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Research Article

Two *TOBAMOVIRUS MULTIPLICATION 2A* homologs in tobacco control asymptomatic response to tobacco mosaic virus

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Abstract

The most common response of a host to pathogens is arguably the asymptomatic response. However, the genetic and molecular mechanisms responsible for asymptomatic responses to pathogens are poorly understood. Here we report on the genetic cloning of two genes controlling the asymptomatic response to tobacco mosaic virus (TMV) in cultivated tobacco (*Nicotiana tabacum*). These two genes are homologous to *tobamovirus multiplication* 2A (*TOM2A*) from Arabidopsis, which was shown to be critical for the accumulation of TMV. Expression analysis indicates that the *TOM2A* genes might play fundamental roles in plant development or in responses to stresses. Consistent with this hypothesis, a null allele of the *TOM2A* ortholog in tomato (*Solanum lycopersicum*) led to the development of bent branches and a high tolerance to both TMV and tomato mosaic virus (ToMV). However, the *TOM2A* ortholog in *Nicotiana glauca* did not account for the asymptomatic response to TMV in *N. glauca*. We showed that TOM2A family is plant-specific and originated from Chlorophyte, and the biological functions of *TOM2A* orthologs to promote TMV accumulation are highly conserved in the plant kingdom—in both TMV host and nonhost species. In addition, we showed that the interaction between tobacco TOM1 and TOM2A orthologs in plant species is conserved, suggesting a conserved nature of TOM1–TOM2A module in promoting TMV multiplication in plants. The tradeoff between host development, the resistance of hosts to pathogens, and their influence on gene evolution are discussed. Our results shed light on mechanisms that contribute to asymptomatic responses to viruses in plants and provide approaches for developing TMV/ToMV-resistant crops.

Introduction

Diseases caused by plant viruses threaten crop production worldwide (Nicaise, 2014). Successful viral infections depend on both viral and host proteins. Interactions between the virus and the host not only affect the replication of the viral genome, cell-to-cell, and long-distance movement, and the

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development of disease symptoms, but also affect host antivirus defense responses (Chisholm et al., 2001; Kushner et al., 2003; Du et al., 2011).

Interactions between tobacco (*Nicotiana tabacum*) and tobacco mosaic virus (TMV) have served as a model system for plant–virus interactions (Scholthof, 2004). TMV was the first virus ever identified, is commonly found in plants and usually causes severe symptoms. Tobacco (2n = 4x = 48) is an allotetraploid derived from the inter-hybridization of two diploids, *Nicotiana sylvestris and Nicotiana tomentosiformis*, that took place ~200,000 years ago (Lim et al., 2004). The tobacco genome was estimated to be 4.5 Gb and to contain a large proportion of repetitive sequences, which hinder genetic studies (Sierro et al., 2014). The reactions of tobacco plants to TMV are diverse and include resistant, susceptible, and asymptomatic responses (Beekwilder, 1999; Yuan et al., 2015). The *N and N′* genes encode Toll/interleukin-1 receptor, nucleotide-binding site and leucine-rich repeat (TIR-NB-LRR) and Coiled-Coil NB-LRR (CC-NB-LRR) proteins that trigger programmed cell death at the sites of TMV infection (i.e. the hypersensitive response, HR) and are largely responsible for resistance in *Nicotiana* species. The asymptomatic response to TMV refers to a phenotype with neither HR at the infected sites nor disease symptom in systematic leaves (Yuan et al., 2015). Asymptomatic response is also found in some tomato (*Solanum lycopersicum*) genotypes when inoculated with *Tobacco mild green mosaic virus* or *Pepper mild mottle virus* (Ishibashi et al., 2009; Yuan et al., 2015). Several host proteins critical for TMV infection have been identified. TOBAMOVIRUS MULTIPLICATION 1 (TOM1) and its homolog TOM3 are required for the accumulation of TMV in Arabidopsis. They are integral membrane proteins containing seven transmembrane helices that were localized to vacuolar membranes and other undefined membranes (Yamanaka et al., 2000, 2002; Fujisaki et al., 2006). TOM2A (AT1G32400) is an integral membrane protein containing four transmembrane helices that accumulate predominantly in the tonoplast that contributes to the accumulation of a Youcai mosaic virus (YoMV, formerly called TMV-Cg) but has little effect on the multiplication of tomato mosaic virus (ToMV) (Tsujimoto et al., 2003; Fujisaki et al., 2008). A small GTP-binding protein ADP-RIBOSYLATION FACTOR-LIKE 8 in the host is also important for the accumulation of TMV (Nishikiori et al., 2011). A PECTIN METHYLESTERASE in tobacco interacts with the movement protein from TMV to facilitate the cell-to-cell movement of TMV (Chen et al., 2000). More recently, members of the SNARE family were also shown to be required for the local accumulation and spread of the TMV virus (Ibrahim et al., 2020).

Although resistant and susceptible responses to viruses have been well characterized, the genetic and molecular mechanisms that contribute to the asymptomatic responses to viruses remain poorly understood. In plants, asymptomatic responses are often induced by endophytic microbes including fungi, bacteria, and virus. Some endophytic microbes may be beneficial to the host by promoting the uptake of nutrients from soil, modulating plant development, and increasing tolerance to biotic and abiotic stress. Other microbes may not contribute any obvious benefit to the host and nonetheless, induce asymptomatic responses (White et al., 2019; Fukuhara et al., 2020). The asymptomatic response to microbes is hard to visualize and might be underestimated in nature (Zheng et al., 2017). The asymptomatic response to a particular pathogen is often universal for all genotypes of a particular species (Martinez-Marrero et al., 2020). However, an asymptomatic response may be just one of the responses of a host species to a particular pathogen. For example, although some people may develop severe or mild symptoms of the Severe Acute Respiratory Syndrome Coronavirus 2, others may have an asymptomatic response (Beck and Aksentijevich, 2020). Although TMV induces asymptomatic responses in particular tobacco genotypes, TMV induces resistant or susceptible responses in other tobacco genotypes (Beekwilder, 1999; Yuan et al., 2015). The genetic and molecular differences responsible for the variation of responses (e.g. asymptomatic and susceptible responses) remain unclear.

In this study, we used bulk segregant analysis (BSA) in combination with next-generation sequencing (NGS) technology to dissect the genetics of the asymptomatic response of tobacco to TMV. Two recessive loci were identified and cloned. We show that the causal genes contribute to the accumulation of TMV. The accumulation of TMV was dramatically reduced in a double mutant that is deficient in both of these genes, which accounts for the asymptomatic response. An evolutionary analysis showed that the causal gene has an ancient origin, dating back to the emergence of algae and that its influence on the accumulation of TMV emerged after the divergence of moss. An RNA-seq analysis indicates that the causal genes affect a number of important pathways in plants. We hypothesize that the biological importance of the causal gene out-weighs the disease caused by TMV, leading to its high conservation in the plant kingdom. Finally, we propose an approach to engineer TMV tolerant crops. The results from this study not only uncover part of the genetic and molecular mechanisms responsible for the asymptomatic response to TMV in tobacco but also provide insight into the origin and evolution of genes that are both biologically important for plant hosts and also critical for the multiplication of pathogenic viruses.

Results

Asymptomatic response to TMV in tobacco genotype TI203 is due to low accumulation of TMV

Tobacco genotype SR1 develops a typical mosaic phenotype after inoculation with TMV. In contrast, genotype TI203 has an asymptomatic response to TMV (Figure 1). TG34, a transgenic plant with the *N* gene from the SR1 background is immune to TMV and develops a HR after inoculation

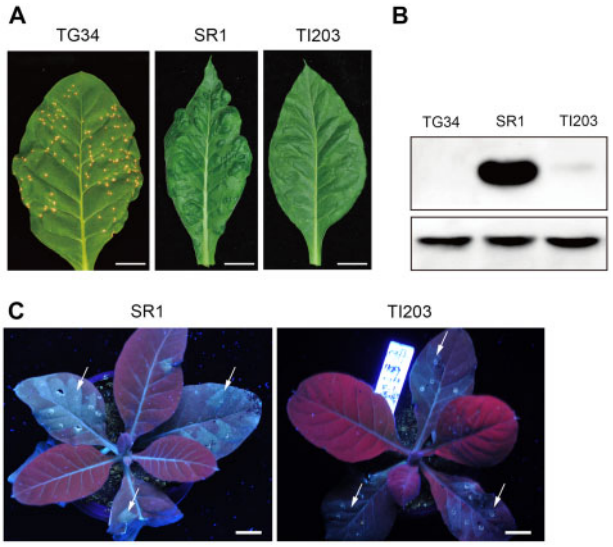

Figure 1 Distinct responses of tobacco genotypes to TMV. A, Responses to TMV. The responses to TMV include HR (left), mosaic (middle), and asymptomatic (right). HR response was at 7 dpi; the mosaic phenotype and asymptomatic responses were from upper noninoculated leaves at 30 dpi. Scale bar, 2 cm. B, TMV levels in leaves. TMV was detected using immunoblotting with an anti-TMV coat protein antibody. TMV accumulated to undetectable (left), high (middle), and low levels (right) in leaves with HR, mosaic and asymptomatic responses, respectively. An immunoblot utilizing an anti-actin antibody was used as a loading control. C, Analysis of TMV accumulation in the inoculated leaves. TMV-GFP infiltration clone was used to infiltrate the second to forth leaves of SR1 and TI203 at five-leaf stage (~4-week-old plants). Photographs were taken at 7 dpi under UV light. White arrows indicated the leaves that were infiltrated with TMV-GFP infiltration clone. Bar, 2 cm.

with TMV (Whitham et al., 1994). To investigate the mechanisms underlying the three distinct responses, we used immunoblotting to quantify the levels of the TMV particles that accumulated in the upper systemic leaves of these plants 30-d post-TMV inoculation (dpi). As expected, no TMV was detected in the resistant genotype TG34, but abundant TMV was detected in the susceptible SR1 (Figure 1). Interestingly, using immunoblotting, we detected a faint band in TI203, which developed an asymptomatic response after inoculation with TMV. To analyze the TMV accumulation in the inoculated leaves, we infiltrate the leaves of SR1 and TI203 with TMV-GFP infiltration clone. We found that the GFP signal in TI203 was much weaker than that in SR1 at 7 dpi (Figure 1), suggesting that TMV replication in TI203 was significantly affected. We concluded that low-level accumulation of TMV particles in the leaves explains the asymptomatic phenotype in TI203.

Two recessive loci synergistically control the asymptomatic phenotype in TI203

To understand the genetics underlying the asymptomatic response to TMV, we crossed TI203 (asymptomatic) with SR1 (mosaic). All of the F_1 plants inoculated with TMV-U1 sap developed mosaic symptoms, similar to the SR1 parent. These data indicate that at least one recessive gene is responsible for the asymptomatic phenotype in TI203. The F_1 plants were then either self-crossed to generate an F_2 population or backcrossed with TI203 to generate a BC_1 population. Of the 216 individuals in the F_2 population, 201 individuals were mosaic and 15 were asymptomatic, which is a Mendelian ratio of 15:1 ($\chi^2 = 0.18$; $P = 0.67$; Supplemental Table S1). The BC_1 population (with 204 plants), on the other hand, had 155 mosaic individuals and 49 asymptomatic individuals, which is a Mendelian ratio of 3:1 ($\chi^2 = 0.1$, $P = 0.75$; Supplemental Table S1). The segregation ratios of mosaic and asymptomatic individuals in both the F_2 and BC_1 populations provide evidence that the asymptomatic phenotype in TI203 was controlled by two recessive genes.

Map-based cloning of a gene controlling the asymptomatic phenotype in TI203

To generate a single gene segregating population, we randomly chose six individuals with mosaic phenotypes from the BC_1 population to backcross with TI203 to generate six BC_2 subfamilies. For each BC_2 subfamily, the phenotypes of at least 40 progeny were initially scored. Among the six BC_2 subfamilies, we observed a mosaic to asymptomatic ratio of 3:1. Thus, both loci appeared to be segregating in these three subfamilies. In contrast, in the remaining three BC_2 subfamilies, we observed a mosaic to asymptomatic ratio of 1:1. Thus, only one of the two loci appeared to be segregating in these three BC_2 subfamilies (Supplemental Table S2).

