Functional Differentiation in the Leucine-Rich Repeat Domains of Closely Related Plant Virus-Resistance Proteins That Recognize Common Avr Proteins

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The *N'* gene of *Nicotiana sylvestris* and *L* genes of *Capsicum* plants confer the resistance response accompanying the hypersensitive response (HR) elicited by tobamovirus coat proteins (CP) but with different viral specificities. Here, we report the identification of the *N'* gene. We amplified and cloned an *N'* candidate using polymerase chain reaction primers designed from *L* gene sequences. The *N'* candidate gene was a single 4143 base pairs fragment encoding a coiled-coil nucleotide-binding leucine-rich repeat (LRR)-type resistance protein of 1,380 amino acids. The candidate gene induced the HR in response to the coexpression of tobamovirus CP with the identical specificity as reported for *N'*. Analysis of *N'*-containing and tobamovirus-susceptible *N. tabacum* accessions supported the hypothesis that the candidate is the *N'* gene itself. Chimera analysis between *N'* and *L*³ revealed that their LRR domains determine the spectrum of their tobamovirus CP recognition. Deletion and mutation analyses of *N'* and *L*³ revealed that the conserved sequences in their C-terminal regions have important roles but contribute differentially to the recognition of common avirulence proteins. The results collectively suggest that *Nicotiana N'* and *Capsicum L* genes, which most likely evolved from a common ancestor, differentiated in their recognition specificity through changes in the structural requirements for LRR function.

The use of plant resistance (*R*) genes to breed resistant crops continues to be an effective strategy for maintaining crop yield. An *R* gene confers effector-triggered immunity (ETI; formerly termed gene-for-gene resistance) to a specific pathogen carrying a corresponding avirulence (Avr) effector gene (Flor 1971; Jones and Dangl 2006). ETI is generally associ-

ated with lesions of programmed cell death in or around the site of pathogen infection, referred to as the hypersensitive response (HR) (Heath 2000). Understanding the mechanisms by which an *R* protein recognizes a pathogen effector, especially the mechanism that determines recognition specificity, can help in the development of new strategies for preventing invasion and the breakdown of useful *R* genes by quickly evolving pathogens.

During the last few decades, a number of plant *R* genes have been isolated from model plants and agronomically important crops (Collier and Moffett 2009). The majority of these *R* genes encode intracellular receptor proteins consisting of three domains: an N-terminal coiled-coil (CC) or Toll interleukin-1 receptor-like (TIR) domain, a nucleotide-binding (NB) domain, and a C-terminal leucine-rich repeat (LRR) domain (Moffett 2009). The NB domain contains a region of homology known as the Apaf1, *R* proteins, and CED4 (ARC) domain that consists of two distinct structural and functional units, ARC1 and ARC2 (Albrecht and Takken 2006; McHale et al. 2006; Raidan and Moffett 2006; van der Biezen and Jones 1998). The LRR domain is the major determinant for recognition specificity (Banerjee et al. 2001; Dodds et al. 2001; Ellis et al. 2000; Moffett et al. 2002; Raidan and Moffett 2006; Shen et al. 2003). An LRR domain contains a series of β -sheets formed by xxLxLxx motifs (where x and L are any amino acid and any aliphatic residue, respectively) and forms the concave face of the horseshoe-shaped domain that is involved in protein–protein interactions (Kobe and Kajava 2001; McHale et al. 2006). Additionally, the solvent-exposed residues of the β -sheets (x in the xxLxLxx motif) in the LRR domain determine the recognition specificity of NB-LRR proteins and LRR receptor-like *R* proteins (McHale et al. 2006).

Several tobamovirus *R* genes have been identified, cloned, and used for breeding resistant crops. The first cloned tobamovirus *R* gene was the tobacco *N* gene encoding a TIR-NB-LRR-type *R* protein that recognizes the helicase domain of *Tobacco mosaic virus* (TMV) RNA polymerase (Erickson et al. 1999; Whitham et al. 1994). The tomato *Tm-2* and *Tm-2*² genes, encoding CC-NB-LRR-type *R* proteins, recognize the movement protein of *Tomato mosaic virus* (ToMV) as an Avr factor (Lanfermeijer et al. 2003, 2005; Meshi et al. 1989; Weber and Pflitzner 1998). Recently, we used map-based cloning and

Nucleotide sequence data can be found in the DDBJ, EMBL, and GenBank databases under accession numbers AB669000 for *N'*, AB669001 for *Nt-n'*, and AB669002 for the *Tobacco mosaic virus* K98 coat protein gene.

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homology-based methods to clone seven *L* gene alleles from *Capsicum* spp. that also encode CC-NB-LRR-type R proteins (Tomita et al. 2011).

