MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 2, pp. 182-189, 1991

A Disease Syndrome Associated with Expression of Gene VI of Caulimoviruses May Be a Nonhost Reaction

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Received 15 August 1990. Accepted 13 December 1990.

Gene VI of cauliflower mosaic virus (CaMV), which specifies a protein (P62) found in virus-induced inclusion bodies, has been implicated as a major determinant for host range and disease induction. In this study, gene VI of two strains of CaMV, CM1841 and D4, and gene VI of one strain of figwort mosaic virus (FMV) were used to transform tobacco (*Nicotiana tabacum*) and *Datura inoxia* and to evaluate the potential of this gene for disease induction. Tobacco, which is not a host for either CaMV or FMV, developed generalized chlorosis or a prominent chlorotic mottling after transformation with gene VI of either virus. *D. inoxia* is not a host for CaMV but is systemically susceptible to FMV. It developed a prominent chlorotic mottling syndrome after transformation with gene VI of CaMV, but no symptoms after transformation with gene VI of FMV. In both tobacco and *D. inoxia* plants that express gene VI of CaMV and tobacco plants that express gene VI of FMV, a positive correlation between the level of gene VI-encoded protein accumulation and disease was found. These experiments and those done previously with transformation of *Nicotiana edwardsonii*, with gene VI of both viruses, suggest that a low level of gene VI-encoded protein is sufficient to cause chlorosis or chlorotic mottling in plants that are not systemic hosts, but not in plants that are systemic hosts for these viruses.

Cauliflower mosaic virus (CaMV) and figwort mosaic virus (FMV) (Shepherd *et al.* 1987) are two members of the caulimovirus group of which CaMV is the type member (Harrison *et al.* 1971). The viruses are characterized by a 50-nm icosahedral virion containing circular double stranded (ds) DNA of approximately 8,000 base pairs (bp). The dsDNA genome is amenable to direct cloning into plasmids and hence can be propagated in bacteria. The full-length clones of CaMV and FMV are infectious when the viral DNA is excised from the plasmid vector and mechanically inoculated to host plants (Howell *et al.* 1980; Richins *et al.* 1987). This technique greatly facilitates the genetic manipulation of these viruses.

The dsDNA genome of CaMV and FMV is organized into six major open reading frames (ORFs) that are conserved to varying degrees (Gardner *et al.* 1981; Richins *et al.* 1987). The aphid-transmission factor, coat protein, reverse transcriptase, and inclusion body protein are associated with ORFs II, IV, V, and VI, respectively. Gene I is probably involved in intercellular movement as suggested by its association with cell walls (Albrecht *et al.* 1988; Lindstead *et al.* 1988). Gene III encodes a 15-kDa structural protein found in virions (Mesnard *et al.* 1990). The large and small intergenic regions contain transcriptional promoters and other regulatory sequences.

Mutagenesis of gene VI of CaMV has suggested this region may be an important factor in disease induction. Small insertions in this gene had a marked consequence on disease symptoms (Daubert *et al.* 1983), and exchanges between gene VI of different strains of CaMV defined ORF VI as a determinant of systemic symptoms (Daubert *et al.* 1984; Schoelz *et al.* 1987; Schoelz and Shepherd 1988).

Several observations suggest that gene VI of the caulimoviruses mediates the host–virus interaction. The mapping of determinants for initial host responses (local symptoms) by constructing hybrid genomes has shown that gene VI controls whether the host reacts with hypersensitivity or compatibility (Schoelz *et al.* 1987).

In the experiments reported here, we have evaluated the effects of gene VI expression in transgenic plants. Our preliminary reports (Goldberg *et al.* 1987, 1989) and two other investigations (Baughman *et al.* 1988; Takahashi *et al.* 1989) reported that transformation of tobacco (*Nicotiana tabacum*) with gene VI of CaMV causes chlorosis, mottling, and necrosis associated with expression of the gene VI-encoded protein.

MATERIALS AND METHODS

Plasmid construction. The bacterium used for routine cloning was *Escherichia coli* TB1 (Vieira and Messing 1982). The vectors were transformed into *E. coli* following the $CaCl_2$ procedure of Cohen *et al.* (1972). Selection and culturing of transformed bacteria, DNA isolation, CsCl purification of plasmid DNA, gel electrophoresis, and other standard molecular biology procedures were done as described by Maniatis *et al.* (1982). Restriction enzymes, purchased from Bethesda Research Laboratories (Gaithersburg, MD) and New England BioLabs, Inc. (Beverly, MA), were used as directed by the supplier.

Gene VI of CaMV and FMV with their homologous promoters (pJS62, pJS65, and pKB27) or a 35S promoter (pKB39 and pKB40) were cloned into gene vectors suitable for transformation mediated by *Agrobacterium*. pKB27 (Kiernan *et al.* 1989; Fig. 1) contains gene VI of FMV

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with its homologous promoter and polyadenylation signal inserted into pGA472 (An *et al.* 1985). The latter contains a neomycin phosphotransferase II (NPTII) gene between the right and left borders of T-DNA that allows positive

