Transgenic *Arabidopsis* Lines Expressing Gene VI from Cauliflower Mosaic Virus Variants Exhibit a Range of Symptom-Like Phenotypes and Accumulate Inclusion Bodies

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Gene VI of cauliflower mosaic virus (CaMV) is an important determinant of symptom expression during infection. We have constructed a series of transgenic *Arabidopsis* lines that express gene VI protein (P6) from two CaMV isolates (Bari-1 and Cabb B-JI) that cause mild and severe symptoms, respectively, in *Arabidopsis*, and from a recombinant virus (Baji-31) with a hybrid gene VI that causes very severe symptoms. From 41 transgenic lines analyzed, 17 showed symptom-like phenotypes that ranged from mild vein chlorosis to severe chlorosis and stunting. P6 levels in transgenic lines varied from undetectable in the lowest expressors to levels greater than those in CaMV-infected plants. There was a strong correlation between phenotype severity and the level of P6, and with the gene VI origin in the order, Baji-31 > B-JI > Bari-1. This was similar to symptom severity in *Arabidopsis* infected with the respective CaMV variant. We also found that transgenic P6 accumulated in inclusion bodies that were similar to those found in infected plants but lacking virions. We conclude that expression of P6, in the absence of virus replication, elicits a subset of the host symptom responses normally observed during infection and that the level, sequence, and possibly the form of P6 are important in potentiating the process.

Additional keyword: transactivator.

Plants generally respond to infections with compatible viruses by developing systemic symptoms. Although there are innumerable potential host-virus combinations, the symptoms that develop often show common characteristics, such as vein-clearing, mosaics, chlorosis, etc. This suggests that a particular virus might initiate a subset of pre-programmed responses from a

suite of possible responses to infection. Presumably, virus and host components must interact to trigger changes in gene expression leading to development of symptoms. Since symptom expression is one of the primary causes of economic losses in virus-infected plants, understanding how viral and host components interact should facilitate the development of strategies to limit symptom development and minimize damage.

In characterizing the components of these interactions, most progress, so far, has been achieved in mapping viral pathogenic determinants, because of the relative accessibility of the viral genome. This is especially so in the case of cauliflower mosaic virus (CaMV) because much is known about its molecular biology (Rothnie et al. 1994), and a wide range of naturally occurring pathogenic variants are available for study (Al-Kaff and Covey 1995). This has enabled the mapping of several viral genome domains that are important in defining specific pathogenic characteristics (Daubert 1988; Schoelz and Shepherd 1988; Stratford and Covey 1989; Anderson et al. 1991; Covey 1991; Anderson et al. 1992; Qiu and Schoelz 1992; Wintermantel et al. 1993; Qiu et al. 1997).

The 8-kbp DNA genome of CaMV has six genes whose functions during the virus propagation cycle have been characterized. One gene (VI) encodes a 60K to 66K polypeptide (P6) involved in regulating viral protein synthesis as a translational transactivator (Rothnie et al. 1994) that is a major component of the virus inclusion bodies found associated with virions in the cytoplasm of infected plants (Covey and Hull 1981). Construction of recombinants between CaMV variants showing different pathological characters has led to the conclusion that the gene VI region also contains pathogenic determinants, including those regulating host specificity (Wintermantel et al. 1993) and symptom type and severity (Daubert et al. 1984; Daubert and Routh 1990; Anderson et al. 1992). We have previously constructed a series of recombinants between a severe CaMV isolate (Cabb B-JI) and a mild isolate (Bari-1), and shown that gene VI contributes determinants affecting symptomatic leaf coloration changes including chlorosis (Stratford and Covey 1989) and expression of a dark green phenotype caused by the Bari-1 isolate (McCallum 1994).

