Members of the ribosomal protein S6 (RPS6) family act as pro-viral factor for tomato spotted wilt orthotospovirus infectivity in *Nicotiana benthamiana*

Tieme A. Helderman1 | Laurens Deurhof2 | André Bertran3 | Manon M. S. Richard1 | Richard Kormelink3 | Marcel Prins1,4 | Matthieu H. A. J. Joosten2 | Harrold A. van den Burg1

**Abstract**
To identify host factors for tomato spotted wilt orthotospovirus (TSWV), a virus-induced gene silencing (VIGS) screen using tobacco rattle virus (TRV) was performed on *Nicotiana benthamiana* for TSWV susceptibility. To rule out any negative effect on the plants’ performance due to a double viral infection, the method was optimized to allow screening of hundreds of clones in a standardized fashion. To normalize the results obtained in and between experiments, a set of controls was developed to evaluate in a consistent manner both VIGS efficacy and the level of TSWV resistance. Using this method, 4532 random clones of an *N. benthamiana* cDNA library were tested, resulting in five TRV clones that provided near complete resistance against TSWV. Here we report on one of these clones, of which the insert targets a small gene family coding for the ribosomal protein S6 (RPS6) that is part of the 40S ribosomal subunit. This RPS6 family is represented by three gene clades in the genome of Solanaceae family members, which were jointly important for TSWV susceptibility. Interestingly, RPS6 is a known host factor implicated in the replication of different plant RNA viruses, including the negative-stranded TSWV and the positive-stranded potato virus X.

**KEYWORDS**
disease susceptibility, forward genetics, genetic screen, tomato spotted wilt virus, Tospoviridae, viral susceptibility factors, virus-induced gene silencing

1 | **INTRODUCTION**
Plant viruses cause major yield losses of crops worldwide (Scholthof et al., 2011). To reduce the problems caused by plant viruses, plant breeders routinely screen different germplasm collections to identify novel (dominant) antiviral resistance (R) traits, which can then be introduced in elite plant material (Hashimoto et al., 2016). However, plant viruses typically have a high mutation rate and thereby often create a plethora of gene variants, of which some eventually escape recognition by cognate R genes. In addition, some DNA viruses recombine in mixed infections, while certain RNA viruses can reassort their genomic segments (Froissart et al., 2005; Lima et al., 2017).
As a consequence, antiviral \( R \) genes are often soon broken on their wide introduction in cultivars due to the emergence of resistance-breaking viral strains, thereby limiting their effectiveness for farmers (García-Arenal & McDonald, 2003).

A potentially more durable strategy to obtain antiviral resistance is the identification of host genes required for the viral infection cycle, as viruses are obligate pathogens that rely on host cells to complete their infection cycle. Genetic variation in such host genes (by induced mutations or natural variation) can provide a genetic source for recessive resistance when the underlying genetic variation interferes with essential viral processes, for example viral replication and/or movement. Such genes are now referred to as susceptibility (\( S \)) genes. Two well-known antiviral \( S \) genes are Eukaryotic translation initiation factor 4E (\( eIF4E \)) (Ruffel et al., 2002) and ty-5 (Pelota) (Lapidot et al., 2015; Pramanik et al., 2021). Loss-of-function mutations in these plant genes provide broad resistance to potyviruses and geminiviruses in different crops (Koeda et al., 2021; Mäkinen, 2020; Robaglia & Caranta, 2006; Wang & Krishnaswamy, 2012). These two cases demonstrate that effective \( S \) genes should turn an otherwise susceptible host plant into a nonhost as a result of an incompatibility between the introduced plant allele and the viral infection cycle (Hashimoto et al., 2016; van Schie & Takken, 2014).

In the past, \( S \) genes have been discovered by biochemical approaches to identify novel interacting host proteins of viral proteins (García-Ruíz, 2018), and by genetic approaches where populations of mutagenized plants are screened (van Schie & Takken, 2014; Yoshii et al., 2009). Biochemical approaches have the caveat that genetic evidence is still needed to demonstrate a role for the corresponding gene(s) in viral replication, while forward genetics strategies are time-consuming and/or challenging in many crops. For example, such strategies require in most cases at least one generation of self-fertilization to fix induced recessive mutations (Kim et al., 2006; Yang et al., 2017) and/or tissue regeneration—a drawback for species with long generation times. For some plant model species, gene edits by RNA-guided nucleases, like CRISPR/Cas9, can be obtained in one plant generation (Ellison et al., 2020; Stuttmann et al., 2021; Vu et al., 2020). In addition, such screens with mutagenized plants often fail to detect plant genes when recessive mutations are functionally complemented by another gene.

Virus-induced gene silencing (VIGS) can circumvent these aforementioned issues to a large extent, as the expression of multiple genes can be simultaneously knocked down with one or more VIGS constructs, and in a matter of days to weeks gene silencing levels can be adequate for evaluating the effect of an induced knockdown phenotype (Baulcombe, 1999; Brodersen & Voinnet, 2006). Improvements in the VIGS methodology (Liu & Page, 2008; Robertson, 2004; Senthil-Kumar & Mysore, 2014) have now made this technique applicable to both monocots and dicots, including Arabidopsis thaliana (Turnage et al., 2002), Solanum species (Brigneti et al., 2004), orchids (Lu et al., 2012), maize, wheat (Zhang et al., 2017), and banana (Tzean et al., 2019). In many plant species, however, the nonuniform pattern of VIGS remains a shortcoming, resulting in a mosaic of silenced and nonsilenced tissue. Different plant species suffer from this patchy gene silencing to various degrees.

Despite the foregoing issues, VIGS has been applied in plants as a method for forward and reverse genetics, thereby revealing the role of different genes in development, metabolic pathways, and defence against pathogens (Burch-Smith et al., 2004; Lu et al., 2003; Senthil-Kumar et al., 2013; Tang et al., 2010; Wangdi et al., 2010). Plant virologists have used VIGS to study host factors involved in viral movement and replication, such as TARF (TMV-associated RING finger protein) in tobacco mosaic virus (TMV) replication (Yamaji et al., 2010), the molecular chaperone SGT1 (Suppressor of the G2 allele of Skp1) in tomato spotted wilt orthotospovirus (TSWV) infection (Qian et al., 2018), and the transcription factor PhOBF1 in tobacco rattle virus (TRV) replication (Sun et al., 2017). To date, a forward genetics screen to identify pro-viral host genes implicated in TSWV disease development has not yet been described.

Tomato spotted wilt orthotospovirus is the type species of the family Tospoviridae. TSWV has a broad host range, infecting over 1000 plant species, both monocot and dicot plants (Parrella et al., 2003). As TSWV causes severe crop losses worldwide and dominant \( R \) genes for TSWV are lacking for most crops, an effective and broadly applicable \( S \) gene is needed for antiviral breeding for TSWV.

In addition, resistance-breaking viral strains are now also known for the two major \( R \) genes applied in pepper (\( Tsw \)) and tomato (\( Sw-5b \)) (Ciuffo et al., 2005; Ferrand et al., 2019; López et al., 2011; de Ronde et al., 2019). A major disadvantage of working with TSWV is that not all hosts show clear disease symptoms when infected with this virus. Recently, a green fluorescent protein (GFP) reporter system based on an infectious clone of TSWV was reported to visualize real-time TSWV infections in planta (Feng et al., 2020). As the infectivity of this TSWV reporter system was found to be \( \pm90\% \) of the wild-type virus, its applicability in large screens is limited due to a relatively large number of escapes (false negatives).

As TSWV disease symptoms are very clear in Nicotiana benthamiana, TSWV rub-inoculations are very robust on this plant, and TRV-mediated gene silencing is relatively uniform in it (Bally et al., 2018), we developed an efficient VIGS workflow to identify plant host factors needed for the TSWV infection cycle. The workflow was based on the sequential infection by TRV and TSWV. This system gave mild TRV symptoms, effective TRV-mediated targeted gene silencing, and strong TSWV symptoms without any escapes. Using this VIGS protocol, we then screened 4532 random clones, which yielded five different clones that each provide some level of resistance to TSWV. In particular one clone, which targeted the entire \( RPS6 \) gene family, provided a strong antiviral resistance to TSWV.