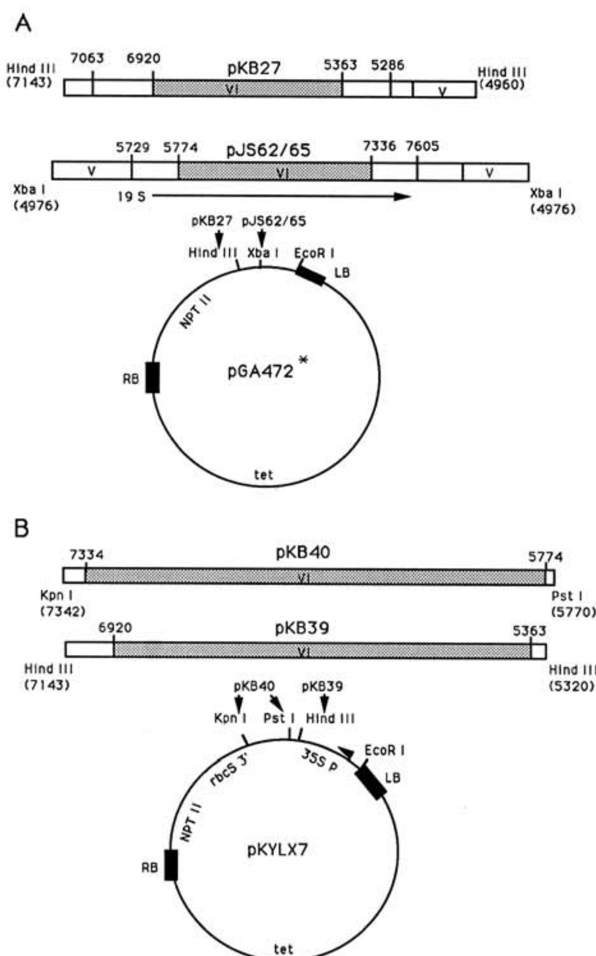

Fig. 1. A illustrates gene VI constructs (top bars) inserted into a binary plasmid gene vector (circular diagram). In each case, the shaded region of the bar diagram indicates the coding region of gene VI. pKB27 contains gene VI of figwort mosaic virus (FMV). The numbers along the top of the diagram indicate the nucleotide coordinates of its genome (Richins *et al.* 1987). pJS62 and pJS65 contain gene VI of cauliflower mosaic virus (CaMV) strains CM1841 and D4, respectively (for details, see Kiernan *et al.* 1989). The numbers along the top of the diagram indicate the nucleotide coordinates of the CaMV genome (Gardner *et al.* 1981). These three plasmids contain gene VI under the control of its homologous promoter. The arrow in the bottom diagram indicates the 19S RNA transcript of CaMV that spans gene VI. A similar transcript is known to be produced for FMV but its coordinates have not been mapped. The circular diagram in **A** indicates the binary gene vector pGA472 described by An *et al.* 1985. The restriction endonuclease sites used for insertion of gene VI of each virus is indicated in the diagram. **B** illustrates constructs in which gene VI of CaMV (pKB40) and FMV (pKB39) are under the control of the 35S promoter of CaMV. The restriction sites and nucleotide coordinates of the CaMV genome are given in the top rectangular diagram (pKB40), and those of FMV (pKB39) are given in the lower rectangular diagram. The circular diagram at the bottom illustrates the transformation vector pKYLX7 used for these constructs. The insertion sites for the 35S-gene VI constructs of each virus are indicated. In each circular diagram, the right (RB) and left (LB) borders of T-DNA are indicated as boxed areas. The neomycin phosphotransferase II (NPTII) and *tet* genes that confer kanamycin and tetracycline resistance, respectively, are indicated.

selection of transformants on kanamycin-containing medium.

The construction of pJS62 and pJS65, gene VI of CaMV CM1841 (Gardner *et al.* 1981) and D4 (Schoelz *et al.* 1986), respectively, was previously described (Kiernan, *et al.* 1989; Fig. 1). Both of these plasmids contain a CaMV CM1841 19S promoter and its homologous polyadenylation signals. These gene VI clones are contained within a modified pGA472 vector that provides the right and left borders of the T-DNA transposon.

Mutagenesis of CaMV CM1841 gene VI. An *Eco*RV fragment of CM1841 CaMV, which contains gene VI (nucleotides 5713-7344) including its start and stop codons, was ligated into the *Sma*I site of pUC119 to create pKB28B. An 18-base oligonucleotide (synthesized by MSAF Services, UK Chandler Medical Center, Lexington, KY) was used for site-directed oligonucleotide mutagenesis (Kunkel 1985) to create pKB28B-16μ that has the complete gene VI ORF with a *Pst*I site immediately upstream of the gene VI ATG. Digestion of this plasmid DNA with *Pst*I and *Kpn*I allowed for insertion of gene VI of CM1841 into a pKYLX7 (Schardl *et al.* 1987) gene vector. pKB40 (Fig. 1) contains a CaMV 35S promoter, all of the coding region of CM1841 CaMV gene VI, an rbcS 3′ terminator, and a kanamycin resistance gene (NPTII) between the right and left borders of T-DNA in pKYLX7.

Mutagenesis of FMV gene VI. A *Hin*dIII fragment of pFMVSc3 (Richins *et al.* 1987), containing gene VI with its homologous promoter and poly(A) signal, was cloned into the *Hin*dIII site of pUC119 to yield pKB29. A 16-base oligonucleotide (synthesized by MSAF Services) was used for site-directed mutagenesis to create a unique *Hin*dIII site between the putative gene VI promoter and the ATG (start codon) resulting in pKB29-6μ. The *Hin*dIII fragment of pKB29-6μ containing FMV gene VI was cloned into pKYLX7 to give pKB39 (Fig. 1), which allowed transcription of an FMV gene VI from a CaMV 35S promoter. Plant transformation vectors were introduced into *Agrobacterium tumefaciens* A281 (Hood *et al.* 1984) by triparental mating (Ditta *et al.* 1980).