Further evidence implicating gene VI as an important CaMV pathogenic determinant comes from experiments in

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which P6 has been expressed as a transgene in plants in the absence of virus infection. Expression of P6 in a transgenic nonhost, *Nicotiana tabacum*, led to development of symptom-like leaf mosaics, mottling, bleaching, and necrotic spots (Baughman et al. 1988; Takahashi et al. 1989; Balazs 1990; Goldberg et al. 1991). However, Goldberg et al. (1991) concluded that this response, elicited by two different caulimoviruses, appeared to be restricted to nonhost plants. Expression of P6 in *Arabidopsis*, a CaMV host species (Balazs and Lebeurier 1981; Melcher 1989), was subsequently shown to elicit a late flowering phenotype and mild chlorosis (Zijlstra and Hohn 1992; Zijlstra et al. 1996). To understand more about the response of host plants to P6, we have now constructed a collection of transgenic *Arabidopsis* lines expressing P6 sequences derived from CaMV isolates Cabb B-JI and Bari-1 and a recombinant (Baji-31) that causes very severe infections in *Arabidopsis* (Cecchini et al. 1997; E. Cecchini, N. Al-Kaff, S. N. Covey, and J. J. Milner, *unpublished*). We show that symptom character elicited in P6-expressing transgenic host plants is dependent upon the level of P6 expression, and upon the P6 sequence itself. We have also found that uninfect P6 transgenic plants contain empty inclusion bodies.

RESULTS

Properties of gene VI-transgenic plants.

T-DNAs from the three constructs pJO-Baji31, pJO-Bari, and pJO-BJI were introduced into three batches of 10 *Arabidopsis* plants by *Agrobacterium*-mediated transformation, using the vacuum infiltration technique. We obtained approximately 50,000 T₂ seed from each batch of plants. For each construct, we screened approximately 10,000 T₂ seedlings for hygromycin resistance. Most of the seedlings were hygromycin-sensitive and stopped growing after the emergence of the cotyledons. A number (20 for Baji-31, 7 for Cabb B-JI, and 16 for Bari-1) manifested hygromycin resistance and continued to grow either normally or at a reduced rate. These T₂ hygromycin-resistant seedlings were transferred to compost and allowed to grow to maturity. DNA was prepared from a single leaf of each T₂ transgenic plant. In polymerase chain reactions (PCRs) with the gene VI primers, DNA from 41 out of 43 plants gave a band at the expected size of 1.6 kbp, indicating that these plants had at least one complete copy of the gene VI coding sequence (data not shown).

Out of the 41 T₂ plants that contained the gene VI coding region, 24 were visibly indistinguishable from the parent. The remaining plants showed a range of abnormal phenotypes, ranging from severe chlorosis, stunting, and altered leaf morphology (in particular, serrated leaf margins), through mild chlorosis and stunting, to vein chlorosis. Figure 1 shows the phenotypes of five representative lines derived from T₂ plants. All plants survived to flowering and set seed successfully, although those with the most severe phenotypes were slower growing and produced fewer seeds. Batches of between 60 and 160 T₃ seedlings were screened for hygromycin resistance. Progeny from 15 plants segregated with approximately the expected 3:1 ratio of resistant to susceptible seedlings (e.g., Baji-A7 100:24, Bari-B5 90:33, BJI-C6 101:34). Progeny of the remaining 26 segregated with apparent ratios of resistant to sensitive seedlings of from 2:1 to <1:10 (e.g., Bari-B6 71:58, Baji-A14 40:75, Baji-A13 8:116). For lines

with particularly low ratios of resistant:sensitive seedlings, even apparently resistant seedlings grew poorly in the presence of hygromycin. However, with two exceptions (see below), we were able to derive, from each of the 41 progenitors, lines that were stably homozygous for the transgene. We believe that poor expression of hygromycin phosphotransferase, rather than any genetic instability at the transgene locus, was responsible for the apparently aberrant segregation ratios.

T₂ plants with a normal (nontransgenic) phenotype produced T₃ progeny that also appeared normal. However, T₂ transgenic plants with a chlorotic phenotype showed a further phenotypic segregation at the T₃ level. Following selection for hygromycin resistance, between two and five T₃ plants out of each batch of 10 to 12 showed a phenotype that was more severe than that of the T₂ parent; the remainder resembled the T₂ parent. An example of this is shown in Figure 2.