2 | RESULTS

2.1 | Optimization of TSWV disease assay in N. benthamiana for performing genetic screens

To be able to routinely assess TSWV disease severity, we carefully evaluated TSWV disease progression under our growth conditions in \( N. \) benthamiana on rub-inoculation with TSWV-infected leaf sap
from *Emilia sonchifolia* plants. Whereas mock-inoculated plants did not develop any viral symptoms (Figure 1a), TSWV-challenged plants developed chlorotic lesions on the primary inoculated leaves 4 days postinoculation (dpi) (de Ávila et al., 1993). At 6 dpi, the first signs of systemic infections became visible with the appearance of leaf rolling and leaf rugosity near the plant apex (Figure 1bII). The virus then spread downwards from the apex, with disease symptoms becoming visible as tissue clearing, starting near the petiole at the base of the lower leaves, which then moved as an expanding chlorotic zone towards the leaf tip, around 11 dpi (Figure 1bIII). As a result of the TSWV infection, plant development became arrested and after 4–6 weeks most infected plants had collapsed due to a strong necrotic response (Figure 1bIV).

Others have previously shown that the TSWV sap inoculum is intrinsically unstable (Black et al., 1963; Tsuda et al., 1991). We also found that the TSWV inoculum deteriorated within hours when placed at room temperature (Figure S1). To avoid the deterioration of the TSWV inoculum that would negatively affect large disease assays, the sap was thereafter always kept on ice in the presence of sodium sulphite in a buffered solution to reduce its deterioration, while it was routinely replaced by fresh inoculum at least every 30 min or after inoculating more than 90 plants.

### 2.2 Optimization of the sequential inoculation of TRV and TSWV

As mixed viral infections can lead to enhanced disease symptoms for either virus (Qian et al., 2018), the impact of combining TRV and TSWV on *N. benthamiana* was also assessed. First, 2-week-old plants were agro-inoculated with an infectious clone of TRV using two different TRV RNA2 variants, TRV::GUS and TRV::00. Whereas TRV::GUS contains a 300-nucleotide (nt) gene fragment of the bacterial gene β-glucuronidase (GUS) from *Escherichia coli*, TRV::00 represents a wild-type clone without an additional gene fragment inserted (Liu, Schiff, & Dinesh-Kumar, 2002; Tameling & Baulcombe, 2007). In line with other reports (Hartl et al., 2008; Tameling & Baulcombe, 2007; Wu et al., 2011), we found that TRV::00 was...
highly aggressive on *N. benthamiana*, resulting in severe stunting, while TRV::GUS caused only mild viral symptoms (Figure S2). For this reason, TRV::GUS was used as negative control for gene silencing in our VIGS experiments.

To infer the optimal moment for inoculating TSWV as a second virus, we also determined the moment that VIGS had spread systemically based on the appearance of photobleaching (tissue whitening) in noninfected tissue as a result of silencing of the gene *Phytoene desaturase* (TRV::PDS) (Liu, Schiff, & Dinesh-Kumar, 2002). Approximately 9 days after TRV::PDS agro-inoculation, the first signs of photobleaching became apparent in the third and fourth leaves above the inoculated leaves (Figure S3a), and after 14 days the apex of the TRV::PDS plants displayed photobleaching (Figure S3b). As the optical densities used for agro-inoculation of TRV differ between reports from an OD$_{600}$ of 0.1 (Velásquez et al., 2009) to 1.0 (Liu, Schiff, & Dinesh-Kumar, 2002), we also evaluated the impact of the bacterial density on the VIGS efficiency under our conditions by quantifying the relative area of photobleaching of the plant canopy. Independent of the density of agro-inoculum (OD$_{600}$ of 0.1–1.0), around 40%–50% of the plant canopy showed photobleaching (Figure S3c). To align with other studies, we opted to use a final OD$_{600}$ of 0.8 for the mixed *Agrobacterium* culture (TRV RNA1+RNA2, mixed in a 1:1 ratio) in our subsequent VIGS experiments. Based on the spread of VIGS, we decided to introduce TSWV (by means of rub-inoculations) 2 weeks after agro-inoculation and compared the TSWV viral titres attained in the dual-infected plants to those in plants infected with only TSWV using a double-antibody sandwich (DAS) ELISA that detects the TSWV nucleocapsid (N) protein (Figure S4a). The levels of the TSWV N protein did not differ over time between the two plant groups.

We then confirmed that VIGS could be used to effectively break TSWV resistance in *N. benthamiana* by targeting the transgene Sw-5b from *Solanum peruvianum* (Stevens et al., 1991). Previously, it was shown that stable expression of Sw-5b in *N. benthamiana* provides full resistance to TSWV (Hallwass et al., 2014). To target Sw-5b for gene silencing, a 300-nt cDNA fragment of Sw-5b was inserted into pYL156TE (TRV::Sw-5b). Two weeks after TRV::Sw-5b agro-inoculation, the Sw-5b *N. benthamiana* plants were exposed to TSWV. As expected, plants preinoculated with TRV::GUS developed necrotic lesions on the inoculated leaves in response to TSWV, which is a classical hypersensitive response seen for Sw-5b-mediated immunity against TSWV (Figure 2a) (Hallwass et al., 2014; Zhu et al., 2017). In contrast, plants preinoculated with TRV::Sw-5b did not develop necrotic lesions in the primary infected leaves, while TSWV disease symptoms became apparent in systemic leaf tissue over the same time course as seen in Figure 1a. The Sw-5b silencing levels and TSWV RNA levels were determined in systemic leaf tissue using reverse transcription-quantitative PCR (RT-qPCR). The Sw-5b transcript levels were reduced by approximately 2-fold in the TRV::Sw-5b agro-inoculated plants in comparison to the TRV::GUS negative control plants, while the TSWV viral titres were increased by 1000-fold in the TRV::Sw-5b agro-inoculated plants (Figure 2b,c). Thus, TRV-mediated gene silencing of Sw-5b resulted in breaking of TSWV resistance, while a mixed infection of TRV and TSWV did not enhance disease symptom development of either virus.

To be able to compare the results obtained between individual large-scale VIGS experiments, a positive control was generated that would provide induced TSWV resistance without altering the plant development. Previous work had identified two plant factors that delayed the TSWV infection, that is, SGT1 on VIGS in *N. benthamiana* (Qian et al., 2018) and the rhd3 mutant in *Arabidopsis* (Feng et al., 2016). As we were searching for a positive control that confers full resistance to TSWV, gene silencing of these two genes cannot be used as a control in our assays. Instead, we examined whether the TSWV viral RNA itself could provide an effective target for VIGS to attain a consistent level of antiviral resistance in *N. benthamiana*. Different studies had already shown that a high level of resistance against different tospoviruses is attained when the RNA interference pathway is primed with antisense/sense viral fragments (Jan et al., 2000; Pang et al., 1993; Prins et al., 1996). Indeed, none of the plants agro-inoculated with the antiviral silencing construct TRV::TSWV N developed TSWV symptoms in the 2-week period after the TSWV inoculation, while in the control group (agro-inoculated with TRV::GUS) all plants became infected with TSWV (Figure 2d). These observations were confirmed by RT-qPCR analyses (Figure 2e). We thus concluded that the TRV::TSWV N construct is suitable as an internal control to normalize the level of resistance attained by agro-inoculation of a random set of VIGS clones over different experiments.