Leaf disk transformation and regeneration of *D. inoxia* and tobacco. Leaf disks of *D. inoxia* and *N. tabacum* 'Burley 21' were transformed via *A. tumefaciens* A281 with gene VI of CaMV strains CM1841 (pJS62 and pKB40) and D4 (pJS65) and FMV gene VI (pKB27 and pKB39). The protocol was essentially the one described by Horsch *et al.* (1985). For *D. inoxia*, 0.225 mg/L of 6-benzylaminopurine (BAP) was used in the medium for shoot regeneration. For tobacco, 1 mg/L of BAP and 0.1 mg/L of 1-naphthaleneacetic acid were used for the same purpose.

Development of FMV gene VI antiserum. A peptide of amino acids 78-103 of a hydrophilic region of FMV gene VI (as predicted from the viral DNA sequence), was used after synthesis by MSAF Services. A female New Zealand white rabbit (about 1.5 kg) was injected with an emulsion of a 1:1 mixture of Freund's complete adjuvant and 100 μg/ml of the synthetic peptide. Approximately 20 dorsal injections of 100 μl each were applied subcutaneously on day 1. At days 19 and 35 emulsions of a 1:1 mixture of Freund's incomplete adjuvant and 100 μg/ml of synthetic

peptide were injected into the hip muscle. The serum was collected at day 49 and stored in 50% glycerol at −20° C.

Purification and Southern blots of plant chromosomal DNA. DNA was isolated from young leaves of regenerated *D. inoxia* plants by using a modified procedure of Grimsley *et al.* (1987) as previously described (Kiernan *et al.* 1989). Generally, the DNA was in excess of 20 kbp and suitable for Southern blot analysis as previously described (Kiernan *et al.* 1989).

Northern blot analysis. RNA was extracted from freshly collected leaves essentially as described by Silflow *et al.* (1979). After purification of total plant RNA, 40 μg was pelleted by centrifugation and treated as described by Maniatis *et al.* (1982) for electrophoresis and transfer to GeneScreen membranes (DuPont, Wilmington, DE).

Prehybridization and hybridization of northern and Southern blots were as described previously (Kiernan *et al.* 1989). Autoradiography was performed at −60° C on Kodak X-Omat AR film (Eastman Kodak, Rochester, NY).

Protein analysis of transgenic plants. Fresh leaf tissue (0.5 or 1 g) was prepared as described (Kiernan *et al.* 1989). The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide (Laemmli 1970). Transfer to nitrocellulose membrane and western blot analysis was as previously described (Kiernan

