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The *N* gene of tobacco confers resistance to tobacco mosaic virus in transgenic tomato

(hypersensitive response/plant disease resistance)

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ABSTRACT **It has been proposed that cloned plant disease resistance genes could be transferred from resistant to susceptible plant species to control important crop plant diseases. The recently cloned *N* gene of tobacco confers resistance to the viral pathogen, tobacco mosaic virus. We generated transgenic tomato plants bearing the *N* gene and demonstrate that *N* confers a hypersensitive response and effectively localizes tobacco mosaic virus to sites of inoculation in transgenic tomato, as it does in tobacco. The ability to reconstruct the *N*-mediated resistance response to tobacco mosaic virus in tomato demonstrates the utility of using isolated resistance genes to protect crop plants from diseases, and it demonstrates that all the components necessary for *N*-mediated resistance are conserved in tomato.**

Genetic resistance is an effective and environmentally benign means of controlling plant pathogens (1). Crop species often lack effective genetic resistance to some of their significant pathogens. Although resistance to these pathogens can be found in other plant species, barriers to interspecific crosses frequently prevent these resistance traits from being introduced by conventional breeding. One type of genetic resistance, termed "gene-for-gene" resistance, is defined by dominant genes that confer resistance to pathogens possessing corresponding dominant avirulence genes (2). Avirulence genes are thought to encode products that are recognized by plants bearing the appropriate resistance genes, where recognition triggers the induction of host defense responses. It has been proposed that cloned resistance genes could be transformed into susceptible species to achieve resistance against pathogens. Recently, the ability to test this hypothesis has been facilitated by the cloning of a number of plant disease resistance genes (3–9).

The *N* gene of tobacco confers a gene-for-gene resistance to the viral pathogen tobacco mosaic virus (TMV) and most other members of the tobamovirus family. *N* is a member of a class of disease resistance genes whose predicted protein products possess a putative nucleotide binding site and leucine-rich repeat region (7). The members of the nucleotide binding site/leucine-rich repeat class of resistance genes confer resistance to taxonomically diverse pathogens, including viruses, bacteria, and fungi (10). Additionally, the amino terminus of *N* possesses similarity to the cytoplasmic domain of the Toll and interleukin 1 receptors. *N*-mediated resistance to TMV is characterized by the formation of localized necrotic lesions at the sites of viral infection, which is known as the hypersensitive response (HR). TMV becomes localized to the cells in and immediately surrounding the HR lesions. The molecular identity of the viral product that triggers the *N*-mediated HR has not been well established. However, the 126-kDa replicase associated protein may be necessary for the induction of the *N*-mediated HR (11).

Some TMV strains can infect over 200 plant species, including most members of the Solanaceae (12, 13). Solanaceous plants, including tomato and pepper, are closely related to tobacco and are agriculturally important crop species. TMV can cause significant yield losses in tomato and pepper, and efforts to identify TMV resistance in these plants have been successful (reviewed in ref. 13). For example, the *Tm-1*, *Tm-2*, and *Tm-2a* loci confer resistance to most strains of TMV in tomato. In pepper, four genes (*L1–L4*) confer resistance to most strains of TMV. Tomato and pepper cultivars that lack these resistance genes are highly susceptible to systemic infection by TMV. Like *N*, the *Tm-2/2a* and *L* genes mediate an HR in response to infection by most viral strains. However, the TMV movement and coat proteins trigger host defense responses in tomato plants that carry *Tm-2/2a* genes (14) and pepper plants bearing the *L3* gene (15), respectively. The TMV encoded elicitors of the *L1-*, *L2-*, and *L4*-mediated resistances in pepper have not been determined. The *Tm-1* gene of tomato does not mediate an HR in response to TMV infection, but it reduces TMV replication in infected cells (16). Significantly, none of the tomato or pepper TMV resistance genes alone are effective against as many strains of tobamoviruses as the *N* gene. To date, only one tobamovirus strain, TMV-ob, can overcome *N*-mediated resistance (17). The ability of TMV-ob to overcome *N* may be conditional, because at temperatures less than 19°C, TMV-ob can induce a HR on plants bearing the *N* gene (17). If the *N* gene retained its resistance properties in tomato or pepper, then it could provide an additional means to combat tobamovirus diseases in these crops. In addition to crop protection, tomato plants expressing a functional *N* gene would provide an excellent genetic system to dissect the signal transduction pathway leading to HR and inhibition of viral replication and movement.

