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Expression of a Viral Avirulence Gene in Transgenic Plants Is Sufficient to Induce the Hypersensitive Defense Reaction

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Tobacco plants containing the N′ resistance gene exhibit a hypersensitive defense reaction when infected with tomato mosaic virus (ToMV); infection results in necrotic lesions at the primary infection sites. In an attempt to investigate the molecular mechanism(s) underlying this plant-pathogen interaction, the ToMV coat protein gene was joined by a transcriptional fusion to the strong constitutive 35S RNA promoter from cauliflower mosaic virus. This chimeric gene was introduced via *Agrobacterium*-mediated transformation into isogenic tobacco cultivars differing only with respect to the N′ gene. Strong necrotic reactions were observed on most emerging calli of the N′ genotype, but never on calli lacking the N′ resistance gene. These data indicate that the coat protein of ToMV is, on its own, sufficient to induce a hypersensitive reaction in tobacco. Thus, recognition of a single viral gene product may be the only prerequisite for the induction of a specific defense reaction in higher plants.

Additional keywords: coat protein expression, *Nicotiana tabacum*, PR-1 proteins.

Resistance in plants against pathogens is, in many cases, dependent on resistance genes that confer protection against a small group of pathogens or only against some strains of one particular microorganism. This interaction between plants and pathogens is very specific. Avirulence genes on the side of the pathogen may induce the plant's defense reaction, which is characterized by a localized necrotic response at the primary infection sites (hypersensitive reaction; Ellingboe 1982). A well-studied example is the interaction of tobacco mosaic virus (TMV) with *Nicotiana sylvestris* Speg. plants containing the N′ resistance gene. Although TMV is able to spread systemically in infected *N. sylvestris* plants, tomato mosaic virus (ToMV), a close relative of TMV, as well as some artificial and naturally occurring mutants of TMV induce the hypersensitive response. Many of these viral mutants have been characterized in detail, and they contain amino acid exchanges in the region of the coat protein gene (Knorr and Dawson 1988; Culver and Dawson 1989; Mundry *et al.* 1990). Similar amino acid changes have also been found in the coat protein of ToMV, thus suggesting that the coat protein gene may be the avirulence gene in the context of the N′ gene. Recombinant viruses were constructed between TMV and ToMV and between TMV and some of the local lesion-producing TMV mutants. The chimeric viruses were inoculated on *N. sylvestris* plants and elicited a hypersensitive response (Knorr and Dawson 1988; Saito *et al.* 1987). These experiments confirmed that the viral coat protein gene seems to be the avirulence gene in the case of the N′ resistance gene.

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For further studies of the specific recognition events between plants and pathogens involved in the induction of the necrotic defense reaction, one must define the gene product of the pathogen that, on its own, is sufficient to trigger this response. Therefore, we investigated whether the coat protein of ToMV fulfills this requirement. In our experimental approach, we expressed ToMV coat protein (Meshi *et al.* 1986), under the control of the strong constitutive cauliflower mosaic virus (CaMV) 35S RNA promoter (pBin19/35SC4; Fig. 1A), in isogenic tobacco cultivars differing with respect to the N′ gene (*Nicotiana tabacum* L. 'Samsun nn' and 'Samsun EN'). For control experiments, transgenic tobacco plants containing the *Escherichia coli* (Migula) Castellani and Chalmers β-glucuronidase reporter gene (GUS) fused to the CaMV 35S RNA promoter (pBin19/35S[GUS]; Fig. 1B) were generated.

N. tabacum is an amphidiploid species that contains the genomes of *N. sylvestris* and *N. tomentosiformis* Goodspeed and should, therefore, express the N′ gene. However, many *N. tabacum* cultivars are systemically infected by TMV and ToMV, so they do not exhibit the N′ phenotype; this suggests that the N′ gene is repressed in these tobacco cultivars. *N. tabacum* 'Samsun nn' is a systemic host for ToMV, whereas *N. tabacum* 'Samsun EN' is a spontaneous mutant (Melchers *et al.* 1966) of *N. tabacum* 'Samsun nn' that displays the N′ phenotype with ToMV infection. This phenotype is inherited in a Mendelian fashion and is correlated with one dominant gene allelic to the naturally occurring N′ gene of *N. tabacum* 'Java' (Melchers *et al.* 1966).