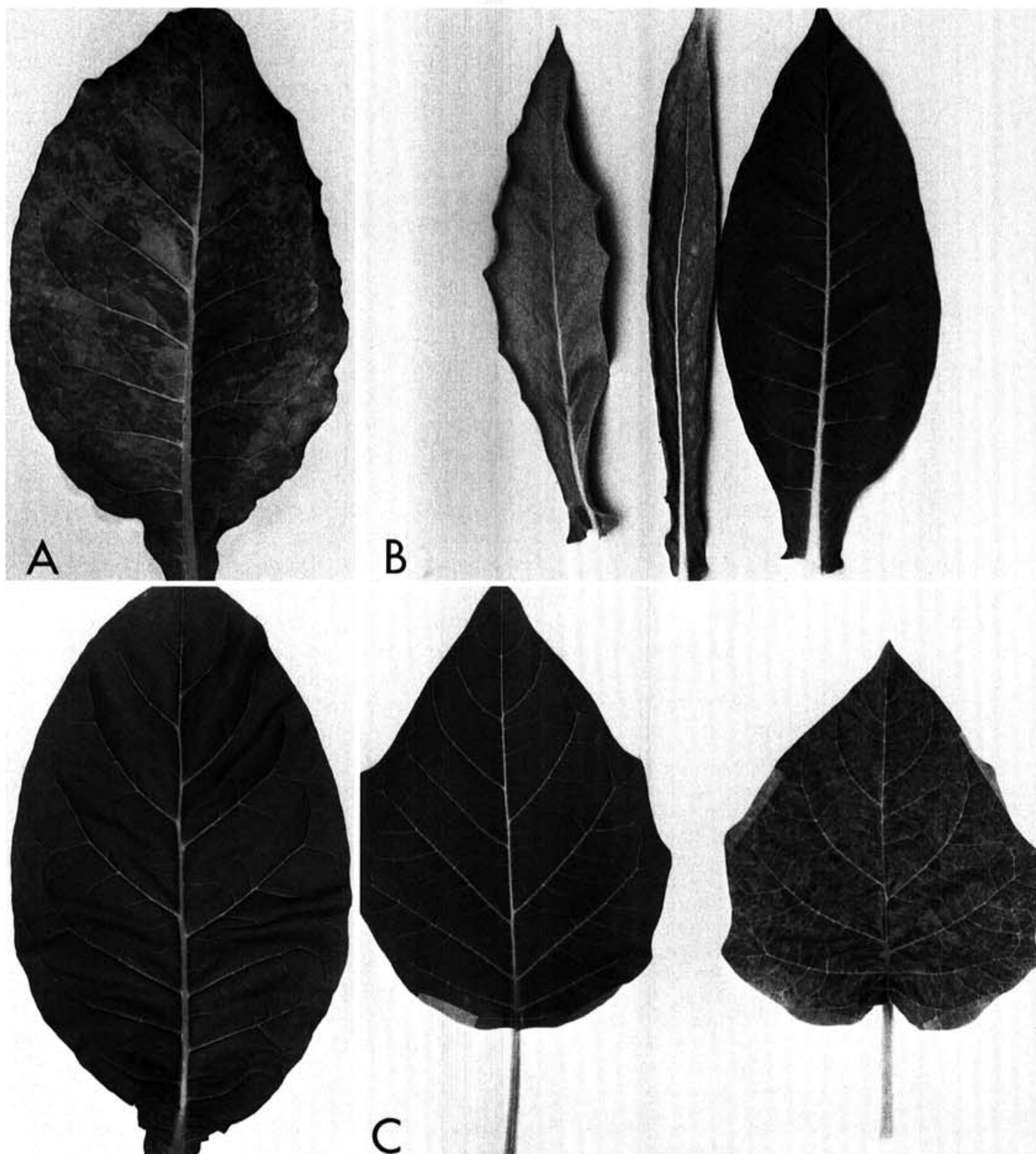

Fig. 2. Symptoms associated with gene VI expression in transgenic plants. **A,** The top leaf is from tobacco transformed with pJS62 (gene VI of cauliflower mosaic virus) showing a prominent chlorotic mottling syndrome. The bottom leaf is from tobacco transformed with the pGA472 gene vector alone. **B,** Leaves on the left showing generalized chlorosis are from tobacco transformed with pKB39 (gene VI of figwort mosaic virus). The leaf on the right was from a plant transformed with the pKYLX7 gene vector without gene VI. **C,** Leaves from transgenic *Datura inoxia*. The leaf on the right was taken from plant 8 transformed with pJS62 (containing gene VI of cauliflower mosaic virus). The leaf on the left was from a plant transformed with the pGA472 gene vector without gene VI.

et al. 1989; Harlow and Lane 1988). Chitinase, sesquiterpene cyclase, β-1,3-glucanase and CaMV gene VI antisera (Young *et al.* 1987) were used at 1:1,000 dilution. The FMV gene VI antiserum was used at a 1:400 dilution for western blot analysis.

RESULTS

Analysis of transgenic tobacco plants. The transformation of tobacco leaf disks with the control plasmid, pGA472, resulted in kanamycin-resistant plants that produced fertile seed. Plants had a normal morphology and were indistinguishable from nontransformed tobacco.

Observation of 19 regenerated kanamycin-resistant tobacco plants after transformation with pJS62 (gene VI of CaMV CM1841; Fig. 1) resulted in six plants that developed chlorotic mottling symptoms (Fig. 2A), 12 plants that developed generalized chlorosis, and one that had a normal appearance. All plants produced fertile seed. The more severely affected plants had narrowed leaves and a somewhat reduced stature compared to healthy plants.

Tobacco plants transformed with pJS65 (gene VI of CaMV D4; Fig. 1) produced fertile seed. Two plants had a completely normal appearance, but 22 others became chlorotic. In some instances, chlorosis was interspersed with areas of normal green tissue to produce a prominent chlorotic mottle. All gene VI-transformed plants were also resistant to kanamycin.

The pJS62- or pJS65-transformed plants were assayed by DNA dot blot and/or Southern blot analysis (data not shown). Plants that were positive for CaMV gene VI DNA also developed a chlorotic mottle or generalized chlorosis. Plants negative for CaMV gene VI DNA had a normal appearance. After northern blot analysis, it appeared that the major transcripts were of a size equivalent to the 19S transcript of CaMV. Moreover, western blot analysis of these plants revealed that the gene VI-encoded protein (P62) was of the same size as that produced in 'Just Right' turnips (*Brassica campestris*) infected with CaMV CM1841 or D4 (Fig. 3A). The level of P62 was positively correlated with the degree of symptom induction; plants showing leaves with complete chlorosis, or a vivid chlorotic mottle, accumulated the highest levels of P62 (Figs. 2A and 3A). The pJS62- and pJS65-regenerated tobacco plants were allowed to self-pollinate, and the F_1 seeds were assayed for kanamycin resistance. At the highest level of antibiotic in the medium (50 mg/L of G418), there was approximately a 3:1 ratio of kanamycin-resistant to kanamycin-sensitive plants, indicating a single dominant gene segregation ratio in the F_1 generation. In addition, the symptoms observed on the original transformed plants (chlorosis or a chlorotic mottle) carried over to the second generation in the same ratio as the kanamycin resistance. This was expected because the gene VI and NPTII genes were genetically linked during the transformation event. The F_2 seeds also showed a high level of kanamycin resistance.

