The nonstructural protein NSs encoded by tomato zonate spot virus suppresses RNA silencing by interacting with NbSGS3

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Abstract
Viral suppressors of RNA silencing (VSRs) are encoded by diverse viruses to counteract the RNA silencing-mediated defence mounted by the virus-infected host cells. In this study, we identified the NSs protein encoded by tomato zonate spot virus (TZSV) as a potent VSR, and used a potato virus X (PVX)-based heterologous expression system to demonstrate TZSV NSs as a viral pathogenicity factor that intensified PVX symptoms in Nicotiana benthamiana. We then used a yeast two-hybrid screen to identify the suppressor of gene silencing 3 protein of N. benthamiana (NbSGS3), a known component of the plant RNA silencing pathway, as an interaction partner of TZSV NSs. We verified this interaction in plant cells with bimolecular fluorescence complementation, subcellular colocalization, and co-immunoprecipitation. We further revealed that the NSs–NbSGS3 interaction correlated with the VSR activity of TZSV NSs. TZSV NSs reduced the concentration of NbSGS3 protein in plant cells, probably through the ubiquitination and autophagy pathways. Interestingly, TZSV infection, but not NSs overexpression, significantly up-regulated the NbSGS3 transcript levels. Our data indicate that TZSV NSs suppresses RNA silencing of the host plant and enhances TZSV pathogenicity through its interaction with NbSGS3. This study reveals a novel molecular mechanism of NSs-mediated suppression of plant host antiviral defence.

Keywords
nonstructural protein NSs, RNA silencing, Suppressor of gene silencing 3, tomato zonate spot virus (TZSV), VSRs

1 INTRODUCTION

RNA silencing or RNA interference (RNAi), also known as posttranscriptional gene silencing in plants, is a natural defence mechanism against viral infections, transgenes, transposons, or other molecular parasites. This pathway is activated by double-stranded RNA (dsRNA) generated from invading nucleic acids, including genomic RNAs of plant viruses. Intracellular dsRNAs are sensed and processed by dsRNA-specific nucleases known as Dicer-like nucleases (DCLs) into smaller RNAs of 21–25 nucleotides (nt), designated as small interfering RNAs (siRNAs). Viral siRNAs, also known as vsiRNAs, are then recruited by the RNA-induced silencing complex
(RISC) and used by the Argonaute (AGO) protein as the specificity determinants to mediate sequence-specific degradation or translational repression of the corresponding viral RNA (Ding, 2010; Li & Wang, 2019; Llave, 2010).

That RNA silencing plays a significant role in defending infected plants against RNA viruses is also evidenced by the fact that many plant viruses encode viral suppressors of RNA silencing (VSRs) to neutralize the RNA silencing defence. VSRs exert their silencing suppressive role through diverse mechanisms (Csorba et al., 2015; Li & Wang, 2019; Yang & Li, 2018). For example, the NS3 protein of rice stripe virus (RSV) was found to bind to 21-nt, single-stranded (ss) siRNAs, siRNA duplex, and long ssRNA, but not long dsRNA (Xiong et al., 2009). By contrast, the dimeric form of RSV p3 binds to dsRNA of 9 base pairs (bp) or longer, and engages long dsRNA with two or more dimeric copies (Shen et al., 2010). The NS3 protein of rice hoja blanca virus binds siRNAs, and its amino acid positions 173–175 are critical for the siRNA binding and silencing suppression activities (Hemmes et al., 2009). Finally, the NSs proteins of tomato spotted wilt virus (TSWV), Impatiens necrotic spot virus (INSV), and groundnut ringspot virus (GRSV) were found to bind to dsRNA, inhibiting DCL-mediated cleavage of long dsRNA in vitro (Hedil et al., 2017; Schnettler et al., 2010; Takeda et al., 2002).

SGS3 is the component of the plant RNA silencing pathway needed for the biogenesis of secondary siRNAs and endogenous trans-acting siRNA (ta-siRNA), and the amplification and intercellular spread of systemic RNA silencing signals (Kumakura et al., 2009; Mourrain et al., 2000; Peragine et al., 2004; Yoshikawa et al., 2013). SGS3 has been found to be targeted by a number VSRs, illustrating a crucial antiviral defence role for this plant protein. Examples of SGS3-targeting VSRs include P2 of RSV (Du et al., 2011), V2 of tomato yellow leaf curl virus (TYLCV) (Glick et al., 2009), P4 of rice stripe mosaic virus (RSMV) (Zhang et al., 2020), and the class 1 RNAse III endoribonuclease (RNase III) of sweet potato chlorotic stunt virus (SPCSV) (Weinheimer et al., 2016), TGB1 of Plantago asiatica mosaic virus (PIAMV) (Okano et al., 2014), and VPg of turnip mosaic virus (TuMV) (Cheng et al., 2017). Additionally, the pC1 protein encoded by the p satellite of TYLCV was found to up-regulate the endogenous RNA silencing suppressor rgs-CaM, the latter mediating the autophagic degradation of SGS3 (Li et al., 2017).