### 2.3 Random VIGS screen revealed ribosomal protein subunit 6 (RPS6) clade C as pro-viral factor for both TSWV and PVX

Using the aforementioned conditions set, a random screen was conducted using two VIGS libraries with random cDNA clones inserted. Two weeks after TRV agro-inoculation, a wide range of developmental deformations was observed, confirming that probably many different genes were targeted by these random clones. The majority (79%, 166/210 clones) of the clones, however, did not induce any apparent change in the plant developmental pattern, that is, their morphology did not differ substantially from the TRV::GUS control (Figure 3a,b). About 12% (26/210) of the randomly silenced plants showed an elongated growth phenotype, while a smaller number of plants displayed severe chlorosis (4%, 8/210 clones), stunted growth (3%, 7/210 clones), or leaf deformations (1%, 2/210 clones). In 0.5% (1/210 clones) of the cases, the VIGS clone triggered severe stunting resulting in plant collapse and these latter clones were excluded from further testing in the screen. Similar percentages were observed for these phenotypes in a related VIGS study by others (Senthil-Kumar et al., 2018).

Including the initial 210 plants, we screened in total 4532 random clones at least once, divided over 10 large experiments (Figure 4). To normalize the attained TSWV resistance levels across the entire screen, the three aforementioned controls
FIGURE 2. Virus-induced gene silencing (VIGS) effectively compromises both Sw-5b-mediated resistance and TSWV susceptibility in *Nicotiana benthamiana*. (a) TSWV disease symptoms visible on 4-week-old Sw-5b transgenic plants on silencing with TRV::Sw-5b (with TRV::GUS as a control). White arrowheads mark necrotic lesions due to the Sw-5b-mediated immune response. (b) Sw-5b transcript levels in the TRV + TSWV-inoculated plants. Four samples were taken from the plants shown in (a). An unpaired Student’s *t* test (**p < 0.01) was performed. The experiment was repeated three times independently with similar results. (c) Experiment similar to (b), except that viral RNA levels of TSWV N (both genomic and transcript RNA) are shown. An unpaired Student’s *t* test (**p < 0.001) was performed. (d) TSWV disease symptoms of 4-week-old plants inoculated with TRV::TSWV N, with TRV::GUS as a control. (e) TSWV N RNA levels in the TRV + TSWV-inoculated plants. Four samples were taken from the plants shown in (d). An unpaired Student’s *t* test (**p < 0.01) was performed. Representative images are shown for (a) and (c). In total, 14 plants were tested for each construct divided over three biological repeats with similar results for each experiment.
were included in each individual experiment to be able to evaluate whether seasonal effects impacted the VIGS efficiency and/or TSWV infectivity. Some plants already showed strong TSWV symptoms 5–7 days after TSWV rub-inoculation, which suggests that the corresponding VIGS clones had turned these plants hypersusceptible to TSWV.

Two weeks after TSWV inoculation, all plants that had become symptomatic for TSWV were discarded except for the control plants. From the remaining symptomless plants and the controls, leaf disks were collected from the youngest fully expanded leaf near the apex to determine the TSWV viral titres with a DAS-ELISA. About 4% of the plants (181/4532 plants) remained free of TSWV symptoms (Figure 5a,b). Surprisingly, 59 of these 181 symptomless plants contained high TSWV titres, suggesting the targeted plant genes support viral symptom development. As we were looking for candidate S genes, the corresponding TRV clones were not included in our further studies, as silencing of the targeted genes had apparently led to loss of viral symptoms without compromising viral replication and/or viral movement of TSWV (i.e., these VIGS clones conferred tolerance to TSWV). The remaining 122 VIGS clones were retested in independent disease trials with seven plants per construct. Of these retested clones, only five clones conferred TSWV resistance in the rescreen, which corresponds to 0.11% of the initially screened clones. The cDNA inserts of these five TRV clones were sequenced and subsequently used as input for a BLAST search (www.nbenth.com) (Nakasugi et al., 2014) and the Sol Genomics Network VIGS tool (Fernandez-Pozo et al., 2015) to identify the corresponding target genes in the *N. benthamiana* genome.

One insert corresponded to Nbv6.1trP1324 (www.nbenth.com), a gene that encodes an isoform of the ribosomal protein subunit 6 (*NbRPS6*) that is part of the 40S ribosome complex (Figure 5c; enlarged pictures are shown in Figure S5). Based on sequence homology and the scores of SGN VIGS software, we found that this 242-nt TRV clone (TRV::RPS6) probably targets four genes simultaneously (Nbv6.1trP1324, Nbv6.1trP1201, Nbv6.1trP68266, and Nbv6.1trP68267) and potentially even targets all six RPS6 homologues in *N. benthamiana* (Table S2). Agro-inoculation of TRV::RPS6 resulted in a pleiotropic effect on plant development, seen as chlorotic leaves, upward leaf curling, and plant growth retardation, as reported by others (Yang et al., 2009). It also strongly compromised TSWV infectivity, that is, 86% of the tested plants remained free of any disease symptoms and no viral proteins could be detected in the systemic leaves (Figure 5d). This result is within the range observed for the positive control, TRV::TSWV N, for which 91% of the silenced plants were resistant (*p > 0.999*, Fisher’s exact test). To determine the silencing levels of the target genes, an RT-qPCR analysis was performed. As the mRNA sequences of the genes Nbv6.1trP1201, Nbv6.1trP1324, Nbv6.1trP68266, and Nbv6.1trP68267 are highly homologous, we were only able to design a primer pair that measures their combined expression levels. We found that TRV::RPS6 reduced the combined mRNA levels of these four genes by about 60% compared to the control (TRV::GUS) (Figure 5e).

Next we investigated both the gene conservation and expansion of the RPS6 gene family in plants based on whole genome sequence data from a diverse set of plant species (Figure 6a and
FIGURE 4  Workflow to identify pro-viral host factors for TSWV using virus-induced gene silencing (VIGS). Fresh Agrobacterium cultures, each carrying a random TRV2 clone, were grown in a 96-well microtitre plate format. TRV1 and TRV2 cultures were mixed 1:1 (final OD₆₀₀ of 0.8 per culture) and the mixtures were toothpick-inoculated onto 14-day-old Nicotiana benthamiana plants by scratching the lower leaf surface twice. In parallel, defective interfering (DI)-free TSWV inoculum was produced in Emilia sonchifolia. Two weeks after TSWV inoculation, sap from systemically infected E. sonchifolia leaves was used to rub-inoculate N. benthamiana plants to obtain sufficient TSWV inoculum for the TRV-infected plants. Inoculum of systemically infected N. benthamiana was then rub-inoculated onto 4-week-old TRV-infected plants. After 14 days, TSWV disease symptoms were scored and the TSWV viral titres were quantified in the plants that lacked any TSWV symptoms, using a double-antibody sandwich (DAS)-ELISA that detects the viral N protein.
Table S3). While in *N. benthamiana* the RPS6 gene family comprises six members, the other plant genomes analysed mostly contained two or three family members. The gene tree revealed that the RPS6 gene tree follows the tree of evolution for most Eudicot species. In the case of the Solanaceae plant family, the gene tree was resolved as three well-defined clades with all species examined having
at least one gene assigned to each clade. Moreover, the clade with Nb6.1trP1324 displayed an extended branch length compared to the other two clades. This observation indicates that this particular RPS6 clade is more divergent and less conserved at the sequence level. The genes in clades A and B (with shorter branch lengths) still show synteny with RPS6 homologues in other plant genomes (Figure S6b), suggesting that NbRPS6a and NbRPS6b represent the ancestral gene state. In contrast, the RPS6c clade appears to have emerged from a more recent gene duplication of RPS6b in a common ancestor of the Solanaceae family, resulting in an insertion of the ancestral gene of RPS6c in a different genomic region in a common ancestor of the Solanaceae.

