Phenotypic Variation in Transgenic Tobacco Expressing Mutated Geminivirus Movement/Pathogenicity (BC1) Proteins

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Tobacco plants were transformed with the movement protein (pathogenicity) gene (*BC1*) from tomato mottle geminivirus (TMoV), using *Agrobacterium*-mediated transformation. Different transgenic tobacco lines that expressed high levels of the *BC1* protein had phenotypes ranging from plants with severe stunting and leaf mottling (resembling geminivirus symptoms) to plants with no visible symptoms. The sequence data for the *BC1* transgene from the transgenic plants with the different phenotypes indicated an association of spontaneously mutated forms of the *BC1* gene in the transformed tobacco with phenotype variations. One mutated transgene associated with an asymptomatic phenotype had a major deletion at the C terminus of 119 amino acid residues with a recombination resulting in the addition of 26 amino acid residues of unidentified origin. This asymptomatic, mutated *BC1* attenuated the phenotypic expression of the symptomatic *BC1* in a tobacco line containing both copies of the *BC1* gene. Another mutated form of the *BC1* gene amplified from an asymptomatic, multicopy transgenic tobacco plant did not induce symptoms when transiently expressed in tobacco via a virus vector. The symptom attenuation in the transgenic tobacco by the asymptomatic *BC1* may involve trans-dominant negative interference.

Transgenic plants expressing viral movement protein (MP) have provided valuable information regarding MP biochemical functions, in defining MP contributions to viral infection (Cooper et al. 1995; Lapidot et al. 1993) and in their potential utilization as novel sources for virus resistance (von Arnim and Stanley 1992b; Lapidot et al. 1993; Beck et al. 1994; Duan et al. 1997). For DNA bipartite geminiviruses, the *BC1* and *BV1* (also known as *BL1* and *BR1* in the literature) MPs are required for viral infectivity and systemic infection (Brough et al. 1988; Etessami et al. 1988; Stanley 1991;

Noueiry et al. 1994; Pascal et al. 1994; Smith and Maxwell 1994). The *BC1* protein is a symptom-inducing element (the determinant of pathogenicity) involved in cell-to-cell and long-distance movement of bipartite geminiviruses (von Arnim and Stanley 1992a; Pascal et al. 1993; Noueiry et al. 1994; Pascal et al. 1994; Smith and Maxwell 1994; Ingham et al. 1995).

Agrobacterium-mediated transformation is a widely used method for inserting genes into plant genomes for both basic and applied studies (Martineau et al. 1994). However, the foreign genes in transgenic plants show varying levels of expressivity/activity in different lines or in siblings with a given transgenic line (Meyer 1995). These variations have been usually attributed to (trans)-gene “silencing,” a common phenomenon that may result from co-suppression or position effects (reviewed by Jorgensen 1992; Flavell 1994; Meyer and Saedler 1996). The homology-dependent gene “silencing” phenomenon is often related to gene inactivation at transcriptional or post-transcriptional levels (Meyer et al. 1993; Dehio and Schell 1994; Mueller et al. 1995). Some of these gene-“silenced” transgenic plants exhibited phenotypic differences that were associated with co-suppression of endogenous genes (Meyer et al. 1987; Elomaa et al. 1995).

Here we present evidence for an alternative mechanism for phenotypic variations observed in transgenic tobacco plants transformed with the *BC1* movement/pathogenicity protein gene of tomato mottle virus (TMoV), a typical bipartite geminivirus (Abouzid et al. 1992). Transgenic plants expressing high levels of the *BC1* gene showed phenotypic differences ranging from severe stunting and leaf mottling (resembling geminivirus symptoms) to no visible symptoms. Sequence analyses of the transgene and co-segregation of the phenotype of progeny with different forms of the transgene showed an association of transgene mutations with phenotype variability in the transgenic plants.

RESULTS

Unexpected phenotype variations in *BC1* transgenic tobacco.

We constructed transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) plants that expressed the *BC1* gene by standard *Agrobacterium*-mediated transformation (Horsch et al. 1985). Surprisingly, a number of plants expressing TMoV *BC1* protein,

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based on protein immunoblot analysis, did not show the expected virus-symptom phenotype of mottling with leaf curl symptoms described for tobacco infected with TMoV (Polston et al. 1993). Only 11 of the 19 transgenic T₀ tobacco plants that expressed the BC1 protein showed disease-like symptoms, which ranged from mild mottling accompanied by slight stunting to mild mottling without stunting. Eight plants expressing the BC1 protein did not show any apparent symptoms. Tobacco transformed with the antisense *BC1* gene or the *BV1* gene had phenotypes indistinguishable from those of nontransformed tobacco (data not shown).

Ten T₀ plants were self pollinated and a segregation in the phenotype was observed in the T₁ and T₂ progeny (Table 1). Among the T₁ progeny, some phenotypes with symptoms more severe than those of any T₀ plant were observed (Table 1). For the T₀ plant designated BC3-11M with a mild mottling symptomatic phenotype, at least three phenotypes were observed in the T₁ generation (Fig. 1). These phenotypes were as follows: type "S" = severe stunting and mottling, more severe than the typical symptoms associated with TMoV infection in tobacco (Fig. 1A and C, plant a); type "M" = mild mottling with no apparent stunting (Fig. 1A and B, plant b/b'); and type "A" = asymptomatic, no visible symptoms (Fig. 1A and B, plant c). Because of greenhouse space limitations, only a limited number of kanamycin-resistant progeny were screened for phenotype segregation (Table 1). Despite the limited sampling of the progeny, marked phenotype segregation was evident in nine of the 10 lines analyzed.