To clone the gene underpinning the asymptomatic response, referred to as *Tobacco Asymptomatic Gene 1* (*TAG1*), one of the BC_2 subfamilies (BC2-01) with a mosaic to asymptomatic response ratio of 1:1 was selected for genetic mapping experiments. A total of 60 mosaic individuals from the BC2-01 subfamily were pooled to generate a "mosaic pool," and 76 asymptomatic individuals were pooled to generate an "asymptomatic pool." RNA was extracted from each pool and sequenced using the Illumina-HiSeq2000 platform. The raw data were mapped to the tobacco genome and SNPs were called. To verify the SNPs and facilitate marker development, we re-sequenced the two parents, SR1 and TI203. Only SNPs detected in both the RNA sequence and re-sequencing data were further considered. The difference in allele frequencies (ΔSNP-index) for each SNP locus in the two pools was calculated. Because tobacco is a tetraploid, the ΔSNP-index may be different than we expect for a diploid species. In this study, all SNPs with ΔSNP-index $\geqslant 0.25$ were considered, and consequently seven single nucleotide polymorphysms (SNP) were identified (Supplemental Table S3). To test whether these seven SNPs were linked to *TAG1*, primers were designed flanking the SNPs and used to amplify PCR products from three bulked pools, each containing eight asymptomatic individuals from the BC2-01 subfamily. The PCR products were sequenced. An SNP locus was considered to be linked with the *TAG1* gene if any of the pools contained TI203 alleles

that were homozygous. Using this criterion, we found that six of the seven SNPs (SNP1–SNP6) were linked to the *TAG1* gene.

Since the tomato reference genome was much better assembled than the tobacco reference genome, we used the tomato genome as a reference genome for the fine mapping and cloning of the *TAG1* gene. First, the tobacco genes that were linked to the *TAG1* gene were used as query sequences in TBLASTX searches of the tomato genome. Three syntenic regions were identified in the tomato genome, on chromosomes 4, 8, and 12. These data provide evidence that translocations occurred after the divergence of *Nicotiana and Solanum* (Figure 2). Contigs from the tobacco genome that are syntenic to these three regions of the tomato genome were retrieved and PCR-based markers were designed based on polymorphic sites (Supplemental Table S4). Using 236 individuals from the BC2-01 subfamily, the *TAG1* gene was mapped between two markers, M3 and M4. These two markers were used to screen 2,517 individuals from the BC2-01 population and 461 asymptomatic individuals from a BC_2F_2 population (i.e. a total of 3,439 informative products of meiosis). Finally, the *TAG1* gene was fine mapped to a region between markers M265 and M248 that corresponds to 58.183–58.307 Mb on chromosome 8 from tomato (Figure 2). The tomato reference genome was derived from the Heinz 1706 cultivar, which is susceptible to TMV. Therefore, we thought that *TAG1* is probably in the tomato reference genome. The 124-kb candidate region in the tomato genome harbors 15 predicted genes (Supplemental Table S5). In this group of 15 genes, *Solyc08g077220* is the only known gene associated with virus susceptibility. *Solyc08g077220* encodes a transmembrane protein with four transmembrane helices that are homologous to the TOM2A from Arabidopsis (Hagiwara et al., 2003; Tsujimoto et al., 2003). We identified a tobacco scaffold (NtomScf100471) containing the candidate gene. Using markers that we developed for this scaffold, we mapped the *TAG1* gene to a region containing six genes (Figure 2). The *TAG1* gene is identical to a gene from *N. tomentosiformis* in the coding region but has 24 polymorphic sites relative to a gene from *N. sylvestris*. These data provide evidence that the *TAG1* gene was derived from a subgenome from *N. tomentosiformis* and therefore, we named the candidate gene *N. tabacum* subgenome *tomentosiformis* *TOM2A* (*NttTOM2A*).

NttTOM2A compromises the asymptomatic response

We amplified and sequenced the *NttTOM2A* gene from the genomes of the two parents. The only difference between *NttTOM2A* in the two parents is a 2-bp deletion in the third exon from the asymptomatic parent TI203 (Figure 2). This 2-bp deletion was predicated bioinformatically to cause frame-shift and premature stop codon, which results in reduced transcript level in TI203 and abolishment of transmembranes 3 and 4 as well as other 98 amino acids in the C-terminal of the TOM2A protein (Supplemental Figure S1). Previous study showed that TOM2A interacts with TOM1 to promote TMV multiplication (Tsujimoto et al., 2003). To test whether the mutant protein in TI203 can interact with TOM1, split-ubiquitin membrane-based yeast two-hybrid assay was performed. The results showed that NttTOM2A failed to interact with the shortened $NtsTOM2A^{SR1}$ (Supplemental Figure S1), indicating that the transmembrane 3, transmembrane 4, or the C-terminal fragment of TOM2A contains important motifs critical for TOM2A's function. This 2-bp InDel co-segregated with the TMV response in all of the individuals that we analyzed.

To independently test its contribution to the TMV response, TI203 was transformed with *NttTOM2A* from the mosaic parent SR1, including 2,075 bp of upstream sequence and 1,002 bp of downstream sequence. Thirty-six transgenic plants were obtained. In contrast to the asymptomatic response of TI203 (i.e. the wild-type), each transgenic plant developed a mosaic phenotype after inoculation with TMV (Figure 3). Based on our immunoblotting assay, TMV accumulated to considerably higher levels in the leaves of the transgenic plants relative to TI203 (Figure 3). Similar results were obtained when the expression of the candidate gene was driven by the cauliflower mosaic virus 35S promoter in stably transformed TI203 (Figure 3). These data indicate that *NttTOM2A* is *TAG1*, which was one of the two genes that contribute to the asymptomatic response to TMV in tobacco.

TAG2 is a homoeolog of *NttTOM2A* in the subgenome *sylvestris* (*NtsTOM2A*)

We speculated that both *TOM2A* homoeologs in the asymptomatic parent TI203 should be nonfunctional because the asymptomatic phenotype is recessive. To test this hypothesis, we amplified and sequenced the genomic DNA of the homoeolog from the *sylvestris* subgenome (*NtsTOM2A*). The only differences between the two parents are a 2-bp deletion in the sixth exon and a point mutation (T5875C) in the fifth exon of the allele from TI203 (Figure 4). The 2-bp deletion in *NtsTOM2A* is located at a different position in *NtsTOM2A* relative to the 2-bp deletion in *NttTOM2A*. This 2-bp deletion was predicted to change the last 18 amino acid of the protein and the mutant protein ($NtsTOM2A^{TI203}$) lost the capacity to interact with TOM1 (Supplemental Figure S1), suggesting that the last 18 amino acid residues of TOM2A might contain motifs critical for TOM2A's function. A polymorphic marker (M360) was designed for the *NtsTOM2A* gene and used to screen a BC_2 subfamily. M360 co-segregated with the phenotype in a population containing 216 individuals. These data indicate that *NtsTOM2A* is the second gene (*TAG2*) required for the asymptomatic response to TMV in tobacco.

To test whether *NtsTOM2A* is required for the asymptomatic response, TI203 was transformed with a complementation vector. A total of 33 transgenic plants (T_0) were obtained, and 30 of them developed mosaic phenotypes after inoculation with TMV (Figure 4). Similar results were found in transgenic plants overexpressing *NtsTOM2A*. Forty

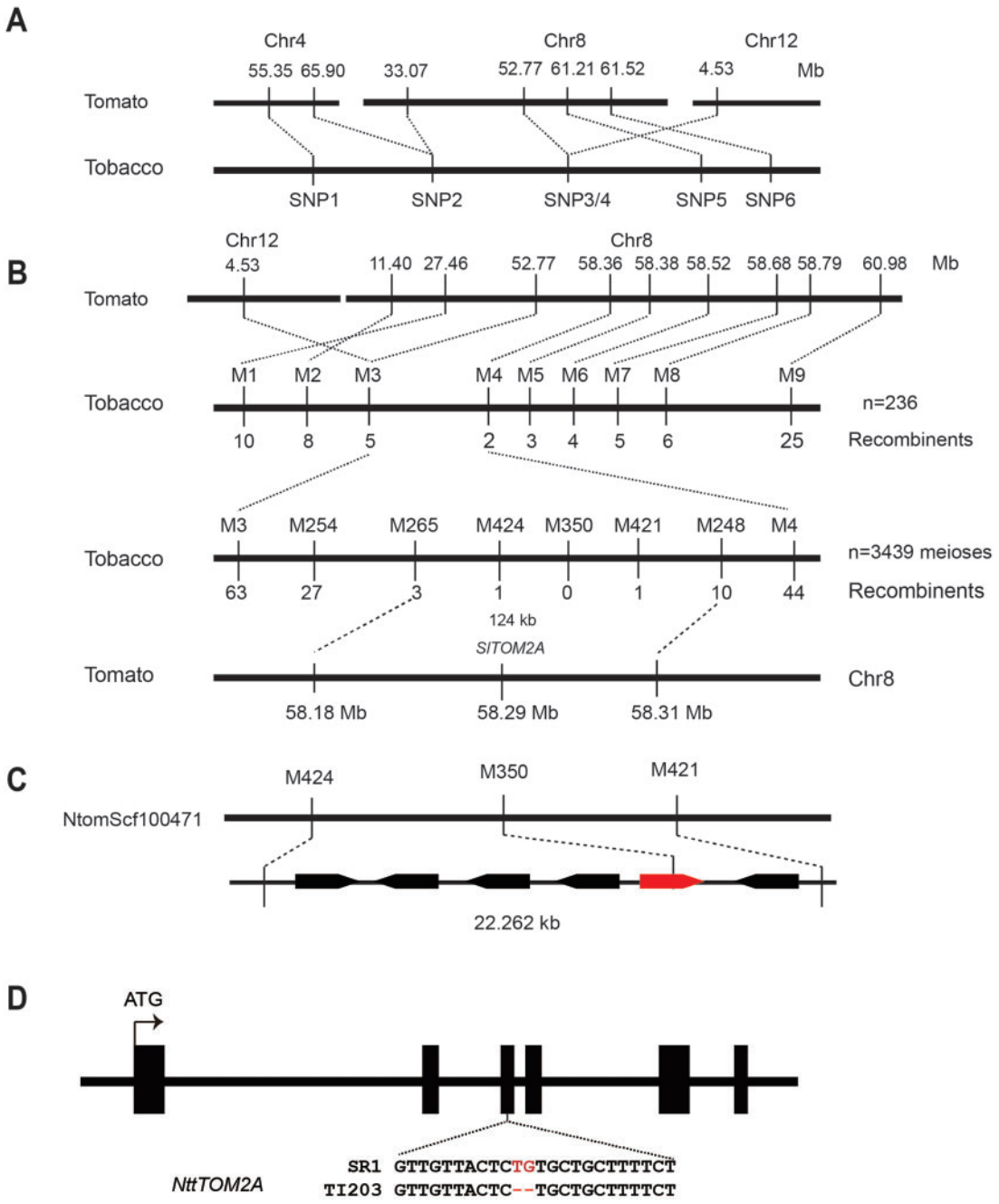

Figure 2 Genetic mapping of *TAG1*. A, Synteny of the *TAG1* region between tobacco and tomato. B, Fine mapping of the *TAG1* gene. C, The *TAG1* gene was mapped to a candidate region containing six genes in tobacco. The candidate gene is indicated with red. D, Sequence variation of the candidate gene in the mosaic (SR1) and asymptomatic (TI203) parents.

of the 43 lines developed mosaic phenotypes after inoculation with TMV. Based on our immunoblotting assay, TMV accumulated at much higher levels in these transgenic lines than in wild-type (TI203; Figure 4). We conclude that a double mutant containing loss-of-function alleles of both *NtsTOM2A and NttTOM2A*, a pair of homoeologous genes in tobacco, can develop an asymptomatic response to TMV in the TI203 background.

