Plants Expressing Tomato Golden Mosaic Virus *AL2* or Beet Curly Top Virus *L2* Transgenes Show Enhanced Susceptibility to Infection by DNA and RNA Viruses

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The *AL2* gene of the geminivirus tomato golden mosaic virus (TGMV) encodes a transcriptional activator protein (TrAP) that is required for efficient expression of the viral coat protein (*CP*) and *BR1* gene promoters. In contrast, *L2*, the positional homolog of *AL2* in the related beet curly top virus (BCTV), is not required for *CP* expression, raising questions about the functional relationship between the AL2 and L2 gene products. In this study, transgenic *Nicotiana benthamiana* and *N. tabacum* var. Samsun plants expressing a truncated *AL2* gene (*AL2$_{1-100}$*, lacking the activation domain) or full-length *L2* were prepared. These transgenic plants showed a novel enhanced susceptibility (ES) phenotype following inoculation with TGMV, BCTV, or tobacco mosaic virus (TMV), an unrelated RNA virus. ES is characterized by a reduction in the mean latent period (from 1 to 9 days) and by a decrease in the inoculum concentration required to infect transgenic plants (ID50 reduced 6- to 60-fold). However, ES does not result in an enhancement of disease symptoms, and viral nucleic acids do not accumulate to substantially greater levels in infected transgenic plants. That both viral transgenes condition ES suggests that their products share the ability to suppress a host stress or defense response that acts against DNA and RNA viruses. The data further indicate that the transcriptional activation activity of AL2 protein is not required for suppression. The nature of the response targeted by the AL2 and L2 gene products is discussed. © 2001 Academic Press

INTRODUCTION

Viruses belonging to the *Geminiviridae* are characterized by a genome consisting of circular, single-stranded DNA (ssDNA) and a unique, twin-icosahedral particle morphology. These viruses multiply in the plant cell nucleus by a rolling circle replication mechanism that employs double-stranded DNA (dsDNA) intermediates as replication and transcription templates (for review see Bisaro, 1996; Gutierrez, 1999; Hanley-Bowdoin *et al.,* 1999). However, despite a similar morphology and mode of replication, family members differ with respect to genome organization and number of genome components, mechanisms of spread within infected hosts, the insect vectors exploited for plant-to-plant transmission, and mechanisms of transcriptional regulation. Of particular interest here, it has been demonstrated that viruses of the genus *Begomovirus* [e.g., *Tomato golden mosaic virus* (TGMV)] conform to the general strategy of DNA virus transcription programs in which early gene products activate the expression of viral genes required later in the replication cycle. In TGMV, transcriptional activator protein (TrAP), the product of the *AL2* gene (also known as *AC2* or *C2*), is required for infectivity because it is necessary for expression of the coat protein (*CP*) and *BR1* movement protein genes (Sunter and Bisaro, 1991,

1992, 1997; Gröning *et al.,* 1994). Consistent with this function, TrAP has been shown to contain an acidic-type transcriptional activation domain (Hartitz *et al.,* 1999). Members of the genus *Mastrevirus* [e.g., *Maize streak virus* (MSV)], however, lack an *AL2* homolog and instead use replication initiator protein (C1 or RepA) to activate *CP* expression (Hofer *et al.,* 1992; Collin *et al.,* 1996). In the case of the third genus [*Curtovirus*; e.g., *Beet curly top virus* (BCTV)], a viral protein required for *CP* expression has yet to be identified. Although the BCTV genome contains a positional homolog of *AL2* called *L2* (or *C2*), *l2* mutants can systemically infect plants and are not impaired in their ability to express *CP* (Stanley *et al.,* 1992; Hormuzdi and Bisaro, 1995). Further, the L2 protein shares only limited amino acid sequence homology with TrAP and lacks an obvious transcriptional activation domain. Thus, the similar position of the *AL2* and *L2* genes on their respective viral genomes does not reflect a similar role in transcriptional regulation.

Despite the apparent absence of a role for L2 in BCTV gene expression, *l2* mutants do have a phenotype in some circumstances. In one study, a large proportion of *Nicotiana benthamiana* plants inoculated with BCTV *l2* mutants appeared to recover from the infection (Hormuzdi and Bisaro, 1995). More specifically, while early disease symptoms were similar in severity to those elicited by wild-type BCTV, later in infection (about 3 weeks after initial symptoms appeared), new shoots produced from axillary buds showed a noticeable reduction in curly

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top symptoms. After further growth, these shoots appeared to lack curly top symptoms altogether, and the recovered tissue contained undetectable or at most very small amounts of viral DNA. Because most *Nicotiana* species show varying degrees of recovery from wild-type BCTV infection (Bennett, 1971), this phenotype was referred to as "enhanced recovery" (Hormuzdi and Bisaro, 1995). However, the enhanced recovery phenotype appears to be dependent on the host as well as the inoculation and/or growth conditions, as it was not observed in sugar beet (*Beta vulgaris*) and is not always observed in *N. benthamiana* plants inoculated with BCTV *l2* mutants (Stanley *et al.,* 1992; unpublished observations).

It is possible that the absence of L2 function attenuates BCTV replication or spread to an extent sufficient to allow *N. benthamiana* plants to more efficiently recover from the infection under some conditions. Alternatively, the enhanced recovery phenotype might reflect a more direct role for L2 in viral pathogenesis. To distinguish between these possibilities, and to further investigate whether any functional similarity exists between the AL2 and L2 gene products, we have examined the response of *N. benthamiana* and *Nicotiana tabacum* plants containing TGMV *AL2* or BCTV *L2* transgenes to inoculation with TGMV, BCTV, and tobacco mosaic virus (TMV), an unrelated RNA virus. The results of these experiments reveal a remarkable function for the products of these viral genes. We report here that expression of *AL2* or *L2* transgenes renders plants more susceptible to infection with both DNA and RNA viruses, suggesting that TrAP and L2 share the ability to suppress a general host stress or defense response. We also present evidence which indicates that the enhanced susceptibility (ES) phenotype does not require direct gene activation by TrAP, indicating that this protein is (at least) bifunctional.

RESULTS

Preparation and analysis of transgenic plants

Transgenic *N. benthamiana* plants containing a truncated TGMV *AL2* gene (*AL2$_{1-100}$*) encoding amino acids 1–100 under control of the cauliflower mosaic virus (CaMV) 35S promoter were prepared by *Agrobacterium*-mediated transformation as previously described (Sunter and Bisaro, 1997). A truncated *AL2* gene was chosen to study the effects of TrAP in the absence of its transcriptional activation domain, which has been mapped to the C-terminal amino acids 115–129, and because of previously observed toxicity of the full-length, wild-type *AL2* gene (Hartitz *et al.,* 1999; unpublished results). The absence of the activation domain also avoids potential synergy due to enhanced transcriptional activation following inoculation of transgenic plants with wild-type TGMV, because TrAP$_{1-100}$ is unable to activate the *CP* promoter (unpublished observations). Several independent lines containing *AL2$_{1-100}$* were established by self-

fertilization of regenerated, transformed plants and three (A471-1, A472-6, and A472-7) were selected for further analysis. Similarly, negative control lines containing a full-length *AL2m* transgene with a mutant start codon (ATG to ACG) were established, and two (A598m-1, A598m-2) were selected for further study. The *AL2m* transgene is expected to express a nontranslatable mRNA. Further, internal ribosome initiation is not likely to produce a functional protein since the only other methionine codon in AL2 specifies residue 116.

