MOLECULAR PLANT-MICROBE INTERACTIONS
VOL. 4, No. 5, pp. 458-463, 1991

Tobacco Mosaic Virus Elicitor Coat Protein Genes Produce a Hypersensitive Phenotype in Transgenic *Nicotiana sylvestris* Plants

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Received 16 April 1991. Accepted 13 June 1991.

The N′ gene hypersensitive response (HR) in *Nicotiana sylvestris* is elicited during virus replication by the coat proteins of specific tobacco mosaic virus (TMV) mutants. In this study, both elicitor and nonelicitor coat protein open reading frames, under the control of the cauliflower mosaic virus 35S promoter, were integrated into the genome of *N. sylvestris*. Transgenic plants expressing a nonelicitor coat protein had a phenotype indistinguishable from nontransformed control plants. Transgenic plants expressing elicitor coat proteins displayed reduced growth and chlorosis and developed necrotic patches that eventually coalesced and collapsed entire leaves. This necrotic response appeared earlier and was more severe in transgenic plants expressing a strong elicitor coat protein than in plants expressing a weak elicitor coat protein. This demonstrates that expression of elicitor coat proteins independently of viral replication can induce the HR in *N. sylvestris*.

For many plant virus diseases, host resistance provides an important means of control. One type of host resistance, termed the hypersensitive response (HR), is expressed in the plant through the development of a necrotic lesion that occurs at the site of virus infection and results in confinement of the virus to this area. The development of the HR is thought to depend on the interaction between a host resistance gene and a pathogen avirulence gene (Flor 1971; Keen 1990). Work done in both fungal and bacterial systems has identified the presence of pathogen avirulence genes responsible for eliciting complementary host resistance genes (Keen 1990), suggesting a requirement for host-pathogen recognition before induction of the HR.

The N′ gene of *Nicotiana sylvestris* Speg. & Comes confers HR resistance against most strains of tobacco mosaic virus (TMV), but not against the U1 strain. Mutants of the systemically infecting U1 strain of TMV with single amino acid substitutions in the coat protein have been found to induce the HR in *N. sylvestris* (Knorr and Dawson 1988; Culver and Dawson 1989a). The induction of the HR in *N. sylvestris* by TMV has been mapped precisely to five different amino acid substitutions in the coat protein (Culver and Dawson 1989a). In addition, a number of other amino acid substitutions in the coat proteins of TMV mutants that induce the HR in *N. sylvestris* have been found (Funatsu and Fraenkel-Conrat 1964; Wittmann and Wittmann-Liebold 1966; Mundry *et al.* 1990). These additional mutations may also be responsible for eliciting the N′ gene HR.

Alteration of the coat protein translational start codon (AUG to AGA) of an HR-inducing TMV mutant resulted in a virus that could not produce coat protein and did not elicit the HR (Culver and Dawson 1989b). Thus, the production of these altered coat proteins during viral replication is required for eliciting the N′ gene HR. However, the complexity of components involved in replication made it impossible to demonstrate that coat protein alone was responsible for inducing this host response. Induction of the HR may have been dependent on interactions between altered coat proteins and other viral components or replication processes. In this paper, we demonstrate that transgenic *N. sylvestris* plants expressing HR eliciting coat proteins develop a phenotype characteristic of the N′ gene HR, while transgenic *N. sylvestris* plants expressing a non-HR eliciting coat protein display a healthy phenotype. Thus, only the expression of elicitor coat proteins is required for the induction of N′ gene HR.

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MATERIALS AND METHODS

Plasmid construction and plant transformation. The *Agrobacterium* binary transformation vector, pMON530 (Rogers *et al.* 1987) was utilized to express three separate coat protein open reading frames (ORFs). TMV coat protein ORFs were obtained from pTMV204, pTMV11, and pTMV25, all previously described (Dawson *et al.* 1986; Culver and Dawson 1989a). TMV nucleotide numbering is from Goelet *et al.* (1982). Virus 3′ end, *Hin*dIII (5081 nt) to *Pst*I (6406 nt), fragments were first subcloned into pUC19. A smaller 731-bp fragment, *Dra*I (5708 nt) to *Eco*RI (6439 nt), containing the coat protein ORF, the virus 3′ nontranslated region, and 33 bp of pUC19 was then ligated into the polylinker region, *Sma*I to *Eco*RI, of pMON530 between the 35S promoter of cauliflower mosaic virus and a nopaline synthase gene polyadenylation sequence to create the transformation vectors pAT204, pAT11, and pAT25 (Fig. 1). The orientation and sequence of each transformation vector was verified by sequence analysis.