Null alleles of *TOM2A* orthologs confer tolerance to TMV in tobacco and tomato

To further study the function of *TOM2A* orthologs and to test whether *TOM2A* is necessary for the multiplication of TMV in tobacco, we knocked out both *NtsTOM2A and NttTOM2A* in SR1 plants using clustered regularly interspaced short palindromic repeats and Cas9 gene-editing technology (CRISPR/Cas9). In the T_4 generation, we obtained

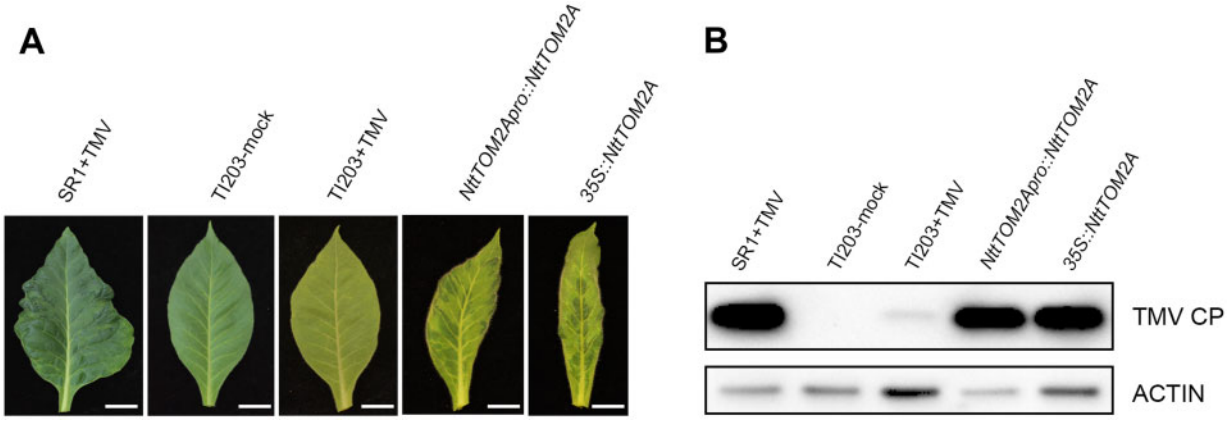

Figure 3 Complementation test with the *NttTOM2A* gene. A, Responses to TMV in the upper noninoculated leaves. Leaves from transgenic TI203 plants that harbor transgenes that use the native promoter or the 35S promoter to express the *NttTOM2A* gene are shown. The mosaic phenotype is apparent in SR1 and the transgenic TI203 but not in the wild-type TI203 control plants. Photographs were taken at 30 dpi. Scale bar, 2 cm. B, TMV coat protein levels in infected leaves. The TMV coat protein was detected by immunoblotting as described in Figure 1B. The protein analyzed in each lane was derived from leaves similar to the leaves shown in Figure 3A as indicated.

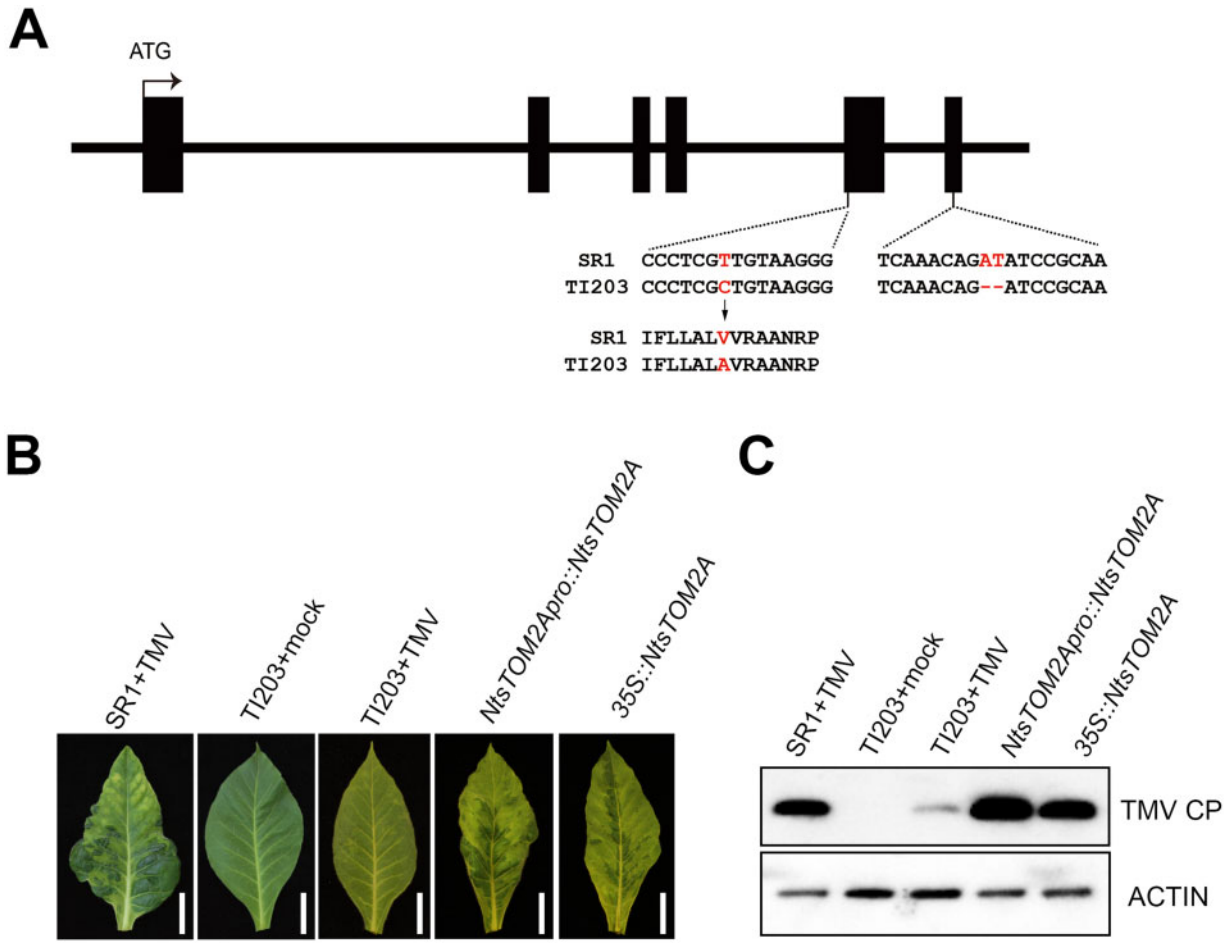

Figure 4 *NtsTOM2A* controls the asymptomatic response A, *NtsTOM2A* sequence differences between SR1 and TI203. The red dashed line represents the 2-bp deletion. B, Responses to TMV in the upper noninoculated leaves. Leaves from transgenic TI203 plants that harbor transgenes that use the native promoter or the 35S promoter to express the *NtsTOM2A* gene are shown. Photographs were taken at 30 dpi. Scale bar, 2 cm. The mosaic phenotype is apparent in SR1 and the transgenic TI203 but not in the wild-type TI203 control plants. C, TMV coat protein levels in infected leaves. The TMV coat protein was detected by immunoblotting as described in Figure 1B. The protein analyzed in each lane was derived from leaves similar to the leaves shown in Figure 4B as indicated.

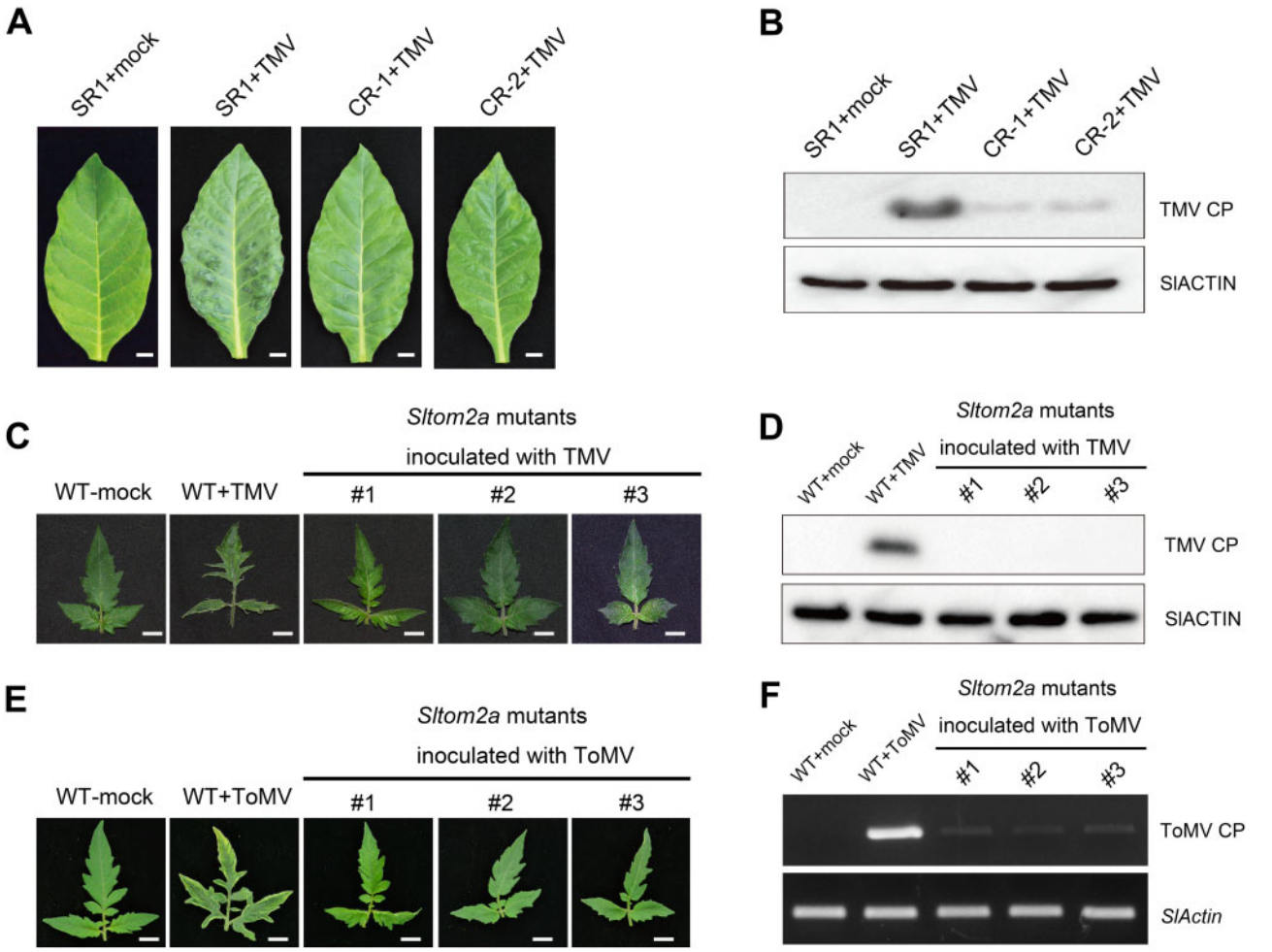

Figure 5 Virus tolerance in the *TOM2A* ortholog knockout mutants of tobacco and tomato. A, Responses to TMV in the upper noninoculated leaves of tobacco. Two double mutants (*CR-1 and CR-2*) deficient in both *NtsTOM2A and NttTOM2A* developed asymptomatic responses to TMV in contrast to the mosaic response of wild-type SR1. Photographs were taken at 10 10 dpi. Scale bars, 2 cm. B, TMV coat protein levels in infected leaves. The TMV coat protein was detected by immunoblotting as described in Figure 1B. The protein analyzed in each lane was derived from a plant shown in Figure 5A as indicated. C, Responses to TMV in the upper noninoculated leaves of tomato. Three knockout lines of *SlTOM2A* developed asymptomatic responses to TMV. In contrast, the mosaic response developed in wild-type. Photographs were taken at 14 dpi. Scale bar, 1 cm. D, TMV coat protein levels in infected leaves. The TMV coat protein was detected by immunoblotting as described in Figure 1B. The protein analyzed in each lane was derived from leaves similar to the leaves shown in Figure 5C as indicated. E, Responses to ToMV inoculation in the upper noninoculated leaves of tomato. The *Sltom2a* mutants developed asymptomatic response to ToMV while the wild-type control developed mosaic response. Wild-type and *Sltom2a* CRISPR/Cas9 mutants are used for control as in Figure 5C. Photographs were taken at 14 dpi. Scale bar, 1 cm. F, ToMV CP levels in infected leaves. RT-PCR was used to detect the ToMV CP RNA levels in the infected leaves. ToMV CP-specific primers were used for RT-PCR. The PCR amplicom analyzed in each lane was derived from leaves similar to the leaves shown in Figure 5E as indicated. The ToMV CP levels in the three independent *Sltom2a* mutants were dramatically reduced compared to the wild-type control. Tomato *Actin* was used for internal control. Three biological replicates were used for each experiment.

two independent homozygous knockout mutants (Supplemental Figure S2). Each mutant was inoculated with TMV and developed an asymptomatic phenotype at 10 dpi (Figure 5). Moreover, the accumulation of TMV in the upper noninoculated leaves was considerably reduced in the knockout plants (Figure 5).

