Determinition of key residues in tospoviral NSm required for Sw-5b recognition, their potential ability to overcome resistance, and the effective resistance provided by improved Sw-5b mutants

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ORIGINAL ARTICLE

Abstract

Sw-5b is an effective resistance gene used widely in tomato to control tomato spotted wilt virus (TSWV), which causes severe losses in crops worldwide. Sw-5b confers resistance by recognizing a 21-amino-acid peptide region of the viral movement protein NSm (NSm21, amino acids 115–135). However, C118Y or T120N mutation within this peptide region of NSm has given rise to field resistance-breaking (RB) TSWV isolates. To investigate the potential ability of TSWV to break Sw-5b-mediated resistance, we mutated each amino acid on NSm21 and determined which amino acid mutations would evade Sw-5b recognition. Among all alanine-scan mutants, NSmP119A, NSmW121A, NSmD122A, NSmR124A, and NSmQ126A failed to induce a hypersensitive response (HR) when coexpressed with Sw-5b in Nicotiana benthamiana leaves. TSWV with the NSmP119A, NSmW121A, or NSmQ126A mutation was defective in viral cell-to-cell movement and systemic infection, while TSWV carrying the NSmD122A or NSmR124A mutation was not only able to infect wild-type N. benthamiana plants systemically but also able to break Sw-5b-mediated resistance and establish systemic infection on Sw-5b-transgenic N. benthamiana plants. Two improved mutants, Sw-5bL33P/K319E/R927A and Sw-5bL33P/K319E/R927Q, which we recently engineered and which provide effective resistance against field RB isolates carrying NSmC118Y or NSmT120N mutations, recognized all NSm21 alanine-substitution mutants and conferred effective resistance against new experimental RB TSWV with the NSmD122A or NSmR124A mutation. Collectively, we determined the key residues of NSm for Sw-5b recognition, investigated their potential RB ability, and demonstrated that the improved Sw-5b mutants could provide effective resistance to both field and potential RB TSWV isolates.

KEYWORDS
alanine scan mutagenesis, resistance breaking, resistance gene, Sw-5b, tomato spotted wilt virus
Tomato spotted wilt orthotospovirus (TSWV) is one of the most harmful plant viral pathogens. It infects more than 1000 plant species, including those of tomatoes (*Solanum lycopersicum*), peppers (*Capsicum annuum*), and peanuts (*Arachis hypogaea*), causing annual economic losses exceeding $1 billion worldwide (Scholthof et al., 2011). Tomato spotted wilt orthotospovirus is the type member of the genus *Orthotospovirus* within the family *Tospoviridae* of the order *Bunyavirales*. It has three genomic RNAs, denoted the large (L), medium (M), and small (S) segments (Kormelink et al., 2021; Zhu et al., 2019). The L RNA encodes an RNA-dependent RNA polymerase (RdRp) (Dehaan et al., 1991). The M and S RNA are ambisense segments. The sense strand of the M RNA segment encodes the nonstructural protein NSm, which plays important roles in cell-to-cell and long-distance movement (Feng et al., 2016; Kormelink et al., 1994; Li et al., 2009), and the antisense strand of the M RNA segment encodes precursors of glycoproteins that are further processed into two mature glycoproteins, Gn and Gc (Kikkert et al., 2001; Ribeiro et al., 2008). The sense strand of the S RNA encodes the nonstructural protein NSs, which functions as an RNA silencing suppressor (Bucher et al., 2003; Schnettler et al., 2010; Takeda et al., 2002), and the antisense strand of the S segment encodes the nucleocapsid (N) protein (Richmond et al., 1998; Uhrig et al., 1999). Sw-5b is the most effective resistance gene used widely in tomato to control a broad spectrum of TSWV isolates (Brommonschenkel et al., 2000; Spassova et al., 2001; Turina et al., 2016). The Nsm protein encoded by TSWV is the avirulence (AVR) determinant in Sw-5b-mediated resistance (Hallwass et al., 2014; Peiro et al., 2014). In a previous study, we found that a 21-amino-acid (NSm21, amino acids 115–135) peptide region of Nsm is sufficient for inducing a hypersensitive response (HR) when coexpressed with Sw-5b in *N. benthamiana* and tomato leaves (Zhu et al., 2017). However, the identities of the residues in the peptide region of NSm21 that are critical for Sw-5b recognition and HR induction remain unclear.