Leaf disks from *N. sylvestris* were cocultivated with *Agrobacterium tumefaciens*, strain LBA4301, carrying either pAT204, pAT11, or pAT25. Transformed plant cells were selected for kanamycin resistance and regenerated into plants as previously described (Horsch *et al.* 1985). Regenerated plants were then potted in soil and grown in growth chambers at 25° C under light (10,000 lux, 12 hr). Seeds

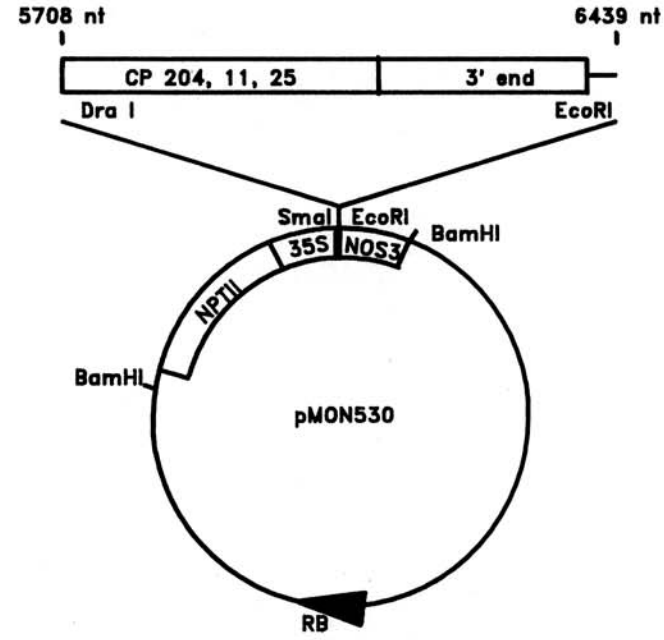

Fig. 1. Construction of tobacco mosaic virus coat protein transformation vectors. Coat protein open reading frames and 3′ nontranslated ends (5708 to 6439 nt), obtained from pTMV204, pTMV11, and pTMV25 (Culver and Dawson 1989a), were ligated into the polylinker region of the *Agrobacterium* binary transformation vector pMQN530 (Rogers *et al.* 1987) to create pAT204, pAT11, and pAT25, respectively. Nucleotide numbering is from Goelet *et al.* (1982). RB, Right border of the Ti plasmid; NPT II, Neomycin phosphotransferase II gene; NOS 3′, nopaline synthase gene polyadenylation sequence; 35S, cauliflower mosaic virus promoter.

from regenerated transformants were germinated on water agar containing 100 μg/ml kanamycin. Green seedlings were transplanted to soil and grown under growth chamber or greenhouse conditions.

DNA extraction and Southern blots. Genomic DNA was extracted from leaves of *N. sylvestris* plants by a method previously described (Dellaporta *et al.* 1983). DNA (20 μg) was digested with *Bam*HI, resolved on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labeled fragment of the coat protein gene (5664–6207 nt), using standard procedures (Southern 1975; Maniatis *et al.* 1982).

Protein extractions and western immunoblotting. Regenerated plants expressing kanamycin resistance were screened for coat protein production by western immunoblot analysis. Proteins were extracted by grinding 3 g of leaf tissue in liquid nitrogen, followed by the addition of 6 ml of grinding buffer (30 mM potassium phosphate, pH 7.5, 0.4 M NaCl, 10 mM 2-mercaptoethanol, and 0.1% Triton X-100). Crude extract was centrifuged for 10 min at 12,000 × *g* and protein concentrations determined (Bradford 1976). Clarified extracts were mixed with equal volumes of sample buffer (Laemmli 1970) and heated for 3 min at 100° C. Protein samples (50 μg) were resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel, transblotted onto nitrocellulose, and incubated with coat protein specific antiserum using methods previously described (Towbin *et al.* 1979; Lehto *et al.* 1990). Nitrocellulose blots were then incubated with anti-IgG alkaline phosphatase conjugate and coat protein bands visualized with nitro blue