Since loss-of-function mutations in *AtTOM2A* in Arabidopsis and *NtTOM2A* in tobacco led to asymptomatic responses to TMV, we speculate that knocking out *TOM2A* orthologs from other TMV susceptible species would also lead to TMV tolerance. To test this hypothesis, we knocked out *SlTOM2A* (Solyc08g077220, see above) in a TMV-susceptible tomato cultivar A57 (*S. lycopersicum* cv Ailsa Craig) using the CRISPR/Cas9 system with two sgRNAs. We obtained three independent mutants harboring distinct null alleles of *SlTOM2A* (Supplemental Figure S2). In the absence of TMV, the only observable phenotype in these knockout mutants was bent vegetative and floral branches (Supplemental Figure S3). After inoculation with TMV-U1 sap, the three mutant lines were asymptomatic. In striking contrast, after inoculation with TMV-U1 sap, the leaves of wild-type

wrinkled and curled (Figure 5). Consistent with this phenotype, based on our immunoblotting assay, leaves from the *sltom2a* mutant lines accumulated barely detectable levels of TMV (Figure 5). In contrast, wild-type accumulated readily detectable levels of TMV (Figure 5).

Previous study showed that Arabidopsis TOM2A contributes to the multiplication of YoMV but has little effect on the multiplication of ToMV (Tsujimoto et al., 2003). To test whether the tomato TOM2A ortholog has any effects on the multiplication of ToMV in tomato, ToMV sap was inoculated on the leaves of a TMV-susceptible tomato cultivar A57 along with its *Sltom2a* CRISPR/Cas9 mutants. Interestingly, unlike Arabidposis *tom2a* mutants, in which ToMV CP accumulated to wild-type levels, *Sltom2a* mutants developed asymptomatic phenotype after ToMV inoculation. The accumulation of ToMV CP was dramatically reduced in the *Sltom2a* mutants compared to that in wild-type cultivar A57 (Figure 5). A previous study showed that TOM2B, a protein distantly related to TOM2A, plays positive roles in the multiplication of both YoMV and ToMV (Tsujimoto et al., 2003). The reduced ToMV level in the *sltom2a* mutant suggests that there is no functional redundancy in tomato genome. Indeed, the most similar protein (Solyc06g061050.2) shows only 37% sequence identity with AtTOM2B, in striking contrast to the 67% identity between SlTOM2A and AtTOM2A, suggesting that the homologs of SlTOM2B in tomato do not support ToMV multiplication.

TOM2A orthologs do not influence asymptomatic responses to TMV in wild *Nicotiana* species

To study the evolution of *TOM2A* orthologs and its role in the response to TMV, we amplified *TOM2A* orthologs from 16 *Nicotiana* species with diverse responses to TMV—six asymptomatic species, six susceptible species, and four resistant species. Sequence analysis showed that all of the *TOM2A* orthologs including those from the six asymptomatic species were highly conserved. The most divergent orthologs encoded proteins with only six different amino acid residues (Supplemental Figure S4). Using these sequences, we constructed a phylogenetic tree that reflects the evolutionary relationships of these species (Figure 6; Clarkson et al., 2004). The *TOM2A* orthologs from the six *Nicotiana* species with asymptomatic responses are randomly distributed in the phylogenetic tree, which provides evidence for the independent origin of their asymptomatic responses to TMV. To test whether TMV does not accumulate in *Nicotiana glauca*—a species with an asymptomatic response to TMV—because of the *TOM2A* ortholog (*NgTOM2A*) (Supplemental Figure S5), we transformed TI203 with the *NgTOM2A* gene. Eleven of the 12 transgenic lines developed mosaic phenotypes (Figure 6). Our immunoblotting assay demonstrated that TMV accumulated to high levels in the transgenic plants overexpressing *NgTOM2A* (Figure 6). We conclude that the asymptomatic phenotype of *N. glauca* is not caused by a loss-of-function allele of the *NgTOM2A* gene.

TOM2A orthologs contribute to TMV multiplication in divergent plant species

We extended the evolutionary study of *TOM2A* orthologs to distantly related plant species. We used the amino acid sequence of AtTOM2A as a query in BLASTP searches of the protein database for 54 species. We found that the *TOM2A* homologs are widely present in all species from monocots, eudicots, basal angiosperms, gymnosperms, lycopodiophyta, bryophyta, and in some species of Chlorophyta, but not in species of Phodophyta or Glaucophyta (Supplemental Table S6). Although most species, including Arabidopsis, have four members, the banana genome contains up to 12 members, probably because of a genome triplication (D'Hont et al., 2012; Supplemental Table S6).

To investigate whether the contribution of *TOM2A* homologs to the replication of TMV is conserved, we obtained the sequences of *TOM2A* orthologs from 16 species including four TMV host species and 12 TMV nonhost species. These species included algae, moss, gymnosperm, monocots, and eudicots (Supplemental Table S6). The coding sequences of the *TOM2A* orthologs were amplified from these species using PCR. However, the coding sequences of *TOM2A* orthologs were synthesized fom *Amborella trichopoda*, *Picea abies*, *Selaginella moellendorffii*, *Physcomitrium patens*, and *Coccomyxa subellipsoidea*. These coding sequences were cloned into pHellsgate 8 downstream of the 35S promoter to drive their expression in plants. TI203 was transformed with these constructs. At least eight stable transgenic plants were obtained from each transformation experiment. Phenotypes were scored at 30 dpi in the T_1 generation. Overexpression of 15 of the 16 *TOM2A* orthologs in TI203 dramatically increased the accumulation of TMV and conferred a mosaic phenotype upon the asymptomatic TI203 (Figure 7). The only exception was that the expression of *CsuTOM2A* from the alga *C. subellipsoidea* did not induce a mosaic phenotype in TI203. Our immunoblotting assay demonstrated that transgenic plants overexpressing the *TOM2A* orthologs accumulated higher levels of TMV than TI203. We conclude that the activity of *TOM2A* homologs that promotes the accumulation of TMV is highly conserved in the plant kingdom but not in algae.

We constructed a phylogenetic tree using TOM2A homologs from nine species including *C. subellipsoidea*, *P. patens*, *S. moellendorffii*, *P. abies*, *A. trichopoda*, *Oryza sativa*, *S. lycopersicum*, *N. tabacum*, and *Arabidopsis thaliana* (Figure 8). These homologs could be divided into two clades based on the topology of the tree. Clade I contains the TOM2A homologs that were experimentally confirmed to be able to support the accumulation of TMV, which are referred to as TOM2A orthologs (Figure 7). Interestingly, the algal ortholog, *CsuTOM2A*, does not have the capacity to support the accumulation of TMV and also belongs to clade I. Members in clade II, on the other hand, probably do not promote the

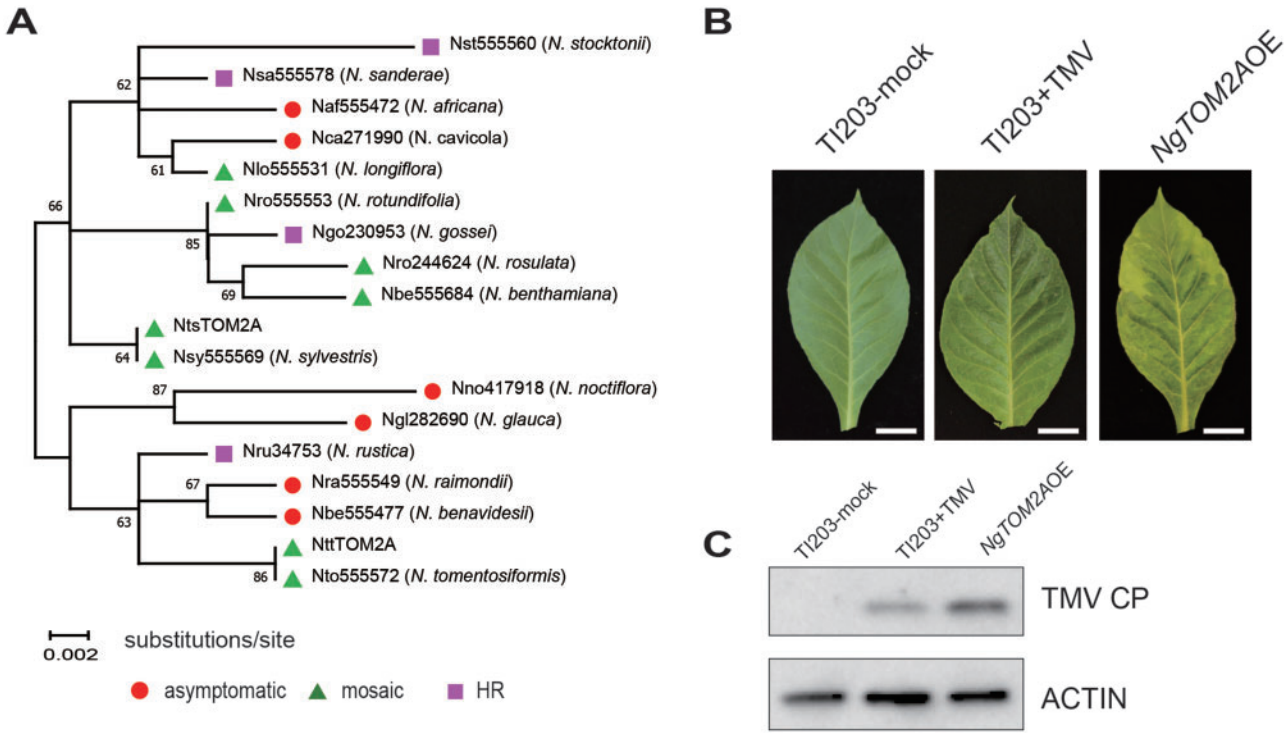

Figure 6 Evolution of the TOM2A orthologs in *Nicotiana* species. A, Phylogeny of TOM2A orthologs from *Nicotiana* species. The six species with asymptomatic responses to TMV are indicated with red circles. Particular sequences are indicated with binomial nomenclature and accession numbers. B, The *NgTOM2A* gene from *N. glauca* compromised the asymptomatic response in TI203. Representative upper noninoculated leaves at 30 dpi are shown. Scale bar, 2 cm. C, TMV coat protein levels in infected leaves. The TMV coat protein was detected by immunoblotting as described in Figure 1B. The protein analyzed in each lane was derived from a leaves similar to the leaves shown in Figure 6B as indicated.

accumulation of TMV because the homologs in clade II from tobacco and tomato (boxed in red) could not complement either *Nts(t)TOM2A* or *SlTOM2A* for the accumulation of TMV. Moreover, the two subclades (II-a and II-b) may represent two ancient duplications. Therefore, our functional and evolutionary studies demonstrate that *TOM2A* homologs acquired the ability to promote the accumulation of TMV after the divergence of *C. subellipsoidea and P. patens*. These data provide evidence that (1) this divergence may have occurred during the transition of plant life from water to land, (2) the functions of the TOM2A paralogs within a genome may have diverged, and (3) only the TOM2A ortholog lineage (Clade I in Figure 8) promotes the accumulation of TMV.