The *L* gene alleles *L*¹ through *L*⁴ are numbered in order of their increasing ability to recognize *Tobamovirus* spp. Correspondingly, tobamoviruses are classified into the pathotypes P₀, P₁, P_{1,2}, P_{1,2,3}, and P_{1,2,3,4} based on their ability to infect systemically *Capsicum* plants carrying different *L* gene alleles (Boukema 1980, 1982, 1984; Genda et al. 2007). TMV and ToMV are classified into pathotype P₀, that can be recognized by all *L* alleles. *Paprika mild mottle virus* (PaMMV) and *Pepper mild mottle virus* (PMMoV) belong to pathotypes P₁ and P_{1,2}, respectively. Some strains of PMMoV with one or a few amino acid substitutions that differ from P_{1,2} and the ability to overcome *L*³-mediated resistance are classified as pathotype P_{1,2,3} (Garcia-Luque et al. 1993; Hamada et al. 2007; Tsuda et al. 1998). To date, two strains classified as the P_{1,2,3,4} pathotype of PMMoV have emerged and are anticipated to spread uncontrollably unless the infected plants are strictly monitored and managed because none of the identified *L* alleles confer resistance to the PMMoV P_{1,2,3,4} pathotype (Antignus et al. 2008; Genda et al. 2007). As shown in a previous study, elicitation of the resistance mediated by the *L* alleles requires a functional coat protein (CP) of tobamovirus as the Avr factor (Berzal-Herranz et al. 1995; Dardick et al. 1999; de la Cruz et al. 1997; Gilardi et al. 1998, 2004; Tomita et al. 2011; Tsuda et al. 1998).

Like the *L* gene alleles, the *N'* gene from *Nicotiana sylvestris* confers resistance to infection by *Tobamovirus* spp. via recognition of viral CP as Avr factors (Culver and Dawson 1989; Saito et al. 1987, 1989). Although cloning *R* genes has become a relatively straightforward procedure, especially after the development of next-generation sequencers, the *N'* gene had not been isolated prior to our investigation. The important feature of the *N'* gene is that it recognizes tobamovirus CP, similar to the *L* gene but with a different recognition spectrum (i.e., *N'* can trigger a resistance response to ToMV, PaMMV, PMMoV, and even pathotype P_{1,2,3,4}, but not to TMV) (Table 1). Cloning and characterization of *N'* would enable us to identify the immutable mechanisms underlying the recognition of specific effectors by different hosts.

Based on their responses to infection with *Tobamovirus* spp., *N'* and *L* proteins were hypothesized to be structurally similar (Culver 2002). In other words, *N'* might be an ortholog of the pepper *L* genes. Similarly, *R* genes sharing common pathogen effectors have been found in different but evolutionarily related plant species (Dangl et al. 1992; Innes et al. 1993; Ronald et al. 1992; Simonich and Innes 1995; Whalen et al. 1991), providing clues about the dynamics of *R* gene evolution. Soybean *Rgp1-b* recognizes a *Pseudomonas syringae*-derived AvrB protein, as does *Arabidopsis RPM1*, but the soybean *R* gene has a distinct origin from *RPM1* (Ashfield et al. 2004). Thus, RPM1 and Rgp1-b provided the first evidence for the convergent evolution of *R* gene function. Analysis of the phy-

logenetic and functional relationships between *Capsicum L* and *Nicotiana N'* genes led to new strategies for identifying *R* genes from different plant species.

In this study, we isolated the *N'* gene and compared it with the *Capsicum L* gene to identify those regions responsible for determining the spectrum of tobamovirus recognition. Our results showed that the *Capsicum L* and *Nicotiana N'* proteins, although encoded by closely related *R* genes, have diverged, leading to distinct recognition specificity. Functional differentiation was achieved by changes in the LRR domains represented by a conserved C-terminal sequence that contributed differentially to the recognition of the same Avr protein.

RESULTS

Isolation of the *N'* gene.

To isolate the *N'* gene, we used a homology-based approach because the N- and C-terminal amino acid sequences of pepper *L* gene alleles are completely conserved (Supplementary Fig. S1) and *N'* was predicted to be homologous to *L* (Culver 2002). In addition, the N- and C-termini of the *L*³ protein were shown to be important for its function (i.e., when fused with epitope tags at either the N or C terminus, the fusion proteins did not function in a transient assay system, although the proteins accumulated to detectable levels as shown by immunoblotting) (Supplementary Fig. S2). We performed polymerase chain reaction (PCR) to amplify *L* gene homologs from the genomic DNA of *Nicotiana* spp. and obtained a PCR product of the *N'* candidate gene from *N. sylvestris* as a single band of approximately 4 kb (Fig. 1A). The nucleotide sequences of three independent clones revealed that the candidate gene of 4,140 nucleotides encoded a CC-NB-LRR-type R protein. Because the sequences of 5' and 3' ends in the initial *N'* candidate clone were those of primers used, we determined the true sequences of these regions by adaptor PCR. We found only a single nucleotide change in the 3' end region between the *N'* candidate and *L*³, and the clones with corrected sequence were hereafter used. The protein product of 1,380 amino acids shared a 68.7% amino acid sequence identity and 89.2% similarity with the *L*³ protein (Supplementary Figs. S3 and S4).