All progeny of plants with the more severe phenotype (from batches of up to several hundred) inherited the phenotype, whereas hygromycin-resistant progeny of plants with the less severe phenotype continued to segregate into mild and severe phenotypes at a ratio of approximately 2:1. These results indicated that the severity of the phenotype might be dose related, and could serve as a marker to distinguish between plants that were hemizygous for the transgene and plants that were homozygous. In one line, Baji-A14, the T₂ (hemizygote) progenitor of which was the most severely chlorotic and stunted, T₃ putative homozygotes segregated out but many failed to survive beyond the eight-leaf stage. For those lines with a nontransgenic phenotype, we were able to select homozygotes by segregation analysis of the progeny, either by PCR to test

Fig. 1. Examples of lines of transgenic plants expressing cauliflower mosaic virus gene VI. **A**, Nontransgenic control. **B**, Baji-A1, symptom severity 1 (for scale, see caption for Figure 3). **C**, BJI-C5, symptom severity 2. **D**, Baji-A16, symptom severity 3. **E**, Bari-B6, symptom severity 4. **F**, Baji-A14, symptom severity 5. Vein chlorosis on the leaves of plants showing mild to intermediately severe phenotypes is indicated by arrows in **C** and **D**. All plants are shown to the same scale.

for the presence of gene VI DNA or, where appropriate, by screening for hygromycin resistance.

Although some lines showed apparently aberrant segregation of hygromycin resistance, with two exceptions all inherited gene VI in a stable mendelian manner. Baji-A16 exhibited a mottled phenotype with vein chlorosis and generated a line that was stably homozygous for hygromycin resistance. However, at each generation, we observed a variable proportion of seedlings with a nontransgenic phenotype, but which were hygromycin resistant. In a second line of plants (Baji-A15), which were severely chlorotic and stunted, we were able to select T_3 plants that were apparently homozygous for the transgene on the basis of the gene VI phenotype and hygromycin sensitivity. However, at each of the T_4 , T_5 , and T_6 generations, 1 to 2% of plants reverted to a normal, nontransgenic phenotype. These revertants were hygromycin sensitive and tested null for the gene VI coding sequence by PCR. We might have expected instability at the transgene locus to produce plants hemizygous for the transgene but, curiously, we never observed any plants segregating out with an intermediate hemizygous gene VI phenotype.

In most of the lines, T_6 plants were phenotypically similar to their T_3 progenitors. However, T_6 homozygous plants of lines Baji-A15 and Baji-A16 showed a decreased phenotypic severity, whereas lines Baji-A5 and Baji-A7 showed an increased severity.

To characterize the transgenes further, DNA from selected lines was analyzed on Southern blots (not shown). Out of 12 lines, seven gave a single band and five lines gave two bands when DNA was digested with *Bam*HI or *Xba*I (which cut the T-DNA at a single site) and hybridized to a gene VI probe. These patterns, taken together with the segregation data, are consistent with the presence in each line of one or two copies of the transgene at either a single locus or at least closely linked loci. Each of the lines that we analyzed had a unique fragment pattern and apparently represents the progeny of an independent transformation event.

Symptom character and transgene expression.

Levels of P6 accumulating in leaves of hemizygous plants from each of the transgenic lines were measured by enzyme-

Fig. 2. Segregation of hygromycin-resistant T_3 plants from line Baji-A15 into hemizygous and homozygous phenotypes. Plants with the homozygous phenotype are indicated by the arrowheads.

linked immunosorbent assay (ELISA). As a control, we also measured levels of P6 in extracts from the leaves of CaMV (Cabb B-JI)-infected turnip plants, harvested 20 days post inoculation. Transgenic lines varied greatly in the levels of P6 accumulation. In 27 lines, the ELISA absorbance was more than twice the background levels (approximately 0.080) obtained with controls of uninfected, nontransgenic *Arabidopsis*; in the remaining 14 lines, the absorbance was similar to or only slightly above the controls. For each of the three constructs, we obtained lines that varied considerably in levels of P6 accumulation. All of the lines with very low levels of P6 were phenotypically indistinguishable from the nontransgenic plants, while lines that were accumulating moderate to high levels of P6 all exhibited the “gene VI” phenotype to a greater or lesser degree of severity. In three lines, Bari-B6, Baji-A14, and Baji-A15, levels of P6 in hemizygous plants were greater than in a sample leaf from a plant infected with the Cabb B-JI isolate of CaMV.

