Systemic Cell Death Is Elicited by the Interaction of a Single Gene in *Nicotiana clevelandii* and Gene VI of Cauliflower Mosaic Virus

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Accepted 7 June 1999.

Cauliflower mosaic virus (CaMV) strains D4 and W260 can be distinguished by the type of symptoms they induce in *Nicotiana clevelandii* and *N. edwardsonii*. W260 induces systemic cell death in addition to a mosaic symptom in *N. clevelandii* and a hypersensitive response (HR) in *N. edwardsonii*, whereas D4 induces a systemic mosaic in both hosts. To determine which W260 genes are responsible for systemic cell death, chimeric viruses were constructed between the D4 and W260 strains. It was found that W260 gene VI was responsible for the elicitation of systemic cell death; previous studies had shown that this same gene elicited HR in *N. edwardsonii*. An immunological analysis of plants infected with W260 or D4 indicated that the systemic cell death symptom was not associated with enhanced levels of either W260 virions or the W260 gene VI product. To investigate the inheritance of systemic cell death, crosses were made between *N. clevelandii* and *N. bigelovii*, a host that reacts with a systemic mosaic symptom upon infection with W260. All F₁ plants developed a systemic mosaic after inoculation with W260, whereas the F₂ generation segregated 3:1 for systemic mosaic versus cell death. The plant gene responsible for cell death was designated *ccd1*, for CaMV cell death gene. These results demonstrate that the systemic cell death symptom in *N. clevelandii* is induced by the interaction between a single host gene and gene VI of CaMV.

The characteristic symptoms induced by plant viruses are likely the result of specific interactions between virus and plant genes. Over the past several years, genes and noncoding nucleotide sequences of plant DNA viruses, RNA viruses, and satellite viruses have been identified that determine symptom formation and host range (for reviews see Daubert 1988; Culver et al. 1991; Dawson and Hilf 1992). On the host side, it has been shown that plant virus infections can trigger substantial changes in host gene expression (Wang and Maule

1995; Geri et al. 1999). There are, however, few examples of host genes that interact in a specific manner with virus sequences to produce the characteristic symptoms of a virus infection.

One symptom that is elicited by many plant viruses is systemic cell death. In some cases, the systemic cell death may represent a failure of the host's hypersensitive defense response to stop the spread of the virus. Thus, it has been noted that *Nicotiana* spp. that contain the *N* gene may develop systemic cell death in response to infection by tobacco mosaic virus (TMV), even when plants are maintained at 17 to 20°C (Dijkstra et al. 1977). Furthermore, single amino acid substitutions within the coat protein of TMV may act as weak elicitors of hypersensitive response (HR) and may actually facilitate systemic cell death in tobacco that contain the *N'* gene (Culver and Dawson 1989). In other cases, the elicitation of systemic cell death by viruses does not appear to be associated with a host resistance gene. For example, both tomato bushy stunt virus and selected satellite RNAs of cucumber mosaic virus (CMV) trigger systemic cell death in their hosts (Scholthof et al. 1995; Talianky et al. 1998), but this has not been correlated with the failure of any form of host resistance.

In this study, we considered that the interaction of cauliflower mosaic virus (CaMV) with *Nicotiana* spp. may be one system in which both host and viral determinants of systemic cell death might be identified. CaMV strain W260 elicits systemic cell death in *N. clevelandii* and only a mosaic in *N. bigelovii* whereas CaMV strain D4 elicits mosaic symptoms in both hosts (Fig. 1). As with many plant viruses, it is possible to construct chimeras between different CaMV strains to identify symptom and host range determinants (Daubert et al. 1984; Schoelz et al. 1986a; Stratford and Covey 1989). This approach has been used to show that gene VI of CaMV strain W260 is responsible for elicitation of an HR in *N. edwardsonii* (Schoelz and Shepherd 1988). On the host side, the interspecific cross between *N. clevelandii* and *N. bigelovii* is fertile, and consequently it has been possible to show that a yellow mosaic symptom induced in *N. bigelovii* by a satellite RNA of CMV is determined by a single, incompletely dominant plant gene (Masuta et al. 1993).

