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 non-synonymous (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 F₂ 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.
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 (*Nu*306637-*N*) 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* parologue in *N. paniculata*.

Like the resistance gene *N* from *N. sylvestris* and the gene *L* 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* 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* (*Pl42334*), and *N. langsdorffii*, as well as TMV-susceptible species *N. alata* (*Pl555474*), *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 *Ng*230953-*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
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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