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ORIGINAL ARTICLE

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

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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 consist 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 nearly 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).

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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, thereafter 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 (Garcia-Ruiz, 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 tiresome 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 ±90% 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) (Figure 1bI) (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

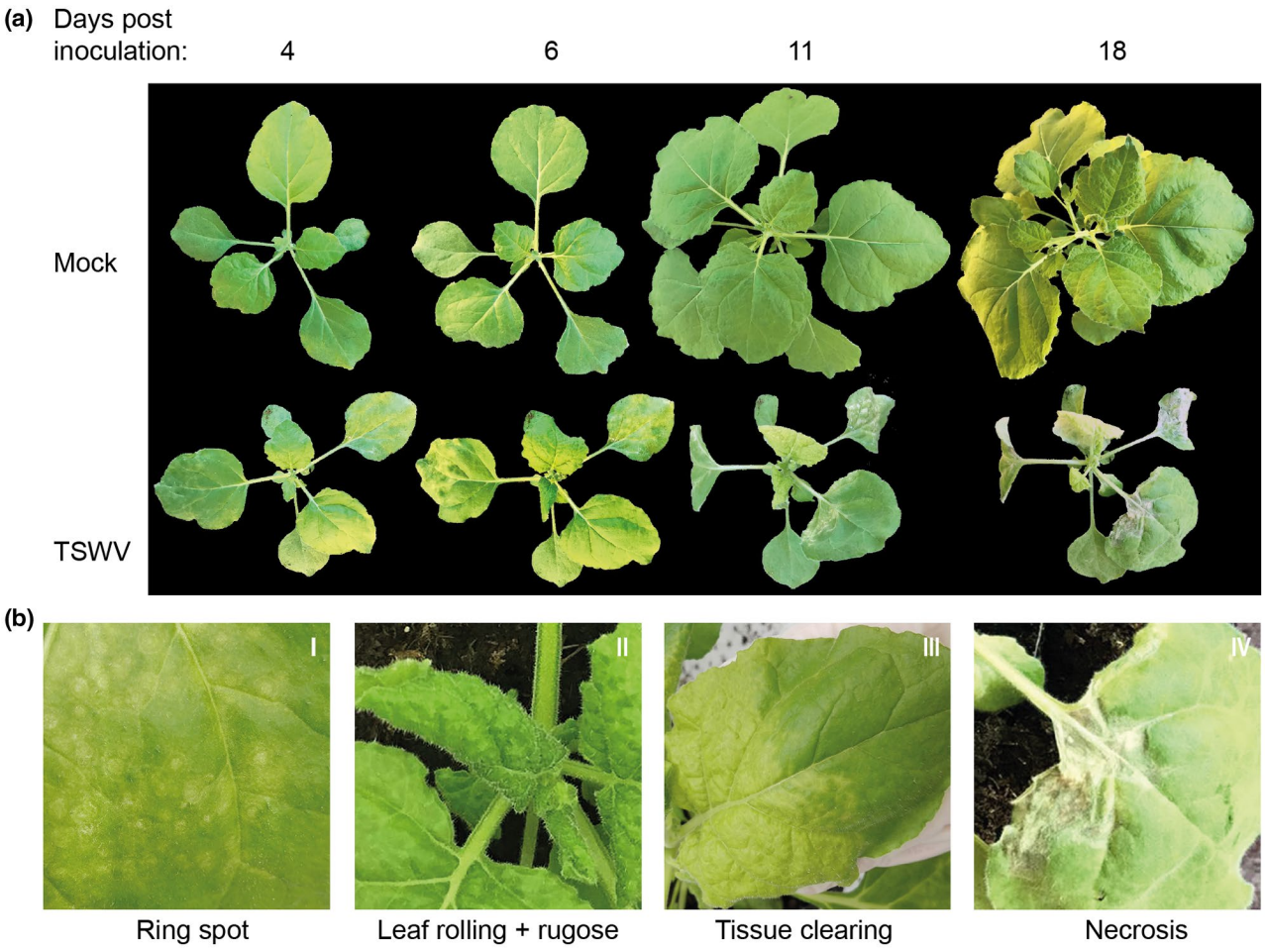

FIGURE 1 Disease symptom development due to tomato spotted wilt orthotospovirus (TSWV) replication and viral spread in *Nicotiana benthamiana*. (a) Time series revealing TSWV symptom development on *N. benthamiana*. (b) Enlargements to reveal typical TSWV symptoms. During early infection, ring spots are visible on the inoculated leaves (I). Subsequently, leaf rolling and leaf rugosity are noticeable in systemically infected leaves (II), followed by tissue clearing (III), and eventually necrotic sectors develop that start near the leaf base and petiole, resulting in full leaf necrosis (IV)

