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Letter to the Editor

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Frequent Gain and Loss of Resistance against *Tobacco Mosaic Virus* in *Nicotiana* Species

Dear Editor,

Resistance (*R*) genes represent one of the most divergent gene families in plants. Novel resistance function might arise through point mutations or sequence exchanges between paralogues (Kuang et al., 2004; Luo et al., 2011, 2012). Sequence exchanges between homologues may generate a large number of distinct genes. In fact, some *R* gene families are extensive chimeras, contributing considerable resistance diversity for a species. In contrast, other *R* gene families are highly conserved between different genotypes. Distantly related plant species may contain resistance against the same pathogen. Such ''conserved'' resistance in different species might be inherited from their common ancestor or result from convergent evolution (Ashfield et al., 2004; Li et al., 2012). How frequently convergent evolution has occurred in nature and its underlying mechanism remain poorly understood. We screened different *Nicotiana* species using *Tobacco Mosaic Virus* (TMV), and the origin and evolution of TMV resistance were studied in detail.

After screening 40 wild *Nicotiana* species using TMV-U1 isolate, 10 species were found that are resistant to TMV and show hypersensitive response (HR) at inoculation sites (Supplemental Table 1). The 10 TMV-resistant species were distributed randomly in the phylogenetic tree for *Nicotiana* species, with only a few exceptions (Supplemental Figure 1).

To check if the resistance genes in the 10 TMV-resistant species recognize the same avirulence gene from TMV, the helicase domain of replicase (P50), the movement protein and coat protein (CP) encoding sequences in TMV were transiently expressed on leaves of the 10 resistant species. Five species (*N. glutinosa*, *N. paniculata*, *N. repanda*, *N. stocktonii* and *N. undulata*) had HR after agroinfiltration of *p50*, while *N. goodspeedii* had obvious HR to *CP*. The other four species had unstable HR to *CP*. Therefore, the TMV resistance genes in the 10 species have different avirulence genes, suggesting their independent origin (convergent evolution).

The TMV resistance in *N. glutinosa* was encoded by the *N* gene (Whitham et al., 1994). The *N* gene was sequenced from seven genotypes of *N. glutinosa*, and two distinct alleles were obtained. The two alleles have 46 polymorphic sites including 43 single nucleotide polymorphisms (SNPs) and three 1-bp indels. Of these, 34 are located in introns (with a total of 3223 bp), while the other 12 are in the coding region and eight of them cause amino acid substitutions (Figure 1A). Using a mutation rate of 1×10^{-8}, the two alleles were estimated to have diverged 1 million years ago.

To better understand the evolution of the *N* family, *N* homologues were PCR amplified from the 10 TMV-resistant and five randomly chosen susceptible species. The number of distinct *N* homologues amplified from a genotype varied from one (*N. goodspeedii*) to 22 (*N. forgetiana* and *N. stocktonii*). The *N* homologues obtained above as well as *N* homologues from the sequenced *Nicotiana* genomes were aligned and a neighbor-joining tree was constructed (Supplemental Figure 2). The *N* gene and nine homologues form a tight clade on the distance tree, showing an obvious orthologous relationship. The ratios of nonsynonymous (*Ka*) to synonymous nucleotide substitutions (*Ks*) between the hypervariable sites of the *N* gene and its orthologues were calculated. The *Ka*/*Ks* ratios between the *N* gene and eight *N* orthologues were higher than 1 (1.40–5.60), showing diversifying selection. Diversifying selection may suggest functional divergence (i.e., they have distinct resistance function). Alternatively, if the orthologues encode the same function as resistance against TMV, diversifying selection among orthologues may indicate an independent origin of their TMV resistance function.

The full resistance function of the *N* gene requires its two alternatively spliced transcripts (Dinesh-Kumar and Baker, 2000). The alternative exon is provided by a solo-LTR (mistakenly annotated as an MITE in a previous study) in intron III of the *N* gene (Kuang et al., 2009). To investigate the origin of the alternative exon of the *N* gene and evolution of the functionally important intron III, intron III of *N* orthologues were amplified from eight *Nicotiana* species (Figure 1A). The intron III sequences vary from 1478 (*Nc555493-N4*) to 6309 bp (*Np555550-N3*). Frequent insertions, deletions, and inversions as well as point mutations were detected among the intron III sequences of *N* orthologues (Figure 1A). The solo-LTR in the *N* gene was not found in any other orthologue, which was further confirmed using a PCR strategy. We conclude that the alternative exon of the *N* gene and its consequent TMV resistance function originated after the speciation of *N. glutinosa*.

