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Disease Symptoms in Transgenic Tobacco Induced by Integrated Gene VI of Cauliflower Mosaic Virus

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Abstract

A chimeric vector (pKR 612B1) containing the neomycin phosphotransferase (APH) gene from the Tn5 transposon under the control of the gene VI promoter of cauliflower mosaic virus (CaMV) and the cloned gene VI region (*Sal*I–*Bst*EII) of the same virus were used to cotransform tobacco protoplasts. Using the polyethylene glycol transformation procedure, a large number of protoplasts were transformed and proved to be resistant to kanamycin (Km). Whole Km-resistant plants were regenerated and shown to contain the integrated foreign genes. DNA from transformed clones was analyzed by Southern blot hybridization, showing the presence of the Tn5-derived gene and the viral gene. Transgenic plants containing the viral gene show mild mosaic patterns and fasciation. The expression of the gene VI product was detected by immunoblots.

Introduction

Knowledge about the genome organization of plant viruses allows us to think much more constructively about possible ways in which viruses might induce symptoms. Following the inoculation of leaves, a systemic host virus moves from the point of inoculation to young leaves 3–4 days after inoculation. Whether macroscopic symptoms develop usually depends upon the strain of virus used and on the host. Symptoms induced by plant viruses can range from necrotic reactions or abnormal growth to the common mosaic pattern on the leaves. Symptom devel-

opment is influenced by different factors: leaf age, environmental conditions, etc. (see 1 for details). The most common symptom on virus-infected plants is the development of light and dark areas, which form a mosaic effect in the systemically infected leaves. Induction of disease by viruses has been reviewed in the past and was recently summarized by Zaitlin and Hull (2). The severity of symptoms does not correlate with the extent of virus accumulation after infection (3). This may be due to the fact that different viruses may induce symptoms by different mechanisms.

All cauliflower mosaic virus (CaMV) strains are able to infect a wide range of cruciferous plants but only a few other plants (4). Most CaMV isolates produce typical viral-type mosaic symptoms in systemically infected leaves (5–7). At the microscopic level, viral inclusion bodies can be detected (7,8). Cytoplasmic inclusion bodies called *viroplasms* are the major site of virion accumulation in the cell and contain a proteinaceous matrix (9). The most abundant viral-coded protein $P_{62\text{-}66}$ encoded by gene VI of CaMV is translated from an independent polyadenylated 19S messenger RNA (10–13). Gene VI is probably responsible for host range control of CaMV, as suggested by Daubert et al. (14). Schoelz and Shepherd (15) have used recombinant genomes of CaMV—namely D4, CM 1841, and Cabb B—to show that a CaMV host range determinant is encoded within the first half of region VI. This recombinant DNA technology has made it possible to understand how this virus causes disease and how its genetic material interacts with the host genome. In order to determine which virus gene or genes have important roles in disease induction, we used this well-characterized virus as a model (16–18). In our experimental system we tried to determine how gene VI of CaMV behaves in plants after integration and expression. The data presented here are compared with a recent publication (19) in which the authors describe similar results on symptomatic phenotypes in transgenic tobacco induced by integrated gene VI.

Materials and Methods

The cloning of DNA fragments was performed essentially according to standard procedures (20). Restriction enzymes and T4 ligase were purchased from New England Biolabs, Boehringer Mannheim, BRL, and Amersham, and used according to the suppliers' instructions. All radioactive materials were purchased from IZINTA (Budapest). The construction scheme for plasmid pKR 612B1 (Fig. 1B) was published earlier (21). The gene VI construct was made directly from a full-length cloned copy of CaMV DNA, isolate Cabb S, by restriction at the *Sal*I–*Bst*EII sites. The restriction fragment containing the 19S promoter, ORF VI, and the polyadenylation site was recovered from the agarose gel and used for transformation experiments (Fig. 1A).