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

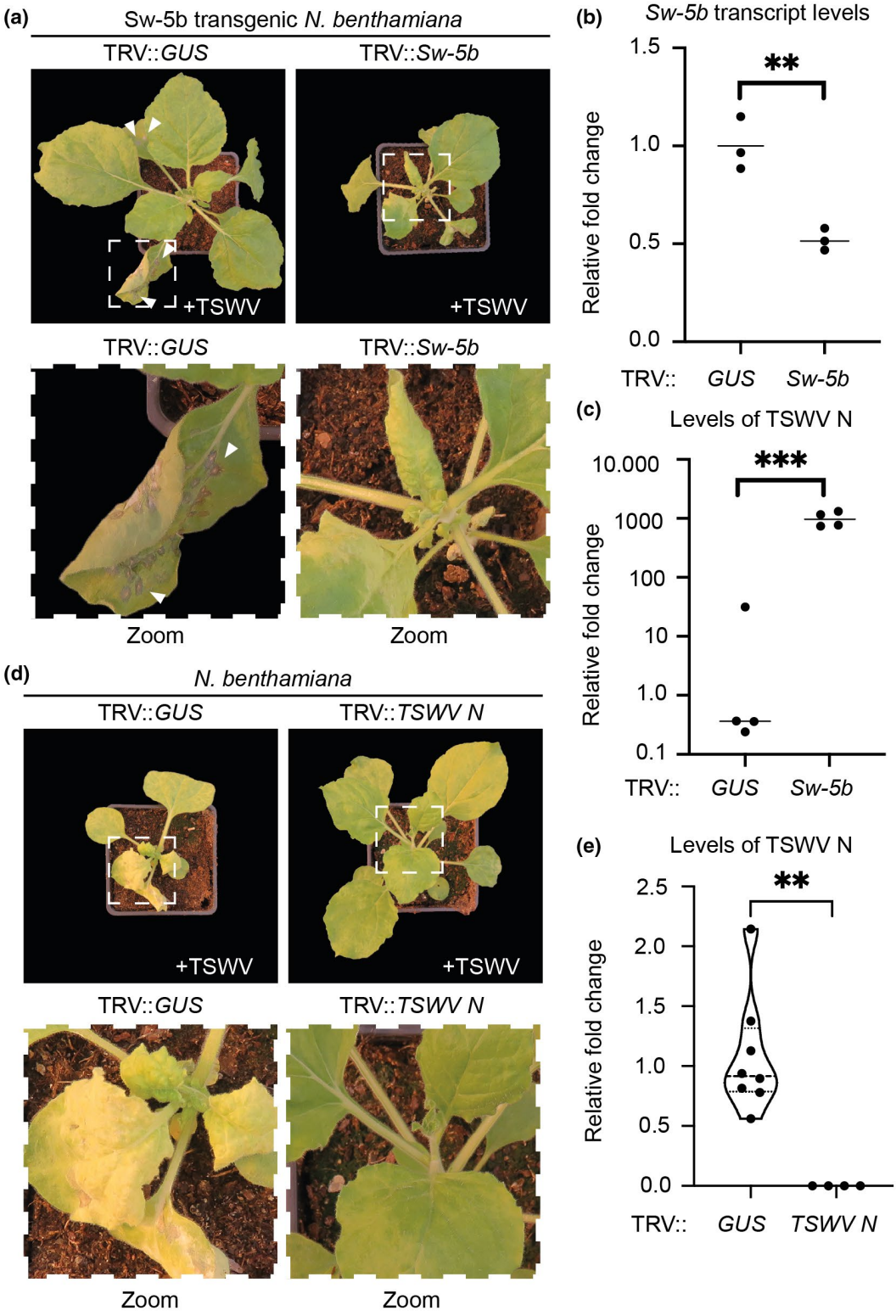

FIGURE 2 Virus-induced gene silencing (VIGS) effectively compromises both *Sw-5b*-mediated resistance and TSWV susceptibility in *Nicotiana benthamiana*. (a) TSWV disease symptoms visible on 4-week-old *Sw-5b* transgenic plants on silencing with TRV::*Sw-5b* (with TRV::*GUS* as a control). White arrowheads 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