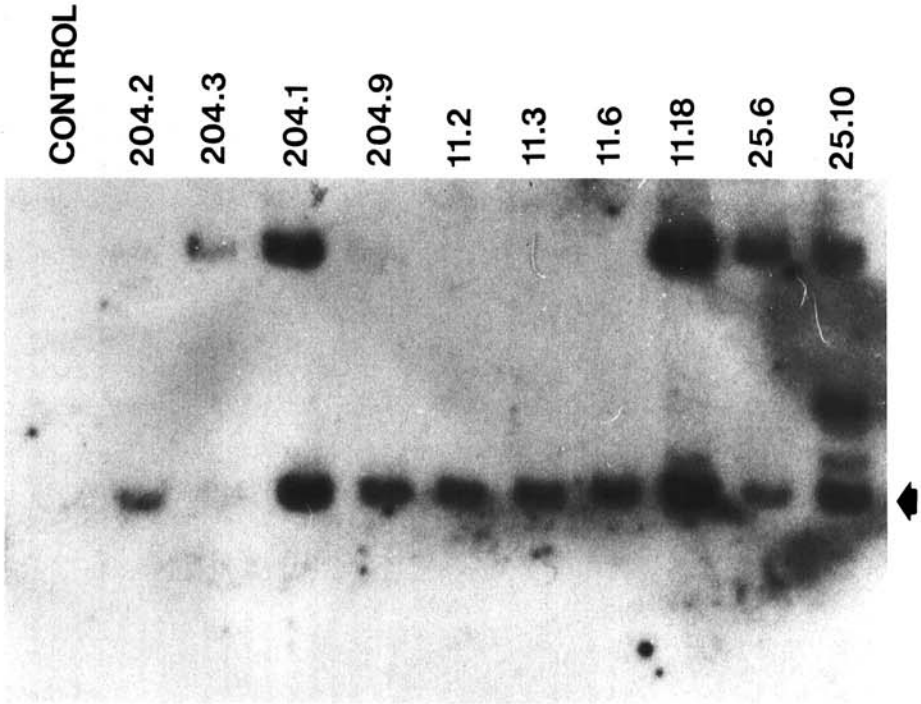

Fig. 2. Southern blot analysis of transgenic *Nicotiana sylvestris* plants. Genomic DNA (20 μg) digested with *Bam*HI was loaded in each lane. The arrow is positioned at a predicted 3.9-kbp fragment (Fig. 1) indicating integration of the 35S promoter, coat protein open reading frame, 3′ nontranslated region, and NOS polyadenylation sequence into the genome of the transformed plants. Higher bands are due to the incomplete digestion of genomic DNA. Numbers before the decimal represent the transformed coat protein, numbers after the decimal represent the individual plant. Lanes 204.2, 204.3, 11.2, 11.3, 25.6, and 25.10 contained DNA from independently regenerated transgenic plants. Lanes 204.1, 204.9, 11.6, and 11.18 contained DNA from the F1 generation of transgenic plants.

tetrazolium (3 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate substrate (1.5 mg/ml) in buffer (10 mM Tris-HCl, pH 9.5,. 0.1 M NaCl, and 5 mM MgCl). Known amounts of virion isolated coat protein (Fraenkel-Conrat 1957) were used as standards.

RESULTS

Phenotypes of selected HR-inducing coat proteins. Three different coat protein ORFs were selected for transformation into *N. sylvestris* (Fig. 1). Transformation vector pAT204 contained the wild-type coat protein ORF from the U1 strain of TMV that does not elicit the HR in *N. sylvestris*. TMV U1 moves systemically and causes the development of a mosaic symptom in *N. sylvestris*. Transformation vectors pAT11 and pAT25 contain the coat protein ORFs of TMV 11 and TMV 25, respectively. Both TMV 11 and TMV 25 are coat protein mutants of TMV U1 that induce the HR in *N. sylvestris*. Each mutant differs from TMV U1 by a single nucleotide substitution in the coat protein ORF that results in amino acid substitutions of Val to Met at position 11 for TMV 11, and Asn to Ser at position 25 for TMV 25 (Culver and Dawson 1989a).