To characterize further the effect of the level of P6 accumulation on the phenotype, plants were scored as follows for severity, on an arbitrary 1 to 5 scale: 1, indistinguishable from the nontransgenic parent; 2, mild vein chlorosis and similar in size to the nontransgenics; 3, mildly chlorotic and slightly smaller than the nontransgenics; 4, moderately chlorotic, much smaller than the nontransgenics, alterations in leaf morphology; 5, severely chlorotic and stunted with small serrated leaves. Figure 1 shows examples of plants in each category. We compared the levels of P6 with the phenotype severity (Fig. 3). For each of the constructs, there was a strong correlation between severity and levels of P6. However, severity was also strongly dependent on which gene VI construct was present, so that for any group of plants with similar amounts of P6, those with Baji-31 exhibited the most severe phenotypes, those with Bari-1 exhibited the mildest phenotypes, and those expressing Cabb B-JI were intermediate between the two. This suggests that the severity of the symptom-like gene VI phenotype depends on both the level

Fig. 3. Relationship between symptom severity and levels of P6 (determined by enzyme-linked immunosorbent assay) in leaves of different transgenic lines. Protein levels are expressed relative to the amount in a control sample of leaf tissue from a plant infected with cauliflower mosaic virus Cabb B-JI. Phenotype “severity” scored on a 1 to 5 scale in which 1 is indistinguishable from nontransgenic (e.g., Baji-A1 in Figure 1), 2 is just discernable (e.g., BJI-C5), 3 is moderate (e.g., Baji-A16), 4 is severe (e.g., Bari-B6), and 5 is very severe (e.g., Baji-A14). Each point represents an individual transgenic.

of P6 accumulation and on the amino acid sequence of the protein. The order of phenotype severity in the transgenic plants was Baji-31 > Cabb B-JI > Bari-1. In CaMV-infected *Arabidopsis* (Ler *gll*), Baji-31 causes a very severe mosaic and severe stunting, Cabb B-JI causes a moderate mosaic and some stunting, and Bari-1 induces only a very mild mosaic (Cecchini et al. 1997; E. Cecchini, N. Al-Kaff, S. N. Covey, and J. J. Milner, *unpublished*). Thus, the order of severity of the symptom-like phenotype from the three constructs was the same as that for severity of symptoms in *Arabidopsis* infected by the three isolates of the virus.

We obtained a low but consistent background level of serological cross-reaction to extracts from nontransgenic control plants, making it difficult to measure accurately the levels of P6 accumulation in those plants that expressed very low levels of the protein. To determine the levels of expression of gene VI mRNA, we carried out Northern (RNA) blots with a gene VI probe on total RNA from selected lines that were accumulating low, medium, and high levels of P6 from the three constructs (Fig. 4). We observed a band of the expected size for the gene VI mRNA (1.6 kb) in all of the plants that accumulated high to moderate levels of P6, and on longer exposure of the autoradiograph, in some of the lines (e.g., Baji-A1) that expressed low levels of P6. However, in some lines (e.g., Bari-B3) we were unable to detect any band of the expected size; these lines must either not express P6 or express it at an extremely low level. In lines Baji-A7, Baji-A14, and Bari-B6, levels of gene VI mRNA were similar to or greater than levels of 19S (gene VI) mRNA in a leaf sample from *Arabidopsis* infected with Cabb B-JI (see Figure 4).

With two exceptions, lines that accumulated the highest levels of P6 also accumulated the highest levels of gene VI mRNA. Baji-A15 and Baji-A16 both expressed lower levels of gene VI mRNA than we might have expected on the basis of P6 accumulation. However, in these two lines, T₅ plants, which were used for mRNA measurements, showed a less severe phenotype than did the T₂ and T₃ plants in which protein levels were assayed.