Tobacco mesophyll protoplasts of *Nicotiana tabacum* L. cv. Petite Havanna line SR 1 were isolated from sterile shoot culture according to Nagy and Maliga (22). Protoplasts were cotransformed as described (23) with *Nru*I linearized plas-

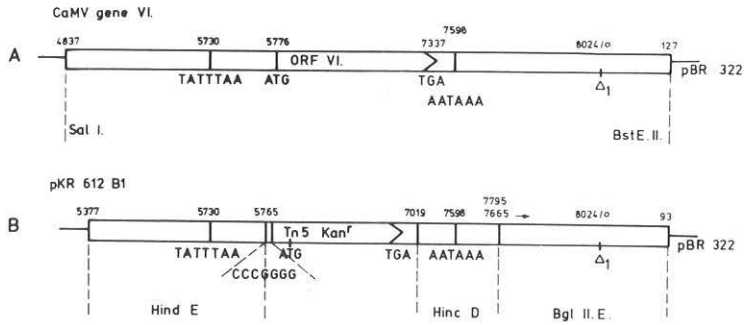

Fig. 1. Maps of plasmids CaMV gene VI (A) and pKR 612B1 (B) under the control of the 19S promoter of CaMV. The numbering system is according to Franck et al. (16).

mid pKR 612B1 and with the viral fragment containing the intact coding region of gene VI of CaMV. Microcalli resistant to kanamycin (Km) were selected in the presence of 200 μg/ml Km and were grown in agarose beads as suggested (24). Km-resistant calli were regenerated to whole plants. DNA was isolated from calli or from regenerated plants after the procedure of Paszkowski et al. (25). Southern blot analysis was performed using 5–10 μg of digested genomic DNA per track (26).

Proteins were electrotransferred from NaDodSO$_4$ PAGE to nitrocellulose membranes at 40 V overnight and treated as described (27). Rabbit antiviroplasm protein serum with a milk-based solution (28) and goat preimmune serum, and peroxidase-coupled goat antirabbit IgG, was used to detect the gene VI product (27,28).

Results and Discussion

After transformation of tobacco protoplasts with a cloned gene VI fragment of CaMV and with the chimeric Km gene and regeneration of Km-resistant plants, 3 of 6 plants showed mild mosaic symptoms (Fig. 2). In several cases we found other viral-type symptoms, including severe stunting and/or fasciation of the transformed plants (Figs. 2C and 2D). Transgenic tobacco plants bearing only the chimeric Km gene were phenotypically normal (Fig. 2A).

Recent data have shown that differences in the type of symptoms are due to the virus isolate used for transformation (19). Gene VI from the Cabb B-JI isolate usually gave a blotchy phenotype, whereas gene VI from the CM 1841 isolate produced a uniform light-green background. In our experiments the mild mosaic symptoms can be attributed to the Cabb S isolate used throughout the transformation series (16).

Baughman, Jacobs, and Howell (19), in a detailed deletion study on gene VI, have clearly shown that an 8-bp frame shifting linker mutation near the start of the