The original TRV::RPS6 clone was predicted foremost to knockdown gene expression of RPS6c1 (Nbv6.1trP1324) as indicated by the Sol Genomics Network VIGS tool (Fernandez-Pozo et al., 2015). This prompted us to investigate whether gene silencing of N. benthamiana RPS6a (Nb6.1trP72816) or RPS6b (Nb6.1trP17350) alone would also compromise TSWV infectivity. To minimize the risk of cross-silencing, two smaller mRNAs fragments (200 nt) were cloned for NbRPS6a and NbRPS6b in pYL156TE. More so than the original TRV::RPS6 clone, agro-inoculation with TRV::NbRPS6a and TRV::NbRPS6b induced both severely arrested plant growth and leaf deformations (Figure 6a; enlarged pictures are shown in Figure S7). To determine the individual expression levels of the RPS6a and RPS6b genes, specific primer pairs were designed for RT-qPCR. Using these primer pairs, we found that TRV::NbRPS6a and TRV::NbRPS6b still caused cross-silencing of the entire RPS6 family (Figure 6b,c) while concomitantly reducing TSWV susceptibility (Figure 6d), similar to the original TRV::RPS6c construct.

As gene silencing of the RPS6 gene family in N. benthamiana compromised the accumulation of TSWV, we investigated whether RPS6-silenced plants also compromised viral accumulation of an unrelated plant virus, in this case potato virus X (PVX, with an infectious clone of PVX expressing the GFP reporter gene). Ten days after PVX inoculation, GFP fluorescence could be observed under UV light in the apex of the RPS6-silenced plants (Figure S8). Interestingly, whereas PVX accumulation was observed by DAS-ELISA, the PVX titres in the RPS6-silenced plants were less than in the mock-silenced plants. This indicates that gene silencing of RPS6 in N. benthamiana reduced the susceptibility towards PVX, albeit to a lesser extent than for TSWV. Thus, the RPS6 gene family combined acts as important pro-viral factor for TSWV and to a lesser extent for PVX too.

3 | DISCUSSION

Different groups have used VIGS to study the role of plant genes in viral replication using dual-inoculation strategies. These viral studies were successful, as they relied on plant-reporter systems that track viral spread in planta (Lozano-Durán et al., 2011; Morilla et al., 2006; Zhu & Dinesh-Kumar, 2008). Here, a VIGS strategy was optimized to identify host factors for TSWV without the use of a reporter construct, and we successfully identified the RPS6 gene family as an important pro-viral factor for TSWV and PVX infectivity. Silencing of this N. benthamiana RPS6 gene family was already known to suppress the accumulation of unrelated positive-stranded RNA viruses, like turnip mosaic virus (TuMV), tomato bushy stunt virus, cucumber mosaic virus, and potato virus A (Rajamäki et al., 2017; Yang et al., 2009). The mechanism by which RPS6 promotes viral accumulation of some, but not all, viruses (such as turnip crinkle virus and TMV) is not yet understood (Rajamäki et al., 2017). However, a plethora of mammalian-infecting viruses is also dependent on the RPS6 protein family for their infection (Li, 2019). RPS6 is one of the best-studied ribosomal proteins (RPs), as for a long time it was the only RP that was shown to undergo phosphorylation (Gressner & Wool, 1974). In A. thaliana and Zea mays (maize), phosphorylation of RPS6 proved to be mediated by ribosomal protein S6 kinase (S6K), thereby enhancing mRNA translation and ribosome biogenesis (Chen et al., 2018; Enganti et al., 2018; Meyuhas, 2015; Williams et al., 2003). S6K activation is regulated by the target of rapamycin (TOR) signaling pathway and the 3-phosphoinositide-dependent kinase (PDK1) (Chen et al., 2018; Enganti et al., 2018; Magnuson et al., 2011; Turck et al., 2004). Both the TOR pathway and the PDK1-activated phosphoinositide 3-kinase (PI3K) pathway monitor changes in the cellular energy homeostasis by using inputs from diurnal patterns, energy metabolism, presence of nutrients, cellular stresses, autophagy, and cell proliferation. These two connected pathways are targeted by many viruses to enhance the RNA translation capacity of virus-infected cells to generate sufficient viral proteins (Li, 2019; Montgomery et al., 2006). Conversely, RPS6 phosphorylation is part of a nonspecific antiviral immune response in humans (Li, 2019).

In Arabidopsis there are two RPS6 homologues that together are essential for plant development (Creff et al., 2010). Single mutants in Arabidopsis for RPS6a or RPS6b do not confer compromised susceptibility towards TuMV (Yang et al., 2009). Whereas many dicots contain at least two RPS6 homologues, the RPS6 gene family appears to have expanded into three clades in the Solanaceae plant family. From the latter three clades, RPS6c was here found to be the most divergent clade, suggesting that this clade evolved under neutral or positive selection pressure compared to the other two clades. In line with this notion, TRV::RPS6a and TRV::RPS6b (which target the two more conserved family members) impacted N. benthamiana plant development more strongly, while all three VIGS constructs suppressed accumulation of all NbRPS6 transcripts.

A primary concern of viral co-infections is that they can cause synergistic interactions, leading to more pronounced viral symptoms. An example of such a synergistic interaction with enhanced disease symptoms is the consecutive inoculation of potato virus Y and PVX, which results in necrotic symptoms that are absent in the single infections (Damirdagh & Ross, 1967). For the combination of TRV and TSWV, as used in this study, we did not observe any synergistic interaction in N. benthamiana, in agreement with reports by others (Qi et al., 2018). In addition, TSWV rub-inoculations onto the TRV-silenced plants did not result in (co-)transfer of any TRV construct between plants, as photobleaching was never observed in plants in the vicinity
Gene silencing with TRV::RPS6a, TRV::RPS6b or TRV::RPS6c suppresses TSWV disease development. (a) Developmental phenotype and TSWV disease symptoms of 6-week-old *Nicotiana benthamiana* plants inoculated with TRV::RPS6a, TRV::RPS6b, and TRV::RPS6c (TRV::GUS, control). Representative images are shown for each TRV construct. (b) Transcript levels of the *RPS6* gene family in the plants inoculated with the indicated TRV clones (*n* = 3). Unpaired Student's *t* test (*p* < 0.05, **p** < 0.01, ***p*** < 0.001, *****p*** < 0.0001). (c) Predicted number of 21-nucleotide (nt) siRNAs obtained with the different RPS6 virus-induced gene silencing (VIGS) clones that specifically target *N. benthamiana* transcripts of RPS6a, RPS6b or RPS6c according to the Sol Genomics Network VIGS tool. (d) Quantification of the TSWV viral titres in systemically infected leaves of plants shown in panel (a) using a double-antibody sandwich ELISA that detects the viral N protein. Analysis of variance followed by a Dunnett multiple comparison test (*****p*** < 0.0001). The experiment was repeated three times independently.
of the TRV::PDS controls. The observed lack of mechanical transfer of TRV::PDS can also, in part, be explained by the observation that both A. thaliana and N. benthamiana can recover from a TRV infection over the time course of 2 weeks (Ma et al., 2014, 2019).

The TRV empty vector (TRV::00) is commonly used as a (negative) control to assess TRV disease symptom development (Hwang et al., 2013; Rajamäki et al., 2017; Zhu et al., 2015). As found by others (Hartl et al., 2008; Tameling & Baulcombe, 2007; Tran et al., 2016; Wu et al., 2011), TRV::00 itself caused strong detrimental effects on N. benthamiana development. By insertion of an exogenous gene fragment (e.g., GUS from E. coli) in TRV, this detrimental effect on plant growth was simply avoided. Besides the original TRV vector we used for the cloning of targeted VIGS constructs, two other TRV2 derivatives exist, that is, a Gateway-recombination-compatible vector that we used for constructing the cDNA library pYL279 (ABRC stock no. CD3-1041) and a Bsal GoldenGate-compatible derivative pTRV2-GG (Addgene stock no. 105349) (Gantner et al., 2018). For other plant species, other viruses might, however, be more suitable than the TRV system used here to initiate VIGS (Dhir et al., 2019). Nevertheless, in this study we established and optimized a VIGS platform in N. benthamiana that can easily be modified and applied for other pathosystems for which no pathogen reporter construct exists.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant growth conditions

N. benthamiana plants, and plants expressing the resistance genes Rx1, Cf-4, or Sw-5b (Table S1), were grown on soil in a climate-controlled greenhouse with a 16 h day length (636–1060 µmol⋅m−2⋅s−1) at 22°C and 8 h of darkness at 20°C, with a relative humidity of 65%. E. chilensis plants were grown in a growth chamber under the same conditions.