Symptoms associated with starch accumulation.

One of the processes involved in plant virus disease induction is the reduction in translocation of fixed carbon out of the leaves (Bos 1970). Starch accumulation in the leaf causes alterations in leaf texture and color. The symptomatic and asymptomatic *BC1* transgenic tobacco as well as TMoV-infected tobacco were tested for altered carbon partitioning, determined by the starch-iodine test (Fig. 1D). Symptomatic transgenic plants showed high levels of starch accumulation while TMoV-infected tissue showed a more localized accumulation. Starch accumulation was not detected in the asymptomatic *BC1* transgenic plants or in uninfected, nontransformed control plants by this test. The mottling symptoms in the leaves as well as the starch accumulation in the symptomatic transgenic tobacco were accentuated by high-light-intensity growth conditions.

Expression of *BC1* in transgenic plants.

High levels of the BC1 protein expression were indicated in the young tissues of all transgenic plants by protein immunoblot analysis, regardless of the phenotype, except for one "A" phenotype T₁ plant (BC3-11-6A) (Fig. 2). Three other plant lines with the "A" phenotype (BC3-31-3A, BC3-6-4A, and BC3-6-3A) had the same size (33 kDa) and similar level of BC1 protein as the "S" phenotype T₁ plant (BC3-11-5S), indicating that the expression level of the BC1 protein apparently was not correlated with the phenotypes (Fig. 2). The heterogeneity of the BC1 protein seen as two bands at ≈33 kDa in sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) with extracts from *BC1* transgenic plants and the TMoV-infected tobacco was also reported in the analysis of the tobacco transgenic for squash leaf curl vi-

rus (SLCV) *BC1* (Pascal et al.1993). Extracts from the "M" phenotype T₁ plant designated BC3-11-2M showed the expected BC1 protein and a trace amount of a smaller, 23-kDa, BC1-related protein. Extracts from the BC3-11-6A plant showed low levels of a smaller, 23-kDa, BC1-related protein.

Instability of the BC1 protein for an "A" phenotype was indicated by the detection of very low protein levels in extracts from older leaves, compared with levels in the "S" phenotype extracts. The detection level of the BC1 protein in various leaves from an "S" phenotype plant (BC3-11-5S) was compared with that from an "A" phenotype plant (BC3-31-1A), by sampling every third leaf from top to bottom in 3-month-old transgenic tobacco plants. BC1 protein was not detected in extracts from older leaves of the "A" phenotype plants whereas the protein was detected in the extracts of the older leaves of the "S" phenotype (Fig. 3).

Transcript analysis.

RNA blots indicated a high steady-state mRNA level for all the transgenic lines (Fig. 4). The "A" phenotype plant shown in Figure 1 (plant c) had a transcript size of ≈0.9 kb (BC3-11-6A) versus the expected ≈1.0-kb transcript seen in the "S" phenotype (BC3-11-5S) or in other "A" phenotype plants (BC3-31-1A, BC3-6-4A, and BC3-6-3A). The expected transcript size assumes that the transcription termination signals of the TMoV *BC1* downstream from the open reading frame (ORF) are active. The smaller transcript for BC3-11-6A is consistent with the smaller BC1 immunoreactive protein seen for the "A" phenotype (Fig. 2, BC3-11-6A). The "M" phenotype showed both the smaller and the expected size transcripts. The transcript level for the "A" phenotype plant expressing the smaller BC1-related protein was high and therefore the low level of BC1-related protein detected in protein immunoblots (Fig. 2) was not related to transcript abundance.

A 1.2-kb transcript, seen in all extracts except for the one with the smaller transcript (Fig. 4), was apparently the result of a readthrough of *BC1* termination signals into the *rbcS* ter-

Table 1. Phenotypes of the T₁ and T₂ progenies of transgenic T₀ lines expressing the *BC1* open reading frame^a

T ₀ plant lines	T ₁ phenotypes ^b			T ₁ plant lines	T ₂ phenotypes		
	S	M	A		S	M	A
BC3-1M ^c	5 ^d	13		BC3-11-5S	45		
BC3-5M	4	10		BC3-11-2M	8	33	10
BC3-11M	8	25	2	BC3-11-6A			52
BC3-18M	2	6		BC3-31-1A			16
BC3-2M		26		BC3-31-3A			14
BC3-13M	6	12		BC3-31-7M		21	
BC3-16M	3	8		BC3-31-8M		18	
BC3-24M	5	2		BC3-6-3A		18	5
BC3-6A		16	24	BC3-6-4A			28
BC3-31A		5	37	BC3-6-7M		11	2

^a Transgenic plant lines were self-pollinated to generate subsequent T₁ and T₂ generations. The seed of the transgenic plants were germinated on MS rooting media containing 100 µg of kanamycin per ml.

^b Phenotypes: S = severe stunting and mottling; M = no apparent stunting but mottling; and A = no visible symptoms.

^c Letters S, M, and A behind each plant line indicate the phenotype of the parent.

^d Numbers refer to progeny plants observed for phenotype description. The limited number of plants observed may not be representative of the segregation ratio.