To clone the TMV resistance gene in *N. paniculata*, an F_2 population segregating TMV resistance was derived from a cross between TMV-resistant (accession PI555550) and TMV-susceptible genotypes (PI241769). At least three *N* homologues were mapped to the *N* locus, and the *N* orthologue *Np555550-N3* (marker M-Np-N3, Supplemental Table 2) cosegregated with the TMV resistance. To verify the function of the *Np555550-N3* gene, its full-length cDNA sequence was cloned. Co-infiltration of *Np555550-N3* and *p50* on leaves of *N. benthamiana* caused HR at the infiltration site, while no HR was found for negative controls (Figure 1B), confirming that the *Np555550-N3* in *N. paniculata* accession PI555550 encodes TMV resistance.

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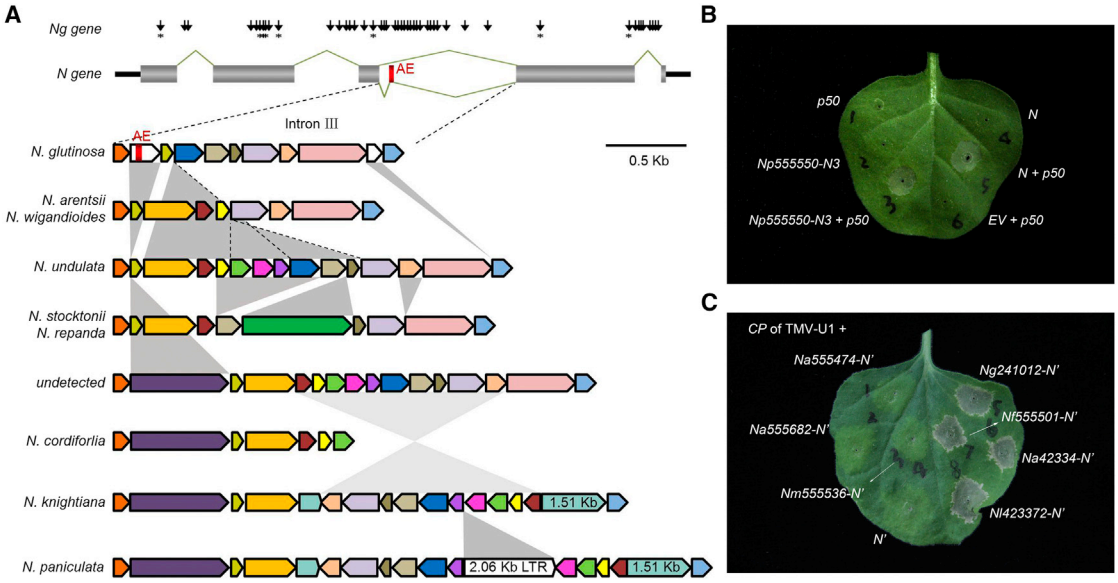

Figure 1. The Evolution of Intron III in *N* Orthologues and Function Assay of Tobacco Mosaic Virus Resistance Genes.

(A) Polymorphisms between the *N* and *Ng* alleles and the evolution of intron III in *N* orthologues. The two *N* alleles have 46 polymorphic sites, as marked by arrows. The eight SNPs causing amino acid substitutions are indicated by asterisks. The boxes in the *N* gene represent exons and AE refers to an alternative exon. The homologous sequences of intron III in different *N* orthologues are illustrated in the same color. The gray/light gray triangles between orthologues represent the insertion/inversion events. The two overlapping triangles are delimited by dashed lines. The scale bar applies to intron III sequences only.

(B) Transient co-expression of *Np555550-N3* with its avirulence (*avr*) gene *p50*. Hypersensitive response (HR) was observed 2–3 days post inoculation (dpi) at an infiltration site when *Np555550-N3* was co-infiltrated with *p50*. *N* + *p50* was used as a positive control, while *p50*, *N*, *Np555550-N3* alone, or empty vector (EV) + *p50* were used as negative controls.

(C) Transient co-expression of *N′* orthologues with coat protein gene (*CP*) of TMV. HR was observed at infiltration sites with *Ng241012-N′*, *Na42334-N′*, *Nf555501-N′,* and *Nl423372-N′* but not at infiltration sites with negative controls *Na555474-N′*, *Na555682-N′*, *Nm555536-N′*, or *N′*.

