Alternatively spliced *N* resistance gene transcripts: Their possible role in tobacco mosaic virus resistance

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The *N* gene, a member of the Toll-IL-1 homology region–nucleotide binding site–leucine-rich repeat region (LRR) class of plant resistance genes, encodes two transcripts, N_S and N_L, via alternative splicing of the alternative exon present in the intron III. The N_S transcript, predicted to encode the full-length N protein containing the Toll-IL-1 homology region, nucleotide binding site, and LRR, is more prevalent before and for 3 hr after tobacco mosaic virus (TMV) infection. The N_L transcript, predicted to encode a truncated N protein (N^{tr}) lacking 13 of the 14 repeats of the LRR, is more prevalent 4–8 hr after TMV infection. Plants harboring a cDNA-N_S transgene, capable of encoding an N protein but not an N^{tr} protein, fail to exhibit complete resistance to TMV. Transgenic plants containing a cDNA-N_S-bearing intron III and containing 3′ *N*-genomic sequences, encoding both N_S and N_L transcripts, exhibit complete resistance to TMV. These results suggest that both *N* transcripts and presumably their encoded protein products are necessary to confer complete resistance to TMV.

Plants resist invading microbial pathogens by mounting rapid defense responses. The genetic control of disease resistance in plants depends on the interaction between a dominant or semidominant resistance (*R*) gene product in the plant and a corresponding dominant avirulence (*Avr*) gene product in the pathogen (1). The *R* gene products are hypothesized to encode receptors that recognize directly or indirectly the pathogen-encoded ligands (elicitors) (2). This recognition is postulated to initiate signaling pathways leading to defense responses. The most ubiquitous plant defense response includes a hypersensitive response (HR) or localized cell death at the site of pathogen ingress. The local HR often leads to a nonspecific general defense response throughout the plant called systemic acquired resistance (3).

In recent years, *R* genes from diverse plant species conferring race-specific resistance to viral, bacterial, fungal, nematode, and insect pathogens have been cloned (4, 5). The *N* gene, cloned from tobacco, is a type member of the Toll-IL-1 receptor homology region (TIR)-nucleotide binding site (NBS)-leucine-rich repeat region (LRR) class of *R* genes and confers resistance to the viral pathogen tobacco mosaic virus (TMV) (6). An interesting characteristic feature of genes that belong to the TIR-NBS-LRR class of *R* genes is that they are predicted to encode multiple transcripts (refs. 6–10; W. Gassman, M. Hinsch, and B. J. Staskawicz, personal communication). The functional role, if any, for these transcripts in the induction of disease resistance is not yet known. In this study we show that the *N* gene from tobacco encodes two transcripts, N_S and N_L, via alternative splicing from a single gene *in vivo*. Our results suggest that alternative splicing of the *N* gene is regulated by TMV-induced signals. The N_S transcript is the more prevalent form before TMV infection and the N_L transcript becomes more prevalent after TMV infection. Our analysis of transgenic plants containing various *N*-cDNA reconstructions suggests that a cDNA-N_S-bearing intron III harboring the alternative exon (AE) and containing 3′ genomic regulatory sequences (GS), encoding both N_S and N_L transcripts, is the minimum sequence required to confer complete resistance to TMV.

Materials and Methods

TMV Inoculation and Phenotypic Analysis. Full-length infectious TMV RNA transcripts were generated by *in vitro* transcription of *Kpn*I-linearized Klenow-filled pTMV004 (University of Florida Citrus Research and Education Center, Lake Alfred) using T7 RNA polymerase (Promega), as described by the manufacturer. *In vitro*-generated TMV transcripts were rub-inoculated onto TMV-sensitive tobacco (SR1) plants, and infected leaves were harvested 10 days after inoculation and reinoculated onto SR1 plants for virus multiplication. The inoculum for plant infection was prepared by grinding SR1-infected leaf tissue in 10 mM sodium phosphate buffer, pH 7.0. The leaf sap with virus was rubbed onto 4- to 5-week-old T0 and T1 plants using a sponge. Plants were scored for development of a resistance or susceptible response to TMV 3–20 days postinfection (d.p.i.).

Plasmid Constructions. All DNA manipulations were performed essentially as described (18). Most constructs used in this study were derived from G38, C18, and C16 clones that have been described (6). *N* cDNAs C18 and C16 described in ref. 6 are referred to as cDNA-N_S and cDNA-N_L, respectively, in this study. Details of each construction are available on request.

Plant Transformation. TMV-sensitive tobacco (SR1::*nn*) plants were transformed with *Agrobacterium tumefaciens* AGL1 carrying various transgene constructs by using a leaf disc transformation procedure (19). Transformants were selected on 150 mg/liter of kanamycin. At least 15 independent transformants were generated for each construct used in this study.