Fig. 1. Phenotypic comparison of transgenic T_1 tobacco plants expressing tomato mottle geminivirus (TMoV) BC1 protein. Transgenic plants were derived from a T_0 plant that showed mild leaf mottling with no apparent stunting. **A**, Plants from left to right: a, Transgenic plant (BC3-11-5S) showing stunting and leaf mottling. Symptoms were more severe than those induced by TMoV infection; b, Transgenic plant (BC3-11-2M), showing mild mottling with no apparent stunting; c, Transgenic plant (BC3-11-6A) with no apparent symptoms; and d, Uninfected, nontransformed tobacco plant. **B**, Plant on the left (b') is representative of the genotype as in b (A) and on the right the same as in c (A). Plants were photographed (A) 45 and (B) 90 days after transplanting. **C**, Transgenic plant a, photographed 120 days after transplanting, showing severe mottling and stunting and delayed senescence. **D**, Starch-iodine test for starch accumulation in tobacco leaves of transgenic for *BC1* or infected with TMoV. Leaves are from tobacco plants in A except for e, which represents a TMoV-infected (systemic) tobacco leaf. Distribution of starch accumulation is scattered in the virus-infected leaf (e) and uniform in the symptomatic, transgenic tobacco leaf (a), except for the area near the petiole, which was under less light intensity (partially shaded by upper leaves).

mination sequences of the pKYLX71:35S² vector (Maiti et al. 1993) (sequence data not shown). This was determined by sequencing products obtained after reverse transcriptase reaction of total RNA extract with oligo(dT) primer and then polymerase chain reaction (PCR) amplification with the specific

Fig. 2. Protein gel blot analysis of the P30 fraction of tissue extracts from transgenic T₁ tobacco plants expressing the *BC1* gene. Young leaf tissue extracts were fractionated into S30 (soluble fraction), P30 (membrane fraction), and P1 (cell wall fraction). Eight microliters of the P30 fractions from transgenic lines was electrophoresed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The BC1 protein was detected with the polyclonal antiserum against bacterial-expressed BC1 protein. Estimated sizes of full-length (33 kDa) and truncated (23 kDa) BC1 proteins are indicated. Lanes represent extracts from plant lines designated as follows: BC3-11-6A, BC3-11-5S, and BC3-11-2M were derived from T₀ line BC3-11 (two copies, data not shown); BC3-6-4A and BC3-6-3A were derived from T₀ line BC3-6 (asymptomatic phenotype; five transgene copies, data not shown); and BC3-31-1A was derived from T₀ line BC3-31 (asymptomatic phenotype; four transgene copies, data not shown). TMoV-inf. represents an extract from tomato mottle geminivirus-infected tissue.

Fig. 3. Protein gel blot analysis of the stability of the BC1 protein in transgenic tobacco plants. Leaves were sampled from the youngest leaf to the oldest leaf of transgenic lines at 76 days after transplanting in the greenhouse. Tissue extracts were from similar leaf positions for (A) and (B). Lanes 1 to 7 represent BC1 protein detection in extracts (P30 fraction), sampling every third leaf starting from the youngest to the oldest leaf; NT = young leaf tissue extract from nontransformed tobacco plant. Eight microliters of each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Membranes were probed with the polyclonal antiserum as in Figure 3. **A**, Protein immunoblot analysis of the leaf tissue extracts from a symptomatic plant (BC3-11-5S). **B**, Protein immunoblot analysis of the leaf tissue extracts from an asymptomatic plant (BC3-31-1A).

5' primer for *BC1* and the oligo(dT) primer. One of the products had a 3' termination sequence expected for the *BC1* gene while the larger product had the expected *BC1* gene sequence plus *rbcS* termination sequence.

Genetic characterization of transgenic lines.

The transgenic T₁ plants were selected on the basis of phenotype and analyzed by DNA blots to verify the presence and segregation of the transgene and to estimate gene copy number (Fig. 5). For the T₁ progeny of the BC3-11M with the “M” phenotype (BC3-11-2M), two copies of the *BC1* gene were

Fig. 4. RNA blot analysis of transgenic plants that express the *BC1* gene, probed with labeled *BC1* DNA. Total RNA, extracted from transgenic plants and a nontransformed plant (NT), was separated on a denaturing agarose gel with formaldehyde, and then transferred to a nylon membrane. *BC1* transgene transcripts were detected by probing with ³²P-labeled *BC1* DNA. Estimated sizes of the two *BC1*-related transcripts for the full-length *BC1* gene and one transcript for a 3'-truncated/recombination form of the *BC1* gene are indicated. Samples indicated are as in Figure 2.

Fig. 5. DNA blot analysis of the T₁ transgenic plants with different phenotypes displayed in Figure 1. DNA samples are from plant lines as indicated in Figure 2. Blots for BC3-31-1A, BC3-6-4A, and BC3-6-3A plant lines with no visible symptom phenotype are shown for comparative purposes; NT = nontransformed plant and V-*BC1* = pKYLX71:35S² vector with the *BC1* open reading frame construct used for transformation. Genomic DNA of the transgenic plants was extracted and digested with *Xba*I. DNA blots were subjected to hybridization with ³²P-labeled *BC1* DNA fragments.

resolved. Other T₁ progeny from this line that had an “S” phenotype (BC3-11-5S) or an “A” phenotype (BC3-11-6A) only had one copy (Fig. 2). For comparative purposes, progeny from three other lines with the “A” phenotype were also examined and found to have three, three, and five copies of the *BCI* gene, respectively (Fig. 2, BC3-31-1A, BC3-6-4A, and BC3-6-3A). The transgene copy numbers identified for the transgenic lines were consistent for genomic DNA digested with either *Xba*I (Fig. 5) or *Eco*RI (not shown).

Transgene sequence analysis.