Tomato zonate spot virus (TZSV) is a negative-sense/ambisense RNA virus in the genus Orthotospovirus, family Tospoviridae. It is transmitted by the thrips Frankliniella occidentalis in a persistent-propagative manner (Oliver & Whitfield, 2016; Rotenberg et al., 2015). TZSV was first discovered in Yunnan province, China, in 2007 (Dong et al., 2008; Zheng et al., 2019). It causes concentric ringspots on tomato fruits and necrotic lesions on leaves of infected plants, resulting in significant economic losses (Dong et al., 2008; Scholthof et al., 2011). Similar to other members of the family Tospoviridae, TZSV has three ssRNA genome segments designated L (large), M (medium), and S (small). The negative-sense L segment encodes the RNA-dependent RNA polymerase (RdRp). The ambisense M segment encodes a nonstructural protein (NSm) in the viral sense and a precursor of the glycoproteins (GPs) in the viral complementary sense. The S segment encodes a nonstructural protein (NSs) in the viral sense and a nucleocapsid protein (N) in the viral complementary sense. Currently, the function of TZSV proteins can only be postulated based on other orthotospoviruses (Dong et al., 2008; Walker et al., 2021).

In the current study, we reported the identification of TZSV-encoded NSs as a VSR. We expressed TZSV NSs in Nicotiana benthamiana plants using a potato virus X (PVX)-based heterologous expression system, and found it to induce significantly intensified infection symptoms. We also found that TZSV NSs directly interacted with NbSGS3 in yeast and plant cells, and this interaction caused accelerated degradation of NbSGS3, providing a novel mechanism for the VSR activity of a tospovirus NSs.

2 | RESULTS

2.1 | TZSV NSs acts as a potent VSR in N. benthamiana

NSs of several tospoviruses have been found to have VSR activities (Bucher et al., 2003; Hedil et al., 2017; Schnettler et al., 2010; Takeda et al., 2002). However, compared to other tospovirus-encoded proteins, the NSs proteins are relatively less conserved (Meng et al., 2015). The amino acid sequence of TZSV NSs is less than 14.2% identical to that encoded by TSWV or INSV (Table S1). Therefore, whether or not TZSV NSs functions as a VSR remains unknown. To resolve this question, we assembled a binary construct that would permit transient expression of TZSV NSs in N. benthamiana leaf cells on Agrobacterium-mediated delivery (agroinfiltration). We then tested whether any of the TZSV-encoded proteins exhibit VSR activity. As shown in Figure 1a, expression of green fluorescent protein (GFP) alone caused an infiltrated patch that was only weakly fluorescent, whereas co-expression of p19, a known VSR encoded by tomato bushy stunt virus (TBSV), enhanced GFP fluorescence intensity dramatically. Notably, co-expression of TZSV NSs, as well as TBSV p19 and TSWV NSs, was corroborated with northern and western blotting that verified a strong increase of GFP mRNA and protein levels, respectively (Figure 1b). These results suggest that the TZSV-encoded NSs is a strong VSR that suppresses ssRNA-triggered RNA silencing.