The coding sequences of the *Np555550-N3* gene (3393 bp) and the *N* gene exhibit 93.9% nucleotide identity. The *Ka*/*Ks* ratio between the hypervariable sites of the *N* and *Np555550-N3* genes is 1.87, and the diversifying selection suggests that their function might have been gained independently (i.e., convergent evolution) although they are obvious orthologues. Their independent origin is consistent with the fact that the indispensable alternative exon of the *N* gene is absent in the *Np555550-N3* gene.

Compared with the *Np555550-N3* gene, its susceptible allele, *Np241769-N3* (from PI241769), has 32 SNPs and a 3-nt deletion. Of them, 21 SNPs led to amino acid substitutions (Supplemental Table 3). Sequence analysis with other *N* orthologues showed that nine of the 22 sites (21 point mutations and one deletion) were due to mutations in the resistant allele, while 13 of the polymorphic sites were caused by mutations in the susceptible allele. If the *Np555550-N3* gene originated after the speciation of *N. paniculata* (see above), the mutation(s) at one or more of the nine sites in the resistant allele should have caused it to gain the TMV resistance function.

A similar genetic approach was applied to the study of TMV resistance in *N. undulata*. The TMV resistance trait in *N. undulata* was also controlled by a single gene, which was genetically mapped to the *N* locus. Interestingly, the *N* orthologue (*Nu306637-N6*) in *N. undulata* is 0.7 cM away from the TMV resistance trait. Therefore, the TMV resistance gene from *N. undulata* was believed to have been generated independently from an *N* paralogue in *N. undulata*.

Like the resistance gene *N′* from *N. sylvestris* and the gene L^3 from pepper (Sekine et al., 2012), both encoding resistance against tomato mosaic virus, the TMV resistance gene from *N. goodspeedii* has HR to the *CP* of TMV. Reciprocal BLAST results showed that the L^3 gene from pepper is not an *N′* orthologue, suggesting that the tobamovirus resistance in pepper and *Nicotiana* was originated independently. To investigate if *N′* orthologues in other *Nicotiana* species encode resistance against TMV, *N′* orthologues were cloned from TMV-resistant *Nicotiana* species as well as randomly chosen susceptible species. Full-length *N′* genes were obtained from TMV-resistant species *N. gossei*, *N. goodspeedii*, *N. forgetiana*, *N. alata* (PI42334), and *N. langsdorffii*, as well as TMV-susceptible species *N. alata* (PI555474), *N. amplexicaulis*, *N. megalosiphon*, and *N. sylvestris*. HR was observed when the *CP* of TMV was co-infiltrated with *N′* orthologues from *N. alata*, *N. forgetiana*, *N. goodspeedii* and *N. langsdorffii*, while the negative control (*Na555474-N′*/*Na555682-N′*/*Nm555536-N′*/*N′* + *CP* of TMV) showed no HR (Figure 1C). In addition, the *Ng230953-N′1* gene from *N. gossei* might be the TMV resistance gene since it triggered unstable HR when co-expressed with *CP* of TMV. This is consistent with the fact that *N. gossei*, *N. goodspeedii*, *N. forgetiana*, *N. alata*, and *N. langsdorffii* are resistant against TMV.

Analysis of hypervariable sites showed that the *N′* gene family was under purifying selection (*Ka*/*Ks* = 0.54). The *Nicotiana* species with TMV-resistant *N′* orthologues spread in a well-supported clade (bootstrap value of 99) on the phylogenetic

tree for *Nicotiana* species (Supplemental Figure 1). Therefore, the common ancestral species of this clade might have a functional TMV-resistant *N′* orthologue; the TMV resistance in *N. goodspeedii*, *N. forgetiana*, *N. alata* (PI42334), and *N. langsdorffii* were inherited from their common ancestor; and some species (such as *N. megalosiphon*) in this clade lost their TMV resistance after speciation.

In conclusion, cloning and functional analysis of six TMV resistance genes from *Nicotiana* species showed that the TMV resistance is encoded by either an *N* (TIR-NBS-LRR) or *N′* (CC-NBS-LRR) homologue. TMV resistance encoded by *N* homologues has originated at least three times in *Nicotiana*. It is possible that additional gain/loss of TMV resistance events may be discovered with more *Nicotiana* genotypes/species investigated in future studies. TMV resistance encoded by *N′* homologues arose independently in pepper and *Nicotiana*. However, the *N′*-encoded TMV resistance was ancient in *Nicotiana*; at least five *Nicotiana* species inherited their *N′*-encoded TMV resistance from this common ancestor, but dozens of species lost their *N′*-encoded TMV resistance after speciation.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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