To test whether *N* could confer TMV resistance in another crop plant species, a TMV-susceptible tomato cultivar was transformed with the *N* gene. Here, we demonstrate that *N* confers resistance to TMV in transgenic tomato. As in tobacco, *N* mediates a HR, a common resistance response characterized by the formation of localized necrosis at infection sites that is correlated with inhibition of viral replication and movement. This is significant for two reasons. First, the transfer of a member of the nucleotide binding site/leucine-rich region class of resistance genes to another species demonstrates the utility of this class of genes in engineering crop resistance. Second, TMV and other related tobamoviruses can be devastating pathogens of solanaceous crops, including tomato and pepper, and we provide an additional, effective means to combat this disease genetically.

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Abbreviations: TMV, tobacco mosaic virus; TMV-U1, U1 strain of TMV; TMV-Cg, crucifer strain of TMV; ToMV, tomato mosaic virus; HR, hypersensitive response.

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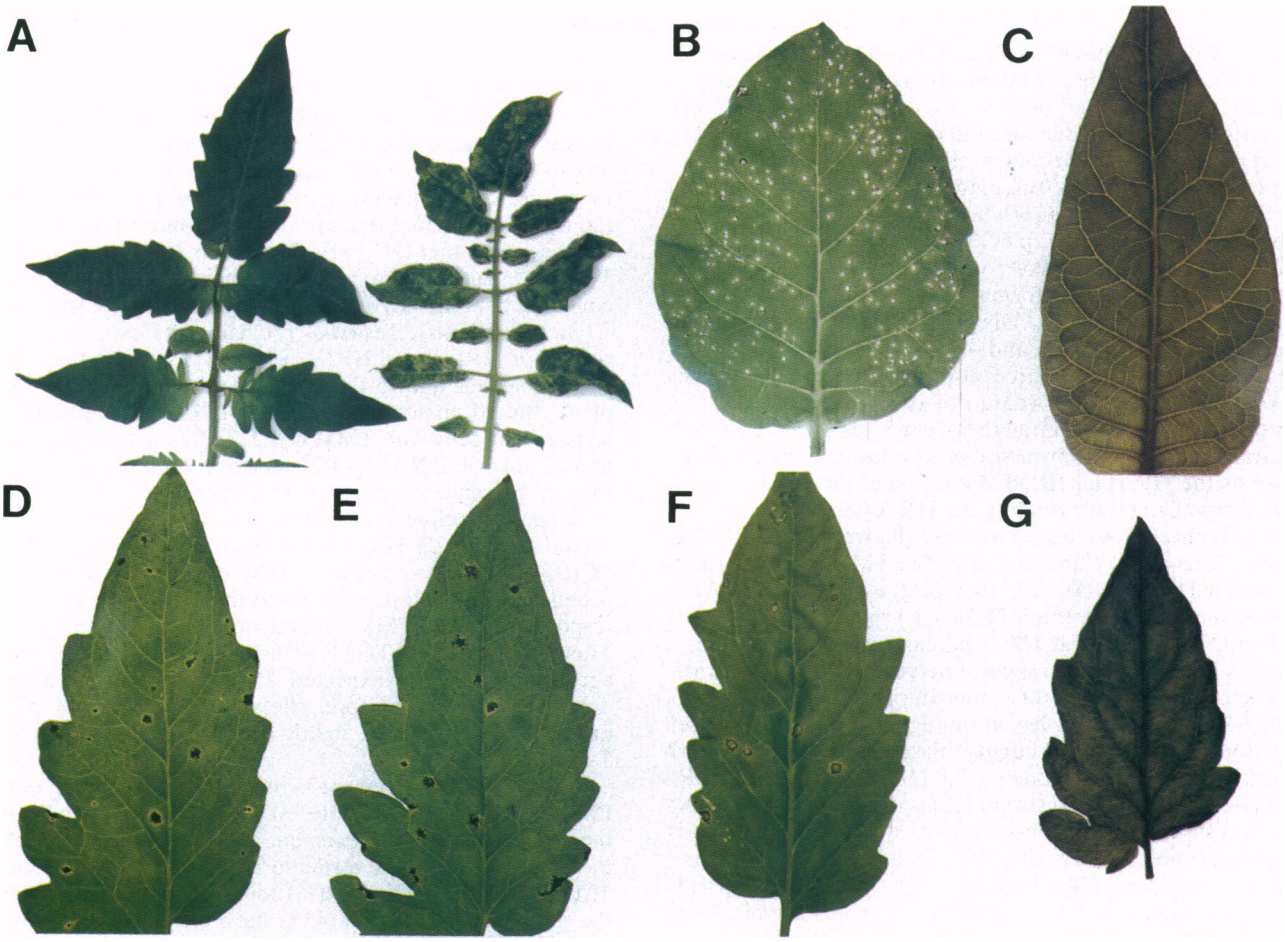