For transformation experiments, plasmids pBin19/35SC4 and pBin19/35S[GUS] were transferred to the *Agrobacterium* strain LBA4404 by triparental mating (Bevan 1984), and the resulting strains were used to infect leaf discs of *N. tabacum* 'Samsun nn' and 'Samsun EN', respectively (Horsch *et al.* 1985; Beilmann *et al.* 1991). After transfer of the leaf pieces to selective medium, petri dishes were monitored every week for the number and the phenotype

of the emerging calli. After 6 wk of selection on kanamycin-containing medium, green calli began to emerge from Samsun nn leaf discs transformed by pBin19/35SC4 (Fig. 2B). Abundant calli emerged at this time from Samsun EN leaf discs transformed by the *Agrobacterium* strain containing plasmid pBin19/35S[GUS] (Fig. 2A). In contrast, leaf discs from *N. tabacum* 'Samsun EN', which had been transformed by the pBin19/35SC4 *Agrobacterium* strain, were significantly retarded in callus formation (Fig. 2C), and the overall number of calli was reduced to approximately 10% as compared to Samsun nn leaf discs (data not shown). After 9 wk of selection, most of these calli developed visible necrotic areas that turned dark brown, and some calli even died (Fig. 2F). On the other hand, Samsun EN calli transformed with the GUS construction (Fig. 2D) and calli from Samsun nn leaf discs transformed with the ToMV coat protein construction (Fig. 2E) continued to grow vigorously and had already started to regenerate shoots. After 12 wk, even more drastic differences between the three transformation experiments were evident (Fig. 2G–I). To examine if the necrotic regions observed with the ToMV coat protein construction in Samsun EN leaf disc calli were confined to the outer cell layers or if larger areas of the developing tissue were affected, we cut several calli in half. As shown in Figure 2J, most calli revealed large brown internal regions of necrosis.

Apart from the N′ gene, the different responses of the two isogenic tobacco cultivars to the presence of the ToMV coat protein could be the result of differences in the amounts of coat protein expressed. We, therefore, estimated the levels of ToMV coat protein formed by western blotting of protein extracts isolated from transformed callus tissue (Pfitzner and Goodman 1987). As shown in Figure 3, the level of expression of the ToMV coat protein is very similar in both tobacco cultivars. In all calli tested, we could detect between 100 and 500 ng of ToMV coat protein per gram fresh weight. Similar results were obtained with 12 other independent transformants (data not shown). Therefore, induction of a necrotic reaction seems to depend solely on the genotype of the tobacco cultivar used for transformation.

During the hypersensitive reaction, many different host proteins are induced in the infected plants. One important class of proteins, the pathogenesis-related proteins (PR proteins), is a group of heterogeneous exoproteins that play a role in the plant defense reaction against pathogens (van Loon 1985). On the other hand, some PR proteins are also induced by other stimuli (e.g., by [acetyl]salicylic acid or by plant hormones during tissue culture procedures) (White 1979; Memelink *et al.* 1987).

To determine if a correlation between the expression of PR proteins and the necrotic reactions in calli transformed with the ToMV coat protein construction exists, we prepared soluble protein extracts. Proteins from *N. tabacum* 'Samsun EN' calli expressing the GUS reporter gene and from *N. tabacum* 'Samsun EN' and 'Samsun nn' calli expressing ToMV coat protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed for the presence of PR-1 proteins, a major group of the PR proteins, by western blotting (Pfitzner and Goodman 1987). Both calli transformed with the ToMV coat protein showed a strong signal (Fig. 4, lanes 2,3). This induction of PR-1 proteins in the transformed tissue is, however, not correlated with the necrotic reaction, because PR-1 proteins, albeit to lower levels, were also observed with extracts from *N. tabacum* 'Samsun nn' calli, which did not exhibit any necrotic regions (Fig. 4, lane 2).