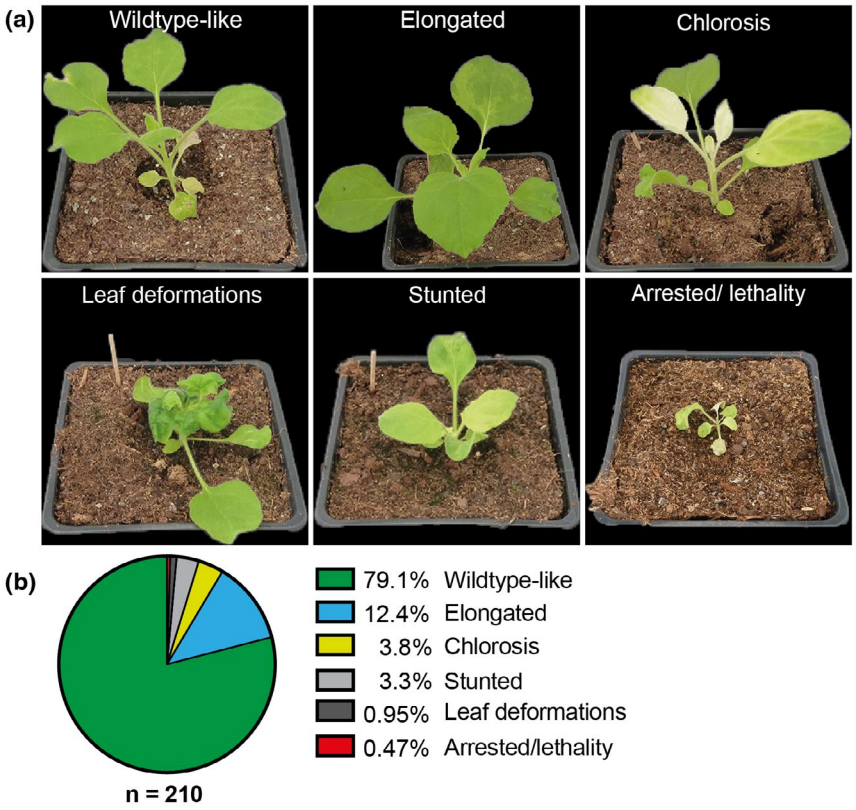

FIGURE 3 Prevalent phenotypes of *Nicotiana benthamiana* on silencing with random cDNA fragments derived from this plant. (a) Plant symptoms were grouped in six well-defined categories: wild-type-like (similar to negative control TRV::*GUS*), elongated, chlorosis, stunted, leaf deformations, and arrested development and/or lethality. (b) Relative group sizes (%) of the categories based on an initial virus-induced gene silencing screen with 210 random clones

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). Agroinoculation 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 S6a and

