 10% acrylamide minigels and then transferred onto a polyvinylidene difluoride membrane (BioRad). Incubations with the NbAGO2 antiserum and the secondary antibody conjugated to horseradish peroxidase (Rockland Immunocchemicals Inc.) were performed following protocols recommended by ECL Plus western blotting detection system (GE Healthcare). Western blots for detection of GFP were performed as described previously (Everett et al., 2010).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number JF815524.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. TBSV infection on N. benthamiana silenced for NbAGO1 or NbAGO4 expression.

Supplemental Figure S2. Complementation of p19-defective TG by coexpression of different viral suppressors.

Supplemental Figure S3. Half-leaf assays showing effect of NbAGO2 silencing on p19-defective TBSV.

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