The objective of the present study was to determine the inheritance of both host and virus genes that condition the systemic cell death symptom in *N. clevelandii*. To identify virus sequences specifying systemic mosaic or cell death in *N. clevelandii*, we utilized chimeric viruses constructed

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between the CaMV strains W260, D4, and CM1841. We also generated crosses between *N. bigelovii* and *N. clevelandii* to identify host genes responsible for the systemic mosaic and cell death traits. We present evidence that an interaction between CaMV gene VI and a single *N. clevelandii* gene determines the development of systemic cell death upon viral infection.

RESULTS

Gene VI of CaMV strain W260 is responsible for the development of systemic cell death in *N. clevelandii*.

CaMV strain D4 causes a systemic mosaic in *N. clevelandii* (Fig. 2A), whereas CaMV strain W260 induces systemic cell death in addition to the mosaic (Fig. 2B). In a typical test, *N. clevelandii* plants inoculated with W260 initially developed a mixture of chlorotic and necrotic lesions on the inoculated leaves 8 to 10 days post inoculation (dpi), and a systemic mosaic about 20 to 23 dpi. A few days after the onset of mosaic symptoms, small necrotic lesions appeared among the mosaic symptoms. These necrotic lesions subsequently became larger and eventually coalesced in the advanced stages of systemic infection. In some cases, entire leaves became necrotized. In contrast, D4-inoculated *N. clevelandii* developed chlorotic lesions and a systemic mosaic; these plants never developed necrotic symptoms.

All W260-inoculated *N. clevelandii* plants developed systemic cell death, but symptom severity was affected by environmental conditions such as light intensity, daylength, and temperature. More severe systemic cell death appeared under conditions of low light intensity, low temperature (18 to 20°C), and short days (9- to 10-h photoperiod). In tests conducted during the winter months, the conditions favoring systemic cell death were best met by maintaining inoculated plants in the greenhouse. In summer, tests were conducted in

growth chambers in order to reproduce winter greenhouse conditions.

To identify regions of the W260 genome that determine systemic cell death in *N. clevelandii*, chimeric viruses were constructed between strains W260 and D4. Previous studies with chimeric CaMV strains have shown that sequences within gene VI can elicit an HR in *N. edwardsonii* and *Datura stramonium* (Schoelz et al. 1986a; Schoelz and Shepherd 1988). To test the possibility that gene VI might also elicit systemic cell death in *N. clevelandii*, the entire gene VI coding region was exchanged between W260 and D4 to form the chimeric viruses H190 and H191 (Fig. 1). H191 induced systemic cell death in *N. clevelandii* (Fig. 2C) and HR in *N. edwardsonii*. In contrast, H190 induced only a systemic mosaic in both *N. clevelandii* (Fig. 2D) and *N. edwardsonii*. We concluded that sequences within the 3' end of gene V, gene VI, and/or the large intergenic region were responsible for the elicitation of systemic cell death in *N. clevelandii*, as well as HR in *N. edwardsonii*.

Two other chimeric viruses, H7 and H35 (Fig. 1), had been used in previous studies to show that gene VI of CaMV strain D4 determines systemic infection of *N. edwardsonii*, and that sequences within the 5' third of W260 gene VI elicit HR in *N. edwardsonii* (Schoelz and Shepherd 1988). In the present study, we confirmed the reactions of H7 and H35 in *N. edwardsonii*, and then used these viruses to investigate whether the 5' third of W260 gene VI was sufficient to elicit systemic necrosis. We found that H7 induced a systemic mosaic in *N. clevelandii*, while H35 induced systemic necrosis in that host (Fig. 1). Thus, the same region of CaMV gene VI responsible for elicitation of HR in *N. edwardsonii* also could elicit systemic cell death in *N. clevelandii*. It is also significant to note that neither H35 nor H191 induced systemic cell death in *N. bigelovii*, a result that is consistent with the reaction of the parent viruses—W260 and D4—in this host (Fig. 1).