Inclusion bodies accumulate in P6-transgenic plants in the absence of infection.

In CaMV-infected plants, P6 accumulates in inclusion bodies within the cytoplasm (Covey and Hull 1981). To determine whether P6 inclusion bodies were present in transgenic plants, leaf tissue sections were made from three lines: two high expressors with a severe phenotype, Baji-A15 and Baji-A7, and a low expressor, Baji-A1, with a nontransgenic phenotype. Sections from Baji-A1 plants were indistinguishable from nontransgenic controls. However, many but not all of the cells from Baji-A15 and Baji-A7 plants contained large, electron-dense inclusion bodies (Fig. 5B). These inclusion bodies were similar in size and appearance to those observed in sections from CaMV-infected plants, and like those in infected plants, contained electron lucent holes, although without virus particles (Fig. 5A). Thus, in the absence of replication, the transgene-derived protein appears to be capable of assembling into inclusion bodies that resemble those formed during infection, at least in plants that accumulate high levels of P6.

To characterize further the inclusion bodies accumulating in uninfected transgenic plants, an inclusion body preparation was made from Baji-A7 transgenic plants and probed for P6 by immunoblotting. This confirmed the presence of a P6-specific polypeptide of about 63K in uninfected gene VI-transgenic plants, similar to that present in CaMV-infected plants (Fig. 6). In fact, the gene VI protein expressed in transgenic *Arabidopsis* line Baji-A7 was from the recombinant virus Baji-31. The Bari-1 P6 has an anomalously lower mobility in polyacrylamide gels (Stratford et al. 1988) that also slightly reduces the mobility of the Baji-31 P6 expressed in transgenic *Arabidopsis* line Baji-A7 (Fig. 6).

DISCUSSION

Symptomatic phenotypes have been observed following transgenic expression of caulimovirus gene VI (Baughman et al. 1988; Takahashi et al. 1989; Balazs 1990; Goldberg et al. 1991) and genes of other viruses (e.g., Van der Wilk et al.

Fig. 4. Hybridization of ³²P-labeled gene VI probe to RNA from plants from 25 transgenic lines plus nontransgenic controls (NT) of uninfected (U) and infected (I) *Arabidopsis*. Each lane contains 5 µg of total RNA. Exposures of the same autoradiograph at (top panel) 3 h, (middle panel) 21 h, (bottom panel) 48 h. For infected controls, RNA was extracted from *Arabidopsis* Col *gll* plants infected with cauliflower mosaic virus B-JI, 20 days after inoculation.

1997). However, for CaMV, these studies were performed essentially in nonhost species, and Goldberg et al. (1991) provided evidence to suggest that these phenotypes were nonhost responses. Although Zijlstra et al. (1996) observed phenotypes of early flowering and alterations in leaf morphology following expression of P6 in a transgenic host, *Arabidopsis*, the relationship between these responses and specific characteristics of the transgenic protein was not established. We have now shown that expression of transgenic P6 in *Arabidopsis* elicits changes in the plant, including development of a subset of symptom characters potentiated by the level of expression and by the gene VI phenotype of the originating viral variant, together with accumulation of empty cytoplasmic inclusion bodies.

Our results indicate that the expression of gene VI in a non-host is not a requirement for the induction of the symptom-like phenotype. Although we cannot exclude the possibility that different hosts may vary in their sensitivity to ectopic expression of P6, the differences between the phenotypes observed by Goldberg et al. (1991) in *N. tabacum*, and by ourselves and Zijlstra et al. (1996) in *Arabidopsis*, may simply reflect differences in levels of expression of the transgene protein, or differences between the amino acid sequences of P6 from the caulimovirus isolates used as the transgene source in the two studies. Although Zijlstra et al. (1996) did not specifically measure the levels of P6 in their transgenic *Arabidopsis* lines, they did report difficulty in regenerating plants from callus following transformation with gene VI expression constructs. Expression of high levels of P6 has a negative effect on plant vigor, and might result in selection against transformants with the highest transcriptional activity. By using vacuum infiltration to transform *Arabidopsis*, we were able to

avoid passage through callus, a stage at which tissue has been reported to be abnormally sensitive to a variety of deleterious agents (Negrutui et al. 1975). This, plus the use of a vector with a highly enhanced promoter, has allowed us to construct transgenic lines with a wide range of P6 expression.