We next tested whether NSs also inhibited dsRNA-triggered RNA silencing. To this end, we infiltrated leaves of N. benthamiana plants with mixtures of A. tumefaciens strains harbouring three different constructs: a GFP-expressing construct (ssGFP), a second construct expressing a dsRNA targeting GFP (dsGFP), and a third construct expressing a potential VSR. As shown in Figure 1c, TZSV NSs rescued GFP expression to levels similar to TBSV p19 and TSWV NSs. These results were also corroborated with northern and western blotting, indicating that TZSV NSs also potently suppressed dsRNA-triggered RNA silencing.
Figure 1  TZSV NSs blocks RNA silencing triggered by single-stranded green fluorescent protein (GFP) and double-stranded GFP. (a) Suppression of GFP silencing in Nicotiana benthamiana 16c plants. N. benthamiana leaf patches were co-infiltrated with Agrobacterium tumefaciens cultures containing GFP (35S-GFP) and empty vector (EV), NSs, NSm, N, NSs (TSWV) or p19 representative leaves were photographed at 6 days postinoculation (dpi) under UV light. (b) Northern blots of GFP mRNA accumulation and western blots (WB) of GFP accumulation in agroinfiltrated leaf patches from the samples indicated in (a). Ethidium bromide staining of rRNA served as loading control for northern blots and Coomassie Brilliant blue (CBB) staining of the large subunit of RuBisCO was used as loading controls for western blots. (c) Suppression of dsGFP silencing in N. benthamiana leaf patches were co-infiltrated with A. tumefaciens cultures containing GFP and dsGFP and EV, NSs, NSs (TSWV) or p19 representative leaf patches were photographed under UV light at 6 dpi. (d) Northern blot of GFP mRNA accumulation and western blot of GFP accumulation in agroinfiltrated leaf patches were performed as indicated in (c). Ethidium bromide staining of rRNA and Coomassie Brilliant blue staining of the large subunit of RuBisCO were used as loading controls for northern blots and western blots, respectively.
2.2 TZSV NSs can enhance the pathogenicity of PVX

We next attempted to determine the role of TZSV NSs in viral pathogenicity by expressing it, along with TZSV NSm and N, from a different virus. The NSs, NSm, and N coding sequences were inserted into a potato virus X (PVX)-based expression vector to produce PVX-NSs, PVX-NSm, and PVX-N, respectively. PVX empty vector (EV) was used as a control. A. tumefaciens strains harbouring PVX EV, PVX-NSs, PVX-NSm, and PVX-N were then introduced into N. benthamiana leaves. Systemic symptoms typical of PVX infections were first observed at 6 days postinoculation (dpi) in plants inoculated with PVX EV, PVX-NSm, and PVX-N. However, the symptoms on plants inoculated with PVX-NSs were visibly more severe, showing leaf crinkling and chlorosis (Figure 2a). These PVX-NSs-caused symptoms continued to intensify, eventually leading to the death of the infected plants by 12 dpi (data not shown).

To assess whether the PVX-NSs-caused systemic symptoms were accompanied by increased accumulation of $\text{H}_2\text{O}_2$, leaves infected systemically with PVX EV, PVX-NSs, PVX-NSm, and PVX-N were stained with 3,3′-diaminobenzidine (DAB), as $\text{H}_2\text{O}_2$ accumulation could induce the polymerization of DAB, producing a dark brown colour that can be visualized after chlorophyll clearing with 95% ethanol. As shown in Figure 2a, PVX-NSs-infected leaves accumulated $\text{H}_2\text{O}_2$ to substantially higher levels than leaves infected with other PVX constructs.

To determine whether the PVX-NSs itself also accumulated to higher levels, we used western blotting to assess the levels of PVX capsid protein (CP) at 6 and 12 dpi (Figure 2b). Compared with PVX EV, PVX-NSm, and PVX-N, PVX-NSs indeed caused PVX CP to accumulate to substantially higher levels at both time points, suggesting that expression of NSs enhanced the replication and/or survival of PVX in the infected plants (Figure 2b). Similarly, northern blotting also detected higher levels of PVX mRNA in PVX-NSs-infected tissues (Figure 2b). Together these results indicate that NSs is a
strong pathogenicity determinant that bolstered PVX infection in *N. benthamiana*.

### 2.3 The VSR activity of TZSV NSs depends on the integrity of its C-terminus

It was recently reported that the C-terminus of the TSWV NSs became truncated at various positions on a single passage in *Capsicum chinensis* plants carrying the *Tsw* gene, and the resulting NSs with C-terminal truncations lost the ability to suppress RNA silencing (Margaria et al., 2014). We wondered if similar deletions in TZSV NSs would cause analogous debilitation. To test this, we produced two different deletion mutants of TZSV NSs by removing 15 and 36 amino acid residues from its C-terminus (Figure 3a). As shown in Figure 3b,c, while deleting the C-terminal 15 amino acids had negligible effect on its VSR activity, removal of the C-terminal 36 amino acids led to a near complete loss of the VSR activity. This indicated that the 21 amino acids between positions 424–443 are required for the VSR activity of TZSV NSs.

We further investigated these two deletion mutants for their effect on viral disease symptoms by infecting them with the PVX expression vector. As shown in Figure 3d,e, the PVX-NSs<sup>(1–444aa)</sup> mutant caused disease symptoms that were indistinguishable from PVX-NSs expressing the intact TZSV NSs, and also led to similarly elevated levels of H<sub>2</sub>O<sub>2</sub>, PVX RNA, and coat protein (CP). By contrast, the disease symptoms, H<sub>2</sub>O<sub>2</sub>, PVX RNA, and CP levels induced by PVX-NSs<sup>(1–423aa)</sup> were nearly identical to PVX EV. These results further illustrated the importance of amino acid positions 424–443 in maintaining both the VSR activity and disease intensification capacity of TZSV NSs.