Fig. 1. Composition of the chimeric cauliflower mosaic virus (CaMV) constructed between W260, D4, and CM1841 to identify W260 regions that influence systemic necrosis and hypersensitive response (HR). Viral genomes are drawn in a linear form to facilitate comparisons. Zero nucleotide position corresponds to left end, while the 8031 nucleotide corresponds to right end, of each map. Functions of the six genes: Gene I, cell-to-cell movement; Gene II, aphid transmission; Gene III, DNA binding protein; Gene IV, coat protein; Gene V, reverse transcriptase; Gene VI, translational transactivation of genes I through V. Restriction enzyme sites used for construction of chimeric viruses are shown where applicable. Sequences of CaMV strains W260, D4, and CM1841 are illustrated by stippled, filled-in, and open regions, respectively.

The systemic cell death symptom is not a consequence of elevated levels of CaMV gene products.

One possible explanation for the systemic necrosis symptom is that the W260 virus replicates to a higher level than the D4 virus. To investigate whether differential levels of virus

replication could be associated with the systemic necrosis symptom, we measured virion titers of D4 and W260 in inoculated and upper, noninoculated leaves of *N. clelandii*. Enzyme-linked immunosorbent assay (ELISA) revealed that the level of D4 virions in both the inoculated and upper, non-

Fig. 2. Systemic symptoms in cauliflower mosaic virus (CaMV)-infected *Nicotiana clelandii* and *N. bigelovii*, and their F₁ and F₂ hybrids. **A–D**, *N. clelandii* infected with D4, W260, H191, and H190, respectively. **E**, *N. bigelovii* infected with W260. **F**, Leaf from an F₁ hybrid plant (*N. bigelovii* × *N. clelandii*) inoculated with W260. F₂ progeny infected with W260 showing **(G)** mosaic symptoms and **(H)** systemic necrosis. Photos were taken 40 to 45 days after inoculation.

inoculated *N. clevelandii* leaves was two to three times higher than that of W260 virions (Table 1). We also measured the level of W260 virions in *N. bigelovii*; this host responds to W260 infection with a systemic mosaic rather than necrosis (Fig. 2E). The W260 titers in both inoculated and upper, non-inoculated *N. bigelovii* and *N. clevelandii* leaves were equivalent (Table 1), further evidence that systemic cell death was triggered by a specific interaction between CaMV strain W260 and *N. clevelandii*.

As CaMV gene VI was found to be responsible for triggering systemic cell death, we compared the levels of W260 gene VI protein in *N. clevelandii* leaves that exhibited moderate levels of cell death with the amount in *N. bigelovii* leaves that exhibited only a mosaic symptom. A Western blot (immunoblot) assay showed that the level of W260 gene VI product in *N. bigelovii* leaves was equivalent to or greater than the amount in *N. clevelandii* (Fig. 3). Consequently, it is unlikely that differential levels of virions or gene VI product could be responsible for the systemic necrosis symptom. Instead, the elicitation of systemic necrosis may be determined by the interaction of the W260 gene VI protein with a host factor in *N. clevelandii*.

The HR of *N. edwardsonii* blocks long-distance movement of CaMV rather than local movement in the inoculated leaf.

The comparison of W260 virion titers in *N. clevelandii* and *N. bigelovii* revealed that this virus moved unimpeded through *N. clevelandii* as it elicited systemic cell death. In contrast, the HR of *N. edwardsonii* appeared to prevent the systemic movement of W260 (Fig. 1; Schoelz and Shepherd 1988). To compare the reaction of *N. clevelandii*, described in the previ-