Transgenic *N. benthamiana* and *N. tabacum* var. Samsun plants containing the full-length BCTV *L2* gene (encoding amino acids 1–173) driven by the 35S promoter were prepared in a similar fashion. Four independent *N. benthamiana* lines (CTL2NB-1, CTL2NB-2, CTL2NB-6, and CTL2NB-8) and three *N. tabacum* lines (CTL2S-3, CTL2S-4, and CTL2S-6) were chosen for further study.

Plants containing the transgenes were phenotypically indistinguishable from nontransgenic plants. However, the presence of the transgene in each of the lines was confirmed by PCR amplification of transgene sequences using primers complementary to flanking T-DNA and/or by Southern blot hybridization (data not shown). Transgene expression was demonstrated by Northern blot hybridization, which showed that plants from each line produce a transcript of the appropriate size and specificity which is not present in nontransgenic plants. In the case of *N. benthamiana AL2$_{1-100}$* plants (Fig. 1A), steady-state transgene transcript levels varied somewhat between lines. A472-1 plants contained nearly twice as much of the 550 nt *AL2$_{1-100}$* transcript as plants from line A472-6, and about fourfold more than A472-7 plants. Plants from independent, transgenic *N. benthamiana* lines harboring the *L2* gene contained roughly equivalent amounts of the 750 nt transgene transcript (Fig. 1B), as did *N. tabacum* plants from the three lines harboring the same transgene (Fig. 1C). A 630 nt transcript corresponding to *AL2m* was present in both *N. benthamiana* lines harboring this transgene (data not shown).

Transgenic plants expressing TGMV *AL2$_{1-100}$* show enhanced susceptibility to geminivirus infection

N. benthamiana is an excellent host for both TGMV and BCTV, and these viruses exhibit distinct and reproducible symptoms in this species. TGMV produces a bright golden mosaic accompanied by leaf deformation, especially in younger leaves. BCTV produces yellow vein clearing, chlorosis, and upward curling of younger leaves that results in stunting, without mosaic symptoms. Three lines (A472-1, A472-6, and A472-7; Fig. 1A) expressing the *AL2$_{1-100}$* transgene were examined for their response to agroinoculation and mechanical inoculation with TGMV, and agroinoculation with BCTV (BCTV is not transmissible by standard mechanical inoculation methods). Both viruses were able to infect the transgenic
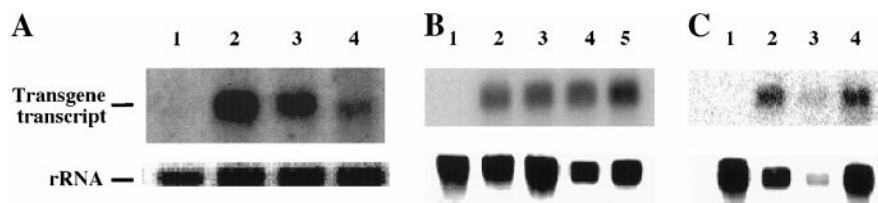
FIG. 1. Steady-state levels of $AL2_{1-100}$ or $L2$ transcripts in transgenic plants. Total RNA was isolated from transgenic and nontransgenic plants and subjected to Northern blot hybridization analysis using a TGMV $AL2$-specific or a BCTV $L2$-specific probe, followed by an 18S ribosomal RNA (rRNA) probe. RNA samples for each transgenic line were obtained from approximately 100 pooled seedlings germinated on MS0 medium containing kanamycin (300 μg/ml) to select for the presence of the transgene. Approximately 2.5 μg of total RNA was loaded in each lane. Relative transgene transcript levels were determined by phosphorimaging and normalized using the internal rRNA control signal. (A) Transgene transcript levels in $N.$ $benthamiana$ $AL2_{1-100}$ plants. Lane 1, RNA from nontransgenic $N.$ $benthamiana$; lanes 2–4, RNA from $N.$ $benthamiana$ lines A472-1, A472-6, and A472-7, respectively (pooled F_3 seedlings). (B) Transgene transcript levels in $N.$ $benthamiana$ $L2$ plants. Lane 1, RNA from nontransgenic $N.$ $benthamiana$; lanes 2–5, RNA from $N.$ $benthamiana$ lines CTL2NB-1, CTL2NB-2, CTL2NB-6, and CTL2NB-8, respectively (pooled F_1 seedlings). (C) Transgene transcript levels in $N.$ $tabacum$ v. Samsun $L2$ plants. Lane 1, RNA from nontransgenic $N.$ $tabacum$; lanes 2–4, RNA from $N.$ $tabacum$ lines CTL2S-3, CTL2S-4, and CTL2S-6, respectively (pooled F_1 seedlings).

plants and produced characteristic disease symptoms. However, transgenic plants proved more susceptible to infection than nontransgenic plants, as judged by a reduction in the time to first appearance of symptoms (mean latent period). As indicated in Table 1, transgenic plants expressing $AL2_{1-100}$ exhibited systemic symptoms typical of TGMV infection 1 to 6 days earlier than nontransgenic plants, depending on the experiment, the line tested, and the method of inoculation. Likewise, symptoms typical of BCTV infection appeared 3 to 4 days earlier on transgenic plants as compared to nontransgenic plants. While some variation is to be expected in experiments of this type, analysis of the data by one-way ANOVA and Dunnett's test confirmed that mean latent period reductions observed in transgenic plant samples were statistically significant in all cases (Table 1). It is important to note that the enhanced susceptibility phenotype is not dependent on the inoculation method employed, because ES was observed regardless of whether TGMV was delivered by agroinoculation or by standard mechanical inoculation of purified viral DNA in the presence of Celite (Table 1). However, transgenic plants which were mechanically inoculated with TGMV DNA displayed disease symptoms somewhat earlier than agroinoculated plants. Why mechanical inoculation resulted in larger reductions in mean latent period is not clear, but this observation effectively rules out the possibility that ES is due to an enhancement of $Agrobacterium$-mediated viral DNA delivery.

These experiments suggest that expression of $AL2_{1-100}$ enhances the susceptibility of $N.$ $benthamiana$ plants to infection with the geminiviruses TGMV and BCTV, as measured by a decrease in the mean latent period. Further, induction of the ES phenotype does not involve

TABLE 1

Mean Latent Period following TGMV and BCTV Inoculation of $N.$ $benthamiana$ Plants Expressing a TGMV $AL2_{1-100}$ Transgene

Virus (inoculation method)	Transgenic generation	$N.$ $benthamiana$ line			
		Nontransgenic	A472-1	A472-6	A472-7
TGMV (agroinoculation)	F2	14.0 ± 1.0^a (48/57)	11.6 ± 0.5^b (56/60)	12.1 ± 0.8^b (50/61)	11.0 ± 0.4^b (56/61)
TGMV (agroinoculation)	F3	12.0 ± 0.5^c (75/80)	10.2 ± 0.3^d (75/75)	10.6 ± 0.4^d (73/74)	10.3 ± 0.4^d (72/79)
TGMV (mechanical)	F2	14.5 ± 1.4^e (12/16)	10.9 ± 1.1^f (12/16)	8.9 ± 0.6^f (13/15)	9.3 ± 0.6^f (15/16)
BCTV (agroinoculation)	F3	19.5 ± 0.7^g (64/98)	15.8 ± 0.7^h (72/87)	15.7 ± 0.6^h (80/104)	17.2 ± 0.6^h (72/99)