The *N'* candidate was introduced into the pBA binary vector (Tomita et al. 2011). A transient expression assay in *N. benthamiana* showed that the *N'* candidate could confer cell death in response to the coexpression of CP from ToMV, PaMMV, and PMMoV, including pathotypes P_{1,2}, P_{1,2,3}, and P_{1,2,3,4}, but not from TMV (Fig. 1B). The cell death was accompanied with accumulation of H₂O₂ and electrolyte leakage, suggesting that this response was an HR resulting from the recognition of tobamovirus CP by the *N'* candidate (Supplementary Figs. S5A and B and S6A). To test whether or not the *N'* candidate could confer resistance to tobamovirus infection, the *N'* candidate gene was coexpressed with a PMMoV vector (Tomita et al. 2011) expressing CP from the PMMoV P_{1,2} and P_{1,2,3,4} pathotypes and TMV. The candidate gene conferred resistance

Table 1. Tobamovirus used in this study

Name	Pathotype	Virus species	Strain	Pathogenicity to resistance gene ^a					References
				<i>N'</i>	<i>L</i> ¹	<i>L</i> ²	<i>L</i> ³	<i>L</i> ⁴	
ToMV	P ₀	<i>Tomato mosaic virus</i>	L	avr	avr	avr	avr	avr	Meshi et al. 1986
TMV	P ₀	<i>Tobacco mosaic virus</i>	K98	vir	avr	avr	avr	avr	This study
PaMMV	P ₁	<i>Paprika mild mottle virus</i>	J	avr	vir	avr	avr	avr	Hamada et al. 2003
PIW	P _{1,2}	<i>Pepper mild mottle virus</i>	Iw	avr	vir	vir	avr	avr	Hamada et al. 2007
PIW-138N	P _{1,2,3}	<i>Pepper mild mottle virus</i>	I	avr	vir	vir	vir	avr	Garcia-Luque et al. 1993;
									Sakamoto et al. 2008
PIW-46R85K	P _{1,2,3,4}	<i>Pepper mild mottle virus</i>	L4BV	avr	vir	vir	vir	vir	Genda et al. 2007

^a Abbreviations: avr = avirulent and vir = virulent.

to PMMoV P_{1,2} and P_{1,2,3,4} but not to TMV (Fig. 1C); therefore, the recognition specificity of the *N'* candidate was consistent with that of the *N'* gene in *N. sylvestris* (Table 1).

To confirm the expression of L and *N'* proteins in the transient assay, we fused these proteins to epitope tags to detect their expression; however, the addition of tag sequences to the 5' or 3' terminus of the *L*³ gene abolished its ability to induce the HR. Therefore, we sought a site to insert the tag sequence in *L*³ without affecting its function. We inserted the epitope tag sequence into regions that were predicted to form turn structures in a secondary structure prediction using the Chou-Fasman method. Eventually, the *L*³ protein was successfully tagged with the hemagglutinin (HA) tag without affecting its ability to confer the HR in response to CP coexpression, and the protein was detectable by immunoblotting (Fig. 1D). Likewise, *N'* was tagged with HA at a similar turn structure in its CC domain. HA-tagged *N'* elicited an HR upon coexpression of avirulent tobamoviruses and was detectable by immunoblotting using an anti-HA antibody (Fig. 1D). Unexpectedly, the *N'* protein migrated faster than *L*³ in sodium dodecyl sulfate (SDS)-polyacrylamide gels, although the coding region of *N'* is longer than that of *L*³. The analysis of cDNA ruled out the possibility

of splicing in *N'*mRNA. The cause for inconsistent migration remains unknown but the difference in distribution of charged amino acids in these polypeptides might affect their electrophoretic mobility, as suggested for some virus CP (Gordon et al. 1988).

Genetic analysis of *N'*

N. tabacum is an amphidiploid generated from crossing *N. sylvestris* and *N. tomentosiformis* that is expected to carry the *N'* gene and show resistance to *Tobamovirus* spp. Although some *N. tabacum* cultivars such as 'Bright Yellow' and 'White Burley' are resistant to ToMV and other *Tobamovirus* spp., other cultivars such as *N. tabacum* 'Xanthi' and 'SR-1' are susceptible to all *Tobamovirus* spp. (Supplementary Fig. S7). In addition, *N. tabacum* 'Ky-57' (Ky) is resistant to all *Tobamovirus* spp. tested, suggesting that it has non-*N'* tobamovirus *R* gene. To clarify the relevance of the *N'* gene to the tobamovirus resistance phenotype in *N. tabacum*, we tried to identify the *N. tabacum N'* (*Nt-N'*) genes and susceptible alleles (*Nt-n'*).

PCR primers designed to amplify the *N'* coding region amplified a single product from the genomic DNA of tobamovirus-resistant cultivars, including *N. sylvestris*. By contrast,

Fig. 1. Isolation of the *N'* gene. **A**, Electrophoresis of polymerase chain reaction (PCR) products amplified from the genomic DNA of *Nicotiana* plant species using the specific primers for the full-length coding region of the *L* gene. PCR products amplified from *Nicotiana sylvestris* (S), *N. rustica* (R), *N. benthamiana* (B), *N. clevelandii* (C), and *N. glutinosa* (G) were analyzed with molecular weight markers (M). **B**, Hypersensitive response (HR) lesions in *N. benthamiana* leaves after coexpression of the candidate *N'* gene and tobamoviruses. Experimental settings are shown in the left diagram, in which six different tobamovirus coat proteins (A to F) were coexpressed with the *N'* candidate gene in *N. benthamiana* leaves. A = Tomato mosaic virus (ToMV), B = Tobacco mosaic virus (TMV), C = Paprika mild mottle virus (PaMMV), and D to F = Pepper mild mottle virus (PMMoV) pathotypes. Four days after agroinfiltration, the leaves were photographed (left panel), cleared with ethanol, and photographed again (right panel). Accumulation of each protein was confirmed by immunoblotting. **C**, Resistance to tobamoviruses in *N. benthamiana* leaves conferred by transient expression of the candidate *N'* gene. HR lesions on the leaves were photographed (left panel). Viral spread was detected by tissue printing immunoblotting (right panel). In each leaf, *L*³ (on the left side) and the *N'* candidate (on the right side) were coexpressed with PMMoV(P_{1,2}), PMMoV(P_{1,2,3,4}), or TMV. **D**, Immunoblot of proteins extracted from *N. benthamiana* leaves transiently expressing β -glucuronidase (GUS)-hemagglutinin (HA) (GUS), *L*³-T3-HA (*L*³), or *N'*-T3-HA (*N'*). Proteins were detected using an anti-HA antibody. In the bottom box, the RubisCO large subunit protein stained with Amido Black is shown as a loading control.