The continuous, wide application of tomato cultivars carrying Sw-5b has resulted in resistance-breaking (RB) TSWV isolates in Spain, Italy, the USA, and other locations around the world (Aramburu & Marti, 2003; Batuman et al., 2017; Ciuffo et al., 2005; Macedo et al., 2019; Oliver & Whitfield, 2016; Turina et al., 2016). In the 2016 epidemic in California, the disease incidence of Sw-5b tomato cultivars by RB TSWV isolates reached 50%–80% (Batuman et al., 2017). RB TSWV isolates contain an amino acid mutation at position C118 to Y (C118Y) or T120 to N (T120N) in the viral movement protein NSm (Lopez et al., 2011). The C118Y and T120N mutations are located within the 115 to 135 amino acid region of Nsm (NSm21) that is critical for Sw-5b recognition; mutation within this region has resulted in a potential new RB isolates of TSWV Nsm21 and investigated the potential ability of TSWV to break Sw-5b-mediated resistance. We found that P119A, W121A, D122A, R124A, and Q126A mutants of TSWV Nsm failed to induce HR when coexpressed with Sw-5b in *N. benthamiana* leaves, whereas other mutations in Nsm were still able to trigger HR. TSWV carrying the NsmD122A or NsmR124A mutation was able to break Sw-5b-mediated resistance and establish systemic infection on Sw-5b-transgenic *N. benthamiana* plants. The two improved versions of the resistance gene, Sw-5bL33P/K319E/R927Q and Sw-5bL33P/K319E/R927A mutants, which provide effective resistance to RB TSWV isolates carrying the NSmC118Y or NSmT120N mutation (Huang et al., 2021), were engineered by a two-step artificial evolution of Sw-5b based on the two-step recognition mechanism. We obtained Sw-5bL33P/K319E/R927A and Sw-5bL33P/K319E/R927Q mutants, which provide effective resistance to RB TSWV isolates carrying the NSmC118Y or NSmT120N mutation (Huang et al., 2021).

In this study, we determined the key residues of NSm required for Sw-5b recognition by alanine scan mutagenesis on the peptide region of NSm21 and investigated the potential ability of TSWV to break Sw-5b-mediated resistance. We found that P119A, W121A, D122A, R124A, and Q126A mutants of TSWV Nsm failed to induce HR when coexpressed with Sw-5b in *N. benthamiana* leaves, whereas other mutations in Nsm were still able to trigger HR. TSWV carrying the NSmD122A or NSmR124A mutation was able to break Sw-5b-mediated resistance and establish systemic infection on Sw-5b-transgenic *N. benthamiana* plants. The two improved versions of the resistance gene, Sw-5bL33P/K319E/R927Q and Sw-5bL33P/K319E/R927A mutants, which provide effective resistance to RB TSWV isolates carrying the NSmC118Y or NSmT120N mutation (Huang et al., 2021), were engineered by a two-step artificial evolution strategy, recognize all alanine substitution Nsm mutants and confer effective resistance against new experimental RB isolates of TSWV with the NSmD122A or NSmR124A mutation. Hence, the artificially improved Sw-5b resistance genes could provide effective resistance to both field RB TSWV isolates and also potential RB isolates.

2 | RESULTS

2.1 | Alanine scan determination of residues required for triggering Sw-5b-mediated HR in 21-amino-acid peptide region of TSWV NSm

Sw-5b is a coiled-coil (CC) nucleotide-binding domain and leucine-rich repeat receptor (NLR) immune receptor (Brommonschenkel et al., 2000; Spassova et al., 2001). In addition to the typical CC-NLR domains, Sw-5b also contains an additional Solanaceae domain (SD) at the N-terminus (van Ooijen et al., 2007). Sw-5b detects the viral movement protein NSm using both the N-terminal SD and the C-terminal LRR domain. The Sw-5b NB-LRR region specifically recognizes NSm and switches from an inactive state to an active state upon NSm recognition. The CC domain suppresses the activation of NB-LRR, and the extra SD domain can also specifically recognize NSm and relieve the inhibitory effects of CC to activate the resistance protein fully. Hence, Sw-5b adopts a two-step recognition mechanism involving both the SD sensor and the NB-ARC-LRR activator (Li et al., 2019; Zhu et al., 2019). To engineer Sw-5b and develop an immune receptor capable of conferring effective resistance against RB TSWV isolates, we recently conducted a stepwise artificial evolution of Sw-5b based on the two-step recognition mechanism. We obtained Sw-5bL33P/K319E/R927A and Sw-5bL33P/K319E/R927Q mutants, which provide effective resistance to RB TSWV isolates carrying the NSmC118Y or NSmT120N mutation (Huang et al., 2021).