$Note.$ Transgenic plants expressing an $AL2_{1-100}$ transgene were agroinoculated using a mixture of $A.$ $tumefaciens$ cells containing Ti plasmids harboring 1.5 copies of the TGMV A or B genome components (OD_{600} = 1.0 each component), or with cells containing a Ti plasmid harboring a tandem dimer of the BCTV genome (OD_{600} = 1.0), as described under Materials and Methods. In one experiment, plants were mechanically inoculated with a mixture of plasmid DNAs containing tandem repeats of TGMV DNA A or DNA B (1 μg each). Plants were scored for the appearance of systemic symptoms typical of TGMV or BCTV infection. The significance of mean latent period differences observed between transgenic plants and nontransgenic plants (mean latent period ± SE days postinoculation) was confirmed by one way ANOVA ($P < 0.05$ for agroinoculations and $P < 0.001$ for DNA inoculation). Lowercase superscripts indicate means that are significantly different from the nontransgenic control mean ($P < 0.05$) as determined by Dunnett's test. Numbers in parentheses indicate infectivity (number of plants infected/number plants inoculated). The data are averages of two and four independent experiments for TGMV agroinoculation of F_2 plants and F_3 plants, respectively, and five independent experiments for agroinoculation of F_3 plants with BCTV. Data for TGMV mechanical inoculation of F_2 plants was obtained from a single experiment. Experiments included 14–32 plants per treatment.

TABLE 2

Mean Latent Period following TGMV and BCTV Inoculation of *N. benthamiana* Plants Expressing a BCTV *L2* Transgene

Virus	*N. benthamiana* line				
	Nontransgenic	CTL2NB-1	CTL2NB-2	CTL2NB-6	CTL2NB-8
TGMV	13.3 ± 1.1^a (24/27)	8.5 ± 0.4^b (28/28)	10.2 ± 0.6^b (24/27)	8.3 ± 0.5^b (11/11)	9.2 ± 0.9^b (12/12)
BCTV	20.8 ± 1.2^c (19/28)	17.3 ± 1.1^d (21/24)	15.5 ± 1.0^d (20/28)	14.4 ± 1.5^d (7/11)	16.0 ± 1.6^d (10/11)

Note. Transgenic plants (F_1 generation) expressing a full-length *L2* transgene were agroinoculated with TGMV or BCTV as described in the legend to Table 1 and under Materials and Methods. Plants were scored for the appearance of systemic symptoms typical of TGMV or BCTV infection. The significance of mean latent period differences observed between transgenic plants and nontransgenic plants (mean latent period $\pm$ SE days postinoculation) was confirmed by one-way ANOVA ($P < 0.05$). Lowercase superscripts indicate means that are significantly different from the nontransgenic control mean ($P < 0.05$) as determined by Dunnett's test. Numbers in parentheses indicate infectivity (number of plants infected/number plants inoculated). The data are from a single experiment or represent the average of two independent experiments, with 11–14 plants per treatment.

direct transcriptional activation, because the $AL2_{1-100}$ transgene encodes a truncated TrAP that lacks an activation domain.

Transgenic plants expressing BCTV *L2* also show enhanced susceptibility

To examine the effect of BCTV *L2* transgene expression on susceptibility, four independent *N. benthamiana* lines containing the full-length *L2* gene were challenged by agroinoculation with TGMV and BCTV. As indicated by the data presented in Table 2, the four transgenic lines tested (CTL2NB-1, CTL2NB-2, CTL2NB-6, and CTL2NB-8; Fig. 1B) also displayed a reduced latent period following inoculation with both viruses. Symptoms characteristic of TGMV infection appeared 3 to 5 days earlier, and BCTV symptoms 4 to 7 days earlier, in transgenic plant samples than in nontransgenic samples. Statistical analysis confirmed that the presence of the *L2* transgene significantly reduced the latent period in all experiments (Table 2).

It was concluded from these experiments that, similar to $AL2_{1-100}$, the full-length BCTV *L2* gene also conditions ES to geminiviruses when expressed in transgenic *N. benthamiana* plants. Here we can exclude the possibility that ES is due to transcriptional synergy because there is no evidence that L2 has a role in activating BCTV gene expression (Stanley *et al.,* 1992; Hormuzdi and Bisaro, 1995), and because L2 cannot activate the TGMV *CP* promoter (Sunter *et al.,* 1994; Sunter and Bisaro, 1997). Thus, these studies with transgenic $AL2_{1-100}$ and *L2* plants establish a common function for the *AL2* and *L2* gene products that does not involve direct transcriptional activation.

Enhanced susceptibility is not limited to geminiviruses

The experiments presented above indicate that ES conditioned by the $AL2_{1-100}$ or *L2* transgenes is not virus specific, as it was observed following inoculation of

transgenic plants with either TGMV or BCTV. To further examine the question of specificity, we asked if ES also occurs in response to inoculation with tobacco mosaic virus (TMV), a tobamovirus with a message sense RNA genome. *N. benthamiana* proved unsuitable for these experiments because this species undergoes severe wilting and dies within a few days of inoculation with TMV. Therefore, transgenic *N. tabacum* var. Samsun lines expressing the BCTV *L2* transgene were established and employed in this study (Fig. 1C). Purified TMV virions were mechanically inoculated to nontransgenic and transgenic *N. tabacum* plants using Celite abrasive. As shown in Table 3, the transgenic lines CTL2S-3, CTL2S-4, and CTL2S-6 clearly showed a reduction in mean latent period following inoculation with TMV. In all cases, a dark green–light green mosaic typical of TMV infection was observed, but disease symptoms ap-

TABLE 3

Mean Latent Period Following TMV Inoculation of *N. tabacum* Plants Expressing a BCTV *L2* Transgene

Virus	*N. tabacum* var. Samsun line			
	Nontransgenic	CTL2S-3	CTL2S-4	CTL2S-6
TMV	25.4 ± 1.5^a (12/32)	20.1 ± 1.4^b (21/32)	18.6 ± 1.3^b (24/32)	16.3 ± 0.9^b (9/11)