4.2 | Construction of VIGS-cDNA libraries

Earlier VIGS-cDNA libraries were generated to study general plant immune responses, which we reused here to screen different target genes. For these, a noninduced VIGS library and an immune-primed library of random cDNA clones of N. benthamiana plants challenged with different immune elicitors were generated. To this end, Agrobacterium tumefaciens expressing (a) GUS, (b) the coat protein from PVX, (c) Avr4, or (d) NRC1D481V were used (Table S1). For the noninduced library, the GUS gene was transiently expressed in planta using A. tumefaciens. To this end, Agrobacterium was grown overnight in YEB medium (5 g/L beef extract, 5 g/L peptone, 1 g/L yeast extract, 5 g/L sucrose, 0.5 g/L MgCl2, pH 7.0) with 175 rpm agitation at 28°C. The bacteria were pelleted and resuspended in infiltration buffer (10 mM MES pH 5.6, 20 g/L sucrose, 5 g/L MS basal salts mixture without vitamins [Duchefa], 200 µM acetosyringone) to an OD600 of 0.5. Agrobacterium expressing GUS or NRC1D481V were separately infiltrated in 3-to 4-week-old wild-type N. benthamiana plants. NRC1D481V was used as it elicits an autoimmune response (Gabriëls et al., 2007). The constructs to deliver PVX coat protein gene (CP) and Avr4 were agro-infiltrated in N. benthamiana expressing the transgenes Strx1 and SlCf-4, respectively. The recognition of Avr4 by Cf-4 induces a defense response by a cell surface-localized immune receptor, whereas detection of PVX CP by Rx1 triggers a defense response by an intracellular immune receptor (Gabriëls et al., 2006; Lu et al., 2003). Two fully expanded leaves were infiltrated per construct. Approximately 24 h after infiltration, leaf material was collected and snap-frozen in liquid nitrogen. The obtained plant material was combined to generate an immune-activated VIGS library. The GUS-infiltrated leaves served as input material for the mock VIGS library.

To construct the VIGS libraries, total RNA was extracted from the plant material using TRIzol LS (ThermoFisher). The libraries were generated by ThermoFisher. In short, the mRNA fraction was isolated using poly-dT resin and double-stranded cDNA was synthesized from 1 mg of mRNA. Through subtractive hybridization, a normalized set of transcripts ranging from 500 to 800 bp in length was isolated, with 10- to 100-fold reduction for the highly abundant transcripts. These short double-stranded cDNA fragments were cloned into the Gateway-ready entry vector pENTR223 using BP clonase II (ThermoFisher). The resulting vectors were recombined with the Gateway-compatible TRV RNA2 vector pYL279 (Liu, Schiff, & Dinesh-Kumar, 2002) using LR clonase II (ThermoFisher). A total of 200 ng of each pYL279 library was introduced into A. tumefaciens C58C1 by electroporation. Transformed bacteria were allowed to recover for 2 h at 28°C with 175 rpm agitation before plating on selective Luria-Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.0, 15 g/L agar), containing 50 µg/ml kanamycin and 5 µg/ml tetracycline (Kan50, Tet5). After 2 days of growth at 28°C, single colonies were transferred to 96-well flat-bottom plates containing 138 µL of YEB medium (supplemented with Kan50 and Tet5) and grown for 72 h at 28°C, 200 rpm. Normalization of the resulting two pYL279 libraries was confirmed by amplifying 100 independent clones by PCR using the primers FP7083/FP7084, followed by digestion with Alul, after which their individual digestion patterns were compared. For long-term storage, 37.5 µl 80% vol/vol glycerol was added to each well before storing the plates at −80°C.

4.3 | VIGS screen

Agrobacterium liquid cultures representing single clones of the VIGS library were started by adding 5 µl of on-ice thawed glycerol stocks to 145 µl of LB (supplemented with Kan50 and Tet5) in a 96-well flat-bottom microtitre plate. In addition, cultures of A. tumefaciens C58C1 carrying the plasmids pTRV1, pTRV2::PDS, pTRV2::GUS (Tameling & Baulcombe, 2007), or pTRV2::TSWV N were started in 10 ml of LB in 50 ml tubes. Agrobacterium was grown at 28°C with 175 rpm agitation for 2 days. The average OD600 of the bacterial cultures of the VIGS library was determined
by measuring 10 random samples. In parallel, the OD$_{600}$ of Agrobacterium carrying pTRV1 was set at 0.8 in infiltration buffer. The cells were collected by spinning the 50 ml tubes for 10 min at 3500 x g and the bacterial pellet was resuspended in infiltration buffer already containing pTRV1 to an OD$_{600}$ of 0.8. The cultures in the 96-well plate were diluted to an OD$_{600}$ of 0.8 with the infiltration buffer already containing Agrobacterium culture carrying pTRV1. The bacterial mixtures were left for 2 h at room temperature prior to agro-inoculation. Sixteen-day-old N. benthamiana seedlings were agro-inoculated by scratching the abaxial leaf epidermis twice parallel to the midvein with a toothpick immersed in the Agrobacterium mixtures. For every 15 plants, one plant was agro-inoculated with TRV::GUS, and three randomly picked plants were agro-inoculated with TRV::PDS.

4.4 Virus inoculation

Throughout this study, plant material infected with the Brazilian isolate BR-01 of TSWV was used (Table S1). Defective interfering (DI)-free viral inoculum was generated from ~80°C frozen stock of TSWV-infected leaf material as described by Inoue-Nagata et al. (1997). The resulting sap in inoculation buffer (10 mM NaPO$_4$, pH 7.0, 10 mM Na$_2$SO$_3$) was rub-inoculated with about five thumb strokes on E. sonchifolia leaves, predested with 500-mesh carborundum. Two weeks later, sap from systemically infected E. sonchifolia was used to inoculate N. benthamiana plants. This procedure provided sufficient TSWV-infected plant material to inoculate a large set of TRV-inoculated N. benthamiana plants. As a rule of thumb, approximately 60 plants (divided over four trays) can be inoculated with the sap produced from one infected plant. Two weeks after TRV inoculation, the same plants were inoculated with the second virus, TSWV. To that end, visually symptomatic TSWV-infected leaf tissue of one N. benthamiana plant was harvested and homogenized in 10 ml of ice-cold inoculation buffer in a precooled mortar and pestle. The silenced plants were lightly dusted with carborundum and chilled leaf homogenate was rubber-inoculated onto the youngest two fully expanded leaves with about three strokes per leaf, using a piece of synthetic sponge. The sap inoculum was replaced every 60–90 inoculated plants by freshly prepared sap (and at least every 30 min). After TSWV inoculation, the carborundum was washed off with filtered rainwater using a watering hose. The transient expression of the PVX-GFP reporter strain was performed as described by Richard et al. (2020).

4.5 Serological detection of TSWV and PVX viral titres

The viral titre in plant tissue was quantified with a DAS-ELISA, as described (Clark & Adams, 1977). To detect TSWV, 96-well plates were coated with polyclonal rabbit antibody raised against the TSWV N protein (1:1000 vol/vol) to bind the antigen (Kikkert et al., 1997). A second rabbit polyclonal antibody raised against the TSWV N protein and conjugated to alkaline phosphatase (de Ávila et al., 1990) (1:1000 vol/vol) was used for the conversion of the phosphatase substrate (Sigma-Aldrich/Merck). The absorbance was measured at 405 nm ($A_{405}$) every 15 min over a period of 1 h using a CLARIOstar Optima plate reader (BMG LABTECH). The detection of PVX titres by DAS-ELISA was performed as described in Richard et al. (2020).