In this study, we determined the key residues of NSm required for Sw-5b recognition by alanine scan mutagenesis on the peptide region of NSm21 and investigated the potential ability of TSWV to break Sw-5b-mediated resistance. We found that P119A, W121A, D122A, R124A, and Q126A mutants of TSWV Nsm failed to induce HR when coexpressed with Sw-5b in *N. benthamiana* leaves, whereas other mutations in Nsm were still able to trigger HR. TSWV carrying the NSmD122A or NSmR124A mutation was able to break Sw-5b-mediated resistance and establish systemic infection on Sw-5b-transgenic *N. benthamiana* plants. The two improved versions of the resistance gene, Sw-5bL33P/K319E/R927Q and Sw-5bL33P/K319E/R927A mutants, which provide effective resistance to RB TSWV isolates carrying the NSmC118Y or NSmT120N mutation (Huang et al., 2021), were engineered by a two-step artificial evolution strategy, recognize all alanine substitution Nsm mutants and confer effective resistance against new experimental RB isolates of TSWV with the NSmD122A or NSmR124A mutation. Hence, the artificially improved Sw-5b resistance genes could provide effective resistance to both field RB TSWV isolates and also potential RB isolates.

2 | RESULTS

2.1 | Alanine scan determination of residues required for triggering Sw-5b-mediated HR in 21-amino-acid peptide region of TSWV NSm

A 21-amino-acid (aa) peptide region in NSm is sufficient for Sw-5b to trigger HR (Zhu et al., 2017). To determine which residues in the NSm 21-aa peptide are critical for triggering HR, we performed alanine scan mutagenesis within the 115–135 aa residues of TSWV NSm. Each amino acid in the NSm21 region was substituted with alanine, except that C118 and T120 were mutated to Y and N as field RB TSWV isolates (Lopez et al., 2011). Each NSm mutant was
coexpressed with yellow fluorescent protein (YFP)-tagged Sw-5b in N. benthamiana leaves. As shown in Figure 1, NSmP119A, NSmW121A, NSmD122A, NSmR124A, and NSmQ126A mutants, as well as NSmC118Y and NSmT120N from field RB isolates, lost the ability to trigger HR by Sw-5b. All other NSm alanine substitution mutants still induced HR when coexpressed with Sw-5b, although the HR index varied among the different mutants, suggesting that they still could be recognized by Sw-5b. Western blotting results further confirmed that protein levels of all NSm mutants had accumulated to similar levels in these leaves. These alanine scan mutagenesis results suggest that P119, W121, D122, R124, and Q126 amino acid residues within the NSm21 peptide region are important for Sw-5b recognition and that NSm carrying an alanine mutation on these sites is unable to induce HR.

### 2.2  Cell-to-cell movement of TSWV eGFP replicon carrying the P119A, W121A, D122A, R124A, or Q126A mutation in NSm and its ability to overcome Sw-5b-mediated resistance

NSm21 is a conserved amino acid region among movement proteins of American-type tosposviruses (Zhu et al., 2017). The residues in this conserved region of NSm recognized by the Sw-5b immune receptor might be critical for viral cell-to-cell movement and systemic infection. To examine whether the five aforementioned noneliciting mutations in NSm have functional effects on viral intercellular movement and systemic infection, we used a recently developed eGFP replicon-based reverse genetics system. To examine whether the five aforementioned noneliciting mutants in NSm TSWV eGFP replicons carrying the five aforementioned noneliciting mutants were still induced HR when coexpressed with Sw-5b, although the HR index varied among the different mutants, suggesting that they still could be recognized by Sw-5b. Western blotting results further confirmed that protein levels of all NSm mutants had accumulated to similar levels in these leaves. These alanine scan mutagenesis results suggest that P119, W121, D122, R124, and Q126 amino acid residues within the NSm21 peptide region are important for Sw-5b recognition and that NSm carrying an alanine mutation on these sites is unable to induce HR.