The variations in the phenotype in transgenic tobacco lines expressing similar levels of BC1 protein indicated possible mutations in the *BCI* gene. The *BCI* gene from transgenic tobacco plants showing the “S” (BC3-11-5) or “A” (BC3-11-6) phenotype was amplified by PCR and sequenced, and the ORF translated into amino acid sequence. The sequence data (for four independent lines) revealed changes (amino acid residue 215 G-S, 219 S-L, and 247 E-G; GenBank no. U65508) near the carboxyl terminus of the BC1 protein (Fig. 6, *BCIS*) for the “S” phenotype (Fig. 1A, plant a) when compared with the viral *BCI* sequence. The *BCIS* sequence was

BC1	1	MDSQLVNPPS	AFNYIESHRD	EYQLSHDLTE	IILQFPSTAS	QLTARLSRSC	50
BCIS							
BC1At/r							
BC1A		FS.....		L.....			
BC1	51	MKIDHCUIEY	RQQVPINATG	SVIVEIHDRK	MTENESLQAS	WTFPIRCNID	100
BCIS							
BC1At/r							
BC1A							
BC1	101	LHYFSASFFS	LKDDPIPKWLY	YRVCDTNVHQ	RTHPAKFGKG	LKLSTAKHSV	150
BCIS							
BC1At/r							
BC1A							
BCIS	151	DIPFRAPTIVK	ILSKQFTDKD	VDFSHVDYGG	WERKPIRCAS	MSRLGLRGPI	200
BCIS							
BC1At/r				PLKLSQ	KCMFWGKIPN	SATFEQTYDG*	
BC1A							
BC1	201	EIRPGESWAS	RSTIGIGHSD	ADSEVENELH	PYRHLNRLGT	GILDPGESAS	250
BCIS			S.....	L.....		G.....	
BC1A							
BC1	251	IVGAQKAESN	ITSMGQLNE	LIRTTVHECI	NSNCKASQTK	SLK*	293
BCIS							
BC1A							

Fig. 6. Predicted amino acid sequences of the tomato mottle geminivirus (TMoV) and transgene-mutated BC1 proteins. BC1 = amino acid sequence of TMoV *BCI* gene from GenBank accession no. L14461. Sequence of the polymerase chain reaction (PCR)-amplified *BCI* open reading frame was verified before and after cloning into the pGEM-T vector. BC1A = amino acid sequence determined for a *BCI* gene from asymptomatic, multicopy transgenic plant that expressed full-length BC1 protein. Sequence was analyzed from the PCR product derived from genomic DNA (BC3-31-3A) (GenBank accession no. U65507). BC1At/r = amino acid sequence determined for *BCI*At/r gene from an asymptomatic, single-copy transgenic plant that expressed a truncated BC1 protein. Transcript for the BC1 truncated/recombination protein (*BCI*At/r) was sequenced after reverse transcription of extracted total RNA, with oligo (dT) primer followed by PCR amplification with a *BCI*-specific primer. Sequence was also verified by sequencing the PCR product from genomic DNA (BC3-11-6A) and from cloned PCR product (GenBank accession no. U65506). BCIS = amino acid sequence determined for *BCIS* from a symptomatic, single-copy transgenic plant that expressed full-length BC1 protein. Sequence was analyzed after reverse transcriptase PCR of total RNA (BC3-11-5S), after PCR amplification of genomic DNA (BC3-11-5S), and after PCR amplification from three different lines with a similar phenotype (GenBank accession no. U65508). Identical amino acid residues are indicated by (.)

identical to that resolved from PCR-amplified product from the *Agrobacterium* culture used for transformation (data not shown). In order to test the fidelity of our sequence analysis, primers used for the *BCIS* analysis were used to sequence a cloned *BCI* PCR-product amplified from TMoV-infected tissue. This sequence was identical to that published for the TMoV *BCI* gene (GenBank no. L14461).

The primers used for the construction of the *BCI* gene failed to amplify any product from the single-copy “A” phenotype line (BC3-11-6A) in PCR reactions. Product representing *BCI* sequence was amplified with the 5′-specific primer and an internal primer, indicating that a partial copy of the *BCI* gene was present in this “A” phenotype line. Several other 3′-end primers, based on the *rbcs* termination sequences of the pKYLX71:35S² vector, also failed. Product suitable for sequencing was obtained after reverse transcriptase (RT) reaction of total RNA extract with oligo (dT) primer and then PCR amplification with the specific 5′ primer for *BCI* and the oligo (dT) primer. The first 612 nucleotide bases were identical to the *BCI* sequence but the 3′ end had 225 unique bases, not including the poly A (data not shown; GenBank no. U65506). The recombinational addition of the unique 225 bases included 81 in phase with the *BCI* ORF, and 144 bases presumably with transcriptional terminator signals. A primer was designed, based on the 3′ terminus of the *BCI*At/r sequence, and used to PCR amplify the mutated transgene from the progenies of BC3-11. The *BCI*At/r transgene was amplified from BC3-11-6A and BC3-11-2M, but not from BC3-11-5S. This verified that the sequence resolved for the 3′ end of the product amplified by RT-PCR was representative of the transgene that had undergone a recombinational event.

The amino acid sequence for the “A” phenotype (resolved for BC3-11-6A, Fig. 2A; *BCI*At/r, Fig. 6) showed a deletion of amino acid residues 175 to 293, and an additional sequence of 26 amino acid residues, from an unidentified recombinational origin, starting after amino acid residue 174. The ORF predicted for the *BCI*At/r sequence was consistent with the detection of a smaller BC1-related protein (≈10 kDa smaller in size than that of the wild type; Fig. 2) in the protein immunoblot and a reduced transcript size in the RNA blot (Fig. 4). The low level of the truncated BC1 protein detection may be due to the loss of epitopes, since 119 amino acid residues were lost at the carboxy end. The slightly less intense 0.9-kb band, compared with 1.0 kb (Fig. 4, BC3-11-2M), may be due to the lack of homology between the 3′ ends of the two different transcripts, since the probe was made to the full-length *BCI* gene. The *BCI* sequence amplified from another line with an “A” phenotype (resolved from a clone derived from PCR amplification from a multicopy line BC3-6-3A; Fig. 6, *BC1A*) showed several changes near the amino terminus (amino acid residue 6 V-F, 7 N-S, and 35 F-L; GenBank no. U65507).