ous section, with the HR of *N. edwardsonii*, we measured the amounts of W260, D4, and selected chimeric viruses in inoculated and upper, noninoculated leaves of *N. edwardsonii*. An ELISA of upper, noninoculated leaves confirmed the visual observations; gene VI of W260 prevented the virus from moving systemically (Table 2). In the inoculated leaves the level of W260 virions was approximately one third of the D4 virion titer (Table 2). This ratio was similar to the W260:D4 virion ratios in inoculated leaves of *N. clevelandii* and *N. bigelovii* (Table 1), even though these plants reacted with systemic cell death and a systemic mosaic, respectively. This comparison indicated that in the inoculated leaf the lower virion titer of W260 relative to D4 was associated with the W260 virus itself, and not due to a specific type of plant reaction against W260. The level of H191 virions was comparable to that of D4 and H190 (Table 2), further evidence that the HR of *N. edwardsonii* did not affect virion concentrations in the inoculated leaves. This study indicated that the HR of *N. edwardsonii* was associated with a block in the long-distance movement of CaMV, but had little effect on local movement in the inoculated leaf.

A single gene in *N. clevelandii* specifies systemic cell death in response to infection by CaMV strain W260.

To observe the inheritance of the systemic cell death and mosaic traits in *N. clevelandii* and *N. bigelovii*, respectively, crosses were made between these two *Nicotiana* spp. It was relatively easy to obtain reciprocal hybrids from conventional crosses of *N. clevelandii* and *N. bigelovii* and both the F₁ and F₂ hybrids were fertile. All F₁ plants displayed a systemic mosaic when inoculated with W260, which indicated that systemic cell death behaved as a recessive trait (Table 3, Fig. 2F). The F₁ plants were selfed and in the F₂ generation the infected plants revealed a 3:1 segregation for systemic mosaic (Fig. 2G) versus necrosis (Fig. 2H), showing that a single, nuclear coded gene in *N. clevelandii* determines the systemic cell death trait (Table 3). Furthermore, we observed a 1:1 segregation (necrosis:mosaic) in the progeny that resulted from a backcross of the F₁ hybrids to *N. clevelandii*, which confirmed that the systemic cell death trait is conditioned by a single gene (Table 3). This plant gene was designated *ccd1*, for CaMV cell death gene.

DISCUSSION

The formation of systemic cell death symptoms in CaMV-infected *N. clevelandii* is caused by an interaction between strain W260 gene VI and a single gene in the host that we have designated *ccd1*. To identify regions of the W260

Table 1. Comparison of cauliflower mosaic virus particle concentrations in *Nicotiana bigelovii* and *N. clevelandii*

Virus	<i>N. bigelovii</i>		<i>N. clevelandii</i>	
	Inoculated ^a	Upper, noninoculated	Inoculated	Upper, noninoculated
D4	6.9 ± 1.1 (2) ^b	10.8 ± 4.4 (23)	2.7 ± 0.7 (2)	11.3 ± 4.6 (24)
W260	1.6 ± 0.1 (2)	5.7 ± 3.0 (21)	1.1 ± 0.1 (2)	4.2 ± 2.7 (29)

^a Micrograms of virions per gram of leaf tissue, as measured by enzyme-linked immunosorbent assay.

^b Number of plants sampled.

Fig. 3. Comparison of levels of the cauliflower mosaic virus (CaMV) gene VI product in *Nicotiana clevelandii* and *N. bigelovii* systemically infected with W260 by Western blot (immunoblot) analysis. Equivalent amounts of *N. bigelovii* and *N. clevelandii* samples were loaded in each lane, whereas one tenth of that amount was loaded in the lane containing CaMV-infected turnip tissue.

Table 2. Comparison of cauliflower mosaic virus particle concentrations in *Nicotiana edwardsonii*

Virus	Host reaction	Inoculated leaf ^a	Upper, noninoculated leaf
D4	Mosaic	1.3 ± 0.2 (4) ^b	4.0 ± 1.5 (6)
H190	Mosaic	1.2 ± 0.5 (8)	2.1 ± 0.3 (4)
H191	Hypersensitive response	1.1 ± 0.5 (8)	0 (4)
W260	Hypersensitive response	0.4 ± 0.1 (3)	0 (6)

^a Micrograms of virions per gram of leaf tissue, as measured by enzyme-linked immunosorbent assay.