RNA Isolation and Reverse Transcriptase (RT)–PCR Analysis. Total RNA was extracted from 4-to 5-week-old wild-type or transgenic plants according to ref. 20. Poly(A)$^+$ RNA was isolated from total RNA using oligo(dT)$_{25}$-cellulose chromatography (Amersham Pharmacia). Poly(A)$^+$ RNA was treated twice with RNase-free DNase (Gene Hunter, Nashville, TN) to remove DNA contamination from RNA. To analyze alternatively spliced *N* gene transcripts, 1 μg of poly(A)$^+$ RNA and primer SP320 (5′-CTAAACTATCGCAACTTCTTAGACC-3′) that anneals to bases 9281–9257 in the *N* gene was used for the synthesis of first-strand cDNA using the Super Script RT preamplification system (GIBCO/BRL). PCR analysis was performed by using

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Abbreviations: *R*, resistance gene; TIR, Toll-IL-1 receptor homology region; NBS, nucleotide binding site; LRR, leucine-rich repeat region; TMV, tobacco mosaic virus; HR, hypersensitive response; SHR, systemic HR; RT, reverse transcriptase; AE, alternative exon; d.p.i., days postinfection; GS, genomic regulatory sequence(s); NOS, nopaline synthase.

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one-tenth of the first-strand cDNA with primers P1 (5′-AAATGCTTGTTCACCTCC-3′) that anneals to bases 7146–7163 and P2 (5′-CTTCAAGATTACTACATTG-3′) that anneals to bases 9146–9128 in the *N* gene. PCR amplification was performed in 50-μl volume using *Taq* DNA polymerase (Promega) and Hot Start *Taq* antibody (New England Biolabs). PCR products were analyzed by electrophoresis on 3% NuSieve GTG agarose gel (FMC).

To quantify the alternatively spliced transcripts generated from the *N* gene, quantitative RT-PCR was performed according to the method described in ref. 11. This technique uses one radio-labeled primer in the PCR, so that the amplified products can be readily quantified. The efficiency of amplification is calculated by using the products generated during the exponential state of amplification, and then by using a regression analysis to calculate the amount of cDNA present at the beginning of the reaction.

Results

The Tobacco *N* Gene Is Alternatively Spliced. In a previous study, we reported isolation of two classes of *N*-cDNAs, cDNA-N_S and cDNA-N_L, from tobacco cDNA library (6). Sequence analysis of the two *N* cDNAs and *N* genomic clone indicated that the *N* gene encodes two alternatively spliced transcripts, N_S and N_L, from a single gene (ref. 6 and Fig. 1*A*). Five exons from the *N* gene are spliced together, resulting in N_S that encodes the putative full-length N protein of 131.4 kDa. The N_L transcript results from the alternative splicing of a 70-bp AE within intron III of the *N* gene (Fig. 1 *A* and *B*). The AE in intron III is flanked by consensus splice acceptor and donor sites (Fig. 1*B*). The inclusion of the AE in N_L results in a shift in the reading frame, leading to premature translation termination after the first LRR in the exon IV. The longer N_L message therefore encodes a putative truncated N (N^{tr}) protein of 75.3 kDa. The first 616 aa of N^{tr} are identical to those of the amino terminus of full-length N protein. N^{tr} contains 36 additional aa at its carboxyl terminus because of the shift in the reading frame (Fig. 1*B*).

In this study, we performed RT-PCR analysis to confirm whether the two *N* transcripts, N_S and N_L, are expressed *in vivo* via alternative splicing. Poly(A)$^+$ RNA was isolated from the *N* transgene containing TMV-resistant (SR1::*NN*) and isogenic TMV-sensitive (SR1::*nn*) plants and used to generate first-strand cDNAs. These cDNAs were used as templates with primers (P1 and P2) flanking intron III of the *N* gene to amplify the region encompassing this intron by PCR (Fig. 1 *A* and *B*). Two products of 252 and 182 bp in length were amplified from the cDNA derived from SR1::*NN* plants (Fig. 1*C*, lane 1). The sizes of the two products were similar to those predicted for N_L and N_S, respectively. Cloning and sequence analysis of these PCR products shows that the 252-bp product is identical to the sequence from N_L and contains 70-bp AE, and the 182-bp product is identical to the N_S sequence. The same primers fail to amplify products from the SR1::*nn* cDNA (Fig. 1*C*, lane 2). These results confirmed that the *N* gene encodes two alternatively spliced products from a single gene *in vivo*.

The RT-PCR analysis indicated that the N_S transcript is more abundant than the N_L transcript (Fig. 1*C*, lane 1). To quantify the relative abundance of N_S to N_L in the *N* gene-containing plants, we performed quantitative RT-PCR according to the method of ref. 11. Our analysis indicates that the ratio of N_S to N_L in the *N* gene-containing plants is approximately 25:1 before TMV infection (Fig. 1*D*). The ratio of these two transcripts changes after TMV infection (see results below).