Tobacco plants transformed with gene VI of FMV with a 35S promoter of CaMV (pKB39) developed a chlorotic mottling syndrome (Fig. 1). Of 27 kanamycin-resistant plants, 10 showed mild to severe chlorosis (Fig. 2B). Southern blot analysis showed that the 10 chlorotic plants possessed the complete FMV gene VI fragment integrated in the plant chromosomal DNA (data not shown). In many plants, one or a combination of the following effects were observed: severe stunting; small, narrow, upturned leaves; leathery leaves; delayed flowering; and development of green islands within the chlorotic leaf tissue. The 10 plants that were both symptomatic and positive by Southern blot analysis were also positive for the presence of a gene VI transcript as established by northern blot hybridization. In addition, immunoblot analysis with the FMV gene VI antiserum revealed that all chlorotic plants accumulated gene VI-encoded protein. In general, the level of expression

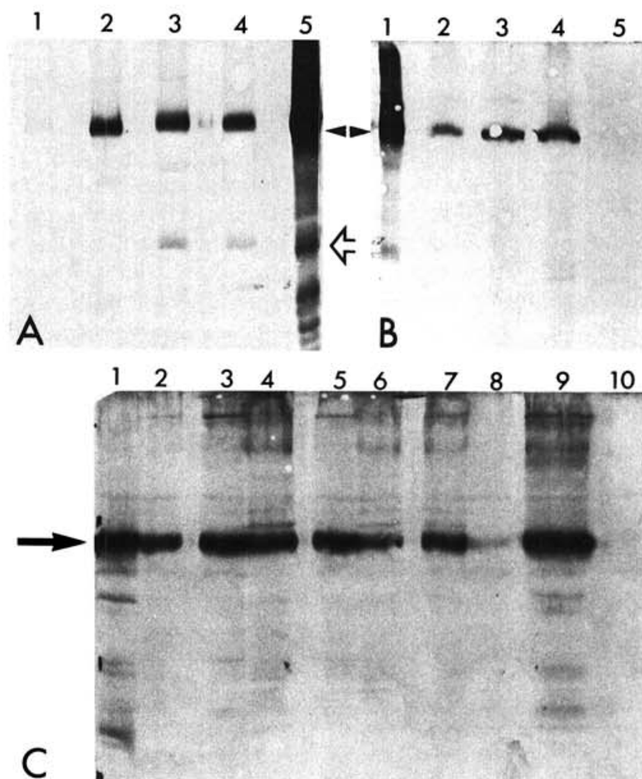

Fig. 3. A, Western blots of tobacco and *Datura inoxia* plants transformed with gene VI of cauliflower mosaic virus (CaMV). Lanes 1 and 2 are from plants 17 and 8 of *D. inoxia* transformed with pJS62 (gene VI of CaMV CM1841), and lanes 3 and 4 are from plants 21 and 25 of tobacco transformed with pJS62 and pJS65, respectively (plasmids with gene VI of CaMV strains CM1841 and D4, respectively). All lanes contained 40 μl of plant extract (0.5 g of leaf homogenized in 1.5 ml of Laemmli buffer); lane 5 has 10 μl of a similar extract of CaMV CM1841-infected turnip leaf. The solid arrowhead indicates the position of P62. The open arrowhead indicates the position of a small immunoreactive protein found in healthy tobacco. **B,** Western blot analysis for P62 accumulation in plants transformed with CaMV gene VI constructs under the control of different promoters. Lane 1 contains 20 μl of extract (0.5 g of leaf per 1.5 ml of Laemmli buffer) of CaMV CM1841-infected turnip; lanes 2 and 3 contain 40 μl of extract of plants 3 and 10 of *D. inoxia* transformed with pKB40; lane 4 contains 40 μl of a similar extract of *D. inoxia* plant 8 transformed with pJS62; and lane 5 contains a similar extract of nontransformed *D. inoxia.* **C,** Western blot analysis of various plants of *D. inoxia* transformed with pJS65 (gene VI of CaMV D4), except for lane 1, which contains extract of plant 8 transformed with pJS62. Lanes 2–9 contain extracts of plants 12, 10, 9, 7, 6, 5, 3, and 1, respectively, transformed with pJS65. Lane 10 contains an extract of a nontransformed *D. inoxia* plant. In each case, the lane contains 50 μl of extract (0.5 g of leaf per 1.5 ml of Laemmli buffer).

of this protein was positively correlated with the severity of the symptoms observed (Figs. 2 and 4B). A sample of plants that represent an array of these effects was maintained for seed collection. Both disease and kanamycin-resistant phenotypes carried over into the next generation.

All but one of the chlorotic plants that gave positive DNA and/or RNA blots also showed a decreased ratio of chlorophyll *a* to chlorophyll *b* compared to healthy tobacco plants (data not shown). The amount of total chlorophyll assayed spectrophotometrically as described by Arnon (1949) in these transgenic tobacco plants was reduced by 24–64% (av. 39%), compared to nontransformed tobacco.

Analysis of *D. inoxia* transgenic plants. The transformation of leaf disks with the plasmid pGA472 (without gene VI) resulted in kanamycin-resistant plants with a normal morphology that produced viable seed.

Transformation of *D. inoxia* leaf disks with gene VI of CaMV CM1841 (pJS62; Fig. 1) resulted in 17 of 19 plants that were resistant to kanamycin (300 mg/L). Among these, two plants showed either a light-green mottle or a more severe chlorotic mottle (Fig. 2C); the remaining plants had a normal appearance. The most severely affected transformant (plant 8) was severely stunted both in stature and in leaf size. The other plant (17) flowered normally and produced fertile seed.