^b Number of plants sampled.

genome that determine systemic cell death in *N. clevelandii*, chimeric viruses were constructed between the wild-type strains W260, D4, and CM1841. Previous research has shown that the source of gene VI in chimeric CaMV strains can determine the ability of the virus to systemically infect solanaceous species (Daubert et al. 1984; Schoelz et al. 1986a). Furthermore, sequences within gene VI specify the development of HR in *D. stramonium* and *N. edwardsonii* (Schoelz et al. 1986a; Schoelz and Shepherd 1988). Interestingly, the same region that elicits HR can also elicit systemic cell death in *N. clevelandii*.

The development of systemic cell death did not appear to be due to elevated titers of W260 virions or its gene VI product. CaMV strain D4 virions accumulated to about twice the level of W260 virions in *N. clevelandii*, and the same difference in virion titer was also evident in *N. bigelovii*, a host that reacts with a systemic mosaic to both of these viruses. Furthermore, Western blot analysis demonstrated that the level of W260 gene VI expression was equivalent or higher in *N. bigelovii* than in *N. clevelandii*. Although it is still possible that other CaMV genes may influence development of cell death, our results indicate that *N. clevelandii* recognizes W260 gene VI in a specific manner and that this recognition event triggers systemic cell death.

To identify the host gene that interacts with gene VI of CaMV for elicitation of systemic cell death, we made crosses between *N. clevelandii* and *N. bigelovii*. The progeny of this interspecific cross are fertile; the two species have the same chromosome number ($n = 24$) and are considered closely related to each other (Goodspeed 1954). In our crosses, all F_1 plants displayed a systemic mosaic when inoculated with W260. In the F_2 generation, infected plants revealed a 3:1 segregation for systemic mosaic versus necrosis. This analysis indicated that a single, recessive, nuclear coded gene in *N. clevelandii* was responsible for the systemic cell death trait. However, it is also possible that *N. bigelovii* might contain a dominant allele that suppresses cell death triggered by CaMV strain W260.

There are few examples in which a specific viral symptom has been shown to be conditioned by an interaction between a single plant gene and a single viral gene. In contrast, gene-for-gene relationships have been well established for host resistance to viral infection. For example, it has been shown that the viral replicase proteins of TMV interact with the *N* resistance gene in tobacco (Padgett and Beachy 1993; Padgett et al. 1997; Witham et al. 1994) and with the *Tm1* resistance gene in tomato (Meshi et al. 1988). Furthermore, the TMV movement protein elicits resistance in *Tm2* tomato (Meshi et al. 1989), and the TMV coat protein elicits resistance in *N'*

tobacco (Knorr and Dawson 1988; Culver and Dawson 1989). In potato, the potato virus X (PVX) coat protein interacts with both the *Nx* and *Rx* resistance genes of potato (Kavanagh et al. 1992), whereas the PVX movement protein elicits HR in potato varieties that have the *Nb* resistance gene (Malcuit et al. 1999). Resistance to CaMV in several Arabidopsis ecotypes has been shown to be conditioned by single genes (Leisner et al. 1993; Callaway et al. 1996), and in one case CaMV gene VI is responsible for overcoming resistance in this host (Agama et al. 1998).

One of the few studies that deals with plant genes influencing virus symptomatology identified host and virus genes that determine severe local chlorosis in tobacco (*N. tabacum*, cv. Ky57) inoculated with CMV strain Y (Takahashi and Ehara 1993). It was shown that severe local chlorosis in CMV(Y)-inoculated Ky57 tobacco is induced by an interaction of two recessive host genes and the virus coat protein gene. Furthermore, these two recessive host genes completely cosegregated with two recessive loci (*yb1* and *yb2* genes) of Ky57 that are known to be responsible for chlorophyll reduction in the stem (Henika 1932). A second study has shown that an incompletely dominant gene in *N. bigelovii* conditions a yellow mosaic symptom in response to infection with satellite Y RNA of CMV (Masuta et al. 1993).