TSWV D122A replicon was still able to move from cell to cell, and the clustered eGFP fluorescence signal was detected in agro-infiltrated N. benthamiana leaves in the presence of Sw-5b at 3 dpi, as well as the RNA TSVW replicon carrying NSm C118Y or NSm T120N. eGFP fluorescence of the TSWV eGFP replicon carrying the NSm P119A, NSm W121A, NSm R124A, or NSm Q126A mutation was detected in a single cell in agro-infiltrated leaves in the presence of Sw-5b (Figure 2b). eGFP accumulation of those replicon mutants was further confirmed by immunoblotting (Figure 2c). The immunoblot results also showed that all of the mutations in NSm had no significant effect on the protein stability of NSm (Figure S1).

### 2.3  TSWV carrying the NSm D122A or NSm R124A mutation established systemic infection in both wild-type and Sw-5b-transgenic N. benthamiana plants

Next, the ability of TSWV carrying the NSm P119A, NSm W121A, NSm D122A, NSm R124A, or NSm Q126A noneliciting mutation to establish systemic infection in WT and Sw-5b-transgenic N. benthamiana plants was investigated using full-length infectious clones of TSWV (L (+)opt + M (+)opt + S (+); Feng et al., 2020). M (+)opt C118Y and M (+)opt T120N carrying mutations from field RB isolates were also used as controls (Figure 3a). Agrobacterium carrying these WT and mutant infectious clones was agro-infiltrated into leaves of 6-week-old WT or Sw-5b-transgenic N. benthamiana, respectively, together with three VSRs, P19, HcPro, and yb. In WT N. benthamiana plants, disease symptoms of rescued (r) WT TSWV were first observed in systemic leaves at 8 dpi. Disease symptoms of rTSWV C118Y, rTSWV T120N, and rTSWVD122A were first observed at 10, 13, and 12 dpi, respectively. At 13-14 dpi, all plants were infected by rTSWV, rTSWVC118Y, and rTSWVD122A; however, rTSWVT120N required 20 days to establish full infection in all treated plants. No disease symptoms were observed for rTSWVP119A, rTSWV W121A, TSWV R124A, and rTSWVQ126A in newly emerged leaves of N. benthamiana plants up to 30 dpi (Figure 3c). However, viral N protein from rTSWVD122A accumulated in systemic leaves of WT N. benthamiana plants (Figure 3d), suggesting that rTSWVP119A, rTSWV W121A, and rTSWVQ126A could not establish systemic infection in WT N. benthamiana plants but that TSWV R124A could.

In Sw-5b-transgenic N. benthamiana plants, viral symptoms in systemic leaves infected by rTSWVC118Y and rTSWVD122A were first observed at 9 dpi. At 16 dpi, 100% of plants were infected. Disease symptoms in systemic leaves infected by rTSWVT120N were first observed at 13 dpi. rTSWVT120N also required about 20 days to infect all Sw-5b-transgenic plants (Figure 3c). rTSWVC118Y, rTSWVT120N, and rTSWVD122A all induced typical symptoms, including leaf curling and stunting, in Sw-5b-transgenic N. benthamiana plants. However, rTSWVP119A, rTSWV W121A, and rTSWVR124A did not infect systemically in Sw-5b-transgenic N. benthamiana plants (Figure 3c). Although rTSWVR124A did not cause any disease symptoms, it still accumulated in systemic leaves of Sw-5b-transgenic N. benthamiana plants (Figure 3d). These data suggest that TSWVD122A and
FIGURE 1 Determination of the key residues required for the hypersensitive response (HR) in the NSm<sup>21</sup> peptide. (a) Schematic representation of NSm and the amino acid (aa) sequence of its 21-aa peptide region, which is sufficient to trigger Sw-5b-mediated HR. (b) Each residue in the NSm 115 to 135 aa region was substituted with alanine, except for C118 and T120, and each mutant was coexpressed with YFP-Sw-5b in Nicotiana benthamiana leaves. The infiltrated area for each treatment is marked with a dotted circle. The infiltrated leaves were photographed at 6 days postinfiltration (dpi), and three independent experiments were performed. The HR index of each sample is shown in (c). The intensity of HR was evaluated according to a scale ranging from 0 (no cell death) to 5 (strong cell death) at 6 dpi. Bars represent the average of 12 replicas for each combination of constructs; error bars represent standard deviation. (d) Western blotting analysis of YFP-tagged Sw-5b coexpressed with NSm<sup>WT</sup> and NSm alanine substitution mutants in N. benthamiana leaves using YFP-specific and NSm-specific antibodies. The RuBisCO large subunit was stained with Ponceau S to indicate sample loading. Protein size is indicated at the left.
TSWV<sub>R124A</sub> were able to establish systemic infection in WT <i>N. benthamiana</i> plants and could overcome Sw-5b-mediated resistance.