FIG. 1. The *N* gene confers a HR to tobamoviruses in transgenic tomato plants. (*A*) Uninfected VF36 (*Left*) and TMV-infected VF36 (*Right*). (*B*) Samsun NN inoculated with TMV-U1 at 24°C. (*C*) Mock-inoculated Samsun NN at 24°C. (*D*) Transgenic VTG34-1 inoculated with TMV-U1 at 24°C. (*E*) Transgenic VTG34-1 inoculated with ToMV at 24°C. (*F*) Transgenic VTG34-1 inoculated with TMV-Cg at 24°C. (*G*) Mock-inoculated transgenic VTG34-1 at 24°C. Leaves of tomato and tobacco plants were inoculated with either the TMV-U1, ToMV, or TMV-Cg tobamoviruses or mock-inoculated without virus.

MATERIALS AND METHODS

TMV Strains and Inoculation Procedures. The U1 strain of TMV (TMV-U1; provided by M. Zaitlin, Cornell University), tomato mosaic virus (ToMV; provided by W. Dawson, University of Florida), and the crucifer strain of TMV (TMV-Cg; provided by S. Naito, Hokkaido University, Japan) tobamoviruses were propagated in the TMV-susceptible tobacco cultivar Petite Havana SR1 (SR1). Viral inoculations were carried out by macerating leaf tissue of infected SR1 plants and diluting the sap in 20 mM sodium phosphate buffer (pH 7.0) and Carborundum 320 Grit (Fisher) (18). The sap suspension was then rubbed on tomato or tobacco leaves. Mock inoculations were performed by rubbing tomato or tobacco leaves with a suspension of phosphate buffer and Carborundum alone. In temperature shift experiments, inoculated plants or excised leaves were inoculated with the TMV suspension then placed at the nonpermissive temperature of 30°C or the permissive temperature of 24°C.

Transformation of Tomato with the *N* gene. A genomic DNA fragment containing the full-length *N* gene was introduced into the TMV-susceptible tomato cultivar VF36 with the pTG34 T-DNA construct by *Agrobacterium* transformation (19), and the four transgenic tomato lines bearing this construct are termed VTG34-1, 2, 3, and 4. pTG34 was constructed by subcloning a 13.0-kb *Xho*I fragment from the genomic DNA clone G34 bearing the *N* gene (7) into the T-DNA vector pOCA28 (20). pTG34 was chosen for these studies because it is the largest of two clones that have been shown to confer TMV resistance in transgenic tobacco plants (S.W. and B.B., unpublished data). There are no significant open reading frames located upstream or downstream of *N* in either of these clones (J. Coleman, S. P. Dinesh-Kumar, and B.B., unpublished data).