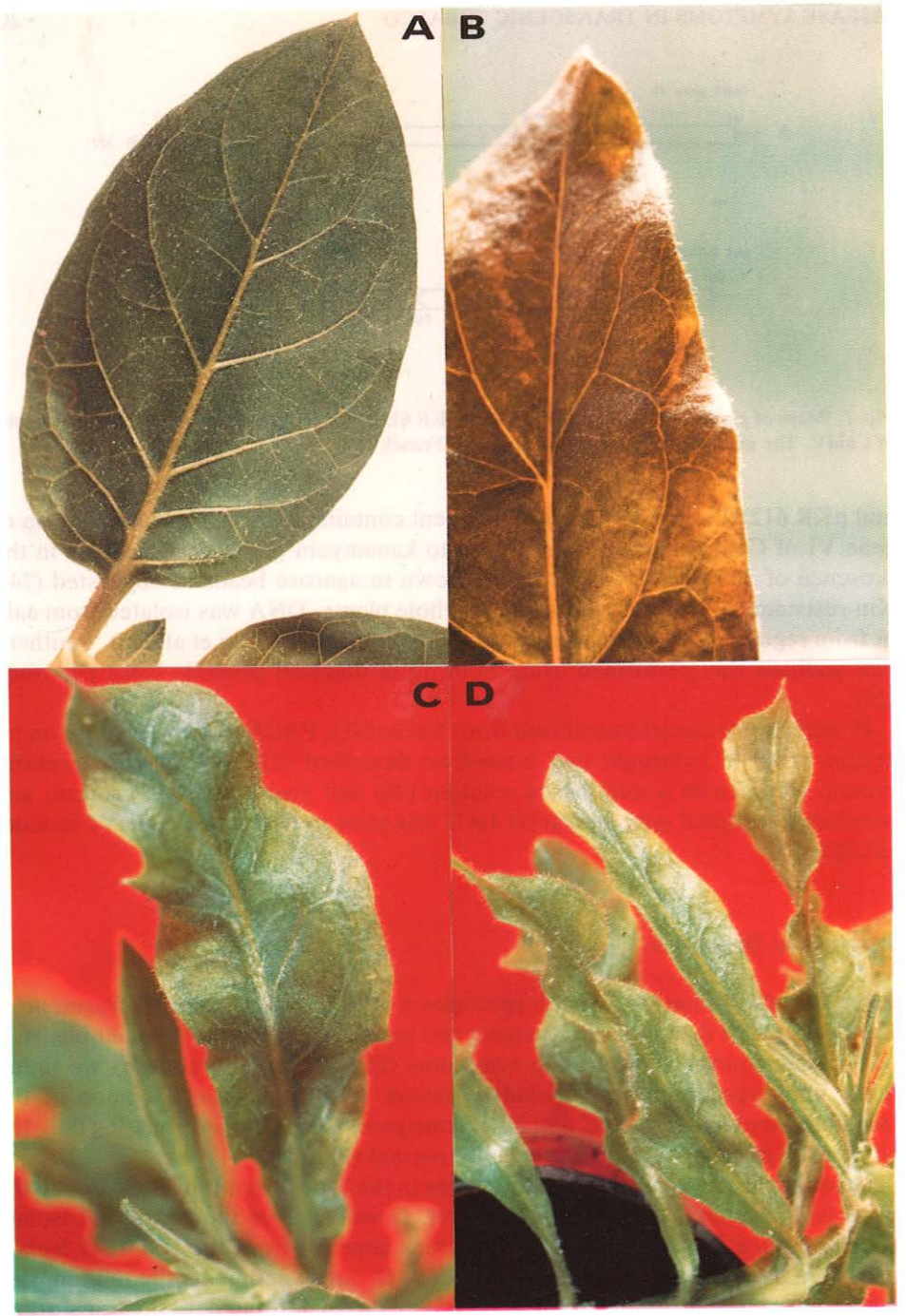

Fig. 2. Symptoms of transgenic plants. *A:* Km-resistant fertile symptomless transformed tobacco leaf. *B:* Fine mosaic symptom induced by integrated gene VI of CaMV. *C,D:* Stunting and fasciation of tobacco induced by integrated gene VI.

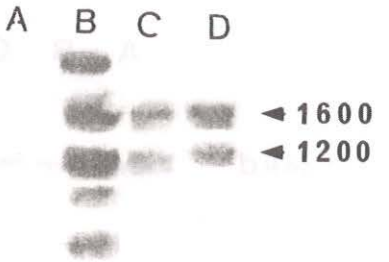

Fig. 3. Southern blot of DNA extracted from tobacco plants regenerated from transformed protoplasts. Lane A, mock-inoculated protoplast line; lane B, sample from plant showing severe fasciation; lanes C,D, samples from independent transformed lines. DNA was extracted from 1 g of plant tissue. Probe was nick translated (α ^{32}P-CaMV). DNA fragments corresponding the integrated genes are 1600 bp and 1200 bp, respectively.

gene VI coding region abolishes the symptomatic phenotype of the transformed plant. Deletion of the 35S transcription unit had no effect on symptom production.