Note. Transgenic plants (F_1 generation) expressing a full-length *L2* transgene were mechanically inoculated with TMV as described under Materials and Methods. Plants were scored for the appearance of systemic symptoms typical of TMV infection. The significance of mean latent period differences observed between transgenic plants and nontransgenic plants (mean latent period $\pm$ SE days postinoculation) was confirmed by one-way ANOVA ($P < 0.05$). Lowercase superscripts indicate means that are significantly different from the nontransgenic control mean ($P < 0.05$) as determined by Dunnett's test. Numbers in parentheses indicate infectivity (number of plants infected/number plants inoculated). The data are from a single experiment, with 11–32 plants per treatment.
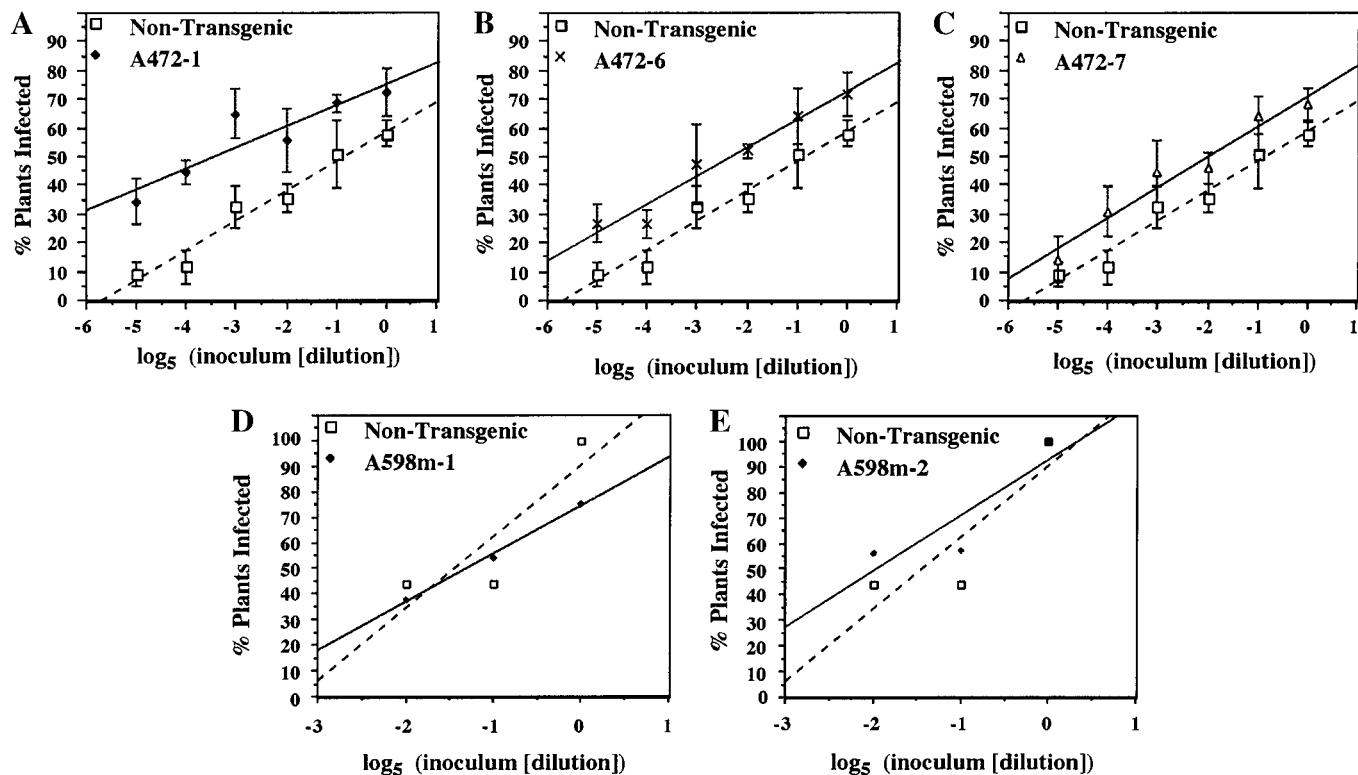
FIG. 2. BCTV ID50, on nontransgenic plants, and transgenic $AL2_{1-100}$ or $AL2m$ plants. Nontransgenic *N. benthamiana* plants and plants from transgenic lines expressing $AL2_{1-100}$ or $AL2m$ were agroinoculated with BCTV, using either a standard dose ($OD_{600} = 1.0$) or serial fivefold dilutions of the standard dose. The fraction of infected plants in each sample was plotted vs the $\log_5$ of the dilution. The BCTV ID50 is calculated as the inoculum dose at which 50% of plants in the sample became infected. Broken lines indicate dilution curves for nontransgenic plants. The data shown in A–C represent the average of three independent experiments with nontransgenic plants and $AL2_{1-100}$ plants, with 16 plants for each inoculum dose in each experiment. The data shown in D–E are from a single experiment with nontransgenic and $AL2m$ plants, with 16 plants used at each inoculum dose. (A) Line A472-1; (B) line A472-6; (C) line A472-7; (D) line A598m-1; (E) line A598m-2.

peared 5 to 9 days earlier in transgenic plant samples as compared to nontransgenic plant samples.

These results indicate that ES is a general phenomenon that extends at least to the ssDNA-containing geminiviruses and ssRNA-containing tobamoviruses and is not limited to a single plant species. In addition, the ES phenotype is observed regardless of whether plants expressing $AL2_{1-100}$ or $L2$ transgenes are infected by agroinoculation, mechanical inoculation of viral nucleic acid, or mechanical inoculation of virions.

ES is also characterized by infection at reduced inoculum doses

To further characterize the ES phenotype, the infectious dose required to infect half the plants (ID50) in samples of transgenic $AL2_{1-100}$ plants and nontransgenic plants was determined. To perform these quantal assays, agroinoculation was used to deliver BCTV to groups of plants beginning with a standard dose of approximately 3×10^7 *Agrobacterium tumefaciens* cells per plant (30 μl of culture at $OD_{600} = 1.0$). Additional groups of plants were inoculated with serial fivefold dilutions of the standard dose. The monopartite BCTV

was selected over the bipartite TGMV for this study because infectivity of the former follows simple one-hit kinetics.

As illustrated in Fig. 2A, the BCTV ID50 in plants from line A472-1 expressing $AL2_{1-100}$ was considerably less than in nontransgenic plants. In this line, the ID50 corresponded to nearly a 250-fold dilution of the standard inoculum, whereas the ID50 was reached at only fourfold dilution in nontransgenic plants. Thus about a 60-fold lower inoculum dose was sufficient to infect 50% of the transgenic plants as compared to nontransgenic plants. Similarly, the BCTV ID50 in lines A472-6 (Fig. 2B) and A472-7 (Fig. 2C) was reached at 40-fold and 25-fold dilution of the inoculum, respectively. These values are 10-fold (A472–6) and 6-fold (A472–7) less than the ID50 in nontransgenic plants.