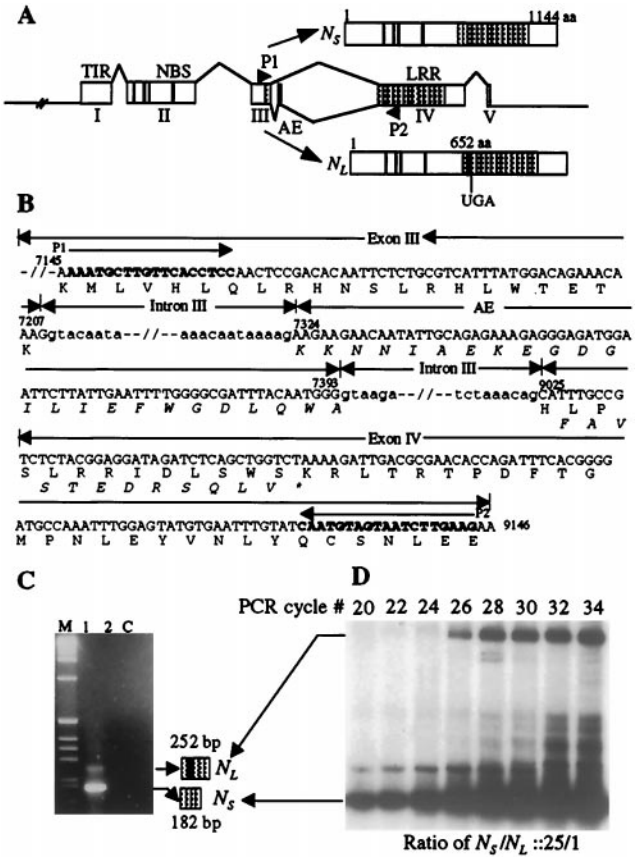

Fig. 1. The *N* gene encodes two alternatively spliced messages. (*A*) Schematic diagram showing the *N* gene with five exons (rectangle boxes) and four introns (lines) and two *N* transcripts, N_S and N_L, that are generated by alternative splicing. The N_S, produced by default splicing, encodes full-length N protein (N) of 1,144 aa. The N_L results from alternative splicing, includes the AE located in intron III, and encodes a truncated N protein (N^{tr}) of 652 aa caused by the premature translational termination (UGA) codon. TIR, NBS, and LRR represent the putative functional domains of the *N* gene. P1 and P2 are primers used in the RT-PCRs. (*B*) Diagram showing the sequence in and around the AE in intron III of the *N* gene. All nucleotide numbers are according to the *N* gene numbering in transcripts (6). The positions of exon III and IV and the AE within intron III are indicated. Part of the protein sequence encoded by N_S and N_L (italics) messages are shown below the nucleotide sequence. The AE nucleotide sequence (nucleotides 7324–7393) within intron III and corresponding protein sequences are shown in italics. Sequences in bold letters correspond to the primer sequences (P1 and P2) used for analysis of two transcripts. (*C*) Ethidium bromide-stained agarose gel showing RT-PCR products amplified by using primers P1 and P2 flanking the intron III sequence. The first-strand cDNA templates were generated from poly(A)$^+$ RNA isolated from TMV-resistant SR1::*NN* (lane 1) and TMV-susceptible SR1::*nn* (lane 2) plants before TMV infection. Lane C is a negative control (for DNA contamination) in which poly(A)$^+$ RNA without RT was used as a template. Lane M, 2 μg of 123-bp DNA ladder from GIBCO/BRL as molecular weight standard. N_S (182 bp) and N_L (252 bp) corresponding products are indicated by arrows. (*D*) Quantitative RT-PCR analysis of the two *N* messages, N_S and N_L, using radiolabeled P1 and unlabeled P2 primers, spanning intron III. The first-strand cDNA template was generated from poly(A)$^+$ RNA isolated from SR1::*NN* plants before TMV infection. Each lane represents the amount of product generated during the exponential phase of amplification (20–34 cycles). The ratio of N_S to N_L, 25:1, is indicated.

The AE Is Required to Confer Complete Resistance to TMV. To determine the functional significance of the AE in intron III of the *N* gene in conferring resistance to TMV, we created a number of *N* gene deletion constructions. These constructions were transformed into TMV-sensitive tobacco (SR1::*nn*) and at least 15 independent transformants for each construction were tested for TMV response from 3 to 20 d.p.i. The resistance response is manifested by the formation of localized HR lesions within 3 d.p.i. and by the containment of TMV to the infection site in *N* transgenic plants (Fig. 2*A*). In susceptible control plants,

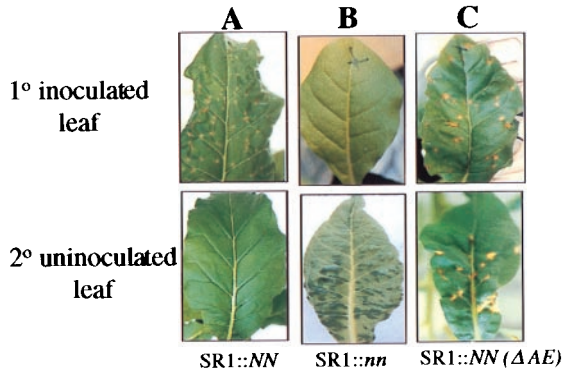

Fig. 2. Response of plants expressing *N* gene and AE deletion constructions to TMV. Primary (1°) TMV-U1-inoculated and secondary (2°) upper uninoculated leaves from transgenic SR1::*NN* (*A*), control SR1::*nn* (*B*), and transgenic SR1::*NN* (ΔAE) (*C*).