### 2.4 Both TZSV NSs and TSWV NSs interact with NbSGS3

To determine how TZSV NSs suppresses RNA silencing and intensifies viral infections in *N. benthamiana*, we next examined whether TZSV NSs could interact with NbSGS3, an important component of the RNA silencing pathway in *N. benthamiana*, as multiple VSRs encoded by plant viruses have been found to target SG3 to achieve silencing suppression. To this end, we cloned TZSV NSs, along with TSWV NSs, into a yeast two-hybrid vector and evaluated its potential interaction with NbSGS3 with the yeast two-hybrid assay.

As shown in Figure 4a, both NSs proteins showed strong interactions with NbSGS3. We further verified both interactions using the bimolecular fluorescence complementation (BiFC) assay in *N. benthamiana* cells (Figure 4b), as well as co-immunoprecipitation (co-IP) using proteins expressed in *N. benthamiana* (Figure 4c,d). Finally, transiently overexpressed TZSV NSs (and TSWV NSs) co-localized with NbSGS3 in the cytoplasm and nuclei of cells co-expressing both. Together these data demonstrated a direct interaction between TZSV NSs and NbSGS3, and suggested that this interaction might be conserved for other tospovirus NSs.

We showed earlier that the VSR activity of TZSV NSs required 21 amino acids spanning positions 424–444 near its C-terminus. To determine whether the NSs–NbSGS3 interaction was integral to the VSR activity of TZSV NSs, we next wondered whether the NSs C-terminal portion was also needed for the NSs–NbSGS3 interaction. To this end, we used BiFC to resolve whether the two deletion mutants of NSs, NSs<sup>(1–444aa)</sup> and NSs<sup>(1–423aa)</sup>, still interacted with NbSGS3. As shown in Figure 4e, the former, but not the latter, retained a strong interaction with NbSGS3, leading to the reconstitution of YFP fluorescence. These results demonstrate that the NSs–NbSGS3 interaction tightly correlates with the VSR activity of NSs, which in turn suggests that the VSR activity of NSs is dependent on its ability to engage NbSGS3 in plant cells.

### 2.5 The VSR activity of TZSV NSs is attenuated by NbSGS3 overexpression

We reasoned that if NSs exerts its suppression on antiviral RNA silencing in *N. benthamiana* by hijacking NbSGS3 through physical binding, then the VSR activity of NSs might be weakened if the relative concentration of NbSGS3 were increased to allow a fraction of NbSGS3 protein to escape from NSs binding. To test this idea, we repeated the NSs VSR assay in the agroinfiltrated *N. benthamiana* leaves in the presence of overexpressed NbSGS3. As shown in Figure 5a (also Figure 1), NSs proteins encoded by both TZSV and TSWV in the absence of overexpressed NbSGS3 consistently suppressed GFP silencing, causing both GFP fluorescence and GFP protein levels to increase dramatically (Figure 5a,b). However, this strong VSR activity was visibly weakened by the presence of overexpressed NbSGS3, weakening the levels of GFP fluorescence and protein levels (Figure 5a,b). These results are consistent with the conclusion that NSs suppresses RNA silencing by binding to NbSGS3 and blocking it from functioning in RNA silencing-mediated defence.

### 2.6 NSs induces the degradation of NbSGS3 protein

To clarify whether TZSV NSs causes accelerated NbSGS3 protein turnover, we co-expressed GFP, the TZSV NSs with a C-terminal HA tag (NSs-HA), and NbSGS3 with a C-terminal FLAG tag (NbSGS3-Flag) in *N. benthamiana* leaf cells, and monitored the protein accumulation levels of GFP, NSs-HA, and NbSGS3-FLAG, with antibodies that recognize GFP, HA, and FLAG epitope tags, respectively. We further used chemical inhibitors of proteosome and autophagy activities, MA132 and E64d, respectively, to reveal potential effects of these two primary protein turnover pathways on NbSGS3 protein levels.