In contrast, the observed BCTV ID50 was similar when nontransgenic plants and plants expressing the $AL2m$ transgene were compared. In these experiments, the ID50 was reached at approximately ninefold dilution of the inoculum in the nontransgenic sample, whereas this value was slightly less in line A598m-1 (8-fold; Fig. 2D) and slightly greater in line A598m-2 (18-fold; Fig. 2E). No
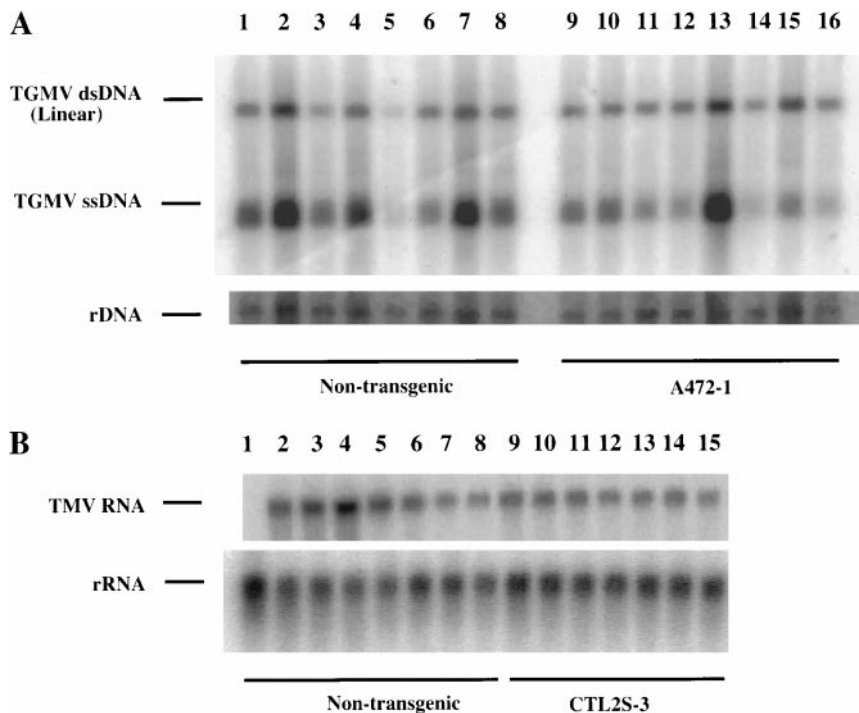
FIG. 3. Gel blot hybridization analysis of viral nucleic acid accumulation in infected, transgenic plants. (A) TGMV accumulation in transgenic *N. benthamiana* plants expressing the $AL2_{1-100}$ transgene (line A472-1). DNA was extracted 14 days postinoculation from systemically infected leaves of individual plants infected with TGMV. DNA samples were digested with *Sca*I to produce linear TGMV DNA fragments (~2.5 kb) from the covalently closed and open circular dsDNA forms of the viral genome. Following electrophoresis and transfer to a nitrocellulose membrane, the blot was hybridized with a probe specific for TGMV DNA A. *Sca*I also generates a convenient 18S rDNA fragment (~9 kb) from plant genomic DNA, which was detected by subsequent hybridization of the blot membrane with an 18S rDNA-specific probe. Approximately 0.5 μg of DNA was loaded in each lane, and all lanes contain DNA from TGMV-infected plants. A hybridization signal was not detected in mock-inoculated plants (not shown). (B) TMV accumulation in transgenic *N. tabacum* plants expressing the *L2* transgene (line CTL2S-3). RNA was extracted 27 days postinoculation from systemically infected leaves of individual plants infected with TMV. Following electrophoresis and transfer to a nitrocellulose membrane, the RNA was hybridized sequentially with probes specific for TMV RNA (~6.4 kb) and 18S rRNA (~1.9 kb). Lane 1 contains RNA from a mock-inoculated plant. The remaining lanes contain RNA from plants infected with TMV. Approximately 2.5 μg of RNA was loaded in each lane.

significant difference in mean latent period was observed when nontransgenic and transgenic lines were compared at the standard inoculum dose (data not shown).

It was concluded from these experiments that ES can be described in at least two ways: by a reduction in mean latent period (i.e., viruses display increased virulence), and by a reduction in the amount of virus required to initiate an infection (i.e., viruses exhibit increased infectivity). Further, because plants containing the *AL2m* transgene, which produces a nontranslatable mRNA, respond in a manner similar to nontransgenic plants following challenge with BCTV, we can also conclude that protein expression is required for the ES phenotype.

ES is not accompanied by large increases in virus accumulation

Although transgenic $AL2_{1-100}$ and *L2* plants display a reduced latent period following virus challenge, the infected plants show no evidence of increased disease severity. In all transgenic lines tested, symptoms typical

of TGMV, BCTV, or TMV infection were observed. Because symptom appearance is at best a subjective measure of virulence, we investigated whether gross changes in virus accumulation accompany the ES phenotype.

TGMV and BCTV accumulation in infected, nontransgenic plants and transgenic plants expressing the $AL2_{1-100}$ transgene was assessed by Southern blot hybridization. DNA extracts were obtained from comparable systemically infected leaves of individual, randomly selected plants approximately 2 days after the mean latent period for each virus (leaves harvested at 14 and 21 days postinoculation for TGMV and BCTV, respectively). Closed and open circular viral dsDNA forms were linearized by restriction endonuclease digestion prior to gel electrophoresis to facilitate quantitation. Quantitation was performed by phosphorimaging, and the data were normalized using an internal rDNA control signal. The Southern blot of extracts obtained from TGMV infected nontransgenic plants and line A472-1 plants is shown in Fig. 3A to illustrate the method. The normalized data for both TGMV and BCTV infected plants (all plants exam-

TABLE 4

Accumulation of Viral Nucleic Acids in Nontransgenic Plants and Plants Expressing TGMV $AL2_{1-100}$ or BCTV $L2$ Transgenes

| Line | Plant no. | Phosphorimager units | | Line | Plant no. | Phosphorimager units |
		TGMV	BCTV			TMV
Nontransgenic	1	4482	249	Nontransgenic	1	1464
	2	5839	319		2	2265
	3	3022	376		3	2628
	4	4675	673		4	1992
	5	1562	286		5	1263
	6	2792	139		6	837
	7	5765	—		7	987
	8	3804	—		Average	1634
	Average	3992	340		Range	837–2628
	Range	1562–5839	139–673			
A472-1	1	4006	209	CTL2S-3	1	940
	2	5754	76		2	921
	3	2223	123		3	1035
	4	2330	57		4	845
	5	5341	83		5	931
	6	2517	104		6	836
	7	3455	—		7	738
	8	2814	—		Average	892
	Average	3555	109		Range	837–2628
	Range	2223–5754	57–209			
A472-7	1	2982	131	CTL2S-4	1	936
	2	1868	254		2	843
	3	2692	121		3	380
	4	1188	158		4	371
	5	2371	144		5	670
	6	—	259		6	461
	7	—	156		7	1060
	Average	2220	174		Average	675
	Range	1188–2982	121–259		Range	371–1060
				CTL2S-6	1	1218
					2	1307
					3	2545
					4	832
					5	915
					6	730
					7	634
					Average	1169
					Range	634–2545

Note. Nontransgenic *N. benthamiana* plants or plants expressing the $AL2_{1-100}$ transgene (lines A472-1 and A472-7) were agroinoculated with the TGMV A and B components, or with BCTV. Nontransgenic *N. tabacum* var. Samsun plants or plants expressing the *L2* transgene (lines CTL2S-3, CTL2S-4, and CTL2S-6) were mechanically inoculated with TMV virions. Viral nucleic acid levels in individual plants were measured as described in the legend to Fig. 3, and normalized using internal rDNA or rRNA signals, as appropriate. Viral nucleic acid signal intensity is expressed in phosphorimager units.

ined, combined ssDNA and dsDNA signals) are presented in Table 4.

The accumulation of TGMV and BCTV genomic ssDNA and dsDNA replicative forms was somewhat variable, as expected, and TGMV accumulated to substantially greater levels than did BCTV (Table 4). However, total viral DNA accumulation in transgenic plants was similar to that observed in nontransgenic plants infected with the same virus, and well within the limits of normal variation.

Northern blot hybridization was used to evaluate TMV accumulation (27 days postinoculation) in infected nontransgenic and transgenic plants expressing the *L2* transgene, and extracts from nontransgenic and line CTL2–3 plants are shown in Fig. 3B. In these experiments, phosphorimager data were normalized using an internal rRNA control signal and the complete data set is also presented in Table 4. As was the case with TGMV and BCTV, genomic TMV RNA levels in infected, transgenic plants were similar to those observed in nontransgenic plants.

While some variability in viral nucleic acid levels is to be expected in a small and random sample of infected plants, these data indicate that ES does not result in increased virus loads. This is consistent with the observation that transgenic $AL2_{1-100}$ plants, transgenic $L2$ plants, and nontransgenic plants show similar disease symptoms following inoculation with TGMV, BCTV, or TMV.