4.6 Cloning of TRV constructs for VIGS

All molecular DNA cloning techniques were performed using standard methods (Sambrook & Russell, 2001). As a template to amplify a Sw-5b gene fragment for VIGS, the plasmid p2300S-Sw-5b was used (Chen et al., 2016). For cloning the silencing construct that targets the TSWV nucleocapsid gene, cDNA of N. benthamiana infected with TSWV strain BR01 was used. A 300-nt cDNA fragment of Sw-5b or from the TSWV nucleocapsid (TSWV N) gene was PCR-amplified using Phusion polymerase (ThermoFisher); all primer sequences are provided in Table S1. The resulting PCR amplicons were subsequently cloned into the Smal restriction site of pYL156TE (GenBank accession MW815519). The TRV2 vector used here is a derivative of pYL156 (Liu, Schiff, Marathe, et al., 2002), except that it contained an additional transposable element (TE) from E. coli outside the transfer DNA (Figure S4b). For constructing the N. benthamiana RPS6a and RPS6b VIGS constructs, a 200-nt cDNA fragment was amplified. The PCR amplicon was cloned into pYL156TE as described above, resulting in the vectors pTRV2::RPS6a and pTRV2::RPS6b. The integrity of all vectors was confirmed by sequencing using the primers FP7083/FP7084 and the constructs were then introduced in A. tumefaciens GV3101.

4.7 Gene expression analysis

To quantify the transcript levels of a gene-of-interest or TSWV N, a total of 100 mg of systemically infected leaf tissue was collected from a 6-week-old N. benthamiana plant at 2 weeks after TSWV inoculation (i.e., 4 weeks after TRV agro-inoculation to initiate VIGS). RNA isolation, transcript quantification, and data analyses were performed as described by Maio et al. (2020). Gene expression data were normalized using N. benthamiana APR as a reference gene (Liu et al., 2012). All primer sets are listed in Table S1.

4.8 Computational analysis and data visualization of the RPS6 gene family

The RPS6 gene fragment in the VIGS clone was compared to the N. benthamiana transcriptome database (v. 6.1) (Nakasugi et al., 2014) and the full-length open reading frames (ORFs) to extract all RPS6 homologues. Full-length ORFs of other plant species were
obtained via Phytozome (v. 12.1.6) (Goodstein et al., 2012). The DNA sequences representing the RPS6 ORFs were aligned with MUSCLE v. 3.8.31 and gaps and/or poorly aligned regions were removed with Gblocks v. 0.91b (Dereeper et al., 2008). The phylogenetic tree was reconstructed via RAxML v. 0.9.0 (Kozlov et al., 2019) using an unpartitioned model following the HKY substitution matrix with a proportion of invariant sites and four gamma-distributed rate categories. Automatic bootstrapping iterations assessed the reliability of internal branches with a bootstopping cut-off of 0.03. Graphical representation of the phylogenetic tree was generated with FigTree v. 1.4.4. The gene synteny between the RPS6 gene family of Solanaceae species was compared with PLAZA v. 4.5 (Family ID: HOM04D001545) (Van Bel et al., 2018).

ACKNOWLEDGEMENTS

We are grateful to Univarm personnel and especially B. Essenstam for taking excellent care of our plants at Wageningen University. H. Lemereis and L. Tikovsky are thanked for excellent plant care at the University of Amsterdam. We would like to acknowledge W. Tameling and H.v.d.B. performed the phylogenetic analysis. T.A.H. and H.v.d.B. acknowledge the NWO-STW (grant 14948) and the partnering breeding companies.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

A.B., L.D., M.R., and T.A.H. did the experimental work. T.A.H., A.B., and H.v.d.B. performed the phylogenetic analysis. T.A.H. and H.v.d.B. wrote the first draft of the manuscript. M.J. and M.P. drafted the original idea of the screen. H.v.d.B., R.K., M.J., and M.P. provided critical feedback to the project. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The pYL156TE vector map and detailed sequence information have been submitted to GenBank at https://www.ncbi.nlm.nih.gov/genbank/ as accession MW815519.

ORCID

Tieme A. Helderman https://orcid.org/0000-0003-0409-2638
Manon M. S. Richard https://orcid.org/0000-0002-7903-024X
Richard Kormelink https://orcid.org/0000-0001-7360-1884
Matthieu H. A. J. Joosten https://orcid.org/0000-0002-6243-4547
Harrold A. van den Burg https://orcid.org/0000-0003-4142-374X

REFERENCES

de Ávila, A.C., de Haan, P., Smets, M.L.L., Resende, R.D.O., Kormelink, R., Kitajima, E.W. et al. (1993) Distinct levels of relationships between tospovirus isolates. Archives of Virology, 128, 211–227.
de Ávila, A.C., Huguenot, C., Resende, R.D.O., Kitajima, E.W., Goldbach, R.W. & Peters, D. (1990) Serological differentiation of 20 isolates of Tomato spotted wilt virus. Journal of General Virology, 71, 2801–2807.

Bally, J., Jung, H., Mortimer, C., Naim, F., Philips, J.G., Hellens, R. et al. (2018) The rise and rise of Nicotiana benthamiana: a plant for all reasons. Annual Review of Phytopathology, 56, 405–426.

Baulcombe, D.C. (1999) Fast forward genetics based on virus-induced gene silencing. Current Opinion in Plant Biology, 2, 109–113.

Black, L.M., Brakke, M.K. & Vatter, A.E. (1963) Purification and electron microscopy of Tomato spotted wilt virus. Virology, 20, 120–130.

Brigñeti, G., Martín-Hernández, A.M., Jin, H., Chen, J., Baulcombe, D.C., Baker, B. et al. (2004) Virus-induced gene silencing in Solanum species. Plant Journal, 39, 264–272.

Brodersen, P. & Voinnet, O. (2006) The diversity of RNA silencing pathways in plants. Trends in Genetics, 22, 268–280.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B. & Dinesh-Kumar, S.P. (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant Journal, 39, 734–746.

Chen, G.-H., Liu, M.-J., Xiong, Y., Sheen, J. & Wu, S.-H. (2018) TOR and RPS6 transmit light signals to enhance protein translation in deetiolating Arabidopsis seedlings. Proceedings of the National Academy of Sciences of the United States of America, 115, 12823–12828.

Chen, X., Zhu, M., Jiang, L., Zhao, W., Li, J., Wu, J. et al. (2016) A multi-layered regulatory mechanism for the autoinhibition and activation of a plant CC-NB-LRR resistance protein with an extra N-terminal domain. New Phytopathologist, 212, 161–175.

Ciuffo, M., Finetti-Sialer, M.M., Galilitelli, D. & Turina, M. (2005) First report in Italy of a resistance-breaking strain of Tomato spotted wilt virus infecting tomato cultivars carrying the Sw5 resistance gene. Plant Pathology, 54, 564.

Clark, M.F. & Adams, A.N. (1977) Characteristics of the microplate method of enzyme-linked immunosorbant assay for the detection of plant viruses. Journal of General Virology, 34, 475–483.

Creef, A., Sormani, R. & Desnos, T. (2010) The two Arabidopsis RPS6 genes, encoding for cytoplasmic ribosomal proteins S6, are functionally equivalent. Plant Molecular Biology, 73, 533–546.

Damirdagh, I.S. & Ross, A.F. (1967) A marked synergistic interaction of Potato viruses X and Y in inoculated leaves of tobacco. Virology, 31, 296–307.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F. et al. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Research, 36, 465–469.

Dhir, S., Srivastava, A., Yoshikawa, N. & Khurana, S.M.P. (2019) Plant viruses as virus induced gene silencing (VIGS) vectors. In: Khurana, S.M.P. & Gaur, R.K. (Eds.) Plant biotechnology: progress in genomic era. Singapore: Springer, pp. 517–526.

Ellison, E.E., Nagalakshmi, U., Gamo, M.E., Huang, P.-J., Dinesh-Kumar, S. & Voytas, D.F. (2020) Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. Nature Plants, 6, 620–624.

Enganti, R., Cho, S.K., Topperzer, J.D., Urquidi-Camacho, R.A., Cakir, O.S., Ray, A.P. et al. (2018) Phosphorylation of ribosomal protein RPS6 integrates light signals and circadian clock signals. Frontiers in Plant Science, 8, 2210.