In order to exclude possible PCR artifacts, the sequence analyses were done in several different ways and with independent reactions. For the “S” phenotype line BC3-11-5S and “A” phenotype line BC3-11-6A, sequences were obtained with independent reactions by sequencing cloned PCR products and by direct sequencing of PCR products from cDNA and from genomic DNA. Three other lines with the “S” phenotype (including one single-copy line and two multiple-copy lines, not shown) were also sequenced after PCR amplifica-

tion of genomic DNA and the sequences were identical to *BC1S*. The sequence for BC3-6-3A line (multiple-copy line) was resolved from two different clones of PCR product amplified from genomic DNA. The sequence for the *BC1* gene in the pKYLX71:35S² vector (identical to the *BC1S* sequence) from the *Agrobacterium* culture used for transformation was done once from a PCR amplification of isolated plasmid DNA (data not shown). In the latter case, we cannot exclude the possibility that other *BC1* gene sequence variations exist in the plasmid population in the *Agrobacterium* culture.

Phenotype of the *BC1A* expressed in tobacco via a PVX vector.

A symptomatic phenotype was associated with the *BC1S* gene expressed alone in transgenic tobacco (Fig. 1, BC3-11-5S). Since we did not resolve the mutated *BC1A* in a single-copy transgenic plant, we tested whether this mutated *BC1* gene could induce symptoms in tobacco during transient expression with the potato virus X vector developed by D. Baulcombe's group (Chapman et al. 1992). *Nicotiana benthamiana* inoculated with the PVX transcript containing the wild-type *BC1* ORF (pGXBC1, see Materials and Methods) showed veinal necrosis and severe mottling, whereas tobacco inoculated with PVX transcripts containing a copy of the *BC1A* ORF (pGXBC1A) showed only a mild mosaic, indistinguishable from those caused by PVX infection alone (Table 2). A serological comparison of the plants inoculated with the PVX vector alone and plants inoculated with the vector expressing either *BC1* from TMoV or the *BC1A* indicated a possible synergism between PVX infection and the wild-type *BC1* gene expression, and a possible interference of the PVX infection with the expression of the *BC1A* (Table 2).

DISCUSSION

Virus-like symptoms associated with *BC1* transgene.

A number of T₀ plants and the subsequent progeny expressing the TMoV *BC1* protein had phenotypes resembling geminivirus-like mottling symptoms. The severity of the mottling was light dependent. Severe stunting and mottling of the plants were seen only in certain progeny of the T₀ plants.

These plants also showed a marked delay in flowering and senescence (Fig. 1C) and reduced seed set. Similarly, tomato plants transgenic for the TMoV *BC1 S* gene have geminivirus-like symptoms characteristic of TMoV infection in tomato (E. Dickstein and E. Hiebert, *unpublished*). This association of pathogenicity with the expression of the TMoV *BC1* gene in transgenic plants is similar to that described for transgenic tobacco expressing SLCV *BC1* (Pascal et al. 1993).

Symptomatic phenotype mimics impaired photoassimilate partitioning.

It has been proposed that virus MPs in plants may significantly disturb source-sink interactions and reduce photoassimilate partitioning in sink tissues, and thus hinder plant growth and development (Leisner and Turgeon 1993; Lucas et al. 1993). Transgenic tobacco plants expressing the tobacco mosaic virus (TMV) 30-kDa MP show an interference with carbohydrate metabolism and its export from the mesophyll tissue (Lucas et al. 1993). In a subsequent study with TMV MP mutants, Olesinski et al. (1995) demonstrated a second function, in addition to the dilation of the plasmodesmata, that involves interaction with cellular processes in carbon metabolism and/or export. The *BC1* transgenic tobacco with the symptomatic phenotype showed starch accumulation (Fig. 1D), presumably due to impaired photoassimilate partitioning, and mimicked the symptoms induced by geminivirus infections. The starch accumulation observed in the tobacco transgenic for the TMoV *BC1* gene appears more pronounced than that associated with the plants transgenic for TMV 30-kDa MP (Lucas et al. 1993).

Unexpected phenotype variations correlated with transgene mutation.

The variations in the phenotype in tobacco plants transgenic for the TMoV *BC1* were unexpected, since similar levels of the *BC1* mRNA and, in most cases, similar levels of the protein (an exception is the progeny of BC3-11-6A) were detected in the asymptomatic as well as in the symptomatic plants. Furthermore, the progeny of nine out of 10 T₀ lines tested revealed a segregation of the phenotype ranging from plants with symptoms more severe than the parent or seen

Table 2. Assessment of the tomato mottle geminivirus (TMoV) *BC1A* expression in tobacco with a PVX vector