We have demonstrated that a plant defense reaction can be triggered by the expression of an avirulence gene in transgenic plants containing the corresponding resistance gene. Thus, at least in the case of the tobacco N′ gene, recognition of a single viral protein, the native ToMV coat protein, may be the only prerequisite for induction of the hypersensitive reaction. Similar observations relevant to our findings have been reported recently by Culver and Dawson (1991). These authors transferred coat protein genes of wild-type and mutant TMV strains, which induced either a systemic infection or a hypersensitive defense reaction, into *N. sylvestris* plants containing the N′ resistance gene. Transformants that expressed elicitor coat proteins exhibited necrosis and reduced growth, whereas control plants expressing the wild-type TMV coat protein displayed a "healthy phenotype" (Culver and Dawson 1991). Thus, very similar results can be obtained by both experimental approaches: expression of one coat protein gene in different tobacco genotypes and expression of mutant coat protein genes in a single tobacco cultivar. One major difference between our results and the results of Culver and Dawson is, however, the severity of necrosis observed with coat protein transformed tissue. Although Culver and Dawson

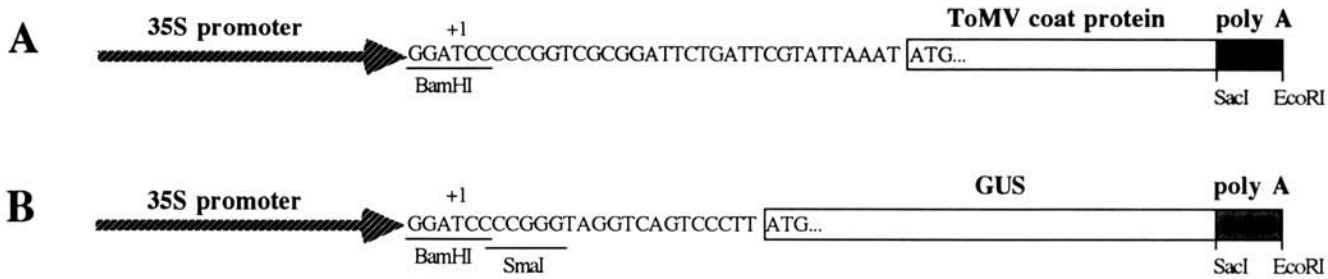

Fig. 1. Structure of chimeric genes used for the plant transformation experiment. Both plasmids contain the cauliflower mosaic virus 35S RNA promoter and the polyadenylation signal from the nopaline synthase gene (NOS). Restriction enzyme sites relevant to the constructs are indicated. **A,** Tomato mosaic virus (ToMV) coat protein expression vector pBin19/35SC4. **B,** For control experiments, pBin19/35S[GUS] was used. It is similar to pBin19/35SC4, except that the coat protein open reading frame is replaced by the β-glucuronidase gene (GUS) from *Escherichia coli.*

were able to regenerate plants expressing the elicitor coat protein and exhibiting various degrees of necrosis during later stages of plant development, we found strong necrotic reactions already at the callus level with tissue expressing coat protein. This may be attributable either to physiological differences between *N. tabacum* 'Samsun EN' and *N. sylvestris* or to the fact that the ToMV coat protein interacts more strongly with the cellular target than the TMV U1 mutant coat proteins. We suggest that our experimental system and that of Culver and Dawson (1991) can