### 2.4 | Artificially improved Sw-5b<sub>L33P/K319E/R927A</sub> and Sw-5b<sub>L33P/K319E/R927Q</sub> could recognize all NSm21 alanine substitution mutants, leading to strong HR

We recently obtained two artificially improved versions of Sw-5b, namely, Sw-5b<sub>L33P/K319E/R927A</sub> and Sw-5b<sub>L33P/K319E/R927Q</sub> (Huang et al., 2021), which are effective against RB TSWV isolates carrying the NSm<sub>C118Y</sub> or NSm<sub>T120N</sub> mutation, as well as other American-type tospoviruses. We examined whether these two improved Sw-5b mutants were also effective at recognizing the above NSm21 alanine substitution mutants. To this end, HR analysis was performed by coexpression of NSm21 alanine substitution mutants and artificially improved Sw-5b mutants in <i>N. benthamiana</i> leaves. Sw-5b<sub>L33P/K319E/R927A</sub> and Sw-5b<sub>L33P/K319E/R927Q</sub> recognized not only NSm<sub>D122A</sub> and NSm<sub>R124A</sub> but also all other NSm mutants and induced strong HR (Figure 4a,b). These data suggest that artificially improved Sw-5b mutants are able to recognize NSm<sub>D122A</sub> and NSm<sub>R124A</sub> noneliciting mutants and trigger a strong defense response.

### 2.5 | Sw-5b<sub>L33P/K319E/R927A</sub> and Sw-5b<sub>L33P/K319E/R927Q</sub> confer effective resistance to RB TSWV carrying the NSm<sub>D122A</sub> or NSm<sub>R124A</sub> mutation

To determine whether Sw-5b<sub>L33P/K319E/R927A</sub> and Sw-5b<sub>L33P/K319E/R927Q</sub> confer resistance against RB TSWV carrying the NSm<sub>D122A</sub> or NSm<sub>R124A</sub> mutation, transgenic <i>N. benthamiana</i> plants carrying empty vector (EV), Sw-5b, Sw-5b<sub>L33P/K319E/R927A</sub>, or Sw-5b<sub>L33P/K319E/R927Q</sub> were inoculated with the infectious clones of TSWV carrying the NSm<sub>WT</sub>, NSm<sub>D122A</sub>, or NSm<sub>R124A</sub> mutation via agro-infiltration. rTSWV<sub>D122A</sub> and rTSWV<sub>R124A</sub> systemically infected EV- and Sw-5b-transgenic plants; however, no viral accumulation was detected for rTSWV<sub>D122A</sub> and rTSWV<sub>R124A</sub> in systemic leaves of the Sw-5b<sub>L33P/K319E/R927A</sub> and Sw-5b<sub>L33P/K319E/R927Q</sub>-transgenic <i>N. benthamiana</i> plants (Figure 5a,b). These data suggest that the artificial, improved Sw-5b<sub>L33P/K319E/R927A</sub> or Sw-5b<sub>L33P/K319E/R927Q</sub> provided effective resistance to potential RB TSWV carrying the NSm<sub>D122A</sub> or NSm<sub>R124A</sub> mutation.

### 3 | DISCUSSION

In previous work, we found that Sw-5b NLR recognizes a conserved 21-aa peptide region of viral movement protein NSm, providing an example of plant NLR immune receptor-mediated resistance through recognition of a small conserved pathogen-associated molecular pattern-like peptide (Zhu et al., 2017). In the present study, we further determined the key residues in the NSm21 peptide for the recognition by Sw-5b NLR, investigated their potential RB abilities, and demonstrated that the artificially improved Sw-5b<sub>L33P/K319E/R927A</sub> and Sw-5b<sub>L33P/K319E/R927Q</sub> mutants provided effective resistance to both field and potential RB TSWV isolates.