Inoculum ^b	Symptoms	ELISA mean value of A ₄₀₅ ^a					
		PVX Ab		TMoV Ab		BC1 Ab	
		Inoc. leaf	Upper leaves	Inoc. leaf	Upper leaves	Inoc. leaf	Upper leaves
PVX vector	Mild mosaic	0.321 ± 0.033	0.352 ± 0.034	0.014 ± 0.004	0.015 ± 0.006	0.018 ± 0.007	0.014 ± 0.005
PVX BC1 ^c	Vein necrosis and severe mottling	0.387 ± 0.040	0.517 ± 0.031	0.012 ± 0.006	0.016 ± 0.001	0.238 ± 0.041	0.266 ± 0.036
PVX BC1A ^d	Mild mosaic	0.370 ± 0.084	0.249 ± 0.068	0.013 ± 0.007	0.012 ± 0.004	0.123 ± 0.057	0.131 ± 0.040
TMoV	Mottling	0.012 ± 0.004	0.014 ± 0.003	0.385 ± 0.032	0.306 ± 0.046	0.142 ± 0.047	0.189 ± 0.037

^a Plate-trapped antigen enzyme-linked immunosorbent assay (ELISA) was done with the PVX and BC1 detection. Indirect ELISA was used to assay the plants at 28 days postinoculation for TMoV infection. Polyclonal antiserum #1110 and monoclonal antiserum 3F7 were used in the indirect ELISA for TMoV (Cancino et al. 1995). Inoculated and upper leaves from individual plants were collected, and homogenized in 1:5 (wt/vol) phosphate-buffered saline for the assay. ELISA readings represent the mean values (A₄₀₅) from 10 different plants. Ab = antibody. Results represent a summary of four separate experiments since it was not possible to conduct all the inoculations at the same time.

^b Tobacco plants (*Nicotiana benthamiana*) were mechanically inoculated with the RNA transcripts derived from the cloned PVX vector. Extracts from TMoV-infected *N. benthamiana* were used as TMoV inoculum.

^c PVX BC1 represents the *BC1* gene amplified from TMoV-infected tissue and it was engineered for expression in the PVX vector kindly provided by D. Baulcombe (Chapman et al. 1992).

^d PVX-BC1A represents the *BC1* gene amplified from the BC3-31-3A line and engineered for expression in the PVX vector.

with TMoV infections to no visible signs, indicating transgene interference in certain lines with attenuated phenotypes.

These variations are not adequately explained by position effects or tissue-culture effects. Tissue-culture-derived progeny containing no transgene as a result of segregation of the transgenic lines were indistinguishable from nontransformed tobacco plants. Neither did we see any similar phenotype variations in our *BV1* and antisense *BC1* or *BV1* transgenic tobacco plants (Duan et al. 1997). Although the transgenic plants harbored different insertions (both insertion position and copy number) of the *BC1* gene (Fig. 5; data for the other lines not shown), similar levels of the *BC1* mRNA and the protein were detected in the asymptomatic as well as symptomatic plants (Figs. 2 and 4; data for the other lines not shown). Furthermore, the phenotype variations of transgenic plants expressing the BC1 protein were observed not only in T₁ progenies but also in T₂ and T₃ (data not shown) generations, and the variation was correlated with the segregation of different forms of the *BC1* transgene, as seen for the progeny of BC3-11.

DNA blot analysis of T₁ (Fig. 5) progeny indicated the "M" phenotype T₀ tobacco selected for this study contained two copies of the *BC1* gene. The "M" phenotype plant described above (Fig. 1, plant b or b') had one copy each of the "S" and "A" forms of the *BC1* gene (Fig. 5, BC3-11-2M). This indicated that the "A" phenotype *BC1* gene attenuated the symptom-inducing element(s) of the "S" phenotype BC1 gene in transgenic plants containing both forms. Apparently, T₀ and a number of T₁ and T₂ transgenic tobacco plants containing copies of both symptomatic and asymptomatic forms of the *BC1* gene resulted in a phenotype with mild mottling and no stunting. Transgene silencing (Meins and Kunz 1995; Baulcombe 1996) was not evident in the two-copy *BC1* plants described above, since protein from each copy was detected in protein immunoblots (Fig. 2, BC3-11-2M). Furthermore, the expression of the symptomatic phenotype in subsequent generations indicated that the symptomatic *BCIS* gene was competent in the asymptomatic phenotype T₀ tobacco parent.

The high levels of BC1 mRNA and protein detected in our transgenic tobacco with phenotype variations may implicate a mechanism known as trans-dominant negative interference. Herskowitz (1987) presented the concept of the functional inactivation of a gene by "dominant negative mutation" and described it as the inhibition of the function of a wild-type gene product by an over production of an inhibitory variant of the same gene product. Von Arnim and Stanley (1992b) have demonstrated the inhibition of African cassava mosaic virus (ACMV) infection by co-inoculation with a modified version of the ACMV genome constructed by replacing the coat protein region with a chimera containing amino-terminal sequences of TGMV *BC1* and a carboxy-terminal sequences of its ACMV homologue *BC1*. They considered this interference with the function of the wild-type gene as characteristic of a dominant negative mutant.

The sequence analyses of the *BC1* gene from transgenic tobacco supported a possible trans-dominant interference mechanism for phenotype attenuation in our transgenic tobacco. Mutations in the *BC1* gene were detected when PCR-amplified products from transgenic plants were sequenced and compared with the viral sequence or with the sequence from the initial PCR clone used in the gene construction for trans-

formation. One *BC1* transgene (*BC1At/r*) showed a major deletion at the 3' end and a recombination event with the addition of ≈225 nucleotides from an unidentified source. This resulted in a modified *BC1* ORF with a deletion of 119 amino acid residues at the carboxy terminus and an addition of 26 amino acids from an unknown source. The progeny of the BC3-11-6A showed resistance to kanamycin, indicating that both borders of T-DNA were inserted into the tobacco DNA and that the recombination event had not resulted in kanamycin gene inactivation.