the same primers amplified two bands from the susceptible cultivars and Ky: one band was identical in size to that amplified from resistant plants and the other was about 1 kb longer (Fig. 2A). These results led us to hypothesize that there were two *Nt-n'* alleles, one with an insertion and another with a point mutation. To test this hypothesis, we performed PCR using another set of primers designed to amplify the region including the 5'-untranslated region (UTR) and 3'-UTR of *N'*. We observed that *Nt-n'* was amplified, unexpectedly, as a single band that was larger than *N'* and *Nt-N'* (Fig. 2B). Sequence analysis revealed that the larger PCR fragment from susceptible cultivars consisted of a complete *N'* coding sequence and a duplicated 902-bp sequence (90 bp of the 5'-UTR region and 812 bp of the *N'* protein coding sequence) (Fig. 2C; Supplementary Fig. S8). The forward primer for amplifying the *N'* coding region was annealed to the two positions in *Nt-n'* (in both copies of duplicated regions) (Fig. 2C, arrows labeled with "a"), resulting in the amplification of two DNA fragments of different sizes. The upstream copy of the duplicated sequences had a stop codon upstream of the complete *N'* coding sequence, a feature likely to compromise the translation of the downstream *N'* gene and to result in plant susceptibility to tobamovirus. The resistance to TMV in Ky, which suggests that Ky has another type of tobamovirus *R* gene, is consistent with our findings that it had *Nt-n'* rather than *Nt-N'* (Fig. 2B). These results support the hypothesis that the candidate gene cloned from *N. sylvestris* using the homology-based method is the *N'* gene.

We examined the phylogenetic relationship between the *N'* and *L* alleles. Analysis of the NB domain-encoding regions from *N'*, *L* alleles, and previously analyzed related sequences (Couch et al. 2006) revealed that the NB-encoding region of *N'* was more closely related to *I2* (Simons et al. 1998), *R3a* (Huang et al. 2005), and *PIH-X*, a paralog of *L³* with unknown function (Tomita et al. 2011), than to the *L* alleles (Supplementary Fig. S9A). Interestingly, analysis of regions encoding the C-terminal half of the LRR domain (LRR2 regions) gave contrasting results. The LRR2 region of *N'* was much more closely related to those of the *L* alleles than to those of *I2* and *R3a*; however, *PIH-X* was more closely related to the *L* alleles than to *N'*.

Identification of domains determining the tobamovirus-recognition specificity of *N'* and *L³* proteins.

We previously showed that the C-terminal halves of the LRR domains from allelic *L* proteins are critical for determining the spectra of *R* proteins recognizing Avr tobamovirus CP. Therefore, we performed a domain swap analysis between *N'* and *L³* to identify those regions contributing to recognition specificity (i.e., TMV recognition by *L³* and *P_{1,2,3,4}* recognition by *N'*).

First, to confirm whether the LRR domains of the *L³* and *N'* proteins determine their own recognition specificity, we constructed chimeras in which *L³* and *N'* were recombined around the junction between the NB and LRR domains. These chimeras, termed *L5N* and *N5L*, respectively, were coexpressed with CP from ToMV, TMV, or PMMoV (*P_{1,2,3,4}*), which were expressed using a PMMoV vector, pBTPIW (Fig. 3A and B). *L5N* conferred the HR in the same pattern as *N'*, suggesting that the LRR domain determines recognition specificity; however, *N5L* conferred the HR to PMMoV (*P_{1,2,3,4}*), unlike *L³*. Raidan and Moffett (2006) showed that the combination of GPA2-ARC2 and Rx-LRR in a chimera of these *R* proteins caused CP-independent HR (autoactivation phenotype). To test whether the *N5L* protein also can exhibit a CP-independent HR, *N5L*, *L5N*, *L³*, and *N'* proteins were coexpressed with a gene-silencing suppressor (p19 from *Tomato bushy stunt virus*). The overexpression of *N5L* induced HR lesions independently of tobamovirus CP, indicating that *N5L* shows the autoactivation phenotype (Fig. 3B) noted that even the coexpression of *N5L* with GFP, which served as a negative control for other *R* protein constructs, induced cell death as manifested by electrolyte leakage. Although it remains unclear whether the LRR domain of *L³* could recognize *P_{1,2,3,4}* CP when combined with the CC domain from *N'*, recognition of *P_{1,2,3,4}* CP is conditioned by the LRR domain from *N'*.