We found that the key residues required for Sw-5b recognition are distributed mainly in the first half of the peptide region. We proposed previously that four polymorphic sites on the Sw-5b LRR domain recognize NSm21 (Zhu et al., 2017). The binding of NSm21 on the LRR domain disrupts the intramolecular interaction between NB and LRR through interference with the R927 residue, thus switch-activating the Sw-5b immune receptor. Based on the new findings here, we further propose that the first half of the NSm21 peptide is responsible for binding onto four polymorphic sites of the Sw-5b LRR domain. These amino acid residues on the NSm21 peptide may also be responsible for disrupting the R927 residue, which plays a critical role in maintaining the autoinhibited state of Sw-5b (Zhu et al., 2017) and in activation of the Sw-5b receptor. Among all of the NSm alanine substitution mutants, the five mutants NSm<sub>P119A</sub>, NSm<sub>W112A</sub>, NSm<sub>D122A</sub>, NSm<sub>R124A</sub>, and NSm<sub>D126A</sub> failed to induce HR when coexpressed with Sw-5b (Figure 1b,c). In addition, four of them (NSm<sub>P119A</sub>, NSm<sub>W112A</sub>, NSm<sub>R124A</sub>, and NSm<sub>D126A</sub>) entirely lost the ability to promote cell-to-cell movement of a viral minireplicon in a transient expression system (Figure 2b). These results suggest that the residues that are important for Sw-5b recognition are also important for virus cell-to-cell movement. Sw-5b was derived from <i>Solanum peruvianum</i> originating in South America and has evolved to confer broad-spectrum resistance against all American-type tospoviruses (Turina et al., 2016; Zhu et al., 2017). We found that most of the amino acid residues studied within the NSm21 peptide are important for viral cell-to-cell movement. This suggests that Sw-5b is a "smart" resistance gene that has evolved to recognize a very important motif in NSm that is critical for virus infection. Most of the amino acid mutations that evolved on NSm21 to evade recognition by Sw-5b are not viable for virus infection. However, a few amino acid mutations in NSm, for example, C118Y, T120N, and D122A, were able to evade recognition by Sw-5b, and viruses containing these mutations could still survive in the plant.

The potential ability of TSWV with NSm21 mutations to overcome the Sw-5b-mediated resistance was investigated further. Rescued TSWV carrying NSm<sub>D122A</sub> or NSm<sub>R124A</sub> could establish systemic infection in WT and Sw-5b-transgenic <i>N. benthamiana</i> plants (Figure 3b–d), indicating that TSWV carrying either of these two NSm mutations has the potential risk to become a new RB isolate in the field. Intriguingly, although NSm<sub>R124A</sub> failed to facilitate the trafficking of a TSWV minireplicon from the primary expression cell to a neighbouring cell (Figure 2b), the full-length infectious clones of TSWV carrying the NSm<sub>R124A</sub> mutation were still able to establish systemic infection in WT and Sw-5b-transgenic <i>N. benthamiana</i> plants (Figure 3a–d). Because the TSWV S minirePLICONS are generated by replacing the NSs gene with the eGFP on the S genomic RNA segment of the full-length infectious clone, the only difference between the <i>S</i><sub>RGFP</sub> replicon and the full-length infectious clone of the S segment is the NSs protein. Therefore, the NSs protein may have helped the NSm<sub>R124A</sub> mutant to establish cell-to-cell and long-distance movement in the plant, although...
NSmR124A itself is defective in viral movement. NSs is a VSR protein. Consistent with this fact, the VSRs from other plant viruses, such as HcPro and 2b, have been previously reported to play a role in viral movement (Cronin et al., 1995; Kasschau & Carrington, 2001; Kasschau et al., 1997; Lewsey et al., 2009; Soards et al., 2002).