In homozygous lines of the highest expressors, e.g., Baji-A7, Baji-A15, and Bari-B6, the levels of gene VI mRNA are greater than levels of *rbcS* mRNA in nontransgenic *Arabidopsis* (E. Cecchini and A. Sohal, unpublished data). Since *rbcS* mRNA is reported to be the most abundant message in green plant tissue, constituting up to 4% of the total mRNA (Thompson et al. 1983), transcription of our high-expressing constructs must be very efficient. In general, lines with the highest levels of gene VI mRNA also accumulated the highest amounts of P6, and vice versa. This implies that variations in the amount of P6 accumulation may be mediated largely at the level of mRNA abundance. Such variation might occur as a

Fig. 6. Immunoblot, using antiserum to P6, following polyacrylamide gel electrophoresis of inclusion body preparations from (H) noninfected turnips, (I) turnips infected with cauliflower mosaic virus isolate Cabb B-JI, (T+) transgenic *Arabidopsis* line Baji-A7, (T-) controls of transgenic *Arabidopsis* containing a green fluorescent protein (GFP) transgene. Approximately twice as much tissue extract was loaded in lane (T+) as in lane (I).

Fig. 5. Electron micrographs of plant sections: a section from a plant of transgenic line Baji-A7; inclusion body-like structure is marked (I); a section from a nontransgenic *Arabidopsis* plant infected with cauliflower mosaic virus Baji-31 showing inclusion bodies (indicated by I). Bar indicates 600 nm.

result of differences in transcriptional activity of the transgene, or through a post-transcriptional silencing mechanism (Baulcombe 1996), although differences in protein turnover cannot be excluded. Although for most lines we did not compare levels of mRNA in plants between hemi- and homozygous plants, in line Baji-A7 hemizygous plants accumulated lower levels of mRNA (data not shown).

With two exceptions, all our transgenic lines inherited the transgene in a stable mendelian manner through the T_6 generation at least. Baji-A15 generated spontaneous loss-of-transgene revertants at a frequency of 1 to 2% per generation. Baji-A16 also generated phenotypic revertants, although these apparently retained hygromycin resistance. Transgene instability at the T_3 to T_6 generations has been reported in *N. plumbaginifolia* (Cherdshewasart et al. 1993). Interestingly, both Baji-A15 and Baji-A16 also showed evidence of reduced transgene mRNA accumulation in later generations. It may be significant that, at the T_2 generation, Baji-A15 was a very high expressor of P6 and had an extremely severe phenotype. Given the potential selective pressure against the slow growth rate and reduced fertility of lines with the most severe phenotypes, it is perhaps surprising that the other high-expressing lines show the stability that they do.

Several lines of evidence lead us to believe that the phenotypes induced by transgene-mediated expression of gene VI result from a response by the plant that in at least some aspects mimics the normal symptom response to CaMV infection. Many of the transgenic lines, in particular those with phenotypes of intermediate severity, e.g., BJI-C5, exhibit the vein clearing and mild chlorosis seen in CaMV-infected *Arabidopsis*. Most importantly, the severity of the transgene-induced phenotype is directly dependent not only on the level of transgene protein, but also on the amino acid sequence of the transgene. The relative severity of the phenotype is strongly correlated with the relative severity of the symptoms caused by infection with the CaMV isolate from which the transgene coding sequence was derived.