TMV multiplies and spreads from the infection site throughout the plant, causing mosaic symptoms within 7–10 d.p.i. (Fig. 2*B*). We found that deletion of introns I, II, and IV from the *N* gene had no effect on the resistance phenotype (data not shown); however, deletion of the 70-bp AE and the flanking splice acceptor and donor sites results in incomplete TMV resistance (Fig. 2*C*). In these plants, the appearance of HR on the primary inoculated leaf was delayed (5 d.p.i. vs. 3 d.p.i.), and TMV continued to spread throughout the plant, resulting in systemic HR (SHR) and mosaic symptoms that appeared at 10 d.p.i in the secondary leaves (Fig. 2*C*). Taken together, these results suggest that the AE sequence within the intron III is required to confer complete resistance to TMV.

Alternative Transcripts and 3′ GS Are Required to Confer Complete Resistance to TMV. To investigate the minimum *N* sequences required to confer complete TMV resistance, we created number of reconstructed *N* cDNAs (Fig. 3) by using genomic and *N*-cDNA clones. These reconstructed *N*-cDNAs were transformed into TMV-sensitive tobacco (SR1::*nn*), and at least 15 independent transformants for each construction were tested for TMV response from 3 to 20 d.p.i. To test whether the cDNA-N_S is sufficient to confer complete TMV resistance, we inserted cDNA-N_S between the *N* promoter and a 1.4-kb 3′ GS (Fig. 3*A*). This construction contains no intron and is incapable of generating the alternative transcript N_L. We used native *N* promoter in all of our constructions, because the *N* gene under the control of cauliflower mosaic virus 35S promoter fails to confer complete resistance to TMV (unpublished work). The 1.4-kb 3′ GS includes sequences downstream of the *N* gene stop codon and contains two putative poly(A) addition sites (see Fig. 1*A*). Transgenic plants containing cDNA-N_S:3′ GS construct showed delayed HR lesions (6 d.p.i.) as opposed to wild-type *N* gene-containing plants (3 d.p.i.) and virus spreads from the infection site, causing SHR and mosaic symptoms throughout the plant (10 d.p.i.) (Fig. 3*A*). This finding suggests that cDNA-N_S, predicted to encode full-length N protein, is not sufficient to confer complete TMV resistance. We also tested the function of N_L in TMV resistance by expressing cDNA-N_L under the control of the native *N* promoter and 1.4-kb 3′ GS (Fig. 3*B*). TMV