To further examine the accumulation of TGMV and BCTV in nontransgenic and transgenic tissue, virus replication was assessed over a 6-day period in leaf discs. Discs obtained from plants expressing the $AL2_{1-100}$ transgene (lines A472-1, A472-6, or A472-7) were agroinoculated with the TGMV DNA A component or with BCTV DNA. Extracts were prepared from leaf discs at various times postinoculation and analyzed by Southern blot hybridization. Viral DNA accumulation (normalized against an internal rDNA standard) was quantitated as described above. As illustrated in Fig. 4, TGMV and BCTV DNAs accumulated with about the same kinetics and to similar levels (variation approximately twofold or less at day 5) regardless of whether nontransgenic or transgenic plants were the source of the leaf discs. It was concluded from these experiments that the ES phenotype conditioned by the $AL2_{1-100}$ transgene does not result in a significant enhancement in the rate or level of virus replication.

DISCUSSION

Comparison of $AL2$ from begomoviruses (e.g., TGMV) with $L2$ from curtoviruses (e.g., BCTV) has presented something of a puzzle. These genes lie in essentially identical positions on their respective genomes, overlapped (in different reading frames) at the 5'-end by the highly conserved $AL1/L1$ genes and at the 3'-end by the highly conserved $AL3/L3$ genes. The AL1/L1 and AL3/L3 proteins of these viruses have similar if not identical roles in viral DNA replication (Bisaro, 1996; Hanley-Bowdoin et al., 1999). In contrast, the $AL2$ and $L2$ genes and their products, TrAP (AL2 protein) and L2 protein, have only limited sequence homology. Further, TrAP is a transcriptional activator (Sunter and Bisaro, 1991, 1992; Hartitz et al., 1999), whereas L2 does not appear to have this function (Stanley et al., 1992; Hormuzdi and Bisaro, 1995). Under some conditions, BCTV $l2$ mutants show a phenotype suggestive of a direct role for L2 in viral pathogenesis (Hormuzdi and Bisaro, 1995). While pursuing this observation, we have apparently discovered an answer to the $AL2/L2$ enigma: when expressed in transgenic plants, the products of both the $AL2$ and the $L2$ genes render plants more susceptible to infection, suggesting that they share a common function which causes plants to serve as more efficient virus hosts. The simplest interpretation of our results is that TrAP and L2 in some way suppress a host stress or defense response. We
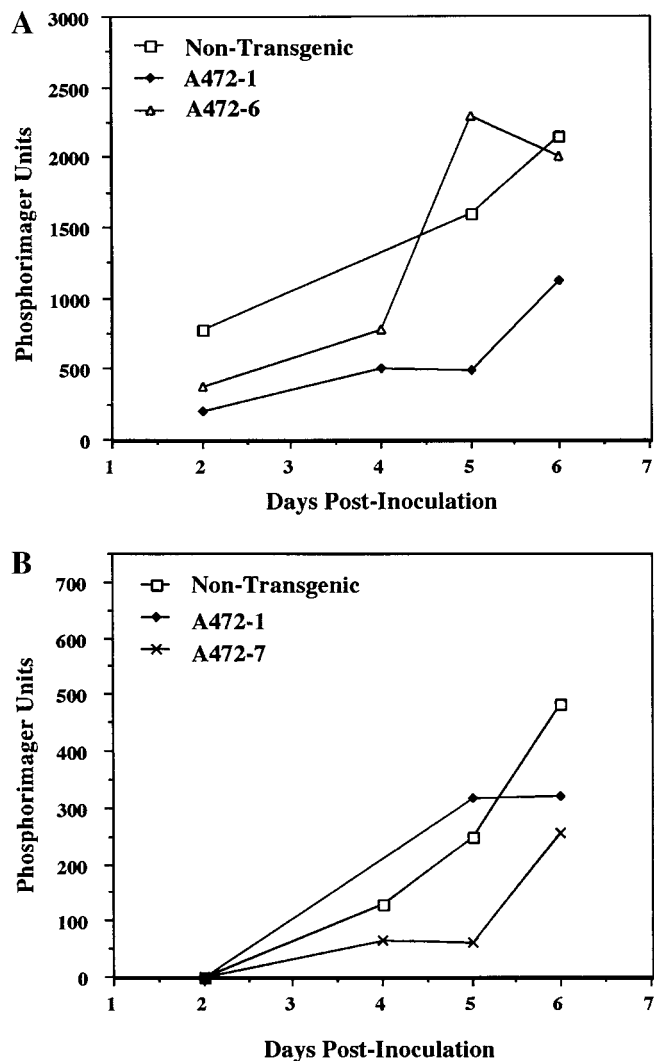

FIG. 4. Replication of TGMV DNA A and BCTV DNA in leaf discs. Amplification of viral DNAs in leaf discs obtained from nontransgenic plants, or plants expressing the $AL2_{1-100}$ transgene, was examined. Discs were agroinoculated with TGMV DNA A or BCTV DNA (OD_{600} = 1) as described under Materials and Methods. Replication was assessed by Southern blot hybridization and quantitated by phosphorimaging. TGMV DNA signals (A) and BCTV DNA signals (B) were normalized using an internal rDNA control. The relative amount of viral DNA (combined ssDNA and dsDNA signals), expressed in phosphorimager units, is plotted vs time (in days) postinoculation. The data were obtained from single experiments using four pooled discs for each time point.

speculate that the common ancestor of begomoviruses and curtoviruses probably had an $AL2/L2$ gene with this function. Further, because enhanced susceptibility can be conditioned by a $AL2_{1-100}$ transgene lacking the C-terminal activation domain, suppression does not require direct transcriptional activation by TrAP. Therefore, transcriptional activation seems to be an independent function that may have been acquired at a later time in the begomovirus lineage. Alternatively, the ancestral gene may also have had a transcriptional activation activity which was later lost in the curtovirus lineage.

The data presented in this report indicate that transgenic plants expressing a full-length $L2$ transgene, or a truncated $AL2_{1-100}$ transgene, show enhanced susceptibility following inoculation with TGMV, BCTV, and the unrelated RNA virus, TMV. The ES phenotype, which was observed in both transgenic *N. benthamiana* and *N. tabacum* var. Samsun plants, is characterized by reduced latent periods following virus inoculation and by a reduction in viral ID50. The extent of reductions in mean latent period conditioned by the two transgenes showed some variation depending on the experiment and the line examined, and also with the virus and method of inoculation (Tables 1, 2, and 3). This is not surprising considering the inherent variability of infectivity experiments, variability in transgene expression levels (Fig. 1), possible differences in transgene expression patterns (not examined), and differences in the natural latent periods of these viruses on nontransgenic plants. Nevertheless, there is an underlying consistency. Expression of the $AL2_{1-100}$ transgene in *N. benthamiana* lines A472-1, A472-6, and A472-7 resulted in a 14–21% reduction in the TGMV latent period and a 12–19% reduction in the latent period of BCTV when agroinoculation was used to deliver these viruses (Table 1). Using the same method of inoculation, mean latent periods were reduced 23–38% for TGMV and 17–31% for BCTV in four *N. benthamiana* lines expressing the $L2$ transgene (Table 2). Thus, even though the natural latent periods of these viruses differ by several days, latent period reductions were proportionally similar, albeit somewhat greater in transgenic *N. benthamiana* plants expressing the $L2$ transgene. In three *N. tabacum* lines expressing the $L2$ transgene, a similar reduction (21–36%) in TMV latent period was observed after mechanical inoculation of purified virus (Table 3).