Feng, M., Cheng, R., Chen, M., Guo, R., Li, L., Feng, Z. et al. (2020) Rescue of Tomato spotted wilt virus entirely from complementary DNA clones. Proceedings of the National Academy of Sciences of the United States of America, 117, 1181–1190.

Feng, Z., Xue, F., Xu, M., Chen, X., Zhao, W., Garcia-Murria, M.J. et al. (2016) The ER-membrane transport system Is critical for intercellular trafficking of the NSm movement protein and Tomato spotted wilt tospovirus. PLoS Pathogens, 12, e1005443.

Fernandez-Pozo, N., Rosil, H.G., Martin, G.B. & Mueller, L.A. (2015) The SGN VIGS tool: user-friendly software to design virus-induced gene silencing (VIGS) constructs for functional genomics. Molecular Plant, 8, 486–488.

Ferrand, L., Almeida, M.M.S., Orillo, A.F., Dal Bó, E., Resende, R.O. & Garcia, M.L. (2019) Biological and molecular characterization of Tomato spotted wilt virus (TSWV) resistance-breaking isolates from Argentina. Plant Pathology, 68, 1587–1601.
Froissart, R., Roze, D., Uzest, M., Galibert, L., Blanc, S. & Michalakis, Y. (2005) Recombination every day: abundant recombination in a virus during a single multi-cellular host infection. *PLoS Biology*, 3, e89.

Gabriëls, S.H.E.J., Takken, F.L.W., Vossen, J.H., de Jong, C.F., Liu, Q., Turk, S.C.H.J. et al. (2006) cDNA-AFLP combined with functional analysis reveals novel genes involved in the hypersensitive response. *Molecular Plant-Microbe Interactions*, 19, 567–576.

Gabriëls, S.H.E.J., Vossen, J.H., Ekenberg, S.K., Ooijen, G.V., Abd-El-Haliem, A.M., Berg, G.C.M.V.D. et al. (2007) An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins. *The Plant Journal*, 50, 14–28.

Gantner, J., Ordon, J., Ilse, T., Kretschmer, C., Gruetzner, R., Löhke, C. et al. (2018) Peripheral infrastructure vectors and an extended set of plant parts for the modular cloning system. *PLoS One*, 13, e0197185.

García-Arenal, F. & McDonald, B.A. (2003) An analysis of the durability of resistance to plant viruses. *Phytopathology*, 93, 941–952.

García-Ruiz, H. (2018) Susceptibility genes to plant viruses. *Viruses*, 10, 484.

Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J. et al. (2012) Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Research*, 40, 1178–1186.

Gressner, A.M. & Wool, I.G. (1974) The phosphorylation of liver ribosomal proteins in vivo: evidence that only a single small subunit protein (S6) is phosphorylated. *Journal of Biological Chemistry*, 249, 6917–6925.

Hallwass, M., de Oliveira, A.S., de Campos Dianese, E., Lohuis, D., Boiteux, L.S., Inoue-Nagata, A.K. et al. (2014) The Tomato spotted wilt virus cell-to-cell movement protein (NSM) triggers a hypersensitive response in Sw-5-containing resistant tomato lines and in *Nicotiana benthamiana* transformed with the functional Sw-5b resistance gene copy. *Molecular Plant Pathology*, 15, 871–880.

Hartl, M., Merker, H., Schmidt, D.D. & Baldwin, I.T. (2008) Optimized virus-induced gene silencing in *Solanum nigrum* reveals the defensive function of leucine aminopeptidase against herbivores and the shortcomings of empty vector controls. *New Phytologist*, 179, 356–365.

Hashimoto, M., Neriya, Y., Yamaji, Y. & Namba, S. (2016) Recessive resistance to plant viruses: potential resistance genes beyond translocation initiation factors. *Frontiers in Microbiology*, 7, 1695.

Hwang, J., Oh, C.-S. & Kang, B.-C. (2013) Translation elongation factor 1B (eEF1B) is an essential host factor for Tobacco mosaic virus infection in plants. *Virology*, 439, 105–114.

Inoue-Nagata, A.K., Kormelink, R., Nagata, T., Kitajima, E.W., Goldbacher, R. & Peters, D. (1997) Effects of temperature and host on the generation of Tomato spotted wilt virus defective interfering RNAs. *Phytopathology*, 87, 1168–1173.

Jan, F.-J., Fagoaga, C., Pang, S.-Z. & Gonsalves, D. (2000) A minimum length of N gene sequence in transgenic plants is required for RNA-mediated tospovirus resistance. *Journal of General Virology*, 81, 235–242.

Kormelink, R., van Poelwijk, F., Storms, M., Kassies, W., Bloksma, H., van Lent, J. et al. (1997) A protoplast system for studying Tomato spotted wilt virus infection. *Journal of General Virology*, 78, 1755–1763.

Kim, Y., Schumaker, K.S. & Zhu, J.-K. (2006) EMS mutagenesis of Arabidopsis. In: Salinas, J. & Sanchez-Serrano, J.J. (Eds.) *Arabidopsis protocols*. Totowa, NJ: Humana Press, pp. 101–103.

Koeda, S., Onouchi, M., Mori, N., Pohan, N.S., Nagano, A.J. & Keshimawati, E. (2021) A recessive gene pepy-1 encoding Pelota confers resistance to begomovirus isolates of *PepyLCIV* and *PepyLCAV* in *Capsicum annuum*. *Theoretical and Applied Genetics*, 134, 2947–2964.

Kozlov, A.M., Darriba, D., Flouri, T., Morel, B. & Stamatakis, A. (2019) RAxML-NL: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics*, 35, 4453–4455.

Lapidot, M., Karniel, U., Gelbart, D., Fogel, D., Evenor, D., Kutsher, Y. et al. (2015) A novel route controlling Begomovirus resistance by the messenger RNA surveillance factor Pelota. *PLoS Genetics*, 11, e1005338.

Li, S. (2019) Regulation of ribosomal proteins on viral infection. *Cells*, 8, 508.

Lima, A.T.M., Silva, J.C.F., Silva, F.N., Castillo-Urquiza, G.P., Silva, F.F., Seash, Y.M. et al. (2017) The diversification of begomovirus populations is predominantly driven by mutational dynamics. *Virus Evolution*, 3, vex005.

Liu, D., Shi, L., Han, C., Yu, J., Li, D. & Zhang, Y. (2012) Validation of reference genes for gene expression studies in virus-infected *Nicotiana benthamiana* using quantitative real-time PCR. *PLoS One*, 7, e46451.

Liu, E. & Page, J.E. (2008) Optimized cDNA libraries for virus-induced gene silencing (VIGS) using Tobacco rattle virus. *Plant Methods*, 4, 5.

Liu, Y., Schiff, M. & Dinesh-Kumar, S.P. (2002) Virus-induced gene silencing in tomato. *The Plant Journal*, 31, 777–786.

Liu, Y., Schiff, M., Marathe, R. & Dinesh-Kumar, S.P. (2002) Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for N-mediated resistance to tobacco mosaic virus. *The Plant Journal*, 30, 415–429.

López, C., Aramburu, J., Galipienso, L., Soler, S., Nuez, F. & Rubio, L. (2011) Evolutionary analysis of tomato Sw-5 resistance-breaking isolates of Tomato spotted wilt virus. *Journal of General Virology*, 92, 210–215.

Lozano-Durán, R., Rosas-Díaz, T., Luna, A.P. & Bejarano, E.R. (2011) Identification of host genes involved in geminivirus infection using a reverse genetics approach. *PLoS One*, 6, e22383.

Lu, H.-C., Hsieh, M.-H., Chen, C.-E., Chen, H.-H., Wang, H.-I. & Yeh, H.-H. (2012) A high-throughput virus-induced gene-silencing vector for screening transcription factors in virus-induced plant defense response in Orchid. *Molecular Plant-Microbe Interactions*, 25, 738–746.