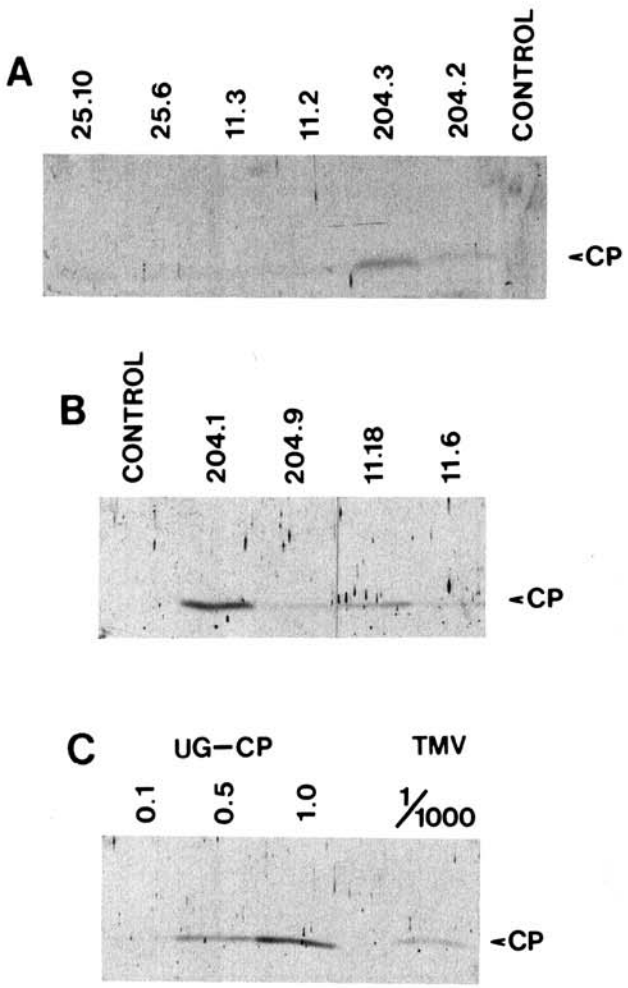

Fig. 3. Western immunoblot analysis of transgenic *Nicotiana sylvestris* plants. Approximately 50 μg of total extracted leaf protein was loaded in each lane of panels A and B. Numbers before the decimal represent the coat protein expressed, numbers after the decimal represent the individual plant. Controls represent the proteins extracted from a nontransformed healthy *N. sylvestris*. **A,** Proteins extracted from six independently regenerated transgenic *N. sylvestris* plants. **B,** Proteins extracted from the F1 generation of transgenic plants. **C,** Known amounts (μg) of purified coat protein and a 1/1,000 dilution of a 280-μg protein sample extracted from a leaf of *N. sylvestris* systemically infected with TMV U1.

Mutants TMV 11 and TMV 25 also differ in their ability to induce HR in *N. sylvestris* (Culver and Dawson 1989a). TMV 11 induces the slow development of necrotic lesions that appear 5–7 days postinfection. Lesions induced by TMV 11 continually expand and lead to the collapse of large portions of leaf tissue. This slow response defines mutant TMV 11 as a "weak elicitor" of the N′ gene HR. In contrast, TMV 25 induces the rapid development of necrotic lesions that appear 2–3 days postinfection and do not expand from the original infection site. This rapid induction of the N′ gene HR defines mutant TMV 25 as a "strong elicitor."

Transgenic plant phenotypes. Regenerated, kanamycin-resistant plants were analyzed for the genomic insertion of a full-length coat protein ORF. Southern blot analyses of these plants revealed the presence of a predicted 3.9-kbp fragment, containing the coat protein ORF (Fig. 2). Regenerated plants were also screened by western immunoblot analysis for coat protein production (Fig. 3). Transgenic plants were then allowed to self-fertilize and F1 generation seedlings screened for maintenance and expression of the coat protein ORF.