For reasons which are not clear, mechanical inoculation of purified TGMV DNA resulted in a somewhat larger reduction in the mean latent period than when agroinoculation was used to deliver this virus to transgenic $AL2_{1-100}$ plants (21–36% vs 14–21%; Table 1). However, while the extent of ES may be affected by the inoculation method, it is clear that the phenotype is observed regardless of the method of virus delivery (agroinoculation, mechanical inoculation of viral DNA, or mechanical inoculation of virions).

The overall conclusion from these experiments must be that expression of the $AL2_{1-100}$ and $L2$ transgenes results in enhanced susceptibility, which can be measured by the more rapid appearance of disease symptoms. The ES phenotype is not host species-specific (at least within the genus *Nicotiana*) and extends to both DNA- and RNA-containing viruses (TGMV, BCTV, and TMV).

While a shorter latent period can be considered an indication of increased virulence, ES was not associated with an obvious enhancement of disease symptoms.

Plants expressing $AL2_{1-100}$ or $L2$ showed the same typical symptoms of virus infection as nontransgenic plants. Consistent with this observation, TGMV, BCTV, and TMV did not accumulate to substantially greater levels in transgenic plants than in nontransgenic plants (Fig. 3 and Table 4). Although we recognize that many factors contribute to the property of virulence, a possible explanation for the absence of symptom enhancement may be that latent period reductions were not large enough to result in a significant difference in pathology. For example, in nontransgenic *N. benthamiana*, the latent period of a severe BCTV strain (CFH) is about 10 days shorter than the more moderate BCTV–Logan strain (Stenger *et al.,* 1994). As a consequence, CFH-infected plants are noticeably more stunted than plants infected with BCTV–Logan. The reductions in mean latent period we observed in $AL2_{1-100}$ or $L2$ transgenic plant samples following inoculation with BCTV–Logan, while significant, ranged from only 3 to 7 days (Tables 1 and 2).

While the $AL2_{1-100}$ and $L2$ transgenes condition what can be described as a moderate increase in virulence, effects on viral infectivity are considerably more striking. Reductions in BCTV ID50 measured in $AL2_{1-100}$ plants were about sixfold in line A472-7, 10-fold in line A472-6, and 60-fold in line A472-1 (Fig. 2). Thus, considerably less virus is needed to infect the transgenic plants than is required to infect nontransgenic plants. In addition, the extent of ID50 reduction correlated with transgene expression levels (Fig. 1), suggesting a direct relationship between the amount of transgene product and the level of ES. Because plants expressing the $AL2m$ transgene, which produces a nontranslatable mRNA, do not display ES (Fig. 2), we can conclude that protein expression is required for the phenotype. That truncated TrAP can be detected in the nuclei of transgenic $AL2_{1-100}$ plants is consistent with this conclusion (data not shown). Native TrAP is known to accumulate in the nucleus following transient expression in tobacco protoplasts or when expressed from a baculovirus vector in insect cells (Sanderfoot and Lazarowitz, 1995), so the truncated protein appears to be correctly localized.

Plants have developed a number of mechanisms to limit or resist pathogens, and it is becoming clear that in some instances plant viruses have developed countermeasures to thwart these mechanisms. The data presented in this report suggest that the begomoviruses and curtoviruses use the products of the $AL2$ and $L2$ genes to suppress a general stress or defense response. In this regard, it is interesting to note that posttranscriptional gene silencing (PTGS) has been proposed as a potentially ubiquitous defense employed by plants against virus infection (Ratcliff *et al.,* 1997; Al-Kaff *et al.,* 1998), and several viruses have been demonstrated to encode proteins that counter this defense by behaving as suppressors of PTGS (Brigneti *et al.,* 1998; Kasschau and Carrington, 1998). Further, recent evidence suggests that

AC2 protein (TrAP) from African cassava mosaic gemini-virus can act as a silencing suppressor (Voinnet *et al.,* 1999). It is reasonable to suppose that the highly homol-ogous TGMV TrAP is likewise an antisilencing protein, and so we speculate that ES conditioned by the $AL2_{1-100}$ transgene may be a reflection of antisilencing activity and that BCTV L2 will ultimately be shown to have anti-silencing activity as well.

On the other hand, there are two observations which appear to argue against this hypothesis. First, ES does not result in an enhancement of disease symptoms or significantly higher levels of virus accumulation (Fig. 3 and Table 4), as might be expected if silencing were impaired in transgenic $AL2_{1-100}$ or *L2* plants. Virus repli-cation also is not enhanced in leaf discs derived from transgenic plants (Fig. 4). In comparison, expression of the tobacco etch potyvirus (TEV) silencing suppressor P1/HC-Pro in transgenic tobacco results in a dramatic increase in TMV symptoms as compared to control non-transgenic plants, and a correspondingly large increase in the accumulation of TMV RNA (more than 20-fold) (Pruss *et al.,* 1997). Second, TGMV *al2* mutants and BCTV *l2* mutants do not show significant replication defects in transfected protoplasts. BCTV *l2* mutants accumulate only about 20% less DNA than wild-type virus in cultured cells (Hormuzdi and Bisaro, 1995). TGMV *al2* mutants accumulate wild-type levels of dsDNA replicative forms, and while ssDNA levels are reduced, this can be attrib-uted to the absence of coat protein (Sunter *et al.,* 1990). In contrast, certain TEV HC-Pro mutants show dramatic replication defects. In particular, amplification levels of class II mutants, which are likely defective in silencing activity, are only 12 to 18% of nonmutant TEV in tobacco protoplasts (Kasschau *et al.,* 1997). Thus, the relationship between PTGS and the ES phenotype is unclear, and work is in progress to resolve this issue. We are also examining whether other defense responses, such as those mediated by salicylic acid, are compromised in $AL2_{1-100}$ or *L2* plants.

MATERIALS AND METHODS

General techniques

The map locations and restriction endonuclease sites cited here refer to the sequences of TGMV (Hamilton *et al.,* 1984) and BCTV-Logan (S. G. Hormuzdi and D. M. Bisaro, GenBank accession number AF379637). All re-striction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers. DNA and RNA manipulations, polymerase chain reaction (PCR), DNA gel blot hybridization, and RNA gel blot hybridization were performed essentially as described by Ausubel *et al.* (1987) unless otherwise stated. DNA sequence alterations were confirmed by sequence anal-ysis performed by the sequencing facility of the Ohio State Biotechnology Center.

Transgene construction

Binary Ti plasmids containing the TGMV *AL2* and BCTV *L2* genes expressed from the cauliflower mosaic virus 35S promoter were generated. A 314-bp *Xba*I/*Bsu*36I fragment of TGMV DNA A encoding a truncated TrAP protein ($AL2_{1-100}$) was obtained from pTGA79, which contains the full-length *AL2* gene (Sunter and Bisaro, 1991). The *Xba*I/*Bsu*36I fragment was subsequently in-serted between the 35S promoter and nopaline synthase polyadenylation signal of the Ti plasmid vector pMON530 (Rogers *et al.,* 1987) to create pTGA472. This construct is capable of expressing the first 100 amino acids of TrAP ($TrAP_{1-100}$) fused at the C-terminus to 11 amino acids from the pMON530 vector.