Amplification of an *N*-Specific PCR Fragment in Transgenic Tomato. The presence or absence of the pTG34 T-DNA construct bearing the *N* gene was determined by isolation of DNA from 48 VF36 × VTG34-1 progeny and analysis by PCR using the *N* specific primers SD1 (5′-GCATCTTCTTCTTCTTCTTC-3′) and SD2 (5′-GAGCCTTTGAGATTGGCCGC-3′) to amplify a 450-bp product. The parameters of the PCRs were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 25 cycles with 250 ng of tomato genomic DNA and 250 ng of each primer.

Isolation of Plant RNA and Detection of TMV. We tested for the presence or absence of TMV RNA in inoculated or upper, uninoculated leaves of tomato and tobacco plants by RNA gel blot hybridization. At 17 days postinoculation, total RNA was extracted from inoculated and upper, uninoculated leaves of tomato and tobacco plants using the RNeasy Plant Total RNA Kit (Qiagen, Chatsworth, CA). Total RNA (1 μg) was electrophoresed and blotted onto Genescreen nylon membrane. RNA gel blots were hybridized with a DNA probe from the 30-kDa movement protein gene of TMV, which was radiolabeled by the random hexamer method (Amersham).

RESULTS

The *N* Gene Mediates a Localized HR to TMV in Transgenic Tomato Plants. Systemic TMV infection of susceptible tomato cultivars is characterized by mottling of the leaves (Fig. 1*A*), stunted growth, yield reduction, and reduced fruit quality (21). To test if the *N* gene could confer resistance to TMV in tomato, we transformed the TMV-susceptible cultivar VF36 (19) with a T-DNA construct (pTG34) bearing the *N* gene, and we recovered four VF36, pTG34 transformants named VTG34-1, -2, -3, and -4. We confirmed the presence of the *N* gene in these four transformants by Southern blot hybridization of genomic tomato DNA with a probe from the *N* gene. The VTG34-1, -2, -3, and -4 tomato lines carried three to four, one, one, and three copies, respectively, of the pTG34 T-DNA construct (data not shown).

In tobacco cultivars bearing the *N* gene, resistance to TMV is characterized by the formation of localized necrotic lesions known as the HR (Fig. 1*B*). TMV becomes localized to cells in and immediately surrounding the HR lesions and does not move systemically. We tested whether the transgenic VTG34 tomato plants could elaborate an HR to TMV by inoculating leaves with TMV-U1. At ≈5–7 days postinoculation, localized necrosis was visible on the VTG34-1 leaves that were inoculated with TMV-U1 (Fig. 1*D*), whereas leaves from untransformed VF36 displayed no signs of necrosis (data not shown). The response of the other three transformants was identical to VTG34-1 with respect to lesion timing and appearance. The HR of tomato developed as circular black lesions with irregular margins surrounded by chlorosis (Fig. 1*D*). By comparison, HR on a leaf from the TMV-resistant tobacco cultivar Samsun NN appears as tan, circular lesions with discrete dark brown margins and no chlorosis (Fig. 1*B*). In contrast to the transgenic tomato plants, TMV-induced HR lesions were observed at ≈48 hr postinoculation on Samsun NN, which is typical for tobacco. Interestingly, transgenic tomato leaves that have been excised from plants, inoculated with TMV-U1, and sealed in a Petri dish on wetted Whatman paper can develop HR lesions at 2–3 days postinoculation, as do tobacco leaves subjected to the same treatment (data not shown). This suggests that tomato has the capacity to mediate HR with approximately the same timing as tobacco. However, onset of the HR of tomato may be influenced by different environmental and physiological factors than tobacco, such as light intensity, water potential, or humidity. Leaves of mock-inoculated Samsun NN and VTG34-1 plants do not develop localized necrosis (Fig. 1 *C* and *G*), demonstrating that HR is dependent on the presence of TMV and is not due to the inoculation procedure.