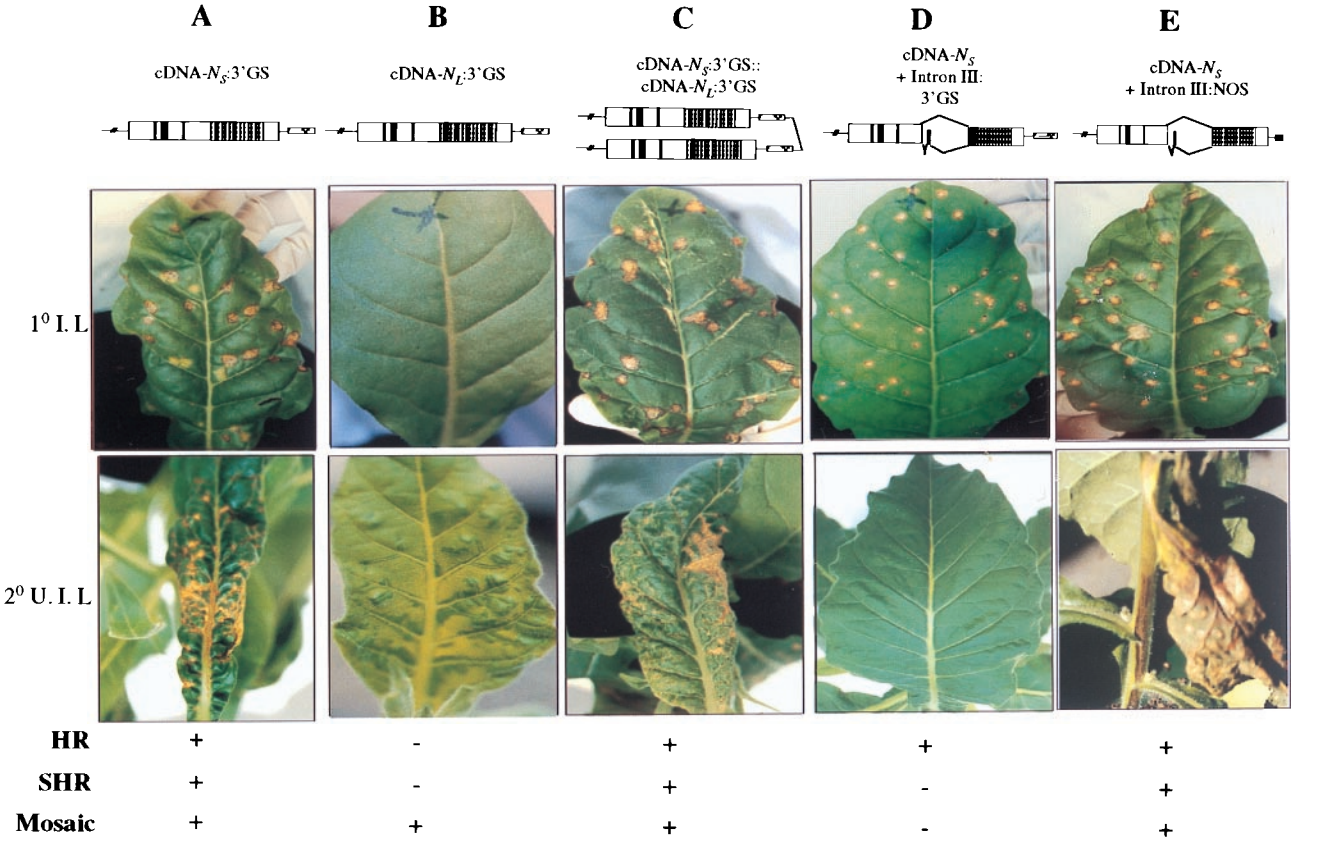

Fig. 3. Response of transgenic plants expressing various *N* cDNA reconstructions to TMV. Primary TMV-U1 inoculated (1° I.L.) and secondary uninoculated upper leaves (2° U.I.L.) from transgenic SR1:*nn* plants bearing cDNA-N_S:3′ GS (*A*), cDNA-N_L:3′ GS (*B*), and cDNA-N_S:3′ GS::cDNA-N_L:3′ GS (*C*), cDNA-N_S + intron III (AE):3′ GS (*D*), and cDNA-N_S + intron III (AE):NOS (*E*) constructions. c-DNA-N_S and cDNA-N_L, alternative *N* messages. AE, AE in intron III of the *N* gene. 3′ GS, 1.4-kb GS (open rectangle with zig-zags) downstream of the *N* gene stop codon. NOS, terminator sequences (filled black rectangle) from the NOS gene.

Table 1. Ratio of N_S to N_L after TMV infection

Transcript	Time												
	0	5 min	30 min	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr	8 hr	9 hr	24 hr
N_S	28	25	25	28	25	20	8	1	1	1	12	28	25
N_L	1	1	1	1	1	1	1	1.5	23	20	1	1	1

infection of transgenic cDNA-N_L:GS plants does not induce detectable HR and TMV spreads, systemically inducing mosaic symptoms (Fig. 3*B*). These results indicate that expression of neither cDNA-N_S nor cDNA-N_L alone can impart complete resistance to TMV.

To examine whether expression of cDNA-N_S and cDNA-N_L together in a single plant is sufficient to confer TMV resistance, we generated transgenic plants containing both cDNA-N_S:GS and cDNA-N_L:GS in the same plant (Fig. 3*C*). We predicted that this construction should produce an equal ratio (1:1) of N_S to N_L transcripts *in vivo*. Transgenic plants containing this construct display the same phenotype as plants transformed with cDNA-N_S:GS alone (compare Fig. 3 *C* and *A*). These plants show HR at 5 d.p.i. and virus spread throughout the plant, causing SHR and mosaic symptoms (10 d.p.i.) (Fig. 3*C*). These results indicate that expression of cDNA-N_S and cDNA-N_L together is not sufficient to confer complete TMV resistance.

Given that the expression of cDNA-N_S and cDNA-N_L in the single plant failed to confer complete resistance to TMV, we hypothesized that the ratio or timing of expression of these two messages might be critical in conferring complete resistance to TMV. To test this theory, we inserted AE containing intron III into cDNA-N_S:GS (Fig. 3*D*). This construct is predicted to encode both N_S and N_L transcripts via alternative splicing in the transgenic plants. Transgenic plants containing this construct exhibited complete resistance to TMV (Fig. 3*D*). Timing of HR appearance and containment of virus to the infection site was similar to that of the wild-type *N* gene-containing plants. These results suggest that cDNA-N_S with AE containing intron III and 3′ GS is the minimum *N* sequence required to confer complete resistance to TMV.

In our *N*-cDNA reconstruction experiments described above we inserted various cDNAs between *N* promoter and 3′ GS. To test whether 3′GS is necessary to confer complete TMV resistance, we substituted nopaline synthase (NOS) terminator sequence for the 3′ GS in cDNA-N_S-bearing intron III construction (Fig. 3*E*). Transgenic plants containing the cDNA-N_S + intron III:NOS construct display delayed HR (5 d.p.i.) and virus spreads out of the inoculated area, resulting in SHR and mosaic symptoms in the secondary leaves (Fig. 3*E*). RT-PCR analysis indicates that these plants express the N_S transcript but lack the N_L transcript (see results below). These results suggest that the 3′ GS of the *N* gene contains a regulatory sequence that is required for proper *N* expression and to confer complete resistance to TMV.