To define more narrowly the region determining recognition specificity, some additional chimeras were constructed by recombining *N'* and *L³* within the stretches of conserved sequence, and these chimeras were tested for their ability to recognize tobamovirus CP (Fig. 3A and C). *L7N* and *N7L* perceived viruses with the same specificity as *N'* and *L³*, respec-

Fig. 2. *N'* alleles in *Nicotiana tabacum* cultivars. **A** and **B**, Electrophoresis of polymerase chain reaction (PCR) products amplified from the genomic DNA of *N. tabacum* cultivars using the specific primer sets shown in **C**. PCR products amplified from *N. tabacum* 'Xanthi' (Xa), 'SR-1' (SR), 'Ky-57' (Ky), 'Bright Yellow' (BY), and 'White Burley' (WB), and from *N. sylvestris* (Ns) were analyzed with molecular weight markers (M). **C**, A diagram for the gene structures of *N'* and the tobamovirus-susceptible allele *Nt-n'*. Arrows show the annealing sites and directions of the primers (a–d). A gray area indicates a duplicated part of *N'*. NB = nucleotide-binding site, CC = coiled coil, and ARC = Apatl, R proteins, and CED4 region.

tively. L9N conditioned the HR to those viruses recognized by N'. These results suggest that the C-terminal half of the LRR of the N' protein determines its specificity to recognize tobamoviruses. In contrast, N9L, L11N, and N11L were not functional, although they accumulated to comparable levels as L³ and N' when they triggered the HR in response to matching CP (Supplementary Fig. S10B). These results suggest that L³ and N' have differentiated to show distinct LRR configuration and, therefore, some subdomains are not interchangeable.

Functional differentiation of the C-terminal regions of N' and L³.

The 5'- and 3'-terminal regions of the N' and L genes are highly conserved and have important roles in resistance because fusion of unrelated sequences in these regions, such as HA or c-Myc tags, abolished their function. Additionally, the C-terminal halves of the LRR domains from N' and L determined their recognition specificity (Fig. 4). These results suggested that the C-terminal conserved sequence has a significant role

in the recognition of common viral proteins by L and N'. To explore the role of the conserved C-terminal regions, we constructed a series of deletion mutants in the C-terminal regions of L³ and N'. In N', deletion of three amino acids totally abolished its ability to recognize all tobamovirus CP. By contrast, in L³, mutants lacking 5 or 10 amino acids were still able to recognize CP from ToMV, TMV, and PMMoV or that of ToMV alone. An L³ mutant lacking the C-terminal 15 amino acids was unable to recognize tobamovirus CP (Fig. 4A and B). Decreasing recognition by the L³ mutants was not the result of decreased protein accumulation because we found no correlation between the recognition spectrum and protein accumulation levels of the deletion mutant proteins. These results collectively indicate that the significance of the C-terminal regions has differentiated between L³ and N' (i.e., the C-terminal region of N' has a pivotal role in its basic function, whereas that of L³ has additive effects).

Because the deletion of 10 amino acids (GRTMFDCECL) but not 5 amino acids (DCECL) compromised TMV recogni-

Fig. 3. Chimeric analysis between the N' and L³ genes. **A**, Summary of the chimeric analysis between the N' and L³ genes. The names of the resistance (R) protein constructs, schemes of chimeric proteins, and phenotypes in the transient expression assay with each of four different tobamovirus coat proteins (CP) are shown. HR, hypersensitive response (HR) induction after coexpression of R proteins and tobamovirus CP; S, lack of visible HR lesion development; HR*, HR induction by the autoactivation of chimeric R protein (see text). **B**, Response of the chimeric R proteins L5N and N5L to different tobamovirus. Experimental settings are shown in the left diagram, in which four different R proteins (L³, N', L5N, or N5L) were coexpressed with *Tomato mosaic virus* (ToMV), *Tobacco mosaic virus* (TMV), *Pepper mild mottle virus* (PMMoV) pathotype (P_{1,2,3,4}), or the gene-silencing suppressor p19 in *Nicotiana benthamiana* leaves. Five days after agroinfiltration, the leaves were photographed. **C**, Response of various chimeric R proteins to different tobamovirus. Experimental settings are shown in the left diagram, in which 10 different R proteins (L³, N', L5N, N5L, L7N, N7L, L9N, N9L, L11N, or N11L) were coexpressed with ToMV, TMV, or PMMoV (P_{1,2,3,4}) in *N. benthamiana* leaves. Five days after agroinfiltration, the leaves were photographed. Accumulation of each protein was confirmed by immunoblotting.