The *N* gene confers resistance to many tobamoviruses and has not been observed to be overcome by any tobamovirus in the field. Thus, the tobamovirus resistance conferred by *N* is extremely durable in tobacco. To test if *N* could confer resistance to other tobamoviruses in tomato, we inoculated transgenic tomato leaves with two additional tobamoviruses, ToMV and TMV-Cg. ToMV is the most common tobamovirus infecting tomato in the field (21). The nucleic acid sequence of ToMV is ≈80% identical to TMV-U1 (22). TMV-Cg is not usually found in tomato, but was used here because it is more distantly related to the TMV-U1 strain than to ToMV. An alignment of the nucleic acid sequence of TMV-Cg (GenBank accession no. D38444) to TMV (GenBank accession no. V01408) and ToMV (GenBank accession no. X02144) using the BESTFIT program (Genetics Computer Group) shows that TMV-Cg is related to both TMV-U1 and ToMV by ≈60% nucleic acid sequence identity. Both ToMV and TMV-Cg elicit HR on tobacco plants bearing *N* (data not shown). We tested ToMV and TMV-Cg for their ability to elicit HR on the leaves of transgenic tomato plants. HR lesions developed at 5–7 days postinoculation and were similar in appearance to those induced by TMV-U1 infection (Fig. 1 *E* and *F*). As observed with TMV-U1 infection, there were no differences in the HR lesion response of the other three tomato transformants (data not shown). However, the onset of HR lesion appearance was more rapid in response to ToMV infection than TMV-U1 or TMV-Cg. ToMV-induced HR could be observed at 6–12 hr before the appearance of HR lesions in response to the other tobamoviruses. This observation may be due to ToMV being more virulent in tomato than other tobamoviruses and thus inducing *N*-mediated defense responses more rapidly.

To confirm that the TMV-dependent HR observed on the VTG34-1 plant was due to the presence of the pTG34 T-DNA construct bearing the *N* gene, we testcrossed TMV-susceptible VF36 to VTG34-1. Testcross progeny were expected to segregate for HR(+) and HR(−) phenotypes, and if the HR(+) phenotype was due to the presence of *N*, then only HR(+) plants should possess the pTG34 T-DNA. We inoculated 48 testcross progeny with TMV-U1; 23 individuals displayed HR and 25 did not. DNA was isolated from each plant and tested for the presence of the pTG34 construct by PCR using *N* gene-specific primers to amplify a 450-bp product. We observed that the 23 HR(+) individuals possessed the 450-bp PCR product, whereas the 25 HR(−) individuals lacked the product. This result demonstrated that the *N* gene is directly responsible for the TMV-dependent HR in transgenic tomato. The segregation of pTG34(+) to pTG34(−) plants is also in agreement with the expected 1:1 segregation for a single dominant gene. Therefore, the multiple copies of pTG34 present in VTG34-1 are tightly linked and comprise a single T-DNA locus.

Systemic TMV Movement Is Blocked in Transgenic Tomato Plants. The formation of HR lesions in tobacco coincides with the inhibition of viral movement and localization of TMV to the inoculated leaf. We tested to see if systemic movement of TMV was abolished in HR(+) tomato plants. A probe from the gene encoding the 30-kDa movement protein (P30) of TMV-U1 was hybridized to RNA gel blots containing total RNA from inoculated and upper, uninoculated leaves of HR(−) VF36 and HR(+) VTG34-1 progeny. In VF36 plants, viral RNA was expected to be detected in both the inoculated and upper, uninoculated leaves of the same plant. However, in HR(+) progeny of VTG34-1, we expected that TMV-U1 RNA would be detected at relatively low levels in the inoculated leaf