As shown in Figure 6a, compared to lane 5 where NbSGS3-FLAG was expressed together with GFP but not NSs-HA, inclusion of
The NSs C-terminal domain is responsible for gene silencing suppression, severe symptoms and necrotic responses. (a) Schematic representation of NSs mutant. (b) Plants were inoculated with green fluorescent protein (GFP) and P19, pBin-NSs, pBin-NSs(1–444aa) or pBin-NSs(1–423aa), and photographed under UV light at 6 days postinoculation (dpi). (c) Northern blot analysis of GFP mRNA and western blot (WB) analysis of GFP. Ethidium bromide staining of rRNA and Coomassie Brilliant blue staining (CBB) of Rubisco were used as loading controls for northern and western blots, respectively. (d) *Nicotiana benthamiana* plants infected with PVX, PVX-NSs, PVX-NSs(1–444aa) or PVX-NSs(1–423aa) were photographed at 6 and 12 dpi. The lower panels show 3,3′-diaminobenzidine-stained leaves of infected plants to show H$_2$O$_2$ induction at 12 dpi. (e) Northern blots and western blots analysis of PVX coat protein (CP) mRNA and PVX CP in systemically infected leaves of the *N. benthamiana* plants. The positions of genomic RNA (gRNA), subgenomic RNA1 and RNA2 (sgRNA1/2), and CP subgenomic RNA (CP sgRNA) are indicated.
FIGURE 4 TSWV NSs or TZSV NSs interact with NbSGS3. (a) Interactions between TZSV NSs and NbSGS3 and between TSWV NSs and NbSGS3 identified by yeast two-hybrid assays. Y2Hgold cells were diluted $10^{-1}$ to $10^{-5}$ and plated onto QDO (SD-Trp-Leu-His-Ade) medium. Controls: AD-T + BD-53 (positive control) or AD-NbSGS3 + BD or AD + BD-NSs or AD + BD-NSs (TSWV) (negative control). (b) Bimolecular fluorescence complementation (BiFC) assays in Nicotiana benthamiana leaves at 72 h postinoculation (hpi). Yellow fluorescence (green) was observed as a consequence of the complementation of the YN and YC tagged with NbSGS3 and NSs or NbSGS3 and NSs (TSWV). Bars, 25 μm. (c) Co-localization of NbSGS3-mCherry with NSs-GFP and NbSGS3-mCherry with NSs (TSWV)-GFP in N. benthamiana leaf cells. Infiltrated leaves were treated at 72 hpi after confocal images. Bars, 25 μm. (d) NbSGS3-3FLAG was co-expressed with TZSV NSs-3HA or TSWV NSs-3HA in N. benthamiana. Total protein was extracted and immunoprecipitated with anti-FLAG magnetic beads. Western blots were carried out using monoclonal anti-FLAG or anti-HA antibodies to explore FLAG-tagged proteins or co-immunoprecipitated HA-tagged protein, respectively. (e) BiFC of NbSGS3-cYFP with NSs-nYFP encoded by TZSV or TSWV were transiently co-expressed in N. benthamiana leaves. Fluorescence was detected by confocal microscopy at 48 hpi. Bar, 25 μm.
NSs-HA (lane 4) caused a dramatic decrease in NbSGS3-FLAG protein levels. Interestingly, both the proteasome pathway and autophagy appear to contribute to the faster NbSGS3 turnover as both MG132 and E64d caused a visible recovery of NbSGS3-FLAG protein levels (lanes 1 and 2). Similar results were also obtained with TSWV NSs (Figure S2).

Finally, to rule out the possibility that NSs down-regulated NbSGS3 expression at the transcriptional level, we measured the endogenous NbSGS3 mRNA levels using reverse transcription quantitative PCR (RT-qPCR) in the presence or absence of NSs. As shown in Figure 6b, addition of TZSV NSs caused a 1.5-fold increase of NbSGS3 mRNA levels. Together these results suggested that NSs, by binding to NbSGS3, accelerates the degradation of NbSGS3 protein by routing the latter to the proteasome and autophagy pathways.