Interaction between different TOM2A orthologs and TOM1 is conserved in plant kingdom

Previous study showed that TOM2A promotes TMV multiplication through interacting with TOM1 (Tsujimoto et al., 2003). We used a split-ubiquitin membrane-based yeast two-hybrid assay to test whether the interaction between TOM2A orthologs and tobacco TOM1 is also conserved in different plant species. TOM2A orthologs from six species that represent the algae, moss, gymnosperm, monocots, and eudicots clade were used for the interaction test. Gene cassettes were constructed to express *NtsTOM1* (*TOM1* on the *N. sylvestris* subgenome) fused at its C-terminal with the Cub-PLV module (NtsTOM1^{SR1}-Cub-PLV; Figure 9) or *TOM2A* fused at its N-terminal with the mutant form of the ubiquitin N-terminal fragment (NubG; e.g. NubG-NtsTOM2A^{SR1}; Figure 9). The NtsTOM1^{SR1}-Cub-PLV construct and each of the NubG-TOM2A constructs was co-introduced to the yeast strain NMY51 strain. Interactions were scored based on transformants growth on the SD medium lacking Trp, Leu, His, and Ade (SD/-Trp/-Leu/-His/-Ade). The results showed that NtsTOM1 interacted with each of the six TOM2A orthologs examined in this study, including the CsuTOM2A from *C. subellipsoidea*, which did not support TMV multiplication in tobacco (see above).

TOM2A orthologs affect the expression of many genes in plants

Reverse transcription-quantitative PCR (RT-qPCR) was used to investigate the expression patterns of *NtsTOM2A and NttTOM2A* in tobacco. We found that the *NtTOM2As* were ubiquitously expressed in leaves, stems, roots and flowers and that the highest expression levels were in roots (Supplemental Figure S6). The expression of *NtTOM2As* did not change after inoculation with TMV (Supplemental Figure S6). Furthermore, the examination of microarray data from the Bio-Array Resource Database (http://bar.utoronto.ca/efp) provides evidence that the Arabidopsis *TOM2A* was also expressed in roots, leaves, stems, flowers, siliques, and developing seeds. Notably, the expression of *AtTOM2A* was

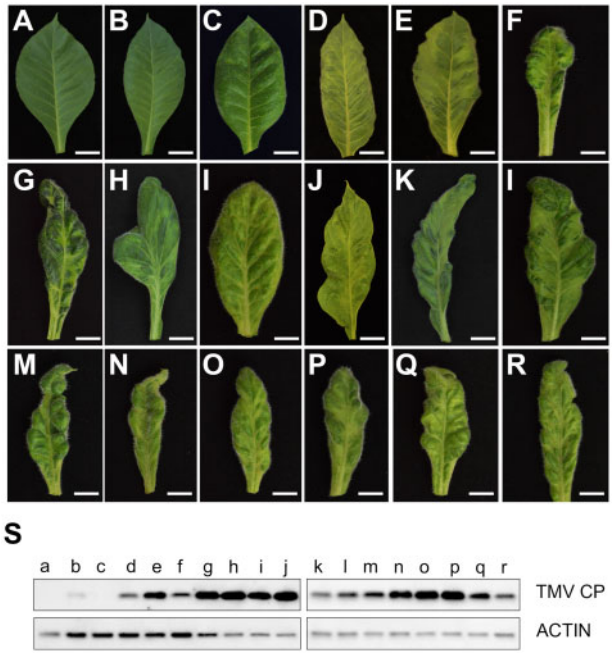

Figure 7 Influence of *TOM2A* orthologs on the accumulation of TMV. Phenotype of upper noninoculated leaves of TI203 treated with H_2O (A) and TMV sap (B). TMV responses of transgenic TI203 lines that expressed *TOM2A* orthologs from *C. subellipsoidea* (C), *P. patens* (D), *S. moellendorffii* (E), *P.abies* (F), *A. trichopoda* (G), *O.sativa* (H), *Zea mays* (I), *Lactuca sativa* (J), *S. lycopersicum* (K), *Populus trichocarpa* (L), *Citrus sinensis* (M), *A. thaliana* (N), *Fragaria vesca* (O), *Cucumis sativus* (P), *Citrullus lanatus* (Q), and *Glycine max* (R). Representative upper noninoculated leaves are shown. Photographs were taken at 30 dpi. Scale bar, 2 cm (S). TMV coat protein levels in infected leaves. The TMV coat protein was detected by immunoblotting as described in Figure 1B. The protein analyzed in each lane was derived from leaves similar to the leaves shown in Figure 7A through Figure 7R as indicated.

elevated in response to particular types of abiotic stress, such as osmotic, salt, and drought stress (Supplemental Figure S7). Therefore, we conclude that in general, *TOM2A* orthologs are constitutively expressed in plants.

Previous study showed that simultaneous knockout of TOM2A and its three homologs resulted in mutant with growth defects and viability deficiencies (Fujisaki et al., 2008). However, the biological function of TOM2A family in plant is still unknown. To gain more insights into the biological functions of TOM2A, we characterized the differentially expressed genes (DEGs) in *TOM2A* mutants that were not inoculated with TMV. We compared TI203 to its NIL that harbored a functional *NtsTOM2A* in the absence of TMV using RNA-seq and found 2,022 DEGs, with 1,516 up-regulated and 506 downregulated genes (Supplemental Dataset S1). To investigate the specific functions of the 2,022 DEGs, we performed a GO term enrichment analysis (Supplemental Figure S8). In the biological process module, the most enriched DEGs were assigned to metabolic process. In the cellular component module, the most enriched GO terms were assigned to membranes. In the molecular function module, the GO terms associated with most DEGs were associated with binding and catalytic activity. These results are consistent with *TOM2A* orthologs playing fundamental roles in plant development, as suggested in Supplemental Figure S2. In conclusion, TMV hijacked TOM2A proteins to accumulate in plant hosts. The important biological functions of TOM2A proteins may have created selection pressure that has maintained *TOM2A* in plant genomes despite its role in promoting the accumulation of TMV.

Discussion

The BSA combined with RNA-seq and comparative genetic mapping may facilitate genetic improvement in polyploidy crops

Bulked segregant analysis combined with high-throughput sequencing had been frequently used for genetic analysis of quantitative traits or identification of candidate genes controlling agronomically important traits in various crops (Dong et al., 2018; Su et al., 2020; Wang et al., 2021). However, successful application of this strategy to positional cloning of genes of interest in tobacco was largely hindered due to the large tobacco genome size, high level of gene redundancy, large proportion of repetitive sequences, low genome diversity, and absence of well-assembled reference genome. In this study, we combined BSA and RNA-seq to efficiently associate *TAG1* to a genetic marker SNP3/4 (Figure 2) which is only ~2 cM away from *TAG1* without using neither prior preliminary mapping information nor tobacco reference genome. We anticipate that narrower mapping interval would be obtained when more individuals were pooled in each bulk (e.g. 200 individuals per pool) and higher sequencing depth was used.

Comparative mapping takes advantage of the presence of microsynteny between the species to be analyzed and its close relatives which have high-resolution genetic map or well-assembled reference genome (Goldberg et al., 2010; Zhang et al., 2010). Although tobacco genome has undergone large-scale structure variations in the genome after speciation, microsynteny with other *Solanaceae* species (e.g. tomato) still exists (Sierro et al., 2014). In this study, we explored the microsynteny regions between tomato and tobacco and fine mapped *TAG1* to a tomato interval with only 15 candidate genes and further successfully pinpointed the candidate gene to a tobacco mapping interval with only six genes, suggesting that comparative mapping is a powerful tool for map-based cloning in tobacco.

Most of the tobacco genome retained after speciation from its two ancestral species (Lim et al., 2007), thus many traits are therefore regulated or controlled by pairs of homoeologous genes (Edwards et al., 2017; Wu et al., 2019). In this study, we showed that the asymptomatic response to TMV, a qualitative trait in tobacco, was also caused by loss-of-function mutations in a pair of homoeologous genes that are required for the efficient accumulation of TMV. Taken together, we demonstrated that, BSA combined with RNA-seq and comparative genetic mapping is a powerful

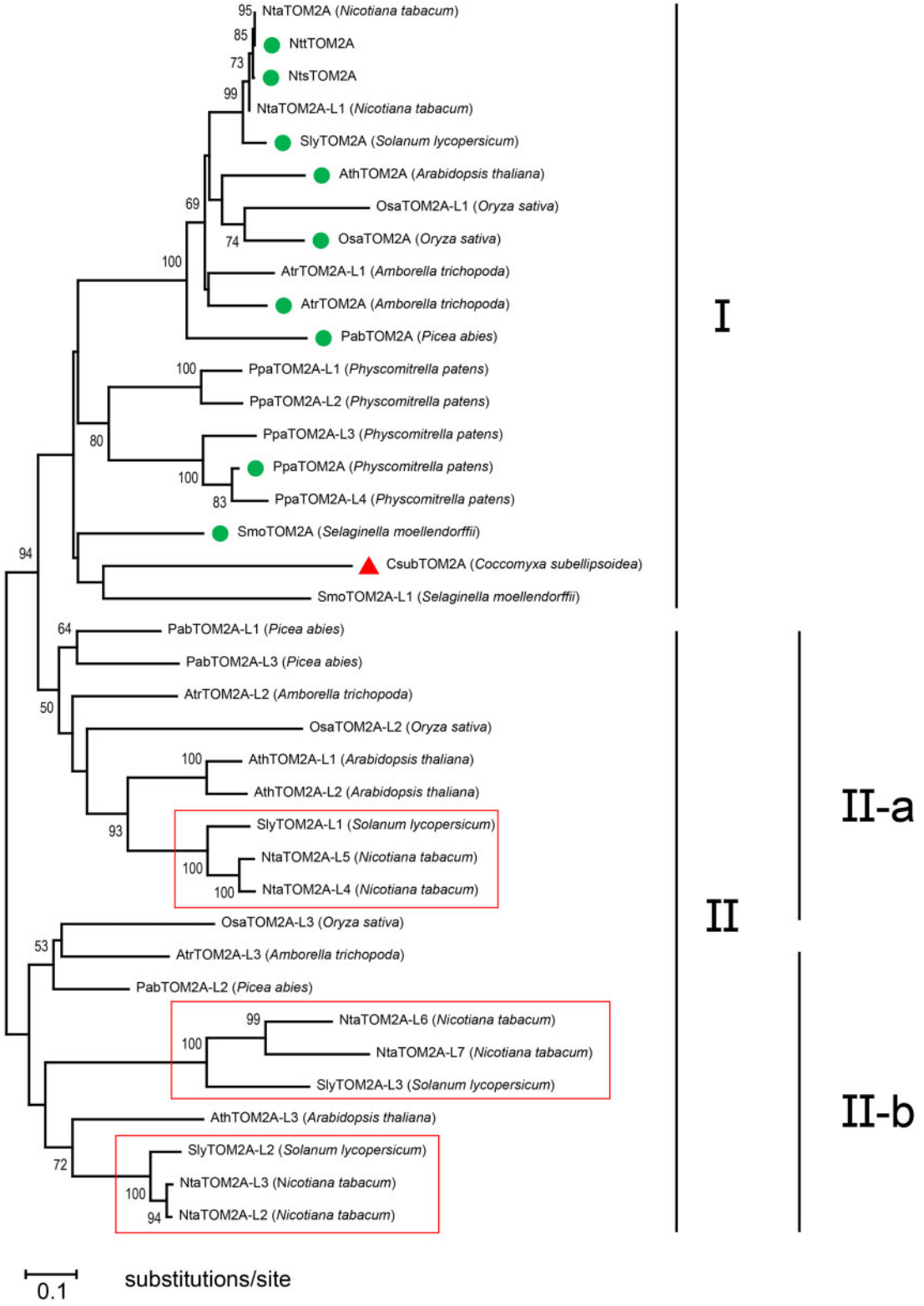

Figure 8 Phylogeny of TOM2A orthologs and paralogs from nine species. Only the amino acid sequences from the conserved domain were used for the phylogenetic analysis. The sequences were named as "*Abc*TOM2A or *Abc*TOM2A-L#," where "A" is the first letter of the genus name, "bc" represents the first two letters from the species name, and "#" represents the serial number. The relevant species names are indicated in parentheses. The functionally characterized genes are indicated with either green circles (promotes the accumulation of TMV) or red triangles (does not promote the accumulation of TMV). Bar represents substitution per site. Genes lacking the capacity to support the accumulation of TMV are indicated with red boxes. The numbers associated with each node are bootstrap values; only bootstrap values >50 are shown.