Pathogens evade the recognition of a plant resistance gene through different mechanisms, including point mutations, recombination, and even deletion of AVR factors. For plant viruses, the AVR factors often serve as a multifunctional protein and play key roles in pathogenicity. This suggests that mutation of the viral AVR protein to evade host resistance may also result...
in a fitness cost. Mutation at the helicase domain of the RdRp can overcome Tm1-mediated resistance, but it affects the viral replication rate in susceptible tobacco protoplasts (Ichishibashi et al., 2012). Changes in the pepper mild mottle virus coat protein overcomes L3 resistance in pepper may result in diminished particle stability (Fraile et al., 2011). Irr RB isolates of raspberry ringspot virus in raspberry reduce its transmission efficiency by the nematode vector and seed transmission rates (Murant et al., 1968). In our study, we compared transmission efficiency by the nematode vector and seed transmission rates of WT TSWV (L(+)opt) or NSm-mutated TSWV medium (M) size genomic RNA segment; SR(+)eGFP, infectious clone of TSWV S minireplicon in which the NSs of the TSWV small genomic RNA segment was replaced by eGFP. (-), viral strand of TSWV genomic RNA; (+), viral complementary strand of TSWV genomic RNA; 2 x 35S. CaMV double 35S promoter; HH, hammerhead ribozyme; RZ, hepatitis delta virus (HDV) ribozyme; NOS, nopaline synthase terminator. (b) Infectious minireplicon clones of WT TSWV (L(+)opt + M(+)opt + SR(+eGFP)) or mutants (L(+)opt + M(+)opt + mut + SR(+eGFP), together with three viral suppressors of RNA silencing (VSRs) (P19, Hc-Pro, and yb), were coexpressed with or without Sw-5b in N. benthamiana leaves by agro-infiltration. C118Y or T120N mutation in NSm from field resistance-isolated was used as controls. Images of eGFP fluorescence foci in agro-infiltrated leaves were taken 3 days postinfiltration (dpi) using inverted fluorescence microscopy. Scale bars = 400 μm. (c) Western blotting analysis of eGFP protein accumulation for various recombination treatments shown in panel (b) at 3 dpi using a GFP-specific antibody. The Rubisco large subunit was stained with Ponceau S.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plasmid construction

The sources of the plasmids L(+)opt, M(+)opt, S(+), SR(+eGFP), and VSRs (P19, Hc-Pro, and yb) were previously described (Feng et al., 2020). M(+)opt C118Y, M(+)opt T120N, Sw-5b L33P/K319E/R927A, and Sw-5b L33P/K319E/R927Q were described by Huang et al. (2021). All NSm mutants were cloned in the pCambia2300S binary vector under the control of the CaMV 35S promoter, and alanine substitution was generated by two-step overlap PCR, as described in Chen et al. (2016). Briefly, the NSm mutation site was directly introduced into the primers for the first round of PCR. The purified NSm fragments were then used as template for the second round of overlap PCR. The mutagenized PCR products were cloned into pCambia2300S digested with KpnI and Sall. All constructs were verified by sequencing.