### 2.7 NbSGS3 plays a critical role in *N. benthamiana* defence to TZSV infection

Having unveiled the NSs-NbSGS3 interaction as a novel mechanism through which TZSV NSs counteracts antiviral RNA silencing in *N. benthamiana*, we wondered whether TZSV infection of *N. benthamiana* would be enhanced by down-regulating NbSGS3 expression. For this purpose, we used a tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) vector to knock down NbSGS3 expression in *N. benthamiana* plants. The NbSGS3-VIGS plants were then inoculated with TZSV 8 days after VIGS induction. We found that TRV-NbSGS3 infection plants showed more severe viral symptoms compared with the TRV infection plants 7 days after TZSV inoculation (Figure S1). As shown in Figure 7a, TRV-NbSGS3 infection caused the NbSGS3 mRNA level to decrease by at least 50%, indicating that NbSGS3 expression was successfully down-regulated with VIGS. Strikingly, down-regulation of NbSGS3 expression caused the accumulation levels of TZSV RNA (Figure 7b), and the TZSV N protein, to increase dramatically (Figure 7c), indicating that a normal level of NbSGS3 expression is critical to keep TZSV infection at a relatively low level, thus suggesting a critical role of NbSGS3 in *N. benthamiana* defence against TZSV. Interestingly, we also found that TZSV infection itself could up-regulate the expression of NbSGS3 (Figure 7d), further corroborating a critical role of NbSGS3 in *N. benthamiana* antiviral defence.
Plant viruses evolved VSRs in order to suppress host antiviral RNA silencing, ensuring successful virus infection. VSRs exert their counterdefence functions using different strategies that include inhibiting vsilRNA biogenesis, binding to siRNA duplex, or decapacitating plant silencing pathway proteins (AGOs, DCLs, RDRs) through direct interactions. Previous studies have found that viruses of the family Tospoviridae, such as TSWV, INSV, and GRSV, use their NSs as a VSR (Du et al., 2011; Hemmes et al., 2009; Schnettler et al., 2010; Takeda et al., 2002; Xiong et al., 2009; Zhang et al., 2015). Although a member of Tospoviridae, TZSV encodes an NSs that shares only limited homology with that of TSWV, INSV, and GRSV; therefore, we set out to find out how TZSV suppresses RNA silencing.

In this study, we first established that TZSV NSs, but not N or NSm, functioned as a strong VSR that suppressed RNA silencing triggered by both ssRNA and dsRNA. We further demonstrated that, similar to many other VSRs, TZSV NSs, when expressed from a PVX vector, dramatically enhanced the plant symptoms induced by PVX. We also found that an intact C-terminus is absolutely required for the VSR function of TZSV NSs. This result is consistent with a recent study revealing the importance of TSWV NSs C-terminus for its VSR function (Margaria et al., 2014).

SGS3 has been found to play a pivotal role in RNA silencing and plant antiviral defence (Dalmay et al., 2000; Glick et al., 2009; Mourrain et al., 2000; Muangsan et al., 2004; Peragine et al., 2004). Many VSRs are reported to interact with SGS3 in order to suppress RNA silencing-mediated antiviral defence of the plant hosts. These include PIAMV TGB1 (Okano et al., 2014), tomato yellow leaf curl China betasatellite (TYLCCNB) βC1 (Li et al., 2017), TYLCV V2 (Glick et al., 2009), TuMV and potato virus A VPg (Cheng et al., 2017; Rajamaki et al., 2014), RSV P2 (Du et al., 2011), SPCSV RNase3 (Weinheimer et al., 2016), and RSMV P4 (Zhang et al., 2020).

Extensive genetic evidence suggests that SGS3 facilitates the conversion of target ssRNA into dsRNA by RDR6, providing a new source for the production of siRNAs (Voornet, 2008). Fukunaga and Doudna demonstrated that SGS3 is a dsRNA-binding protein that preferentially recognizes dsRNA substrates containing a 5′ single-stranded overhang at one or both ends to trigger gene silencing (Fukunaga & Doudna, 2009). Our data indicated that TZSV NSs, as well as TSWV NSs, interacts with the SGS3 ortholog in N. benthamiana—NbSGS3—and that this interaction correlated with its VSR activity. This implies a possible strategy that NSs binding may inactivate SGS3 by inhibiting the binding of SGS3 to target dsRNA, thereby blocking the SGS3-mediated silencing pathway. Interestingly, similar to a few previously characterized VSR–SGS3 interactions, the turnover of SGS3 could be accelerated by various VSRs, such as P4 encoded by RSMV and VPg of TuMV, via enhanced ubiquitination and autophagy (Cheng et al., 2017; Zhang et al., 2020). Separately, the βC1 protein encoded by TYLCCNB was found to enhance SGS3 degradation by up-regulating the expression of rgs-CaM, an endogenous RNA silencing suppressor rgs-CaM, the latter routing SGS3 into the autophagosomes (Li et al., 2017). Therefore, we speculated that TZSV NSs might also enhance the degradation of NbSGS3. TZSV NSs also accelerated the protein degradation of NbSGS3, probably through a combination of protein ubiquitination and autophagy. Finally, we established that TZSV infection, and also transient expression of TZSV NSs, up-regulated NbSGS3 mRNA levels, thus ruling out transcriptional repression as a possible mechanism for the VSR activity of TZSV NSs. In summary, our study identifies SGS3 as a novel target.
of a tospoviral VSR, and suggests that tospoviral VSRs may target multiple silencing pathway components to achieve effective suppression of the RNA silencing-mediated antiviral defence.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials and virus