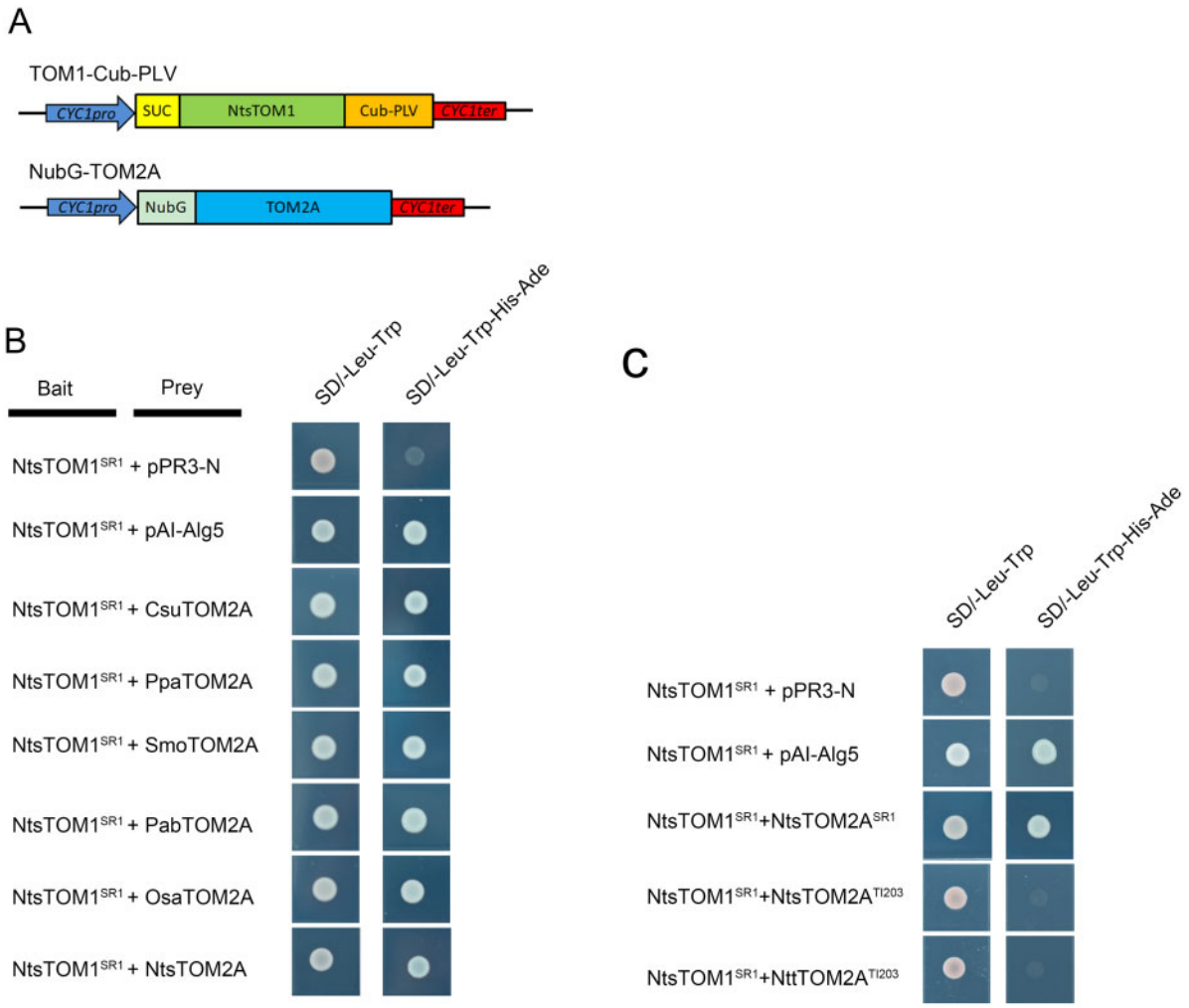

Figure 9 Split-ubiquitin system showing interaction between TOM1 and TOM2A orthologs. A, Schematic diagram of bait and prey constructs. The NtsTOM1 was fused at its C-terminal by Cub-PLV and serves as bait construct; the TOM2A was fused at its N-terminal by the mutant form of ubiquitin N-terminal fragment (NubG) and servers as prey construct. *CYC1pro*, cytochrome C-1 (*CYC1*) promoter; *CYC1ter*, *CYC1* terminator; SUC, cleavable signal sequence from yeast SUC2 gene; Cub, C-terminal fragment of ubiquitin; PLV, a protein A-LexA-VP16 reporter polypeptide; NubG, N-terminal fragment of mutant form of ubiquitin. B, Yeast membrane-based Split-Ubiquitin protein–protein interaction analysis of $NtTOM1^{SR1}$ with TOM2A orthologs. The bait construct ($NtsTOM1^{SR1}$-Cub-PLV) was co-transformed with prey constructs using standard lithium transformation method. Co-transformation of $NtsTOM1^{SR1}$-Cub-PLV with either pPR3-N or pAI-Alg5 serves as negative and positive control, respectively. After transformation, the yeast was grown on vector selective medium (SD/-Trp/-Leu) and protein interaction selective medium (SD/-Trp/-Leu/-His/-Ade). Photographs were taken 3 d after plating.

strategy for map-based cloning in allotetraploid species with complex genome. Given the fact that many agronomically important crops are polyploid and many of them lack well-assembled genome, the strategy demonstrated here would facilitate the isolation of agronomically important genes and thereby accelerate genetic improvement in polyploidy crops.

High conservation of *TOM2A* orthologs with an unknown function in plants

Our previous study showed that in addition to some cultivated tobacco, such as TI203, six wild *Nicotiana* species also develop asymptomatic responses to TMV infection (Yuan et al., 2015). In this study, we showed that the asymptomatic response to TMV in *N. glauca* was probably not caused by the loss of *TOM2A* function, indicating that other genes in addition to TOM2A are involved in the asymptomatic phenotype in the *Nicotiana* species. The *TOM2A* orthologs were highly conserved not only in the six wild *Nicotiana* species with asymptomatic responses but also in all land plants. The finding that TOM2A-like genes are present in some species of Chlorophyte (*C. subellipsoidea*, *O. tauri*, *O. lucimarinus*, *C. variabilis*, *B. prasinos*, and *V. carteri*) but not in Rhodophyte or Glaucophyte or other species out of plant kingdom (Supplemental Table S6) indicates that the TOM2A-like genes are restricted to the plant kingdom and had an ancient origin, possibly originated from Chlorophyte. It had

been reported that AtTOM2A promotes TMV multiplication through interacting with AtTOM1 (Tsujimoto et al., 2003). Our results showed that tobacco TOM1 could interact not only with tobacco TOM2A but also with TOM2A orthologs from other distantly related species, suggesting that TOM2A–TOM1 module is highly conserved in plant kingdom.

These data also provide evidence that the function of *TOM2A* is highly conserved and that strong selective pressure has prevented the accumulation of loss-of-function mutations in *TOM2A*. Consistent with this hypothesis, Arabidopsis mutants deficient in all three *TOM2A* homologs grew more slowly than wild-type (Fujisaki et al., 2008). Our expression analysis also showed that the DEGs were enriched in some important and fundamental pathways (Supplemental Figure S8). Moreover, in tomato, a *SlTOM2A* knockout mutant developed bent branches. In the future, it will be interesting to investigate the biochemical function of this protein in plant hosts and its association with virus replication. Additionally, the expression of *TOM2A* was elevated in response to abiotic stresses, such as salt stress and dehydration stress (Supplemental Figure S7). We hypothesize that *TOM2A* and its homologs have important but currently undetermined functions and that the fundamental role of TOM2A in plant hosts outweighs the damage suffered during TMV infection, leading to its high conservation in the plant kingdom.

Promising target for antivirus resistance breeding

Pathogens replicate in their hosts. Theoretically, disruption of host pathways necessary for the replication of particular pathogens would yield plants resistant to particular pathogens. However, the pathways employed by the pathogens are maintained in the host plants simply because they are indispensable for the survival of the host. For example, the loss of function of a NAC (NAM, ATAF, and CUC) domain-containing protein named RICE DWARF VIRUS MULTIPLICATION 1 (RIM1) in rice confers high tolerance to the rice dwarf virus; however, root growth is inhibited in the *rim1* mutant (Yoshii et al., 2010). Although the *Atmlo2 Atmlo6 Atmlo12* triple mutant in Arabidopsis provides resistance to powdery mildew, its roots develop abnormally and its pollen tubes overgrow (Consonni et al., 2006). The Arabidopsis *ssi* mutant is resistant to bacteria, oomycetes, and CMV but is dwarf with an abnormal cell death phenotype (Sekine et al., 2004). Therefore, development of plants with resistance to the pathogens without introducing other detrimental effects is generally preferred.

We showed that knocking out *TOM2A* orthologs leads to TMV-tolerant tobacco and tomato. Unlike other pathogen-associated genes, knocking out individual *TOM2A* genes in Arabidopsis and its orthologs in tobacco did not produce a major phenotype. Indeed, we observed only a minor phenotype in tomato—bent branches. Therefore, although *TOM2A* seems to be dispensable in agricultural production, it may be important during evolution. We predict that the *TOM2A* ortholog knockout mutants might produce durable resistance against TMV, in contrast to the resistance provided by many disease resistance genes, which may be overcome in one or a few years after large scale use of disease-resistant germplasm (Ashfield et al., 2004; Ishibashi et al., 2007; Strasser and Pfitzner, 2007).

Tobamoviruses can multiply in *TOM2A* mutants of Arabidopsis, tomato, and tobacco, though the multiplication may be severely affected. Therefore, there are other components or pathways parallel to TOM2A for virus multiplication in plant genomes. Different viruses may prefer different pathways for multiplication in a species. YoMV may prefer the TOM2A pathway in Arabidopsis, but ToMV may prefer the alternative pathway since the mutation of TOM2A had little effect on ToMV multiplication in Arabidopsis. We found that all tobamoviruses investigated in this study including ToMV prefer the TOM2A pathway for multiplication, since ToMV multiplication in tomato *sltom2a* mutants was severely affected (Figure 5).

TMV infects all of the important crops from the Solanaceae family, including tomato, potato, pepper, and eggplant. Our evolutionary analysis provides evidence that promoting the accumulation of TMV is a highly conserved function among the *TOM2A* orthologs in plants. Therefore, we conclude that the *TOM2A* orthologs are promising targets for the engineering of improved resistance to TMV in Solanaceae species and other economically important crops and that knocking out *TOM2A* orthologs has limited effects on other agriculturally important traits.