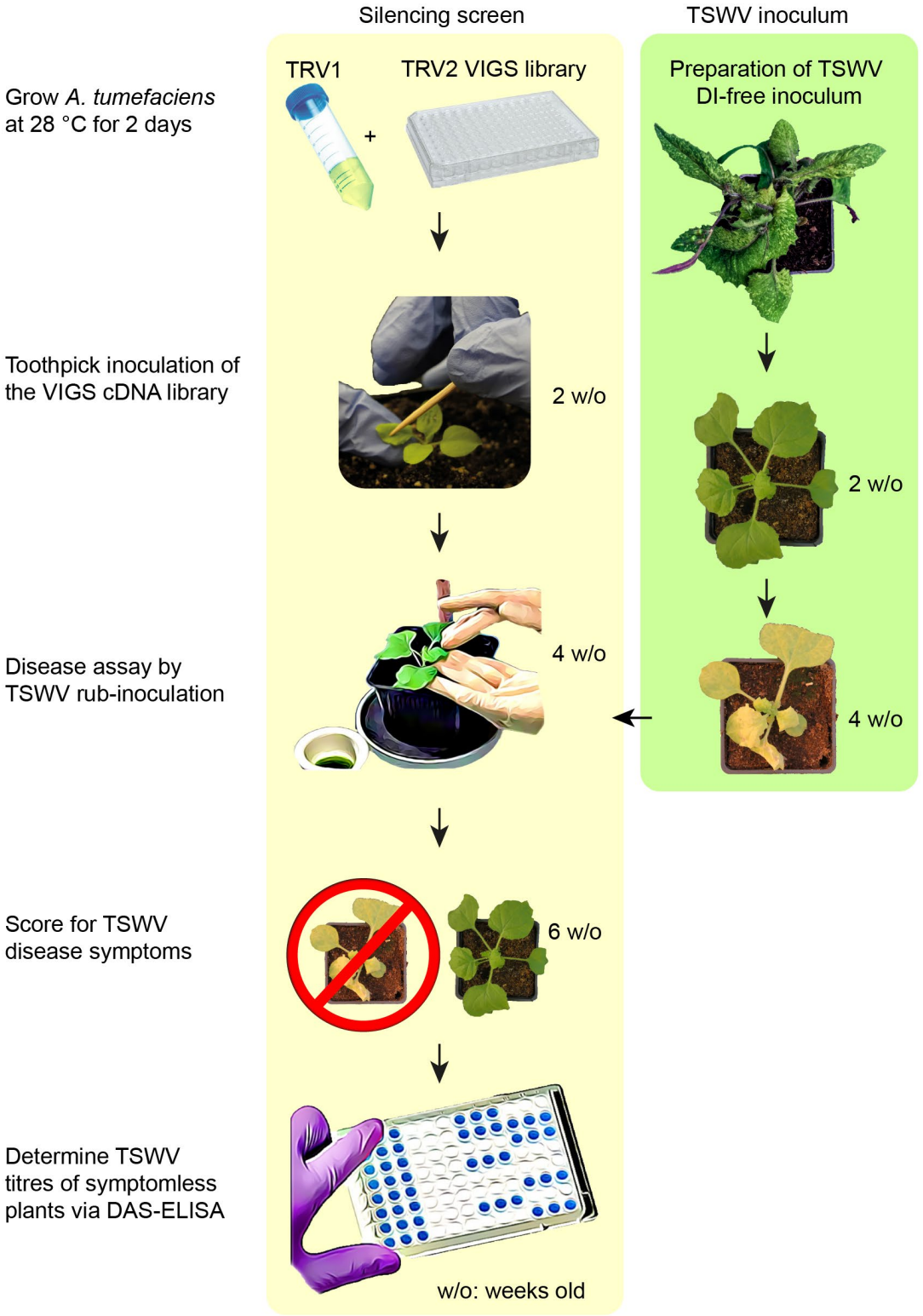

FIGURE 4 Workflow to identify pro-viral host factors for TSWV using virus-induced gene silencing (VIGS). Fresh *Agrobacterium* cultures, each carrying a random TRV2 clone, were grown in a 96-well microtitre plate format. TRV1 and TRV2 cultures were mixed 1:1 (final OD_{600} of 0.8 per culture) and the 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