Fig. 4. Deletion, insertion, and substitution mutant analysis of the C-terminal regions in N' and L³. **A**, Summary of the deletion, insertion, and substitution mutagenesis analysis of the C-terminal region in N' and L³. In the table, the names of the mutant resistance (R) proteins, C-terminal amino acid sequences, and their phenotypes under coexpression with each of the four different coat proteins from *Tomato mosaic virus* (ToMV), *Tobacco mosaic virus* (TMV), *Paprika mild mottle virus* (PaMMV), and *Pepper mild mottle virus* (PMMoV) are shown. HR, hypersensitive response (HR) induction after the coexpression of the combination of wild-type or mutant R proteins; S, susceptible, defined by the lack of visible HR lesion development. **B**, Responses of the C-terminal mutants of L³. Experimental settings are shown in the left diagram, in which β -glucuronidase (GUS), wild-type L³ (L³), L³dC5, L³dGRTMF, L³dC10, or L³dC15 were coexpressed with ToMV, TMV, PaMMV, or PMMoV (P_{1,2}) in *Nicotiana benthamiana* leaves. **C**, Responses of the C-terminal mutants of N'. Experimental settings are shown in the left diagram, in which L³, N'iGRTMF, wild-type N' (N'), N'dC3, N'dC5, or N'dC10 were coexpressed with ToMV, TMV, PaMMV, or PMMoV (P_{1,2}) in *N. benthamiana* leaves. **D**, Responses of the C-terminal mutants of N'. Experimental settings are shown in the left diagram, in which L³, L³dPEIYI, L³D1324A, or L³LRR32AIAIA were coexpressed with ToMV, TMV, PaMMV, or PMMoV (P_{1,2}) in *N. benthamiana* leaves. All of the leaves were photographed at 5 days postagroinfiltration. Accumulation of each protein was confirmed by immunoblotting.

tion, we hypothesized that N' cannot recognize TMV because it lacks the GRTMF sequence (Fig. 4A and B). To test this hypothesis, we assessed the recognition specificity of GRTMF-inserted N' (N'IGRTMF) (Fig. 4A and C) and GRTMF-deleted L³ (L³dGRTMF) (Fig. 4A and B). Unexpectedly, GRTMF-inserted N' still showed no function, whereas the GRTMF-deleted L³ mutant recognized TMV CP, although the latter did not recognize PMMoV CP. This result indicates that the GRTMF sequence, although it is present in the L³ that recognizes TMV CP and absent in the N' that does not recognize TMV CP, does not have a crucial role in TMV CP recognition. In addition, the result suggests that the length of the C-terminal region is more important than the context of the amino acid sequence for determining recognition specificity in L³. To examine this possibility, we tested whether the deletion of five different amino acids (PEIYI from the upstream region) affected only the recognition of PMMoV CP (L³dPEIYI) (Fig. 4A and D). The PEIYI-deletion mutant was compromised in the recognition of not only PMMoV CP but also PaMMV CP, suggesting that both the length of and the sequence context in the C-terminal region play important roles in the determination of recognition specificity.

Importance of the C-terminal-most copy of the xxLxLxx β -sheet motif in recognition.

Next, we analyzed the significance of the C-terminal DCECL sequence that was perfectly conserved between the L alleles and N'. N' was mutated by alanine scanning, in which the 1376D, 1377C, 1378E, 1379C, or 1380L of N' was substituted with A to generate N'D1376A, N'C1377A, N'E1378A, N'C1379A, or N'L1380A, respectively. N'C1377A and N'C1379A showed normal function that was indistinguishable from that of wild-type N' (Fig. 5B). In contrast, N'D1376A lost the ability to recognize all of the assayed viral CP (Fig. 5B). N'E1378A and N'L1380A induced a delayed HR, characterized by a statistically significant decrease in ion leakage (Fig. 5C).

In contrast to N'D1376A protein, substitution of 1324D to A in L³ protein (L³D1324A) (Fig. 4A and D) did not affect its recognition specificity. It should be noted that, in the N' protein, the 1376D corresponds to x, a putative solvent-exposed residue, in the xxLxLxx β -sheet motif, but the 1324D of the L proteins does not. The PEIYI-deletion mutant of L³ (L³dPEIYI) (Fig. 4A and D) that failed to recognize PaMMV and PMMoV CP had lost a copy of this motif. The results suggest that muta-

Fig. 5. Alanine scanning analysis in the C-terminal region of N'. **A**, Summary of alanine scanning analysis in the C-terminal region of N'. The names of the C-terminal single amino acid substitution mutant N' proteins, C-terminal amino acid sequences, and phenotypes induced by their coexpression with the coat proteins (CP) of *Tomato mosaic virus* (ToMV), *Paprika mild mottle virus* (PaMMV), or *Pepper mild mottle virus* (PMMoV) (P_{1,2}) are shown. HR, hypersensitive response (HR) induction after the coexpression of resistance (R) protein and CP; DHR, HR induction that was slower than that by combination of wild-type N' and CP; S, no induction of visible HR lesions. **B**, Responses of the C-terminal single amino acid substitution mutants to tobamovirus CP. Experimental settings are shown in the left scheme, in which PIH-X, wild-type N', N'D1376A, N'C1377A, N'E1378A, N'C1379A, or N'L1380A were coexpressed with ToMV, PaMMV, or PMMoV (P_{1,2}) in *Nicotiana benthamiana* leaves. At 3 or 5 days postagroinfiltration (dpi), the leaves were photographed. **C**, Electrolyte leakage was measured in *N. benthamiana* leaves at 4 dpi to express an R protein with *Tomato mosaic virus*. Ratios of leaked electrolyte to total electrolyte were calculated, and error bars indicate the standard error. Detectable accumulation of each protein was confirmed by immunoblotting.

tions of solvent-exposed residues in the C-terminal-most copy of the β -sheet motif affect the recognition function (i.e., they totally abolish the function of N' and reduce the recognition spectrum of L^3). To test this possibility, the C-terminal-most copy of the β -sheet motif in the LRR of L^3 (PEIYIGR) was altered to PAIAIAR, designated as L^3 LRR32AIAIA (Fig. 4A and D). L^3 LRR32AIAIA did not induce the HR when coexpressed with PMMoV CP, suggesting that the C-terminal-most copy of the β -sheet motif is important for recognition.