Summary and conclusions

In summary, we showed that the asymptomatic response to TMV in tobacco is due to low accumulation of TMV and is controlled by simultaneous mutations of two TOM2A homeologues. We provide a successful example of map-based cloning in tobacco without using any prior mapping information by combining BSA, RNA-seq, and comparative genetic mapping, which could accelerate the gene cloning in other polyploidy species. We provided evidence that TOM2 family was ancient, plant-specific, and likely originated from Chlorophyte. We show that the function of TOM2A in supporting TMV multiplication as well as the TOM1–TOM2A module is highly conserved in plant kingdom, suggesting a conserved nature of TOM2A function. Further work is required to explore the biological function of TOM2A homologs in plants. In addition, we demonstrated that TOM2A could be a promising target for developing TMV-resistant crops by generating TMV-resistant tobacco and both TMV and ToMV-resistant tomato plants using CRISPR/Cas9 technology.

Materials and methods

Plant materials and growth conditions

Cultivated tobacco (*N. tabacum*) SR1, TI203, and other *Nicotiana* plants were obtained from the Germplasm Resources Information Network (https://www.ars-grin.gov) or provided by the Yunnan Tobacco Academy of China.

Unless indicated otherwise, all of the plants used in this study were grown in a growth room at 24 \tf="TT5843c571"\char"8B 2°C in a photoperiod containing 16 h of light and 8 h of dark.

The nearly isogenic lines (NILs) for RNA-sequencing were obtained by backcrossing the F_1 hybrid with TI203 7 times (BC_7). Two genetic markers named M315 and M373 (Supplemental Table S4) that flank *NtsTOM2A* and are 0.45 and 0.30 cM from *NtsTOM2A*, respectively, were used to assist the development of the NILs.

Virus and virus inoculation

TMV-U1 was propagated and maintained in SR1, which is susceptible to TMV. ToMV sap was kindly provided by Professor Feng Li (Huazhong Agricultural University, China). Virus sap was prepared by grinding systemically infected plant leaves in sterile water (1 g tissue: 2 mL sterile water). Virus inoculation was performed by the rub-inoculation of virus sap onto the third and fourth true leaves of plants with 5–6 true leaves (i.e. ~4- to 5-week-old plants). Inoculated plants were grown at 22–24°C in a photoperiod containing 16 h of light and 8 h of dark.

Mapping population and phenotype scoring

To map *TAG1 and TAG2*, two BC_2 segregating populations that segregated alleles of only *TAG1* or *TAG2* were constructed by backcrossing SR1 with TI203. All populations and their parents were grown in a greenhouse at Huazhong Agricultural University, Wuhan, China. Phenotypes of plants post TMV inoculation were scored by visual inspection of upper noninoculated leaves at 10–30 dpi depending on the plant growth rate. Relative levels of TMV accumulation in the un-inoculated upper leaves of plants were measured using an immunoblotting assay with an anti-TMV coat protein antibody. The specificity of the TMV antibody was tested before use.

Plasmid construction and plant transformation

A 9,048-bp DNA fragment containing 2,075 bp from the promoter region, all exons, and introns, and 1,002-bp downstream of the stop codon was amplified from SR1 using a nested PCR method. Similarly, a 10,010-bp DNA fragment containing *NtsTOM2A* was obtained that includes 2,338 bp from the promoter, all exons and introns, and 1,005-bp downstream of the stop codon. PCR products were gel purified (CWBIO, CN) and cloned into pH7LIC14 using a Ligation Independent Cloning method.

The putative coding sequences of *TOM2A* orthologs from different plant species were either amplified from cDNA or synthesized by the Tsinke Company. PCR products that contained the adapter and coding sequence were purified and cloned into XhoI-XbaI-digested pHellsgate 8 using homologous recombination kits (Vazyme, Nanjing, China) following the manufacturer's instructions to generate overexpression constructs.

All binary vectors were introduced into the GV3101 strain of *Agrobacterium tumefaciens* using the heat shock method and used to transform TI203. Tobacco transformation was performed using the leaf disc method as described previously (Gallois and Marinho, 1995). For each construct, at least eight T_0 transgenic lines were obtained. Tomato (*S. lycopersicum*) transformation was performed as described previously (Sun et al., 2006). The phenotype was scored at 10–30 dpi depending on the plant growth rate. All primers used for making constructs are listed in Supplemental Table S7.

The split-ubiquitin system

Plasmids pBT3-SUC and pPR3-NubG were used to make bait and prey constructs, respectively. The full-length coding sequence of *NtsTOM1*^{SR1}, *NtsTOM2A*^{SR1}, *NtsTOM2A*^{TI203}, *NttTOM2A*^{TI203}, and *TOM2A* orthologs from six other species were amplified from their corresponding overexpression constructs. PCR fragments containing appropriate adapters at both ends were purified and subcloned into *Sfi*I sites of pBT3-SUC (for bait construct) or pPR3-N (for prey construct) using homologous recombination method. The yeast strain NMY51 was used to express the bait and prey vectors. Self-activation of the bait construct (pBT3-NtsTOM1^{SR1}-Cub-PLV) was tested on SD/-Trp/-Leu/-His/-Ade selective medium. Co-transformation of bait and prey constructs, pPR3-N (negative control), pAI-Alg5 (positive control) into yeast strain NMY51 was performed using the standard lithium acetate/SS-DNA/PEG method (Grefen et al., 2009). After transformation, the yeast was grown on vector selective medium (SD/-Trp/-Leu) and incubated at 30°C for 3 d. At least three independent yeast clones were pooled, resuspended in 0.9% NaCl, and diluted in a series of 10, 100, and 1,000 times. For protein interaction test, 3 μL of each diluted liquid culture was dropped on the interaction selective medium (SD/-Leu/-Trp/-His/-Ade) and incubated at 30°C for 3 d. All primers used to make bait and prey constructs were listed in Supplemental Table S7.

DNA-seq/RNA-seq and bioinformatics analysis

For BSA RNA-seq, plants were grown at 25°C in a photoperiod that contained 16 h of light and 8 h of dark. Total RNA from the fifth true leaves of TTSL2-NIL and TI203 was extracted using the TransZol reagent (Transgene, China) following the manufacturer's protocol. The nondirectional paired-end RNA-seq sequencing library was prepared using the Illumina TruSeq RNA sample preparation kit (version 2). The libraries were sequenced on an Illumina HiSeq 2000 platform to obtain 100-bp paired-end reads. For DNA-seq, genomic DNA from TI203 and P50 (a transgenic plant from the SR1 genetic background) were extracted from 30-d-old plants and sent to the company for library construction and paired-end sequencing.

Raw NGS reads were filtered using Trimmomatic version 0.33 (Bolger et al., 2014). Filtered reads were then aligned to the combined tobacco reference genome using HISAT2 (Kim et al., 2015) for RNA-seq data or using BWA (Li and Durbin, 2009) for DNA-sequencing data. The combined tobacco reference genome was generated by combing the

subgenomes from *N. sylvestris* and *N. tomentosiformis* (Sierro et al., 2013). SNPs were derived from a comparison of bulk pools made from TI203 and TG34. An SNP allele frequency difference (ΔSNP-index) was determined for each SNP using custom Perl scripts. DEGs were defined as genes with at least a two-fold difference in expression levels and a false discovery rate of <0.05 using the package DESeq2 in R (Love et al., 2014).

RT-qPCR

For RT-qPCR, total RNA was extracted from different fresh tissues using the RNA ISO Plus kit (Transgene) following the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of RNA using the HiScrip II first-strand cDNA synthesis kit (TRANSGEN Biotech, China). Primers used for RT-qPCR were developed using primer3 with annealing temperatures ranging from 59°C to 63°C and amplification product lengths ranging from 100 to 300 bp. *Actin* (Asano et al., 2005), a housekeeping gene, was used as an internal control. RT-qPCR was performed with a LightCycler 480 system with the following protocol: initial denaturation at 50°C for 5 min and 95°C for 3 min followed by 39 cycles of 95°C for 15 s and 60°C for 40 s, followed by a final step at 95°C for 10 s. All primers used for RT-qPCR are listed in Supplemental Table S7. All experiments were performed with three biological replicates. Relative gene expression levels under each treatment were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Identification of TOM2A orthologs from tobacco and other plant species

The *TOM2A* orthologs from wild tobacco were amplified using primers listed in Supplemental Table S7 and sequenced. *TOM2A* homologs from other plant species were retrieved by performing database searches with BLASTP using the AtTOM2A amino acid sequence as a query. We concluded that these amino acid sequences were encoded by *TOM2A* homologs if (1) the BLASTP evalue $\leqslant$1e-5 and (2) the sequences were predicted to contain four transmembrane helices using the TPCON software (Tsirigos et al., 2015).

Phylogenetic analysis

The putative full-length protein sequence from each TOM2A was aligned using MUSCLE with default parameters (Edgar, 2004). The multiple sequence alignment was manually examined using BioEdit version 7.1.3 (Hall, 1999). The phylogenetic trees were generated with MEGA X, using the Neighbor-joining statistical method with 1,000 bootstrap replicates (Kumar et al., 2018).

CRISPR/Cas9 gene editing in tobacco and tomato

For gene editing in tobacco, two gRNAs that are conserved in *NttTOM2A* and *NtsTOM2A* were designed in the first exon of *NtTOM2A*. PCR products that contain appropriate adapters and gRNAs were assembly into pKSE401 (for tobacco) or pTX (for tomato) using a homologous recombination kit (Vazyme). Relative positions of gRNAs in tobacco and tomato are shown in Supplemental Figure S1. After confirming that there were no mutations, GV3101 was transformed with these plasmids. Transgenic plants were produced using an *Agrobacterium*-mediated plant transformation procedure.

Positive first-generation (T_0) transgenic lines were screened for the presence of the Cas9 transgene using the primers listed in Supplemental Table S7. At least three different leaves from transgenic T_0 plants were collected for detection of all possible alleles generated by the Cas9 protein. A target region was amplified with primers flanking the two gRNAs. PCR products were first directly sequenced. If double peaks or attenuated signals were detected in the sequence data, TA cloning was performed. At least six different colonies were repeatedly sent for sequencing until no additional alleles were identified. Homozygous mutant lines were identified by screening the T_1 population with PCR-based markers that were developed based on the polymorphisms between the mutant alleles and wild-type alleles. These mutants were independently analyzed using sequencing. Primers used for making CRISPR/Cas9 constructs and for genotyping are listed in Supplemental Table S7.

Accession numbers

The NGS data from this study have been deposited in the NCBI Sequence Read Archives and are associated with project accession PRJNA689122. RNA-Seq data from the two bulks, TI203 and its NIL, were deposited in Genbank and associated with the biosample accession numbers SAMN17199923 and SAMN17199924, SAMN17199927-SAMN17199930. Genome resequencing data for SR1 and TI203 were deposited in NCBI and associated with biosample accession numbers SAMN17199925 and SAMN17199926. *TOM2A* ortholog sequences that were amplified from *Nicotiana* species were deposited in Genbank and associated with accession numbers MW465313–MW465330. The *TOM2A* orthologs that were used for complementation tests in TI203 were deposited in GenBank and associated with accession numbers MW465331–MW465336, XM_005644316, XM_024511869, XM_024677129, XM_006849963, NM_001157355, XM_023903125, AK323532, XM_006485344, NM_001036050, and XM_004148450.

Supplemental data

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

Supplemental Figure S1. Comparative sequence, expression, and interaction analysis between $TOM2A^{SR1}$ and $TOM2A^{TI203}$.

Supplemental Figure S2. CRISPR/Cas9 knockout of *NtTOM2A and SlTOM2A*.

Supplemental Figure S3. Phenotype of *Sltom2a* CRISPR/Cas9 mutants.

Supplemental Figure S4. Amino acid sequence alignment of *Nicotiana* TOM2As.

Supplemental Figure S5. Asymptomatic phenotype of *N. glauca* upon TMV inoculation.

Supplemental Figure S6. Expression profile of *TOM2A* in tobacco.

Supplemental Figure S7. Elevated *AtTOM2A* expression under abiotic stress.

Supplemental Figure S8. GO term analysis of DEGs.