Eleven of 22 regenerated *N. sylvestris* plants transformed with pAT204 expressed detectable levels of coat protein (data shown for regenerated transgenic plants 204.2 and 204.3) (Fig. 3A). Regenerated transgenic plants expressing the wild-type U1 coat protein displayed normal development when compared with nontransformed *N. sylvestris* plants (Fig. 4A3). However, some necrosis on older leaves of both the wild-type coat protein transformed plants and nontransformed control plants did appear 4–5 mo after transplanting. Plants derived from the F1 generation of pAT204 transformed plants (data shown for the F1 generation of transgenic plants 204.1 and 204.9) (Fig. 3B) also expressed coat protein and displayed the same normal phenotype (data shown for the F1 generation of transgenic plant 204.1) (Fig. 4B3 and C3).

Of 30 regenerated plants transformed with pAT11, 14 expressed detectable levels of the TMV 11 weak elicitor coat protein (data shown for regenerated transgenic plants 11.2 and 11.3) (Fig. 3A). Transgenic plants expressing the TMV 11 coat protein displayed slight to moderate reductions in growth. These transgenic plants also developed varying degrees of necrosis across fully expanded leaves. In general, other than slowed growth, these plants developed normally for the first month after being transplanted. After 1 mo, chlorotic patches and small necrotic spots developed on older expanded leaves. Necrotic areas slowly expanded over a 4- to 8-wk period and eventually coalesced, resulting in the collapse of entire leaves (Fig. 4A2). In addition, many of the transgenic lines showed slight chlorosis in young plants and in leaves not yet fully expanded. Regenerated transgenic plants that did not express coat protein 11 did not display the necrotic phenotype and developed normally. The F1 generation (data

shown for the F1 generation of transgenic plants 11.6 and 11.18) (Fig. 4B and C) displayed similar reductions in growth along with the development of chlorosis and necrosis in older leaves of plants expressing the weak elicitor coat protein (Fig. 3B).

Detectable levels of the TMV 25 strong elicitor coat protein were expressed in two out of 17 pAT25 transgenic plants (Fig. 3A). These two plants, 25.6 and 25.10, were severely stunted and developed large necrotic areas that led to the collapse of entire leaves before their full expansion (Fig. 4A1). Necrotic development occurred on these plants within 2 mo after transplantation to soil and the necrosis then spread into the vascular tissue, resulting in the death of the plants. A third regenerated transgenic plant also