Amino acid substitution in N'D1376A totally abolished the recognition of tobamovirus CP. In contrast, L^3 LRR32AIAIA did not recognize PMMoV CP but did recognize ToMV, TMV, and PaMMV, although these two mutants share the feature that one or a few solvent-exposed residues in the C-terminal-most copy of the xxLxLxx β -sheet motif were changed to alanine. These results again indicate that the C-terminal region of N' is important for the overall function of the protein but that of L^3 is additive.

DISCUSSION

Homology-based cloning of N' .

Phylogenetic analysis suggested that the tobacco N' and pepper L genes evolved from a common ancestor. The $I2$ family genes, to which L and N' belong, were reported to be monophyletic, to have diversified within the Solanaceae over 14 million years, and to have evolved through a slow birth-and-death process (Couch et al. 2006). In contrast to other types of genes, such as those encoding specific enzymes, it is not easy to use PCR to amplify and clone functional copies of an R gene based on its homology to a previously characterized R gene because plant genomes contain many R gene copies and their sequences have diverged through evolution (Gao et al. 2007). The exceptions to this generally are the allelic R genes, that branched in a comparatively recent time (Lanfermeijer et al. 2005; Tomita et al. 2011). The N- and C-termini of N' and L are likely conserved due to functional constraints. Thus, we were able to amplify the N' gene by PCR using primers designed from the L^3 sequence (Fig. 1), although these R genes are not allelic, and from different taxa.

Remarkably, *Pseudomonas* R genes, *Arabidopsis* *RPM1*, and soybean *Rpg1-b* share pathogen-recognition specificity but exhibit low amino acid sequence similarity (Ashfield et al. 2004). In contrast, allelic R genes can recognize distinct pathogens; for example, *Arabidopsis* *RPP8*, *HRT*, and *RCY1* are alleles and their protein products share approximately 95% amino acid sequence similarity; however, they confer resistance to *Hyaloperonospora parasitica*, *Turnip crinkle virus*, and *Cucumber mosaic virus*, respectively (Cooley et al. 2000; McDowell et al. 1998; Takahashi et al. 2002). Although these facts explain the difficulty in using PCR to obtain specific R genes, the use of a cloning strategy based on conserved sequences has recently become a more feasible approach to identify R gene homologs with the same recognition specificity from phylogenetically related plants. A recent approach called allele mining enabled the isolation of novel R gene alleles with desired function from different species by large-scale comparative analysis of structurally related genes (Bhullar et al. 2009, 2010; Wang et al. 2008). More recently, Jordan and associates (2011) identified *TmMla1* through intergeneric allele mining, in which a diploid wheat bacterial artificial chromosome library was screened using a probe to the N-terminal region of the barley *HvMla1* LRR. Together with the study by Jordan and associates (2011), our results are encouraging that new functional homologs of a given R gene can be identified from a different genus or a more distant taxon.

Differentiation of N' and L in their mode of tobamovirus CP recognition.

Chimera analysis revealed that the recognition specificity of N' and L^3 is determined by their LRR domains (Fig. 3). The LRR domains of these two proteins were interchangeable (Fig. 3, L7N and N7L), although the first 200 amino acids of the LRR domains regulate the switching between active and inactive states, as suggested by the autoactivation of the N5L chimera (Fig. 3B). Nonetheless, a series of observations suggested that the LRR domains of N' and L^3 have functionally differentiated.

First, the N5L chimera showed autoactivation activity accompanied by the induction of the HR without recognition of CP (Fig. 3B). This phenomenon might be caused by an inappropriate intramolecular interaction between the NB and LRR domains (Rairdan and Moffett 2006).

Second, three chimeras (N9L, N11L, and L11N) were not functional, indicating that some of the LRR subregions are not interchangeable between N' and L^3 . In the case of the chimeras for the L alleles, the LRR subregions were interchangeable and, thus, we could narrow down the regions required for broad-spectrum recognition (Tomita et al. 2011). The LRR domains of N' and L^3 share 81.5% similarity, whereas the L proteins share 98.7 to 100.0% similarity. In addition, the LRR region of N' is 50 amino acids longer than those of the L proteins. These findings support the idea that L^3 and N' may be structurally different.

Third, mutational analysis of the C-terminal regions of N' and L^3 revealed that the regions fulfill quite different functions (Figs. 4 and 5). These results also indicate that different LRR subregions of the N' and L proteins contribute to the recognition of their common Avr effectors. Perhaps different sequences or secondary or tertiary structures of CP are recognized by the N' and L gene alleles, although a bundle of four α helices was reported to be important for recognition by both R proteins (Dardick et al. 1999).