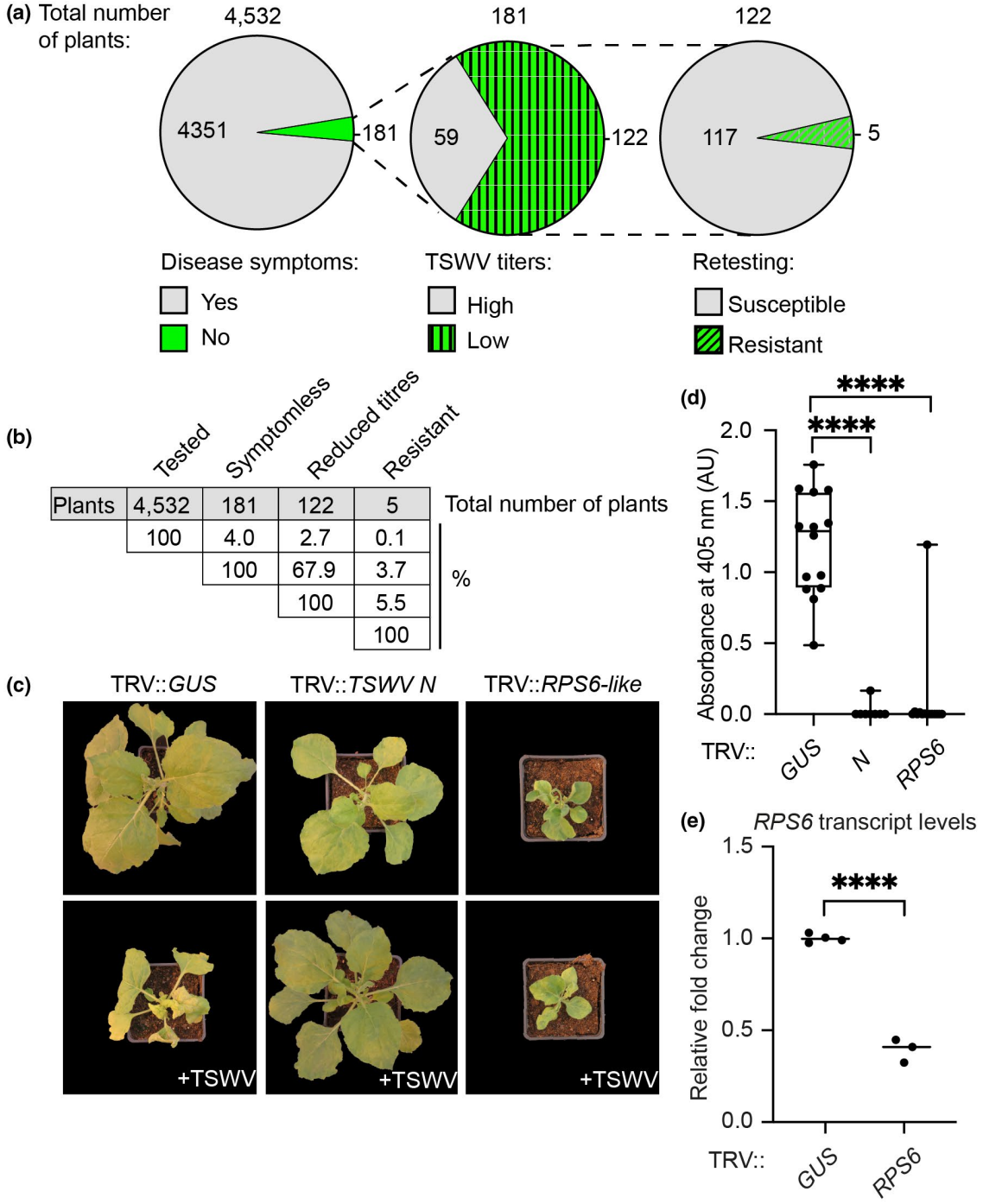

FIGURE 5 A random screen to identify pro-viral host factors for TSWV using virus-induced gene silencing (VIGS). (a) Pie charts displaying from left to right: number of random cDNA fragments screened in Round 1 (including the clones resulting in no disease symptoms), number of TSWV-free plants amongst the symptomless plants based on double-antibody sandwich (DAS) ELISA, and the number of confirmed TRV clones resulting in resistant plants in an independent disease assay ($n = 7$ per construct in one experimental repetition). (b) Table displaying the efficacy of the screen in each round, expressed in absolute numbers or in percentages of the clones retained. (c) Loss of TSWV disease symptoms due to TRV::*RPS6*. Shown *Nicotiana benthamiana* plants were 6-week-old plants (controls: TRV::*GUS* and TRV::*TSWV N*). (d) Quantification of the TSWV viral titres in the plants shown in (c) using a DAS-ELISA that detects the viral N protein in systemically infected leaves. Analysis of variance followed by a Dunnett multiple comparison test (****$p < 0.001$). Similar findings were observed in four independent biological repeats. (e) Transcript levels of *RPS6* in the plants inoculated with the indicated TRV clones ($n = 4$). Unpaired Student's *t* test (****$p < 0.001$)

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 *Nbv6.1trP1324* 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 knock-down 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* (*Nbv6.1trP72816*) or *RPS6b* (*Nbv6.1trP17350*) 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) signalling 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 (Qian 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