Supplemental Table S1. Genetic analysis of asymptomatic phenotype in F_2 and BC_1 population.

Supplemental Table S2. Genetic analysis of asymptomatic phenotype in BC_2 population.

Supplemental Table S3. SNP allele frequency between the two RNA-sequencing bulks.

Supplemental Table S4. Genetic markers used for genetic mapping.

Supplemental Table S5. Annotation of 15 genes in the tomato mapping interval.

Supplemental Table S6. Identification of TOM2A homologs in plant species.

Supplemental Table S7. Primers used in this study.

Supplemental Dataset S1. List of DEGs.

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References

Asano M, Satoh R, Mochizuki A, Tsuda S, Yamanaka T, Nishiguchi M, Hirai K, Meshi T, Naito S, Ishikawa M (2005) Tobamovirus-resistant tobacco generated by RNA interference directed against host genes. FEBS Lett **579**: 4479–4484

Ashfield T, Ong LE, Nobuta K, Schneider CM, Innes RW (2004) Convergent evolution of disease resistance gene specificity in two flowering plant families. Plant Cell **16:** 309–318

Beck DB, Aksentijevich I (2020) Susceptibility to severe COVID-19. Science **370:** 404–405

Beekwilder KM (1999) The Inheritance of Resistance to Tobacco Mosaic Virus in Tobacco Introductions. Master thesis. Virginia Polytechnic Institute and State University, Blacksburg, VA

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30:** 2114–2120

Chen MH, Sheng J, Hind G, Handa AK, Citovsky V (2000) Interaction between the *tobacco mosaic virus* movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. EMBO J **19:** 913–920

Chisholm ST, Parra MA, Anderberg RJ, Carrington JC (2001) Arabidopsis *RTM1* and *RTM2* genes function in phloem to restrict long-distance movement of tobacco etch virus. Plant Physiol **127:** 1667–1675

Clarkson JJ, Knapp S, Garcia VF, Olmstead RG, Leitch AR, Chase MW (2004) Phylogenetic relationships in *Nicotiana* (Solanaceae) inferred from multiple plastid DNA regions. Mol Phylogenet Evol **33:** 75–90

Consonni C, Humphry ME, Hartmann HA, Livaja M, Durner J, Westphal L, Vogel J, Lipka V, Kemmerling B, Schulze-Lefert P, et al. (2006) Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. Nat Genet **38:** 716–720

D'Hont A, Denoeud F, Aury JM, Baurens FC, Carreel F, Garsmeur O, Noel B, Bocs S, Droc G, Rouard M, et al. (2012) The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. Nature **488:** 213–217

Dong W, Wu D, Li G, Wu D, Wang Z (2018) Next-generation sequencing from bulked segregant analysis identifies a dwarfism gene in watermelon. Sci Rep **8**: 2908

Du P, Wu J, Zhang J, Zhao S, Zheng H, Gao G, Wei L, Li Y (2011) Viral infection induces expression of novel phased microRNAs from conserved cellular microRNA precursors. PLoS Pathog **7:** e1002176

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32:** 1792–1797

Edwards KD, Fernandez-Pozo N, Drake-Stowe K, Humphry M, Evans AD, Bombarely A, Allen F, Hurst R, White B, Kernodle SP, et al. (2017) A reference genome for *Nicotiana tabacum* enables map-based cloning of homeologous loci implicated in nitrogen utilization efficiency. BMC Genomics **18:** 448

Fujisaki K, Kobayashi S, Tsujimoto Y, Naito S, Ishikawa M (2008) Analysis of tobamovirus multiplication in *Arabidopsis thaliana* mutants defective in *TOM2A* homologues. J Gen Virol **89:** 1519–1524

Fujisaki K, Ravelo GB, Naito S, Ishikawa M (2006) Involvement of *THH1*, an *Arabidopsis thaliana* homologue of the *TOM1* gene, in tobamovirus multiplication. J Gen Virol **87:** 2397–2401

Fukuhara T, Tabara M, Koiwa H, Takahashi H (2020) Effect of asymptomatic infection with southern tomato virus on tomato plants. Arch Virol **165**: 11–20

Gallois P, Marinho P (1995) Leaf disk transformation using *Agrobacterium tumefaciens*-expression of heterologous genes in tobacco. Methods Mol Biol **49**: 39–48

Goldberg MT, Spigler RB, Ashman TL (2010) Comparative genetic mapping points to different sex chromosomes in sibling species of wild strawberry (Fragaria). Genetics **186**: 1425–1433

Grefen C, Obrdlik P, Harter K (2009) The determination of protein-protein interactions by the mating-based split-ubiquitin system (mbSUS). Methods Mol Biol **479**: 217–233

Hagiwara Y, Komoda K, Yamanaka T, Tamai A, Meshi T, Funada R, Tsuchiya T, Naito S, Ishikawa M (2003) Subcellular localization of host and viral proteins associated with tobamovirus RNA replication. EMBO J **22**: 344–353

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser **41**: 95–98

Ibrahim A, Yang X, Liu C, Cooper KD, Bishop BA, Zhu M, Kwon S, Schoelz JE, Nelson RS (2020) Plant SNAREs SYP22 and SYP23 interact with *Tobacco mosaic virus* 126 kDa protein and SYP2s are required for normal local virus accumulation and spread. Virology **547**: 57–71

Ishibashi K, Masuda K, Naito S, Meshi T, Ishikawa M (2007) An inhibitor of viral RNA replication is encoded by a plant resistance gene. Proc Natl Acad Sci USA **104**: 13833–13838

Ishibashi K, Naito S, Meshi T, Ishikawa M (2009) An inhibitory interaction between viral and cellular proteins underlies the resistance of tomato to nonadapted tobamoviruses. Proc Natl Acad Sci USA **106**: 8778–8783

Kim D, Langmead B, Salzberg SL (2015) HISAT: a fast spliced aligner with low memory requirements. Nat Methods **12:** 357–360

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol **35**: 1547–1549

Kushner DB, Lindenbach BD, Grdzelishvili VZ, Noueiry AO, Paul SM, Ahlquist P (2003) Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. Proc Natl Acad Sci USA **100**: 15764–15769

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics **25:** 1754–1760

Lim KY, Kovarik A, Matyasek R, Chase MW, Clarkson JJ, Grandbastien MA, Leitch AR (2007) Sequence of events leading to near-complete genome turnover in allopolyploid *Nicotiana* within five million years. New Phytol **175:** 756–763

Lim KY, Matyasek R, Kovarik A, Leitch AR (2004) Genome evolution in allotetraploid *Nicotiana*. Biol J Linn Soc **82:** 599–606

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25**: 402–408

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol **15**: 550

Martinez-Marrero N, Avalos-Calleros JA, Chiquito-Almanza E, Acosta-Gallegos JA, Ambriz-Granados S, Anaya-Lopez JL, Arguello-Astorga GR (2020) A new begomovirus isolated from a potyvirus-infected bean plant causes asymptomatic infections in bean and *N. benthamiana*. Arch Virol **165:** 1659–1665

Nicaise V (2014) Crop immunity against viruses: outcomes and future challenges. Front Plant Sci **5:** 660

Nishikiori M, Mori M, Dohi K, Okamura H, Katoh E, Naito S, Meshi T, Ishikawa M (2011) A host small GTP-binding protein ARL8 plays crucial roles in tobamovirus RNA replication. PLoS Pathog **7:** e1002409

Scholthof KB (2004) *Tobacco mosaic virus*: a model system for plant biology. Annu Rev Phytopathol **42:** 13–34

Sekine KT, Nandi A, Ishihara T, Hase S, Ikegami M, Shah J, Takahashi H (2004) Enhanced resistance to *Cucumber mosaic virus* in the *Arabidopsis thaliana ssi2* mutant is mediated via an SA-independent mechanism. Mol Plant Microbe Interact **17:** 623–632

Sierro N, Battey JN, Ouadi S, Bakaher N, Bovet L, Willig A, Goepfert S, Peitsch MC, Ivanov NV (2014) The tobacco genome sequence and its comparison with those of tomato and potato. Nat Commun **5:** 3833

Sierro N, Battey JN, Ouadi S, Bovet L, Goepfert S, Bakaher N, Peitsch MC, Ivanov NV (2013) Reference genomes and transcriptomes of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. Genome Biol **14:** R60

Strasser M, Pfitzner AJ (2007) The double-resistance-breaking *Tomato mosaic virus strain* ToMV1-2 contains two independent single resistance-breaking domains. Arch Virol **152**: 903–914

Su W, Tao R, Liu W, Yu C, Yue Z, He S, Lavelle D, Zhang W, Zhang L, An G, et al. (2020) Characterization of four polymorphic genes controlling red leaf colour in lettuce that have undergone disruptive selection since domestication. Plant Biotechnol J **18:** 479–490

Sun HJ, Uchii S, Watanabe S, Ezura H (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol **47:** 426–431

Tsirigos KD, Peters C, Shu N, Kall L, Elofsson A (2015) The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. Nucleic Acids Res **43:** W401–407

Tsujimoto Y, Numaga T, Ohshima K, Yano MA, Ohsawa R, Goto DB, Naito S, Ishikawa M (2003) *Arabidopsis TOBAMOVIRUS MULTIPLICATION (TOM) 2* locus encodes a transmembrane protein that interacts with TOM1. EMBO J **22**: 335–343

Wang Z, Yu A, Li F, Xu W, Han B, Cheng X, Liu A (2021) Bulked segregant analysis reveals candidate genes responsible for dwarf formation in woody oilseed crop castor bean. Sci Rep **11:** 6277

White JF, Kingsley KL, Zhang Q, Verma R, Obi N, Dvinskikh S, Elmore MT, Verma SK, Gond SK, Kowalski KP (2019) Review: endophytic microbes and their potential applications in crop management. Pest Manag Sci **75:** 2558–2565

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene *N*: similarity to toll and the interleukin-1 receptor. Cell **78:** 1101–1115

Wu X, Gong D, Xia F, Dai C, Zhang X, Gao X, Wang S, Qu X, Sun Y, Liu G (2019) A two-step mutation process in the double *WS1* homologs drives the evolution of burley tobacco, a special chlorophyll-deficient mutant with abnormal chloroplast development. Planta **251:** 10

Yamanaka T, Imai T, Satoh R, Kawashima A, Takahashi M, Tomita K, Kubota K, Meshi T, Naito S, Ishikawa M (2002) Complete inhibition of tobamovirus multiplication by simultaneous mutations in two homologous host genes. J Virol **76**: 2491–2497

Yamanaka T, Ohta T, Takahashi M, Meshi T, Schmidt R, Dean C, Naito S, Ishikawa M (2000) *TOM1*, an *Arabidopsis* gene required for efficient multiplication of a tobamovirus, encodes a putative transmembrane protein. Proc Natl Acad Sci USA **97**: 10107–10112

Yoshii M, Yamazaki M, Rakwal R, Kishi-Kaboshi M, Miyao A, Hirochika H (2010) The NAC transcription factor *RIM1* of rice is a new regulator of jasmonate signaling. Plant J **61:** 804–815

Yuan X, Yan C, Wu Z, Ren F, Zhang H, Baker B, Chen J, Kuang H (2015) Frequent gain and loss of resistance against *Tobacco Mosaic Virus* in *Nicotiana* species. Mol Plant **8:** 1813–1815

Zhang H, Guan H, Li J, Zhu J, Xie C, Zhou Y, Duan X, Yang T, Sun Q, Liu Z (2010) Genetic and comparative genomics mapping reveals that a powdery mildew resistance gene Ml3D232 originating from wild emmer co-segregates with an NBS-LRR analog in common wheat (Triticum aestivum L). Theor Appl Genet **121:** 1613–1621

Zheng Y, Gao S, Padmanabhan C, Li R, Galvez M, Gutierrez D, Fuentes S, Ling KS, Kreuze J, Fei Z (2017) VirusDetect: an automated pipeline for efficient virus discovery using deep sequencing of small RNAs. Virology **500**: 130–138