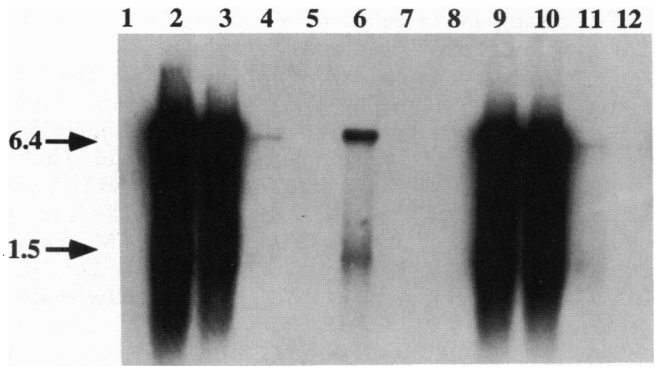

FIG. 2. Systemic movement of TMV is inhibited in the VTG34-1 line. Lane 1, VF36 mock-inoculated leaf; lane 2, VF36-inoculated leaf; lane 3, VF36 upper, uninoculated leaf; lane 4, VTG34-1 progeny plant-inoculated leaf; lane 5, VTG34-1 progeny plant upper, uninoculated leaf; lane 6, VTG34-1 progeny plant-inoculated leaf; lane 7, VTG34-1 progeny plant upper, uninoculated leaf; lane 8, SR1 tobacco mock-inoculated; lane 9, SR1 tobacco-inoculated leaf; lane 10, SR1 tobacco upper, uninoculated leaf; lane 11, Samsun NN-inoculated leaf; and lane 12, Samsun NN upper, uninoculated leaf. Total RNA was isolated from tomato and tobacco leaf tissue collected at 17 days postinoculation. RNA gel blots were hybridized with a DNA probe corresponding to the movement protein gene of TMV-U1. The 6.4-kb genomic RNA and a subgenomic RNA of TMV-U1 identified by the movement protein probe are indicated.