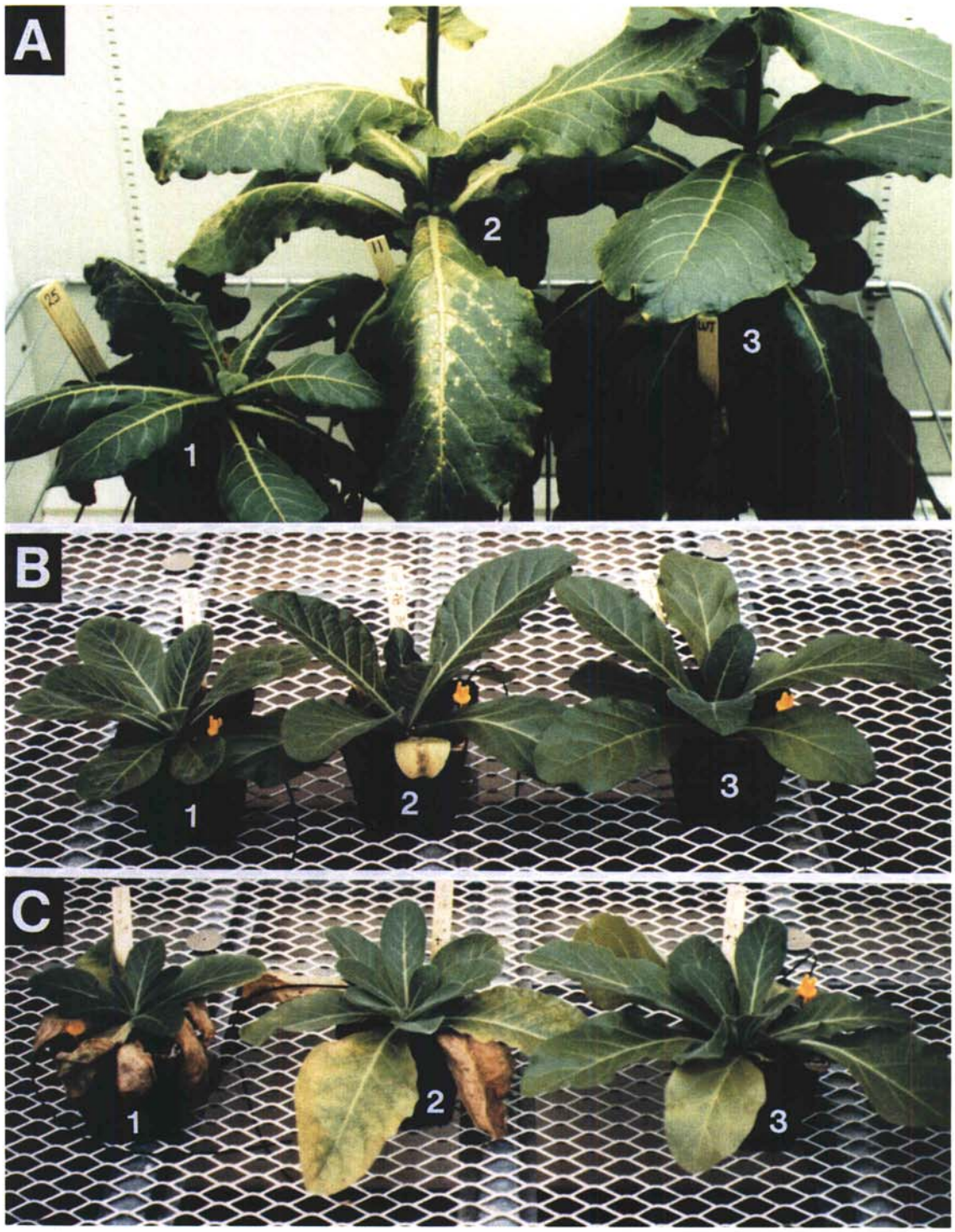

Fig. 4. Phenotypes displayed by transgenic *Nicotiana sylvestris* plants. **A,** Independently regenerated transgenic plants 1, 25.6; 2, 11.2; and 3, 204.3, 3 mo after transplantation to soil. **B,** The F1 generation of transgenic plants 1, 11.6; 2, 11.18; and 3, 204.1 after 1.5 mo of growth. **C,** The F1 generation of transgenic plants 1, 11.6; 2, 11.18; and 3, 204.1 after 3 mo of growth.

had a similar necrotic phenotype but did not express detectable levels of coat protein. Due to the necrosis and death of transgenic plants expressing the TMV 25 strong elicitor coat protein, no F1 generation was obtained. Other pAT25 transgenic plants that did not express detectable levels of coat protein failed to display the necrotic phenotype.

Coat protein production in transgenic plants. Protein samples were obtained from expanded leaves of approximately the same developmental stage. Levels of coat protein were estimated from western immunoblots of samples containing 50 μg of total extracted leaf protein (Fig. 3).

The maximum accumulation of coat protein was found in plants transformed with the wild-type U1 coat protein. Wild-type coat protein accumulation reached a maximum of approximately 2% of the total extracted leaf protein for the F1 generation of transgenic plant 204.1 (Fig. 3B). The highest level of coat protein accumulation found in transgenic plants expressing the TMV 11 weak elicitor coat protein was obtained from the F1 generation of transgenic plant 11.18 and was estimated to be 1% of the total extracted leaf protein (Fig. 3B). A range of lower coat protein levels was also obtained for plants transformed with either the wild-type or weak elicitor coat proteins, with approximately half of the total regenerated plants accumulating a detectable level of coat protein. In contrast, only low levels of coat protein accumulated in the two transgenic plants expressing the TMV 25 strong elicitor coat protein (Fig. 3A). Transgenic plants, 25.6 and 25.10, accumulated the strong elicitor coat protein at a level of less than 0.2% of the total extracted leaf protein. None of the transgenic plants accumulated coat protein at levels near that found in a systemic wild-type TMV infection (Fig. 3C).

F1 generation transgenic plants expressing the TMV 11 weak elicitor coat protein did not show a correlation between coat protein accumulation and the severity of the HR phenotype. For example, the HR phenotype displayed by transgenic line 11.18 was milder than that displayed by transgenic line 11.6 (Fig. 4B and C) even though line 11.18 accumulated more coat protein (Fig. 3B). This type of variation was observed for a number of the transgenic lines expressing the weak elicitor coat protein, but not for all. Transgenic plants accumulating low levels of the TMV 25 strong elicitor coat protein displayed a faster and more severe HR than transgenic plants accumulating higher levels of the TMV 11 weak elicitor coat protein. This reflects the differences observed in the development of necrotic lesions by the two mutant viruses.