In the present study, the monogenic systemic cell death trait revealed in W260-infected *N. clevelandii* was not linked to any visible phenotype in the uninoculated host. Therefore, at this point one can only speculate on the function of the systemic cell death gene and on the way it interacts with the gene VI product of the invading virus. For instance, the systemic cell death gene might be a defective copy of a cellular protectant (antioxidant) gene such as glutathione-S-transferase or glutathione peroxidase. Products of such genes are believed to block plant cell and tissue necrosis (Levine et al. 1994; Tenhaken et al. 1995). More likely, this recessive *N. clevelandii* gene could be a positive regulator of cellular protectant gene expression, which fails to carry out its function during systemic viral infection, enabling necrosis to develop. The same or a similar defective regulator gene might be responsible for the development of spontaneous necrosis in *N. clevelandii* plants that express a fenthion-sensitivity gene from tomato (Rommens et al. 1995). In fact, a positive regulator of cellular protectants has been identified in the weed *Conyza bonariensis* as a single gene with Mendelian inheritance (Shaaltiel et al. 1988). Also, several monogenic, recessive mutants exist in Arabidopsis that are unable to control the spread of necrosis during pathogenesis and aging (Greenberg et al. 1994). One of these mutants shows more severe, aging-related necrosis

Table 3. Segregation of systemic necrosis and mosaic in crosses of *Nicotiana clevelandii* (Nc) and *N. bigelovii* (Nb) after inoculation with cauliflower mosaic virus strain W260

Host	Systemic mosaic	Systemic necrosis	Observed ratio ^a	Expected ratio ^a	χ^2
Nc	0	45	0 : 45		
Nb	52	0	52 : 0		
$F_1[Nc \text{♀} \times Nb \text{♂}]$	195	0	195 : 0		
$F_1[Nb \text{♀} \times Nc \text{♂}]$	32	0	32 : 0		
$F_2[\text{selfed } F_1(Nc \text{♀} \times Nb \text{♂})]$	133	41	3.2 : 1	3 : 1	0.122
$F_2[\text{selfed } F_1(Nb \text{♀} \times Nc \text{♂})]$	22	6	3.6 : 1	3 : 1	0.322
$F_1[Nc \text{♀} \times Nb \text{♂}] \times Nc$	18	24	0.75 : 1	1 : 1	0.881

^a Systemic mosaic : systemic necrosis.

under low light intensity (Dietrich et al. 1994), the same light-dependent response observed during CaMV-elicited systemic necrosis in *N. clelandii*.

Alternatively, the recessive *N. clelandii* gene that interacts with the CaMV gene VI product during systemic cell death could potentially code for a plant protein that is part of the host translation machinery. One or more of these proteins must interact with the gene VI product to facilitate the translational transactivation of genes I through V during viral pathogenesis (Bonneville et al. 1989; Gowda et al. 1989). The copy of such a protein present in *N. clelandii* might interact with CaMV gene VI in a manner that alters translational transactivation and triggers host responses that result in the development of systemic cell death. However, the relationship between translational transactivation, the molecular function assigned to gene VI, and the ability of gene VI to elicit systemic cell death or HR remains unclear.

MATERIALS AND METHODS

CaMV wild-type strains and construction of chimeric viruses.

The wild-type CaMV strains W260 and D4 have been described previously (Gracia and Shepherd 1985; Schoelz et al. 1986b). Both viruses have been cloned in infectious form into the *SalI* site of the plasmid vector pBR322 to form pCaMVW260 and pCaMVD4, respectively (Schoelz et al. 1986b; Schoelz and Shepherd 1988). The chimeric virus clones pH190 and pH191 were made by exchanging *SalI*-*BstEII* restriction enzyme-generated DNA fragments between pCaMVW260 and pCaMVD4. The construction of chimeric viruses H7 and H35 has been described previously (Daubert et al. 1984; Schoelz and Shepherd 1988).