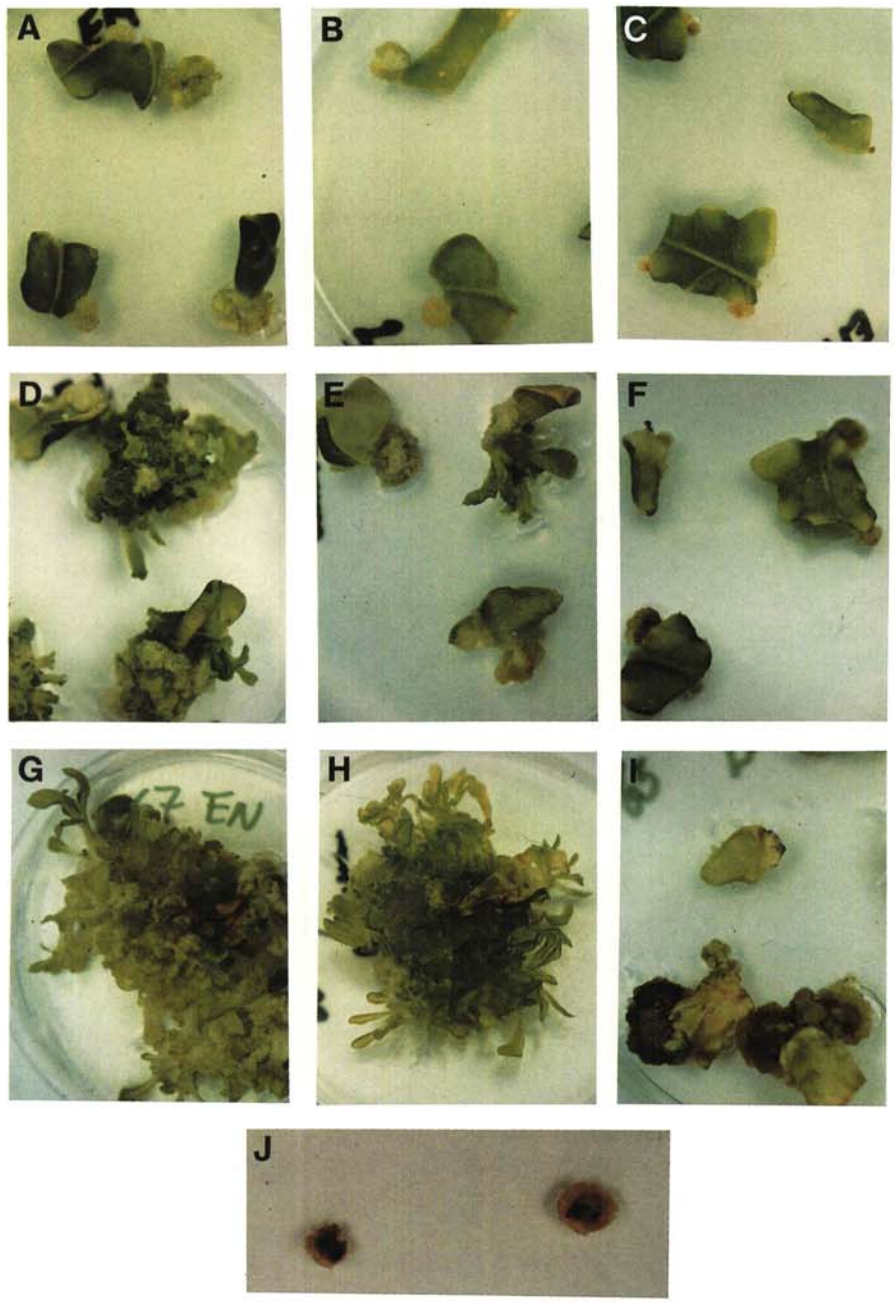

Fig. 2. Phenotype of calli and shoots developing from different *Nicotiana tabacum* genotypes as a result of expression of the tomato mosaic virus (ToMV) coat protein. **A, D, G,** Control transformation of *N. tabacum* 'Samsun EN' leaf discs with pBin19/35S[GUS] after 6 (A), 9 (D), and 12 wk (G) of selection, respectively. **B, E, H,** Transformation of *N. tabacum* 'Samsun nn' leaf discs with the ToMV coat protein expression vector pBin19/35SC4. No significant differences were observed when compared to the control transformation after 6 (B), 9 (E), and 12 wk (H) of selection, respectively. **C, F, I,** Transformation of *N. tabacum* 'Samsun EN' leaf discs containing the N′ resistance gene with the coat protein expression vector pBin19/35SC4. Retardation of callus growth and browning of the tissue are visible after 6 (C) and 9 wk (F) of selection, respectively. Even after 12 wk of propagation, shoot formation was not observed in general (I). **J,** Cross sections of calli as shown in I. Calli, which do not exhibit major areas of brown tissue on the outside, generally not contain large regions of necrotic tissue inside. Representative results are shown.

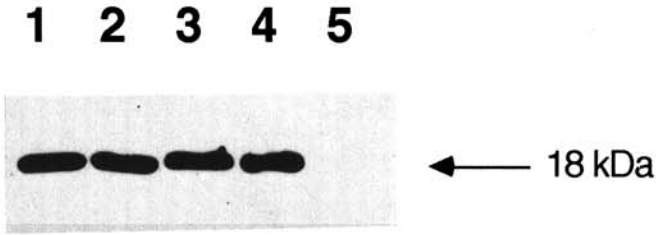

Fig. 3. Expression of tomato mosaic virus (ToMV) coat protein in transformed callus tissue. Proteins were extracted 9 wk after transformation from a callus of *Nicotiana tabacum* 'Samsun nn' transformed with pBin19/35SC4 (lane 2), from a callus of *N. tabacum* 'Samsun EN' transformed with pBin19/35SC4 (lanes 3,4), and from a callus of *N. tabacum* 'Samsun EN' transformed with pBin19/35S[GUS] (lane 5). Equal amounts of protein from the extraction