Transgenic plants differed in the severity of the symptoms. This may be due to variation in expression of the CaMV DNA segment associated with different numbers of copies or sites of integration in the genome. Using immunoblot detection, they found a near-perfect correlation between the appearance of symptoms and the presence of $P_{62\text{-}66}$ protein. Our transgenic plants were analyzed by Southern blot to detect integrated sequences. In all cases tobacco plants bearing the gene VI fragment of the CaMV genome (Fig. 3) showed mild mosaic symptoms.

When plants exhibited fasciation, we often found several integrated fragments differing in size. This may be due to rearrangement of the viral sequences. Plants showing mosaic symptoms contained only the integrated gene VI and the marker Km gene (1600 bp and 1200 bp, respectively).

To explain the origin of the fasciation and abnormal growth, we would like to point out a similarity between the experiment of Schmülling et al. (29) and our data. While studying independent and synergistic activities of *rol* loci of Agrobacterium rhizogenes in stimulating abnormal growth in plants, Schmülling et al. found that changes in the mode of expression obtained by replacing promoters of the *rol* B and *rol* C genes with the 35-S promoter of CaMV elicited new and distinct developmental alterations. They concluded that regulation of expression plays a crucial role in generating morphological alterations in plants. Their observations are in good agreement with our results when transgenic tobacco showed fasciation and growth retardation. This can be attributed not only to the different viral fragment that was integrated, but also to the use of a different vector and

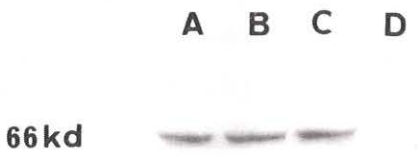

Fig. 4. Immunoblot of proteins extracted from callus tissue and reacted with viroplasm antiserum. A,B,C lanes, proteins extracted from transformed-protoplast-derived callus; D lane, Km-resistant callus extract.

promoter. The ratio of cotransformation of the nonselectable marker and the selectable marker was 1:2. We deduced this ratio from the number of regenerated Km-resistant and phenotypically mosaic plants. When we include the data with other types of symptoms, as many as 70% were double transformants. Such a high efficiency of cotransformation was also obtained by Schocher et al. (30), using direct gene transfer with zein, nopaline-synthase, and Km genes. In our experiment we were not able to demonstrate a clear correlation between the presence of $P_{62\text{-}66}$ and the appearance of the symptoms (Fig. 4). In all cases, when we obtained mild mosaic symptoms we observed different amounts of $P_{62\text{-}66}$ hr. In addition, Km-resistant calli bearing the viral gene VI had a slower growth rate than the single Km transformants. They also produced fewer viable shoots than the Km-resistant plants, and most of the young plantlets died. This may be due to the overexpression of gene VI.

Earlier it was demonstrated that gene VI of CaMV is associated with disease symptoms in turnip plants (14,15,31). In these experiments genomic CaMV hybrids were used and introduced. By using an in-frame insertion mutant of gene VI, altered symptoms were obtained (14). Changing gene VI regions of different isolates proved that gene VI encodes a host range determinant. Therefore it is conceivable that it might be possible to infect our transgenic tobacco plants, a non-host for CaMV, with wild-type virus. For this reason we inoculated our plants showing mild mosaic symptoms (apparently expressing gene VI) with CaMV, but we could not detect infection in either the inoculated or in the upper leaves using ELISA or quantitative rocket immunoelectrophoresis. Therefore we conclude that gene VI alone is not enough for recognition of the host plant. This is in accordance with a recent publication of Schoelz and Shepherd (15), where they argue that $P_{62\text{-}66}$ may have to interact with other viral protein(s) to determine the host range.

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