Wild-type and GFP transgenic (line 16c) *N. benthamiana* lines were used in this study. The TZSV isolate was collected from tomato plants in Yunnan (Dong et al., 2008). The isolate was stored in liquid nitrogen or −80°C for long-term storage. Freshly TZSV-infected leaves of *N. benthamiana* were ground in inoculation buffer (0.2% Na₂SO₄, 2% polyvinylpyrrolidone [PVP]-15, 0.1 M Na₂PO₄, 0.1 M Na₂HPO₄, pH 7.0) and this crude extract was mechanically inoculated onto plant leaves. All experimental plants were cultivated in a growth room maintained at 25°C under a day/night cycle of 16 h/8 h.

4.2 | Plasmid construction

In-Fusion cloning technology (Vazyme) was used to generate all the plasmid constructs. The gene sequences including TZSV NSs (GenBank KC133530.1), NSm (GenBank EF552434.1), N (GenBank MG656995.1), and TSWV NSs (GenBank JF960235.1) were amplified by PCR using Phanta Max Super-Fidelity DNA polymerase (Vazyme) with specific primers (Table S2) and then were cloned individually to generate pBin-NSs, pBin-NSm, pBin-N, pBin-NSs (TSWV), PVX-NSs, PVX-NSm, PVX-N, pGBK-NSs, pGBK-NSs (TSWV), pCV-nYFP-NSs, pCV-nYFP-NSm, pCV-nYFP-N, pCV-nYFP-NSs (TSWV), pCV-nYFP-NSm.
4.3 | Agroinfiltration and chemical treatments

The recombinant plasmids were electroporated into *A. tumefaciens* GV3101. After incubating on YEP solid medium (with 50 mg/L kanamycin, 25 mg/L rifampicin) for 48 h at 28°C, a single colony was cultivated in YEP broth (with 50 mg/L kanamycin and 25 mg/L rifampicin) at 28°C overnight with shaking at 220 rpm, and then harvested by centrifugation, resuspended in an infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl₂, 100 μM acetylsyringone), and adjusted to an optical density (OD₆₀₀) of 0.5–1.0 before infiltration into *N. benthamiana* plants. After incubation at room temperature for 3 h, the mixture was agroinfiltrated into 4-week-old *N. benthamiana* plants. For chemical treatments, phosphate-buffered saline (PBS) containing 2% dimethyl sulphoxide (DMSO) (control) or an equal volume of DMSO with 100 μM MG132 (Selleck) for inhibition or transferred onto nitrocellulose membranes (GE Healthcare Life Sciences) for detection with polyclonal antibodies specific to the GFP (1:5000; Invitrogen), T茨SV N (1:3000), HA (1:4000; Invitrogen), FLAG (1:500; Invitrogen) or PVX CP (1:4000; Hua An) proteins. Secondary reactions were carried out with goat anti-rabbit IgG horse-radish peroxidase conjugate (1:10,000; Invitrogen). Protein signals were detected by chemiluminescence reaction using Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen). Protein bands were visualized using a NEWTON 7.0 Bio plus (VILBER) and analysed with ImageJ software.

4.4 | Northern blot assay

Total RNA was extracted from *N. benthamiana* leaves using TRIzol reagent (Invitrogen). The RNA probes for GFP or PVX CP were labelled with digoxigenin (DIG) using the DIG Northern starter kit (Roche) following the manufacturer’s instructions. Four micrograms of total RNA was separated in 1.5% formaldehyde agarose gel. The RNA was transferred to a Hybond-N nylon membrane (GE Healthcare Life Sciences) and UV cross-linked (1500 μJ/cm²) to the membrane. Membranes were incubated with GFP-specific or PVX CP-specific RNA probes. The detection signals were performed using CDP-star reagent in the kit. RNA bands were visualized using a NEWTON 7.0 Bio plus (VILBER).