Infections with cloned viral DNA were initiated by inoculating turnips (*Brassica rapa* cv. Just Right) with viral DNA excised from the plasmid vector with the appropriate restriction enzyme as previously described (Howell et al. 1980; Lebeurier et al. 1980). Viral DNA was purified from infected plants following the procedure of Gardner and Shepherd (1980) and identification of chimeric viruses was confirmed by mapping key restriction enzyme sites. Wild-type and chimeric viruses were stored in lyophilized turnip leaf tissue at 4°C to serve as inoculum for experiments.

Analysis of host symptoms and virus detection.

Virus inoculum was prepared from infected turnips as described by Schoelz et al. (1986a) or as purified virions (Hull et al. 1976). Reciprocal hybrids of *N. bigelovii* and *N. clelandii* were obtained from conventional crosses. All *Nicotiana* spp. and hybrids were inoculated 5 to 6 weeks after seeds were sown. In the case of *N. clelandii* and the F₁ and F₂ hybrids, seeds were soaked in 2.0% NaOCl for 15 to 20 min prior to sowing, as described by Burk (1957), to overcome dormancy. Inoculated plants were maintained for 45 to 50 days either in growth chambers set for a 10-h day with a light intensity of 100 to 180 $\mu\text{E s}^{-1} \text{m}^{-2}$ at 18°C or in the greenhouse during the months of October through April. Symptoms were recorded daily. The presence of CaMV virions in leaves showing systemic symptoms was verified by ELISA (Clark and Adams 1977) as described previously (Anderson et al. 1991).

SDS-polyacrylamide gel electrophoresis and Western blot analysis of CaMV gene VI protein.

Samples were prepared by grinding infected tissue with a mortar and pestle at a 1:5 (wt/vol) dilution in 0.02 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged for 30 s at 12,000 $\times g$. To 200 μl of the clarified extract, an equal volume of 2 $\times$ sodium dodecyl sulfate (SDS) treatment buffer (0.125 M Tris [pH 6.8], 4% [wt/vol] SDS, 20% [vol/vol] glycerol, and 10% [vol/vol] 2-mercaptoethanol) was added. The samples were heated for 4 min at 100°C, then reduced and alkylated (Lane 1978). The equivalent of 2 mg of tissue was then loaded into a well for the respective sample of a 10% acrylamide mini-gel (Laemmli 1970). Gels were stained with Coomassie blue R-250 to confirm that equal amounts of protein were loaded or they were transferred to nitrocellulose membranes for Western analysis.

Proteins were transferred to nitrocellulose membranes for 1.5 h at 300 mA (constant current) (Towbin et al. 1979) and blocked overnight in blocking buffer (phosphate buffered saline [PBS; pH 7.4], 5% [wt/vol] nonfat dry milk, 1% [wt/vol] bovine serum albumin [BSA], 0.01% [vol/vol] Antifoam A). Membranes were incubated at room temperature for 1.5 h with a 1:500 dilution of anti-CaMV gene VI IgG (in PBS Tween buffer [pH 7.4] containing 2.0 g of BSA per liter as the blocking agent), followed by an incubation for 1.5 h at room temperature with a 1:7,500 dilution of alkaline phosphatase-labeled goat-anti-rabbit IgG (Fisher, St. Louis, MO). Between each step the membranes were washed with PBS (pH 7.4), 0.05% Tween 20. Protein bands were visualized by addition of 10 ml of alkaline phosphatase color developer (100 mM Tris [pH 9.1], 100 mM NaCl, 5 mM MgCl₂, 0.4 mM nitro blue tetrazolium chloride, 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate D-toluidine salt, which was allowed to react for 10 to 20 min.

ACKNOWLEDGMENTS

This research was supported by a grant from the Food for the 21st Century program at the University of Missouri and by U.S. Department of Agriculture/National Research Initiative Competitive Grant No. 98-35303-6711. This is a contribution from the Missouri Agricultural Experiment Station, Journal Series No. 12,922.

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