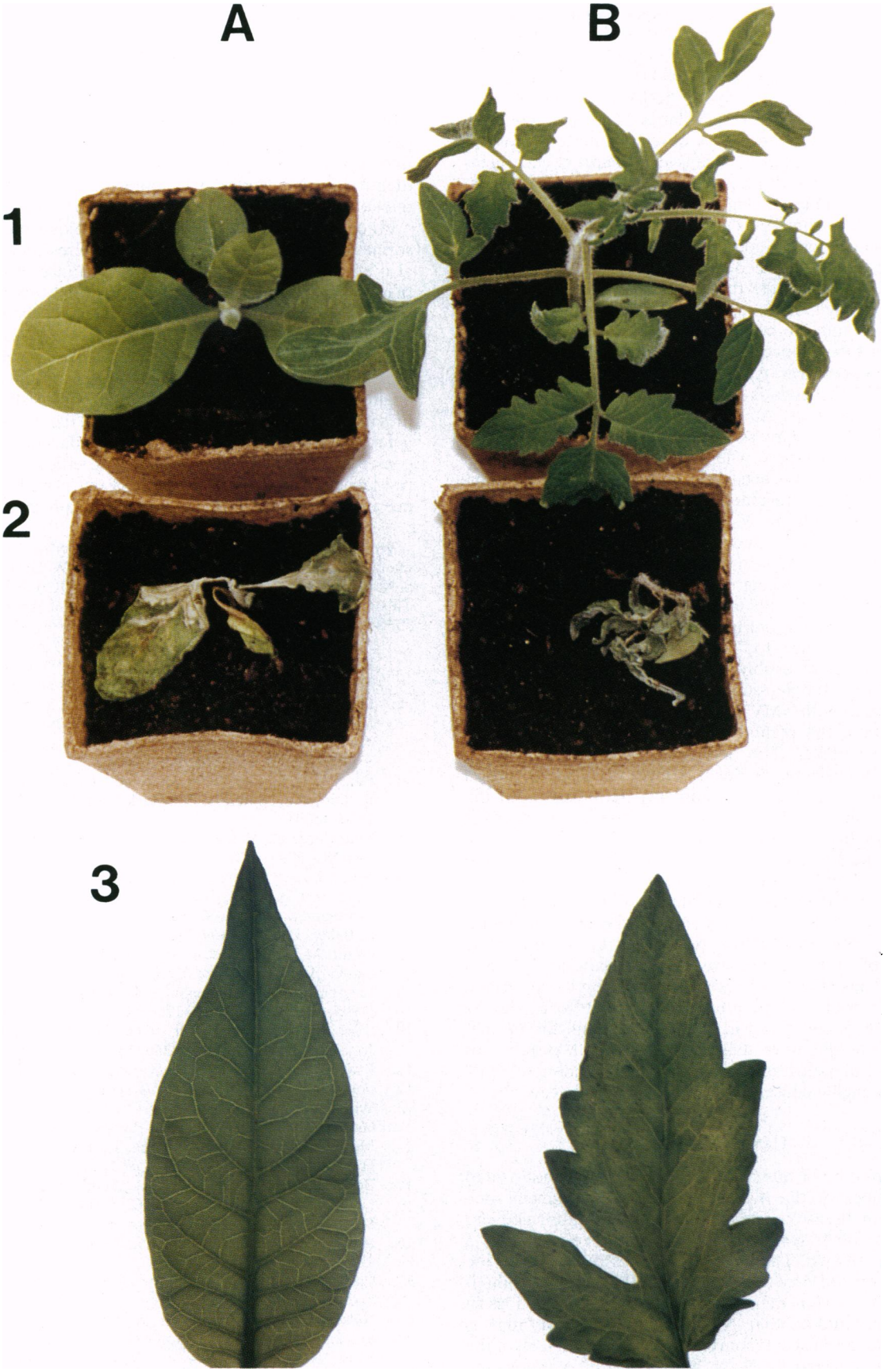

FIG. 3. The *N*-mediated HR of tomato is reversibly inactivated at high temperatures. (*A1*) TMV-susceptible SR1 tobacco 7 days post-temperature shift. (*B1*) TMV-susceptible VF36 tomato 7 days post-temperature shift. (*A2*) *N*-containing Samsun NN tobacco 7 days post-temperature shift. (*B2*) A transgenic VTG34-1 plant 7 days post-temperature shift. (*A3*) A close-up of a Samsun NN leaf inoculated with TMV-U1 at 30°C. (*B3*) A close-up of a VTG34-1 leaf inoculated with TMV-U1 at 30°C. Tobacco and tomato plants with and without *N* were inoculated with TMV-U1 at the nonpermissive temperature of 30°C. At 4 days postinoculation, the plants were shifted to the permissive temperature of 23°C to restore the *N*-mediated HR and induce systemic necrosis.

and would be absent in upper, uninoculated leaves. We observed no hybridization of the P30 probe to total RNA isolated from a mock inoculated VF36 plant (Fig. 2, lane 1), whereas copious amounts of TMV-U1 genomic and subgenomic RNAs were observed in RNA of inoculated and upper, uninoculated leaves (Fig. 2, lanes 2 and 3). In contrast, very little P30 hybridization was observed in RNA isolated from inoculated leaves of two transgenic plants (Fig. 2, lanes 4 and 6) and no hybridization was observed in upper, uninoculated leaves (Fig. 2, lanes 5 and 7). The results are indistinguishable from P30 hybridization to total RNA from inoculated and upper, uninoculated leaves of TMV-susceptible and TMV-resistant tobacco cultivars (Fig. 2, lanes 8–12). These results demonstrate that expression of the *N* gene in tomato effectively inhibits replication and systemic movement of TMV, as in tobacco bearing the wild-type *N* gene.