4.5 | Co-immunoprecipitation and western immunoblot analysis

Total protein was extracted from 2 g of leaf tissue and resuspended in 2 ml of extraction buffer (50 mM Tris-MES pH 8.0, 0.5 M sucrose, 1 mM MgCl₂, 10 mM EDTA, 5 mM dithiothreitol [DTT], protease inhibitor cocktail [Roche]) for co-immunoprecipitation as described previously (Liu et al., 2010). After centrifugation (15 min at 3000 × g), the supernatant was captured by adding anti-FLAG magnetic beads (Millipore) and then shaking at 4°C for 4 h. The beads were recovered at 14,000 × g for 10 s and washed with cold Tris-buffered saline (TBS; 10 mM Tris, 0.15 M NaCl, pH 7.5) five times. The agroinfiltrated *N. benthamiana* leaf tissue was collected and homogenized in lysis buffer (1.5 mM Tris-HCl pH 6.8, 10% sodium dodecyl sulphate [SDS], β-mercaptoethanol) for immunoblot analysis. Proteins were separated on SDS-PAGE gels followed either by Coomassie Brilliant blue staining or transferred onto nitrocellulose membranes (GE Healthcare Life Sciences) for detection with polyclonal antibodies specific to the GFP (1:5000; Invitrogen), T茨SV N (1:3000), HA (1:4000; Invitrogen), FLAG (1:500; Invitrogen) or PVX CP (1:4000; Hua An) proteins. Secondary reactions were carried out with goat anti-rabbit IgG horse-radish peroxidase conjugate (1:10,000; Invitrogen). Protein signals were detected by chemiluminescence reaction using Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen). Protein bands were visualized using a NEWTON 7.0 Bio plus (VILBER) and analysed with ImageJ software.

4.6 | DAB staining

Hydrogen peroxide (H₂O₂) in leaves of *N. benthamiana* was detected visually in leaves using the DAB uptake method as described previously (Sharma & Ikegami, 2010). Briefly, the plant leaves were excised in a DAB solution (1 mg/ml, pH 3.8) at 25°C for 8–16 h. Then the samples were immersed with 95% ethanol in boiling water for 5–10 min and preserved in 70% ethanol and photographed.

4.7 | Yeast two-hybrid assay

To confirm interaction between NbSGS3 and T茨SV NSs, we used the yeast two-hybrid system according to standard protocols. Briefly, a clone of yeast strain Y2Hgold was selected and incubated in 50 ml of yeast peptone dextrose adenine agar (YPDA) at 30°C with shaking until the culture reached an OD₅₆₀ of 0.6–0.8. The culture was collected by centrifugation and used to prepare competent yeast cells. Then, 1 μg of prey vector (pGBD-NbSGS3) and 1 μg of bait vector (pGBK-NSs) were used to cotransform 200 μl of freshly competent yeast with the assistance of single-stranded carrier DNA incubated in a 42°C water bath for 15 min. Finally, the mixture was plated onto selection plates of double dropout (DDO; SD-Trp-Leu) and quadruple dropout (QDO; SD-Trp–Leu–His–Ade) medium and incubated for 3–4 days at 30°C. Yeast cells were co-transformed with AD-Nb SG3 and BD, AD and BD-NSs, AD-T and BD-Lam as negative controls, and AD-T and BD-PS3 as a positive control.

4.8 | Confocal microscopy

For BiFC assays, the entry clones pCV-nYFP-NSs, pCV-cYFP-NSs (TSVV), pCV-cYFP-NbSGS3, and empty vector controls were...
transformed into A. tumefaciens GV3101. Cultured cells were harvested and resuspended in infiltration buffer and then infiltrated into leaves of N. benthamiana. For co-localization analysis, the mixture of NSs-GFP and NbSGS3-mCherry was infiltrated into leaves of N. benthamiana. After 48 h of infiltration, images were observed using confocal microscopy (Nikon C2 plus) with the preset sequential scan settings for YFP with Ex 514 nm, Em 568–585 nm, GFP with Ex 488 nm, Em 510–550 nm, and for mCherry with Ex 543 nm, Em 590–630 nm. Collected images were analysed with the NIS-Element software (Nikon C2 plus).

4.9 | RT-qPCR analysis

The total RNA was extracted using TRizol reagent (Invitrogen) and dissolved in 50 μl of double-deionized water, then cDNA was synthesized from 1 μg total RNA using MonScript RTIII All-in-one Mix with dsDNase (Monad). Real-time qPCR was conducted using the MonAmp SYBR Green qPCR Mix (Monad). The NbActin gene was selected as an internal control for the assays. All experiments were repeated at least three times and the relative gene expression levels were calculated using the 2−ΔΔCt method for analysis (Livak & Schmittgen, 2001).

4.10 | Statistical analysis

Unless otherwise stated, all experiments were performed with at least three biological replicates in all cases. Significant differences between samples in gene expression were statistically analysed with Student’s tests in GraphPad Prism 8.0 software; values of p < 0.05 and p < 0.01 were taken as statistically significant.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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