Symptom attenuation was also evident in other transgenic lines with multiple *BC1* gene copies, since some of these asymptomatic, transgenic T₀ tobacco plants also revealed segregation in the T₁ and T₂ progeny, as indicated by the appearance of several symptomatic plants in the progeny (Table 1). *BC1* transgene sequence variations other than that seen for *BC1At/r* may also result in an asymptomatic phenotype. The *BC1* gene sequence resolved from a clone selected from the PCR amplification of DNA from a transgenic plant (BC3-6-3A) containing five copies of *BC1* revealed three changes near the amino terminus of the BC1 protein (Fig. 6, *BC1A*). Possible variations in the *BC1* gene in the other transgenic lines with asymptomatic phenotypes have not been analyzed because single-copy transgene plants have not been identified in these lines.

It is not known whether the mutations identified in *BC1A* were involved in phenotype attenuation, and whether there is the epistasis of *BC1A* over *BCIS* or the incomplete dominance of *BCIS*. However, the transient expression of this *BC1A* gene in tobacco, utilizing a PVX expression vector (Chapman et al. 1992), indicated that the *BC1A* gene lacked the symptom-inducing elements associated with the wild-type *BC1* gene (Table 2). Unexpected, apparent interaction of the wild-type *BC1* or the mutated *BC1A* genes with the PVX infection (Table 2) complicated the assessment of the possible symptom-inducing effects of the *BC1A* during transient expression in tobacco. Complementation of apparent movement function in RNA and DNA viruses is possible in heterologous combinations, as seen in mixed virus infections of bean golden mosaic virus (BGMV) and TMV (Carr and Kim 1983).

The pathogenic effects of BC1 protein may be a factor in the recovery of attenuated phenotype plants during transformation. Pascal et al. (1993) indicated growth retardation of transgenic plants expressing SLCV *BC1*; five out of 17 transgenic lines were symptomless, and others showed a range of symptomatic phenotypes. Transgenic cells expressing the symptomatic *BC1* gene may result in cell death or growth too slow to be selected during regeneration. There was a marked reduction in plant regeneration with our *BC1* transformation experiments compared with the antisense variations of this gene or the TMoV *BV1* gene (data not shown), and the severe symptom phenotypes were only seen in the T₁ and subsequent progenies. With one exception, all the nucleotide changes identified in the *BC1* transgenes resulted in an amino acid change. This high correlation of nucleotide changes with amino acid changes may be due to selection involved in mollifying the toxic effects of nonmutated BC1 protein.

Deletions, recombination events, and point mutations in nonselected genes during recombinant DNA propagation have been reported infrequently. Donson et al. (1993) observed a 1.4-kbp insert, an IS10-like transposable element, during the

propagation of a TMV 183-kDa replicase ORF in a plasmid. Stephens et al. (1996) reported that a mutation in the β -glucuronidase (GUS) gene in a stock of *Agrobacterium tumefaciens* clone LBA 4404/pBI121 resulted in loss of GUS activity in transformed tobacco plants. They concluded that non-selected genes within the T-DNA region may undergo mutations in *Agrobacterium*. Hong and Stanley (1996) proposed that selection against a functional transgene may have occurred during plant transformation and regeneration to explain the failure of their transgenic tobacco lines for ACMV *ACI* in complementation experiments with ACMV defective in *ACI*. The sequence of the *BCIS* transgene (Fig. 6) was identical to the sequence resolved for the PCR product amplified from our *Agrobacterium* culture used for transformation but differed from the sequence of the viral *BCI* gene (Fig. 6).

We have presented an association of "spontaneously" mutated transgenes with phenotypic variations in transgenic plants. Some of these transgenic plants also showed resistance to TMoV and to a distantly related cabbage leaf curl geminivirus (Duan et al. 1997). These mutants should enable study of the pathogenicity elements of this gene, study of the cytological localization and physiological effects of this protein in planta, and identification of the elements in the mutated, transgene *BCI* responsible for the resistance to virus infection.

MATERIALS AND METHODS

Tobacco transformation.

The sequence between nucleotides 1278 and 2308 of TMoV-DNA B component containing *BCI* ORF and the putative translational leader and transcriptional termination signals was amplified from the extracts of infected tomato plants by PCR, cloned into pGEM-T vector (Promega, Madison, WI), and subcloned into the unique *HindIII* site of pKYLX71:35S² vector (Maiti et al. 1993; Duan et al. 1997). The *BCI* sequence in the pGEM-T vector and in the pKYLX71:35S² vector after *Agrobacterium* transformation was verified by sequencing the PCR products generated after the cloning experiments. The sense clone pKYs*BCI* and the antisense clone pKYas*BCI* were transformed into cells of *Agrobacterium tumefaciens* LBA 4404. *Nicotiana tabacum* cv. Xanthi was transformed by the leaf disk method and transformants were identified as described previously (Duan et al. 1997). A total of 19 T₀ plants containing pKYs*BCI* and 19 plants containing pKYas*BCI* were selected and self-pollinated for T₁ progeny seeds. The seeds of T₀ lines were germinated individually on Murashige and Skoog (MS) medium containing 100 μ g of kanamycin per ml and the T₁ seedlings were transplanted into soil a month after germination.

Starch-iodine staining.

Newly expanded leaves were sampled from TMoV-infected (30 days post inoculation), transgenic, and control tobacco plants in the morning. Leaves were decolorized in 70% ethanol, and stained with iodine-potassium iodide-lactic acid mixture (stock IKI was 2% iodine and 6% KI, mixed with 85% lactic acid in 1:20 ratio) as described by Lindner et al. (1959).