Effect of virus replication on transgenic phenotypes. To determine if additional viral components or processes would affect the HR phenotype expressed by the transgenic plants, infectious RNA of a mutant, TMV [-CP] was inoculated onto the leaves of F1 generation transgenic plants expressing the weak elicitor coat protein. Mutant TMV [-CP] contains an altered coat protein translational start codon (AUG to AGA) and does not produce coat protein, but does replicate and move from cell-to-cell in *N. sylvestris* (Culver and Dawson 1989b). TMV [-CP] infections were used to provide all other components of a TMV infection *in trans* to the coat protein produced by the transgenic plants. The replication of TMV [-CP] in inoculated leaves of transgenic plants expressing the weak elicitor coat protein was determined by western immunodetection of the viral 126K protein (data not shown). TMV [-CP] infections were established in transgenic plants at times before and after the display of a visible necrotic phenotype. The presence of a TMV [-CP] infection did not alter the development of the HR phenotype expressed by the elicitor transgenic plants at any of the inoculation times. Thus, viral processes and components other than coat protein that are present during replication do not affect the development of the HR phenotype.

DISCUSSION

To determine if elicitor coat proteins expressed in the absence of viral replication would elicit the N′ gene HR, transgenic *N. sylvestris* plants expressing elicitor and non-elicitor coat proteins were produced. Plants that expressed elicitor coat proteins were found to display necrosis and reduced growth, while control plants expressing the wild-type coat protein displayed a "healthy phenotype." This demonstrated that elicitor coat proteins alone were sufficient to induce the N′ gene HR.

The ability to obtain surviving *N. sylvestris* plants that constitutively express an elicitor to the N′ gene HR, suggests that the expression of this resistance response is developmentally regulated. Khan and Jones (1988) showed that *N. sylvestris* plants could be regenerated from callus tissue containing high titers of an HR-inducing strain of TMV. These regenerated plants developed necrosis on expanded leaves, similar to the phenotype displayed by transgenic plants expressing elicitor coat proteins. Thus, *N. sylvestris* callus tissue and immature plants have a reduced ability to express the HR. Only upon the development of more mature plant tissue does a visible HR appear.

A systemic infection by TMV can result in the accumulation of high levels of coat protein, up to 10% of the dry weight of the leaf lamina (Fraser 1987). Levels of coat protein produced by transgenic plants demonstrated that substantially lower amounts of coat protein can lead to the induction of the N′ gene HR. However, the development of a necrotic response in transgenic plants expressing elicitor coat proteins was marginal, requiring weeks to months for the development of a necrotic response. In comparison, viral infections that produce high levels of elicitor coat proteins induce the development of necrosis in only a few days. Thus, the rapid induction of HR required for resistance appears to depend on high expression levels of elicitor coat proteins in developed leaf tissue.

No correlation existed between the level of coat protein accumulation and the severity of necrosis in different transgenic plants expressing the weak elicitor coat protein. This may indicate a difference in the cell- and tissue-specific expression of the coat protein ORF in different transgenic plants, possibly as a result of chromosomal position. Previous studies have identified large variations in the tissue-specific expression of integrated genes under the control of the CaMV 35S promoter (Benfey *et al.* 1989; Hanley-Bowdoin *et al.* 1989). Display of the HR phenotype in transgenic plants may therefore depend on the accumulation of elicitor coat proteins in specific cell or tissue types.

The strength of the elicitor coat protein appears to play

a major role in determining the severity of the HR phenotype. Low levels of strong elicitor coat protein produced a severe plant response, while higher levels of the weak elicitor coat protein induced a mild plant response. It was also difficult to obtain transgenic plants expressing the TMV 25 strong elicitor coat protein, compared with transgenic plants that expressed the wild-type or weak elicitor coat proteins. This may indicate that the stronger elicitor coat proteins have a higher affinity for interacting with the N′ gene product than do the weaker elicitor coat proteins.

ACKNOWLEDGMENTS

This work was supported by grant 90-37262-5384 from the U.S. Department of Agriculture.

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