Of 14 plants assayed by dot- and Southern-blot analyses, only the two mentioned above were positive (data not shown); the remaining 12 plants had no symptoms and were not positive for CaMV gene VI DNA. Southern blot analysis showed that one of the DNA fragments observed for a pJS62 transformant (plant 17) was smaller than predicted, but the deleted portion probably was near the left border

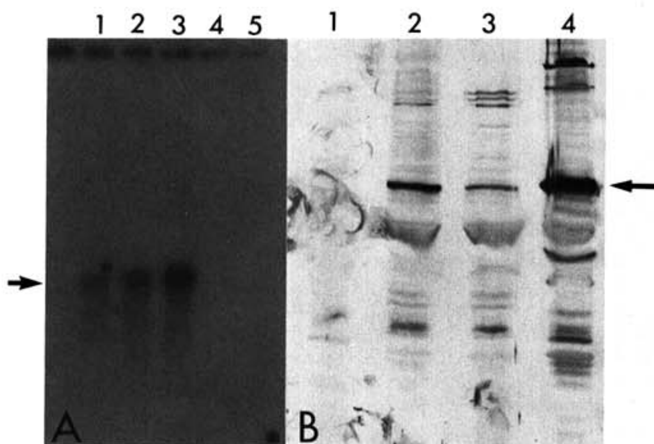

Fig. 4. Analysis of plants transformed with gene VI of figwort mosaic virus (FMV). **A,** Northern blot of *Datura inoxia* transformed with pKB27. Lanes 1–4 contain 40 μg of RNA isolated from transformed plants 16, 12, 8, and 5, respectively. Lane 5 is RNA from a plant transformed with the gene vector pGA472. The arrow indicates the position for a fragment of approximately 1,800 nucleotides similar to that expected from plants infected with FMV. **B,** Western blot for the FMV gene VI-encoded protein from transformed tobacco. Lane 1, extract from nontransformed tobacco plants; lanes 2 and 3, extracts from tobacco plants 19B and 9B, respectively, transformed with pKB39; and lane 4, extract from FMV-infected *D. stramonium*. All except lane 4 contain 50 μl of extract (1 g of leaf homogenized in 1.5 ml of Laemmli buffer). Lane 4 contains 25 μl of a similar extract. The arrow indicates the location of the gene VI-encoded product.

and not within gene VI. The CaMV gene VI-encoded protein (P62) was detected only in the two plants that tested positive for gene VI DNA insertions. The product of gene VI (P62) was of the predicted size (Fig. 3A–C). The amount of P62 produced was much lower than that in a turnip cultivar Just Right infected with CaMV CM1841. There was about a 10-fold difference between the expression of P62 by pJS62-transformed *D. inoxia* plants (8 and 17) and virus-infected turnips; plant 8 had a very severe chlorotic mottle and plant 17 had a mild mottle. The levels of CaMV gene VI-encoded product in these plants were similar to that observed with transgenic tobacco (pJS62 and pJS65; Fig. 3A) and transgenic *D. inoxia* (pJS65 and pKB40; Fig. 3B and C) plants.

The transformation of *D. inoxia* with gene VI of CM1841 under the control of a CaMV 35S promoter (pKB40) resulted in 13 kanamycin-resistant plants. Four of these also tested positive for DNA of gene VI. Two of the latter had striking symptoms and high levels of gene VI-encoded protein accumulation. Plant 3 was stunted and had necrotic leaves. Plant 10 was chlorotic and of reduced stature. The level of gene VI RNA transcripts of these plants was similar, but about five to 10 times less than the gene VI transcript level in the pJS62 transformant 8 of *D. inoxia* referred to above (not shown). It was observed that when pKB40 transformant 3 was placed under different environmental conditions (12–14 hr days and 30–35° C temperatures) the accumulation of gene VI RNA and gene VI-encoded protein declined and symptoms abated. For example, during midsummer this plant developed new leaves with a normal, healthy morphology and had low levels of gene VI-encoded protein accumulation. In contrast, *D. inoxia* transformant 8 (pJS62) had symptoms of a severe chlorotic mottle that persisted throughout the year; its level of accumulation of P62 was uniformly high as well. Severe symptoms developed on pKB40 transformants 3 and 10 of *D. inoxia*, maintained at 20° C with a 10-hr day, in association with expression of gene VI-encoded protein. The level of P62 accumulation in plant 10 was comparable to plant 8 of *D. inoxia* transformed with pJS62 (Fig. 3B). The other two plants that accumulated very low levels of P62 had a normal appearance.

Western blot analysis of pKB40 transformant 3 and pJS62 transformant 8 of *D. inoxia* plants, which had severe symptoms, revealed that these plants expressed stress-related proteins. Blots were positive for the presence of chitinase, β-1,3-glucanase, and sesquiterpene cyclase in addition to gene VI-encoded protein (Fig. 5). These host defense-related proteins were not detected in healthy *D. inoxia* or other transgenic plants that had milder symptoms.

Transformation of *D. inoxia* plants with pJS65 (Fig. 1) resulted in nine of 12 plants that expressed the CaMV D4 gene VI-encoded protein (Fig. 3C). Of these 12 plants, nine displayed a chlorotic mottle and were resistant to kanamycin. One of the three symptomless plants that was assayed by western blot had very low levels of P62 (gene VI-encoded product) (pJS65, transformant 3). Plants with symptoms showed accumulation of at least fivefold more P62 than those without symptoms. Hence, there was a positive correlation between the amount of P62 accumulation and the disease syndrome.