DNA and RNA extractions.

DNA was extracted for Southern blot analyses as described previously (Duan et al. 1997).

RNA was isolated by a modification of the DNA isolation method (Cocciolone and Cone 1993). Samples were prepared and extracted as above until just before the isopropanol addition. After the addition of an equal volume of isopropanol to the supernatant and chilling on ice for 2 min, a heavy precipitate formed. The soluble fraction was removed from the heavy precipitation with a pipette. The soluble fraction containing a large amount of RNA and a trace amount of DNA was precipitated by centrifugation at $16,000 \times g$ for 10 min after incubation at -20°C for 1 h. The pellet was washed with 70% ethanol and resuspended in H₂O for direct use, or resuspended in a 0.4-ml solution containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 0.5% Sarkosyl, extracted once with phenol:chloroform, and stored at -20°C after the addition of 0.1 vol of 3 M sodium acetate and 2 vol of 100% ethanol.

DNA and RNA blot analyses.

Ten micrograms of genomic DNA was digested for 3 to 6 h with restriction enzymes *EcoRI* or *XbaI*; both have unique sites within the vector T-DNA (Maiti et al. 1993). The restriction sites of these enzymes within the transformation cassette are shown in Figure 1 of Duan et al (1997). The digested DNA was separated by electrophoresis on a 0.75% agarose gel and transferred to a nylon membrane with alkaline transfer buffer. A probe was made by random primed ³²P-labeling of TMoV *BCI* DNA fragment (nucleotides 1278 to 2308) with the Prime-a Gene labeling kit (Promega). Hybridization (overnight at 65°C) of blots was in $6\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), $5\times$ Denhardt's solution, and 0.5% SDS, 0.1 mg of salmon sperm DNA per ml. Blots were washed twice in $2\times$ SSC, 0.1% SDS at room temperature for 10 min, and once in $1\times$ SSC, 0.1% SDS and in $0.1\times$ SSC, 0.1% SDS at 65°C for 15 min, respectively.

Ten to 15 μ g of total RNA was separated on a 1.0% agarose gel with formaldehyde and blotted onto nylon membranes. Hybridization was carried out as described above for DNA gel blots.

Protein gels and immunoblots.

The subcellular fractions of the leaf extracts from transgenic and control plants were prepared as described by Pascal et al. (1993) and analyzed as described previously (Duan et al. 1997).

Cloning of the potato virus X (PVX) expression vector.

The PVX vector (pP2S2C) used for transient expression of the TMoV *BCI* genes in tobacco is a derivative of pGC3 (Chapman et al. 1992). The two clones used in this work, pGXBC1 and pGXBC1A, consisted of the PVX vector containing full-length copies of the wild-type *BCI* and a *BCIA* gene containing point mutations, respectively. The coding sequence of the wild-type *BCI* was amplified from TMoV-infected *Nicotiana benthamiana* by PCR and cloned as a blunt-ended fragment into the unique *EcoRV* site (nucleotide 5677) of pP2S2C. The coding sequence of the *BCIA* was amplified from the *BCIA* clone derived from an "A" phenotype plant (BC3-31-3A) and cloned as a blunt-ended fragment into the unique *EcoRV* site (nucleotide 5677) of pP2S2C. Primers for the amplification of the *BCI* and *BCIA* ORFs were 5'-CATTATATGGATTCTCAGTTAG-3' or 5'-GCAACAATGGATTCTCAGTTAG-3' and 5'-GAATTCTTATTTTAATGATT

TCGTCTG-3'. Transcripts of pGXBC1 and pGXBC1A were made following standard protocols. Inoculation of *N. benthamiana* was performed by spotting a portion of the transcript mixture on Carborundum-dusted leaves and gently rubbing the inoculum on the leaf with a glass inoculating rod.

Sequence analysis.

The *BCI* transgene was amplified by PCR from genomic DNA with specific primers for the TMoV *BCI* gene (EH 262, 5'-CCCAAGCTTCGAGTTCGAAACTGC-3' and EH 263, 5'-CCCAAGCTTAACGAAGTGTGTTTGAC-3') and for the *rbcS* terminator sequence (EH 274, 5'-TCGATTGATGCATG TTGTC-3'). Reactions were run in a thermocycler (Biometra, Tampa, FL) for 30 cycles with a touch down program composed of a 2-min denaturation at 92°C, 30-s annealing time at 60 to 50°C (-1°C each cycle), and 1-min extension at 72°C, for the first 10 cycles, and a 1-min denaturation at 92°C, 30-s annealing time at 50°C, 1-min extension 72°C, for the next 20 cycles. RT-PCR was carried out from total RNA by SuperScript reverse transcriptase (GIBCO BRL, Gaithersburg, MD) with oligo(dT) primer and *Taq* DNA polymerase (Promega) with EH 262 primer for the *BCI*. To confirm the *BCI*At/r sequence resolved from the cDNA, the transgene sequence was amplified by PCR from genomic DNA with primers EH 262 and EH 278, 5'-GAGCTCAGAAATAATTT GGAAAC-3' designed on the basis of the cDNA sequence at the 3' end. PCR products were directly sequenced, or were cloned into pGEM T-vector (Promega) and then sequenced. Sequencing was done in both directions and repeated at least once with an independent reaction. DNA samples were sequenced by the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR). The DyeDeoxy Terminator Cycle Sequencing protocols developed by Applied Biosystems with fluorescent-labeled dideoxynucleotides and primers were used. The sequences were analyzed by the University of Florida ICBR Computer Core, which has the University of Wisconsin Genetics Computer Group software.

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