TMV Infection Regulates Alternative Splicing of the *N* Gene. The results described above suggest that AE containing intron III is required for expression of complete resistance to TMV and that the regulation of the ratio of N_S to N_L may be critical for *N* gene-mediated resistance. We therefore set out to determine the effect of TMV infection on the ratio of these two messages. Leaves from the *N* gene-containing plants were inoculated with a high-titer TMV that produces 400–500 lesions per leaf. We used a high titer of TMV so that most of the cells in a single leaf would be infected with TMV. Poly(A)$^+$ RNA was isolated from these leaves at different time points 0–48 h postinfection. This RNA was used for quantitative RT-PCR analysis. The ratio of N_S to N_L before TMV infection (zero time) and 3 hr after TMV infection was approximately 28:1 (Table 1), confirming our previous results. However, the ratio of the two transcripts changed dramatically 4 hr after TMV infection (Table 1). The ratio of N_S:N_L, approximately 1:1.5 hr after infection compared with 28:1 before TMV infection, was completely reversed (1:23) 6 hr after TMV infection, and returned to the original state (28:1) 9 hr after infection. Similar results were obtained in four different experiments. These results indicate that a TMV signal may trigger alternative splicing of the *N* gene. Although the ratio of two messages changes upon TMV infection, the total amount of *N* message (N_S + N_L) remains the same before and after TMV infection (data not shown).

The *N* Gene 3′ GS Influences Alternative Splicing. Our results indicate that the 3′ GS of the *N* gene plays a role in conferring resistance to TMV. To investigate whether the 3′ GS plays a role in alternative splicing of the *N* gene, we analyzed the level of N_S and N_L messages in transgenic plants that contain various synthetic *N* cDNAs described in Fig. 3. Poly(A)$^+$ RNA was isolated from these transgenic plants and used for RT-PCR analysis using primers P1 and P2 spanning intron III of the *N* gene (Fig. 1 *A* and *B*). Results of these analyses are shown in Fig. 4 *A* and *B*. RT-PCR analysis of transgenic plants harboring either cDNA-N_S alone (Fig. 3*A*) or cDNA-N_L alone (Fig. 3*B*) revealed the expected 182-bp (Fig. 4*A*, lane 1) and 252-bp (Fig. 4*A*, lane 2) amplified products, respectively. RT-PCR analysis of plants containing the AE deletion construct revealed only the 182-bp amplified fragment as expected (Fig. 4*A*, lane 3). Plants expressing cDNA-N_S and cDNA-N_L from a single vector (Fig. 3*C*) revealed an expected equal amount of 182- and 252-bp products (Fig. 4*A*, lane 4 and 5). Interestingly, transgenic plants containing cDNA-N_S with intron III and NOS terminator (Fig. 3*E*) revealed only the 182-bp fragment (Fig. 4*A*, lane 6) and no 252-bp product. Transgenic plants containing reconstructed cDNA-N_S with intron III and 3′ GS (Fig. 3*D*) revealed both the 182- and 252-bp alternatively spliced fragments (Fig. 4*A*, lane 7). The ratio of the two messages in these plants is similar to that of wild-type *N* gene containing plants (Fig. 4*A*, compare lanes 7 and 8). These results clearly indicate that the 3′ GS influences the generation of the *N* gene alternatively spliced products. To exclude the possibility that the ethidium bromide-stained gel was not sensitive enough to detect a low level of alternatively spliced 252-bp product generated from plants that contain reconstructed cDNA-N_S with intron III and NOS terminator, we performed RT-PCR analysis using radioactively labeled primers. Using this approach, we were unable to detect an alternatively spliced 252-bp product from transgenic plants that contain a NOS terminator (Fig. 4*B*, *Right*) compared with plants bearing the cDNA-N_S + intron III:3′ GS construct (Fig. 4*B*, *Left*). Taken together these results indicate that the 3′GS influences the generation or stability of the *N* gene alternatively spliced products.