Transformation of *D. inoxia* plants with gene VI of FMV (pKB27 and pKB39; Fig. 1) resulted in plants with a normal appearance. The transgenic plants produced abundant amounts of viable seed. A total of 22 plants were kanamycin-resistant, and 11 of these were also transformed with gene VI of FMV. Southern and northern hybridization analyses of pKB27 plants 8, 12, and 16 indicated that the complete gene VI coding region was inserted into chromosomal DNA and a single major RNA component similar in size to the CaMV 19S RNA hybridized to a ^{32}P-labeled FMV gene VI probe (Fig. 4A), respectively. Two of the 20 kanamycin-resistant, symptomless pKB39 transformants (plants 10 and 20) expressed the FMV gene VI-encoded protein, as determined by western blot analysis. Comparison of these two pKB39 transformants of *D. inoxia* (data not shown) and pKB39 Burley 21 (Fig. 4B) revealed similar levels of gene VI-encoded protein accumulation (data not shown), although the transformants of Burley 21 had a striking chlorotic mottle and distorted leaves.

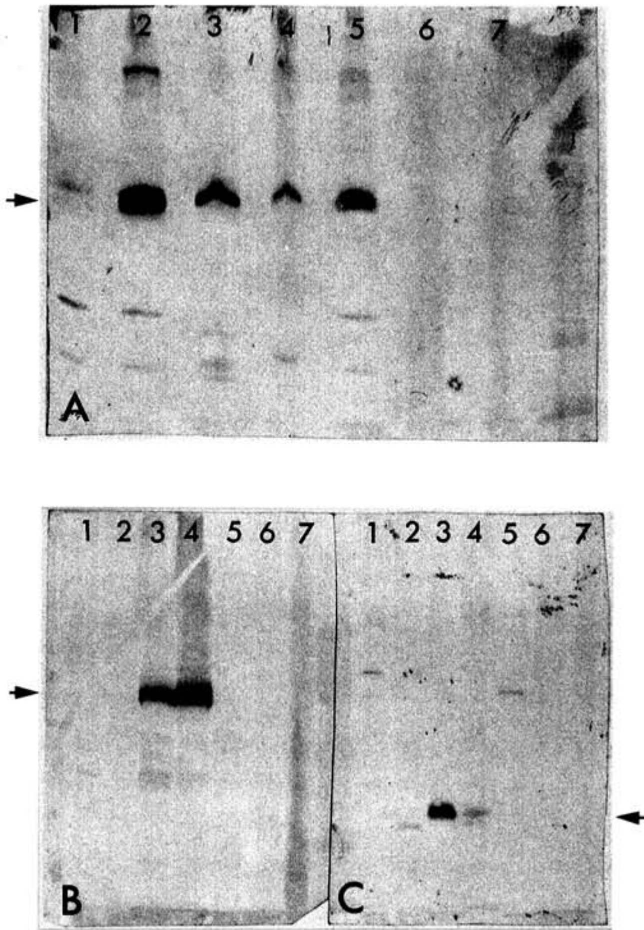

Fig. 5. Western blots for detection of stress-induced proteins in plants transformed with gene VI of cauliflower mosaic virus. The lanes of each of the three gels shown (**A**, **B**, and **C**) contain protein extracts from the same transgenic plants. **A** has been immunoblotted against the antibody to P62 (gene VI-encoded protein of cauliflower mosaic virus). **B** has been immunoblotted against the antibody to sesquiterpene cyclase. **C** has been immunoblotted against the antibody to β-1,3-glucanase. Lane 1 in each gel contains extract from plant 6B of tobacco transformed with pKB39. Lane 2 contains extract from plant 14 of tobacco transformed with pJS65. Lane 3 has extract from plant 8 of *Datura inoxia* transformed with pJS62. Lane 4 has extract of plant 3 of *D. inoxia* transformed with pKB40. Lane 5 contains extract from plant 10 of *Nicotiana edwardsonii* transformed with pKB40. Lanes 6 and 7 contain extracts of nontransformed *D. inoxia* and *N. edwardsonii*, respectively. The extracts in lanes of gel **A** represented about 13 mg of fresh leaf tissue. The extracts in **B** and **C** represent about 6.5 mg of fresh tissue per lane. The plants tested showed the following symptoms: Lanes 1 and 2, severe chlorosis; lane 3, severe chlorotic mottle; lane 4, necrotic spots; and lane 5, mild chlorosis. Lanes 6 and 7 represent plants with no symptoms. Chitinase expression was also detected in plant 8 of *D. inoxia* transformed with pJS62.

DISCUSSION

Baughman *et al.* (1988) reported that tobacco plants transformed with gene VI of CaMV showed virallike disease symptoms. The CaMV 19S transcription unit plus the coding region of gene VI were required for symptom production in transgenic plants. Frameshift mutants of the gene VI coding region did not induce symptoms in transgenic plants indicating that the native gene VI-encoded protein (P62) was necessary for disease induction. This was confirmed by finding a nearly perfect correlation between the development of symptoms and detectable levels of P62. In similar experiments with CaMV gene VI transferred to tobacco, Takahashi *et al.* (1989) found that about 40% of the transformants (kanamycin-resistant plants) expressed immunodetectable levels of P62 and that this corresponded with the development of symptoms. Chlorosis, necrosis, and suppressed growth were associated with P62 accumulation. We have confirmed and extended the observations made in both laboratories. At least one other plant, *D. inoxia*, which is also not a systemic host of CaMV, reacts in the same manner as tobacco when transformed with gene VI of this virus. As with tobacco, there is also a close correlation between symptoms and gene VI expression in *D. inoxia*.