An ~400-bp DNA fragment containing the full-length TGMV *AL2* coding region was obtained by PCR using pTGA26 (Sunter *et al.,* 1990) as template with the primers 5′-GGACTTTCCATAACGCGAAATTCGTC and 5′-GCG-GAGCTCCTATTTAAATAAGTTCTC. The underlined nucle-otides create a mutation of the *AL2* start codon (ATG to ACG). Plasmid pTGA26 contains 1.5 copies of TGMV DNA A. The PCR fragment was inserted between the 35S promoter and nopaline synthase polyadenylation signal of pMON530 to create pTGA598m. This construct con-tains a mutant *AL2* gene (*AL2m*) which is expected to produce a nontranslatable mRNA.

A 522-bp DNA fragment containing the full-length BCTV *L2* coding region was obtained by PCR using pCT8 (Hormuzdi and Bisaro, 1993) as template with the prim-ers 5′-GCGCCATGGAAAACCACGTG and 5′-GCGGATC-CTTATCCAAGTATATCTC. Plasmid pCT8 contains 1.5 cop-ies of the BCTV (strain Logan) genome. The PCR frag-ment was inserted into the *Sma*I site of pMON530, between the 35S promoter and nopaline synthase poly-adenylation signal in the vector, to create pCTL2. This construct is capable of expressing the full-length L2 protein (amino acids 1–173).

Construction of transgenic plants

Ti plasmid constructs pTGA472, pTGA598m, and pCTL2 were transferred into *A. tumefaciens* strain GV3111SE containing the disarmed Ti plasmid pTiB36SE by triparental mating, and cultures were used to trans-form *Nicotiana benthamiana* or *N. tabacum* var. Samsun leaf discs (Horsch and Klee, 1986). Transformed plants were selected on the basis of kanamycin resistance (300 μg/ml), and the presence of the transgene was con-firmed by PCR amplification of integrated sequences and/or Southern blot hybridization (Sunter and Bisaro, 1997). Seeds derived from transgenic plants were sur-face sterilized and germinated on MSO medium (MS salts, B5 vitamins, 30 g/l sucrose) containing kanamycin (300 μg/ml) for up to 35 days and used to isolate RNA or were transferred to soil for infectivity analysis.

Infectivity experiments

Plasmids containing tandemly repeated copies of the TGMV A and B genome components in both pUC118 (pTGA26 and pTGB40) (Sunter et al., 1990; Brough et al., 1992) and Ti plasmid vectors (pMON337 and pMON393) (Elmer et al., 1988a) have been previously described. A plasmid harboring tandemly repeated copies of BCTV-Logan in a Ti plasmid vector (pLogan) has likewise been described (Stenger et al., 1991).

Healthy, nontransformed N. benthamiana plants, or transgenic plants expressing TGMV $AL2_{1-100}$ or the full-length BCTV L2 gene, were agroinoculated with A. tumefaciens harboring Ti plasmids containing tandemly repeated copies of the TGMV or BCTV genomes (Grimsley et al., 1987; Elmer et al., 1988a; Briddon et al., 1989). In all cases, transgenic seeds were germinated on MSO medium containing kanamycin (300 μg/ml) to ensure the presence of the transgene before seedlings were transferred to soil. Plants were inoculated 30 to 40 days postgermination by injecting 10 μl of A. tumefaciens culture, at a known OD_{600} (1 OD_{600} unit $\sim 10^9$ cells/ml), into the base of each of three leaves using a Hamilton syringe. In the case of TGMV, approximately equal numbers of cells containing DNA A or DNA B were used. In some experiments, TGMV DNAs A and B were mechanically inoculated as a mixture of plasmids pTGA26 and pTGB40 in 50 mM sodium phosphate buffer, pH 7.0, containing Celite abrasive.

Healthy, nontransformed N. tabacum var. Samsun plants, or transgenic plants harboring the BCTV L2 transgene, were mechanically inoculated with TMV virions in 50 mM sodium phosphate buffer, pH 7.0 in the presence of Celite. TMV (strain U1) was kindly provided by Dr. D. C. Stenger.

Inoculated plants were scored for the appearance of disease symptoms typical of TGMV, BCTV, or TMV on systemic leaves. Latent periods (mean $\pm$ SE days postinoculation until onset of visible symptoms) were recorded and statistical analysis performed by ANOVA and Dunnett's test as described by Zar (1974). Experiments to determine the dose at which 50% of plants become infected (ID50) were performed by agroinoculating BCTV to groups of nontransgenic and transgenic ($AL2_{1-100}$ or $AL2m$) N. benthamiana plants with serial fivefold dilutions of the standard inoculum dose ($OD_{600} = 1.0$).

Leaf disc assay for virus replication

The leaf disc assay for the transient analysis of geminivirus replication in N. benthamiana has been described (Elmer et al., 1988b). Briefly, discs were obtained from nontransgenic plants or plants expressing the $AL2_{1-100}$ transgene and agroinoculated with TGMV DNA A (pMON337) or BCTV (pLogan) using a standard inoculum dose ($OD_{600} = 1.0$). Two days after agroinoculation, discs were plated on selective medium containing kanamycin (300 μg/ml) and carbenicillin (500 μg/ml). DNA samples were prepared 2–6 days postinoculation and analyzed by Southern blot hybridization as described below and in the text.

Blot hybridization experiments

To determine transgene expression levels, total RNA was purified from approximately 100 transgenic seedlings germinated on MSO medium containing kanamycin (300 μg/ml). Nontransgenic seedlings were germinated in the same medium lacking kanamycin. Seedlings were frozen in liquid nitrogen and ground in RLT buffer containing guanidine isothiocyanate (Qiagen, Valencia, CA), extracted with phenol and chloroform/isoamyl alcohol, and nucleic acids precipitated with ethanol. The precipitate was dissolved in water and RNA precipitated by adding LiCl to a final concentration of 2 M. The RNA pellet was dissolved in water, treated with DNase I, extracted with phenol and phenol/chloroform, and reprecipitated with ethanol. RNA gel blot analysis was carried out following fractionation of total RNA samples in formaldehyde-agarose gels. ^{32}P-labeled probes were prepared by random primer labeling (Ambion, Austin, TX) of PCR fragments generated using primer sets specific for the full-length TGMV AL2 (pTGA26 template), BCTV L2 (pCT8 template), or 18S ribosomal RNA (N. benthamiana genomic DNA template) genes.

To analyze the accumulation of TMV RNA in infected plants, total RNA was isolated from systemically infected leaves 27 days postinoculation using the RNeasy Plant Mini Kit (Qiagen) and subjected to RNA gel blot analysis as described above. Leaves 6 through 8 above the inoculated leaf were used in this study. A TMV-specific, ^{32}P-labeled probe was prepared from purified TMV RNA by the random primer method using AMV reverse transcriptase (Promega, Madison, WI).

To analyze TGMV accumulation in infected plants, total DNA was isolated from systemically infected leaves 14 days postinoculation. Leaves 4 and 5 above the inoculation site were chosen for this analysis. Total DNA was isolated from BCTV-infected plants 21 days postinoculation from leaves 7 and 8 above the inoculation site. DNA samples were subjected to DNA gel blot hybridization. ^{32}P-labeled TGMV DNA A or BCTV DNA probes were prepared by random primer labeling of pTGA26 and pCT8, respectively. Gel blot signals were quantitated by phosphorimaging (Bio-Rad Molecular Imager FX, Hercules, CA) and normalized using internal 18S rRNA or 18S rDNA control signals, as appropriate.

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