Because our data suggest that the 1.4-kb 3′ GS plays a critical role in alternative splicing of the *N* gene and influences the TMV-resistance response, we determined whether this region is transcribed. RT-PCR analysis was performed by using nuclear RNA isolated from *N* gene-containing plants with P3 primer that anneals to exon V and primers P4-P9 that anneal to various regions in the 1.4-kb 3′ GS of the *N* gene (Fig. 4*C*). Primer P3

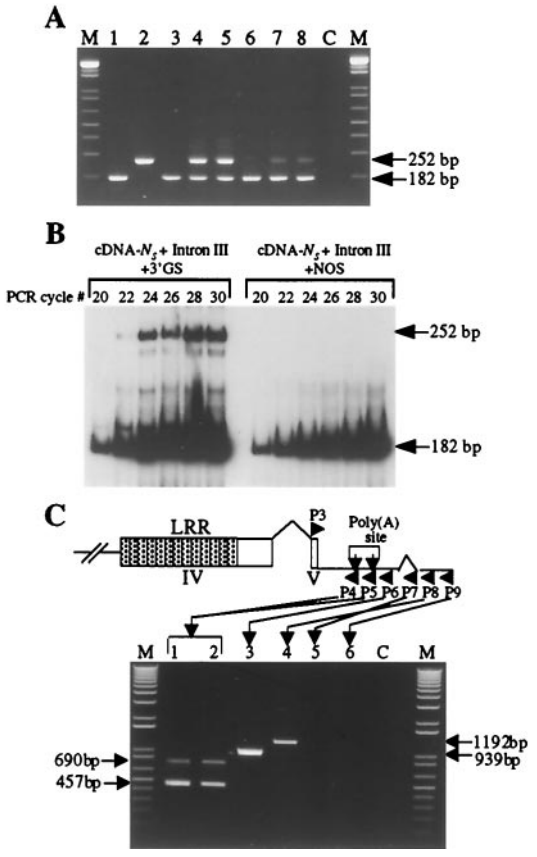

Fig. 4. The *N* gene 3′ GS is transcribed and influences alternative splicing. (*A*) Ethidium bromide-stained agarose gel showing RT-PCR products amplified by using primers P1 and P2 flanking intron III sequence. The first-strand cDNA was generated from poly(A)$^+$ RNA extracted from plants expressing cDNA-N_S:3′ GS (lane 1), cDNA-N_L:3′ GS (lane 2), AE deletion (lane 3), cDNA-N_S:3′ GS::cDNA-N_L:3′ GS (lanes 4 and 5), cDNA-N_S + intron III (AE):NOS (lane 6), cDNA-N_S + intron III (AE):3′ GS (lane 7), and SR1::*NN* (lane 8) plants. Lane C is a negative control (for DNA contamination) in which poly(A)$^+$ RNA without RT was used as a template. Lane M, 2 μg of 123-bp DNA ladder from GIBCO/BRL as molecular weight standard. (*B*) Autoradiograph of a polyacrylamide gel, showing radiolabeled RT-PCR products from the two *N* messages, N_S and N_L, generated by using radio-labeled P1 and unlabeled P2 primers that flank intron III. The first-strand cDNA was generated from poly(A)$^+$ RNA extracted from cDNA-N_S + intron III (AE):3′ GS (*Left*) or cDNA-N_S + intron III (AE):NOS (*Right*) plants. Each lane represents products generated during the exponential phase of amplification (20–30 cycles). (*C*) Ethidium bromide-stained agarose gel showing 3′ rapid amplification of cDNA ends (RACE) PCR products amplified by using primer P3 that anneals to exon V and primers P4-P9 that anneal to sequences downstream of the *N* gene stop codon. The first-strand cDNA was generated from nuclear RNA extracted from *N* gene-containing plants and used as templates for 3′ RACE. Lane C is a negative control (for DNA contamination) in which poly(A)$^+$ RNA without RT was used as a template. Lane M, 2 μg of 123-bp DNA ladder from GIBCO/BRL as molecular weight standard. Arrows to the right and left of the gel indicate sizes in bp of 3′ RACE products.

with P4 and P5 [anneals to the predicted poly(A) addition sites] in the same reaction results in the amplification of the expected 457- and 690-bp products (Fig. 4*C*, lanes 1 and 2). A 939-bp amplified product was detected with the primer P6 that anneals to the region (bases 11644 to 11621) beyond the poly(A) addition site (Fig. 4*C*, lane 3), and the P8 primer that anneals downstream of the P7 primer was capable of generating a 1,192-bp fragment (Fig. 4*C*, lane 4). However, no amplified products were detected with primer P7 (Fig. 4*C*, lane 5). Sequence analysis of the 1,192-bp product generated from P3 and P8 primers suggests that there is an intron that is spliced from the 3′ GS. Taken together, these results indicate that the 3′ GS is transcribed and contains an intron that is processed in the precursor RNA.