4.2 | Transient expression, virus inoculation, and plant growth

Six- to eight-week-old plants of WT N. benthamiana were used for transient expression analyses. Sw-5b, Sw-5b L33P/K319E/R927A, and Sw-5b L33P/K319E/R927Q transgenic N. benthamiana plants (Chen et al., 2016; Huang et al., 2021) were used for TSWV infectious clone inoculation and resistance testing. DNA constructs were transformed individually into Agrobacterium tumefaciens GV3101 through electroporation. The cells were grown in Luria Bertani broth medium at 28°C until the OD600 reached 0.8–1.0. The Agrobacterium cultures were pelleted by low-speed centrifugation and resuspended in infiltration medium (10 mM MES, pH 5.6, 10 mM MgCl2, 150 mM acetosyringone) to a final concentration of OD600 = 0.3. After 3 h of incubation at 28°C, the cell suspensions were infiltrated into the abaxial side of N. benthamiana leaves using a needleless syringe. Agrobacterium-mediated inoculation of minireplicons or full-length infectious clones of TSWV and derivatives was performed as previously described (Huang et al., 2021). The infiltrated plants were grown inside a greenhouse under white fluorescent light (c.150 μmol·m⁻²·s⁻¹, 16 h light and 8 h dark) at day/night temperatures of 25°C/23°C.
FIGURE 3  Viral systemic infection analysis of TSWV carrying noneliciting mutations in wild-type (WT) or Sw-5b-transgenic Nicotiana benthamiana plants. (a) Diagrams of the full-length infectious cDNA clones of TSWV WT (L_opt^+M_opt^-S^+) and mutants (L_opt^+M_opt^-mutant^-S^+) carrying the C118Y, P119A, T120N, W121A, D122A, R124A, or Q126A mutation in NSm. (b) Infectious clones of WT or noneliciting mutants of TSWV were coexpressed with viral suppressors of RNA silencing (P19, Hc-Pro, and γb) in WT or Sw-5b-transgenic N. benthamiana leaves by agro-infiltration. Disease development was analysed and photographed when typical symptoms including leaf curling were present in all plants. For each sample, the assay was repeated for 10 N. benthamiana plants. (c) Line chart of disease symptom development of WT or Sw-5b-transgenic N. benthamiana plants infected with WT and mutant viruses at different days postinoculation (dpi). (d) The N protein accumulation level in systemic leaves of various treated plants shown in panel (b) detected using an N-specific antibody. The RuBisCO large subunit was stained with Ponceau S to indicate sample loading.
FIGURE 4  Hypersensitive response (HR) induction analysis of the NSm21 alanine substitution mutants coexpressed with Sw-5bL33P/K319E/R927A and Sw-5bL33P/K319E/R927Q mutants. YFP-tagged Sw-5bL33P/K319E/R927A (a) or Sw-5bL33P/K319E/R927Q (b) was coexpressed with each NSm21 alanine substitution mutant in Nicotiana benthamiana leaves. The infiltrated leaves were monitored for HR from 1 to 7 days postinfiltration (dpi) and photographed at 7 dpi. Three independent experiments were repeated for each mutant. Values scored at 7 dpi are plotted. The HR index is shown in the lower panel. Bars represent the average of 12 replicas for each combination of constructs; error bars represent standard deviation.
FIGURE 5  Analysis of artificial evolved Sw-5b\(^{L33P/K319E/R927A}\) and Sw-5b\(^{L33P/K319E/R927Q}\) mutant-mediated resistance against resistance-breaking (RB) TSWV carrying the NSm\(^{D122A}\) or NSm\(^{R124A}\) mutation in transgenic Nicotiana benthamiana plants. (a) Full-length infectious clones of wild-type (WT) TSWV (L\(^{(+)}\)opt + M\(^{(-)}\)opt + S\(^{(+)}\)) and RB type with NSm\(^{D122A}\) or NSm\(^{R124A}\) mutation, respectively, coexpressed with three viral suppressors of RNA silencing (VSRs; P19, Hc-Pro, and γb) in p2300S (empty vector [EV]) control transgenic N. benthamiana plants and Sw-5b, Sw-5b\(^{L33P/K319E/R927A}\), and Sw-5b\(^{L33P/K319E/R927Q}\) transgenic plants. Viral infection and symptoms in systemic leaves of various agro-infiltrated plants were monitored from 10 to 30 days postinfiltration (dpi). (b) Immunoblot detection of N protein in systemic leaves of various treated plants shown in (a) using an N-specific antibody. The RuBisCO large subunit was stained with Ponceau S to indicate sample loading. Protein size is indicated on the left.
4.3 | Fluorescence microscopy

Leaf discs were collected from *N. benthamiana* leaves and mounted in water between a glass slide and coverslip. GFP was excited at 488 nm, and fluorescence was detected using a GFP barrier filter with an inverted fluorescence microscope (IX-71-F22 FL/DIC; Olympus). The captured images were analysed further using Image-Pro software (Olympus).

4.4 | Immunoblotting analysis

Total protein was extracted from 1 g agro-infiltrated *N. benthamiana* leaf tissue in 2 ml extraction buffer, as previously described (Zhu et al., 2017). After centrifugation at 18,000 g and 4°C for 30 min, 10 µl supernatant was mixed with 5 µl 3x SDS loading buffer and then subjected to 12.5% SDS-PAGE. For YFP-Sw-5b detection, 1.5 ml supernatant was incubated with GFP Trap beads (Chromotek) at 4°C for 90 min. The beads were heated in 30 µl 1x SDS loading buffer at 95°C for 10 min, and protein samples were separated by 10% SDS-PAGE. The proteins were transferred through electroblotting from the gel to a polyvinylidene fluoride membrane (GE Healthcare), which was blocked with 5% skim milk solution for 1 h and incubated with anti-TSWV N (1:5000, produced in our laboratory), anti-TSWV NSm (1:5000, produced in our laboratory), or anti-YFP (1:5000, produced in our laboratory) primary antibodies for 1.5 h at room temperature. After incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000, Sigma-Aldrich) for 1 h, the blots were detected using the ECL Substrate Kit (Thermo Scientific) by the ChemiDoc Technology Innovation Program (to X.T.).

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CONFLICT OF INTEREST

The authors declare that they have no competing interest.

DATA AVAILABILITY STATEMENT

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

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