N-Mediated Resistance to TMV Is Reversibly Inactivated by Elevated Temperatures. A useful property of the *N*-mediated HR to TMV in tobacco is that it is reversibly inactivated at elevated temperatures. At temperatures of 28°C and above, *N*-mediated HR is suppressed and TMV moves systemically (23). The HR is restored when the temperature is reduced below 28°C and necrosis occurs throughout the plant, presumably due to systemic movement of TMV, followed by massive cell death mediated by *N*. The property of temperature sensitivity of the *N*-mediated HR was exploited to identify *N* loss-of-function mutations in tobacco, which led to the cloning of *N* by transposon tagging. If this property is conserved in tomato, then it could be employed in a genetic selection scheme to isolate mutations in other loci that are necessary for *N*-mediated resistance to TMV. To test whether the *N*-mediated HR was reversibly inactivated at elevated temperatures in tomato, SR1, Samsun NN, VF36, and VTG34-1 plants were inoculated with TMV-U1 and placed at 30°C. At 4 days postinoculation, the plants were shifted to the permissible temperature of 23°C. The SR1 tobacco and VF36 tomato plants were not affected by this treatment because they lack a functional *N* gene (Fig. 3 *A1* and *B1*). However, both the Samsun NN and VTG34-1 plants developed systemic necrosis within 12–18 hr following the temperature shift and were completely dead at 7 days following the shift (Fig. 3 *A2* and *B2*). Fig. 3 *A3* and *B3* shows a close-up of Samsun NN and VTG34-1 leaves that were inoculated with TMV-U1 at 30°C and displayed no sign of HR lesions. Following the temperature shift to 23°C, the HR was restored as evidenced by the formation of systemic necrosis on the Samsun NN and VTG34-1 plants (Fig. 3 *A2* and *B2*). These results confirmed that the reversible, temperature-sensitive nature of the *N*-mediated HR is conserved in tomato. Although the mechanism of the temperature sensitive block is not known, this result further demonstrates that the pathway leading to TMV resistance is highly conserved in tomato and tobacco.

DISCUSSION

The major finding of this study is that we have demonstrated that a member of the nucleotide binding site/leucine-rich region class of disease resistance genes can be transformed into another crop plant species, where it confers resistance to a significant pathogen. The *N* gene mediates resistance to the viral pathogen TMV by the formation HR lesions and inhibition of TMV movement in transgenic tomato, as it does in tobacco. The finding that N-mediated TMV resistance is reconstituted in transgenic tomato demonstrates that all of the components required by *N* for both TMV recognition and signal transduction are conserved in tomato. Tomato and tobacco are closely related by virtue of being members of the plant family Solanaceae. There are a number of *N* homologues in tomato that might function in disease resistance (24), which may explain why the signal transduction components required for *N*-mediated resistance are present in tomato. It will be interesting to test if *N*-mediated resistance to TMV can reconstituted in plant species more distantly related to tobacco, such as the crucifers, which can also be hosts for tobamovirus infection. Significantly, *N* confers resistance to TMV in a genetically tractable species where genetic tools (25–28) and a powerful selection for loss-of-function mutations using the temperature shift protocol (7) can be used to identify genes encoding the components of the signal transduction pathway leading to HR and resistance to TMV.

Another resistance gene, *Pto,* the unique member of the serine-threonine kinase class of disease resistance genes, has also been demonstrated to confer resistance in heterologous plant species. *Pto* confers resistance to *Pseudomonas syringae* bacterial strains possessing the avirulence gene *avrPto* in tomato. *Pto* has been shown to encode resistance to *P. syringae* strains expressing avrPto in the heterologous species *Nicotiana benthamiana* and *Nicotiana tabacum* (29, 30). The ability to engineer plant species with new pathogen resistance specificities indicates that, within the Solanaceae, the molecular constituents of pathogen induced signal transduction pathways are conserved. This work demonstrates the efficacy of exchanging resistance genes between members of this plant family for engineering genetic resistance to destructive pathogens.

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