Although many of the phenotypes are symptom-like in character, the severe chlorosis and alterations in leaf morphology associated with lines expressing the highest levels of P6 from Baji-31 are not usually associated with CaMV infections. Conversely, symptom characters, such as the leaf crinkling and distortions typically observed in *Arabidopsis* infected with severe CaMV strains (Cecchini et al. 1997; E. Cecchini, N. Al-Kaff, S. N. Covey, and J. J. Milner, *unpublished data*) are absent in the transgenic plants. Levels of P6 in those lines that are severely chlorotic are higher than in infected plants. Plants may not perceive transgene-derived P6, which is expressed ectopically and throughout all stages of development, in the same way as P6 synthesized during a spreading infection, because particular cells or cell types may escape infection, or fail to support virus replication (Al-Kaff and Covey 1995, 1996). Timing of P6 expression may be also important, and gross measurements of P6 accumulation may not accurately reflect the physiological dose perceived by the plant. Most likely, high levels of P6 have a deleterious effect on a number of intracellular processes. Nevertheless, this effect may still be related to the effect of P6 expression during virus infection, since gene VI constructs from Baji-31 were much more effective than those from Bari-1 in inducing extreme chlorosis. In turnip infected with recombinant hybrids

of CaMV, symptom characters associated with leaf coloration (e.g., chlorosis, dark greening etc.) all mapped to the gene VI region (Daubert et al. 1984; Stratford and Covey 1989; McCallum 1994), whereas stunting, leaf distortions, and symptom timing mapped variously to genes I, III, IV, and V. It is not unexpected, therefore, that transgenic plants expressing only gene VI do not show leaf crinkling and distortion.

Transgenic P6 appears to accumulate in cytoplasmic, electron-dense, vacuolated inclusion bodies similar to those formed during infection, those in transgenic plants lacking virions (Fig. 5). The function of inclusion bodies during infection is not fully understood. They could be sites of virion synthesis or bodies regulating free virion levels in cells (Covey and Turner 1991). It is also not known if free P6 or that in inclusion bodies is responsible for symptom expression during infection or in noninfected P6-transgenic plants. Transgenic plants have significant levels of both forms (data not shown). Even so, the presence of such inclusion bodies indicates that neither replication nor any other viral product is necessary for their formation.

Symptom expression in virus-infected plants most likely results from a combination of effects including nonspecific toxicity of viral products, metabolic overload, and specific interactions between viral and host factors. It is these latter interactions, which could initiate programmed host responses via signal transduction pathways, that should be most accessible for manipulation. That such host programs exist is suggested from observations in which plants appear to respond by selectively regulating synthesis of a number of host genes (Wang and Maule 1995; Aranda et al. 1996). We suggest the term viral pathogene (v-pathogene) to describe virus genes such as CaMV gene VI, the products of which appear to play a critical role in eliciting symptoms. Such proteins might perhaps interact specifically with the product of a host gene (which might be termed an h-pathogene). Evidence for such an interaction has been provided by the recent demonstration by Wittman et al. (1997) that the VPg protein from turnip mosaic virus interacts specifically with the translational initiation factor eIF(iso)4E from *Arabidopsis*. The ability to induce disease-like symptoms by transgene-mediated expression of a virus protein provides a model system with which to study host-pathogen interactions during symptom expression and could provide a route to isolating h-pathogenes in the absence of infection.

MATERIALS AND METHODS

Virus and plants.

CaMV isolates Cabb B-JI and Bari-1 were propagated (Al-Kaff and Covey 1995) in turnip (*Brassica rapa-rapifera* L. cv. Just Right) or *Arabidopsis thaliana* Col-gll plants following mechanical inoculation with purified virus, sap, or cloned viral DNA. Plants were grown at 18 to 22°C in a glasshouse or growth chamber. Glasshouse-grown plants had light supplemented to a 16-h photoperiod. *Arabidopsis* plants were grown under continuous illumination, or for infection studies in a 10-h photoperiod.

Constructs.

Three plasmids, pCa24GS, pBB1.1 (Stratford and Covey 1989), and pBaji-31 (McCallum 1994), containing the P6

open reading frames from CaMV isolates Cabb B-JI and Bari-1 and recombinant Baji-31 were used as sources of the gene VI coding region. In Baji-31, nucleotides 6103 to 7027 of the Cabb B-JI genome (encoding amino acids 112 to 419 of P6) have been replaced by the corresponding region of P6 coding sequence from Bari-1 (McCallum 1994). The coding sequences for P6 were PCR-amplified by *Pfu* polymerase, with a forward primer ACATCTCTGGACCTGAGAAAATCAGACCTC and a reverse primer CTTCTTATCTAGAGGAAGG GT, each complementary to the sequences flanking the P6 coding sequence from Cabb B-JI, but modified to introduce internal *KpnI* and *XbaI* restriction sites, respectively. These "gene VI primers" were designed to be complementary to those regions of the flanking sequences that had the most sequence homology between Cabb B-JI and Bari-1.