Finally, differences in the N' and L^3 proteins in their modes of pathogen recognition are supported by different patterns in emergence of resistance-breaking virus variants. The tobamovirus recognition spectrum of N' does not follow the hierarchical interactions between L gene alleles and *Tobamovirus* spp. PMMoV pathotypes P_{1,2,3} and P_{1,2,3,4} differ from pathotype P_{1,2} in one or two amino acids in the CP. L^3 and L^4 are overcome by both and the latter, respectively; however, N' is effective against any pathotypes of PMMoV. In contrast, a single amino acid substitution enables an avirulent variant of TMV CP to escape N' recognition because N' can confer a resistance response to single amino acid substitution mutant CP of TMV (Taraporewala and Culver 1996). Although the L gene alleles are not durable against PMMoV, L genes are known to be durable R genes against TMV (i.e., no L gene-breaking TMV has been identified to date) (Janzac et al. 2009). Given that the N' and L proteins share some CP structures for recognition, these R gene proteins should respond in the same way to CP mutations; however, this is not the case. Taken together, it is most likely that N' and L^3 have functionally differentiated during evolution.

At the beginning of this study, we hypothesized that the N' gene would be an ortholog of the L gene because these two R genes confer resistance to *Tobamovirus* spp. and share structural similarities. However, phylogenetic analysis suggested that the NB domain of N' is closer to *I2* and *R3a* than to L , whereas the C-terminal half of the LRR domain of N' is closer to L than to *I2* and *R3a*. The results suggest that the N' and L alleles have evolved through a complex pathway that likely includes multiplication and diversification of an ancestral gene and recombination or gene conversion. Although the evolution

of the *N'* and *L* alleles remains to be studied in detail, it is highly unlikely that the *N'* and *L* alleles evolved by convergence. Moreover, chimera analysis of *N'* with *L*³ and comparative mutational analysis revealed that the LRR domains of *N'* and *L*³ have functionally differentiated during evolution. Therefore, it is unclear whether the *N'* and *L* alleles are orthologs. At present, we have little information on the relationship between *N'* and *L* because we isolated *N'* by a homology-based method and not through linkage analysis. There are two clues that would help clarify the genetic relationship between *N'* and *L* and reveal the evolutionary processes leading to their divergence. One is to identify the *N'* locus in the *N. sylvestris* genome to determine if there is synteny with the *Capsicum L* loci. Another is to identify other Tobamovirus *R* genes that recognize the same pathogen effector from different genera of Solanaceae and compare them with *N'* and *L*. One Tobamovirus *R* gene has been reported in eggplant (Dardick and Culver 1997).

In this study, we molecularly identified the *N'* gene, which has long been known and well studied but had not been cloned. We report here that *N'* is structurally close to the *L* gene alleles but differs in its structural requirements for pathogen recognition. Further comparative analysis of these *R* proteins will lead us toward understanding the precise mechanisms underlying pathogen recognition by plants in ETI.

MATERIALS AND METHODS

Plants and viruses.

Nicotiana plants were grown in pots containing a commercial soil mixture (Sakata Seed Co., Yokohama, Japan) in a glasshouse at 25 ± 5°C. After agroinfiltration, the plants were transferred to growth chambers and conditioned under a 22°C cycle of 16-h days and 8-h nights. The tobamoviruses and CP genes used in this study are shown in Table 1. TMV was isolated from a commercial field of sweet pepper in Kochi Prefecture, Japan, characterized as TMV under standard procedures, and confirmed to be TMV by CP gene sequencing.

Transient expression assay.

Plasmid construction for a transient expression assay is described in Supplementary Methods. The plasmids for the expression of *R* proteins were transformed into *Agrobacterium tumefaciens* LBA4404. Agroinfiltrations were conducted as described before (Tomita et al. 2011). The tobamovirus CP were expressed alone by pGT vector or expressed within virus genome by pBTPIW vector (Tomita et al. 2011). Green fluorescent protein (GFP) was expressed by pBTPIW vector as a control and *Tomato bushy stunt virus* p19 was expressed using the 35S:p19 construct (Voinnet et al. 2003). *N. benthamiana* leaves were agroinfiltrated (absorbance at 600 nm [*A*₆₀₀] = 0.5 for *R* gene constructs and *A*₆₀₀ = 0.1 for CP/virus and p19) and then harvested at 3 to 5 days postinfiltration.

Electrolyte leakage measurement.

Electrolyte leakage was measured as previously described (Tomita et al. 2011). Three independent leaves were used for each measurement and the measurements were repeated three times for the transient expression of each *R* protein, with representative data. Statistical analysis was performed with Student's *t* test.

Detection of virus infection.

Press blotting was performed as described previously (Hamada et al. 2007) using anti-ToMV, anti-PaMMV, anti-PMoV, or anti-TMV antisera (Japan Plant Protection Association, Tokyo). To assess the tobamovirus resistance in *N. taba-*

cum plants, fully expanded leaves of 6-week-old plants were mechanically inoculated with purified virus particles at 10 µg/ml as previously described (Tomita et al. 2011).

Western blot analysis.

Virus CP were detected by immunoblotting according to Taraporewala and Culver (1996) using anti-ToMV, anti-PaMMV, anti-PMoV, or anti-TMV antisera. Transiently expressed HA-tagged proteins were detected by immunoblotting as described previously (Sekine et al. 2008). For SDS polyacrylamide gel electrophoresis (PAGE) to separate HA-tagged *R* proteins, NuPAGE Novex 3 to 8% Tris-Acetate Gels (Life Technologies, Carlsbad, CA, U.S.A.) were used. As an internal control, post-transfer membranes were stained with 0.1% Amido Black in 45% methanol and 10% acetic acid followed by destaining in 90% methanol and 2% acetic acid, and the bands of the RubisCO large subunit were photographed.

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