Lu, R., Malcuit, I., Moffett, P., Ruiz, M.T., Peart, J., Wu, A.-J. et al. (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO Journal*, 22, 5690–5699.

Ma, X., Nicole, M.-C., Meteignier, L.-V., Hong, N., Wang, G. & Moffett, P. (2014) Different roles for RNA silencing and RNA processing components in virus recovery and virus-induced gene silencing in plants. *Journal of Experimental Botany*, 66, 919–932.

Ma, X., Zhou, Y. & Moffett, P. (2019) Alterations in cellular RNA decapping dynamics affect tomato spotted wilt virus (TSWV) cap snatching and infection in Arabidopsis. *New Phytologist*, 224, 789–803.

Magnuson, B., Ekim, B. & Fingar, D.C. (2011) Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochemical Journal*, 441, 1–21.

Maio, F., Helderman, T.A., Arroyo-Mateos, M., van der Wolf, M., Boeren, S., Prins, M. et al. (2020) Identification of tomato proteins that interact with replication initiator protein (Rep) of the geminivirus *TYLCV*. *Frontiers in Plant Science*, 11, 1069.

Mäkinen, K. (2020) Plant susceptibility genes as a source for potyvirus resistance. *Annals of Applied Biology*, 176, 122–129.

Meyahus, O. (2015) Ribosomal protein S6 phosphorylation: four decades of research. *International Review of Cell and Molecular Biology*, pp. 320, 41–73.

Montgomery, S.A., Berglund, P., Beard, C.W. & Johnston, R.E. (2006) Ribosomal protein S6 associates with alphavirus nonstructural protein 2 and mediates expression from alphavirus messages. *Journal of Virology*, 80, 7729–7739.

Morilla, G., Castillo, A.G., Preiss, W., Jeske, H. & Bejarano, E.R. (2006) A versatile transreplication-based system to identify cellular proteins involved in geminivirus replication. *Journal of Virology*, 80, 3624–3633.

Nakasugi, K., Crowhurst, R., Bally, J. & Waterhouse, P. (2014) Combining transcriptome assemblies from multiple de novo assemblers in the allo-tetraploid plant *Nicotiana benthamiana*. *PLoS One*, 9, e91776.

Pang, S.-Z., Slightom, J.L. & Gonsalves, D. (1993) Different mechanisms protect transgenic tobacco against tomato spotted wilt and...
Nature Biotechnology, 11, 819–824.

Parrella, G., Gognalons, P., Gebre-Selassie, K., Vovlas, C. & Marchoux, G. (2003) An update of the host range of tomato spotted wilt virus. Journal of Plant Pathology, 85, 227–264.

Pramanik, D., Shelake, R.M., Park, J., Kim, M.J., Hwang, I., Park, Y. et al. (2021) CRISPR/Cas9-mediated generation of pathogen-resistant tomato against tomato yellow leaf curl virus and powdery mildew. International Journal of Molecular Sciences, 22, 1878.

Prins, M., de Oliveira Resende, R., Anker, C., van Schepen, A., de Haan, P. & Goldbach, R. (1996) Engineered RNA-mediated resistance to Tomato spotted wilt virus is sequence specific. Molecular Plant-Microbe Interactions, 9, 416–418.

Qian, X., Xiang, Q., Yang, T., Ma, H., Ding, X. & Tao, X. (2018) Molecular co-chaperone SGT1 is critical for cell-to-cell movement and systemic infection of tomato spotted wilt virus in Nicotiana benthamiana. Viruses, 10, 647.

Rajamäki, M.-L., Xi, D., Sikorskaite-Gudziuniene, S., Valkonen, J.P.T. & Richard, M.M.S., Knip, M., Aalders, T., Beijaert, M.S. & Takken, F.L.W. (2006) Translation initiation factors: a weak link in plant RNA virus infection. Trends in Plant Science, 11, 40–45.

Robertson, D. (2004) VIGS vectors for gene silencing: many targets, many tools. Annual Review of Plant Biology, 55, 495–519.

de Ronde, D., Lohuis, D. & Kormelink, R. (2019) Identification and characterization of a new class of Tomato spotted wilt virus isolates that break Tsw-based resistance in a temperature-dependent manner. Plant Pathology, 68, 60–71.

Velázquez, A.C., Chakravarty, S. & Martin, G.B. (2009) Virus-induced gene silencing (VIGS) in Nicotiana benthamiana and tomato. Journal of Visualized Experiments, e1292.

Vu, T.V., Das, S., Tran, M.T., Hong, J.C. & Kim, J.-Y. (2020) Precision genome engineering for the breeding of tomatoes: recent progress and future perspectives. Frontiers in Genome Editing, 2, 612137.

Wang, A. & Krishnaswamy, S. (2012) Eu karyotic translation initiation factor 4E-mediated recessive resistance to plant viruses and its utility in crop improvement. Molecular Plant Pathology, 13, 795–803.

Wangdi, T., Uppalapati, S.R., Nagaraj, S., Ryu, C.-M., Bender, C.L. & Mysore, K.S. (2010) A virus-induced gene silencing screen identifies a role for Thylakoid formation in Psedomonas syringae pv. tomato symptom development in tomato and Arabidopsis. Plant Physiology, 152, 281–292.

Williams, A.J., Werner-Fraczek, J., Chang, I.-F. & Bailey-Serres, J. (2003) Regulated phosphorylation of 40S ribosomal protein S6 in root tips of maize. Plant Physiology, 132, 2086–2097.

Wu, C., Jia, L. & Goggin, F. (2011) The reliability of virus-induced gene silencing experiments using Tobacco rattle virus in tomato is influenced by the size of the vector control. Molecular Plant Pathology, 12, 299–305.

Yamaji, Y., Hamada, K., Yoshinuma, T., Sakurai, K., Yoshii, A., Shimizu, T. et al. (2016) Virus-induced gene silencing in Nicotiana benthamiana. Virus Research, 245, 167–172.

Yoshii, M., Shimizu, T., Yamazaki, M., Higashi, T., Miyao, A., Hirochi, H. et al. (2009) Disruption of a novel gene for a NAC-domain protein in rice confers resistance to Rice dwarf virus. The Plant Journal, 57, 615–625.

Zhang, J.J.U., Yu, D., Zhang, Y.I., Liu, K., Xu, K., Zhang, F. et al. (2017) Vacuum and co-cultivation agroinfiltration of (germinated) seeds results in Tobacco rattle virus (TRV) mediated whole-plant
virus-induced gene silencing (VIGS) in wheat and maize. *Frontiers in Plant Science*, 8, 393.

Zhu, F., Deng, X.-G., Xu, F., Jian, W., Peng, X.-J., Zhu, T. et al. (2015) Mitochondrial alternative oxidase is involved in both compatible and incompatible host-virus combinations in *Nicotiana benthamiana*. *Plant Science*, 239, 26–35.

Zhu, M., Jiang, L., Bai, B., Zhao, W., Chen, X., Li, J. et al. (2017) The intracellular immune receptor Sw-5b confers broad-spectrum resistance to tospoviruses through recognition of a conserved 21-amino-acid viral effector epitope. *The Plant Cell*, 29, 2214–2232.

Zhu, X. & Dinesh-Kumar, S.P. (2008) Virus-induced gene silencing as a tool to identify host genes affecting viral pathogenicity. In: Foster, G.D., Johansen, I.E., Hong, Y. & Nagy, P.D. (Eds.) *Plant virology protocols: from viral sequence to protein function*. Totowa, NJ: Humana Press, pp. 641–648.

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

**How to cite this article:** Helderman, T.A., Deurhof, L., Bertran, A., Richard, M.M.S., Kormelink, R., Prins, M., et al (2022) Members of the ribosomal protein S6 (RPS6) family act as pro-viral factor for tomato spotted wilt orthotospovirus infectivity in *Nicotiana benthamiana*. *Molecular Plant Pathology*, 23, 431–446. [https://doi.org/10.1111/mpp.13169](https://doi.org/10.1111/mpp.13169)