Discussion

In this study using RT-PCR analysis, we confirm that two alternatively spliced *N* messages, N_S and N_L, are expressed *in vivo* from a single gene in tobacco. Our *N* gene deletion analysis revealed that the AE sequence might play an important role in the TMV-resistance response. Deletion of the AE sequences and the flanking splice sites leads to partial TMV resistance in the form of SHR, where the virus escapes from the infection site and spreads systemically, inducing HR throughout the plant. Our analysis of transgenic plants containing various reconstructed *N*-cDNAs suggests that cDNA-N_S-bearing intron III harboring the AE and containing 3′ GS, encoding both N_S and N_L transcripts, is the minimum sequence required for proper expression of the two *N* messages and to confer complete resistance to TMV. TMV infection of transgenic plants expressing only cDNA-N_S, capable of encoding only full-length N product, induces an aberrant HR insufficient to arrest TMV spread from the infection site and leads to SHR. In contrast, the expression of cDNA-N_L alone, encoding only the N^{tr} product fails to induce TMV-dependent HR and results in virus spread and mosaic disease symptoms. In addition, expression of both *N* messages at a 1:1 ratio in a single plant does not impart complete resistance to TMV. This finding suggests that the ratio of N_S to N_L mRNAs, before and after TMV infection, is critical for complete resistance to TMV. This hypothesis is supported by our data from experiments in which the inclusion intron III into cDNA-N_S, capable of encoding both transcripts, is sufficient to confer complete resistance to TMV. Furthermore, both transcripts are expressed in these plants at a ratio comparable to that of wild-type *N* gene-containing plants.

Our results indicate that the relative ratio of two *N* messages in plant cells is regulated by TMV signals. Before TMV infection and during the early stages of infection (0–3 hr), N_S is prevalent relative to N_L (25:1). However, N_L predominates over N_S (from 1:8 to 1:23) from 4 to 8 hr after infection. This shift in the ratio toward the N_L form is temporary and reversible, and the ratio returns to the original 25:1 state 9 hr after TMV infection. The relative ratio of the two *N* messages before and after TMV infection is important because the expression of only one transcript or both transcripts at a 1:1 ratio is not sufficient to confer TMV resistance. This finding suggests that perturbation of the capacity of the plant to express the proper ratio of the N_S and N_L transcripts can lead to loss of TMV resistance.

How do TMV signals regulate the ratio of the two *N* transcripts or splicing of the *N* gene? TMV is a single-stranded, positive-sense RNA virus that encodes at least four proteins. Two replicase proteins of 126 and 183 kDa are translated from genomic TMV RNA; the 30-kDa movement protein and 17.5-kDa coat proteins are translated from two subgenomic RNAs. The helicase region of the 126-kDa replicase protein is the elicitor for the *N* gene-induced disease resistance response (12–14). The *N* gene elicitor, the TMV replicase, localizes to the cytoplasmic face of the endoplasmic reticulum (ER) (15). If the *N* gene elicitor is at the cytoplasmic face of the ER, then how does the N protein-TMV elicitor interaction induce splicing changes that occur in the nucleus or changes in mRNA stability? One possibility is that the N protein, encoded by N_S transcript, the more abundant form early in infection, may interact directly or indirectly with the TMV elicitor. This interaction may induce a signaling cascade that regulates the alternative splicing of the *N* gene, resulting in the generation of N_L. There are many examples where alternative splicing is regulated in response to extracellular or physiological stimuli such as growth factors, hormones, cytokines, extracellular pH, heat shock, Ca^{2+} secondary messenger, and protein kinase (ref. 16 and references

therein). The *N*-TMV interaction is known to initiate rapid induction of reactive oxygen species, nitric oxide, Ca^{2+} and ion fluxes, serine/thronine protein kinase, mitogen-activated protein kinase cascade, and defense response genes (17). Because rapid, second messenger-induced cellular changes and protein kinases play an important role in alternative splicing in other systems, it is possible that *N*-TMV-induced secondary messengers or other unknown factors may function as signals to induce alternative splicing of the *N* gene after TMV infection.

Like the *N* gene, other plant TIR-NBS-LRR resistance genes (L^6, *M*, *RPP5*, and *RPS4*) have been shown to encode two or more transcripts (refs. 7–10; W. Gassman, M. Hinsch, and B. J. Staskawicz, personal communication). This finding suggests that the encoded protein products from these transcripts play a role in disease resistance. Our results from the *N*-TMV system indicate that the alternative N_L transcript and presumably the encoded truncated N^{tr} protein product have important roles in TMV resistance, which contrasts with the results published for L^6 flax resistance (9). Transgenic flax plants with an L^6 synthetic construct that fails to produce alternative transcripts induces a resistance response to rust that is similar to wild-type L^6-containing plants. However, those authors (9) cannot rule out possible functions provided by alternatively spliced transcripts of other *L* alleles or genes at the *M* locus in the transgenic plants, because no flax line is available that lacks other *L* alleles or genes at *M* locus.

Finally, our results suggest that the 3′ GS, 2.5 kb downstream from the AE, plays a crucial role in the regulation of the *N* gene alternative splicing or stability. The 3′GS is necessary for the generation or stability of the alternative N_L transcript and represents a distant control element for regulation of pre-mRNA alternative splicing or RNA stability. In many eukaryotic systems, the alternative splicing of pre-mRNA is influenced by cis sequences, cis structures, and trans-acting factors. Specifically, intron length, intron sequences, sequences in and around exons, exon size, and RNA secondary structures have major effects on alternative splicing. Future studies should focus on identification of precise cis sequences and trans-acting factors that are required for alternative splicing of the *N* gene and TMV resistance. In addition, detection and localization of N and N^{tr} proteins in the plant cells are required to elucidate their functions in plant defense.

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