of approximately 20 mg of callus tissue were separated on a 15% sodium dodecyl sulfate polyacrylamide gel and analyzed for expression of the ToMV coat protein by western blotting with the enhanced chemiluminescence (ECL) detection system. As a reference, 10 ng of ToMV was loaded onto the gel in lane 1. Similar results were obtained with 12 other independent transformants. Although the phenotype of the calli appeared very different, no differences could be observed in the level of expression of the coat protein.

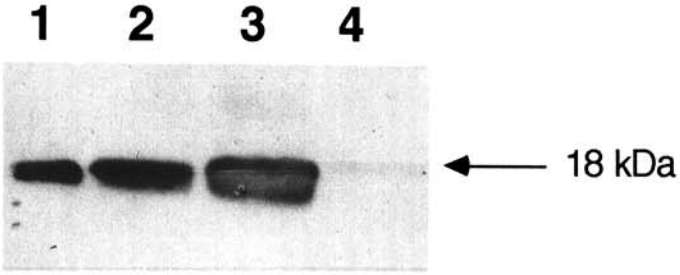

Fig. 4. Expression of pathogenesis-related proteins (PR-1) in transformed callus tissue. Proteins were extracted 9 wk after transformation from a callus of *Nicotiana tabacum* 'Samsun nn' transformed with pBin19/35SC4 (lane 2), from a callus of *N. tabacum* 'Samsun EN' transformed with pBin19/35SC4 (lane 3), and from a callus of *N. tabacum* 'Samsun EN' transformed with pBin19/35S[GUS] (lane 4). Equal amounts of protein from the extraction of approximately 20 mg of callus tissue were separated on a 15% sodium dodecyl sulfate polyacrylamide gel and analyzed for the expression of PR-1 proteins by western blotting. As a reference, 100 ng of purified PR-1 proteins was loaded onto the gel in lane 1. Representative results are shown. Both tobacco cultivars expressing the tomato mosaic virus (ToMV) coat protein show a strong induction of the endogenous PR-1 proteins, although necrotic reactions could be observed only with *N. tabacum* 'Samsun EN' callus tissue.

be used as a model system for the study of interactions between avirulence genes and resistance genes in higher plants.

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