We have also found that tobacco responds with chlorosis and mottling when transformed with gene VI of FMV, a second caulimovirus, showing that the phenomenon is not unique to gene VI of CaMV. Tobacco is also not a systemic host for this virus.

We have also transformed two other species of *Nicotiana*, *N. edwardsonii* (Kiernan *et al.* 1989) and *N. bigelovii*, with pJS65 (gene VI of CaMV D4). These two additional species were symptomless in contrast to the chlorosis and mottling observed with pJS65 transformants of tobacco and *D. inoxia*. However, the amount of accumulation of gene VI-encoded protein was similar to that observed with the lower levels of expression of pJS62 and pJS65 *D. inoxia* transformants (see Kiernan *et al.* 1989; Fig. 3A and C). *N. bigelovii* and *N. edwardsonii* are hosts of both CaMV D4 and FMV. Consequently, one may speculate that symptom induction is a response of nonhosts to perturbation by the gene VI-encoded proteins of FMV and CaMV. The fact that *D. inoxia* develops prominent chlorotic mottling when transformed with gene VI of CaMV but not with gene VI of FMV supports this notion. *D. inoxia* is a systemic host for FMV but not for CaMV. The expression levels of the gene VI-encoded proteins of each virus in *D. inoxia* were readily detectable in each case. Even at low levels of gene VI (P62) expression of pJS62 in *D. inoxia* and tobacco plants, mild symptoms were

evident. Similar effects were noted for pJS62 in *N. edwardsonii* (Kiernan *et al.* 1989). These observations suggest that there is a fundamental difference in the way the two gene VI-encoded proteins interact with *D. inoxia*. Further, one is led to speculate that the manner of this interaction may be a factor that accounts for the systemic development of FMV, but not of CaMV, in this plant.

We have previously suggested that gene VI appears to be at least one factor involved in the systemic mobilization of CaMV in its hosts (Daubert *et al.* 1984). Although gene I of these viruses is probably involved in virus movement (Albrecht *et al.* 1988; Linstead *et al.* 1988), gene VI has been found to control the hypersensitive (versus compatible) reaction of the host (Schoelz *et al.* 1987; Schoelz and Shepherd 1988). Hence, gene VI is also a major determinant that mediates the host–virus interaction of the caulimoviruses.

To test if higher levels of gene VI could result in disease development in species that are systemic hosts of these viruses, we used the strong 35S promoter of CaMV with some of our gene VI constructs. *D. inoxia* and tobacco showed the highest levels of CaMV gene VI expression whether the 19S or the 35S CaMV promoter was used (Fig. 3A–C). In transgenic *D. inoxia*, the 19S and 35S promoters brought about similar levels of expression. However, the same constructs in *N. edwardsonii* suggested the 35S promoter was considerably stronger as determined by western blot analysis of P62 accumulation (Goldberg *et al.* 1989). FMV gene VI expression with its homologous promoter or a CaMV 35S promoter resulted in symptomless transgenic *D. inoxia* plants.

It is apparent from these results and previously those of Baughman *et al.* (1988), Takahashi *et al.* (1989), and Kiernan *et al.* (1989) that low concentrations of the gene VI-encoded proteins of CaMV and FMV alone can perturb plants to cause disease. However, it is not clear whether gene VI alone is adequate to cause disease in plants that are systemic hosts of these viruses. In virus-infected plants, gene VI is expressed at much higher levels than in transformants mediated by *Agrobacterium*. This difference is generally 20- to 100-fold in our experience. The higher levels of expression in virus-infected plants are probably because of the much higher copy number of actively transcribing material in the nucleus.

Higher levels of gene VI-encoded protein in a host might possibly result in symptom development. This is similar to the effect of a toxin. At high levels a toxin can affect all cells; the threshold level is important. In this case, we observe that in a nonhost of CaMV or FMV their respective gene VI-encoded protein can cause symptoms with low levels of gene VI-encoded protein accumulation. In a systemic host no effect is observed with similar levels of gene VI expression.

One can also speculate that other viral genes probably contribute to disease induction in virus-infected plants because gene VI has been shown to activate the expression of downstream cistrons of the full-length polycistronic transcript of these viruses (Gowda *et al.* 1989; Bonneville *et al.* 1989). Previous observations with CaMV that implicate gene VI in the development of chlorosis and mottling (Daubert *et al.* 1984; Stratford and Covey 1989) may be explained by more efficient transactivation of other viral genes by particular versions of gene VI. Other types of disease symptoms have been mapped to regions other than gene VI (Stratford and Covey 1989).

It is also interesting to speculate on how gene VI expression alone stimulates a disease response in transgenic plants. Perhaps the protein activates downstream cistrons of plant messenger RNAs to cause expression of genes that are ordinarily silent in healthy plants under the same conditions.

ACKNOWLEDGMENTS

We are grateful to Amy Wilson for skillful assistance in the laboratory and to Jim Schoelz and Herman Scholthof for helpful discussions. We also thank Arthur Hunt for providing the pKYLX7 vector and Joseph Chappell for the generous gift of antisera to the stress reaction proteins. This work was supported by the U.S. Department of Agriculture CRGP grants 88-37263-3847 and 87-CRCR-1-2528. We gratefully acknowledge Kentucky State THRI project 5-41086 for support and use of its facilities.

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