The PCR products were digested with *KpnI* and *XbaI*, and ligated into pUC19 to give three intermediate plasmids, pUC-BJI, pUC-Bari, and pUC-Baji-31. These plasmids were digested with *KpnI* and *XbaI* and the inserts ligated between the same cloning sites of pJO530 (J. Ogas, *unpublished*), a Ti plasmid expression vector derived from pBIB but modified to include the enhanced CaMV 35S promoter from pRTL2 and an octopine synthase termination/polyadenylation sequence (Becker 1990). This vector is designed to maximize expression of coding sequences placed behind the enhanced promoter. The three recombinant Ti plasmids, pJO-BJI, pJO-Bari, and pJO-Baji-31, each contain the P6 coding sequence plus 27 bp of sequence upstream of the ATG start codon and 71 bp downstream of the TGA stop codon.

Construction of transgenic plants.

Ti plasmids were introduced into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell 1986) by freeze-thaw transformation (Hookyas 1988). *Agrobacterium*-mediated transformation was used to transfer each of the three constructs into *Arabidopsis*, *Landsberg erecta*, carrying a glabrous (*gll*) mutation. Pots (100 mm in diameter) containing 10 plants were vacuum infiltrated with *A. tumefaciens* containing the appropriate construct, according to the method of Bechtold et al. (1993). Seeds from the T₁ plants were collected and screened for hygromycin resistance by sowing them in batches of approximately 1,000 onto petri plates (120 mm in diameter) containing MS (Murashige and Skoog) agar plus 20 or 30 µg of hygromycin per ml. For segregation analysis, plates were scored for the percentage of hygromycin-resistant seedlings. Hygromycin-resistant seedlings were selected and transferred to compost. Seeds from lines homozygous for the transgene were usually germinated directly on the surface of moist compost, without selection. Plants were grown in an environmental chamber at 22°C under continuous light.

Nucleic acid and protein analysis.

For Northern and Southern blot analyses, total RNA and DNA were purified from plants with Purescript and Puregene Kits, respectively (Flowgen, Lichfield, UK). The 1,657-bp *KpnI-XbaI* fragment from pUC-Baji31 was labeled with ³²P, using a Random Priming Kit (Pharmacia, Uppsala, Sweden), and used as a probe for gene VI sequences. PCR was carried out in capillaries, with an Idaho 1605 Air Thermo-Cycler (Idaho Technology, Idaho Falls, ID).

Rabbit antiserum to CaMV P6 (S-isolate), was a kind gift

from A. J. Maule (John Innes Centre). The production of the antiserum has been described (Harker et al. 1987). Individual leaves from *Arabidopsis* plants were ground in PBS (100 mM Na phosphate pH 7.2, 100 mM NaCl) containing 2% (wt/vol) sodium dodecyl sulfate (10 µl per mg of fresh weight). The extract was incubated at 65°C for 10 min, allowed to cool, and centrifuged in a micro-centrifuge at 13,000 × g for 10 min (Ismail et al. 1987). The supernatant was stored at -20°C until required. The extracts were diluted in PBS and P6 protein levels were determined by indirect ELISA essentially as described by Ismail et al. (1987).

Inclusion bodies were prepared from plants as described by Covey and Hull (1981) and polypeptides were analyzed by gel electrophoresis and immunoblotting as described by Stratford et al. (1988), with the P6 antiserum described above.

Electron microscopy.

Leaf tissue sections were cut, embedded in LR White resin, stained, and examined in a JEOL electron microscope (JEOL, Tokyo) as described by Ismail et al. (1987).

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