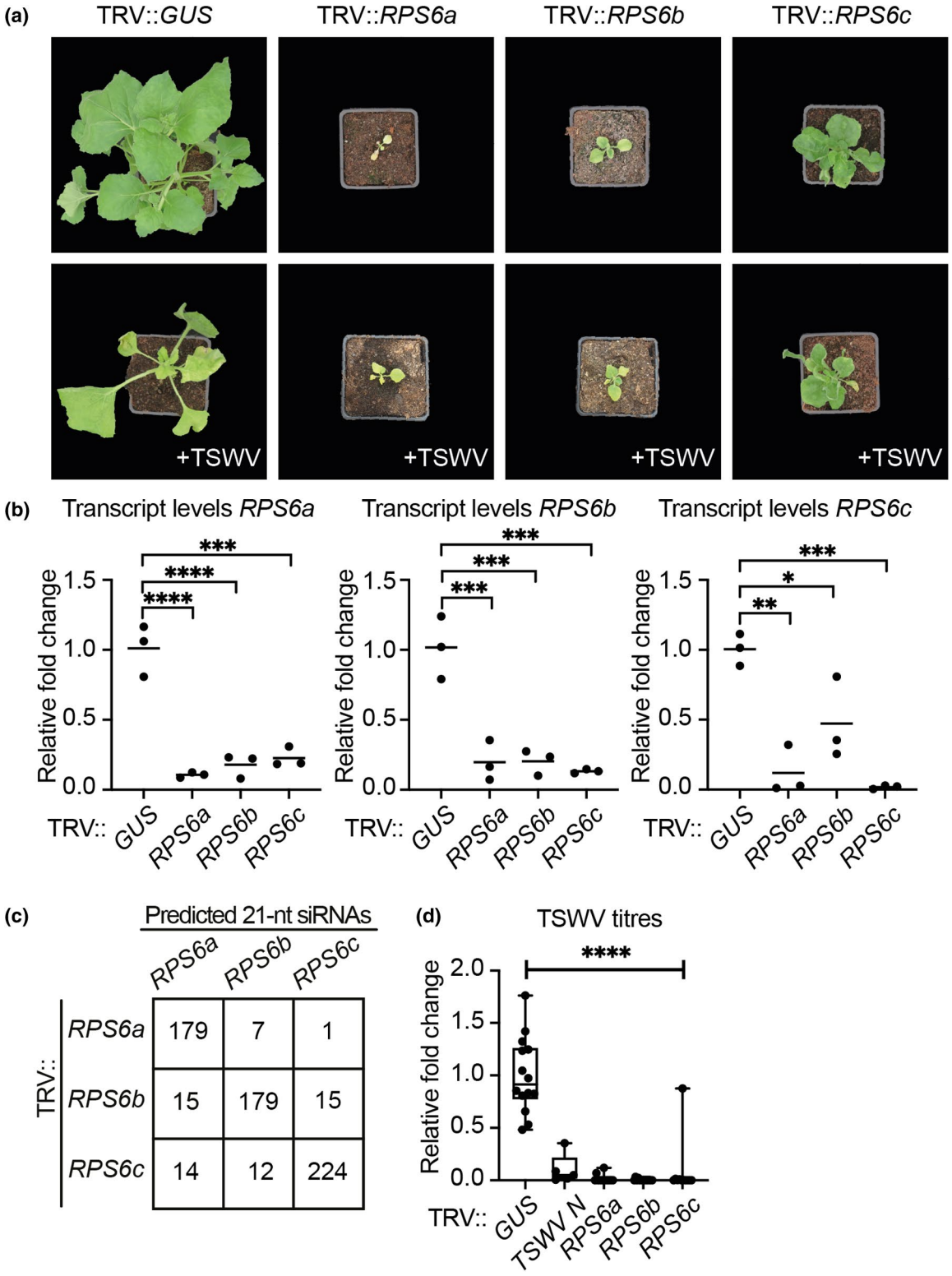

FIGURE 6 Gene silencing with TRV::*RPS6a*, TRV::*RPS6b* or TRV::*RPS6c* suppresses TSWV disease development. (a) Developmental phenotype and TSWV disease symptoms of 6-week-old *Nicotiana benthamiana* plants inoculated with TRV::*RPS6a*, TRV::*RPS6b*, and TRV::*RPS6c* (TRV::*GUS*, control). Representative 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 *Bsa*I 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 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C and 8 h of darkness at 20°C, with a relative humidity of 65%. *E. sonchifolia* 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) *NRC1*^{D481V} 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 $MgCl_2$, 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 OD_{600} of 0.5. *Agrobacterium* expressing *GUS* or *NRC1*^{D481V} were separately infiltrated in 3-to 4-week-old wild-type *N. benthamiana* plants. NRC1^{D481V} 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 defence response by a cell surface-localized immune receptor, whereas detection of PVX CP by Rx1 triggers a defence 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 Lurai-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 *Alu*I, 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 OD_{600} 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 × *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_2SO_3) was rub-inoculated with about five thumb strokes on *E. sonchifolia* leaves, predusted 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 rub-inoculated onto the youngest two fully expended 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 *Sma*I 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).

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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.

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